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TECHNIQUES

Development of High-Concentration Lipoplexes for In Vivo Gene Function Studies in Vertebrate Embryos

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Here we report that highly concentrated cationic lipid/helper lipid-nucleic acid complexes (lipoplexes) can facilitate reproducible delivery of a variety of oligonucleotides and plasmids to chicken embryos or to mouse embryonic mesenchyme. Specifically, liposomes composed of N,N-dioleyl-N,N-dimethylammonium chloride (DODAC)/1,2 dioleoyl glycero-3-phosphorylethanolamine (DOPE) prepared at 18-mM concentrations produced high levels of transfection of exogenous genes in vivo and in vitro. Furthermore, we report sufficient uptake of plasmids expressing interference RNA to decrease expression of both exogenous and endogenous genes. The simplicity of preparation, implementation, and relatively low toxicity of this transfection reagent make it an attractive alternative for developmental studies in post-gastrulation vertebrate embryos. ***Developmental Dynamics 240:2108–2119, 2011.*** **© 2011 Wiley-Liss, Inc.**

Key words: chicken embryo; mouse embryo; limb; face; mesenchyme; expression; transfection; liposome; cationic lipids; interference RNA; Morpholino; micromass culture

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INTRODUCTION

The ability to express DNA or to knock down genes in vivo is essential to characterizing gene function. Transgenesis can be achieved with physical, chemical, or viral methods but, as we will discuss, each procedure has its problems depending on the parameters of the gene and tissue being studied (Table 1). The physical method most commonly used in embryos is electroporation. Here a pulse of electrical current permits passage of DNA through the plasma membrane (Sauka-Spengler and Barembaum, 2008). Although onset of gene expression is rapid, there are several disadvantages including difficulty controlling the field of electroporation and preventing the DNA from diffusing away from the tissue of interest, unless there is a natural cavity, which will act as a reservoir. Furthermore, it is a challenge to electroporate embryos older than E2.5 (stage 15) in the egg due to growth of chorioallantoic membrane over the top of the embryo. In addition, the proximity of the heart to regions such as the pharyngeal arches and face reduces survivorship. Some of these problems can be overcome with ex-ovo manipulations but even in the best case scenario, survival to skeletogenesis stages is poor (Luo and Redies, 2005). Electroporated tissues such as neural folds can be grafted into host embryos in order to extend survival (Creuzet, 2009). However, the grafting itself presents its own challenges. Thus although electroporation is currently the most favoured method of in vivo transgenesis, there remain significant limitations (Table 1).

Viral vectors are a very efficient way to transfect specific regions of embryos and are compatible with longer-term experiments. In avian embryos, the replication competent retrovirus RCASBP can be used to deliver both coding sequences and interference RNA in a stage-and

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TABLE 1. Comparison of Different Methods Used to Deliver Plasmids In Vivo

Transfection method	Advantages	Disadvantages
Electroporation	High amounts of plasmid can be delivered. Transient and stable transfection possible. Effective in targeting the neural tube, somites, and eyes (Inoue et al., 2001; Scaal et al., 2004; Baeriswyl et al., 2008; Sauka-Spengler and Barembaum, 2008). A variety of plasmids and nucleic acids can be delivered. No limit to insert size.	There is a rapid onset of gene expression but embryo survival beyond 48 hr is poor. Some tissues cannot take up DNA with this method including mesenchyme, the vasculature, and many organs. The effective range of electroporation is controlled by the size of the electrodes and the larger the area the greater damage to the tissue, which ultimately reduces survival.
RCASBP retrovirus	Global misexpression over a region of the embryo is possible. Can deliver both coding sequences and interference RNA. It is possible to localize the gene of interest as well as virus. Low biohazard risk since it is ecotropic (Sauka-Spengler and Barembaum, 2008; Gordon et al., 2009).	Large vector, restrictions on the size of insert. Variable onset of expression. Replication competent so it will continue to spread. Preparing high titre virus is laborious.
Lentivirus	Enters genome very efficiently in post-mitotic cells (Brugmann et al., 2007; Howarth et al., 2010).	High biohazard risk since it is amphotropic. Preparing high titre virus is laborious.
Adenovirus	Enters cells very efficiently including in post-mitotic cells (Brugmann et al., 2007; Howarth et al., 2010).	Lower efficiency of transfection. Transient transfection that diminishes with time. Preparing high titre virus is laborious.
DODAC/DOPE liposomes	Enters cells easily. Low toxicity. Works well in mesenchyme. Targeted delivery of a variety of nucleic acids is possible. Can result in focal transfection of cells allowing cell behaviour to be studied in vivo. Sufficiently high expression levels can be achieved to knock down endogenous gene expression.	Transient transfections work well for the first 2 days after delivery, hence other means are required to induce stable expression in chicken embryos such as the use of transposable elements.

site-specific manner (Sauka-Spengler and Barembaum, 2008; Gordon et al., 2009). However, onset of expression is gradual, the size of insert is restricted to 2.5 kb, and coordination of viral expression with the developmental process being studied is sometimes difficult (Table 1).

The most common method of in vitro transfection involves the use of cationic lipids to facilitate passage of nucleic acid into cells. Cationic liposomes condense nucleic acids into lipid-nucleic acid complexes (lipoplexes) that interact with the negatively charged cell membrane (Liu et al., 2003). Although several groups have published technical papers showing that liposomes can work in vivo, they are still not commonly used. In avian embryos, the results are variable depending on the tissue and stage being transfected (Muramatsu et al., 1997; Dickinson et al., 2002; Bollérot et al., 2006; Decastro et al., 2006; Albazerchi et al., 2007). In addition, some of the carrier systems are complicated to prepare, which can pose a barrier to widespread adoption by developmental biologists (Dickinson et al., 2002; Decastro et al., 2006). Despite these caveats, of all the methods described, lipoplexes hold the most promise of achieving localized transgenesis in vivo, particularly of the mesenchyme. The potential of lipoplexes to work better than established methods motivated us to develop these reagents further.

RESULTS AND DISCUSSION

Our first aim was to find a cationic/helper lipid combination that would work better in vivo than off-the-shelf commercial reagents or customized reagents published by others for use in the chicken embryos (Muramatsu et al., 1997; Dickinson et al., 2002; Bollérot et al., 2006; Decastro et al., 2006). To start with, we tested three cationic lipids, DOTAP (Bollérot et al., 2006), DOTMA, and DODAC.

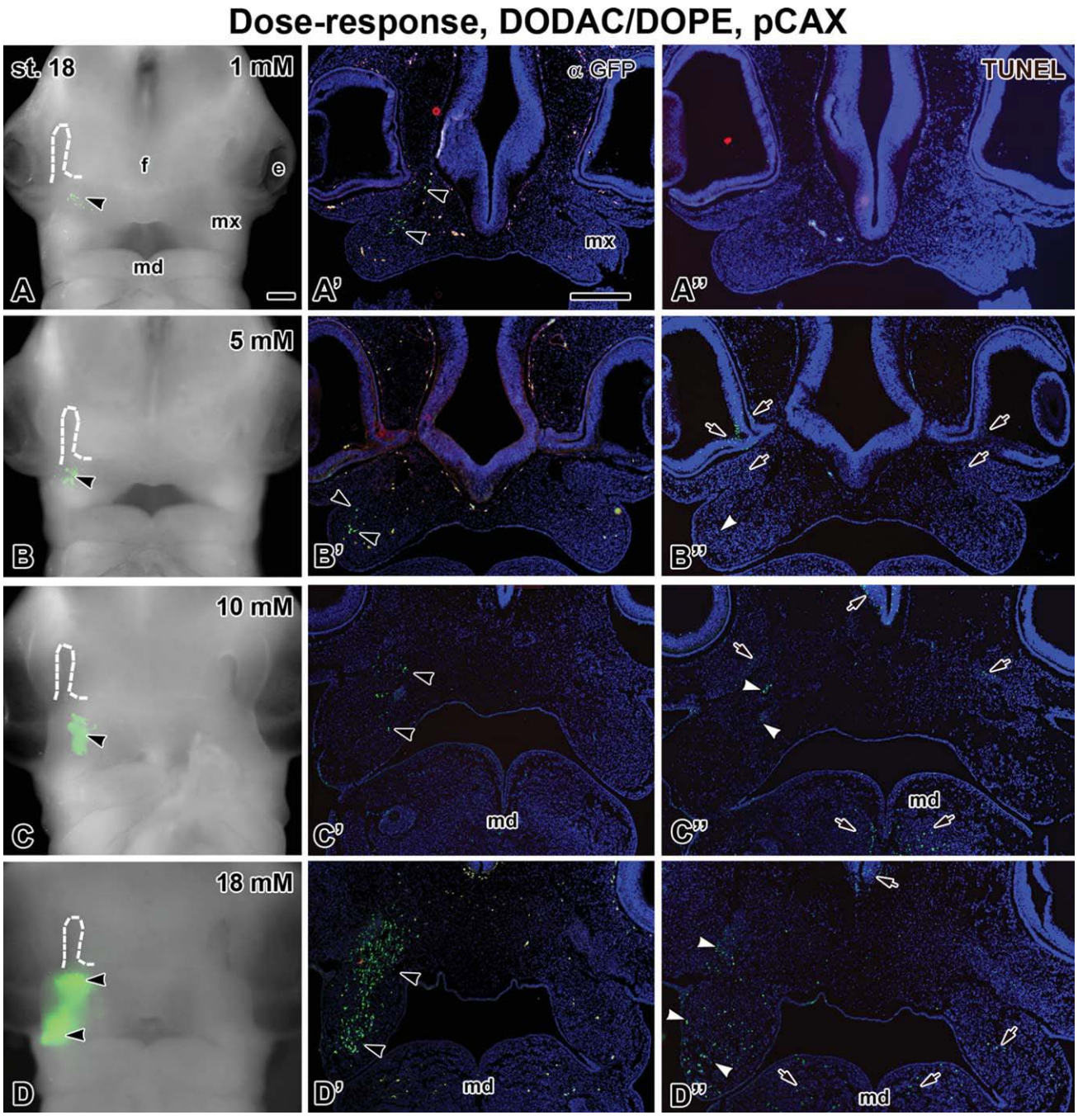

Fig. 1. Maxillary prominence transfection with DODAC/DOPE. All chicken embryos were injected at stage 18 in the right maxillary prominence with varying concentrations of DODAC/DOPE (1–18 mM) complexed with pCAX-EGFP and fixed 16 hr after transfection at approximately stage 24. For wholemount embryos, separate photographs were taken in bright field and fluorescence illumination and combined in Adobe Photoshop. Immunofluorescence and TUNEL pictures were photographed with DAPI, FITC, and Rhodamine illumination and the channels were combined in Adobe Photoshop. In this way, yellow-red autofluorescence from blood cells can be easily distinguished from green, FITC signal. **A–D**: Increased levels of fluorescence with increasing lipid molarity are seen in the right maxillary prominence (black arrowheads, nasal slit outlined with a dashed line). The strongest signal is seen with 18 mM DODAC/DOPE (D). **A′–D′**: Anti-GFP antibody has detected transfected cells in the mesenchyme (black arrowheads) on frontal sections through the face. **A″–D″**: TUNEL staining on near-adjacent sections shows normal apoptosis (arrows with tails) occurring in cells inferior to the eye (B″, C″, D″), the optic stalks (B″), ventral floorplate of the telencephalon (C″), and midline of the mandibular prominence (C″, D″). The sections from the 1-mM specimen had almost no detectable cells (A″). Increased numbers of apoptotic cells in 18-mM specimen are concentrated under the lateral ectoderm of the maxillary prominence (white arrowheads, D″). The GFP and TUNEL domains partially overlap (D′, D″) but there are fewer TUNEL-positive cells than GFP-positive cells. e, eye; f, frontonasal mass; md, mandibular prominence; mx, maxillary prominence. Scale bar = 250 μm.

All cationic lipds are similar, they contain a positive headgroup and a hydrophobic tail (Hafez and Cullis, 2001; Hafez et al., 2001). DOTMA was the first cationic lipid ever reported (Felgner et al., 1987). DOTAP is another commercially available cationic lipid so it was easy to obtain this lipid for our study. DODAC was

developed by our group (Hafez et al., 2000) and had been shown to be a good transfection reagent in vitro (Mok and Cullis, 1997; Hope et al., 1998) Each of these cationic lipids was combined with the most commonly used helper lipid, DOPE (Hafez and Cullis, 2001; Hafez et al., 2001). Furthermore, DOPE has been used by others for in vivo transfections (Bollérot et al., 2006; Decastro et al., 2006) and it is a component of the original Lipofectamine™ from Invitrogen (Carlsbad, CA).

The initial tests were carried out in stage-18 chicken embryos and transfections were directed to the face (Fig. 1) or limb bud (Fig. 2) mesenchyme. We chose the chicken as opposed to mouse embryo for our tests because avian embryos are accessible to microinjection at many stages and development can continue until hatching if desired. We selected the post-migratory, neural crest–derived facial mesenchyme as this region is particularly hard to transfect in vivo with electroporation. We also tested the reagents in limb mesenchyme in order to compare our results to those obtained with electroporation.

Of the three, DODAC/DOPE (1:1) gave the most promising initial results so we continued to develop this formulation. We tested a range of concentrations of lipid from 1–25 mM to determine the optimal working concentration. A molarity of 1 mM resulted in minimal or no transfection in a majority of embryos (Table 2, Figs. 1A,A′, 2A,A′). At the other end of the spectrum, the 25-mM DODAC/DOPE was incompatible with gene delivery. The solution was too viscous to inject and caused DNA to precipitate (Table 2, data not shown).

The intermediate concentrations all resulted in embryos with some transfection (Fig. 1B–D′; Table 2) and sections stained with anti-GFP antibody confirm that transfected cells were present in the mesenchyme (Figs. 1A′–D′, 2A′–D′). The 18-mM concentration gave the most reproducible transfection of mesenchyme (Figs. 1D′, 2D′). Virtually all of the embryos had strong, externally visible GFP expression within 16 hr as compared to variable results with the other concentrations (Table 2). The commercial lipid, Lipofectamine LTX™ (Invitrogen) gave variable results with only a small number of cells transfected in a majority of embryos (72%, Table 2, see Supp. Fig. S1A–D, which is available online).

We next investigated whether the lipoplex induced apoptosis. In the maxillary prominence of the face, there is little endogenous apoptosis and there was no increase following transfection with 1–10 mM DODAC/DOPE (Fig. 1A″–C″). There is an increase in TUNEL-positive cells in the 18-mM transfection experiments particularly under the ectoderm in the lateral maxillary prominence (Fig. 1D″); however, this level of apoptosis does not affect the size of the prominence (Fig. 1D, also see Figs. 4A, C, E, 5A–C).

In the limb bud, similar to the face, there was increasing effectiveness of transfection with increasing molarity of the lipid (Fig. 2A–D′). The level of apoptosis was very low (Fig. 2A″–C″) with the exception of the 18-mM lipoplex in which there are large numbers of positive cells in many regions of the limb bud (Fig. 2D″). However, at this stage (stage 24) there are normally apoptotic cells in the centre of the limb bud where cartilage will condense, in the apical ectodermal ridge, and in the anterior and posterior necrotic zones (Fig. 2E,G,I,K; Sanz-Ezquerro and Tickle, 2000; Bastida et al., 2009). To separate out the effects of delivering high concentrations of plasmid from those of the liposomes themselves, we carried out additional controls injecting lipids only (Fig. 2F,H,J,L). The liposomal reagent delivered on its own without plasmid did not alter the normal patterns of apoptosis in concentrations of 1–10 mM. With 18 mM DODAC/DOPE, there were ectopic TUNEL-positive cells under the ectoderm (Fig. 2L) similar to limbs transfected with plasmid. Although the liposome itself leads to a small increase in apoptosis, this does not affect overall limb development (Fig. 2A–D).

In comparison to other liposomal reagents published for chicken embryo work (Dickinson et al., 2002; Bollérot et al., 2006; Decastro et al., 2006), DODAC/DOPE (18 mM) appears to result in a qualitatively higher level of transfection, especially in the normally difficult to transfect mesenchyme. The lipoplexes also appear to give equivalent levels of limb mesenchyme transfection compared to electroporation of stage-15 lateral plate mesoderm (Sato et al., 2007; Boehm et al., 2010; Gros et al., 2010; Casanova et al., 2011). There are no direct comparisons where facial mesenchyme has been transfected using electroporation. We have shown that it is possible to direct plasmid to one region of the face in a reproducible manner but other areas can be similarly targeted.

Our formulation is also effective in delivering nucleic acid(s) to the neural tube, neural crest cells, somites, and endothelial cells (Supp. Fig. S2A–C, data not shown). In the case of the neural crest (Betancur et al., 2010; Rinon et al., 2011) and dermomyotome (Scaal et al., 2004; Gros et al., 2009; Wang et al., 2011), electroporation appears to result in a greater number of cells being transfected compared to our liposomes (Supp. Fig. S2). Our system is perhaps bettered suited for studies on individual cell behaviour.

Further experiments were required to quantify the number of transfected cells and the effect on cell survival. However, since this is difficult to do in vivo (due to variability in the volumes injected), a more standardized approach was needed. We, therefore, used cell cultures in which transfections can be standardized. To maintain context, we used micromass culture in which mesenchymal cells are isolated from the embryo and grown at high density (Richman and Crosby, 1990; Weston et al., 2000). These cultures recapitulate the early stages of chondrogenesis and are often used to study cell differentiation in vitro. Uptake of plasmids in primary cell culture is poor using commercial reagents such as Lipofectamine 2000. However, transfection with DODAC/DOPE leads to a 2.7-fold greater numbers of positive cells per field ($n = 4$, 455 ± 54 cells for DODAC/DOPE versus 172 ± 15; Supp. Fig. S3A, B). In addition, transfection with DODAC/DOPE was twice as efficient as Lipofectamine 2000 in fibroblastic cell lines (chicken DF1 fibroblasts, Supp. Fig. S3C,D; $n = 4$, $29 \pm 9\%$ transfected with DODAC/DOPE versus $14 \pm 1\%$ with Lipofectamine 2000).

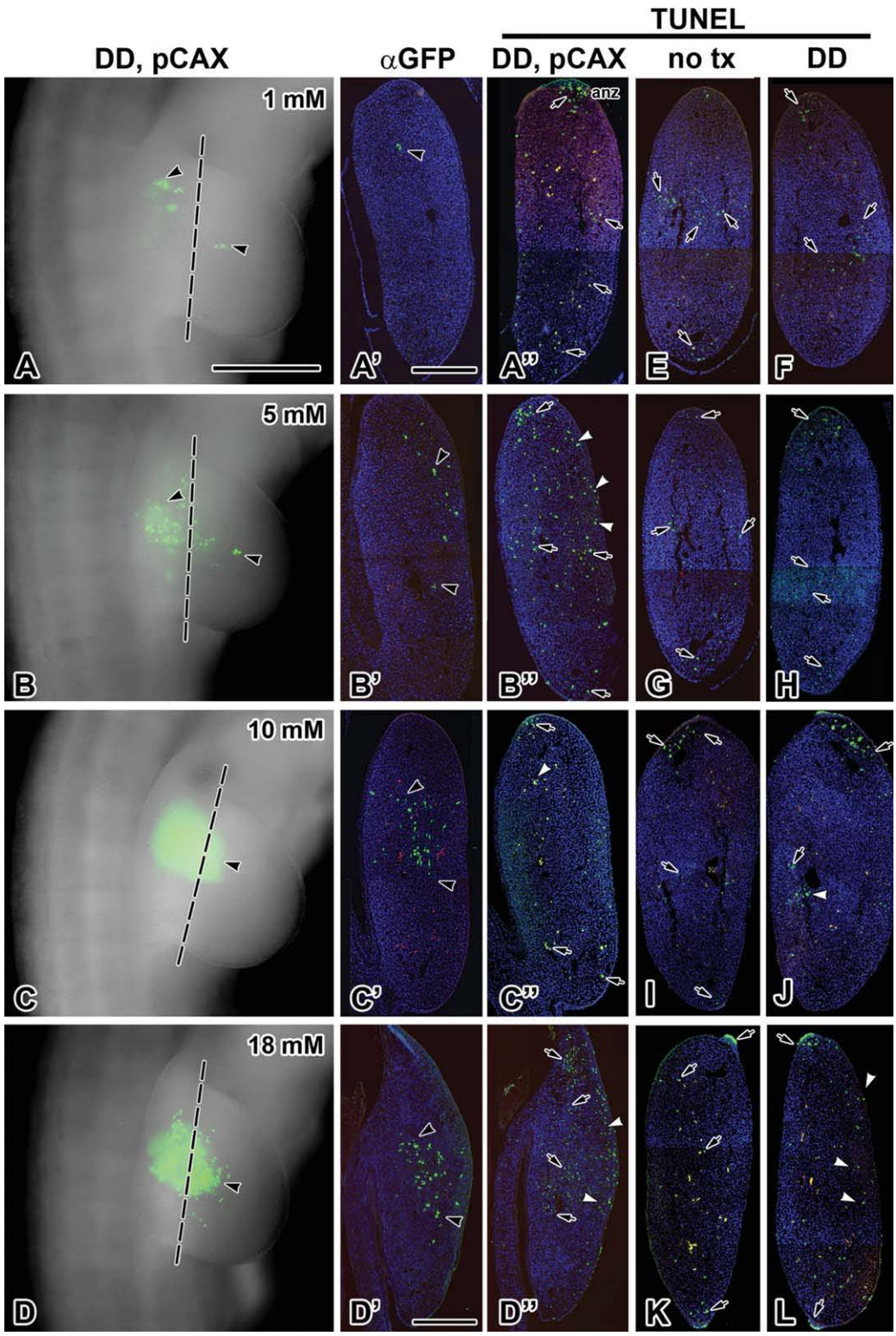

Fig. 2. Transfection of pCAX-EGFP plasmid in to the limb mesenchyme using various concentrations of DODAC/DOPE. Injections were carried out on stage-18 chicken embryo forelimb buds. Embryos were photographed 16 hr after transfection at stage 24. Immunofluorescence and TUNEL were performed on near-adjacent sections with the dorsal surface facing the right side of the figure (plane of section indicated with dashed line). **A,A′**: Minimal transfection is observed in limb mesenchyme with 1 mM DODAC/DOPE (black arrowheads) and this is increased with higher molarities of lipid (**B, B′, C, C′, D, D′**). Normal regions of apoptosis are seen in the anterior necrotic zone, apical ectodermal ridge, and central limb mesenchyme (**A″–D″**, arrows with tails). Ectopic TUNEL-positive cells are mainly localized close to the dorsal ectoderm (B″–D″, white arrowheads). DODAC/DOPE on its own does not induce apoptosis. To determine the effect of lipids, different concentrations of DODAC/DOPE liposomes without plasmid were injected into the limb bud mesenchyme. Embryos were fixed after 16 hr at stage 24, sectioned, and the TUNEL assay performed. **E,G,I,K**: The contralateral, uninjected limb buds show the normal extent of apoptosis for this stage of development including clusters of cells near the centre of the limb bud (future chondrogenic region), apical ectodermal ridge, and anterior and posterior necrotic zones (arrows with tails). **F,H,J,L**: The treated buds from the same embryos are in right-most column. DODAC/DOPE injections (**F, H, J, L**) lead to ectopic TUNEL-positive cells under the dorsal ectoderm (white arrowheads). Central apoptotic cells are considered part of the normal distribution (arrows with tails). Scale bar = 500 μm for A–D, 200 μm for limb bud sections except for D′, which is 250 μm and applies to D″.

TABLE 2. Lipid Combinations Tested for Transfection Ability in the Limb Buds of Chicken Embryos With the pCAX- EGFP Expression Plasmid[a]

Cationic lipid	Helper lipid	Charge ratio	Molarity of lipids (mM)	Number with good transfection efficiency (%)	Number with no or minimal transfection (%)
DODAC	**DOPE**	**Neutral**	1 (n = 11)	1 (10)	10
			5 (n = 13)	1	12
			10 (n = 18)	2	16
			18 extruded (n = 12)	11 (92)	1 (8)
			18 non-extruded (n = 15)	14 (93)	1 (7)
			25 (n = 7)	0	7 (100)
DODAC	**DOPE**	**Positive**	18 (n = 8)	0	8 (100)
DOTAP	**DOPE**	**Neutral**	18 (n = 13)	0	13 (100)
DOTMA	**DOPE**	**Neutral**	18 (n = 9)	0	9 (100)
Lipofectamine			Unknown (n = 25)	7 (28)	18 (72)

[a]For brevity, only limb injections are listed here. Face injection data were similar.

In quantitative assays, our formulation showed significantly higher luciferase when compared to other commercial reagents in both chicken face and mouse limb bud micromass cultures ($P < 0.05$, Supp. Fig. S3E). The excellent correlation between in vitro and in vivo efficacy of DODAC/DOPE contrasts with previous studies. Most of the reagents that work well in vitro usually produce less than optimal results when tested in vivo in chicken embryos (Muramatsu et al., 1997; Toy et al., 2000; Bollérot et al., 2006; Decastro et al., 2006). This suggests that micromass cultures are a good way to screen rapidly for reagents that may be useful for in vivo applications.

Since numbers of TUNEL-positive cells vary in tissue sections, we used the MTT cell proliferation assay (Invitrogen) on micromass cultures where the absorbance at a particular wavelength is proportionate to the number of viable cells (Mosmann, 1983). An increase in concentration of transfection mix above what is appropriate resulted in reduced survival rate as expected (Supp. Fig. S3F). However, at the optimal concentration of 0.5 μg plasmid (0.2 μl of lipid), our formulation had significantly lower toxicity compared to Lipofectamine 2000 ($P < 0.05$). Therefore, on highly sensitive primary mesenchyme cells, the survival rate for 18 mM DODAC/DOPE is superior to some commercial reagents. The MTT results are generally consistent with the TUNEL data, although the TUNEL assay was able to better localize the ectopic apoptotic cells. We did not compare Lipofectamine 2000 to our reagent in TUNEL assays but the MTT data suggest there would be more apoptosis than with 18 mM DODAC/DOPE.

We next investigated in more detail the onset of gene expression to see if it was comparable to electroporation. GFP is detected as early as 6 hr (Fig. 3A) and the signal peaks between 12 and 24 hr after injection similar to electroporation (Fig. 3B,C; (Sato et al., 2007). Levels are maintained at 48 and 72 hr (Fig. 3D,E). Unexpectedly, it is occasionally possible to detect external expression for up to 6 days (144 hours, n=2/7, Fig. 3F). Two reasons for the long-lived expression are that some populations of cells have slower cell cycle times and that some of the differentiated cells may have become post-mitotic since incorporating the GFP. Indeed, we found upon sectioning such limbs there were weakly fluorescent cells in the cartilage (Fig. 3F′). These were likely chondrocytes that had differentiated a day or so after transfection and thus retained the GFP protein.

A third perhaps more important reason for the persistent GFP signal is due to the stability of the protein. In order to determine how long RNA is expressed, we probed embryos with an antisense digoxygenin-labeled probe to GFP. There is very close concordance between the localization of the RNA signal and GFP fluorescence up to 48 hr (Fig. 3G,G′). The drop off in expression is very steep, however, such that by 72 and 96 hr only a very few cells can be detected with the riboprobes (Fig. 3H–I′). In summary, DODAC/DOPE-mediated transient transfections work well for the first 2 days but other means are required to induce stable expression. The most promising means to integrate foreign DNA without using viruses is the use of transposable elements. Co-electroporation of a plasmid expressing the transposase enzyme with another plasmid expressing a gene of interest (flanked by appropriate recombination sites) results in long- term expression (Sato et al., 2007; Takahashi et al., 2008; Wang et al., 2011). The transposable element approach should also work with lipoplexes.

Aside from using a small expression plasmid expressing GFP, we also wanted to test whether DODAC/DOPE would work with other types of nucleic acid molecules. Indeed, size of the plasmid is not a limiting factor since large proviral DNA (13 kb) can enter cells just as efficiently as the small pCAX plasmid. RCAS::*EGPF* expression was visible 48 hr post-injection (Fig. 4A, B; n=7/9), which is a similar time-frame to expression from concentrated viral particles (Gordon et al., 2009). Thus, with our method, instead of creating stocks of high-titre virus, it is only necessary to inject the proviral DNA. This method is applicable to any retroviruses that do not need helper cell packaging.

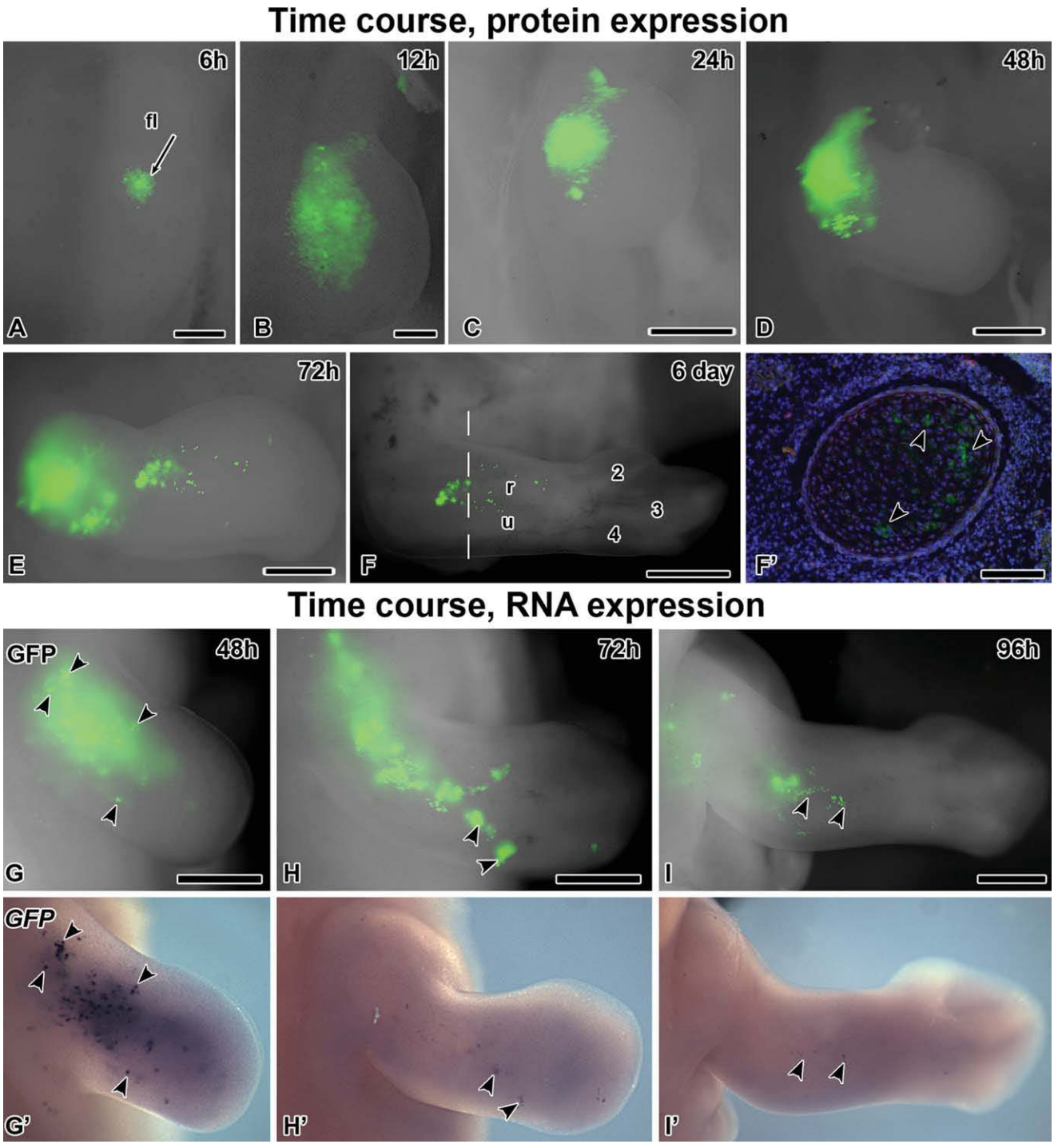

Fig. 3. Onset and stability of expression of a GFP expression plasmid delivered with DODAC/DOPE. GFP protein expression. Serial photographs of the same embryo taken at various time points following transfection using 18 mM DODAC/DOPE, pCAX-EGFP. Injections were directed into the forelimb at stage 17. Embryo movement during the photography has caused the blur in A–E. **A**: GFP localization is first detected as early as 6 hr and persists at 12 hr (**B**), 24 hr (**C**), 48 hr (**D**), 72 hr (**E**), and 6 days (144 hr) post-transfection (**F**). **F′**: Sections through the 6-day limb indicated with dashed lines in F limb stained with anti-GFP antibody shows weakly positive cells in the cartilage. RNA expression. G–I′: Fresh views showing native GFP fluorescence and photographs of the same embryos after in situ hybridization with digoxygenin-labelled probe to *GFP*. **G,G′**: At 48 hr, comparable localization of the RNA signal and GFP fluorescence (black arrowheads). In contrast at 72 (**H, H′**) and 96 hr (**I, I′**) only a few cells continue to express *GFP* RNA. 2,3,4 are the digit numbers; fl, fore limb bud; r, radius; u, ulna. Scale bar = 250 μm for A–F, 100 μm for F′, 500 μm for G–I′.

Since gene knockdown is necessary to understand function, we next tested the ability of DODAC/DOPE to deliver siRNA (short interfering RNA duplexes) and antisense morpholinos. A tagged siRNA duplex could be visualized within 2 hr and remained detectable in mesenchyme for 24 hr (Fig. 4C,D; face, n=15/16; limb, n=4/4). Morpholinos that are modified, single-stranded DNA oligonucleotides, were also

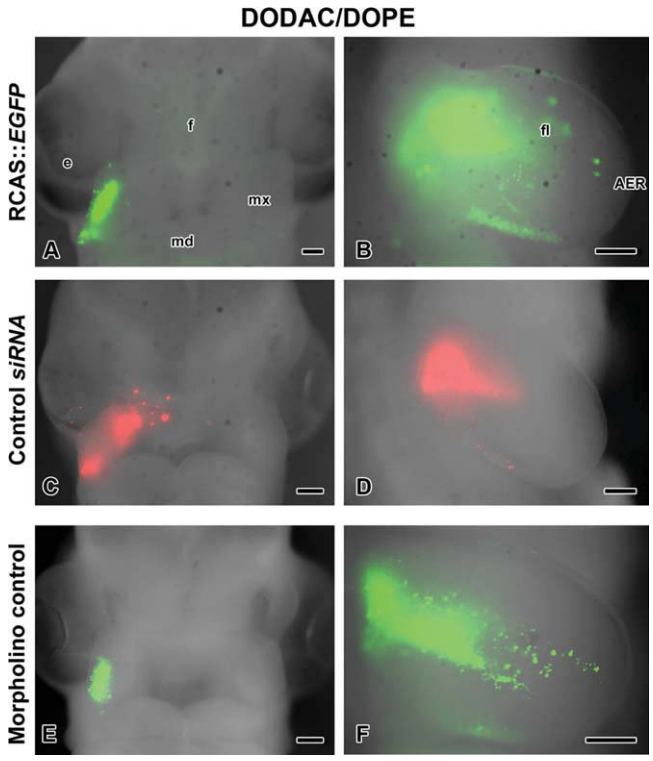

Fig. 4. DODAC/DOPE can deliver a variety of nucleic acid moieties. All nucleic acids were complexed with 18 mM DODAC/DOPE. Embryos in A, B were injected at stage 15 and fixed 48 hr later at stage 24. Embryos in C–E were injected at stage 18 and fixed 24 hr later at stage 24. **A, B**: Fluorescence indicates incorporation and expression of the avian retrovirus, RCASBP::*EGFP*. **C, D**: the TYE 563-tagged control siRNA from Integrated DNA Technologies (IDT) is localized in the maxillary prominence (C) and in forelimb (D). **E, F**: Uptake of the FITC-tagged, control Morpholino from Genetools. AER, apical ectodermal ridge; e, eye; f, frontonasal mass; fl, forelimb bud; md, mandibular prominence; mx, maxillary prominence. Scale bars = 250 μm.

Fig. 5. Reduction of exogenous and endogenous RNA expression using shRNA-targeting plasmids delivered with 18 mM DODAC/DOPE. All embryos were injected at stage 18 with lipoplexes made with 18 mM DODAC/DOPE and fixed 16 hr later at stage 24. **A, A′**: The same embryo was photographed with GFP and DiI filter sets showing the overlap of both pCAX plasmid and the empty reporter plasmid, pRFPRNAi-*RFP* expression. **B**: RFP localization seen shows that sufficient transfection has taken place in this embryo. There is a significant decrease in expression of pCAX-derived EGFP after pRFPRNAi-*shEGFP* injection (**B′**). **C**: A control embryo injected with pRFPRNAi-*shEGFP* and hybridized to the *WNT11* probe. No change in endogenous expression is seen in the area where the RFP reporter is expressed in frontal view (**C**, black arrowheads) or lateral view (**C″**). The non-injected side demonstrates the expression domains typical for this gene (C‴). **D**: An embryo injected with the sh*WNT11* targeting construct showing localization of the plasmid to the maxillary prominence. **D′**: Frontal view showing slightly reduced expression on the right side of the face (white arrowhead) in the area transfected with the plasmid. **D″**: The lateral view illustrates more clearly that the endogenous RNA in the maxillary prominence and inferior to the eye is reduced (white arrowheads) compared to the control side (**D‴**). Scale bar = 500 μm.

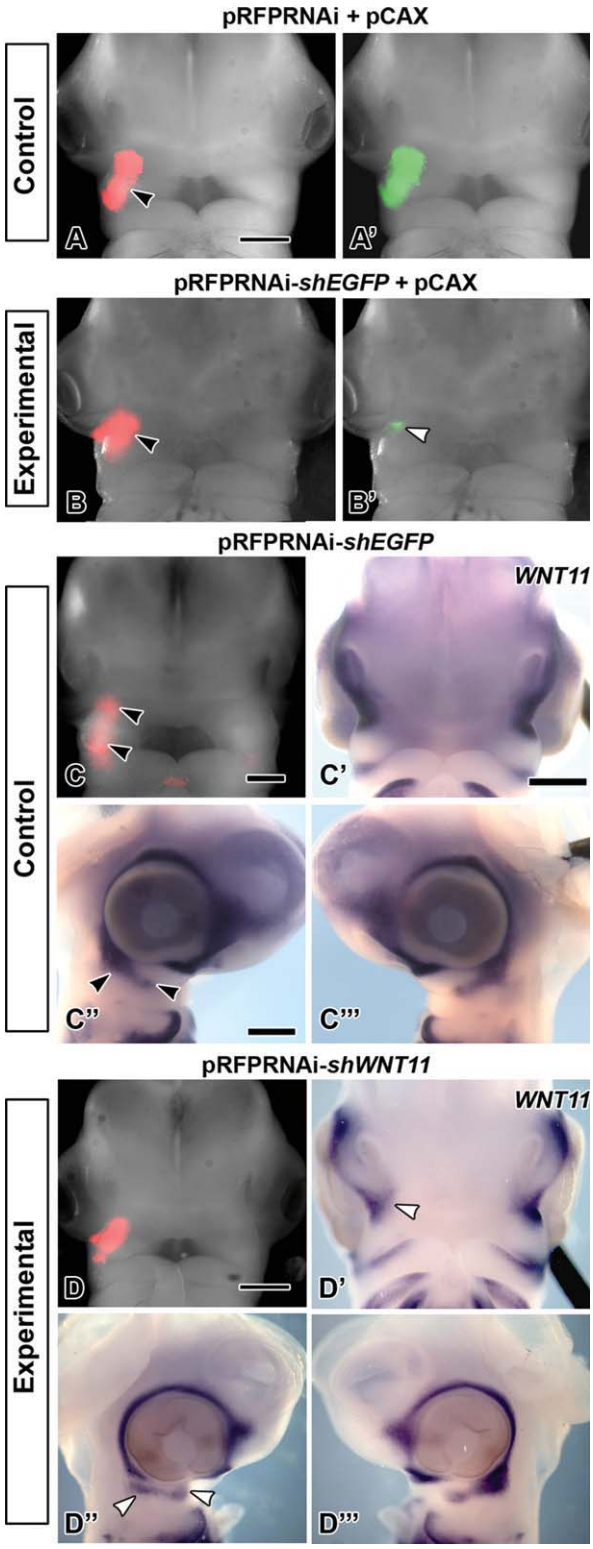

Fig. 5.

delivered efficiently, perhaps more so than the proprietary reagent sold by Genetools (Philomath, OR) (Toy et al., 2000); Fig. 4. E, F; face n=12/12; limb, n=10/12). Our delivery system opens up the possibility of studying endogenous gene function in a wider variety of stages and tissues than is currently possible with electroporation. Furthermore, if short-term live cell imaging experiments are carried out, the control morpholino is a good way to visualize cells almost immediately after transfection.

We next performed some functional tests to determine whether gene-specific knockdown could be achieved in vivo. We tested the plasmid-containing targeting sequences for GFP (Das et al., 2006), which also includes the red fluorescent protein (RFP) reporter in the same vector. In control experiments, co-transfection with pCAX and the empty targeting vector revealed very good overlap of the GFP and RFP signals (Fig. 5A,A′, n=12/12). In embryos co-transfected with the shGFP targeting construct and pCAX, the majority of exogenous GFP signal was greatly reduced (Fig. 5B,B′, n=9/9).

Since the ultimate goal of any transfection method is to be able to manipulate the levels of endogenous gene expression, we tested whether we could knockdown a gene that is highly expressed in the face, *WNT11*. Our previous study showed that *WNT11* is expressed in the dorso-cranial portion of the maxillary prominences as well as more proximally, under the eye (Geetha-Loganathan et al., 2009). Here we used a plasmid that others have shown to successfully target gallus *WNT11* in electroporation experiments (Gros et al., 2009). Control pRFPRNAi-*shEGFP* (Fig. 5C–C‴, n=5/5) or empty vector (data not shown, n=3/3) had no effect on *WNT11* expression. In contrast, decreased maxillary expression was detected in the majority of embryos injected with the targeting construct pRFPRNAi-*shWNT11* (Fig. 5D–D‴, n=10/13). Others had reported off-target effects of the pRFPRNAi plasmid delivered to stage-5–6 chicken embryos using electroporation (Mende et al., 2008). They observed an increase in apoptosis that was specific to this plasmid and not seen with electroporation of other nucleic acids. We have analyzed sections at several depths in the face to confirm these results (Supp. Fig. S4A–D″ and data not shown). The difference in results between our two studies is likely due in part to older embryos tolerating the plasmid better as well as the less invasive nature of microinjection. A second off-target effect reported by Mende et al. (2008) was a decrease in *PAX2* expression in embryos electroporated with a targeting construct against *NOTCH, EYA2*, and with the same *GFP* targeting sequence we used. We cannot rule out such off-target changes in gene expression in our study. Therefore, in future work it would be important to compare the target gene effects to those achieved with another, non-plasmid-based method such as Morpholino knockdown.

We have developed a novel cationic lipid formulation that results in sufficiently high levels of expression to be experimentally useful in a variety of ways. Processes such as AER regression or even anterior posterior patterning begin later in development and thus cannot be studied using electroporation. Our method is compatible with a wide range of embryo stages and so could be used to study other developmental processes. In addition, stable integration is possible using the transposase system, which should permit long-term fate-mapping studies (Sato et al., 2007) or cell behavior studies (Gros et al., 2010; Wang et al., 2011). The other clear application is that easy transfer of multiple types of nucleic acid (plasmids, siRNAs, Morpholinos) into cells is possible. This makes it feasible for networks of genes to be studied in vivo. Additionally since our method can deliver any plasmid, it is possible to report activity of signalling pathways by fusing response elements to destabilized versions of EGFP as has been reported recently for the canonical Wnt pathway (Rios et al., 2011).

While we focused on the avian embryo in the present study, we hope that these reagents or ones of a similar nature will be developed to target late-stage zebrafish or Xenopus embryos. Indeed, the mouse micromass culture results suggest that lipid-based transfection reagents could be an important adjunct to gene targeting studies in mouse. In conclusion, use of high-concentration liposomes will be a useful addition to current transgenesis techniques.

EXPERIMENTAL PROCEDURES

Plasmids and Oligonucleotides

pCAX-EGFP (5,500 bp provided by Mime Kobayashi, C.E. Krull) and RCASBPA containing GFP (13,000 bp, provided by S. Gaunt) were used. For the majority of experiments, we used the pCAX plasmid but other plasmids we have tested also work equally well (e.g., N1-EGFP, Clontech, Mountain View, CA; pRFPRNAi, Das et al., 2006).

The siRNA construct targeting gallus *WNT11* used in Gros et al.'s report (2009) was used in this study. The siRNA targeting construct directed against GFP was cloned in our lab into pRFPRNAi following the sequences and strategy described in Das et al. (2006). The Renilla luciferase reporter plasmid, pGL3, was obtained from Promega (Madison, WI). Cy3-labelled control siRNAs and carboxyfluorescein-tagged control Morpholino (MO) were obtained from Integrated DNA Technologies (IDT, Coralville, IA) and Gene Tools, LLC respectively.

Generating Liposomes

The combinations of lipids tested are listed in Table 2. The following cationic lipid was tested: *N*-*N*-dioleyl-*N,N*-dimethylammonium chloride (DODAC, synthesized as described in Hafez et al., 2000), 1,2-Dioleoyl-3-trimethylammonium-propane (DOTAP, Avanti Polar Lipids, Alabaster, AB), and *N*-(1-(2,3-dioleyloxy propyl)-*N,N,N*-trimethylammonium chloride (DOTMA, Avanti). The neutral helper lipid used was dioleyl phosphatidyl ethanolamine (DOPE, Avanti).

In order to prepare liposomes, cationic and helper lipid were first dissolved in chloroform and equimolar amounts of each lipid were added to the same 10-ml round-bottomed glass tube. Lipids were dried to a thin film using nitrogen gas and further dried by high vacuum for 2–3 hr. The lipid film was rehydrated with 1 ml of PBS (pH 7.0) or HEPES-saline (5 mM HEPES, 150 mM NaCl, pH 7.4) and briefly agitated using a vortex mixer. The solution was mixed until it became translucent and there was no solid lipid visible at the bottom of the tube. Buffering is important since the same lipid formulated in H_2O gave poor transfection.

In some cases cationic/helper lipid combinations were extruded through two polycarbonate filters with a 100-nm pore size. Others were used

without extrusion. In this case suspended vesicles can also be sonicated to produce small, variably sized, unilamellar vesicles. It is important to note that extrusion and sonication were not absolutely required for successful delivery of DNA in our study (Table 2), however vortexing was important. Extrusion may be important in other experimental systems where uniform particle size is required. All reconstituted liposomes were stored at 4°C.

Preparation of Lipoplexes

Neutral cationic lipoplexes were prepared by gentle mixing plasmid and lipid in a ratio of 2.74 μg DNA to 1.0 μl of the lipid mix (stock concentration of DNA should be a minimum of 1 μg/μl). The lipid stock consisted of different molarities of DODAC and DOPE mixture (Table 2). These DNA/Lipid mixing ratios yielded lipoplexes at a neutral (1:1) charge ratio, based on the number of lipid amines and nucleic acid phosphates (Felgner et al., 1987). For example, the final lipid concentration of 18 mM lipid mixed with 2.74 μg of DNA at a concentration of 1 μg/μl is 4.81 mM. To create negatively charged lipoplexes (0.5:1, positive to negative charge ratio), 5.50 μg of pCAX plasmid was mixed with 1 μl of lipid. Positively charged lipoplexes (1.5:1) were prepared by mixing 1.83 μg of DNA with 1 μl of lipid. Note that once lipoplexes are formed, vigorous vortexing must be avoided to prevent disruption of the complexes.

For preparing lipoplexes containing the tagged siRNA duplexes, 10 μl of siRNA (2 nM) was mixed with 1.15 μl of 18 mM DODAC/DOPE (1:1). For using Morpholinos, 1 μl of MO (500 μM) was mixed with 1.39 μl of 18 mM DODAC/DOPE. All lipoplexes were allowed to stand at room temperature for at least 15–30 min before injection. The lipoplexes were gently mixed by pipetting prior to loading them into the microinjection pipette.

Lipofectamine LTX with Plus™ Reagent (cat. Nos. 15338 and 11514, Invitrogen) was used as follows. For 2.5 μg DNA, 2.5 μl Plus reagent was added first and then incubated for 5–10 min. Following this, 6.25 μl of Lipofectamine LTX was added and then the mixture was incubated for 45 min.

Embryo Manipulations

Fertilized White leghorn eggs were obtained from the University of Alberta and incubated to the appropriate stage (Hamburger and Hamilton, 1951). Serological monitoring is regularly carried out on this flock and the birds do not harbour most of the common avian pathogens. Experiments were performed at different stages of the embryos depending on the region to be transfected and substance to be delivered. Electrolytically pulled needles were created from micropipettes and injection of liquids was controlled with a Picospritzer II (Parker Hannifin Corp.). On average 3-5 pulses of the Picospritzer were used per injection site. Microinjections into the following regions were carried out: (1) the post-optic mesenchyme at stage 15 to target the maxillary prominence or (2) the fore or hind limb fields at stage 17 to target the limb buds, (3) the dorsal neural folds of stage-10 embryos to target the cranial neural crest cells, (4) the dorsal somites of the trunk to target the dermomyotome. Embryos were incubated from 2 hr to 6 days, covering initiation to extinction of expression. Where embryo position permitted, serial photographs were taken in ovo using a stereofluorescence microscope (MZFLIII, Leica). Other embryos were removed from the egg prior to fluorescence photography.

In Situ Hybridization, GFP Immunofluorescence, and TUNEL Reaction

Embryos were removed from the egg, fixed overnight at 4°C in 4% buffered paraformaldehyde (PFA), and then processed into 100% methanol. Wholemount in situ hybridization with digoxygenin-labelled probes was carried out using an Intavis in situ hybridization robot. C. Marcelle provided the gallus *WNT11* cDNA for this study. The GFP probe was cloned by us into pGEM-T-easy.

To obtain tissue localization of the transfected cells, embryos were fixed, embedded in wax, and sectioned at a thickness of 7 μm. Sections were steamed with 10 mM sodium citrate (pH 6.0) for 10 min. Primary antibody for anti-rabbit GFP (Synaptic Systems, 1:2,000) was applied overnight at 4°C, rinsed in PBS, and then incubated in FITC (Jackson) secondary donkey anti-rabbit antibody (1:500) at room temperature for 30 min.. Slides were cover slipped using Prolong Gold (Invitrogen), which contains DAPI to counterstain the nuclei. Sections were photographed using a Zeiss wide-field fluorescence microscope using DAPI, FITC, and Rhodamine filter sets.

For the TUNEL assay, paraffin sections were dewaxed, treated with proteinase K (20 μg/ml) for 15 min at room temperature, post-fixed in 4% PFA for 20 min, rinsed in PBS, and then incubated with terminal deoxynucleotide transferase (TdT buffer) for 5 min. Labelling was carried out under coverslips with 0.3 U/μl TdT and 10 μM dig-dCTP (Chemicon, Temecula, CA, Apoptag kit) at 37°C for 1.5 hr. Signal was amplified using the FITC-conjugated anti-dig antibody contained in the kit. Slides were post-fixed in 4% PFA, rinsed, and cover slipped with Prolong Gold (Invitrogen).

Fibroblast Cell Culture, Primary Mesenchymal Cultures, Luciferase, and MTT Assays

DF1 fibroblasts were obtained from ATCC. Cells were grown to 70% confluency and transfected in the dish by removing the serum containing medium adding 100 μl of Optimem mixed with the lipoplex mixture (0.5–2.5 μg of plasmid complexed with 0.182 μl–0.91 μl of lipid). High-density micromass cultures were established from chicken maxillary prominences of stage-24 embryos and E11.5 mouse limb buds as described previously (Richman and Crosby, 1990; Weston et al., 2000). A cell suspension with a density of 2×10^7 cells/ml was used and cultures were plated as 10 μl spots each containing 2×10^5 cells. Lipoplex containing DODAC/DOPE or Lipofectamine LTX with Plus™ Reagent (Invitrogen) or Effectine™ (Cat no. 301427, Qiagen, Chatsworth, CA) was mixed with 0.5 μg pGL3-Renilla luciferase (Promega, Madison, WI) per each 10 μl spot. Serum-free media was used for Lipofectamine LTX and Effectine (Optimem,

Invitrogen). Complete media containing 10% serum was used for 18 mM DODAC/DOPE. Cells were gently mixed with the lipoplex mixture and then 10 μl of the cell suspension was dropped onto each well of a 24-well tissue culture plate (Nunc). The micromass spots containing the transfection mixture were allowed to adhere for 1 hr at 37°C in a CO_2 incubator. After this period, 0.5 ml of complete culture medium consisting of 60% F12/40% DMEM and supplemented with 10% FBS (Qualified, Invitrogen) was added to each well. Cultures were grown for 48 hr, following which extracts for luciferase analysis were collected. All luciferase assays were performed in triplicates and repeated using three distinct preparations of primary cells. Analysis of reporter gene activity was carried out using the Luciferase Assay System according to the manufacturer's instructions (Promega). Renilla luciferase activity was measured in a Molecular Devices (Sunnyvale, CA) luminometer.

MTT Proliferation Assay

Cell viability in micromass cultures was determined by using a Vybrant MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-3 diphenyl tetrazolium bromide) Cell Proliferation Assay Kit (V-13154, Invitrogen). Relative viability was measured at increasing amounts of 18 mM DODAC/DOPE while maintaining a constant DNA/lipid ratio of 2.74 μg:1 μl of lipid. Thus for 2.5 μg of DNA, 0.9 μl of DODAC/DOPE was added to the well, which contained 100 μl of assay buffer. Our formulation was compared with Lipofectamine LTX with Plus reagent (Invitrogen) using high-density primary culture from the chick face. The MTT assay was carried as per the manufacture's instructions (Invitrogen) on 48-hr, maxillary mesenchyme micromass cultures. To increase accessibility to the reagent, the cultures were trypsinized, dissociated, and resuspended in fresh media before adding MTT stock solution.

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REFERENCES

Albazerchi A, Cinquin O, Stern CD. 2007. A new method to transfect the hypoblast of the chick embryo reveals conservation of the regulation of an Otx2 enhancer between mouse and chick extraembryonic endoderm. BMC Dev Biol 7:25.

Baeriswyl T, Mauti O, Stoeckli ET. 2008. Temporal control of gene silencing by in ovo electroporation. Methods Mol Biol 442:231–244.

Bastida MF, Sheth R, Ros MA. 2009. A BMP-Shh negative-feedback loop restricts Shh expression during limb development. Development 136:3779–3789.

Betancur P, Bronner-Fraser M, Sauka-Spengler T. 2010. Genomic code for Sox10 activation reveals a key regulatory enhancer for cranial neural crest. Proc Natl Acad Sci USA 107: 3570–3575.

Boehm B, Westerberg H, Lesnicar-Pucko G, Raja S, Rautschka M, Cotterell J, Swoger J, Sharpe J. 2010. The role of spatially controlled cell proliferation in limb bud morphogenesis. PLoS Biol 8: e1000420.

Bollérot K, Sugiyama D, Escriou V, Gautier R, Tozer S, Scherman D, Jaffredo T. 2006. Widespread lipoplex-mediated gene transfer to vascular endothelial cells and hemangioblasts in the vertebrate embryo. Dev Dyn 235:105–114.

Brugmann SA, Goodnough LH, Gregorieff A, Leucht P, ten Berge D, Fuerer C, Clevers H, Nusse R, Helms JA. 2007. Wnt signaling mediates regional specification in the vertebrate face. Development 134:3283–3295.

Casanova JC, Uribe V, Badia-Careaga C, Giovinazzo G, Torres M, Sanz-Ezquerro JJ. 2011. Apical ectodermal ridge morphogenesis in limb development is controlled by Arid3b-mediated regulation of cell movements. Development 138: 1195–1205.

Creuzet SE. 2009. Regulation of pre-otic brain development by the cephalic neural crest. Proc Natl Acad Sci USA 106: 15774–15779.

Das RM, Van Hateren NJ, Howell GR, Farrell ER, Bangs FK, Porteous VC, Manning EM, McGrew MJ, Ohyama K, Sacco MA, Halley PA, Sang HM, Storey KG, Placzek M, Tickle C, Nair VK, Wilson SA. 2006. A robust system for RNA interference in the chicken using a modified microRNA operon. Dev Biol 294:554–563.

Decastro M, Saijoh Y, Schoenwolf GC. 2006. Optimized cationic lipid-based gene delivery reagents for use in developing vertebrate embryos. Dev Dyn 235:2210–2219.

Dickinson ME, Murray BA, Haynes SM, Waters CW, Longmuir KJ. 2002. Using electroporation and lipid-mediated transfection of GFP-expressing plasmids to label embryonic avian cells for vital confocal and two-photon microscopy. Differentiation 70:172–180.

Felgner PL, Gadek TR, Holm M, Roman R, Chan HW, Wenz M, Northrop JP, Ringold GM, Danielsen M. 1987. Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. Proc Natl Acad Sci USA 84:7413–7417.

Geetha-Loganathan P, Nimmagadda S, Antoni L, Fu K, Whiting CJ, Francis-West P, Richman JM. 2009. Expression of WNT signalling pathway genes during chicken craniofacial development. Dev Dyn 238:1150–1165.

Gordon CT, Rodda FA, Farlie PG. 2009. The RCAS retroviral expression system in the study of skeletal development. Dev Dyn 238:797–811.

Gros J, Hu JK, Vinegoni C, Feruglio PF, Weissleder R, Tabin CJ. 2010. WNT5A/JNK and FGF/MAPK pathways regulate the cellular events shaping the vertebrate limb bud. Curr Biol 20:1993–2002.

Gros J, Serralbo O, Marcelle C. 2009. WNT11 acts as a directional cue to organize the elongation of early muscle fibres. Nature 457:589–593.

Hafez IM, Cullis PR. 2001. Roles of lipid polymorphism in intracellular delivery. Adv Drug Deliv Rev 47:139–148.

Hafez IM, Ansell S, Cullis PR. 2000. Tunable pH-sensitive liposomes composed of mixtures of cationic and anionic lipids. Biophys J 79:1438–1446.

Hafez IM, Maurer N, Cullis PR. 2001. On the mechanism whereby cationic lipids promote intracellular delivery of polynucleic acids. Gene Ther 8:1188–1196.

Hamburger V, Hamilton H. 1951. A series of normal stages in the development of the chick embryo. J Morphol 88:49–92.

Hope MJ, Mui B, Ansell S, Ahkong QF. 1998. Cationic lipids, phosphatidylethanolamine and the intracellular delivery of polymeric, nucleic acid-based drugs (review). Mol Membr Biol 15:1–14.

Howarth JL, Lee YB, Uney JB. 2010. Using viral vectors as gene transfer tools (Cell Biology and Toxicology Special Issue: ETCS-UK 1 day meeting on genetic manipulation of cells). Cell Biol Toxicol 26:1–20.

Inoue T, Krumlauf R. 2001. An impulse to the brain–using in vivo electroporation. Nat Neurosci 4 Suppl:1156–1158.

Liu D, Ren T, Gao X. 2003. Cationic transfection lipids. Curr Med Chem 10: 1307–1315.

Luo J, Redies C. 2005. Ex ovo electroporation for gene transfer into older chicken embryos. Dev Dyn 233:1470–1477.

Mende M, Christophorou NA, Streit A. 2008. Specific and effective gene knockdown in early chick embryos using morpholinos but not pRFPRNAi vectors. Mech Dev 125:947–962.

Mok KW, Cullis PR. 1997. Structural and fusogenic properties of cationic liposomes in the presence of plasmid DNA. Biophys J 73:2534–2545.

Mosmann T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65: 55–63.

Muramatsu T, Mizutani Y, Ohmori Y, Okumura J. 1997. Comparison of three nonviral transfection methods for foreign gene expression in early chicken embryos in ovo. Biochem Biophys Res Commun 230:376–380.

Richman JM, Crosby Z. 1990. Differential growth of facial primordia in chick embryos: responses of facial mesenchyme to basic fibroblast growth factor (bFGF) and serum in micromass culture. Development 109:341–348.

Rinon A, Molchadsky A, Nathan E, Yovel G, Rotter V, Sarig R, Tzahor E. 2011. p53 coordinates cranial neural crest cell growth and epithelial-mesenchymal transition/delamination processes. Development 138:1827–1838.

Rios AC, Denans N, Marcelle C. 2011. Real-time observation of Wnt beta-catenin signaling in the chick embryo. Dev Dyn 239:346–353.

Sanz-Ezquerro JJ, Tickle C. 2000. Autoregulation of Shh expression and Shh induction of cell death suggest a mechanism for modulating polarising activity during chick limb development. Development 127:4811–4823.

Sato Y, Kasai T, Nakagawa S, Tanabe K, Watanabe T, Kawakami K, Takahashi Y. 2007. Stable integration and conditional expression of electroporated transgenes in chicken embryos. Dev Biol 305:616–624.

Sauka-Spengler T, Barembaum M. 2008. Gain- and loss-of-function approaches in the chick embryo. Methods Cell Biol 87:237–256.

Scaal M, Gros J, Lesbros C, Marcelle C. 2004. In ovo electroporation of avian somites. Dev Dyn 229:643–650.

Takahashi Y, Watanabe T, Nakagawa S, Kawakami K, Sato Y. 2008. Transposon-mediated stable integration and tetracycline-inducible expression of electroporated transgenes in chicken embryos. Methods Cell Biol 87: 271–280.

Toy J, Bradford RL, Adler R. 2000. Lipid-mediated gene transfection into chick embryo retinal cells in ovo and in vitro. J Neurosci Methods 104:1–8.

Wang H, Bonnet A, Delfini MC, Kawakami K, Takahashi Y, Duprez D. 2011. Stable, conditional, and muscle-fiber-specific expression of electroporated transgenes in chick limb muscle cells. Dev Dyn 240: 1223–1232.

Weston AD, Rosen V, Chandraratna RA, Underhill TM. 2000. Regulation of skeletal progenitor differentiation by the BMP and retinoid signaling pathways. J Cell Biol 148:679–690.

Developmental Dynamics