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# Efficient, Low-Cost Nucleofection of Passaged Chondrocytes

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**Abstract**

Nucleofection of chondrocytes has been shown to be an adequate method of transfection. Using Amaxa's nucleofection system, transfection efficiencies up to 89% were achievable for vector (pmaxGFP) and 98% for siRNA (siGLO) into passaged chondrocytes. However, such methods rely on costly commercial kits with proprietary reagents limiting its use in basic science labs and in clinical translation. Bovine-passaged chondrocytes were plated in serum reduced media conditionsand then nucleofected using various in laboratory-produced buffers. Cell attachment, confluency, viability, and transfection efficiency was assessed following nucleofection. For each parameter the buffers were scored and a final rank for each buffer was determined. Buffer denoted as 1M resulted in no significant difference for cell attachment, confluency, and viability as compared to non-nucleofected controls. Nucleofection in 1M buffer, in the absence of DNA vectors, resulted in increased col2, ki67, ccnd1 mRNA levels, and decreased col1 mRNA levels at 4 days of culture. Flow cytometry revealed that the transfection efficiency of 1M buffer was comparable to that obtained using the Amaxa commercial kit. siRNA designed against lamin A/C resulted in an average reduction of lamin A and C proteins to 19% and 8% of control levels, respectively. This study identifies a cost-effective, efficient method of nonviral nucleofection of bovine-passaged chondrocytes using known buffer formulations. Human-passaged chondrocytes could also be successfully nucleofected in 1M buffer. Thus this method should facilitate cost-efficient gene targeting of cells used for articular cartilage repair in a research setting.

**Keywords**

transfection, nucleofection, chondrocytes, cartilage, gene therapy

## Introduction

Monolayer expanded chondrocytes have been approved for clinical use by the US Food and Drug Administration for autologous cell-based repair of damaged articular cartilage. However, a major drawback in using passaged chondrocytes is the unstable phenotype leading to dedifferentiation and production of fibroblast matrix, rich in type I collagen (col1), rather than articular cartilage matrix, rich in type II collagen (col2) and aggrecan (acan). Studies have determined that gene targeting either through knockdown of specific molecules (such as fibroblastic matrix molecule col1,[1] or matrix degrading enzymes cathepsin k[2]) or the overexpression of genes such as master transcriptional regulator, sox9,[3] aid in redifferentiation of passaged cells.

In order to translate such findings into practical utility, the introduction of DNA into cells to either knockdown or overexpress specific genes is important for gene therapy. Although viral methods have been shown to effectively introduce DNA into chondrocytes,[4,5] these rely on viral particles that have potential complications such as immunogenicity. Thus nonviral methods are not only safer for such applications but also rely on simpler methods of vector synthesis and modification. Chondrocytes are notoriously difficult to transfect through chemically based methods[6]; however, successful nonviral physical transfection of chondrocytes through electroporation has shown to be promising.[7] Utilization of Amaxa nucleofector technology has been utilized successfully for difficult to transfect cells[8] and nucleofection of chondrocytes results in

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transfection efficiencies of up to 70%.[9] Unfortunately, these methods are costly and rely on proprietary commercial buffers limiting its therapeutic utility and use in research labs. To circumvent these issues, various characterized buffer formulations have emerged for use in other cell types.[10,11] In endothelial cells, nucleofection in phosphate-buffered saline resulted in transfection efficiencies of 61%[11] and nucleofection of T-lymphocytes in formulated, laboratory generated buffers have revealed efficiencies approaching 45%.[12] The objective of this study was to identify a characterized buffer for successful nucleofection of passaged chondrocytes. This was defined as no change to cell attachment, spreading or cell viability, and high transfection efficiency with the ability to change expression of the gene of interest. For cell-based repair therapies, passaged chondrocytes nucleofection should not negatively affect phenotype and the ability to deposit articular cartilage matrix under redifferentiating conditions.

**Table 1.** Composition of In-Laboratory-Generated Nucleofection Buffers Used in Study.

| Buffer | Composition |
|---|---|
| PBS | 2.7 mM KCl; 137 mM NaCl; 10 mM $Na_2HPO_4$; 1.8 mM $KH_2PO_4$ |
| PBSM | 2.7 mM KCl; 137 mM NaCl; 10 mM $Na_2HPO_4$; 1.8 mM $KH_2PO4$; 50 mM mannitol |
| 1M | 5 mM KCl; 15 mM $MgCl_2$; 120 mM $Na_2HPO_4$/$NaH_2PO_4$; 50 mM mannitol |
| 2M | 5 mM KCl; 15 mM $MgCl_2$; 15 mM HEPES; 150 mM $Na_2HPO_4$/$NaH_2PO_4$; 50 mM mannitol |
| 3P | 5 mM KCl; 15 mM $MgCl_2$; 90 mM NaCl; 10 mM glucose; 0.4 mM $Ca(NO_3)_2$; 40 mM $Na_2HPO_4$/$NaH_2PO_4$ |
| 3H | 5 mM KCl; 15 mM $MgCl_2$; 90 mM NaCl; 10 mM glucose; 0.4 mM $Ca(NO_3)_2$; 20 mM HEPES; 75 mM Tris/HCl |

## Materials and Methods

### Cells and Cell Culture

Articular cartilage was harvested from bovine metacarpophalangeal stifle joints or adult human hip or knee as previously described.[13] Adult human articular cartilage was obtained from unaffected regions of osteoarthritic cartilage from joints resected from patients undergoing total joint replacements. These tissues were obtained with patient consent and the use of these tissues was approved by the Mount Sinai Research Ethics Board. Mouse ATDC5 cells were obtained from Sigma-Aldrich (Oakville, Ontario, Canada).

Both bovine and human cartilage was harvested under aseptic conditions using similar methodology. Chondrocytes were liberated from matrix using sequential digestion in 0.5% protease for 1 hour followed by 0.1% collagenase A (Roche, Canada) for approximately 18 hours at 37°C and 5% $CO_2$. The tissue digests were filtered through a 40μm filter to remove undigested pieces. Cell filtrates were washed twice in Ham's F12.

To expand cell numbers, chondrocytes were seeded in monolayer culture at a density of $1.5 \times 10^3$ cells per $cm^2$ in complete media as previously described.[14] For bovine as well as ATDC5 cells complete media was defined as Ham's F12 medium supplemented with 5% fetal bovine serum (FBS; Hyclone, Logan, CT). For human cells complete media was defined as DMEM medium supplemented with 20% FBS. Cells were cultured until approximately 80% to 90% confluency, at which point cells were harvested using 0.5% trypsin/ethylenediamine tetraacetic acid (EDTA). Cells were then washed in medium, pelleted at 600*g* for 6 minutes, and resuspended in respective complete media. At this point cells were designated as passage 1 (P1) chondrocytes. P1 chondrocytes were plated at the aforementioned density of $1.5 \times 10^3$ cells/$cm^2$. Cells harvested from this passage were designated as passage 2 (P2) chondrocytes. Either P1 or P2 chondrocytes were utilized for nucleofection experiments.

### Nucleofection Protocol

Laboratory-produced nucleofection buffers designated PBS, PBSM, 1M, 2M, 3P, and 3H were prepared as described.[11,12] The composition of the buffers are listed in **Table 1**. To obtain a pH of 7.2 to 7.4, either NaOH or HCl were added drop wise to buffers until pH was attained.

Passaged chondrocytes from culture were trypsinized and reseeded at an approximate density of $6 \times 10^5$ cells/$cm^2$ in serum reduced (0.5% FBS) media. Following 2 days of serum starvation, cells were harvested using trypsin, washed with complete media, pelleted, resuspended in complete media, and then counted. Approximately $2 \times 10^6$ cells, were repelleted, and resuspended in 0.1 mL of either commercial (human chondrocyte kit; Amaxa Biosystem, Cologne, Germany) or laboratory-produced nucleofection buffers. For GFP (green fluorescent protein) transfection, 5 μg of either pmaxGFP (Amaxa), CignalGFP (Qiagen, Mississauga, Ontario, Canada), or pMX-GFP (Cell Biolabs, San Diego, CA, USA) was utilized. For siRNA experiments, 100 pmol of siGLO red transfection indicator siRNA, siGENOME Lamin A/C siRNA, or siGENOME nontargetting siRNA (GE Healthcare, Ottawa, Ontario, Canada) was added to the nucleofection buffer-cell mixture. The resulting nucleofection buffer-cell-DNA mixture was then placed into cuvettes (2-mm gap width) and nucleofected in an Amaxa Nucleofector II using program U24 as per manufacturer's recommendations for chondrocytes. Cells were removed from cuvettes using a syringe with an 18g needle and resuspended in serum-rich media (Ham's F12 + 20%FBS for bovine; Dulbecco's

modified Eagle's medium (DMEM) + 20% FBS for human). Cells were pipetted up and down to ensure even cell suspension within the media and then aliquoted into flasks at a density of approximately $5.0 \times 10^4$ cells/cm$^2$. Cells were incubated at 37°C and 5% $CO_2$. After 4 hours, nonadherent cells were removed from culture by removal of media and remaining cells were transferred to complete medium.

### Flow Cytometry

Chondrocytes were harvested from culture vessels using trypsin. After 5 minutes, cells were recovered in PBS supplemented with 2%FBS. GFP or red fluorescent protein (RFP) expressed by cells was analyzed in chondrocytes using flow cytometry (EPICS XL FACS) and Kaluza analysis software (Beckman Coulter).

### DNA Assay

Four hours after nucleofection, cultures were rinsed in serum-free PBS to remove nonadherent cells. Adherent cells were harvested with trypsin and pelleted at 600*g* for 6 minutes. Cells were digested for 24 hours in 0.2 mL of papain digestion solution (consisting of 40 μg/mL papain in 20 mM ammonium actetate, 1 mM EDTA, and 2 mM dithiothreitol at pH 6.2) at 65°C. The samples were then diluted 1:5 in PBS. DNA Assay dye solution buffer (consisting of 0.1 μg/mL Hoechst 33258 in 1 mM Tris, 1 mM EDTA, and 0.1 mM NaCl at pH 7.4) was added to the samples. DNA was quantified using fluorometry (excitation and emission wavelengths 365 and 458 nm, respectively). The DNA standard curve was generated using calf thymus DNA (Sigma-Aldrich).

### Cell Confluency Measurement and Live/Dead Assay

One day after nucleofection, cells on culture vessels were stained with calcein AM and ethidium homodimer (Life Technologies, Burlington, Ontario, Canada) in PBS at 37°C for 20 minutes, rinsed with PBS and visualized using fluorescent microscopy. The proportion of cells stained for calcein and ethidium homodimer were manually counted. To measure cell confluency, cultures were photographed and images were transformed into 8-bit. At least 2 randomly chosen fields were analyzed from 3 different sets of cells. Percentage confluency was defined as the area of pixels occupied by cells nucleofected in experimental buffers over area of pixels occupied by control cells multiplied by a factor of 100.

### Three-Dimensional Redifferentiation

Following nucleofection, bovine-passaged chondrocytes were plated onto monolayer polystyrene for 2 days. Cells were then trypsinized, pelleted, counted and seeded within an agarose mold. Agarose molds were created by pipetting 3 mL of molten agarose in PBS in the well of a 12-well plate and left at room temperature. Following gelation, 4-mm diameter core of agarose was removed from the center of the well using a biopsy punch (Acuderm, Fort Lauderdale, FL, USA). Cores were removed using sterile forceps and the remaining agarose was rinsed with PBS ensuring that no residual agarose remained on the polystyrene at the bottom of the core as this prevents cell attachment. Passaged chondrocytes were seeded at a density of $1.6 \times 10^7$ cells/cm$^2$ within the defect in the molds in complete media. After 24 hours, complete media was replaced with redifferentiation media (DMEM, ITS+ (BD Bioscience, Bedford, MA), proline (40 mg/mL), pyruvate (110 mg/mL), dexamethasone (0.1 mM), and ascorbate-2-PO4 (50 mg/mL). Cells were fed every 2 days until harvested.

### RNA Extraction and Relative Real-Time RT-PCR

Total RNA was extracted using TRIzol (Life Technologies) following the manufacturer's protocol with slight modifications. Briefly, samples were harvested in TRIzol and homogenized. Chloroform was added to facilitate phase separation and samples centrifuged at 14,000 rpm for 15 minutes. Following centrifugation, the top aqueous phase containing the RNA was removed and the RNA precipitated using isopropyl alcohol for 15 minutes at room temperature. The samples were centrifuged at 14000 rpm for 15 minutes to form the RNA pellet, and washed with 75% ethyl alcohol. The wash was discarded, the pellet was dried, and then resuspended in 20 μL molecular-grade water. Total RNA was quantified on a Nanodrop 1000 and reverse transcribed to cDNA using SuperScript III (Life Technologies), as described by the manufacturer. For each reaction mixture, 20 ng of cDNA per polymerase chain reaction (PCR) reaction. Relative real-time PCR was performed using Express SYBR GreenER (Life Technologies) with the primer sequences listed in Supplementary Table S1 (available at http://car.sagepub.com/content/by/supplemental-data). Thermal cycling and fluorescent detection was performed using a LightCycler 96 Real-Time PCR System (Roche, Mannheim, Germany). mRNA levels were derived from Ct values according to the Pfaffl mathematical model for relative real-time PCR.[15] mRNA levels were normalized to controls, which had been set to 100%.

### Western Blotting

Total protein was harvested from cultured cells using RIPA extraction buffer (50 mM Tris HCl, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS]) supplemented with mini protease inhibitor tablet (Roche) and quantified with the Pierce BCA assay kit (ThermoFisher

**Table 2.** Ranking of In-Laboratory-Generated Nucleofection Buffers Based on Sum of Scores for Cell Attachment, Confluency, Viability, and Transfection Efficiency.

| | Attachment | | Confluency | | Live Cells | | Efficiency | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Buffer | % of Control | Score | % of control | Score | % Cells Live | Score | % GFP +ve | Score | Sum of Scores | Final Rank |
| PBS | 21 ± 5 | 6 | 24 ± 4 | 6 | 88 ± 2 | 6 | 51 | 1 | 19 | 5 |
| PBS-M | 25 ± 4 | 5 | 26 ± 4 | 5 | 90 ± 1 | 5 | 34 | 6 | 21 | 6 |
| 1M | 70 ± 10 | 1 | 77 ± 20 | 1 | 97 ± 1 | 1 | 45 | 2 | 5 | 1 |
| 2M | 60 ± 12 | 2 | 71 ± 11 | 3 | 96 ± 1 | 2 | 37 | 5 | 12 | 2-3 |
| 3P | 49 ± 12 | 3 | 73 ± 12 | 2 | 94 ± 3 | 3 | 41 | 4 | 12 | 2-3 |
| 3H | 41 ± 6 | 4 | 40 ± 6 | 4 | 92 ± 4 | 4 | 42 | 3 | 15 | 4 |

Scientific, Waltham, MA, USA). Equal proportions of protein extracts were loaded on polyacrylamide gels. Lamin A/C and GAPDH were separated on 8% or 12% SDS-polyacrylamide gels by electrophoresis, respectively. Gels were loaded with 20 µg of protein. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Life Technologies) by wet transfer in blotting buffer (39 mM glycine, 48mM tris base, 0.05% w/v SDS, 20% methanol) at 80 V for 1.5 hours. The membrane was incubated in 5% nonfat milk for 30 minutes prior to overnight incubation at 4°C with primary antibody reactive with lamin A/C (1:500; Millipore, Billerica, MA, USA) or GAPDH (1:2000; Millipore). The membrane was then washed 3 times for 5 minutes in wash buffer (0.0001% Tween 20 in PBS), and incubated with goat anti-mouse or goat anti-rabbit horse radish peroxidase–conjugated secondary antibody (Abcam, Toronto, Ontario, Canada) for 1 hour at room temperature. The membrane was washed 3 times for 5 minutes in wash buffer, developed using ECL Prime Western Blotting Detection Reagent (GE Life Sciences, Baie D'Urfé, Quebec, Canada), and visualized on X-ray film. Densitometry of the resultant bands was performed using Image J software.

### *Statistical Analysis*

Each condition was performed in triplicate and repeated 3 to 4 times in independent experiments. *T* tests were used to analyze differences between 2 groups. Univariate analysis of variance (ANOVA) was performed when an analysis between several was required and Dunnett's *post hoc* test was used to detect differences between groups.

Buffers were scored according to 4 different categories: cell attachment, confluency, viability, and nucleofection efficiency (**Table 2**). Buffers were scored from best "1" to worst "6." The scores from each of the 4 categories were summed. The final rank of the buffers were assigned based on the sum of these scores. The buffer with the lowest sum of scores was determined to be the most suitable.

## Results

### *Bovine and Human-Passaged Chondrocytes Can Be Successfully Nucleofected Using the Commercial Kit*

To test the efficacy of nucleofection on passaged chondrocytes, bovine- and human-passaged chondrocytes were transfected with pmaxGFP, a vector supplied with the commercial kit. GFP expression was examined using flow cytometry three days post nucleofection. pmaxGFP was seen expressed in 89% of nucleofected passaged bovine cells (**Fig. 1A** and **B**). Similarly human cells had transfection efficiencies of approximately 87% when transfected with pmaxGFP (**Fig. 1B**). There was no apparent difference in transfection efficiency between passage 1 or 2 bovine chondrocytes (data not shown). In bovine-passaged chondrocytes, 75% efficiency was achieved with the nucleofection of Cignal GFP vector. The transfection efficiency with siGLO red indicator was 98% (**Fig. 1C**).We have also utilized our nucleofection protocol to successfully transfect mesenchymal ATDC5 cells with pmaxGFP (supplementary Fig. S1 available at http://car.sagepub.com/content/by/supplemental-data).

### *Successful Nucleofection of Passaged Cells with Formulated In-Laboratory-Produced Buffers*

To investigate the efficiencies of the various characterized buffers on nucleofection of cells, passaged bovine cells were nucleofected in the different buffers and then seeded in monolayer culture. Control cells were treated identically and maintained in PBS but were not nucleofected. The proportion of cells that attached to culture vessels following nucleofection in the different buffers was estimated through analysis of DNA content in cells that were attached to polystyrene after four hours.

PBS and PBSM had the lowest proportion of adherent cells following nucleofection. Cells nucleofected in PBS

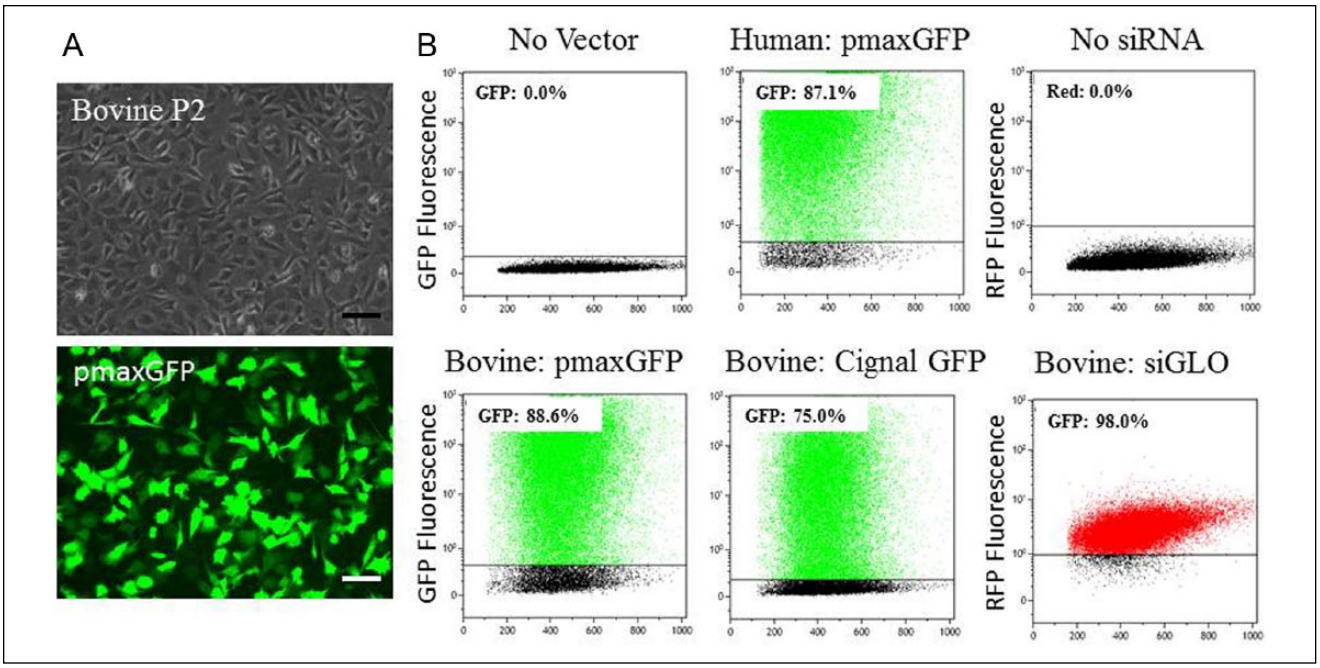


**Figure 1.** Successful nucleofection of passaged chondrocytes using Amaxa buffers. (A) Phase contrast and fluorescent images of bovine-passaged cells transfected with pmaxGFP. Scale bars = 100 µm (B) Flow cytometry of chondrocytes transfected with no vector (bovine), pmaxGFP (human or bovine), and Cignal GFP (bovine) measured by green fluorescent protein (GFP) fluorescence and no siRNA (bovine) or siGLO transfection indicator (bovine) measured by red fluorescent protein (RFP) fluorescence.

and PBSM had significantly less attachment then control (**Fig. 2A** and **B**; **Table 2**). Although there was a trend toward less attachment of cells nucleofected in 1M, 2M, 3P, and 3H these were not statistically different from controls.

Chondrocytes that have been passaged in culture acquire the ability to spread and this was observed in control P2 cells. Cell spreading appeared to be buffer dependent as nucleofection in PBS led to a large proportion of small rounded cells as compared to cells in buffer 1M (**Fig. 2A**). Following nucleofection in the various buffers, cell confluency was evaluated through Image J analysis to quantify cell spreading. While PBS, PBSM, and 3H were significantly less confluent than controls, buffers 1M, 2M, and 3P were not statistically different (**Fig. 2C**; **Table 2**). The effect of nucleofection buffer on viability was determined, 1M and 2M had the greatest amount of live cells at 97% and 96%, respectively (**Fig. 2D**; **Table 2**).

In order to assess transfection efficiencies, pMX-GFP was transfected into bovine cells. Transfection efficiencies ranged from 34% to 51% for in-laboratory-generated buffers. Nucleofection of cells in PBS had the greatest transfection at 51%. Buffer 1M had the second most cells transfected with GFP at 45% (**Fig. 2E**; **Table 2**).

Based on the aforementioned findings the buffers were scored according to attachment, confluency, percent cell death, and transfection efficiency (**Table 2**). Buffer 1M was the most suitable for transfection of bovine-passaged chondrocytes. Bovine primary chondrocytes (Supplementary Fig. S2 available at http://car.sagepub.com/content/by/supplemental-data) and human-passaged chondrocytes (Supplementary Fig. S3 available at http://car.sagepub.com/content/by/supplemental-data) could also be successfully nucleofected using buffer 1M.

## *Nucleofection in Buffer 1M Resulted in Efficient Transfection Comparable to Commercial Kit*

Previously reported transfection efficiencies in chondrocytes have demonstrated that efficiencies of 70% were possible using pmaxGFP supplied with the Amaxa kits,[9] we determined that efficiencies up to 89% were achievable as shown in **Figure 1**. However, during our screen of buffers it was determined that the efficiency of pMX-GFP transfection using 1M buffer was much lower (45%) than the efficiency of pmaxGFP transfection using the Amaxa kit. To determine whether this was due to buffers or to the vector used in our transfections, a direct comparison between the commercial buffer and in house made buffer 1M was done using the pmaxGFP. Nucleofection with pmaxGFP resulted in higher transfection efficiency with 1M buffer, approaching 75%, which was similar to commercial buffer at 79% (**Fig. 3A** and **B**). Chondrocytes transfected with pmaxGFP had a larger proportion of cells expressing GFP (**Fig. 3B**) than cells transfected with pMX-GFP (**Fig. 2E**).

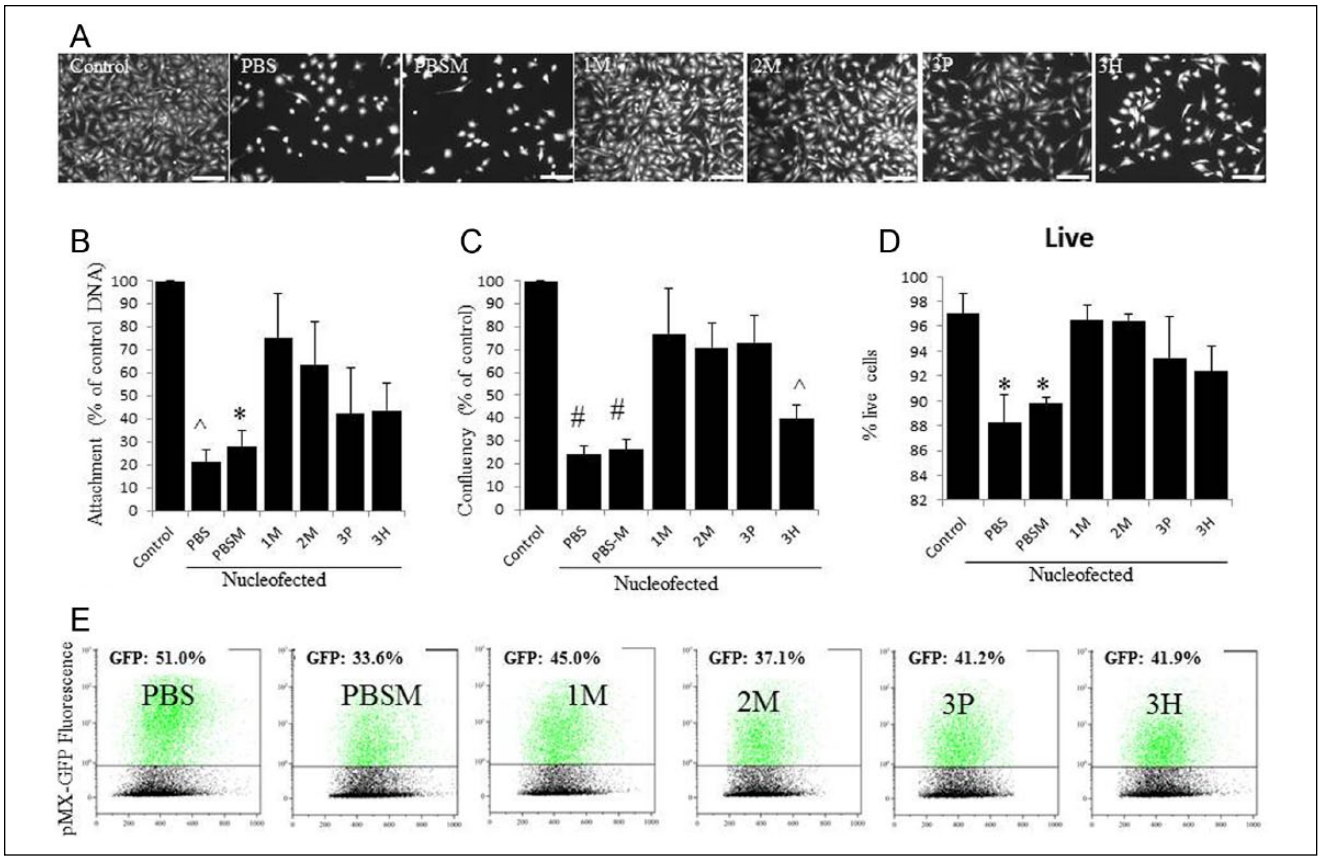


**Figure 2.** Nucleofection of bovine-passaged chondrocytes using specific formulated buffers. (**A**) Florescent microscopic images of calcein-AM stained chondrocytes 1day following nucleofection. Scale bars = 100 μM. (**B**) Attachment of cells as a percentage of non-nucleofected control cells as determined by DNA assay. (**C**) Confluency of cells as a percentage of non-nucleofected control cells calculated through image j analysis. (**D**) Proportion of live cells as determined by live/dead assay counts. (**E**) Efficiency of nucleofection for buffers as determined through flow cytometry. $^{*}P < 0.05$; $^{\wedge}P < 0.01$; $^{\#}P < 0.001$, versus control.

To demonstrate whether the 1M buffer could be used for gene targeting, nucleofection of siRNA for lamin A/C was performed. There was a decrease in lamin-A and -C protein levels to 19% and 8% of the control protein, respectively, after approximately 2 days (**Fig. 3C** and **D**). Knockdown of lamin did not result in any significant differences in gene expression for other genes tested (Supplementary Fig. S4 available at http://car.sagepub.com/content/by/supplemental-data).

## *Nucleofection in 1M Buffer Resulted in Specific Gene Modulation and Nucleofected Bovine-Passaged Cells Retained Their Ability to Form Tissue*

To determine whether nucleofection itself had an effect on passaged cell gene expression, RT-PCR analysis was performed for expression of genes related to fibroblast (col1) or cartilage (col2, acan) matrix, chondrogenic transcription factor (sox9), proliferation (ki67, ccnd1), and apoptosis (casp3) gene expression. At 2 days post-nucleofection, col1 and col2 gene expression were significantly downregulated (**Fig. 4A**). None of the other genes analyzed were significantly modulated at this time point, however there was a trend toward downregulated aggrecan and upregulated ccnd1. At four days following nucleofection, col1 gene expression remained significantly downregulated, while col2 and sox9 was significantly upregulated (**Fig. 4B**). This resulted in an increased ratio of col2 to col1 mRNA levels from 0.8 at day 2 to 1.7 at day 4. Proliferation markers ki67 and ccnd1 were also significantly upregulated. There was a trend toward upregulated aggrecan but this was not statistically significant. Casp3 expression remained unchanged.

Passaged bovine chondrocytes are capable of redifferentiation when grown in 3D culture in the presence of redifferentiation media.[16] To determine if nucleofection affected the ability of cells to produce tissue, cells were nucleofected and then placed in redifferentiation conditions (3D culture and redifferentiation media).

After 20 days, nucleofected passaged cells produced a continuous layer of cartilagenous tissue that had an average

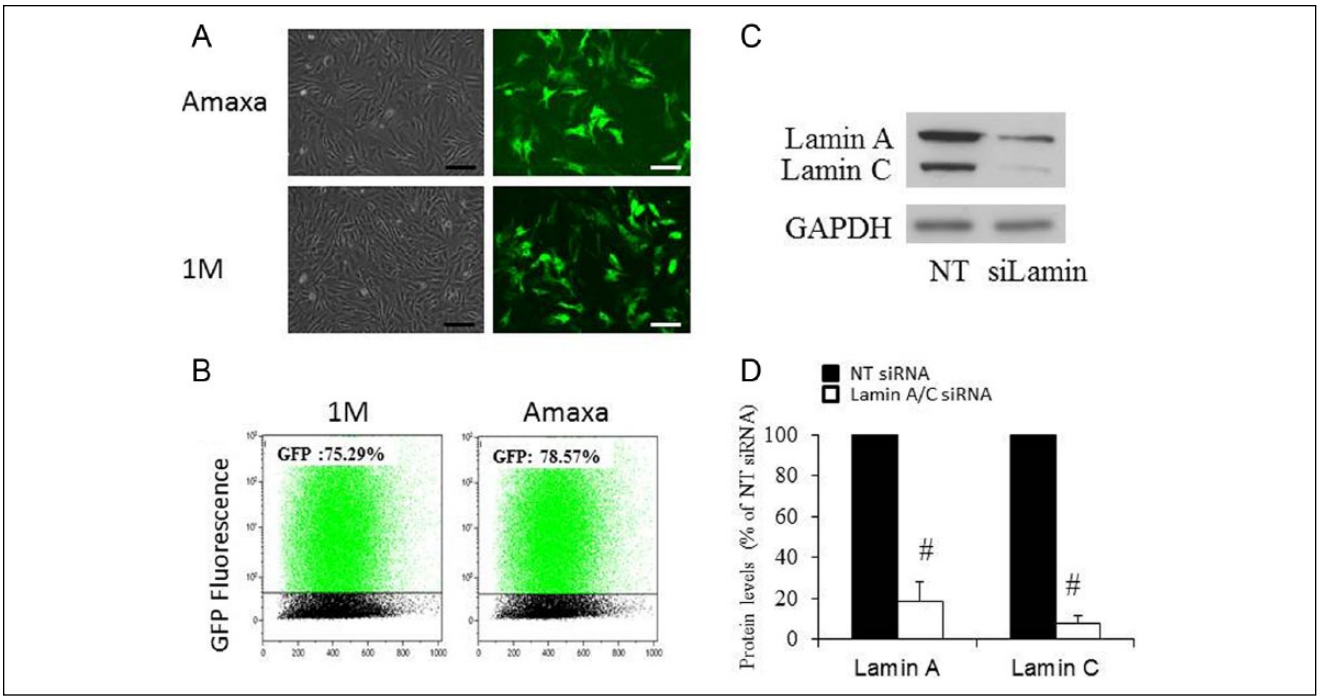


**Figure 3.** Nucleofection of bovine-passaged chondrocytes with pmaxGFP using buffer 1M is similar to transfection with Amaxa buffers as demonstrated through (**A**) phase contrast and fluorescent microscopy images of passaged chondrocytes (scale bars = 100 μm) and (**B**) flow cytometry. Passaged bovine chondrocytes nucleofected with no template siRNA (NT) or siRNA designed against lamin A/C. Levels of lamin A and C are evaluated by Western blot analysis (**C**) and densitometry (**D**). $^{\#}P < 0.001$, versus NT siRNA.

height of approximately 1.7 mm (**Fig. 4C**) and stained for proteoglycans (**Fig. 4D**). While there was a trend toward decreased collagen content in nucleofected passaged chondrocytes, collagen and glycosaminoglycan (GAG) accumulation was not significantly different from non-nucleofected controls (**Fig. 4E**).

## Discussion

Consistent with the literature this study demonstrates that nucleofection is an appropriate way of introducing DNA into chondrocytes.[9] Additionally nucleofection of passaged chondrocytes is possible using buffer 1M, which was generated within the laboratory.

Cell preparation is critical for successful nucleofection of chondrocytes. Enzymatic digestion of cells prior to nucleofection in pronase and collagenase has been shown to be critical for nucleofection of cells presumably by removing the pericellular matrix that develops around chondrocytes.[9] However this reportedly results in 20% cell death. We found that placing bovine-passaged chondrocytes in serum-reduced conditions prior to nucleofection was suitable preparation for transfection and maintained high (97% in 1M buffer) cell viability. This condition was critical for efficient transfection (data not shown), and may limit the amount of pericellular matrix surrounding the cells rendering them amenable to nucleofection. It also must be noted that for passaged chondrocytes enzymatic matrix digestion may not be required as these cells do not accumulate as much matrix as primary chondrocytes in media containing serum.[17]

Although transfection efficiency was similar between the buffers tested, bovine cell attachment and viability was buffer dependent. Bovine cell attachment and viability was highest when buffers 1M and 2M were used for nucleofection. Cell death following nucleofection could be due to colloid-osmotic lysis. Colloid-osmotic lysis occurs post-electroporation as cell membranes are electrically perforated allowing the entrance of small ions within the cell.[18] As a result of ion entrance, an influx of water occurs leading to increased cytosolic pressure, cell swelling, membrane rupture and eventually cell death. This process appeared to be prevented by mannitol, a sugar-alcohol, which is included in buffers 1M and 2M. Mannitol raises the osmolality of the extracellular environment, balancing the osmotic pressure within and outside the cell preventing colloid-osmotic lysis.[19] This may explain why buffer 1M and 2M was preferred over other buffers.

In addition to colloid-osmotic lysis, another concern with nucleofection or other electroporation techniques is the potential effects on cell behavior and health.[20,21] In this study, chondrocyte health did not appear compromised following nucleofection in 1M buffer. While apoptosis is a

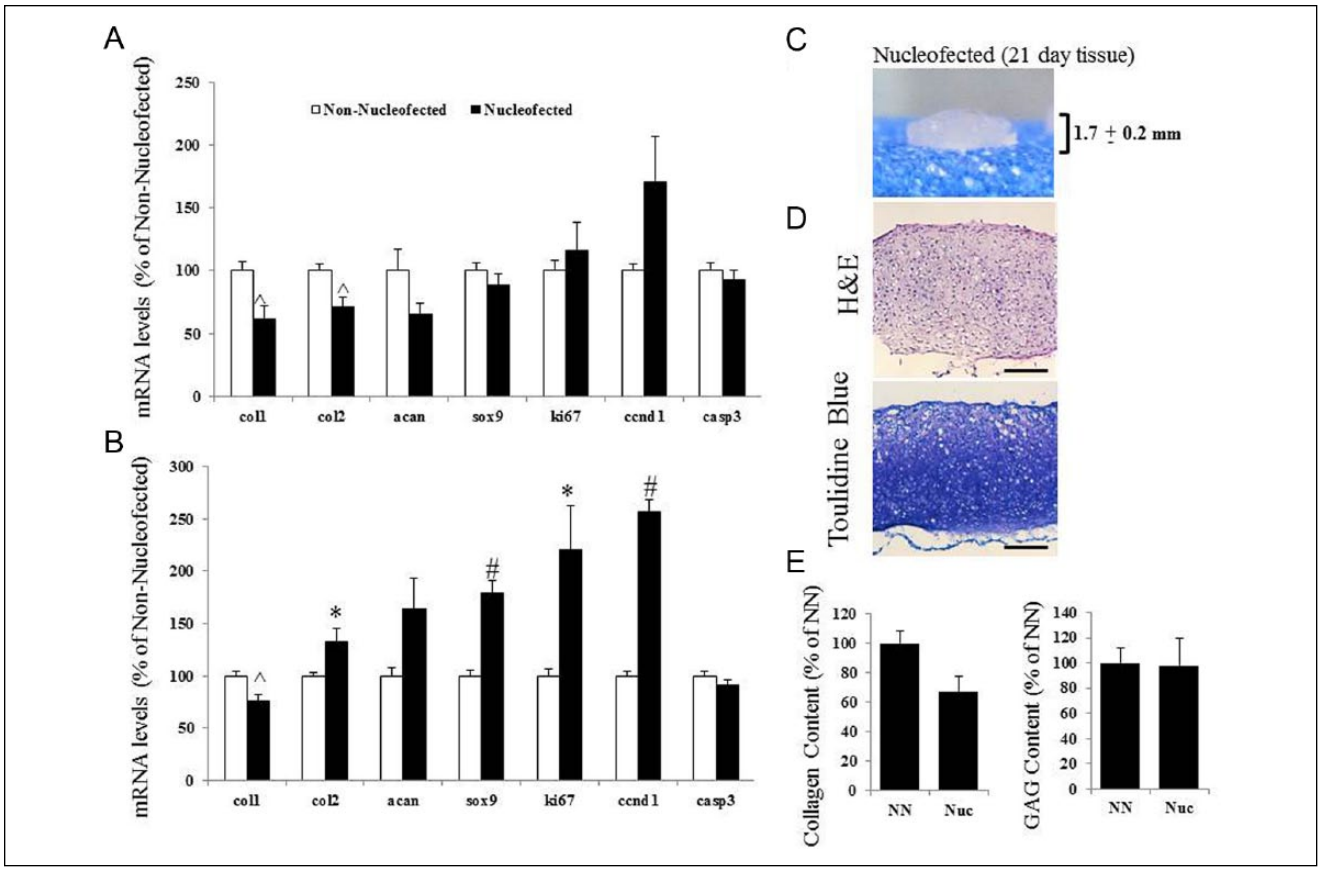


**Figure 4.** Effect of nucleofection on bovine-passaged chondrocyte gene expression at (**A**) 2 and (**B**) 4 days following nucleofection as compared to non-nucleofected control. Gross appearance of tissue that formed 21 days after nucleofection (**C**). Histological examination by light microscopy showed a continuous layer of cartilage that is rich in proteoglycan (**D**). (**E**) No differences were detected in glycosaminoglycan (GAG) and collagen accumulation by passaged chondrocytes that were nucleofected (Nuc) as compared to non-nucleofected (NN) cells. (C, hematoxylin and eosin; D, toluidine blue; scale bars = 0.5 mm). $^{*}P < 0.05$; $^{\wedge}P < 0.01$; $^{\#}P < 0.001$, versus control.

concern with electroporation,[21] caspase 3 gene expression, which has been shown to increase in apoptotic chondrocytes,[22] was not significantly different in nucleofected versus non-nucleofected passaged chondrocytes. Intriguingly, proliferation genes, ccnd1 and ki67, were upregulated. This was in keeping with findings that cells may be more proliferative after electrical stimulation.[23] Furthermore, while nucleofection initially reduced col1 and col2 gene expression after 2 days of culture, col2 and sox9 became upregulated after 4 days. This is in agreement with other studies showing that electrical stimulation could upregulate chondrogenic gene expression[23,24] and suggests that nucleofection may aid in redifferentiation of chondrocytes. The effects of nucleofection may either be limited to gene expression changes or alternatively could be transient as nucleofected cells cultured for 20 days in redifferentiating conditions produced similar matrix, in terms of collagen and glycosaminoglycan, by nucleofected as compared with non-nucleofected passaged chondrocytes. Nevertheless, nucleofection in 1M did not adversely affect chondrocyte phenotype and is a suitable buffer for transfection with efficiencies similar to Amaxa commercial buffers.

The transfection efficiency obtained with nucleofection was DNA dependent. Nucleofection of siRNA had high transfection efficiencies and similar to previous studies was a suitable way to enable targeted gene knockdown.[25] As compared with siRNA, plasmid vectors had lower transfection efficiencies. This could be due to the size of the particles transfected into passaged chondrocytes as siRNA is much smaller than vectors. In support of size effecting efficiencies, we found a higher transfection efficiency with the smaller 3.5-kb pmaxGFP than the larger 5.3-kb pMX-GFP construct. Additionally, pmaxGFP is under the control of the CMV promoter whereas pMX-GFP is under the control of MuMV-LTR which has been found to be less potent than CMV in transfection of 293T cells.[26] Thus, size of vectors and the promoter used to drive genes should be taken into consideration during vector design for gene targeting into

passaged chondrocytes. Furthermore, the amount of construct DNA utilized can also affect the ability of cells to be effectively nucleofected. Human-passaged chondrocytes are capable of transfection but this required 10 µg of Cignal GFP (Supplementary Fig. S3) as nucleofection with 5 µg Cignal GFP resulted in a smaller proportion of cells expressing GFP (data not shown).

For the highest possible efficiency of nucleofection, other parameters require careful optimization such as the percentage of serum during monolayer expansion, which should be reduced to enhance nucleofection ability. Preliminary results indicated that monolayer expansion of bovine chondrocytes in Ham's F12 supplemented with 20% FBS as opposed to 10% FBS led to decreased nucleofection efficiency in 1M buffer (data not shown). This effect was evident despite the two day growth in serum reduced conditions (0.5% FBS) prior to nucleofection.

In summary, nucleofection of passaged chondrocytes in 1M buffer is a suitable means of nonviral transfection. Within the laboratory we have also utilized this protocol to successfully transfect other cell types known to be difficult to transfect such as ATDC5 cells and intervertebral disc cells (data not shown). This provides research labs with a low-cost alternative to commercial kits for nonviral transfection and a buffer of known formulation with potential therapeutic utility in gene transfer for clinical applications.

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## Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

## Ethical Approval

Ethical approval for the use of human cells for this study was obtained from Mount Sinai Hospital Research Ethics Board 05-0071-E.

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