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An effective and efficient method of transfecting primary human chondrocytes in suspension

Mohammad Shahidul Makki¹, Nahid Akhtar^{1,2}, and Tariq M Haqqi^{1,3}

¹Department of Anatomy and Neurobiology, Northeast Ohio Medical University, Rootstown, Ohio 44272

Abstract

Human chondrocytes accumulate an ECM-rich matrix by secreting matrix macromolecules during monolayer culture, which makes them difficult to transfect efficiently. Here we report a non-viral based protocol to transfect the primary human chondrocytes with high efficiency in suspension. Chondrocyte cultures were digested using Pronase and Collagenase and transfected in suspension. Transfection efficiencies of more than 80% were achieved routinely using the protocol described. The viability of siRNA transfected or un-transfected chondrocytes was not affected and resulted in 80–90% knockdown of the target mRNA levels. This protocol may be useful in gene knockdown, and ectopic overexpression studies in chondrocytes.

Keywords

Chondrocytes; Transfection; siRNA; Osteoarthritis

Osteoarthritis (OA) is a degenerative disease of the cartilage in the articulating joints. Seventy percent population over the age of 60 years suffers from this disease, which has tremendous adverse socio-economic impact on the population [1, 2]. Cartilage is a resilient tissue which absorbs the mechanical stress on the joints, however due to the post-mitotic nature of chondrocytes it has very limited ability to repair the damage. Chondrocytes are the only cell type in the cartilage and responsible for the synthesis and maintenance of extracellular matrix (ECM) primarily composed of aggrecan and type II collagen. The wear-and-tear process in articulating cartilage that contributes to OA pathogenesis occurs due to changes in the catabolic and anabolic genes expression in chondrocytes [3]. Therefore, extensive studies are required to understand the molecular functions of OA associated regulation of gene expression in chondrocytes. The *in-vitro* transfer of siRNA, anti-sense RNA or ectopic overexpression of cDNA vector into chondrocytes provides a powerful approach for the study of various basic cellular and genetic processes.

³To whom correspondence should be addressed: 4209 St. Rt. 44, RGE 238, Rootstown, OH 44272. Tel.: 330-325-6704; thaqqi@neomed.edu.

²Current Address: Eidactis and Strabismus Research Foundation 2000 Van Ness Avenue, Medical Arts Building, Suites 209-210 San Francisco CA, 94109-3023

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Transfection of nucleic acids into primary chondrocytes has always been hampered by the low transfection efficiencies of the protocols currently in use. Although some protocols have been successfully used for the gene transfer but they suffer from various shortcomings indicating low efficiency of transfection and cell viability. Viral vectors have shown high efficacy in transforming primary chondrocytes but the procedures are labor intensive and require extreme precautions. Some viral vectors need dividing cells for integration and may also induce host immune response [4, 5].

Non-viral based transfection methods which includes chemical and physical means of introducing nucleic acids into the chondrocytes are widely used [6,15]. Various lipid based transfection reagents such as Lipofectamine, TransFast, Fugene, Cellfectin etc. generally enjoy low toxicity but the transfection efficiency ranges only from 2–30% [5, 7]. Fugene was found to be most efficient with transfection efficacy of around 7–40% depending on the species of origin of the chondrocytes [7]. These liposomal or non-liposomal formulations offer excellent biocompatibility in performing various biochemical assays and at the same time they are also biodegradable. Therefore, these formulations are excellent choice in the gene manipulation studies in chondrocytes but the studies suffer due to low transfection efficiencies.

In this study we explored the use of Oligofectamine and Lipofectamine to transfect siRNA and plasmid DNA respectively into primary chondrocytes with high efficiency in order to manipulate the gene function. We successfully established the method of nucleic acids transfection into the chondrocytes in suspension. This novel and simple approach reproducibly provides ~80% knockdown of mRNA and proteins in the transfected chondrocytes. We believe our approach is optimized and could facilitates gene function studies not only in chondrocytes but also in other hard to-transfect cells efficiently and in a cost-effective way.

With the Institutional Review Board (IRB) approval, discarded cartilage samples were obtained from the donors who underwent total knee joint replacement surgery at Summa St Thomas Hospital, Akron, OH. Pieces of cartilage were resected using a sterile #10 Feather blade and chondrocytes were extracted essentially as described previously [8, 9]. For the siRNA or plasmid DNA transfection of primary chondrocytes in suspension, half a million chondrocytes were cultured for 2 days in a 6-well plate in DMEM supplemented with 10% FBS (Thermo Fisher Scientific, Waltham, MA, cat#SH30243FS). For transfection experiments chondrocytes were first digested for 2.0 hrs at 37°C in 1 ml mixture of Pronase (Roche Diagnostics, Indianapolis IN, cat#11459643001) (1mg/ml), Collagenase (Roche Diagnostics, Indianapolis IN, cat#11088815001) (1 mg/ml) and 4U/ml Hyaluronidase (Sigma-Aldrich, St Louis, MO, cat#H3506) prepared in DMEM. Solution was swirled every 20 min for quick and efficient digestion of extracellular matrix. After digestion chondrocytes were centrifuged at 100 xg for 5 min and supernatant was discarded and the cell pellet was washed twice with PBS. Oligofectamine complex was prepared by diluting the siRNA (final concentration of 100nM) into 175µl Opti-MEM I reduced serum medium (Thermo Fisher Scientific, Waltham, MA, cat#11058021) lacking serum and antibiotics in one tube. In a second tube 4µl Oligofectamine reagent (Thermo Fisher Scientific, Waltham, MA, cat#12252011) was diluted into 15 µl Opti-MEM I medium. Components of each tube were

gently mixed and incubated for 15 min at room temperature. Diluted siRNA and Oligofectamine reagents were then combined and washed chondrocytes were resuspended in the complex followed by addition of 4U/ml hyaluronidase. Chondrocytes in suspension were then incubated for 2.0 hr at 37°C with gentle shaking every 15 min. Following the transfection chondrocytes were supplemented with 500µl DMEM and seeded into 6 wells plate and further incubated for 2 hrs without antibiotic or serum. After 2 hrs 350µl DMEM supplemented with 3X serum was added to the culture medium and chondrocytes were grown for an additional 36 hrs and then stimulated with 5ng/ml IL-1β for 24 hrs. For MCP1 encoding plasmid DNA transfection 2.0 million chondrocytes were used. Reagents were scaled up for 6 cm petri dish format and 10µg plasmid DNA was diluted into 200µl Opti-MEM I Reduced Serum Medium. In a separate tube 25 µl Lipofectamine (Thermo Fisher Scientific, Waltham, MA, cat#L3000-015) was mixed with 200 µl Opti-MEM I Reduced Serum Medium. The two mixtures were combined and incubated for 15 min at room temperature. Chondrocytes were resuspended in the mixture and 4U/ml hyaluronidase was added and incubated for 2.0 hr at 37°C with gentle shaking every 15 minutes. Chondrocytes were supplemented with 2ml DMEM and seeded into 6cm plate and incubated for 2 hrs without antibiotic or serum as above. After 2 hrs, 1ml DMEM with 3X serum was added and chondrocytes incubated for further 36 hrs before analysis.

We first transfected the siGLO oligonucleotides (Dharmacon, GE Healthcare, Lafayette, CO, cat#D-001630-02-05) that emit red fluorescence upon excitation to easily monitor the transfection efficiency using fluorescence microscope. We counted the transfected versus total number of chondrocytes in three different microscopic fields. Transfection of chondrocytes with siGLO using this method was highly efficient as transfection efficiency of over 90% was achieved (Figure 1A and C). As a comparative analysis we also transfected adherent chondrocytes using manufacture's protocol and obtained 40–50% transfection efficiency (Figure 1B and C) which was also reported previously [6, 7, 10]. Further to explore whether this method have any effect on the chondrocytes marker gene expression we assess the expression of COL2A1 and ACAN Marker gene expression was compared between adherent chondrocytes and chondrocytes transfected in suspension. Expression of COL2A1 and ACAN were comparable and no difference was noted (Figure 1D).

TUT1 (Terminal Uridylyl Transferase 1) and TUT2 encodes a nucleotidyl transferase that functions as both a terminal uridylyltransferase and a nuclear poly(A) polymerase. The encoded enzyme specifically adds and removes nucleotides from the 3' end of small nuclear RNAs and select mRNAs and may function in controlling gene expression and cell proliferation [11, 12]. Chondrocytes were transfected in suspension using 100nM concentration of siRNA targeting TUT1(Dharmacon, GE Healthcare, Lafayette, CO, cat#L-014221-02-0005) or TUT2 (Dharmacon, GE Healthcare, Lafayette, CO, cat#L-018505-01-0005). After IL-1β stimulation of chondrocytes mRNA expression was measured by TaqMan assay. Expression of TUT1 mRNA was reduced by ~75% compared to the non-targeted (NT) siRNA transfected chondrocytes (Figure 2A). Similarly, expression of TUT2 was ~80% reduced compared to control chondrocytes (Figure 2B). This suggests that siRNA transfection of chondrocytes in suspension is a highly efficient and feasible method for gene expression analysis.

Next we wondered whether this transfection protocol and the reagents were toxic to chondrocytes. To address this issue, we performed Trypan blue exclusion assay after transfecting chondrocytes with TUT1 or TUT2 siRNA and compared their viability with mock transfected or non-targeted siRNA transfected chondrocytes. Comparing mock versus siRNA transfected chondrocytes no significant difference was noted ($p>0.05$). Similarly, no noticeable difference was observed in the viability of siRNA transfected or un-transfected chondrocytes either. This suggest that this protocol is safe to perform transfection in chondrocytes (S1). Next we validated the method by investigating the IL-6 (interleukin-6) gene regulation by MCPIP1 in human chondrocytes. Earlier it has been demonstrated that IL-6 is a target of MCPIP1 that reduce the level of IL-6 mRNA in different cell types including chondrocytes [8, 9]. We employed this previously established gene regulatory network for the protocol validation. Transfection of chondrocytes using MCPIP1 siRNA (Origene, Rockville, MD, cat# SR312813) with this protocol decreased the levels of MCPIP1 mRNA and protein by 80% (S2-A and -B). Moreover, expression of IL-6 was 1.5-fold increase compared to the non-targeted cells as previously reported albeit transfected by a different method (S2-C) [9]. Contrary to siRNA knockdown overexpression of MCPIP1 cDNA significantly decreased the expression of IL-6 in IL-1 β treated chondrocytes (S2-D and -E).

Proper preparation of chondrocyte is crucial for the successful completion of the transfection procedure. We found that in this procedure 2.0 hrs of pronase/collagenase digestion of chondrocytes primary cultures was sufficient for efficient transfection compared to previous report of 3–5 hrs pronase/collagenase digestions and using nucleofection [13]. Our procedure was carried out without compromising the efficiency or the viability of the primary chondrocytes. However, overall this transfection approach is lengthy and time consuming compared to other chemical or electroporation based procedure. Previously, transfection efficiency of adherent rabbit articular chondrocytes was increased several fold by adding hyaluronidase, an ECM digesting enzyme, before and during the transfection protocol using the calcium phosphate method [14]. In another study comparisons were made using different liposomal or non-liposomal based transfection reagents for articular chondrocytes [7]. Use of Hyaluronidase before and during the transfection of chondrocytes resulted in 41% transfection efficiency. However this technique differs from our approach in that Fugene 6 was used and chondrocytes remained adherent [7]. In the current procedure we employed same amount of hyaluronidase only during the transfection procedure which gives ~90% transfection efficiency. The large variation could be attributed to the fact that in suspension chondrocytes are fully exposed and transfection occurs in a multi-dimensional fashion.

Overexpression or knockdown of target molecules is a useful approach for studying the role of diverse biological molecules involved in multiple signaling pathways [9, 11, 12]. Three different siRNA duplexes were transfected and were able to knock down 80–90% of the target transcripts as well as protein levels, implying that the protocol we describe was not gene specific but could be useful in knocking down diverse molecules. MCPIP1 was previously shown to regulate IL-6 expression by targeting IL-6 mRNA (9). Hence overexpression of MCPIP1 suppress the levels of IL-6 mRNA as well as its protein. Using the current method of transfection described in this report we successfully showed that when

expression of MCP1P1 was knocked down, expression of IL-6 was enhanced as shown previously (9).

In conclusion, this study demonstrates the efficiency of transfection of primary chondrocytes in suspension using a regular transfection reagent (Oligofectamine or Lipofectamine) and result in high level of knock-down or transgene overexpression with apparent no toxicity. This is an economical approach with easy to follow procedure and could be beneficial in a regular lab setting without purchasing expensive lab equipment. In addition to functional studies this method may also be useful in a wide array of translational studies where high efficiency of transfection is needed such as cartilage tissue engineering, ex vivo gene therapy or *in vitro* drug testing.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Transfection efficiency of the established method. Primary human chondrocytes were transfected in suspension (A) or in adherent (B) condition using 100nM red fluorescent siRNA oligonucleotides. Twenty-four hrs post-transfection red and blue fluorescence signals were acquired using confocal microscopy. Blue signal represents the DAPI, staining the nuclei. Representative field of the fluorescence microscopic view is shown. (C) Counting from three different fields demonstrated that approximately 90% chondrocytes were transfected in suspension compare to 50% in adherent culture. (D) Expression of COL2A1 and ACAN determined by TaqMan assay. mRNA expression was normalized against β -actin expression. n=3.

Fig. 2. siRNA mediated knockdown of TUT1 and TUT2 expression in primary human OA chondrocytes transfected in suspension. OA chondrocytes were transfected with 100nM TUT1 (A) or TUT2 (B) siRNAs in suspension. Thirty-six hrs post-transfection OA chondrocytes were stimulated with 5ng/ml IL-1 β for additional 24 hrs. OA chondrocytes were harvested and RNA was isolated. Gene expression analysis was performed using gene specific TaqMan Assay. mRNA expression was normalized against β -actin expression. n=3. **p< 0.005.