Selective loading of exosomal HULC and miR-372 is responsible for chondrocyte death during OA pathogenesis

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ABSTRACT
Exosomes are membranous vesicles containing various biomolecules, including non-coding RNAs (ncRNAs). ncRNAs are secreted from several cell types and are involved in various biological functions, including cellular communication. The aim of this study was to identify and illustrate the significance of the osteoarthritis (OA)-specific packaging of exosomal ncRNAs. In this study, we hypothesized that selective packaging of ncRNAs into exosomes would reflect the cellular response to chondrocyte death during OA pathogenesis. Exosomal HULC level significantly decreased in OA exosomes, whereas exosomal miR-372-3p level significantly increased in OA exosomes. In addition, chondrocytes with high HULC levels in the cytosol showed lower overall proliferation and higher apoptotic cell death than normal chondrocytes, whereas chondrocytes with high miR-372-3p in the cytosol showed higher overall proliferation and lower cell death than OA chondrocytes. Among the signaling molecules known to be involved in OA pathogenesis, GSK is one of the regulators of the selective exosomal packaging observed in OA chondrocytes. Inhibition of GSK observed in OA chondrocytes was responsible for enriched uploading of miR-372-3p and suppressed uploading of HULC during OA pathogenesis. In conclusion, we show that selective ncRNAs observed in OA play a critical role in chondrocyte proliferation/apoptosis.

1. Introduction
Recent techniques such as genomic tiling arrays and cDNA sequencing have broadened the resolution of transcription and have demonstrated that a surprisingly small amount (~2%) of the human genome actually encodes protein, although more than 90% of the human genome is transcribed into RNA. Most transcripts appear as non-coding RNA (ncRNA), such as microRNAs (miRs, miRNAs) and long non-coding RNAs (lncRNAs; Mercer et al. 2009; Geisler and Coller 2013; Kang et al. 2016). miRNAs are small non-coding RNA molecules that act as a novel class of endogenous negative regulators of gene expression. miRNAs have the ability to bind to specific regions in the 3’UTR of mRNAs and repress gene expression through interaction with the RNA induced silencing complex (RISC; Finnegan and Pasquinelli 2013). lncRNAs are noncoding RNAs that consist of sequences longer than 200 nucleotides (Mercer et al. 2009; Geisler and Coller 2013; Kang et al. 2016) and are classified as intergenic, intragenic/intronic, or antisense IncRNAs based on their position relative to protein-coding genes (Mercer et al. 2009). Although the control of cellular functions has classically been considered the responsibility of proteins, research over the last decade has elucidated many roles for ncRNAs in the regulation of not only the proteins that control cellular functions, but also the cellular functions themselves. The biological functions of ncRNAs are still being elucidated, but many recent studies have shown that they are involved in the regulation of chromatin modification, gene transcription, mRNA translation, and protein function (Mercer et al. 2009; Wang and Chang 2011). Moreover, greater knowledge of the function and role of ncRNAs has helped to improve our understanding of the regulatory mechanisms of various pathogeneses (Esteller 2011; Wapinski and Chang 2011). Therefore, many recent studies have suggested the strong potential of ncRNAs as novel biomarkers for diagnosis and prognosis, and as a powerful surveillance system as part of a new therapeutic platform (Qi and Du 2013; Crea et al. 2014). Moreover, the...
pathophysiological significance of exosomes carrying ncRNAs is beginning to be defined in diseases, including cancer (Ling et al. 2013) and arthritis (Song et al. 2015b) and the data suggest a role for these exosomes as mediators in cell-to-cell communication (Ramachandran and Palanisamy 2012). Here, we investigated the expression of a panel of ncRNAs in exosomes isolated from normal and osteoarthritis (OA) chondrocytes and selected two molecules with high (miR-372-3p) and low expression (HULC) that modulate the survival/apoptosis of human articular chondrocytes.

2. Materials and methods

2.1. Human patient-derived chondrocyte culture

Human cartilage specimens were obtained from patients undergoing TKR (Total Knee Replacement) and chondrocytes were isolated from severely damaged areas of OA cartilage (designated as OA chondrocytes). Normal chondrocytes were obtained from biopsy samples of normal cartilage. Tissue collection was approved by the Human Subjects Committee of Wonkwang University (Iksan, Chunbuk, Korea). Chondrocytes were seeded at 1.5 × 10^4 cells/cm^2 in chondrocyte growth media (Cell Applications, San Diego, CA, USA) with 100 units/mL penicillin and 100 µg/mL streptomycin (Gibco Invitrogen, Carlsbad, CA, USA).

2.2. Exosome isolation

Chondrocytes were cultured in DMEM with 10% exosome-depleted FBS (System Biosciences, Palo Alto, CA, USA) and 1% penicillin/streptomycin (Gibco) for 24 h at 37°C in a 5% CO₂ incubator. ExoQuick-TC (1 mL) was added and cells were incubated for 12 h at 4°C and then centrifuged at 1500×g for 30 min to obtain pelleted exosomes.

2.3. miRNA profiling

miRNA expression profiles were examined using the miScript System (Qiagen, Germantown, MD, USA). All procedures were performed according to the manufacturer’s instructions. Briefly, the reverse transcription (RT) reaction contained 1 µL of miScript RT mix, 4 µL of 5X miScript RT buffer, and 15 µL of RNase-free water. The reaction mix was incubated at 37°C for 60 min and 95°C for 5 min, then diluted to 100 µL with water. The qRT-PCR mixture was composed of 10 µL of SYBR Green PCR Master Mix, 2 µL of miScript universal primer, 2 µL of specific primer, 1 µL of cDNA, and 5 µL of RNase-free water. Amplification was performed with one cycle of 95°C for 15 min, followed by 40 cycles using the ABI StepOnePlus PCR system (Applied Biosystems, Foster City, CA, USA). Each reaction was performed in triplicate and normalized to the expression levels of RNU6. Gene expression data were analyzed and visualized using GenEx software (Weigenstephan, Germany).

2.4. lncRNA profiling

PCR for lncRNA-specific expression was performed using specific primers from LncRNA Profiler (System Biosciences). Total RNA was isolated using RNAiso Plus (TaKaRa, Shiga, Japan). cDNA was synthesized according to the manufacturer’s protocols and RT-PCR was carried out with an initial denaturation step at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min, using Maxima SYBR Green/ROX qPCR Master Mix 2X (Thermo Scientific, Pittsburgh, PA, USA). Data were analyzed and visualized using GenEx software.

2.5. Lentivirus packaging

HULC, miR-372-3p, and negative control lentiviruses were produced using the 3rd Generation Packaging Mix (Applied Biological Materials, Inc.; ABM, Richmond, BC, Canada). Plasmids were transfected into Lenti-X 293 T cells (Clontech, Mountain View, CA, USA) using Lentifectin (ABM) in Opti-MEM I medium (Gibco Invitrogen) and cultured overnight. The supernatant was collected and lentiviral particles were concentrated using Lenti-X Concentrator (Clontech) and stored at −80°C. Cells were infected with lentivirus supernatant for 2 days with 5 µg/mL polybrene in a 37°C humidified CO₂ incubator.

2.6. Apoptosis assay

Cells were washed twice with 1x phosphate buffered saline (PBS) and resuspended in 1% bovine serum albumin (BSA; Sigma Aldrich, St. Louis, MO, USA). Apoptotic cell counts were obtained with Muse Annexin V & Dead Cell Assay kit (Merck EMD Millipore, Billerica, MA, USA). Assays were performed according to the manufacturer’s instructions. Briefly, 1 × 10^5 cells in 1% BSA were added to 100 µL of Muse Annexin V & Dead Cell reagent (2x dilution). Samples were incubated at room temperature for 20 min and then analyzed using the MUSE Cell Analyzer (Merck EMD Millipore).

2.7. Proliferation assay

Cell proliferation assays were performed using the Quick Cell Proliferation Colorimetric Assay Kit (BioVision, Milpitas, CA, USA), according to the manufacturer’s
instructions. Briefly, cultured cells were seeded in 96-well culture plates (1 x 10^4 cells/well) and grown at 37°C in a CO₂ incubator. At each time point, assay reagent was added, samples were incubated for 4 h at 37°C, and absorbance at 460 nm was measured using a microplate reader, with a reference wavelength of 650 nm.

### 2.8. Western blotting

Cells were lysed with RIPA buffer (50 mM Tris-HCl, pH 7.4, containing 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 0.1% deoxycholic acid, 10 mM NaF, 10 mM Na₃P₂O₇, 0.4 mM Na₃VO₆, and protease inhibitors) for 30 min on ice. Total proteins were electrophoresed and transferred to nitrocellulose membranes (GE Healthcare, Marlborough, MA, USA). Each membrane was probed with specific antibodies for GSK-3β (BD Transduction Laboratories, San Jose, CA, USA) and GAPDH (Santa Cruz Biotechnology Inc, Dallas, TX, USA). Blots were developed using an HRP-conjugated secondary antibody and then immunoreactive proteins were detected with an ECL system (Dogen, Seoul, Korea).

### 2.9. Ethical approval

All procedures in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee.

## 3. Results

Consistent with previous reports (Song et al. 2015a), we observed a significant increase in apoptotic cell death and a significant decrease in cell proliferation in OA articular chondrocytes (n = 10) compared to that in normal chondrocytes (n = 10, Figure 1). To identify the key regulatory ncRNAs in exosomes responsible for stimulating apoptotic cell death or suppressing chondrocyte proliferation, we assessed the expression patterns of miRNAs using exosomes isolated from normal (n = 10) and OA chondrocytes (n = 10; Figure 2). The average value of 10 different normal chondrocytes isolated from normal biopsies was compared to the average value of 10 different OA chondrocytes isolated from OA cartilage, and the results were expressed as a heat-map. According to Ingenuity Pathway Analysis (IPA; Qiagen, Redwood City, CA, USA), miRNAs whose expression were altered significantly in OA chondrocytes could affect several molecular and cellular functions, such as cellular development, cellular growth and proliferation, cell death and survival, and the cell cycle (Figure 2A). Among these miRNAs, we selected miRNAs previously reported as OA-miRs including miR-146, miR-34, miR-9, and miR-373 (Wang and Chang 2011; Song et al. 2015a) and re-confirmed their expression levels. The expression level of miR-372-3p dramatically increased in exosomes of OA chondrocytes (average of 2.2-fold) isolated from OA cartilage of OA patients (n = 10) compared to that in exosomes of normal chondrocytes isolated from biopsy cartilage samples of normal patients (n = 10). Moreover, the cytoplasmic level of miR-372-3p was significantly reduced in OA chondrocytes compared to that in normal chondrocytes (Figure 2C). To identify the key regulatory IncRNAs in exosomes during OA pathogenesis, we analyzed the

![Figure 1](image-url)  
**Figure 1.** Stimulation of cell death and suppression of cell proliferation during OA pathogenesis. OA chondrocytes isolated from OA cartilage (n = 10) and normal chondrocytes (n = 10) isolated from normal biopsy cartilage were cultured and apoptosis and cell proliferation were analyzed. Results are shown as the mean, with error bars representing the 95% CI (lower/upper limit); *, p < 0.05.
expression patterns of IncRNAs using exosomes isolated from normal (n = 10) and OA chondrocytes (n = 10, Figure 2D). The average value of 10 different normal chondrocytes isolated from normal biopsies was compared to the average value of 10 different OA chondrocytes isolated from OA cartilage and results were expressed as a heat-map. Expression of several IncRNAs, such as Zfas1, KRASP1, SNHG6, Air, Sox2pt, IPW, WT1-AS, HULC, and NRON was high in exosomes secreted by OA chondrocytes (Figure 2D). The expression level of HULC was significantly decreased in exosomes of OA chondrocytes isolated from OA cartilage of OA patients compared to exosomes of normal chondrocytes isolated from biopsy cartilage sample of normal patients (Figure 2E). Moreover, cytoplasmic levels of HULC were significantly increased in OA chondrocytes compared to that in normal chondrocytes (Figure 2F).

To determine whether miR-372-3p and HULC are involved in molecular and cellular functions, such as cellular development, cellular growth and proliferation, cell death and survival, and the cell cycle, as suggested in IPA, we treated normal chondrocytes with OA exosomes. After treatment with OA exosomes, cell proliferation significantly decreased, whereas apoptotic cell death significantly increased (Figure 3A & D). As we treated normal chondrocytes with concentrated OA exosomes, we observed a more drastic suppression in cell proliferation compared to cell proliferation inhibited in OA chondrocytes.

Overexpression of HULC in normal chondrocytes dramatically suppressed cell proliferation and stimulated apoptotic cell death (Figure 3B & D). Conversely, overexpression of miR-372-3p in OA chondrocytes significantly increased cell proliferation and suppressed apoptotic cell death (Figure 3C & D). These data suggest a negative role of HULC and a positive role of miR-372-3p in cell growth and proliferation.

To identify the signaling regulator in secreting OA-specific exosomes with high miR-372-3p and low HULC, we modulated signaling pathways that are known to be involved in maintaining cartilage homeostasis. Among them, exosomes isolated from normal chondrocytes treated with a GSK inhibitor featured high miR-372-3p and low HULC expression (Figure 4A). Moreover, treatment of normal chondrocytes with the GSK inhibitor suppressed cell proliferation and stimulated apoptotic cell death up to 2-fold compared to controls (Figure 4B). We also confirmed that GSK was dramatically inhibited at the translational level in OA chondrocytes (Figure 4C).

Figure 2. Upregulation of miR-372-3p and downregulation of HULC in exosomes of OA chondrocytes. (A) miRNA profiles were analyzed with exosomes isolated from normal or OA chondrocytes and IPA was performed. (B) The exosomal level of miR-372-3p was analyzed in human OA patients (n = 10) compared to normal patients (n = 10). (C) The cytoplasmic level of miR-372-3p was analyzed in human OA patients (n = 10) compared to normal patients (n = 10). (D) IncRNA profiles were analyzed with exosomes isolated from normal or OA chondrocytes and IPA was performed. (E) The exosomal level of HULC was analyzed in human OA patients compared to normal patients. (F) The cytoplasmic level of HULC was analyzed in human OA patients compared to normal patients. Results are shown as the mean, with error bars representing the 95% CI (lower/upper limit); *, p < 0.05.
4. Discussion

Many studies have confirmed that exosomes can interact with recipient cells (Ramachandran and Palanisamy 2012; Song et al. 2015b) and regulate the function of target cells by binding to their surface, fusing with their cell membrane, or internalizing though receptor-mediated endocytosis (Keller et al. 2006; Raposo and Stoorvogel 2013). Secreted exosomes have emerged as promising reservoirs of diagnostic and prognostic biomarkers in various diseases, owing to the presence and differential abundance of ncRNAs (Properzi et al. 2013). Exosomes are known to be involved in a novel form of cell-independent communication by activating cell surface receptors on target cells and transferring bioactive signaling molecules (Lakkaraju and Rodriguez-Boulan 2008; Camussi et al. 2010). The role of exosomes in oncogenesis has been studied intensively. Selective enrichment of the let-7 miRNA family has been shown in exosomes derived from highly metastatic gastric cancer cells. As members of the let-7 miRNA family have tumor suppressor activity by targeting oncogenes, such as RAS and HMGA2, highly invasive tumor cells release let miRNAs through exosomes into the extracellular milieu to maintain the tumorigenic phenotype (Ohshima et al. 2010). The level of exosomal miR-372 was reported to be higher in receptor-negative and more aggressive breast carcinomas (Eichelser et al. 2014) and the level of exosomal miR-21 is significantly higher in patients with esophageal squamous cell cancer than in patients with benign diseases, with or without systemic inflammation (Tanaka et al. 2013). Besides cancer, some studies have reported a different signature of exosomes during pathogenesis of other diseases, including OA. In this study, we screened OA-specific exosomal ncRNAs. Compared to non-OA exosomes, miR-372-3p was the most highly enriched molecule in OA exosomes, whereas HULC was significantly lower in OA exosomes. This represents the first report describing selective loading of ncRNAs into...
Exosomes during OA pathogenesis. However, the mechanisms of this selective packaging and releasing are not well established.

Recently, Rabinowits and colleagues (2009) suggested that the content of tumor cell-derived exosomes is correlated with miRNA levels in primary tumors, and Fabbri and colleagues (2012) showed that tumor-secreted miR-21 and -29 act as ligands for human toll-like receptor (TLR)-8 and mouse TLR-7 in immune cells and this interaction between miRNA and TLR mediates a prometastatic inflammatory response (Fabbri et al. 2012). Besides the regulation of immune function, cancer cells may transfer miRNAs to surrounding cells via exosomes to stimulate cell proliferation and tumor growth (Liu et al. 2006). In this study, we also found that two ncRNAs, presented here as OA-specific exosomal ncRNAs, miR-372-3p and HULC, are involved in the apoptotic death of chondrocytes. Overexpression of miR-372-3p in chondrocytes significantly stimulated chondrocyte proliferation and inhibited apoptotic death of chondrocytes, whereas overexpression of HULC in chondrocytes significantly inhibited chondrocyte proliferation and stimulated apoptotic death of chondrocytes, as seen in Figure 5. Our data suggest that the selective packaging of miR-372-3p and prevention of HULC packaging into exosomes occurred during OA pathogenesis and affected apoptotic cell death. Unlike a previous study (Valadi et al. 2007) suggesting that exosomal miRNAs were transferred and caused inhibition of target genes, we found that selective active and negative packaging of beneficial ncRNAs were involved in cell survival and/or proliferation during OA pathogenesis.

It has been suggested that differential expression of ncRNAs in exosomes may be related to biological functions (Zhang et al. 2015). However, although some studies have recently shed light on this matter, regulatory mechanisms underlying differential packaging of exosomal molecules are not well known. It has been shown that constitutive MAPK activation in BCPAP and TPC1 cells increases the number of exosomes released per cell (Agarwal et al. 2015). In this study, we found that GSK signaling may be the key regulator of selective packaging observed during OA pathogenesis. However, further work is needed to explore the exact mechanisms of selective loading into exosomes during OA pathogenesis.

**Figure 4.** GSK-3β may be responsible for selective packaging of OA exosomes. (A) Cells were treated with 5 µM GSK-3β inhibitor and exosomal levels of miR-372-3p and HULC were analyzed. (B) Cells were treated with 5 µM GSK-3β inhibitor and cell proliferation and apoptosis were analyzed. Results are shown as the mean, with error bars representing the 95% CI (lower/upper limit); *, p < 0.05. C. GSK-3β protein levels were analyzed by western blotting using normal and OA chondrocyte cell lines.

**Figure 5.** Schematic diagram showing the selective packaging of exosomal ncRNA in normal and OA chondrocytes.
5. Conclusion

In summary, these data indicate that increased levels of HULC and decreased levels of miR-372-3p in OA exosomes may be responsible for stimulating chondrocyte apoptosis, and this OA-specific exosomal expression is regulated by GSK signaling.

Disclosure statement

No potential conflict of interest was reported by the authors.

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