Function of Heterogeneous Nuclear Ribonucleoprotein U and related MicroRNAs in Human Coronary Artery Endothelial Cells and Endothelial Microparticles

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I dedicate this work to my wife,

He Wang, and the rest of my family.

Contents

List of abbreviations

CAD	**coronary artery disease**
ACS	**acute coronary syndrome**
HCAEC	**human coronary artery endothelial cells**
MP	**microparticles**
EMP	**endothelial cell-derived microparticles**
MMP	**monocyte/macrophage microparticle**
PMP	**platelet microparticle**
miRNP	**microRNA binding protein**
RISC	**RNA-induced silencing complex**
RBP	**RNA binding protein**
HnRNP	**heterogeneous nuclear ribonucleoprotein**
HnRNP U	**heterogeneous nuclear ribonucleoprotein U**
BrdU	**5-bromo-2'-deoxyuridine**
DAPI	**4′,6-diamidino-2phenylindole**
EV	**extracellular vesicles**
AGO	**Argonaute**
miRNAs	**microRNAs**
microRNA-30c	**miRNA-30c**
microRNA-24	**miRNA-24**

1. Introduction

1.1 Motivation

Cardiovascular diseases and coronary artery disease (CAD) represent the leading cause of death worldwide, even though, since 1968, the mortality rate has fallen (Mozaffarian, Benjamin et al. 2016). Atherosclerosis is the developmental process of atheromatous plaques and predominant pathology in coronary artery disease. Endothelial cell dysfunction is regarded as the classic stimulus for the development of atherosclerotic lesions. Also, the inflammatory process plays a significant role in the etiology of atherosclerosis. Circulating microparticles (MP) are released in abundance in patients with cardiovascular diseases and contribute to the initiation and development of atherosclerosis and its complications (accumulating in atherosclerotic lesions). Circulating microparticles play a role in the pathogenesis of atherosclerosis and could be a biomarker for the early diagnosis of coronary artery disease (Koganti, Eleftheriou et al. 2016).

Endothelial-derived microparticles (EMP) are released into the circulatory system from activated or apoptosis endothelial cells and could influence cardiovascular disease pathogenesis via autocrine/paracrine signaling. EMP represent novel biological markers of endothelium dysfunction or injury as well as vasomotion disorders that are involved in the pathogenesis of cardiovascular, metabolic, and inflammatory diseases.

The heterogeneous nuclear ribonucleoproteins (hnRNPs) bind to a specific region of, and actively regulate, target protein translation (Kim, Lee et al. 2017). HnRNPs specifically bind miRNAs through the recognition of specific motifs and control miRNAs loading into extracellular vesicles (Villarroya-Beltri, Gutierrez-Vazquez et al. 2013). However, the exact function of hnRNP U and its related miRNAs are unknown. With this in mind, this study focuses on the role of the protein hnRNP U and its specific binding of miRNAs in human coronary artery endothelial cells (HCAEC).

1.2 Coronary Artery Disease, Endothelial Cells, and Atherosclerosis

The most important function of the endothelium is to prevent blood cell adhesion and inhibit clot formation. With extensive endothelial cell damage, injury, and apoptosis (due to classical cardiovascular risk factors, such as hypertension and smoking), the endothelium loses its integrity. In this sense, endothelium permeability increases and

inflammatory cells migrate from the blood vessel. Endothelial function is decisively influenced by the degree of endothelial cell apoptosis or death, such as endothelial denudation and plaque rupture, which result in acute coronary syndromes (ACS) (Lusis 2000).

Endothelial cells (EC) also play a crucial role in atherosclerosis progression and its clinical manifestations in the coronary artery (Lusis 2000). At the cellular level, endothelial dysfunction and progressive atherosclerosis are based on a gradual loss of endothelial cells (Werner and Nickenig 2006). Experimental and clinical studies show that endothelial dysfunction is closely related to the determinants of atherosclerosis (Libby 2001) and could predict adverse events in CAD patients (Schachinger, Britten et al. 2000, Heitzer, Schlinzig et al. 2001); in addition, it can be quantitatively assessed by the measurement of MP plasma levels (Werner, Wassmann et al. 2006, Bulut, Maier et al. 2008). The relationship between MP with CAD and atherosclerosis will be introduced in section 1.3.

1.3 The definition, history, and function of EMP

Microparticles (MP, also known as microvesicles or circulating microvesicles) are fragments of plasma membrane ranging from 100 nm to 1000 nm that are shed from various cell types following apoptosis or activation. Wolf described microparticles in 1967 (Wolf 1967) and, at that time, they were thought of as a kind of cellular debris. Over the last 50 years, however, numerous studies have demonstrated how circulating MP play a role in intercellular communication and transport mRNA, miRNA, and proteins between cells (Ratajczak, Miekus et al. 2006, McCarthy, Wilkinson et al. 2016). Circulating MP originate directly from the plasma membrane of the cell and reflect the antigenic content of the cells from which they arise. Thus, through flow cytometry, they can be classified and measured, for example, as endothelial microparticles (EMP), monocyte/macrophage microparticles (MMP), and platelet microparticles (PMP). MP contain cytoplasm and surface markers of their maternal cells of origin, such as CD31, CD144, and CD146 for endothelial cells, CD42 and CD61 for platelets, and CD45 for monocyte/macrophage cells (Hugel, Martinez et al. 2005, Prokopi, Pula et al. 2009). EMP are released into the circulation from activated and/or apoptosis endothelial cells and reflect disease severity and vascular and endothelial dysfunction (Distler, Pisetsky et al. 2005, Mause and Weber 2010, Sierko, Sokol et al. 2015, McCarthy, Wilkinson et al. 2016). Under

physiological conditions, the concentration of EMP in the blood is clinically insignificant (Sierko, Sokol et al. 2015). Endothelial microparticles (EMP) have been developed as a promising new method of assessing endothelial injury. Circulating levels of EMP are thought to reflect a balance between cell stimulation, proliferation, apoptosis, and cell death (Berezin, Zulli et al. 2015). Plasma EMP levels increase in patients with cardiovascular diseases (VanWijk, VanBavel et al. 2003, Boulanger 2010, Shantsila, Kamphuisen et al. 2010, Helbing, Olivier et al. 2014, Berezin, Zulli et al. 2015, Koganti, Eleftheriou et al. 2016); specific cardiovascular medications also affect plasma EMP levels (Amabile, Rautou et al. 2010). EMP are also biomarkers for the early detection of cardiovascular disease and its progression (Werner and Nickenig 2006, Sinning, Losch et al. 2011, Koganti, Eleftheriou et al. 2016, McCarthy, Wilkinson et al. 2016). Another function of EMP is to remove misfolded proteins, cytotoxic agents, and metabolic waste from the cell.

In summary, EMP have diverse functions in cardiovascular disease and could represent novel biomarkers in cardiovascular risk assessment.

1.4MicroRNA: definition, discovery, biogenesis, and function

MicroRNAs are tiny (21-24 nucleotides, about 22 nucleotides) non-coding RNA molecules found in plants, animals, and some viruses that regulate gene expression predominantly at the post-transcriptional level (Economou, Oikonomou et al. 2015). Most miRNAs are located within the cell, while some miRNAs, known as circulating miRNA or extracellular miRNA, have been found in the extracellular environment, including various biological fluids and cell culture media (Sohel 2016).

A recent study indicates that miRNAs are involved in many different biological processes as well as innovative diagnostics and therapeutic approaches for diseases such as atherosclerosis and coronary artery disease (especially in myocardial infarction) (Economou, Oikonomou et al. 2015). MiRNAs have been found not only in cardiac tissue but also in circulating blood. Thus, miRNAs are involved in intercellular communication and have been shown to circulate in the bloodstream in stable forms (Economou, Oikonomou et al. 2015). Pioneering studies describe how down-regulated miRNAs or elevated miRNAs in plasma could be a diagnostic biomarker for patients with coronary artery disease (FichtIscherer, De Rosa et al. 2010, Wang, Zhu et al. 2010). A few analyses have revealed that miRNAs play an essential role during heart development (Zhao, Ransom et al. 2007,

Chen, Murchison et al. 2008). More specifically, miRNA expression profiling studies have demonstrated that expression levels of specific miRNAs change in diseased human hearts, pointing to their involvement in cardiomyopathies (van Rooij, Sutherland et al. 2006, Tatsuguchi, Seok et al. 2007, Thum, Galuppo et al. 2007). Animal experiments have shown that particular miRNAs play a central role not only in heart development, but also in several pathological conditions, such as cardiogenesis, hypertrophic growth response, and cardiac conductance (Zhao, Samal et al. 2005, Care, Catalucci et al. 2007, Yang, Lin et al. 2007, Zhao, Ransom et al. 2007, Wagschal, Najafi-Shoushtari et al. 2015).

The RNA-induced silencing complex (RISC) is a multiprotein complex, specifically a ribonucleoprotein, which could incorporate miRNA (Filipowicz, Bhattacharyya et al. 2008). The mature 22 nucleotide miRNA products are incorporated into RISC. The mature miRNA bind RISC complex and regulate mRNA silencing in some ways, such as mRNA translation repression, mRNA degradation, heterochromatin formation, and DNA elimination.

1.5The RBP: definition, biogenesis, and function

In circulating blood, there are abundant RNases, which rapidly degrade circulating RNAs in plasma. Why plasma miRNAs are relatively stable is unknown. Non-coding RNAs which include miRNAs almost always function as ribonucleoprotein complexes and not as naked RNAs (Matera, Terns et al. 2007). Evidence shows that miRNAs bind RNA binding proteins (RBPs) in the circulation or are protected by incorporation in MP (Connerty, Ahadi et al. 2015). Further, high-throughput sequencing data demonstrates that miRNA expressions are significantly different in MP and their parental cells, and thus miRNAs seem to be selectively packaged from cells into MP (Diehl, Fricke et al. 2012).

RNA binding proteins are kinds of distinct cytoplasmic and nuclear proteins which contain a modular structure composed of RNA binding domains or motifs. These binding domains or motifs could bind the double or single stranded RNA which includes mRNA and non-coding RNA in cells. The RNA binding domain is composed of 80 amino acids and could bind to a short single stranded RNA sequence. Numerous binding motifs or domains have been found, such as RNA recognition motif, dsRNA binding domain, and zinc finger (Lunde, Moore et al. 2007).

RNA binding proteins have an important role in the post-transcriptional control of RNAs, such as through splicing, polyadenylation, mRNA stabilization, and mRNA localization and translation. Although RBPs play a crucial role in the post-transcriptional regulation of gene expression and cellular function, relatively few RBPs have been studied systematically (Glisovic, Bachorik et al. 2008, Hogan, Riordan et al. 2008). Most RBPs exist as complexes of protein and pre-mRNA in the nucleus and quickly export mature RNA from the nucleus to the cytoplasm. These kinds of RBPs are also called heterogeneous ribonucleoprotein particles (hnRNPs) and will be discussed in section 1.6.

1.6 The hnRNPs family and hnRNP U: definition, biogenesis, and function.

Heterogeneous nuclear ribonucleoproteins (hnRNPs) are multi complexes of RNA and protein present in the cell nucleus and cytoplasm. Their most important function is to bind the 3′- and 5′-UTRs of mature mRNAs, transport them from the nucleus to the cytoplasm, and promote protein synthesis (Geuens, Bouhy et al. 2016). Moreover, they play important roles in multiple aspects of nucleic acid metabolism, such as the packaging of nascent transcripts, mRNA stabilization, alternative splicing, and translational regulation.

The hnRNP family have different molecular weights ranging from 34 to 120 kDa, and they have been named alphabetically from A to U. All the above genes belong to the subfamily of ubiquitously expressed heterogeneous nuclear ribonucleoproteins and their name and binding domain or motif, as shown in Table 1.

The hnRNPs are not only related to influence pre-mRNA processing and other aspects of mRNA metabolism and transport but are also related to non-coding RNA, such as microRNA. Recently, several results have shown that hnRNPs bind several microRNAs in cell or exosomes. HnRNP A1 binds miR-18a *in vivo* (Guil and Caceres 2007). HnRNPA2B1 specifically binds miRNA-198 and miRNA-601 in exosomes *in vivo* (Villarroya-Beltri, Gutierrez-Vazquez et al. 2013). HnRNP K binds miR-122 with a nanomolar dissociation constant *in vitro* (Santangelo, Giurato et al. 2016). HnRNP Q directly binds miR-3470a, miR-194-2-3p, miR-6981-5p, miR-690, miR-365-2-5p, and miR-29b-3p in exosomes *in vitro* (Fan, Sutandy et al. 2015). Also, Laura Santangelo illustrated how hnRNPA2B1 binds miR-3470a, miR-194-2-3p, miR-365-2-5p, miR-6981-5p, miR-690, and miR-125b-5p *in vitro* (Fan, Sutandy et al. 2015).

Further, Konishi's results revealed that hnRNP A1 binds miR-26a, 29a, 29b, 107, 584, and 1229* (Konishi, Fujiya et al. 2015).

Name	RNA Binding domain or motif
HnRNP A (A0, A1, A1L1, A1L2, A3)	RRM, RGG, Glycine rich
hnRNP AB (AB, A2B1)	RRM, RGG, Glycine rich
hnRNP B1	RRM, RGG, Glycine rich
hnRNP C (C, CL)	RRM, Acidic rich
hnRNP D (D, DL)	KH
hnRNP F	qRRM, Glycine rich
hnRNP G	RRM, Glycine rich
hnRNP H (H1, H2, H3)	qRRM, Glycine rich
hnRNP K	KH, Proline-rich, others
hnRNP I	RRM
hnRNP L (L, LL)	RRM, Glycine rich
hnRNP M	RRM
hnRNP P	RRM, RGG, Glycine rich
hnRNP Q	RRM, Acidic rich
hnRNP R	RRM, RGG, Acidic rich, others
hnRNP U (U, UL1, UL2, UL3)	RGG, Acidic rich, others

Table 1: The name and structure of the hnRNPs family.

HnRNPs - heterogeneous nuclear ribonucleoproteins: RRM, RNA recognition motif; RGG, RNA-binding domain consisting of Arg-Gly-Gly repeats; qRRM, quasi-RNA recognition motif; KH, K-homology domain.

Figure 1 sets out a schematic diagram showing the way in which hnRNPs specifically sort miRNAs into EMP and secretion.

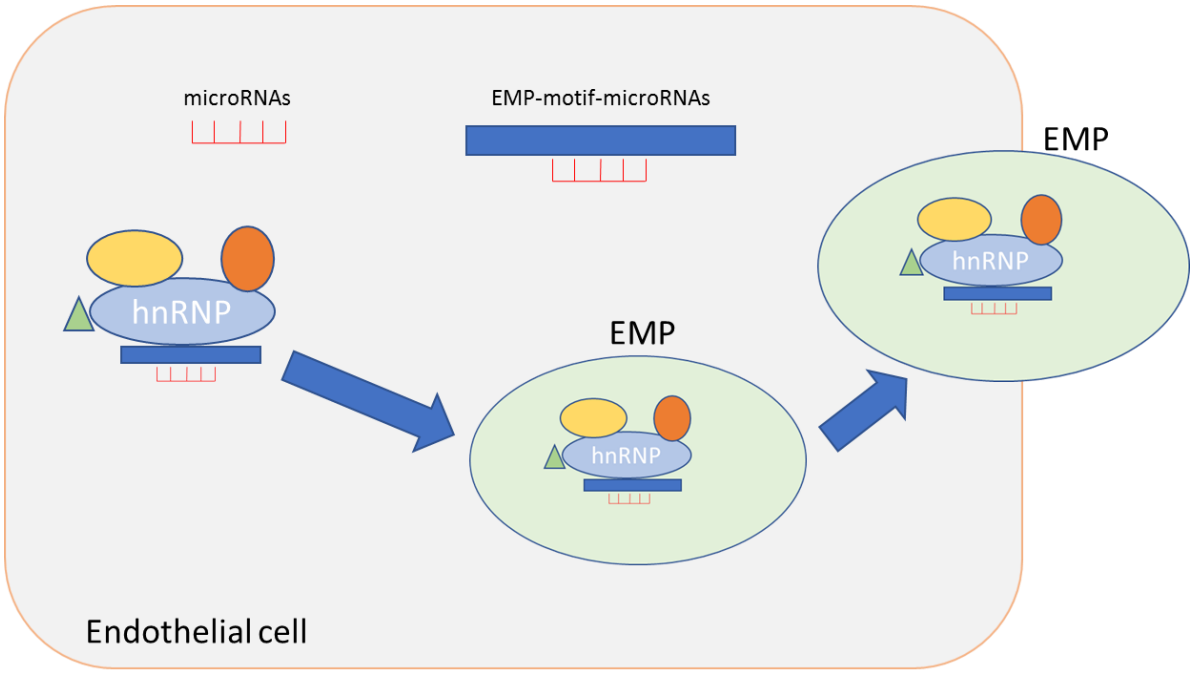


Figure 1: *Schematic diagram showing how hnRNPs specifically sort miRNAs into EMP and secretion.*
Modified from Santangelo, L., et al., The RNA Binding Protein SYNCRIP Is a Component of the Hepatocyte Exosomal Machinery Controlling MicroRNA Sorting. Cell Rep, 2016. ***17****(3): p. 799–808.*

HnRNP U (also known as scaffold attachment factor SAF-A, AFA, HNRPU, SAF-A, U21.1, hnRNP U, HNRNPU-AS1) is one of the most abundant hnRNPs in the heart and belongs to the family of hnRNPs. The hnRNP U protein has the highest molecular weight in the hnRNP family. The hnRNP U protein has two conserved

binding domains that could bind DNAs and RNAs (Hegde, Banerjee et al. 2012). The

N-terminal domain of hnRNP U mediates its binding to DNA and the C-terminal with the arginine-glycine-glycine motif is responsible for its RNA binding activities (Kim and Nikodem 1999). HnRNP U has several roles in mRNA metabolism, including the packaging of nascent mRNAs, alternative splicing, and regulation of translation (Han, Tang et al. 2010). The hnRNP U protein is an important regulator of cellular

processes, such as mRNA stability and de-stabilization, mRNA transport, mRNA transcription, and protein translation (Han, Tang et al. 2010). The hnRNP U protein plays an essential role in the development and function of mice's hearts (Ye, Beetz et al. 2015). HnRNP U-deficient hearts also cause cardiomyocyte disarray, leading to sudden death in mice (Ye, Beetz et al. 2015). Recent data shows that hnRNP U interacts with all classes of regulatory non-coding RNAs in the nucleus, including all small nucleolar RNAs (snRNAs) (Xiao, Tang et al. 2012).

The alleged role of hnRNP U in HCAEC has, according to present knowledge, not yet been addressed. In this regard, the present study hypothesizes that hnRNP U could bind microRNA and influence cellular function *in vitro*.

1.7 Aims

In this study, we aim to explore the role of hnRNP U and its associated miRNA in HCAEC and endothelial cell-derived MP.

2. Materials and methods

2.1 Cell culture and EMP generation

Human coronary artery endothelial cells (HCAEC, Promo Cell, Heidelberg, Germany) were cultured in an EC growth medium with endothelial growth medium Supplement Mix (Promo Cell, Heidelberg, Germany) under standard cell culture conditions (37°C, 5% CO_2). Cells of passage 7~8 were used when 90% confluent. Endothelial microparticles were generated from HCAEC, as previously described, with minor changes (Jansen, Yang et al. 2013). Briefly, HCAEC were starved by subjecting to basal medium without growth medium supplements for 24 hours to induce apoptosis. After starvation, all supernatant medium was collected and centrifuged twice (1500 × g, 15 min and 20 000 × g, 40 min) at 4 degrees Celsius. The obtained EMP were washed in sterile PBS (pH 7.4) and pelleted again at 20 000 × g.

Microparticles derived from si-hnRNP U and scrambled siRNA-treated HCAEC were defined as $EMP^{si\text{-}hnRNP\ U}$ and $EMP^{siRNA\ negative\ control}$. By generating $EMP^{si\text{-}hnRNP\ U}$ and $EMP^{siRNA\ negative\ control}$ derived from HCAEC transfected si-hnRNP U and scrambled siRNA, 80% confluent HCAEC were stimulated with 20 mM si-hnRNP U and scrambled-siRNA for 48 hours (Federici, Menghini et al. 2002) and then subjected to a basal medium without growth medium supplements for 24 hours in order to generate EMP. Isolated EMP were re-suspended in sterile PBS and used fresh.

2.2 Western blot

HCAEC were lysed in a RIPA buffer (Sigma, cat#0278, USA) with protease inhibitor cocktail tablets (Roche, cat#1071140, Germany) on ice for 30 minutes. Then lysates were ultrasonicated 3 times for 15 seconds and centrifuged at 13000 rpm for 15 min. After this, a Lowry protein assay (Bio-Rad, Munich, Germany) was performed to measure protein concentration. Equal amounts of protein (15-20 µg) and SDS-loading buffer were mixed and boiled, run into an 8% SDS electrophoresis buffer, and transferred onto polyvinylidene difluoride (PVDF) membranes. Membranes were then blocked in a 5% BSA-PBS for one hour at room temperature. Next, blot membranes were incubated overnight at 4 degrees Celsius with the appropriate primary antibodies, such as anti-hnRNP U (Abcam Biotechnology, ab10297), hnRNP K (Abcam Biotechnology, ab39975), hnRNP A2B1(Abcam Biotechnology, ab6102), and anti-GAPDH (Hytest,cat#:5G4Mab6c5). The membranes were washed three times with a 0.1% PSB-T and then incubated with HRP-conjugated secondary

antibodies (goat anti-mouse peroxidase, Thermo Scientific, cat#31446) for one hour at room temperature. Proteins were revealed by chemiluminescence using the ECL™ primer western blotting detection kit (GE Healthcare, cat#2232, UK). GAPDH was the loading control. Image J software was used to analyze the grayscale.

2.3 EMP mass spectrometry

Proteins were in-gel digested using a previously described protocol (Bonzon-Kulichenko, Perez-Hernandez et al. 2011). Briefly, EMP were isolated and lysis by RIPA buffer was performed and then loaded into a SDS-PAGE gel. A protein band was stained with *Coomassie* brilliant *blue* and digested with trypsin. Ammonium bicarbonate extracted the resulting tryptic peptides. Peptide identification was performed in cooperation with the Institute of Biochemistry and Molecular Biology, University of Bonn. The MS/MS raw files were searched according to the Human Swiss-Prot database (http://www.uniprot.org/uniprot UniProt release, 20168 sequence entries for human). SEQUEST results were confirmed using the probability ratio method (Martinez-Bartolome, Navarro et al. 2008), and false discovery rates were calculated using the refined method (Navarro and Vazquez 2009). Peptide and scan-counting were performed assuming as positive events those with an FDR equal to or lower than 5%.

2.4 RNA immunoprecipitation (RIP)

Each 10 cm dish of endothelial cells was lysed in 1ml of RIPA buffer and incubated for pre-clearing with pre-washed Protein G/A Dynabeads (Roche, REF#11719416001/11719408001, Germany) (40 µl per 10 cm plate cells) (1 h, 4°C). A total of 40 µl of Dynabeads were washed twice and re-suspended in 200 µl containing 10 µg mouse anti-hnRNP U (Abcam, ab10297), or mouse anti-IgG control (Abcam, ab200699), and then they were incubated for one hour at room temperature. Mouse Ig G was used as an isotype control. Lysates were incubated with antibody-conjugated Dynabeads (1.5 h, 4°C). Antibody-target protein conjugated Dynabeads complex was washed three times with an RIPA buffer. After this, the pellets were an hnRNP U- anti hnRNP U antibody-Dynabeads complex, and this was divided equally and transferred to clean tubes.

For protein detection, an SDS-loading buffer was mixed and boiled at 95°C for 10 min and processed for western blot, according to section 2.2.

For microarray and RT-PCR analysis, 500 µl of Trizol lysis reagent (Ambion Life technology, REF15596018) was added and samples were vortexed. Then, the total RNA was isolated from the hnRNP U- anti hnRNP U antibody-Dynabeads complex by Trizol extraction method, according to sections 2.5 and 2.6.

2.5 Taqman microRNA array

RNA isolated from $HCAEC^{hnRNP\ U\text{-}IP}$ and $HCAEC^{Ig\ G\text{-}IP}$ was converted to cDNA by priming with a mixture of looped primers (Human MegaPlex Primer Pools; Applied Biosystems). The miRNA profiles were performed with TaqMan® Array MicroRNA Cards for a total of 384 different assays specific to human miRNAs under standard real-time PCR conditions. Quantitative real-time PCR was conducted by Applied Biosystems 7900HT thermocycler using the manufacturer's recommended program Detailed analysis of the results was performed with DataAnalysis v3.0 Software (Applied Biosystems). All CT values above 40 were defined as undetectable. More than a four-fold difference was considered indicating a significant change.

2.6 The expression of mRNA and miRNA

Human coronary artery endothelial cells and EMP were lysed in Trizol (Ambion Life technology, REF15596018). Total RNA was extracted according to the manufacturer's instructions and quantified using a NanoDrop spectrophotometer.

For mRNA expression, 1 µg (HCAEC) or 200ng (EMP) of the extracted total RNA was reversely transcribed to cDNA. An Omniscript@RT Kit (Qiagen, Germany) was used according to the manufacturer's protocols. The single-stranded cDNA was amplified by a real-time polymerase chain reaction with the TaqMan system (ABI-7500 (Life Technologies, Germany) Fast PCR System). All primers were bought from Life Technologies (hnRNP U, Hs00244919, Life Technologies, Germany; GAPDH, Hs03929097, Life Technologies, Germany). GAPDH was used as an endogenous control. The delta Ct method was used to quantify relative mRNA expression.

For miRNA expression, 10 ng of total RNA was reversely transcribed to cDNA. TaqMan®microRNA Reverse Transcription kit (Applied Biosystems, Life Technologies, Germany) was used according to the manufacturer's protocols. Then, quantitative real-time PCR was performed and Taqman miRNA assays (Applied Biosystems) were used to measure miRNA-30c, miRNA-100, and miRNA-24 levels on a 7500 HT Real-Time PCR machine (Applied Biosystems). All primers were bought from Life Technology (hsa-miR-30c-5p, Hs000419; hsa-miR-24, Hs000402;

hsa-miR-100, Hs000437). RNU-6b served as an endogenous control. MiRNA-30c and miRNA-24 mimics were used to create the standard curve and calculate the copy number. Copy numbers were used to quantify absolute miRNAs expression.

2.7 Transfection in HCAEC

To generate miRNA-30c-overexpressing and miRNA-30c-down-expressing HCAEC lines, HCAEC were transfected with microRNA-30c mimic, microRNA-30c inhibitor, and microRNA control (100pM, all from Applied Biosystems, miRNA-30c Cat # A25576) using Lipofectamine 2000 (Invitrogen, Life Technology, REF#1168-019) for 24 hours. Functional assays were performed in 48 hours.

To generate miRNA-24-overexpressing and miRNA-24-down-expressing HCAEC lines, HCAEC were transfected with microRNA-24 mimic, microRNA-24 inhibitor, and microRNA control (100pM, all from Applied Biosystems, miRNA-24 Cat. # A25576) using Lipofectamine 2000 (Invitrogen, Life Technology, REF#1168-019) for 24 hours. Functional assays were performed in 48 hours.

2.8 The protein of hnRNP U silencing

To generate hnRNP U down-expressing HCAEC lines, HCAEC were transfected with si-hnRNP U. Scrambled siRNA served as a negative control. HCAEC were transfected with si-hnRNP U (Ambion, cat#AM16708, ID 145414) and negative control siRNA (Ambion, cat#AM4611, ID 145414). HCAEC were transfected with a pool of single-stranded siRNAs targeting hnRNP U for 48 hours. Cells were incubated in a 10 cm dish and 6-well plate, separately adding 300pM and 100pM si-hnRNP U and siRNA negative control.

2.9 Flow cytometry analysis

In order to count the number of EMP, Trucount™ tubes were used (BD Biosciences, cat#340334) and analyzed with FACS BD LSR II. Annexin V positive (AnnV+) with CD 31+ positive EMP were counted using Trucount beads. The formula for calculating for EMP concentrations was as follow:

$$\frac{\text{number of events for annexin V}}{\text{number of events in Trucount bead region}} \times \frac{\text{number of Trucount beads per test}}{\text{test volume}}$$

EMP was used at a concentration of 2000 AnnV+ EMP/µl for all experiments. Nile red particles sized from 0.7 to 0.9 µm (Spherotech, USA) were used as reference beads and set the gate for EMP. Briefly, 100 µl of EMP was re-suspended in PBS, adding 4 µl CD31+APC antibody (Invitrogen, Cat#A16224, USA) in each tube, and incubated for 45 minutes in a dark environment at room temperature. Next, 5 µl of Annexin V-FITC (BD Biosciences, Cat#556419, USA) was added to each tube. After incubation for 15 min in a dark environment at room temperature, PBS was washed twice and centrifuged for 20 minutes at 20 000 g. Pelleted EMP were re-suspended in a 200 µl annexin V-binding buffer (10mM HEPES, pH 7.4, 140mM NaCl, 2.5 mM $CaCl_2$), and were measured by flow cytometry and analyzed with FACS BD LSR II.

2.10 Boyden chamber assay

HCAEC (passage 7) were seeded onto the upper compartment of Boyden chambers (BD Falcon, Germany) with Transwell polycarbonate inserts (8.0µm pore size) for one hour. EMP were added to the lower well of the Boyden chamber and given a soft shaking, then incubating for six hours to allow for cell migration. The insert was removed and the upper side of the insert was scraped off with a rubber cell lifter. Inserts were fixed with 4% fresh paraformaldehyde and stained with DAPI. Cell migration was quantified by counting the cells of five random microscopic fields (×100) in each well.

2.11 Wound scratch assay

Passage 7 HCAEC were used when grown to confluence in a 6-well plate, and a sterile pipette (100 ul) was used to make a scratch. After the scratch, EMP were added to the cells. Cells were photographed in a marked position at 0, 6, and 10 hours. The remaining cell-free area was measured and correlated (in percentage) to the initially scratched area.

2.12 Proliferation assay

HCAEC in the basal medium were deprived of growth medium supplements and coincubated with EMP for six hours. The cells were then pulsed with BrdU (10µM, BD) for six hours. Cells were fixed and denatured. BrdU incorporation was detected using rat anti-BrdU (Abcam, ab53435) and secondary antibody anti-rat cy3 (Jackson ImmunoResearch, cat#709-165-149, USA). Nuclei were stained with DAPI (Vector Laboratories, CA94010, USA). All photographs were taken using a Zeiss Axiovert 200M microscope and AxioVision software.

2.13 Statistical analysis

All data is expressed as the Mean ± SD or Mean ± SEM. Means between two categories were compared using a two-tailed unpaired Student's *t*-test. A one-way ANOVA test was applied for comparisons of categorical variables when the data fitted the homogeneity of variance. For post hoc analysis, a Bonferroni test was used. Statistical significance was assumed. All *P*-values less than 0.05 were considered a statistically significant difference. All statistical analyses were performed with GraphPad Prism 5.

3. Results

3.1 hnRNPs were expressed in HCAEC

Endothelial microparticles (EMP) regulate several processes in cardiovascular biology by transferring proteins or nucleic acids and can act as cell-to-cell messengers. To determine which proteins are expressed in EMP, *mass spectrometry* (MS) assay was done, and the RNA binding protein database (http://rbpdb.ccbr.utoronto.ca/) was used for further analysis. The MS results showed that there were nearly 2500 different kinds of proteins in EMP, out of which 125 were RNA binding proteins, as illustrated in Figure 3.1.1. Table 1 lists the twenty highest expressed RNA binding proteins in EMP; from this, hnRNP U, hnRNP K, and hnRNP A2B1 are the top three expressed RNA binding proteins.

Also, the total HCAEC and EMP proteins were lysed and a western blot was performed. Unfortunately, hnRNP U, K, and A2B1 could not be found in EMP. However, hnRNP U showed higher expression than hnRNP K and hnRNP A2B1 in HCAEC, as illustrated in Figure 3.1.2 (in this sense, hnRNP U bands are stronger than hnRNP K and A2B1). Therefore, hnRNP U was focused upon as a target protein for further experiments.

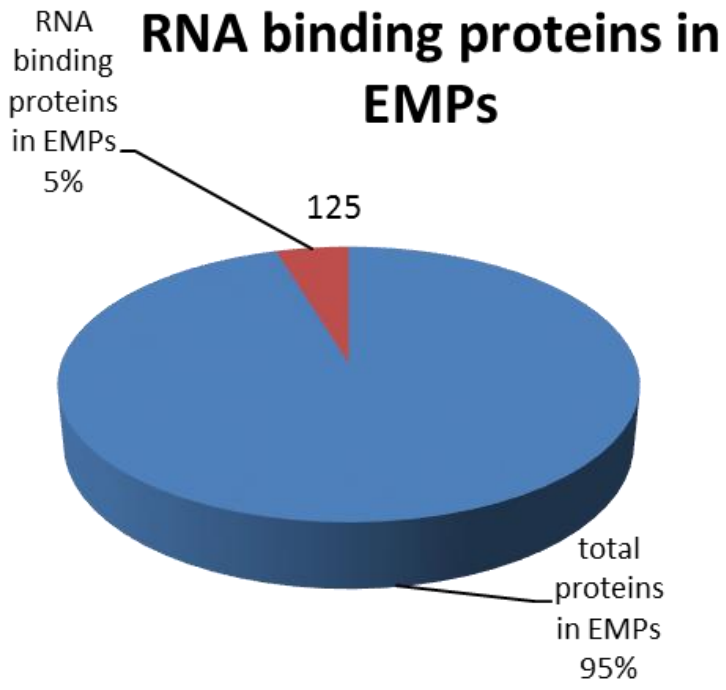


***Figure 2 3.1.1**: EMP- Mass Spectrometry results.*
125 types of RNA binding proteins were predicted in EMP.

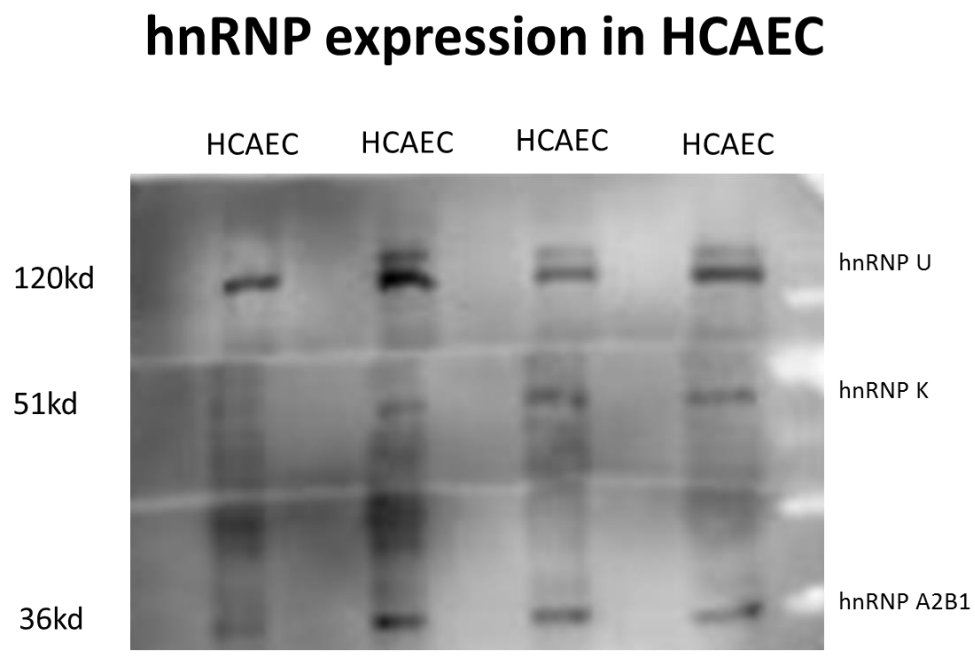


***Figure 3 3.1.2**: The expression of hnRNPs in HCAEC.*
The proteins hnRNP U, K and A2B1 were confirmed by the western blot. The bands of hnRNP U were strongest in the three bands. Unluckily, three kinds of hnRNP could not be detected in EMP by the western blot.

Accession	Protein	Area	Description
Q00839	HNRNPU	5,091E8	Heterogeneous nuclear ribonucleoprotein U OS =Homo sapiens GN=HNRNPU PE=1 SV=6 - [HNRPU_HUMAN]
P22626	HNRNPA2B1	5,023E8	Heterogeneous nuclear ribonucleoproteins A2/B1 OS =Homo sapiens GN=HNRNPA2B1 PE=1 SV=2 - [ROA2_HUMAN]
P61978	HNRPK	3,904E8	Heterogeneous nuclear ribonucleoprotein K OS =Homo sapiens GN=HNRNPK PE=1 SV=1 - [HNRPK_HUMAN]
P07910	HNRPC	3,672E8	Heterogeneous nuclear ribonucleoproteins C1/C2 OS =Homo sapiens GN=HNRNPC PE=1 SV=4 - [HNRPC_HUMAN]
Q14103	HNRPD	3,661E8	Heterogeneous nuclear ribonucleoprotein D0 OS =Homo sapiens GN=HNRNPD PE=1 SV=1 - [HNRPD_HUMAN]
P04406	G3P	3,336E8	Glyceraldehyde-3-phosphate dehydrogenase OS =Homo sapiens GN=GAPDH PE=1 SV=3 - [G3P_HUMAN]
P52272	HNRPM	3,101E8	Heterogeneous nuclear ribonucleoprotein M OS =Homo sapiens GN=HNRNPM PE=1 SV=3 - [HNRPM_HUMAN]
Q15365	PCBP1	3,092E8	Poly(rC)-binding protein 1 OS=Homo sapiens GN =PCBP1 PE=1 SV=2 - [PCBP1_HUMAN]
P31943	HNRH1	3,089E8	Heterogeneous nuclear ribonucleoprotein H OS =Homo sapiens GN=HNRNPH1 PE=1 SV=4 - [HNRH1_HUMAN]
P23246	SFPQ	3,008E8	Splicing factor, proline- and glutamine-rich OS =Homo sapiens GN=SFPQ PE=1 SV=2 - [SFPQ_HUMAN]
P55795	HNRH2	2,864E8	Heterogeneous nuclear ribonucleoprotein H2 OS =Homo sapiens GN=HNRNPH2 PE=1 SV=1 - [HNRH2_HUMAN]
Q15366	PCBP2	2,851E8	Poly(rC)-binding protein 2 OS=Homo sapiens GN =PCBP2 PE=1 SV=1 - [PCBP2_HUMAN]
P52597	HNRP	2,026E8	Heterogeneous nuclear ribonucleoprotein F OS =Homo sapiens GN=HNRNPF PE=1 SV=3 - [HNRPF_HUMAN]
P09651	A1	2,002E8	Heterogeneous nuclear ribonucleoprotein A1 OS =Homo sapiens GN=HNRNPA1 PE=1 SV=5 - [ROA1_HUMAN]
Q15233	NONO	1,980E8	Non-POU domain-containing octamer-binding protein OS =Homo sapiens GN=NONO PE=1 SV=4 - [NONO_HUMAN]
P43243	MATR3	1,860E8	Matrin-3 OS=Homo sapiens GN=MATR3 PE=1 SV=2 - [MATR3_HUMAN]
P11940	PABP1	1,767E8	Polyadenylate-binding protein 1 OS =Homo sapiens GN=PABPC1 PE=1 SV=2 - [PABP1_HUMAN]
P26599	PTBP1	1,441E8	Polypyrimidine tract-binding protein 1 OS =Homo sapiens GN=PTBP1 PE=1 SV=1 - [PTBP1_HUMAN]
O14979	HNRDL	1,408E8	Heterogeneous nuclear ribonucleoprotein D-like OS =Homo sapiens GN=HNRPDL PE=1 SV=3 - [HNRDL_HUMAN]
P51991	HNRNPA3	1,370E8	Heterogeneous nuclear ribonucleoprotein A3 OS =Homo sapiens GN=HNRNPA3 PE=1 SV=2 - [ROA3_HUMAN]

Table 2: The twenty highest expressed RNA binding proteins in EMP.

HnRNP U, hnRNP K, and hnRNP A2B1 were the top three expressed RNA binding proteins.

3.2 MiRNA-30c and miRNA-24 strongly bind to cellular hnRNP U

According to the western blot results depicted in Figure 3.1.2, hnRNP U showed the highest expression of the three candidate proteins in HCAEC. Thus, we focused on the hnRNP U function in HCAEC.

HnRNP U is an important regulator of cellular processes, such as mRNA transport, mRNA transcription, and protein translation (Han, Tang et al. 2010). As hnRPN U is an RNA-binding protein, the aim of this study is to assess whether miRNAs are bound to hnRNP U and might mediate its cellular processes and functions.

To assess whether some miRNAs specifically bind to the hnRNP U protein, hnRNP U-RIP-microarray analysis from HCAEC was conducted. The immunoprecipitation of hnRNP U was performed in HCAEC and subsequent western blot analysis revealed a proper isolation of hnRNP U from the precipitant (Figure 3.2.1). Total RNA was eluted from isolated hnRNP U. Microarray analysis showed that miRNA-30c, miRNA-100, and miRNA-24 were the miRNAs bound to hnRNP U with the highest expression compared to the control. RT-PCR analysis confirmed the higher expression of miRNA-30c and miRNA-24 in isolated hnRNP U compared to the control (Figure 3.2.2: 17.32- and 3.24- fold higher; p-values of 0.04 and 0.10 respectively). However, the result for miR-100 showed no significant differences (Figure 3.2.2). Overall, the microarray and RT-PCR results revealed that hnRNP U binds miRNA-30c and miRNA-24. Therefore, the following experiments focused on the relationship between miRNA-30c and miRNA-24 with hnRNP U.

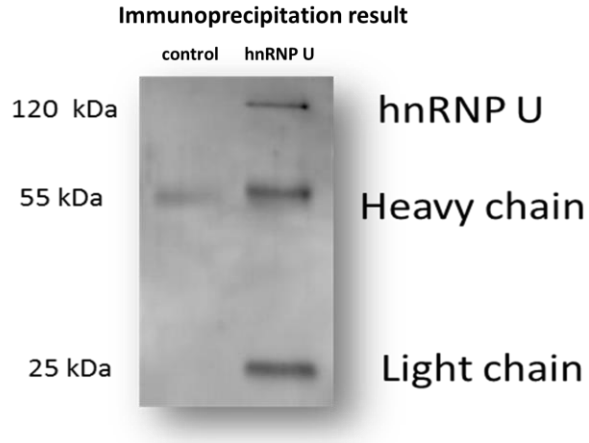


Figure 4 3.2.1: *Immunoprecipitation-western blot result.*

Western blot analysis revealed a proper isolation of hnRNP U from the precipitant. Ig G antibody as control.

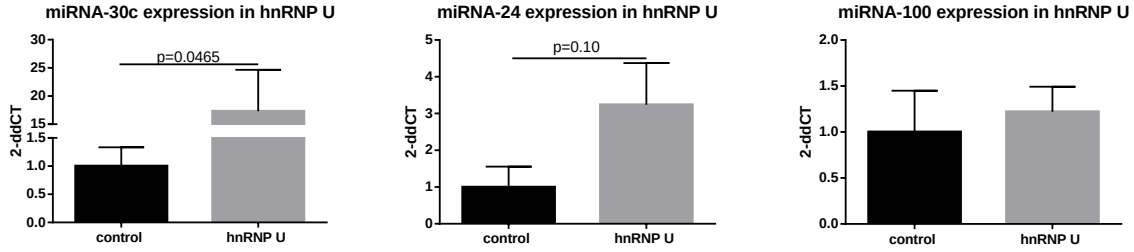


Figure 5 3.2.2: *MicroRNAs expression in protein hnRNP U.*

The RT-PCR analysis confirmed the higher expression of miRNA-30c and miRNA-24 in isolated hnRNP U compared to the control. However, the result of miR-100 showed no significant differences.

3.3 The expression of hnRNP U, miRNA-30c, and miRNA-24 in HCAEC and EMP

To investigate the relationship between hnRNP U with miRNA-30c and miRNA-24, hnRNP U was down-regulated in HCAEC by si-RNA. The RT-PCR and western blot analyses confirmed the inhibitory efficacy of hnRNP U at the mRNA and protein levels. RT-PCR results revealed that the hnRNP U mRNA level decreased when hnRNP U-siRNA was transfected for 24 hours (Figure 3.3.1: siRNA-negative control vs. si-hnRNP U,1.00 ± 0.19 vs. 0.57 ± 0.12, n=8, p=0.07). The western blot results showed that hnRNP U-siRNA significantly decreased the protein levels of hnRNP U after being transfected with hnRNP U-siRNA for 48 hours (Figure 3.3.2: siRNA-negative control vs. si-hnRNP U, 1±0 vs. 0.63±0.02, n=3, p=0.001). Furthermore, miRNA-30c and miRNA-24 both showed lower expression in the hnRNP U down-regulation group than in the control group (Figure 3.3.3: miRNA-30c copy number, si-hnRNP U vs. siRNA-negative control, 1.52 ± 0.33 vs. 0.75 ± 0.72 $\times 10^6$, n=8, p=0.04; miRNA-24 copy number, si-hnRNP U vs. siRNA-negative control, 7.22 ± 0.70 vs. 9.54 ± 0.75 $\times 10^6$, n=8, p=0.046).

As we know, EMP are released into the circulation from activated endothelial cells and reflect disease severity, including vascular and endothelial dysfunction, which could influence disease pathogenesis via autocrine/paracrine signaling. Subsequently, this study focused on whether the decrease in the hnRNP U protein could affect the EMP released and miRNA levels in EMP.

EMP was isolated and total RNA was extracted. The RT-PCR result showed that the expression of miRNA-30c and miRNA-24 in $EMP^{si\text{-}hnRNP\ U}$, derived from si-hnRNP U

transfected HCAEC, were both expressed higher than the $EMP^{siRNA\ negative\ control}$, which were derived from si-RNA negative control transfected HCAEC (Figure 3.3.3: miRNA-30c copy number, $EMP^{si\text{-}hnRNP\ U}$ vs. $EMP^{siRNA\ negative\ control}$, 3.15± 0.98 vs. 0.86 ± 0.24 $\times 10^4$, n=6, p=0.04; Figure 3.3.4 miRNA-24 copy number, $EMP^{si\text{-}hnRNP\ U}$ vs. $EMP^{siRNA\ negative\ control}$, 5.28± 1.58 vs. 11.50 ± 2.1 $\times 10^4$, n=6, p=0.03).

Furthermore, the flow cytometry result revealed that si-hnRNP U transfected HCAEC might have no influence on EMP secretion (Figure 3.3.5: EMP numbers per microliter, si-hnRNP U vs. siRNA-negative control, 5.4 ± 0.77 vs. 4.60 ± 0.38$\times 10^3$, n=5, p=0.37).

In summary, the data showed that miRNA-30c and miRNA-24 levels decreased consistently with the hnRNP U mRNA and protein level downregulation. Thus, the association of miRNA-30c and miRNA-24 with hnRNP U was confirmed. However, with the decrease of hnRNP U, the level of miRNA-30c and miRNA-24 were both expressed higher in $EMP^{si\text{-}hnRNP\ U}$ when compared with the $EMP^{siRNA\ negative\ control}$.

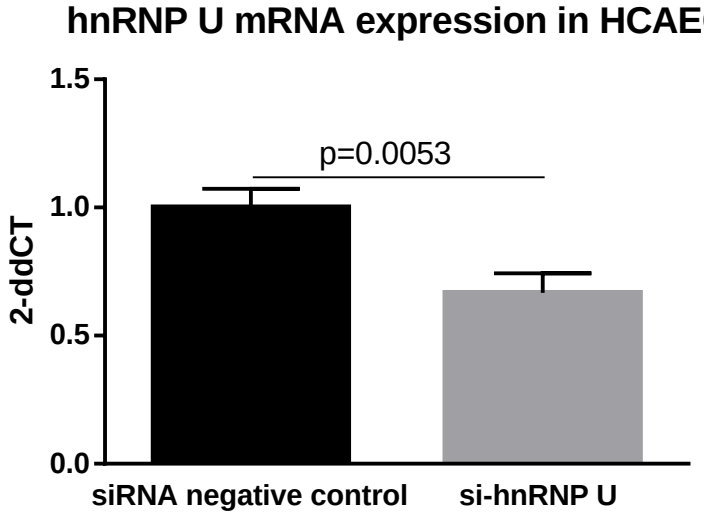
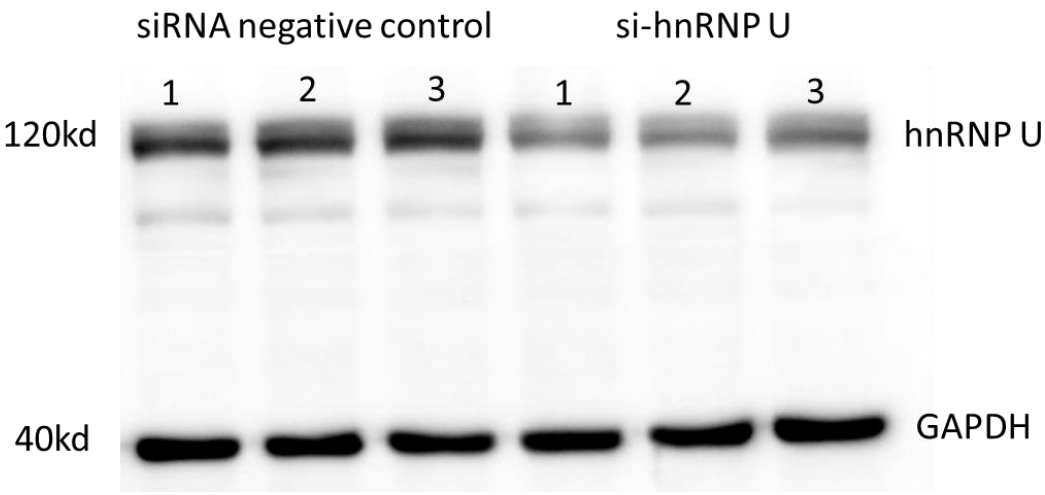


Figure 6 3.3.1: *The mRNA expression of hnRNP U in HCAEC.*
RT-PCR result revealed that the hnRNP U mRNA level decreased when transfected si-hnRNP U 24 hours. n=11.



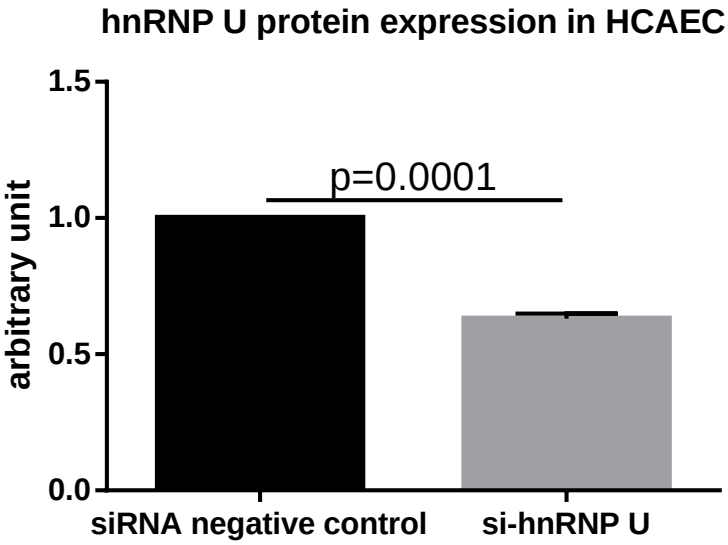


Figure 7 3.3.2**:** *The protein of hnRNP U expression in HCAEC.*
The western blot result revealed the protein level of hnRNP U was significantly decreased when transfected si-hnRNP U 48 hours. n=3.

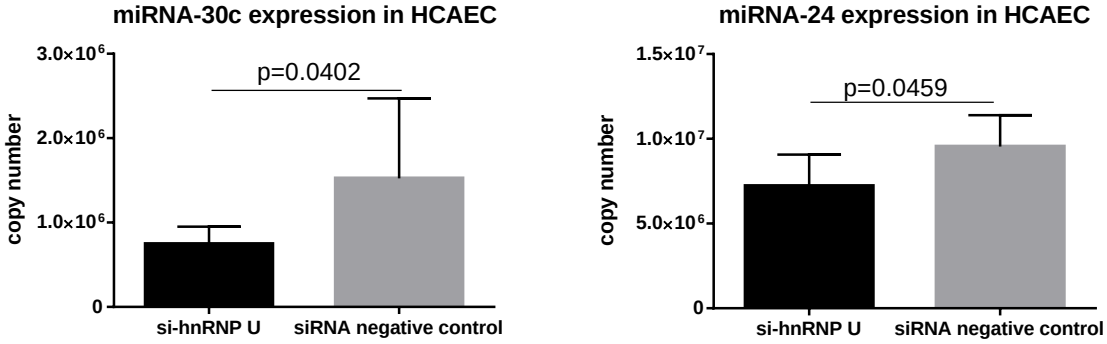


Figure 8 3.3.3**:** *MiRNA-30c and miRNA-24 expression in HCAEC$^{si\text{-}hnRNP\ U}$ and the HCAEC$^{siRNA\ negative\ control}$.*

RT-PCR result revealed that the copy number value of miRNA-30c and miRNA-24 were significantly decreased in HCAEC$^{si\text{-}hnRNP\ U}$ when compared with the HCAEC$^{siRNA\ negative\ control}$. MiRNA-30c mimic and miRNA-24 mimic were used to make the standard curve and calculate the copy number. n=8

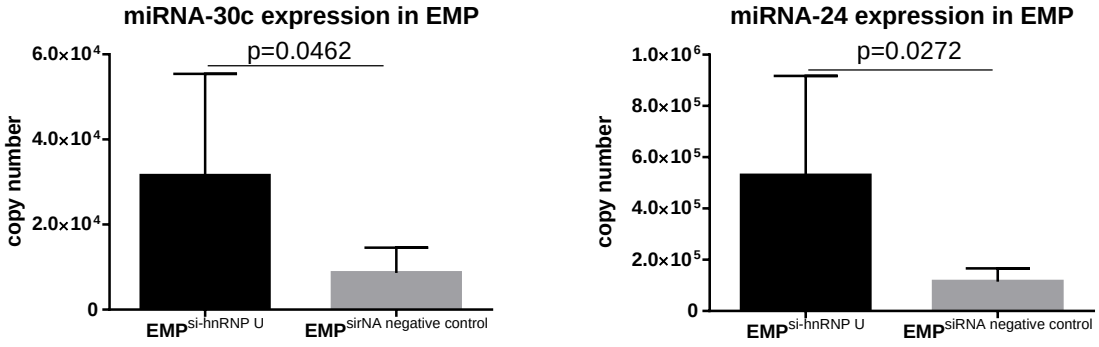


Figure 9 3.3.4: *MiRNA-30c and miRNA-24 expression in EMP$^{si\text{-}hnRNP\ U}$ and EMP$^{siRNA\ negative\ control}$.*

RT-PCR result revealed that the copy number value of miRNA-30c and miRNA-24 were significantly increased in EMP$^{si\text{-}hnRNP\ U}$ and the EMP$^{siRNA\ negative\ control}$. MiRNA-30c mimic and miRNA-24 mimic were used to make the standard curve and calculate the copy number. n=6.

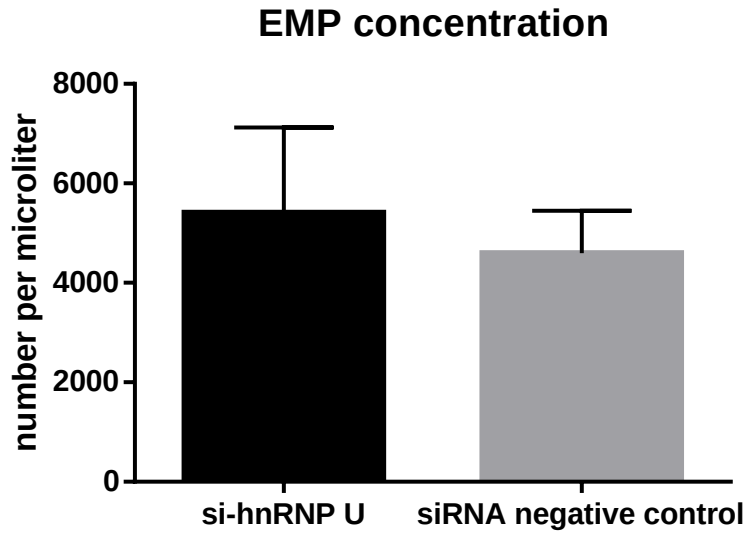


Figure 10 3.3.5: *EMP concentration results.*

The flow cytometry result revealed that si-hnRNP U transfected HCAEC might have no influence on the EMP secretion. n=5.

3.4MiRNA-30c and miRNA-24 expression in supernatant.

To explore the underlying mechanisms of the controversial miRNA expression, such as in hnRNP U-negative HCAEC and EMP, the miRNA-30c and miRNA-24 expression in the supernatant was measured.

The results showed that in the HCAEC supernatant, miRNA-30c, and miRNA-24 showed higher expression after hnRNP U downregulation when compared with the control group, whereas there was no difference in cells (Figure 3.4.2: miRNA-30c copy number, si-hnRNP U supernatant vs. siRNA-negative control supernatant, 16.85 ± 2.26 vs. 4.52 ± 0.92×10^5, n=6, p<0.01; Figure 3.4.4: miRNA-24 copy number, si-hnRNP U supernatant vs. siRNA-negative control supernatant, 5.83 ± 1.08 vs. 49.46± 8.33 ×10^5, n=6, p<0.01).

The data confirms that si-hnRNP U HCAEC might secrete miRNA-30c and miRNA-24 into the supernatant.

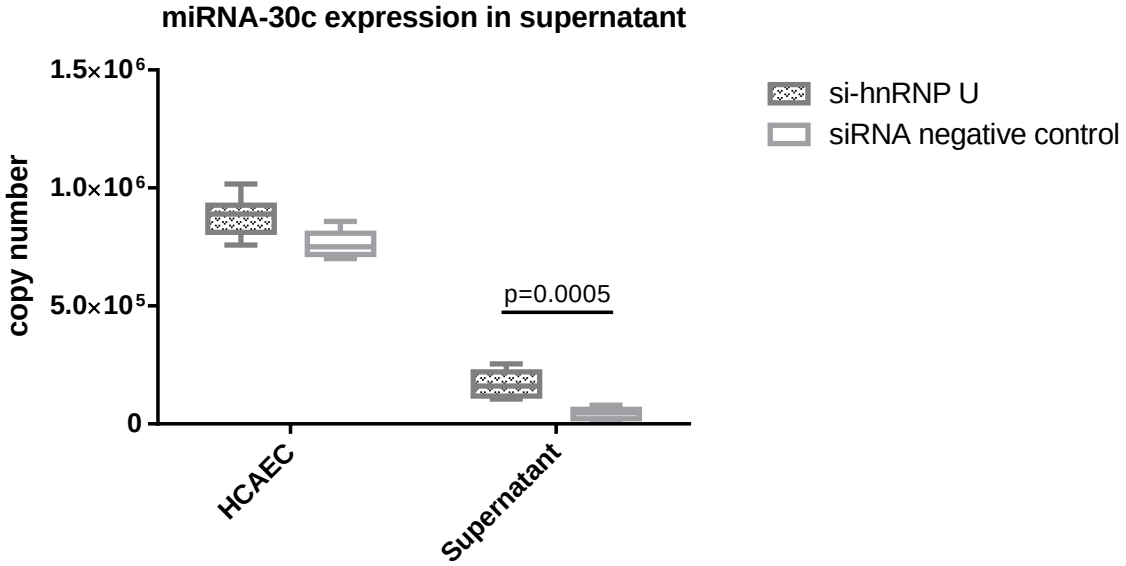


Figure 11 3.4.2: *MiRNA-30c expression in the supernatant.*
MiRNA-30c were expressed more highly in the si-hnRNP U cell supernatant than the siRNA-negative control supernatant. n=6. Supernatant, HCAEC culture supernatant.

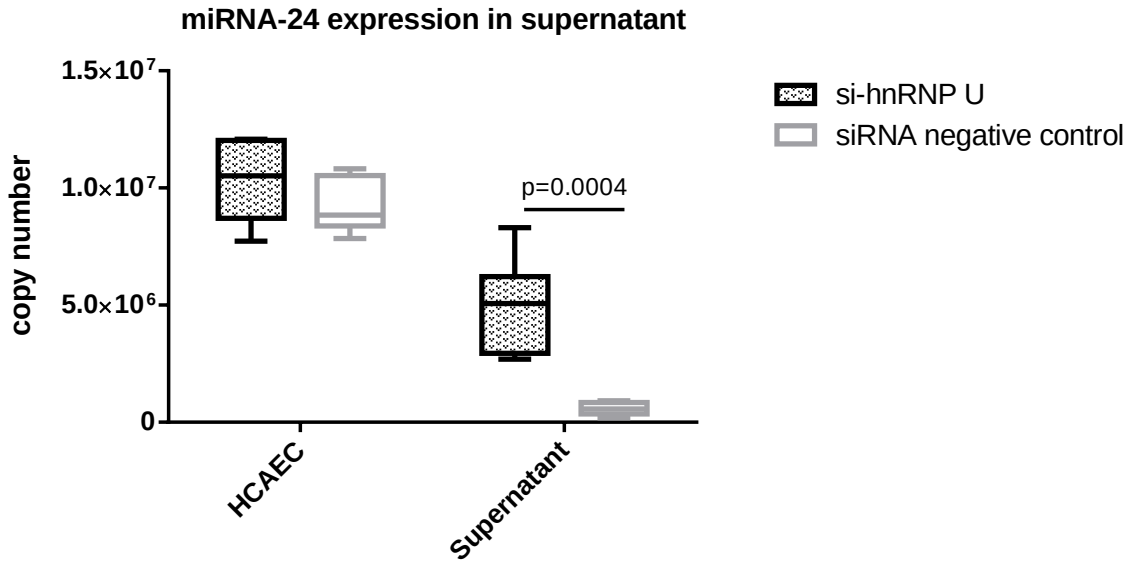


Figure 12 3.4.4: *MiRNA-24 expression in the supernatant.*

MiRNA-24 were expressed more highly in the si-hnRNP U cell supernatant than the siRNA-negative control supernatant. n=6. Supernatant, HCAEC culture supernatant.

3.5 $EMP^{si\text{-}hnRNP\ U}$ inhibits HCAEC migration and promotes HCAEC proliferation

The protein hnRNP U could actively regulate the packaging of specific miRNA into extracellular vesicles, and also seems to regulate miRNA-30c and miRNA-24 release into the cell supernatant, as shown in Figure 3.3.4. In this way, miRNA-containing EMP represent cell-to-cell messengers and regulate the function of HCAEC.

To observe the function of $EMP^{si\text{-}hnRNP\ U}$ or the $EMP^{siRNA\ negative\ control}$ on HCAEC, migration assay and proliferation assay were performed. Wound scratch assay results indicated that the $EMP^{si\text{-}hnRNP\ U}$ group obviously inhibited while the $EMP^{siRNA\ negative\ control}$ group promoted the migration abilities of HCAEC cells (Figure 3.5.1: $EMP^{si\text{-}hnRNP\ U}$ vs. $EMP^{siRNA\ negative\ control}$, 6 and 10 hours, 40.73. ± 1.92 % vs. 55.49 ± 3.21%, 60.68 ± 2.80% vs. 78.76 ± 3.86%, n=12, $p<0.01$ and $p<0.01$). Moreover, a transwell migration assay was applied to study the influence of $EMP^{si\text{-}hnRNP\ U}$ and the $EMP^{siRNA\ negative\ control}$ on the migration of HCAEC cells (Figure 3.5.2: $EMP^{si\text{-}hnRNP\ U}$ vs. the $EMP^{siRNA\ negative\ control}$, 159.7 ± 8.46 vs. 216.3 ± 19.43, n=6, p=0.02). In line with the wound scratch assay results, the transwell migration revealed a promigratory effect of $EMP^{si\text{-}hnRNP\ U}$. The proliferation assay results indicated that the $EMP^{si\text{-}hnRNP\ U}$

group promoted HCAEC proliferation when compared with the $EMP^{siRNA\ negative\ control}$ group (Figure 3.5.3: $EMP^{hnRNP\ U\text{-}siRNA}$ vs. $EMP^{crambled\text{-}siRNA}$, 9.99 ± 0. 31% vs. 8.92 ± 0.35%, n=6, p=0.04).

Taken together, $EMP^{si\text{-}hnRNP\ U}$ inhibits HCAEC migration and promotes HCAEC proliferation.

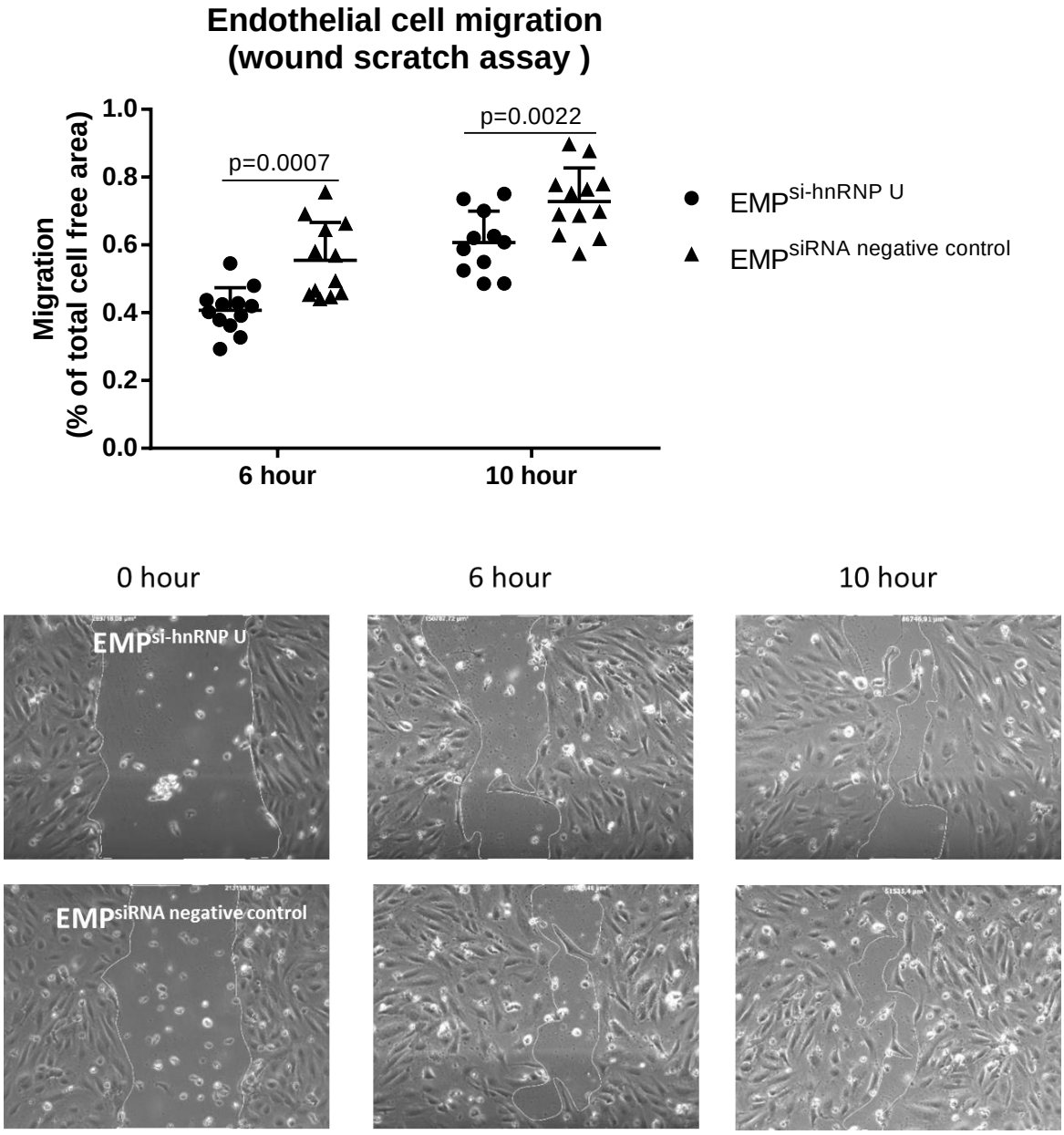


***Figure 13 3.5.1**: $EMP^{si\text{-}hnRNP\ U}$ inhibits HCAEC migration (wound scratch assay).*
HCAEC were coincubated with $EMP^{si\text{-}hnRNP\ U}$ or the $EMP^{siRNA\ negative\ control}$. The area of migration was shown at 0, 6, and 10 hours respectively. Data was shown as the mean ± SD. Culture medium: 20% complete medium with 80% basal medium. All experiments were repeated three times. n=12.

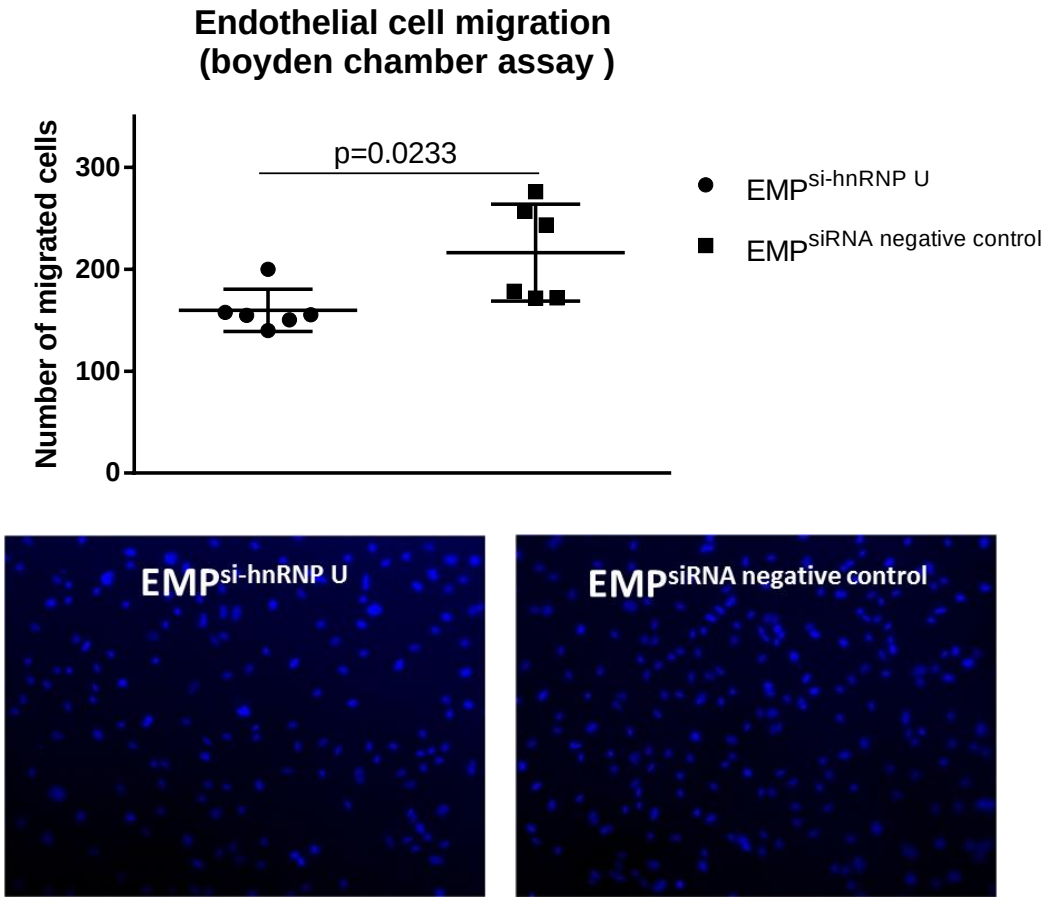
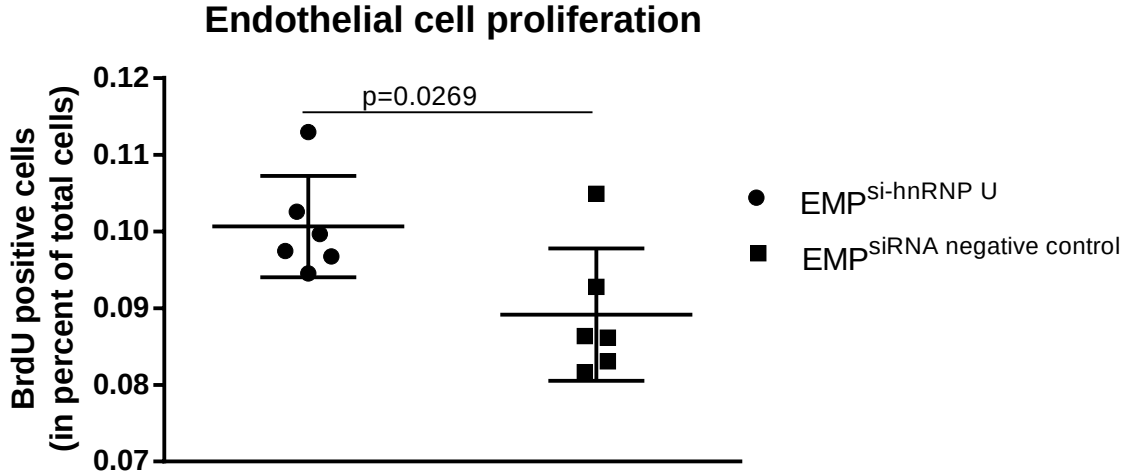


***Figure 14 3.5.2**: EMP$^{si\text{-}hnRNP\ U}$ inhibits HCAEC migration (transwell migration assay).*

HCAEC were coincubated with EMP$^{si\text{-}hnRNP\ U}$ or the EMP$^{siRNA\ negative\ control}$. Data was shown as the numbers of migrated cells. All experiments were repeated three times. n=6



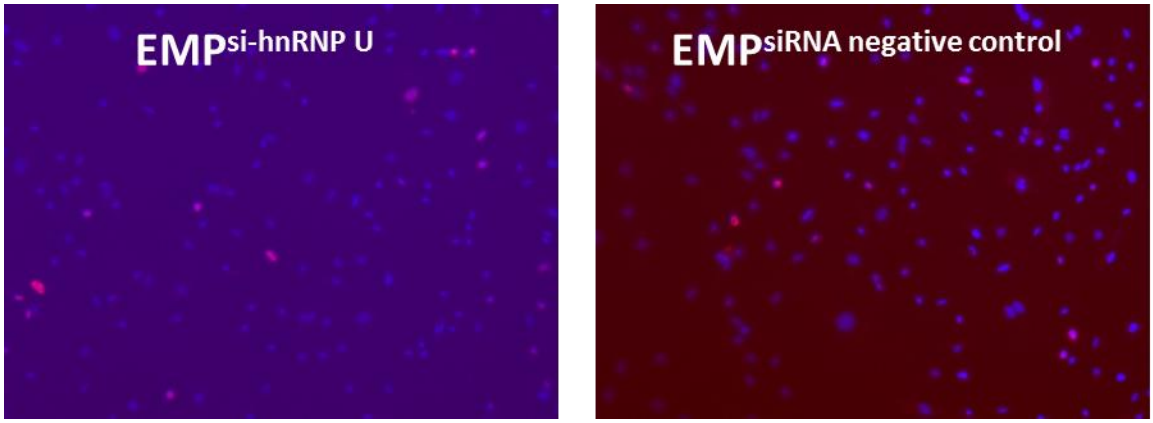


***Figure 15 3.5.3**: EMP$^{si\text{-}hnRNP\ U}$ promotes HCAEC proliferation.*

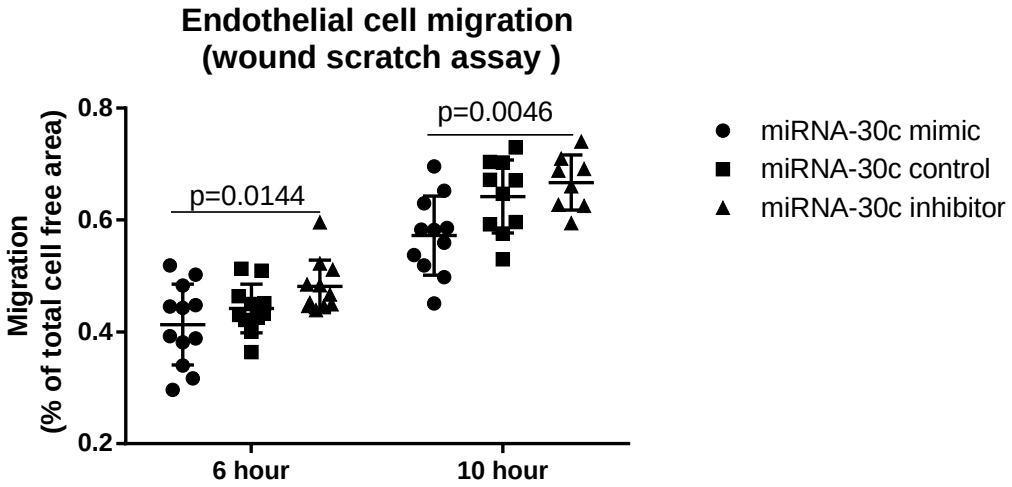
HCAEC in the completed medium were stimulated with EMP$^{si\text{-}hnRNP\ U}$ or the EMP$^{siRNA\ negative\ control}$ for 18 hours and then incubated with BrdU for 6 hours. Immunofluorescence assay was performed. BrdU and nuclei were stained with Cy3 (red) and DAPI (blue) respectively. BrdU staining cells were counted by Image J software and the percentage of BrdU-positive cells in total cells was calculated and analyzed. Magnification ×200, n=6.

3.6 Overexpression of miRNA-30c inhibits HCAEC migration and promotes HCAEC proliferation

To clarify the function of miRNA-30c in HCAEC, HCAEC with miRNA-30c mimic/control/inhibitor was transfected. The overexpression of miRNA-30c markedly improved proliferation and inhibited the migration of HACEC cells, whereas the inhibition of endogenous miRNA-30c significantly suppressed proliferation and promoted the migration of HCAEC when compared with the negative control. Wound scratch assay results indicated that the overexpression of miRNA-30c obviously inhibited HCAEC migration, while the downregulation of miRNA-30c promoted the migration abilities of HCAEC (Figure 3.6.1: mimic vs. control vs. inhibitor, 6 and 10 hours, 41.29 ± 2.09% vs 44.18 ± 1.31% vs. 48.14 ± 1.41%, 57.20 ± 2.13% vs. 64.20 ± 2.06% vs. 66.70 ± 1.74%, n=12, p=0.02 and p=0.01). Moreover, a transwell migration assay was applied in order to study the influence of miRNA-30c on the migration of HCAEC cells (Figure 3.6.2: mimic vs. control vs. inhibitor, 133.3± 7.41 vs. 151.3 ± 5.87 vs. 193.9 ± 5.15, n=6, p<0.01). In line with the wound scratch assay results, the transwell migration revealed a promigratory effect of miRNA-30c. The proliferation assay result indicated that overexpression of miRNA-30c obviously

promoted HCAEC proliferation, while downregulation of miRNA-30c inhibited the proliferation abilities of HCAEC (Figure 3.6.3: mimic vs. control. vs. inhibitor 15.72± 0.42% vs. 12.71 ± 0.57% vs. 11.94 ± 0.60%, n=6, p<0.01).

In this sense, the results showed that up-regulated miRNA-30c expression promotes HCAEC proliferation and inhibits HCAEC motility *in vitro*.



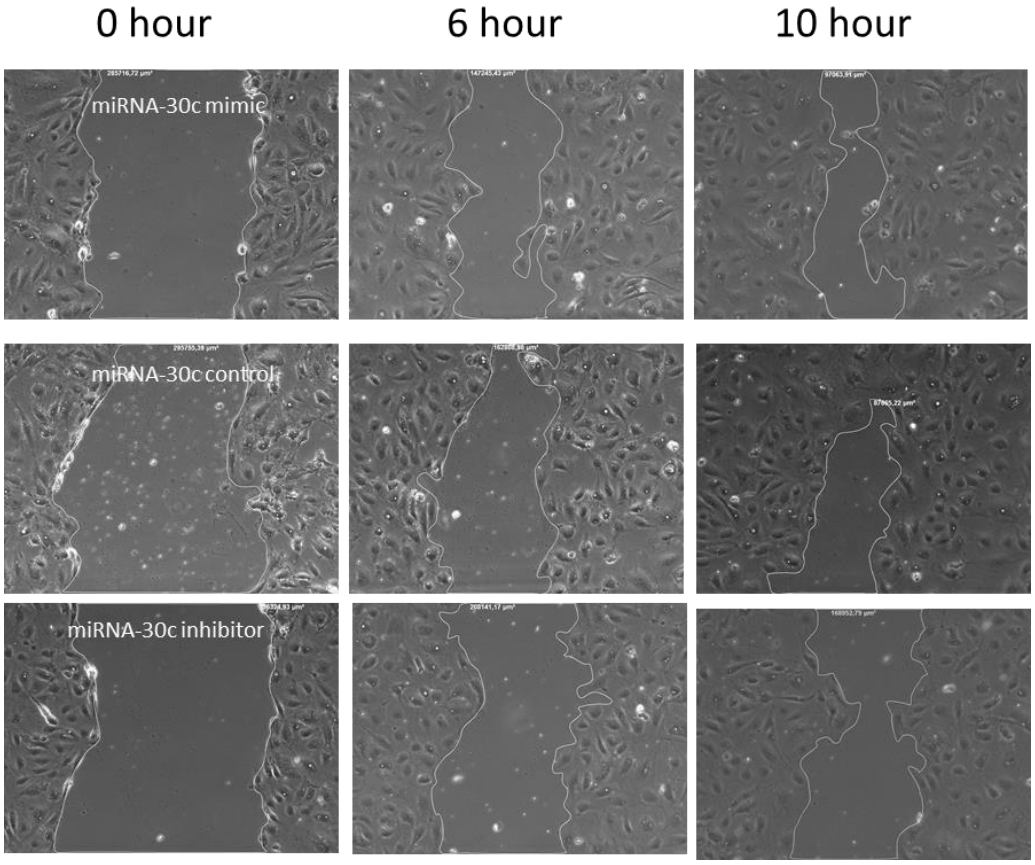


Figure 16 3.6.1: *Overexpression of miRNA-30c inhibits endothelial cell migration (wound scratch assay).*

Confluent HCAEC in the basal medium were coincubated with miRNA-30c mimic, control or inhibitor. The area of migration was shown at 0, 6, and 10 hours respectively. Quantitative analysis of migration was measured as a percentage of the total cell-free area. Data was shown as the mean ± SD. Culture medium: 20% complete medium with 80% basal medium. All experiments were repeated three times. n=12.

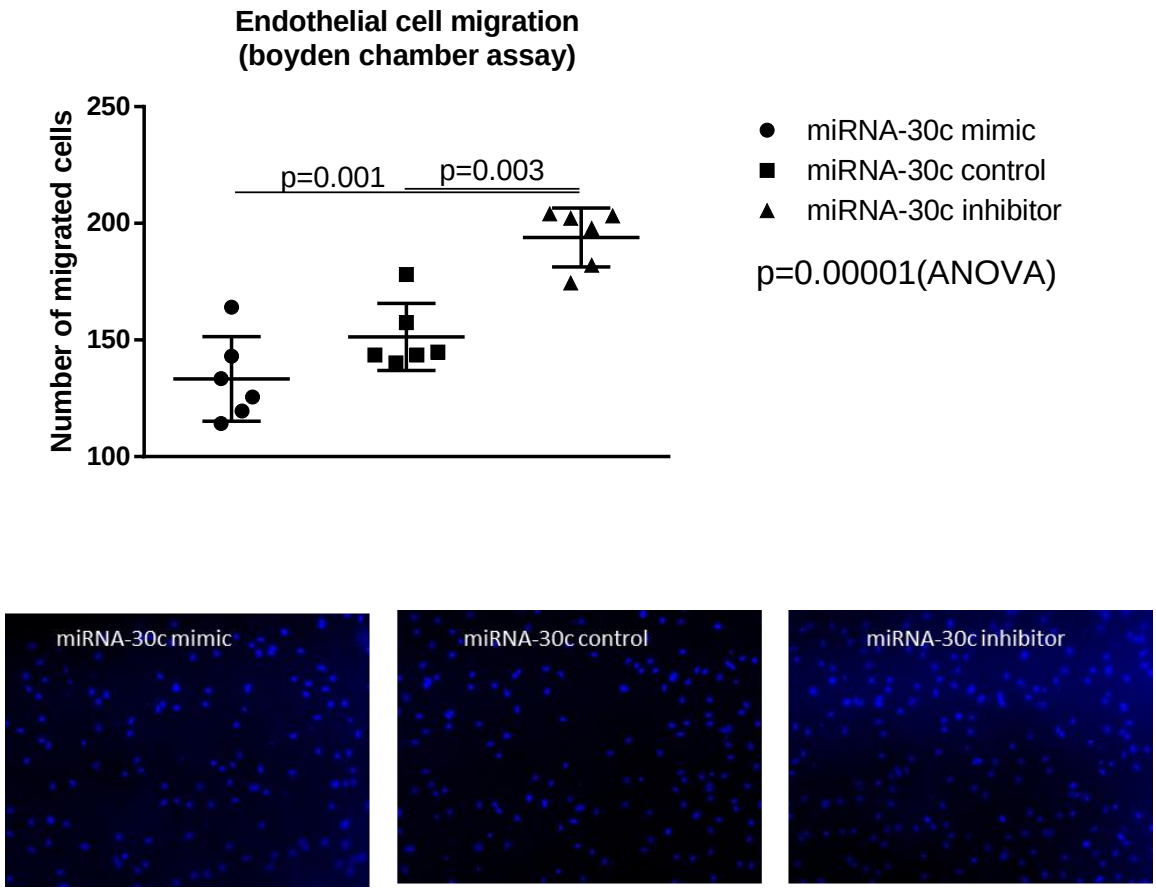


Figure 17 3.6.2: *Overexpression of miRNA-30c inhibits endothelial cell migration (transwell migration assay).*

HCAEC were treated with miRNA-30c mimic, control or inhibitor. Data represent the numbers of migrated cells. All experiments were repeated three times. n=6.

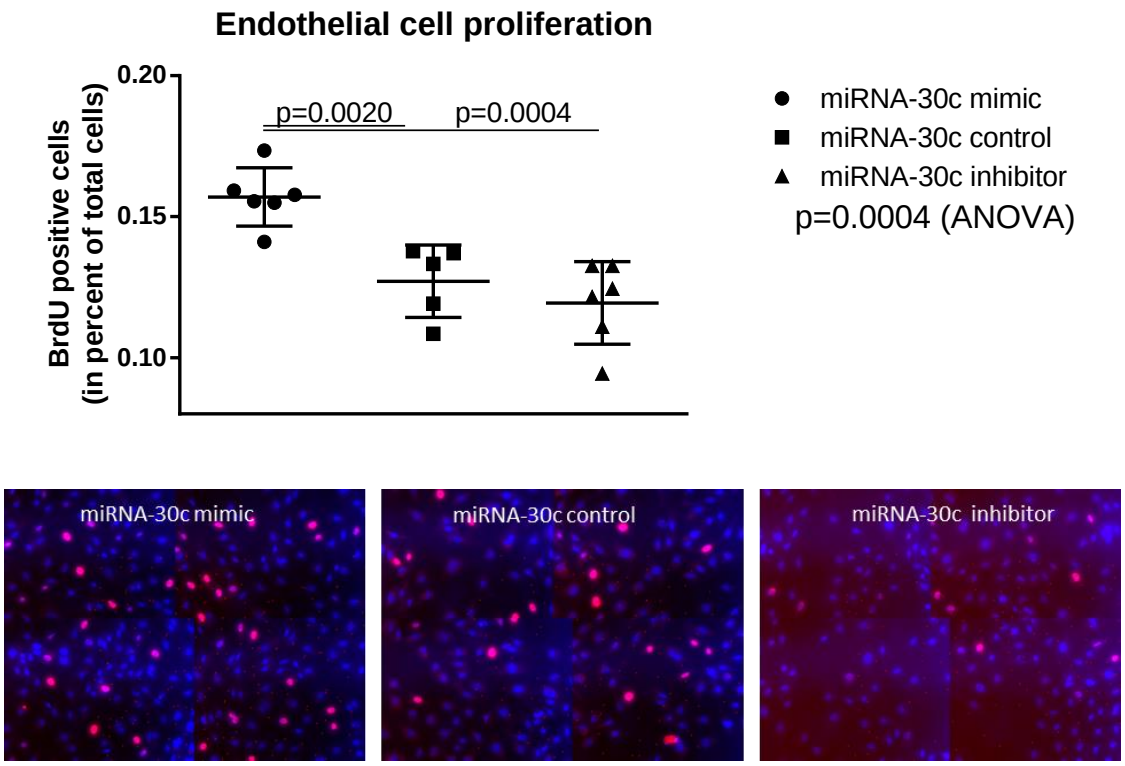


Figure 18 3.6.3: *Overexpression of miRNA-30c promotes endothelial cell proliferation.*

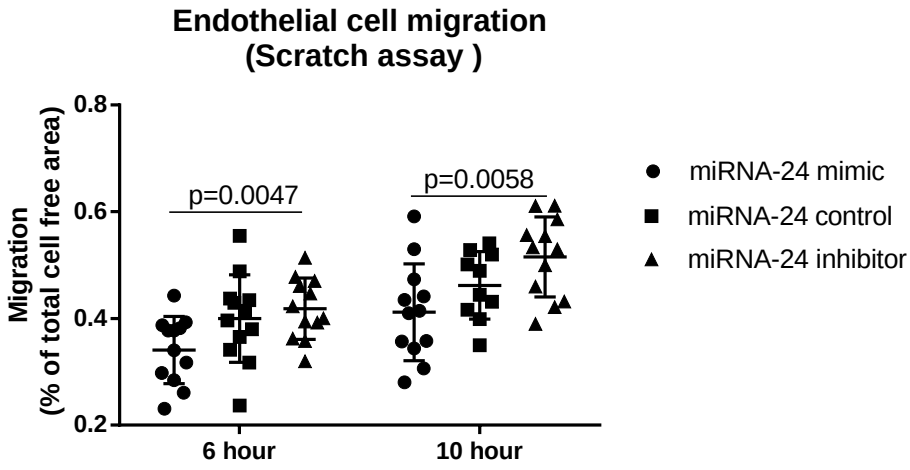
BrdU incorporation was determined by immunofluorescence (red). Nuclei were stained with DAPI (blue). The percentage of BrdU-positive cells was compared. Magnification, ×200. BrdU, indicates bromodeoxyuridine; DAPI, 4',6-diamidino-2-phenylindole; and HCAEC, human coronary artery endothelial cell. n=6.

3.7 Overexpression of miRNA-24 promotes HCAEC proliferation and inhibits HCAEC migration

To clarify the function of miRNA-24 in HCAEC, HCAEC with miRNA-24 mimic/control/inhibitor was transfected. The overexpression of miRNA-24c markedly promoted proliferation and inhibited the migration of HACEC, whereas the inhibition of endogenous miRNA-24 significantly suppressed proliferation and promoted the migration of HCAEC when compared with the negative control. Wound scratch assay results indicated that the overexpression of miRNA-24 was obviously inhibited, while the downregulation of miRNA-24 promoted the migration abilities of HCAEC cells (Figure 3.7.1: mimic vs. control vs. inhibitor, 6 and 10 hours, 34.08 ± 1.82 % vs. 39.95 ± 2.37 % vs. 41.82 ± 1.66 %, 41.16 ± 2.62 % vs. 46.20 ± 2.00 % vs. 51.52 ± 2.17%, p=0.02 and p=0.01). Moreover, a transwell migration assay was applied in

order to study the influence of miRNA-30c on the migration of HCAEC cells (Figure 3.7.2: mimic vs. control vs. inhibitor, 137.8± 6.02 vs. 177.7 ± 12.59 vs. 189.1 ± 11.07, p=0.01). In line with the wound scratch assay results, transwell migration revealed a promigratory effect of miRNA-24. The proliferation assay results indicated that the overexpression of miRNA-30c was obviously promoted, while the downregulation of miRNA-30c inhibited the proliferation abilities of HCAEC (Figure 3.7.3: mimic vs. control vs. inhibitor 14.02± 0. 63 % vs. 12.36 ± 0.61 % vs. 11.01 ± 0.49 %, p<0.01).

Taken together, it was found that miRNA-24 was likely to play a major role in cell migration, invasion, and proliferation.



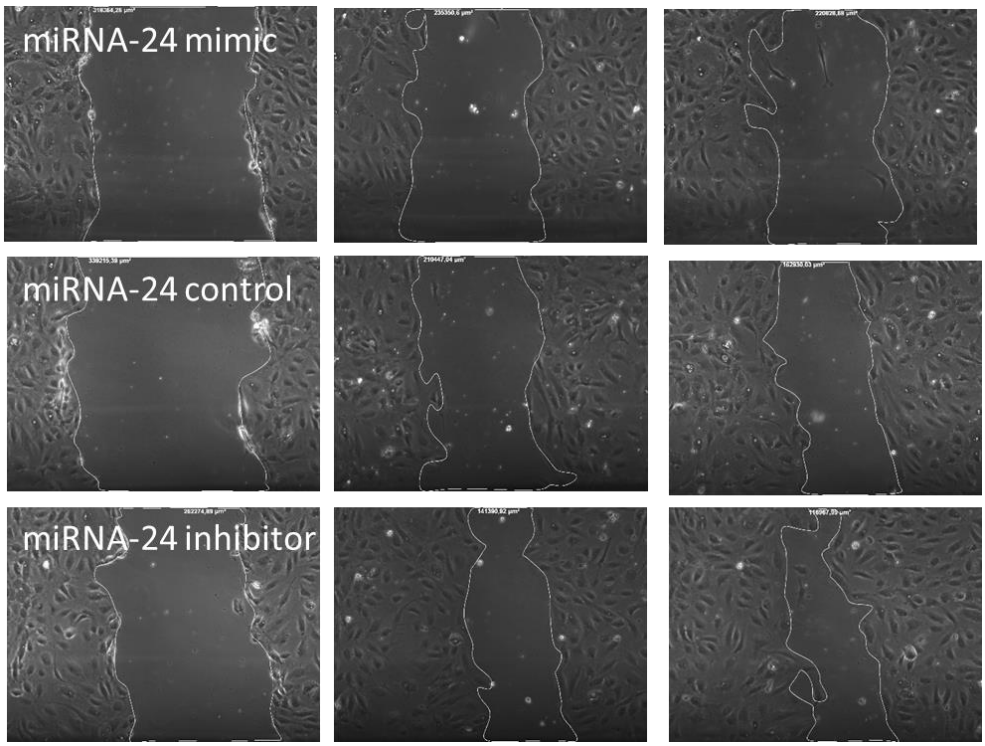


Figure 19 3.7.1**:** *Overexpression of miRNA-24 inhibits endothelial cell migration (wound scratch assay).*

Confluent HCAEC in the basal medium were coincubated with miRNA-24 mimic, control or inhibitor. The area of migration was shown at 0, 6, and 10 hours respectively. Quantitative analysis of migration was measured as a percentage of the total cell-free area. Data was shown as the mean ± SD. Culture medium: 20% complete medium with 80% basal medium. All experiments were repeated three times. n=12.

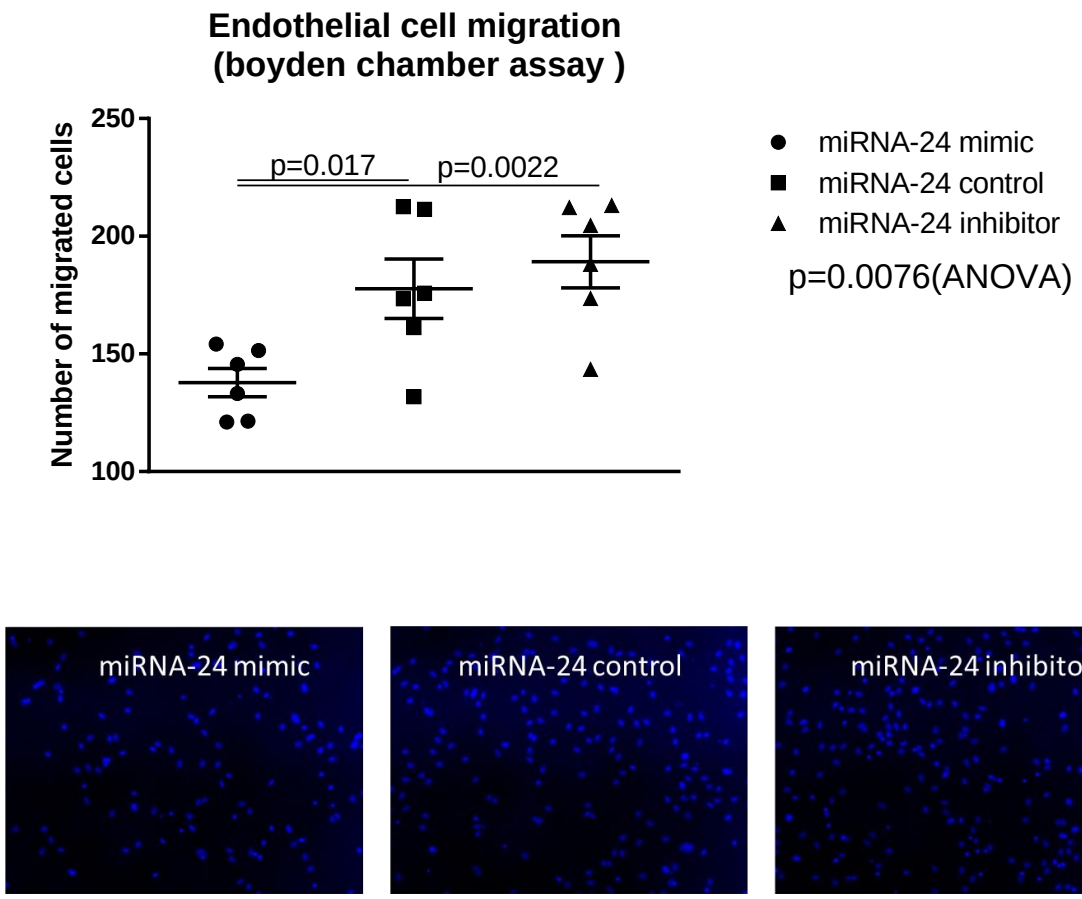


Figure 20 3.7.2: *Overexpression of miRNA-24 inhibits endothelial cell migration (transwell migration assay).*

HCAEC were treated with miRNA-24 mimic, control or inhibitor. Data represent the numbers of migrated cells. All experiment were repeated three times. n=6.

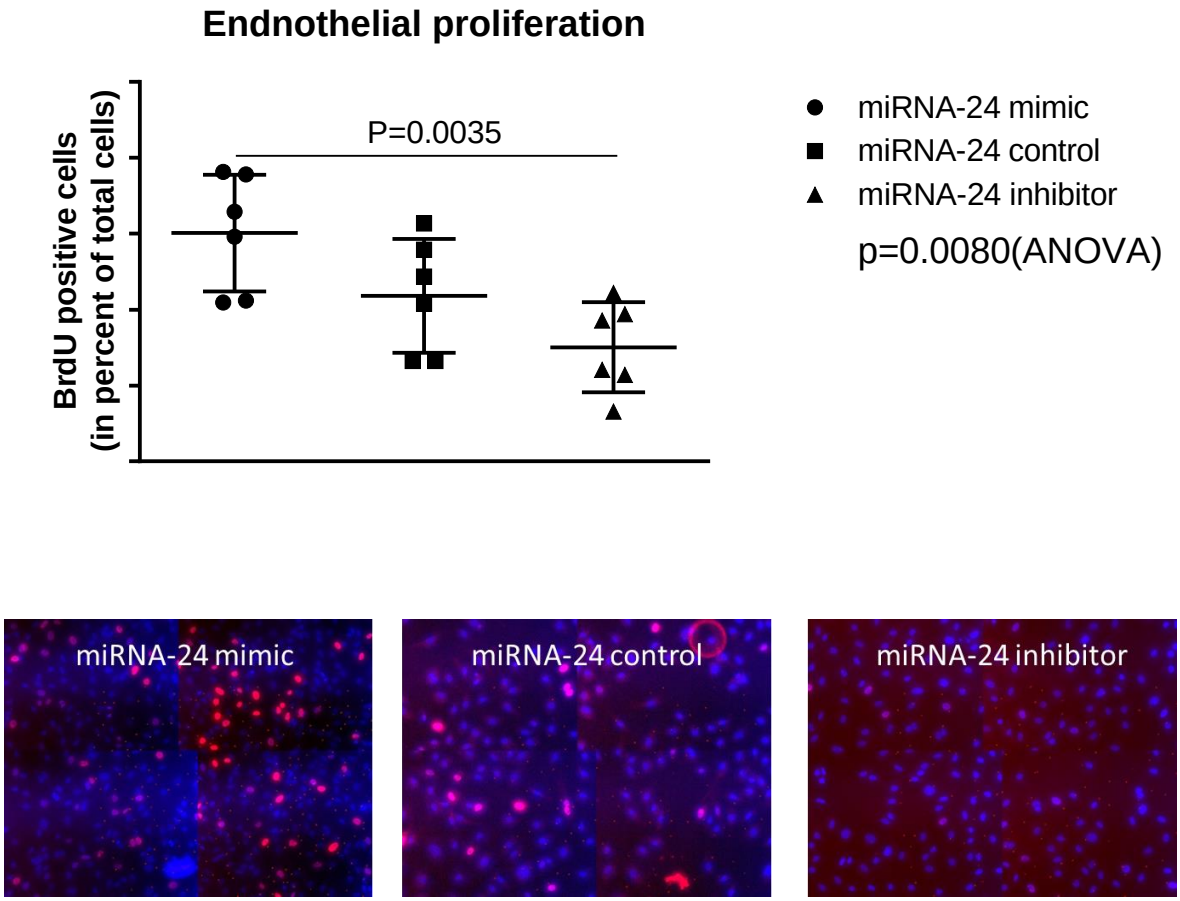


Figure 21 3.7.3: *Overexpression of miRNA-24 promotes endothelial cell proliferation.*

BrdU incorporation was determined by immunofluorescence (red). Nuclei were stained with DAPI (blue). The percentage of BrdU-positive cells was compared. Magnification, ×200. BrdU, indicates bromodeoxyuridine; DAPI, 4',6-diamidino-2-phenylindole; and HCAEC, human coronary artery endothelial cell. n=6.

4. Discussion

This report has shown that the hnRNP U protein binds to miRNA-30c and miRNA-24 in HCAEC cells *in vitro*. Furthermore, it has been demonstrated that the downregulation of hnRNP U in HCAEC also decreases the levels of both miRNA-30c and miRNA-24. No differences in EMP secretions between HCAEC transfected with either si-hnRNP U or the siRNA negative control were observed. miRNA-30c and miRNA-24 expression levels in HCAEC were higher in the cell culture supernatant when transfected with si-hnRNP U compared to a transfection with the siRNA negative control. EMP derived from HCAEC transfected with si-hnRNP U inhibit HCAEC migration and promote proliferation. Furthermore, upregulation of miRNA-30c and miRNA-24 levels in HCAEC also inhibit HCAEC migration and promote proliferation.

Recent studies have shown that hnRNP A1 (Guil and Caceres 2007, Konishi, Fujiya et al. 2015), hnRNP A2B1 (Villarroya-Beltri, Gutierrez-Vazquez et al. 2013, Fan, Sutandy et al. 2015), hnRNP Q (Santangelo, Giurato et al. 2016), and hnRNP K (Fan, Sutandy et al. 2015) could bind to several miRNAs in cells and exosomes. However, there has been no report exploring whether miRNAs are specifically binding to the hnRNP U protein. In this study, microarray and RT-PCR results confirmed that hnRNP U specifically binds to miRNA-30c and miRNA-24 in HCAEC *in vitro* (Figures 3.2.2 and 3.2.3). Furthermore, the expression levels of miRNA-30c and miRNA-24 were decreased with the downregulation of hnRNP U in HCAEC (Figures 3.3.3 and 3.3.4). Together, this data demonstrates that hnRNP U specifically binds miRNA-30c and miRNA-24 in HCAEC *in vitro*. As shown in Figure 1.5.1, in the hnRNP U protein there are many binding domains, such as the acid-rich domain, the Gly-rich domain, the RGG box (consisting of Arg-Gly-Gly repeats), and other binding domains (Bi, Yang et al. 2013, Vu, Park et al. 2013, Geuens, Bouhy et al. 2016). The hnRNP U protein binds RNA through these binding domains and transfers RNA from nucleus to cytoplasm. In several reports, hnRNP U protein was demonstrated to specifically bind to the mRNAs or miRNAs (Vu, Park et al. 2013, Geuens, Bouhy et al. 2016). Unfortunately, the exact binding domain and sequence for the hnRNP U interaction with miRNA-30c and miRNA-24 is unknown. Further experiments in this direction need to be performed.

Because miRNAs in EMPs determine cellular biological effect, it was also decided to detect the miRNA-30c and miRNA-24 expression in EMP. The results indicated that both the miRNA-30c and miRNA-24 expression levels in EMP were higher in EMPs derived from HCAEC transfected with si-hnRNP U than in the siRNA negative control (Figures 3.3.3 and 3.3.4). However, both the miRNA-30c and miRNA-24 expression levels were lower in HCAEC transfected with si-hnRNP U than in the siRNA negative control. To clarify why the levels of miRNA-30c and miRNA-24 were inverted between EMP and HCAEC after transfection, the miRNA-30c and miRNA-24 levels in the cell culture medium were measured. The miRNA-30c and miRNA-24 were both expressed higher in the si-hnRNP U cell supernatant than in the siRNA negative control medium (Figures 3.4.1 and 3.4.2).

RNA sequencing (RNA-seq) and microarray data have shown that miRNAs are differentially enriched in extracellular vesicles (EV) compared to their producer cells (Valadi, Ekstrom et al. 2007, Skog, Wurdinger et al. 2008, Gibbings, Ciaudo et al. 2009, Simons and Raposo 2009, Guduric-Fuchs, O'Connor et al. 2012, Nolte-'t Hoen, Buermans et al. 2012, Villarroya-Beltri, Gutierrez-Vazquez et al. 2013, Vu, Park et al. 2013). Interestingly, specific miRNAs are enriched in EVs in a cell type-dependent fashion. All of the above findings suggest that the sorting of specific miRNA species to extracellular vesicles may be actively regulated. However, the underlying mechanisms for how miRNAs are sorted into microparticles and the significance of miRNA transfer to acceptor cells remain largely unknown. Villarroya-Beltri et al. showed that sumoylated hnRNPA2B1 could be used to monitor the sorting of miRNAs into exosomes through binding to specific motifs (GGAG) (Villarroya-Beltri, Gutierrez-Vazquez et al. 2013). Thus, chaperone proteins might interact with specific motifs or sequences in certain miRNAs and guide the miRNA sorting into EVs (Villarroya-Beltri, Gutierrez-Vazquez et al. 2013). In addition, Squadrito et al. demonstrated that microRNA sorting into exosomes is modulated by the levels of endogenous natural targets (Vu, Park et al. 2013). Squadrito has also shown that not only miRNAs and their targeted transcripts promote bidirectional miRNA relocation from the cytoplasm to multivesicular bodies, but they also modulate miRNA sorting to exosomes (Squadrito, Baer et al. 2014).

It has been demonstrated here that miRNA-30c and miRNA-24 were both expressed higher in the si-hnRNP U basal cell supernatant than in the siRNA negative control

supernatant. There was no difference in HCAEC expression levels of miRNA-30c and miRNA-24 between cells transfected with si-hnRNP U and those transfected with the siRNA negative control. However, the miRNA-30c and miRNA-24 expression levels in the supernatant were expressed higher for the si-hnRNP U than for the siRNA negative control in the same cells (Figures 3.4.1 and 3.4.2). The machinery how microRNAs specifically secretion and packing into EMP remains to be determined in future endeavors.

Flow cytometry results showed that there were no differences in the amounts of EMP secretions between the HCAEC transfected with si-hnRNP U vs. the siRNA negative control (Figure 3.4.3). Thus, the downregulation of hnRNP U did not influence the secretion of EMP.

The next step in the project was to determine the role of EMP derived from HCAEC transfected with either si-hnRNP U or the siRNA negative control. The data showed that the $EMP^{si\text{-}hnRNP\ U}$ inhibits HCAEC motility and promotes HCAEC proliferation (Figures 3.5.1-3). It also showed that up-regulated miRNA-30c and miRNA-24 in HCAEC inhibited HCAEC motility and proliferation (Figures 3.6.1-3 and Figures 3.7.1-3). Thus, it can be speculated that the miRNA-30c and miRNA-24 in EMP might affect HCAEC motility and proliferation. Earlier studies have revealed that miRNA-30c (Zhou, Xu et al. 2012, Xia, Chen et al. 2013, Ling, Han et al. 2014, Wu, Zhang et al. 2015, Zhang, Yu et al. 2015) and miRNA-24 (Amelio, Lena et al. 2012, Fiedler, Stohr et al. 2014, Zhu, Shan et al. 2015, Li, Wang et al. 2016, Yang, Chen et al. 2016, Ehrlich, Hall et al. 2017) inhibit cell motility *in vivo* and *in vitro*. However, another study reported that miRNA-30c and miRNA-24 have inverse functionality in cell motility and proliferation, in that miRNA-30 (Liu, Li et al. 2016) and miRNA-24 (Ma, She et al. 2014, Zhao, Hu et al. 2016, Yu, Jia et al. 2017) promote cell motility. Thus, miRNA-30c and miRNA-24 play different functions in different cell lines. This study's data showed upregulation miRNA-30c and miRNA-24 inhibit HCAEC motility *in vitro*.

Several studies have shown that miRNA-30c (Zhou, Xu et al. 2012, Ling, Han et al. 2014, Zhong, Chen et al. 2014, Xing, Zheng et al. 2015) and miRNA-24 (Giglio, Cirombella et al. 2013, Xu, Liu et al. 2013, Ma, She et al. 2014, Lu, Wang et al. 2015, Zhao, Liu et al. 2015, Sun, Xiao et al. 2016, Zhao, Hu et al. 2016, Yu, Jia et al. 2017)

promote cell proliferation. Furthermore, other studies have reported that miRNA-30c (Tanic, Yanowsky et al. 2012, Dobson, Taipaleenmaki et al. 2014) and miRNA-24 (Lal, Navarro et al. 2009, Amelio, Lena et al. 2012, Song, Yang et al. 2013, Fiedler, Stohr et al. 2014, Inoguchi, Seki et al. 2014, Zhang, Zhang et al. 2015, Zhu, Shan et al. 2015, Li, Wang et al. 2016, Yang, Chen et al. 2016, Ehrlich, Hall et al. 2017) inhibit cell proliferation in cells. Fiedler reported that miRNA-24 had no effect on cell cycle progression in HASMCs (Fiedler, Stohr et al. 2014) and Xia demonstrated that miRNA-30c also had no effect on proliferation in the A549 cell line (Xia, Chen et al. 2013). The data in this study indicates that miRNA-30c and miRNA-24 promote HCAEC proliferation *in vitro*.

Unfortunately, no research to date has focused on miRNA-30c and miRNA-24 in HCAEC's motility and proliferation. In this study, it has been confirmed that miRNA-30c and miRNA-24 could inhibit HCAEC migration and promote HCAEC proliferation.

Although there were several discoveries during these studies, there are also many questions remaining as well as limitations regarding the work performed. First, hnRNP U has many motifs and binding domains which could interact with many different miRNAs. This experiment only focused on two types of miRNAs according to our RIP-microarray result. Therefore, other miRNAs which also bind to hnRNP U are sure to be missed. These miRNAs also have several key functions in HCAEC and EMP, and only a few of them have been explored. Second, Electrophoretic mobility shift assay (EMSA) is also critical in confirming miRNA binding to hnRNP U *in vitro.* EMSA is a common affinity electrophoresis technique used to study protein–RNA interactions performed *in vitro*. This procedure can determine whether a protein is capable of binding to a given RNA sequence and can sometimes indicate if more than one protein molecule is involved in the binding complex. Third, experiments in the downregulation and upregulation of hnRNP U in HCAEC are both necessary in studying the function of hnRNP U in HCAEC, and the upregulation experiments have not yet been conducted. Fourth, *in vivo* animal experiments are also necessary. In the coming months, all of these experiments will be performed in order to confirm these preliminary results.

In summary, it has been identified that the hnRNP U protein binds to miRNA-30c and miRNA-24 *in vitro*. EMP derived from HCAEC cells transfected with si-hnRNP U

negatively regulates HCAEC's motility and positively promotes proliferation.

This study has highlight that:

1) hnRNP U binds to miRNA-30c and miRNA-24 *in vitro*.

2) miRNA-30c and miRNA-24 levels decreased with the transfection of si-hnRNP U in HCAEC.

3) miRNA-30c and miRNA-24 levels increased in $EMP^{si\text{-}hnRNP\ U}$ and the cell culture medium.

4) $EMP^{si\text{-}hnRNP\ U}$ inhibits HCAEC's migration and promotes HCAEC proliferation *in vitro*.

5) Overexpression of miRNA-30c and miRNA-24 inhibits endothelial cell migration and promotes proliferation *in vitro*.

5. Abstract

The protein heterogeneous nuclear ribonucleoprotein U (hnRNP U) plays an essential role in the development and function of the heart. The hnRNP U protein in human coronary artery endothelial cells (HCAEC) mediates its function by binding microRNAs (miRNAs). Endothelial microparticles (EMP) derived from HCAEC regulate several processes in cardiovascular biology by transferring miRNAs to target cells. In this study, we aimed to explore the function of hnRNP U in HCAEC and EMP.

We found that the hnRNP U protein binds to microRNA-30c (miRNA-30c) and microRNA-24 (miRNA-24) in HCAEC *in vitro*. Furthermore, the downregulation of hnRNP U in HCAEC decreases the levels of both miRNA-30c and miRNA-24. Downregulation of hnRNP U did not affect the number of EMP released from HCAEC. The miRNA-30c and miRNA-24 expression levels were higher in both EMP and the supernatant when transfected with si-hnRNP U compared to transfection with the siRNA negative control. EMP derived from HCAEC transfected with si-hnRNP U inhibit HCAEC migration and promote proliferation. Furthermore, upregulated miRNA-30c and miRNA-24 expression both inhibit HCAEC motility and promote proliferation *in vitro*.

HnRNP U protein binds to miRNA-30c and miRNA-24 *in vitro*. EMP derived from HCAEC cells transfected with si-hnRNP U negatively regulate HCAEC motility and positively promote proliferation.

6. List of Figures

7. List of Tables

8. Reference

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