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Running Title: Multiplexed targeting of microRNA-derived EVs

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ABSTRACT

Methods used to restore damaged cells or tissue function via regeneration, repair or replacement in regenerative medicine ultimately use stem cells or their derivatives to induce response to disease and dysfunction. Stem cell-derived extracellular vesicles (EVs) are recognized as an attractive source of enriched exogenous microRNAs (miRNAs) by targeting pathological cells and overcome the obstacles faced by current cell therapy agents. However, there are some limitations that need to be addressed before using miRNA-enriched EVs derived from stem cells for multiplexed therapeutic targeting in many diseases. Here, we review the various strategies to induce effective and stable functional improvement of stem cell-derived EVs. In addition, we analyze the implications of several miRNA-enriched EV therapies improved by multiplexed targeting in diseases involving the circulatory system and nervous system. This systematic review may offer potential insights into the role of stem cell-derived therapeutics via multiplexed targeting mechanisms.

INTRODUCTION

Regenerative medicine has the potential to facilitate spontaneous regeneration of damaged tissues and organs, as well as restore birth defects. Thus, regenerative medicine strategies are increasingly being improved using novel materials and regenerative cells, as well as their combinations (1). Cell therapy is an attractive option available to induce in situ tissue regeneration via transplantation of host cells and stem cells. In particular, stem cells derived from fetuses or adults are attracting increased attention due to their potential for clinical application based on self-renewal, multipotent or pluripotent properties as undifferentiated cells. Stem cells can be classified into three categories: embryonic stem cells (ESCs), pluripotent stem cells (PSCs), and mesenchymal stem cells (MSCs) (2). Among them, MSCs are tissue-specific without ethical concerns associated with ESCs, and show less tumorigenicity compared with PSCs, are therefore widely used in tissue repair and regenerative medicine.

Despite the multiple benefits of stem cell transplantation, it is limited by abnormal cell differentiation and low engraftment rate at damaged sites. Studies to address these limitations are needed given the paracrine effects of stem cells mediated via release of growth factors and cytokines (3). In vitro stem cell therapies resulted in nutritional imbalance at the transplant site. The need for hundreds of millions of stem cells, the generation of a large number of stem cells that cannot be delivered to the transplant site, and carcinogenic potential are the other limiting factors of stem cell therapeutics hindering clinical application in regenerative medicine (4-8). In the ischemic heart, 1% of cells survived after 24 hours and less than 0.44% on day 4 after MSC transplantation (9, 10). Furthermore, most of the administered stem cells did not penetrate the blood-brain barrier (BBB) and showed a therapeutic effect due to the limited characteristics of the BBB in the ischemic brain (11).

Stem cell-free therapies that can ameliorate the shortcomings of cell therapies are emerging as safe and effective treatment options compared with conventional cell therapies. The MSC

secretome induced by paracrine effect via culture mediums, protein, RNA, and lipids represent a cell-free therapeutic strategy for physiological or pathological improvement of tissue damage. The effect of MSC secretome is mediated by extracellular vesicles (EVs), which are biochemical components associated with a large number of activation signals, rather than single growth factors or cytokines. EVs play a role in delivering functional genetic material to other cells, and transmission of information to neighboring cells (microenvironment), thereby resulting in cell-to-cell interaction (12). In particular, microRNA (miRNA), a short RNA molecule that regulates several cellular processes via post-transcriptional gene silencing, mediates the communication between neighboring cells. Notably, it has been reported recently that miRNAs silencing multiplexed targets can be delivered via EVs in the microenvironment (13, 14).

In this review, we mainly focus on the potential role of stem cell-free therapies in regenerative medicine to attenuate cardiovascular and brain tissue damage. We also discuss multiplexed targeting strategies using miRNA-enriched EVs.

EXTRACELLULAR VESICLES DERIVED FROM STEM CELLS

EVs are cellular derivatives measuring 40-1000 nm in size. They are composed of adhesion molecules and soluble mediators. They exchange cellular biological information and deliver contents using soluble mediators including growth factors, cytokines and small molecules as small extracellular membraneous particles. EVs are mainly classified into exosomes, microvesicles (MVs), microparticles, ectosomes, oncosomes, apoptotic bodies, and others according to their size and biogenesis. According to the 2014 Minimal Information for Studies of Extracellular Vesicles (MISEV) guidelines proposed by the International Society for Extracellular Vesicles (ISEV), they are collectively referred to as EVs (12, 13, 15).

Origin and size

EVs are largely divided into exosomes, MVs and apoptotic bodies according to their biogenesis, release pathway, size, content, and function. Although the specific protein markers that distinguish EVs have yet to be established, each protein profile is different. Exosomes are released to the outside of the cell in the form of microvesicular endosomes measuring 40-150 nm in diameter. They mainly express CD63 (CD81/CD9). MV is generated by the outward budding of the plasma membrane, and represents a subunit of EV with a typical diameter of 100-1000 mm. They express predominantly annexin A, a cytoskeletal component such as actin and microtubule. Apoptotic bodies have a diameter of about 50-5000 nm and are released into the extracellular space in the form of active amphisomes during apoptotic cell death, and via hydrostatic pressure in passive cell death. In general, it carries double-stranded DNA or histones as markers (16, 17). The diameter of stem cells is about 15 μm, whereas the capillary is smaller than 8 μm, and is clogged during intravenous cell transplantation. However, since EVs containing exosomes and MVs have a diameter of 40-1000 nm, they can migrate peripherally (Fig. 1).

Constituents

Since the exosome formation and transport are regulated via endosomal sorting complexes required for transport (ESCRT) signaling, the exosomes carry signal-regulating proteins such as Alix, TSG101, HSC70, and HSP90β. Other components include exosome marker proteins such as tetraspanin family membrane proteins (CD63, CD51 and CD9) (17, 18). Although tetraspanins were originally thought to be specific markers of exosomes, they have recently been identified in MVs and apoptotic bodies as well (16, 18). MVs contain cytoskeletal proteins, integrin family proteins for adhesion, and proteins responsible for translational modification.

Specifically, the glycan-binding protein is responsible for interaction with other cells. Annexins A1 and A2, which are abundant membrane-associated proteins classified as exosomal constituents, are recognized as novel constituents of MV. Apoptotic bodies contain Annexin A5 protein that specifically binds to the negative curvature-specific lipid phosphatidylserine, and due to the presence of specific organelles, proteins related to the nucleus, mitochondria, or endoplasmic reticulum can be observed (e.g., histones, heat shock protein, glucose-regulated protein, etc.) (18, 19).

IMPROVEMENT OF EXTRACELLULAR VESICLES

Cell-derived EVs contain many physiologically active substances (20, 21). Specific substances with potential therapeutic efficacy have been identified via proteomic profiling in EVs. However, for the induction of cell-derived EVs carrying an abundance of tissue- or target-specific substances, extrinsic substances must be expressed in high concentrations and maintained at constant levels in EVs. In the context of regenerative medicine, RNA modification of cell-derived EVs can facilitate genetic communication and transfer of genetic information to target cells, while simultaneously altering target gene regulatory networks to accelerate disease recovery.

Strategies for enhancing information within the extracellular vesicles

Among the many types of RNAs, mRNAs carry information for new proteins and tissue regeneration. The mRNAs measuring less than approximately 1 kb in length can be included and delivered to EVs (22). Cuesta et al. suggested that human pulmonary artery smooth muscle cell-derived EVs overexpressing TGF-β1 and BMP4 may contribute to vascular remodeling

and endothelial-mesenchymal transition (EndoMT) during pulmonary hypertension (23). To efficiently generate glial cell-derived neurotrophic factor (GDNF), which can be used to improve kidney damage, the mRNA was inserted into MSCs via a lentiviral transfection system and EVs were isolated. In the hypoxia/serum deficiency (H/SD) model, GDNF-enhanced MSC-derived EVs showed a cytoprotective effect on human umbilical vein endothelial cells (HUVECs) against damage by stimulating migration and angiogenesis and inducing resistance to apoptosis (24). GLO-1, the major rate-limiting enzyme of the glyoxalase system, is known to decrease the excessive accumulation of toxic end products due to oxidative stress in cells via glycolysis. GLO-1 was overexpressed to produce GLO-1-enhanced ASC-derived EVs. Under in vitro and in vivo conditions, GLO-1-enhanced EVs improved type 2 diabetes via activation of the eNOS/AKT/ERK/P-38 signaling pathway, inhibition of AP-1/ROS/NLRP3/ASC/Caspase-1/IL-1β, and increased secretion of various growth factors (25).

Unlike mRNA, transfecting miRNAs into EVs facilitates simultaneous targeting of a variety of cellular signals. The miRNA is a short, noncoding and single-stranded RNA molecule that regulates the expression of protein-coding genes by promoting degradation and interfering with translation based on the mRNA-miRNA complementary sequence (26, 27). Compared with siRNA, miRNAs cannot inhibit protein coding completely. However, they can lead to approximately 60% inhibition via a similar mechanism and facilitate efficient transformation due to their small size and low weight (28). The miRNAs released from EVs play a key role in regenerative medicine due to internalization in donor cells and effects on neighboring genes by altering cell-cell communication, and thus used as cell-free therapy (29). To our knowledge, this function is mediated via direct or indirect effects on neighboring cells or tissue microenvironment via paracrine effects of stem cells at the transplanted site.

Izarra et al. observed highly expressed miRNA-133a via analysis of purified exosomal fraction after transfecting miRNA-133a, which is involved in cardiac development and

pathophysiology, into SCA-1+ Lin− adult cardiac progenitor cells (CPCs) (30). The miRNA-133a-enriched CPCs clearly improved cardiac function in a murine model of myocardial infarction by reducing fibrosis and hypertrophy and increasing vascularization and cardiomyocyte proliferation. Despite indirect treatment of EVs, this study showed a correlation with upregulated expression of several relevant paracrine factors and secretion of miRNA-133a via EV (30). Also, other studies demonstrated the regulatory effects of exogenous miRNA in endothelial cells (ECs) on smooth muscle cell (SMC) turnover. Biotinylated miR-126 transfected into ECs completely affected SMC co-culture in the same medium, and the neointimal lesion formation of carotid arteries by cessation of blood flow was inhibited by regulating the levels of FOXO3, BCL2, and IRS1 via direct EC-to-SMC transmission (31, 32).

In addition, an increasing number of experimental studies demonstrated the relevance of direct miRNA delivery using EVs in various physiological environments. For example, in neuronal to astrocyte signaling, EV-mediated miRNA-124a modulated glutamate transporters, especially excitatory amino acid transporter 2 (EAAT2, rodent analog GLT1), using SOD G93A mouse, an end-stage animal model of amyotrophic lateral sclerosis (ALS). It was recognized that miRNA-124a controls perisynaptic function via neuron-astrocyte communication (33). Furthermore, Salvucci et al. showed that adipose tissue macrophages (ATMs) derived from obese mice secreted miRNA-155-containing exosomes (Exos) that induced glucose intolerance and insulin resistance when administered to lean mice via direct inhibition of the target gene peroxisome proliferator-activated receptor (PPAR) γ. This suggests that these EV regulatory systems can be delivered to insulin target cell types via paracrine or endocrine regulatory mechanisms that strongly influence insulin sensitivity and overall glucose homeostasis (34).

An antisense oligonucleotide (ASO) construct antagonizing oncogenic miR-125b was induced in red blood cells (RBCs), suggesting the potential for treatment of leukemia cells using RBC-EVs. RBCs lacking nuclear and mitochondrial DNA were treated with ASO-

miRNA-125b, demonstrating a safe and scalable platform without the risk of horizontal gene transfer (35).

Strategies for loading EV cargo

To our knowledge, two approaches can be used to generate EVs containing miRNAs for multiplexed targeting. First, overexpression can be induced by directly injecting miRNA into cells. The easiest and simplest method of miRNA loading is incubation with miRNA-secreting cells. Migration to intracellular vesicles by concentration gradient represents a natural integration mechanism that does not threaten cell survival. However, this strategy cannot be used to predict the concentration of miRNAs that diffuse into EVs, and the low efficiency due to miRNA instability.

Chemical transfection can be used to stably introduce miRNA into EVs. As mentioned earlier, stem cells are basically loaded with paracrine molecules, and EVs inside the cells represent the best tool for the biogenesis and delivery of the desired miRNAs. It is used to transduce miRNA itself or a specific plasmid/virus-based construct designed to activate cellular miRNA and induce ectopic expression and incorporation into EVs (36, 37).

Transient micropores can be induced in EV membranes or membrane recombination sites to promote miRNA incorporation visa sonication and electroporation. Ultrasound has been used to develop solid images using sound waves. However, it is possible to apply a low level of mechanical shear force to increase the membrane permeability transiently. Lamichhane et al. found that miRNA capable of oncogene knockdown was introduced into EVs more than 3 times via sonication, yielding significantly better results than siRNA and single-strand RNA (38). Electroporation has been widely used for gene delivery into cells but can also be used for transfection of miRNA EV. Liang et al. used electroporation (1000 V, 10 ms and 2 pulses), while simultaneously treating cells with miRNA-21 and chemotherapeutics to control drug

resistance in colon cancer. EV-derived miR-21 induced cell cycle arrest and apoptosis by simultaneously regulating phosphatase and tensin homolog (PTEN) and mutant DNA mismatch repair (hMSH2) (39). However, since the extracellularly ejected EV or the miRNA derived from the EV may aggregate or be destroyed, it is necessary to calculate the appropriate parameters before proceeding with the experiment. In addition, since it is difficult to distinguish between the results of direct packaging of miRNA via biogenesis in EV and the results of overexpression of miRNA via external processing, it is necessary to understand these approaches. (40, 41).

Second, it is a direct method to isolate EVs from stem cells, concentrate them, and transfer miRNAs. Zhang et al. newly developed a modified calcium chloride-mediated transfection method to produce miRNA-15a-loaded EVs. EVs isolated from macrophages were mixed with miRNA, followed by application of calcium chloride and heat shock, establishing their functional role in recipient cells (42). Naseri et al. isolated EVs from bone marrow-derived mesenchymal stem cells, and LNA-anti-miRNA-142-3p was transfected into EVs via electroporation. LNA-antimiRNA-142-3p-incorporated EVs reduced colonization and tumor formation in breast cancer stem cells (43). Although the yield of these techniques was increased compared with that of miRNA transfection into cells, the difficulty in determining the mechanism of packaging miRNAs within EVs is a significant limitation (40, 41). Therefore, according to a recent study, the protons in EVs generate a pH gradient across the EV membrane that can be used to enhance vesicle loading of nucleic acids containing miRNAs. This is one of the new strategies that can be achieved without introducing external energy (44).

REGENERATIVE MEDICINE FOR TISSUE REPAIR

A recent study reported improved regeneration by introducing stem cell-mediated miRNA into EVs inside the cell (13, 14). In this section, we discuss the case of multiplexed targeting of various signals related to cell/tissue improvement by stem cell-derived EVs with increased miRNA used in heart and brain diseases.

Coronary artery disease, a representative cardiovascular disease, is caused by atherosclerosis and contributes to arrhythmias and myocardial infarction. Stem cell-derived EV therapy can be used to maintain the homeostasis of various cells (cardiac cells, fibroblasts, lymphocytes, mast cells, and macrophages) in the cardiovascular system and facilitate cardiac regeneration and repair (14), especially using EV-derived miRNAs via multiplexed targeting.

Song et al. conducted a multiplexed targeting study with positive effects on cardiac and vascular cells, while maintaining the advantages of stem cells, but a negative effect on myocardial fibroblast proliferation. Using a lentivirus, miRNA-EV system was constructed to stably generate miRNA-210, which plays a pivotal role in cardiovascular cells, in adult stem cell EVs. The miRNA-210 in stem cell-derived EVs controlled cardiac cell death by targeting protein tyrosine phosphatase 1B (PTP1B) and death-associated protein kinase 1 (DAPK1), and also controlled angiogenesis of vascular cells by targeting Ephrin A (EFNA3), without affecting cardiac fibroblasts (14). Similarly, substances targeted by miRNA-133a expressed in adult CPC were discovered via bioinformatics analysis of BMF (Bcl-2 modifying factor) and BIM (Bcl2l11), which are potent proapoptotic factors, a serine threonine kinase STK4 (formerly Mst1) activated by oxidative stress, and Foxo1 that is a critical promotor of cardiomyocyte survival under oxidative stress conditions. EVs derived from miR-133-CPC have been shown to protect cardiac function and prevent hypertrophy as well as increase vascularization (30). Peng et al. also identified exosomal miRNA-25-3p targeting the proapoptotic protein enhancer of zest homologue 2 (EZH2). Bone marrow MSC-derived miRNA-25-3p generally targets phosphatase and tensin homologue deleted on chromosome 10 (PTEN) and Fas ligand (FasL),

which are proapoptotic genes, and contributes to the cardioprotective effect of EZH2. The cardioprotective and inflammatory effects on induced cardiomyocytes were confirmed under oxygen-glucose deprivation (OGD) (45). Ferguson et al. found multitarget candidates inducing cardiomyocyte proliferation after isolating MSC exosomes in virus-free miRNA loading conditions based on a miRNA prediction system. Results suggested a total of 22 targeting miRNA-199a, including retinoblastoma protein 1(RB1), which leads to cell cycle arrest, liver kinase B1 (LKB1), specific loss of which results in cellular proliferation, and NEUROD1, were associated with cell cycle arrest (46).

Stem cell-derived EV-based miRNA studies investigating multiplexed targeting in CNS and cardiovascular diseases have yet to be reported on a large scale. In order to control Rett syndrome (RTT), one of the neurodevelopmental disorders, Pan et al. transfected miRNA-21-5p into urine-derived stem cells to establish the association with EVs. This study also confirmed that miRNA-21-5p directly targeted Eph receptor A4 (EphA4) after incorporating EVs into neuronal stem cells and was negatively correlated with TEK receptor tyrosine kinase (TEK or Tie2) receptor signaling (47). Spinal cord injury (SCI) due to loss of motor and sensory function has been mostly studied using stem cell therapy (48). In order to effectively improve SCI while ensuring their paracrine effect, Huang et al. confirmed the EV effect after loading miRNA-126 into MSCs. In the case of miRNA-126, the functional recovery following direct treatment was already established by the same research group (49). In MSC-derived MV, the levels of miRNA-126 were enhanced by increasing angiogenesis and neurogenesis and inhibiting apoptosis via multiple targeting of sprouty-related EVH1 domain-containing protein 1 (SPRED1) and phosphoinositide-3-kinase regulatory subunit 2 (PIK3R2). Thus, this result was established in the therapeutic study investigating the multiple targeting effects of EV-derived miRNA-126, which confirmed the stem cell effect (50).

CONCLUSION

Therapeutic studies investigating regenerative medicine using stem cells have reported enormous progress. Investigations into stem cell-derived EV are also currently in progress, however it is necessary to establish their therapeutic efficacy and safety in vivo. Stem cell-derived EVs with increased specificity and multiplexed targeting of miRNAs represent powerful cell-derived therapeutic agents that can overcome the limitations of existing studies.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

FIGURE LEGENDS

Figure 1. Size comparison of extracellular vesicles.

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Table 1. A summary of enhancing information for cell-derived extracellular vesicles.

Types of RNAs	Target	Cell source of EVs	Effects	Reference
mRNA	TGF-β1 BMP-4	human pulmonary artery smooth muscle cells	Vascular remodeling EndoMT	23
	GDNF	human adipose mesenchymal stem cells	Angiogenesis Decrease of renal fibrosis	24
	GLO-1	Adipose derived stem cells	Angiogenesis Improvement of T2DM	25
miRNA	miRNA-133a	Adult cardiac progenitor cells	Indirect EV treatment Anti-apoptosis Improvement of cardiac function	30
	miRNA-126	Endothelial cells	Indirect EV treatment SMC turnover Atheroprotective laminar shear stress	31
	miRNA-124a	Neuron	Neuron-astrocyte communication Improvement of ALS	33
	miRNA-155	Adipose tissue macrophage	Glucose tolerance Insulin sensitivity	34

	ASO-miRNA-125b	Red blood cells	Treatment of leukemia cells	35

Table 2. A summary of stem cell-derived EV therapies mediating multiplexed targeting by miRNAs

Disease	miRNA	Target	Cell source	Effects	Reference
Ischemic heart	miRNA-210	Ptp1b Dapk1 Efna3	Human adipose-derived stem cells	Regulation of apoptosis and angiogenesis Heart regeneration	14
	miRNA-133a	Bmf Bim Stk4 Foxo1	Cardiac progenitor cells (SCA-1+ Lin−)	Anti-apoptosis Improvement of cardiac function	30
	miRNA-25-3p	Fasl Pten Ezh2	Bone marrow mesenchymal stem cells	Cardioprotection Anti-inflammation	45
Heart	miRNA-199a	22 genes included Rb1 Lkb1 Neurod1	Mesenchymal stem cells	Cell death/proliferation Cell cycle regulation (Target Prediction)	46
Rett syndrome	miRNA-21-5p	EphA4 Tek	Urine-derived stem cells	Facilitation of early nerve formation Neurogenesis	47
Spinal cord	miRNA-126	Spred1 Pik3r2	Mesenchymal stem cells	Angiogenesis Neurogenesis	50

injury

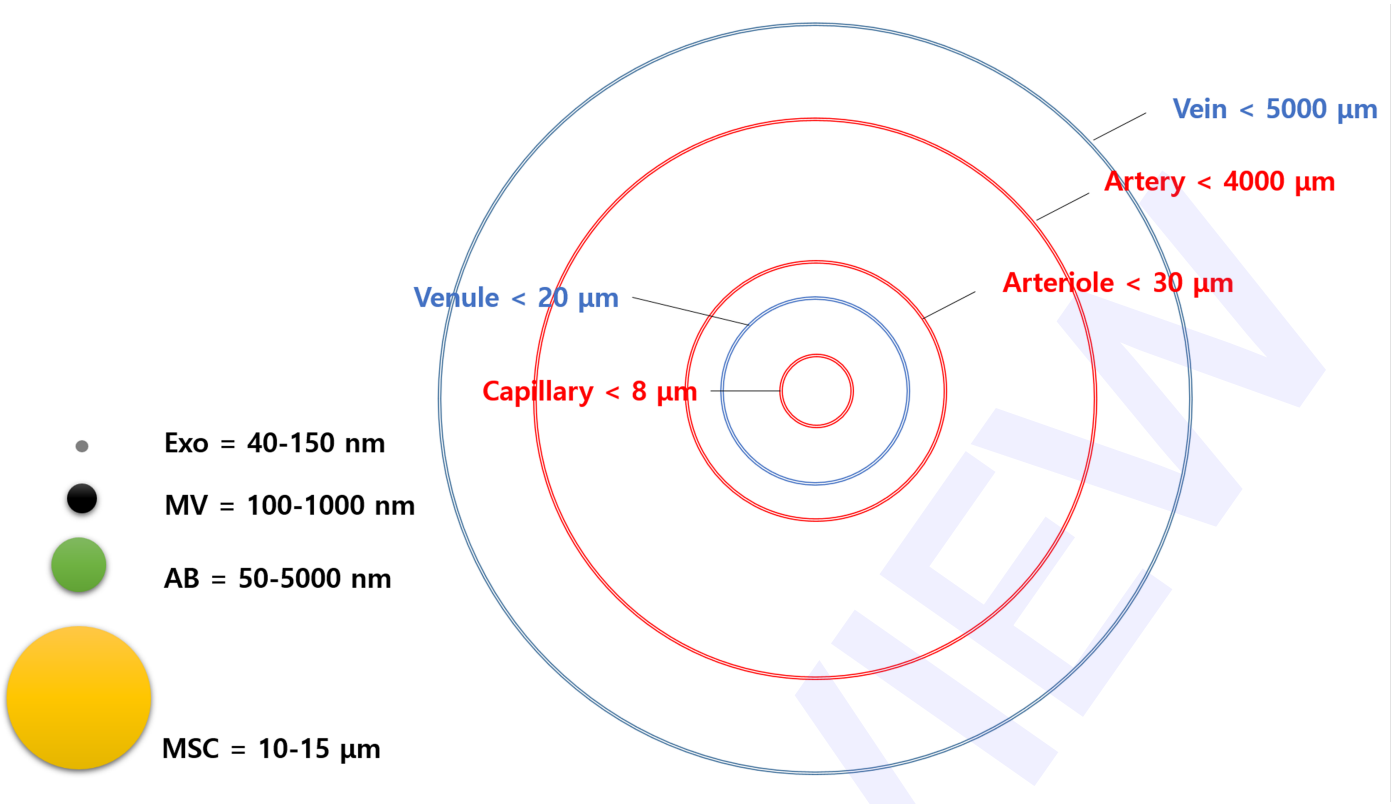

Fig. 1.