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## REVIEW

# The therapeutic potential of mesenchymal stem cell-derived extracellular vesicles

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Extracellular vesicles (EVs), membrane vesicles that are secreted by a variety of mammalian cell types, have been shown to play an important role in intercellular communication. The contents of EVs, including proteins, microRNAs, and mRNAs, vary according to the cell type that secreted them. Accordingly, researchers have demonstrated that EVs derived from various cell types play different roles in biological phenomena. Considering the ubiquitous presence of mesenchymal stem cells (MSCs) in the body, MSC-derived EVs may take part in a wide range of events. In particular, MSCs have recently attracted much attention due to the therapeutic effects of their secretory factors. MSC-derived EVs may therefore provide novel therapeutic approaches. In this review, we first summarize the wide range of functions of EVs released from different cell types, emphasizing that EVs echo the phenotype of their parent cell. Then, we describe the various therapeutic effects of MSCs and pay particular attention to the significance of their paracrine effect. We then survey recent reports on MSC-derived EVs and consider the therapeutic potential of MSC-derived EVs. Finally, we discuss remaining issues that must be addressed before realizing the practical application of MSC-derived EVs, and we provide some suggestions for enhancing their therapeutic efficiency.

Received: August 16, 2012

Revised: October 4, 2012

Accepted: October 22, 2012

**Keywords:**

Cell biology / Exosome / Extracellular vesicle / Mesenchymal stem cells / Microvesicle / Therapeutics

## 1 Introduction

The presence of membrane vesicles in the extracellular space was observed as early as in 1960s [1], but their significance remained obscure for a long time. These vesicles were later

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**Abbreviations:** **A $\beta$** ,  $\beta$ -amyloid peptide; **AD**, Alzheimer's disease; **ADSC**, adipose tissue-derived mesenchymal stem cell; **AKI**, acute kidney injury; **BM**, bone marrow; **CM**, conditioned medium; **DC**, dendritic cell; **EBV**, Epstein-Barr virus; **EC**, endothelial cell; **EGFR**, epidermal growth factor receptor; **ESC**, embryonic stem cell; **EV**, extracellular vesicle; **HLSC**, human liver stem cell; **IFN**, interferon; **IL**, interleukin; **iPSCs**, induced pluripotent stem cells; **MCAo**, middle cerebral artery occlusion; **MIR**, myocardial ischemia/reperfusion injury; **miRNA**, microRNA; **MSCs**, mesenchymal stem cells

classified into two types according to their secretory processes, namely, exosomes and shedding vesicles [2, 3]. Exosomes were discovered around 30 years ago as small vesicles that were released when multivesicular endosomes fused with the plasma membrane [4, 5]. These vesicles, however, were long regarded as cellular garbage cans for discarding unwanted molecular components [2, 6–8]. Meanwhile, shedding vesicles were also found in many biological processes as vesicles that directly bud from the cell plasma membrane [9]. However, shedding vesicles were also considered for a long time to be inert cellular debris resulting from cell damage or dynamic plasma membrane turnover [3, 10]. It is only recently that major advances have been made in the identification of their biological significance as tools of intercellular communication.

Some confusion exists in the literature regarding the terms “exosomes” and “microvesicles.” The difference between these two terms is generally based on size: exosomes are in the range of 10–100 nm, and microvesicles are in the range of 100–1000 nm. However, because this research area

is still in its infancy, these definitions are flexible. Some researchers use these terms according to strict definitions, whereas others use the terms interchangeably. In particular, the term microvesicles have been often used more widely for membrane vesicles regardless of their intracellular origin, including exosomes and shedding vesicles. Recently, the International Society for Extracellular Vesicles have recommended researchers to use the term “extracellular vesicles (EVs)” as an umbrella term for all types of vesicles present in the extracellular space, including exosomes, shedding vesicles, melanosomes, prostasomes, and apoptotic bodies. Following this recommendation, we use the term EVs throughout the paper.

EV research has dramatically changed because of two major breakthroughs, and it is now attracting much interest from various fields. The first breakthrough occurred in 1996 when Raposo's group found that EVs derived from immune cells function as activators of the immune system [11]. Many groups have since reported that EVs derived from certain cell types contain functional proteins that can activate biological events. These findings have established the novel concept that EVs serve as carriers for intercellular communication. The second breakthrough came with the findings that EVs shuttle functional mRNAs and microRNA (miRNA). In 2006, Ratajczak's group found that mRNA could be delivered by EVs to target cells and translated into the corresponding proteins [12]. In the next year, Lötvoll's group found that EVs contain not only mRNA but also miRNA [13]. Furthermore, in 2010, three groups independently demonstrated that the miRNAs contained in EVs also traveled between cells and suppressed the expression of target genes in recipient cells [14–16]. EV function, the mechanisms of EV biogenesis and secretion, and the molecular composition of EVs, including proteins, lipids, and nucleic acids, have been comprehensively studied [17]. For example, release of exosomes and shedding vesicles are regulated by calcium-dependent manner [3, 18]. On the other hand, Simon's group revealed that ceramide triggered budding of exosome vesicles into multi-vesicular endosomes, providing evidence for an alternative pathway to ESCRT (endosomal sorting complex required for transport) machinery-independent pathway for exosome biogenesis [19]. Consistent with this observation, Kosaka et al. demonstrated that decreased activity of neutral sphingomyelinase 2 (nSMase2), a rate-limiting enzyme in ceramide biosynthesis, resulted in the reduced secretion of miRNAs [16]. In addition, Théry's group demonstrated that Rab27 isoforms play essential roles in the exosome secretion pathway [20].

One major interest in this research area is the potential for various EV functions in therapeutic applications. While secreted EVs exhibit some common, shared contents, they also express molecules that reflect the originating cells [21]. Accordingly, there are many reports showing that functions of EVs reflect, at least in part, those of the originating cells despite the differences between the contents of EVs and those of the originating cells. Furthermore, it should be noted that EV-derived molecules can still help the parent cells play their

roles even in a noncanonical manner [22]. From these evidences, the hypothesis can be drawn that cells utilize their EVs to fulfill their roles. This suggests that EVs released from cells that are capable of repairing damaged tissue may also have therapeutic ability. Some of the most promising candidate parent cells are mesenchymal stem cells (MSCs). A rapidly increasing number of reports have suggested that using MSC-derived EVs in treatment for several diseases is feasible. In this article, we first summarize the wide range of functions among EVs released from different cell types and highlight evidence showing that EVs reflect the phenotype of their parent cell. Then, we describe the various therapeutic effects of MSCs, with particular emphasis on the significance of the MSC paracrine effect. In subsequent sections, we survey the latest reports on MSC-derived EVs and discuss their therapeutic potential. Finally, we point to issues that require attention before realizing the practical application of EVs, and we provide some suggestions for enhancing therapeutic EV effects.

## 2 The functionality of EVs is origin-dependent

EVs have functions that depend on the phenotype of their parent cell. Because cells package cellular material into EVs, it is reasonable to speculate that EV content echoes that of its parent cell. Indeed, the molecular contents of EVs, including proteins, mRNAs, and miRNAs, are reported to imitate, at least in part, their parent cells. Selective enrichment of specific molecules in EVs, however, is also observed. The mechanisms underlying the EV packaging process require further study. Nonetheless, it should be noted that as described below, various types of cells produce EVs with functions that mirror those of their parent cells.

### 2.1 EVs in the immune system

The first evidence for EV functionality came from immunology studies. In 1996, Raposo et al. revealed that B-lymphocytes secreted antigen-presenting EVs [11]. They found that EVs released from human and murine B-lymphocytes induced an antigen-specific major histocompatibility complex class II-restricted T cell response. Further, they later demonstrated that dendritic cells (DCs) secreted antigen presenting EVs that expressed major histocompatibility complex class I and class II proteins and T-cell costimulatory molecules [23]. Importantly, the DC-derived EVs served as a novel cell-free vaccine. Tumor peptide-pulsed, DC-derived EVs primed specific cytotoxic T lymphocytes *in vivo* and eradicated murine tumors. Furthermore, mast cell-derived EVs were also reported to participate in immune reactions. Skokos et al. showed that mast cell-derived EVs activated B- and T-lymphocytes [24] and induced phenotypic and functional maturation of DCs [25].

Recent reports have revealed EV-mediated miRNA transfer between immune cells. Pegtel et al. demonstrated that

EV-mediated miRNAs secreted by Epstein-Barr virus (EBV) infected B cells were transferred to uninfected recipient cells such as DCs [14]. The internalized EBV miRNAs repressed target genes including CXCL11, an immunoregulatory gene. Of note, they found that in peripheral blood mononuclear cells from patients with increased EBV load, EBV miRNAs were present not only in B cells but also in uninfected non-B cells, suggesting *in vivo* miRNA transfer. Mittelbrunn et al. also reported EV-mediated unidirectional transfer of miRNAs from T cells to antigen-presenting cells [26]. Interestingly, Mittelbrunn et al. found that immune synapse between these two cell types significantly increased the efficiency of EV-mediated delivery of the miRNAs.

## 2.2 Tumor cell-derived EVs in cancer immunity

In addition to immune cells, tumor cells also release EVs that are related to cancer immunity. At the early stages of carcinogenesis, cell-intrinsic barriers to tumor development seem to be associated with stimulation of an active antitumor immune response, a process known as cancer immunosurveillance [27, 28]. Cancer cells avoid immunosurveillance through the outgrowth of poorly immunogenic tumor-cell variants and through subversion of the immune system, which allows cancer cells to achieve immunotolerance [27, 28]. Tumor cell-derived EVs have been reported to play roles in both immunosurveillance and immunotolerance.

Tumor cell-derived EVs can contribute to tumor rejection by the host immune system. The first functional study on tumor-derived EVs by Zitvogel's group reported that tumor cell-derived EVs transferred tumor antigens to DCs, which resulted in CD8<sup>+</sup> T cell-dependent anti-tumor effects [29]. Accordingly, Théry's group highlighted the importance of EVs for an efficient anti-tumor immune response [30]. They showed that tumor cells secreting an antigen in an EV-associated form induced antitumor immune responses more efficiently than those secreting the same antigen as a soluble protein. Furthermore, tumor cell-derived EVs also played an important role in activation of the innate immune system by stimulating migratory and cytolytic activity of natural killer cells [31].

In contrast to the above reports, tumor cell-derived EVs have also been reported to promote tumor immune resistance. Andreola et al. reported that melanoma cell-derived EVs induced T-cell apoptosis [32]. They showed that this effect could be ascribed to EV-bound Fas ligand, a transmembrane protein belonging to the tumor necrosis factor family that plays a pivotal role in the induction of Fas receptor-mediated apoptosis. A similar effect was confirmed for prostate cancer cell-derived EVs [33]. Clayton et al. showed that mesothelioma cell-derived EVs selectively impaired lymphocyte response to IL-2 by suppressing cytotoxic T lymphocytes and natural killer cells [34]. Intriguingly, these EVs did not impair regulatory T-cell response to IL-2. Instead, their inhibitory function was enhanced by tumor EVs. These ef-

fects worked in concert to contribute to the tumor immune resistance. Consistently, Chalmin et al. found that tumor cell-derived EVs activated the immunosuppressive activity of myeloid-derived suppressor cells, which are thought to contribute to tumor progression [35].

## 2.3 Tumor cell-derived EVs can modulate the surrounding environment

In addition to cancer immunity, tumor cell-derived EVs seem to have various functions that affect tumor invasiveness, tumor cell proliferation, and the formation of pro-metastatic and pre-metastatic niches. Many studies have reported that EV-mediated effects occur through EV cargo proteins including growth factors/cytokines and membrane-bound receptors such as epidermal growth factor receptor (EGFR). In addition, recent reports have suggested a pathological significance for EV-mediated transfer of tumor cell-derived miRNAs.

Tumor cell-derived EVs can interact with surrounding cells and promote malignancy in an autocrine/paracrine-dependent manner. Gutwin et al. reported that ovarian carcinoma cell-derived EVs mediated the secretion of the L1 adhesion molecule (CD171) that is overexpressed in human ovarian and endometrial carcinomas and is associated with a poor prognosis [36]. The authors found the presence of L1 on the EV surface, and the membrane-bound L1 was subsequently cleaved into a soluble form that triggered cell migration and phosphorylation of ERK. Higginbotham et al. revealed a novel, EV-mediated pathway for EGFR ligands [37]. Higginbotham et al. found that human breast and colorectal cancer cells released EVs containing full-length, signaling competent EGFR ligands. Interestingly, these EVs increased the invasiveness of recipient breast cancer cells over an equivalent amount of the soluble form of EGFR ligands. In addition, an oncogenic form of EGFR, EGFRvIII, can be transferred via this EV-mediated pathway to recipient cells. In aggressive human brain tumors, gliomas, only a small percentage of cells possess the EGFRvIII gene; however, most of these cells exhibit a transformed, carcinogenic phenotype [38]. Al-Nedawi et al. showed that EVs containing EGFRvIII were released from glioma cells, merged with the plasma membranes of cancer cells lacking EGFRvIII, and led to the transfer of oncogenic activity, including the activation of transforming signaling pathways, changes in expression of EGFRvIII-regulated genes, morphological transformation, and an increase in anchorage-independent growth capacity [39]. Furthermore, Skog et al. demonstrated the presence of EGFRvIII mRNA in EVs extracted from primary glioblastoma cells [40]. Their observation that recipient cells translated messages delivered by EVs supports the idea that EGFRvIII delivery among glioblastoma cells involves EV-mediated transfer of both its message and the translated product.

Tumor cell-derived EVs are delivered not only to tumor cells but also to neighboring and/or distant normal cells. These EVs provide a supportive environment for

tumor progression. Several groups have reported that tumor cell-derived EVs can be transferred to vascular endothelial cells (ECs) where they promote angiogenesis. Zöller's group found that tetraspanin 8 (Tspan8)-expressing tumor cells enhanced tumor growth by inducing angiogenesis and that Tspan8-bound EVs also promoted *in vitro* angiogenesis [41]. They also showed that Tspan8-CD49d association contributed to EV binding to ECs and that internalized EVs modulated the fate of ECs and EC progenitors [42]. In the same manner as described above [39], EV-mediated EGFR transfer also occurred between tumor cells and recipient ECs, which resulted in the onset of vascular endothelial growth factor expression in ECs, and autocrine activation of its key signaling receptors, i.e. vascular endothelial growth factor receptor2 [43]. Sheldon et al. found that a Notch ligand delta-like 4 was expressed on tumor cell EVs, and transferred to ECs *in vitro* and *in vivo* [22]. Intriguingly, transferred delta-like 4 did not activate Notch signaling in the ECs, but instead inhibited the pathway. As a consequence, tumor-derived EVs switched the recipient ECs to tip cell phenotype, and induced angiogenesis. This finding highlights the notion that EV-derived molecules can help the parent cells play their roles even in a noncanonical manner. In addition to neighboring cells, tumor cell-derived EVs can reach distant cells. In 2005, Kaplan et al. demonstrated that vascular endothelial growth factor receptor1+ bone marrow (BM) progenitor cells migrated to and conditioned a premetastatic lung niche in response to systemically administered melanoma-derived conditioned medium (CM) [44]. This group recently reported that this effect was, at least in part, due to secreted EVs. Metastatic melanoma-derived EVs altered BM progenitor cells toward a prometastatic phenotype [45]. Moreover, Jung et al. reported that CD44v6 expressing tumor cell-derived CM is essential for premetastatic niche formation in lymph nodes and lung tissue [46]. Their data suggested that tumor cell-derived EVs, when assisted by the soluble fraction of CM, promoted premetastatic niche formation.

## 2.4 EVs in other biological phenomena

EV-mediated intercellular communication is not limited to the immune system and cancer pathology. Rather, it seems that most cell types produce EVs, and their functions vary accordingly. Ratajczak et al. demonstrated that embryonic stem cell (ESC)-derived EVs may contribute to cell-fate determination and may be a critical component in the self-renewal and expansion of stem cells [12]. Deregibus et al. reported that endothelial progenitor cell-derived EVs activated an angiogenic program in ECs via transfer of mRNA [47]. Recently, our group reported that in contrast to tumor cells, noncancerous cells secreted EVs containing tumor-suppressive miRNAs and inhibited the proliferation of tumor cells [48]. This finding suggests that EV-derived tumor-suppressive miRNAs act as an inhibitory signal for cancer cells in a cell-competitive process.

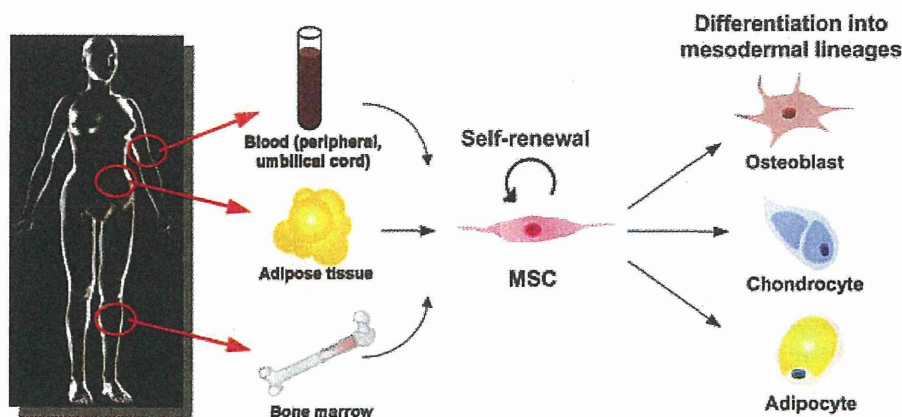
## 3 MSCs and their therapeutic potential

The possibility of cell therapy using pluripotent stem cells has attracted much attention from researchers and the general public alike, but such technology is not yet within reach. Although pluripotent ESCs have long been predicted as a cell source for regenerative medicine, use of ESCs has been hampered by the possibility of immune rejection and ethical issues. Induced pluripotent stem cells (iPSCs) have recently gained increasing attention as a cell source that can circumvent the problems associated with ESCs. However, despite their promising potential, many hurdles must be overcome before human iPSC-based therapy will appear in clinics (reviewed in [49]). For example, similar to the case with ESCs, therapeutic application of iPSCs involves the risk of teratoma formation. Therapy with iPSC also involves genetic modification, which could possibly give rise to various obstacles [50, 51]. Thus, there still exists a need for an alternative to iPSCs in order to make cell therapy a viable option.

MSCs, a type of adult stem cell, have emerged as a very attractive candidate for cell therapy applications [52–55]. MSCs can be isolated from adult connective tissue such as BM and adipose tissue and can differentiate into mesodermal cell lineages. MSCs have already been clinically applied during breast reconstruction following conservative surgery for breast cancer, and research is now underway on clinical applications for MSCs in disorders such as chronic heart disorders, acute myocardial infarction, and stroke [56]. Moreover, in the last decade, it has been demonstrated that MSCs have many other functional properties. They can differentiate into cells from unrelated germline lineages, resist immunosurveillance, home to injured tissue, and secrete factors with immunosuppressive, anti-apoptotic, and trophic effects [52–55]. These clinically useful features of MSCs have provoked enthusiasm for their application in a wide range of clinical situations.

### 3.1 What are MSCs?

MSCs are multipotent stem cells present in mesodermal tissue [52–55]. In the 1970s, Friedenstein et al., using BM, first isolated spindle-shaped, clonogenic cells in monolayer cultures that could differentiate into colonies resembling small deposits of bone or cartilage [57, 58]. Other groups extended the observations of Friedenstein et al. throughout the 1980s [52] and established that these cells were multipotent and could differentiate into osteoblasts, chondrocytes, adipocytes, and even myoblasts. To date, similar cells have also been isolated from a variety of other connective tissues such as adipose tissue, periosteum, perichondrium, cartilage, umbilical cord blood and tissue, amniotic membrane, and synovial tissue [52–55]. These cells are currently referred to as MSCs because of their ability to differentiate into mesenchymal-type cells (Fig. 1).



**Figure 1.** Origin and characteristics of mesenchymal stem cells. MSCs can be isolated from various tissues, including bone marrow, adipose tissue, and blood including peripheral and umbilical cord blood. MSCs have the capacity to self-renew and differentiate into multiple cell lineages including three major mesodermal lineages: osteoblasts, chondrocytes, and adipocytes.

The surface marker profiles for MSCs differ between species and are dependent on methods of isolation and culture, making a common standard and a precise definition for an MSC difficult. The current and widely accepted definition for MSCs was proposed in 2006 by the International Society for Cellular Therapy [59]. First, an MSC must be plastic adherent when maintained in standard culture conditions. Second, an MSC must express CD105, CD73, and CD90 and lack expression of CD45, CD34, CD14, or CD11b, CD79a, or CD19 and HLA-DR surface molecules. Third, an MSC must differentiate to osteoblasts, adipocytes, and chondroblasts *in vitro*.

Although MSCs initially attracted interest for their ability to differentiate into cells of mesodermal lineage *in vitro* and *in vivo*, the beneficial effects of MSCs appear to be due to other properties. MSCs can transdifferentiate into other lineages besides mesoderm, and they migrate to injured tissue beds, interact with injured host cells, and secrete paracrine-soluble and growth factors that modulate immune responses and alter the responses of the endothelium or the epithelium to injury (Fig. 2).

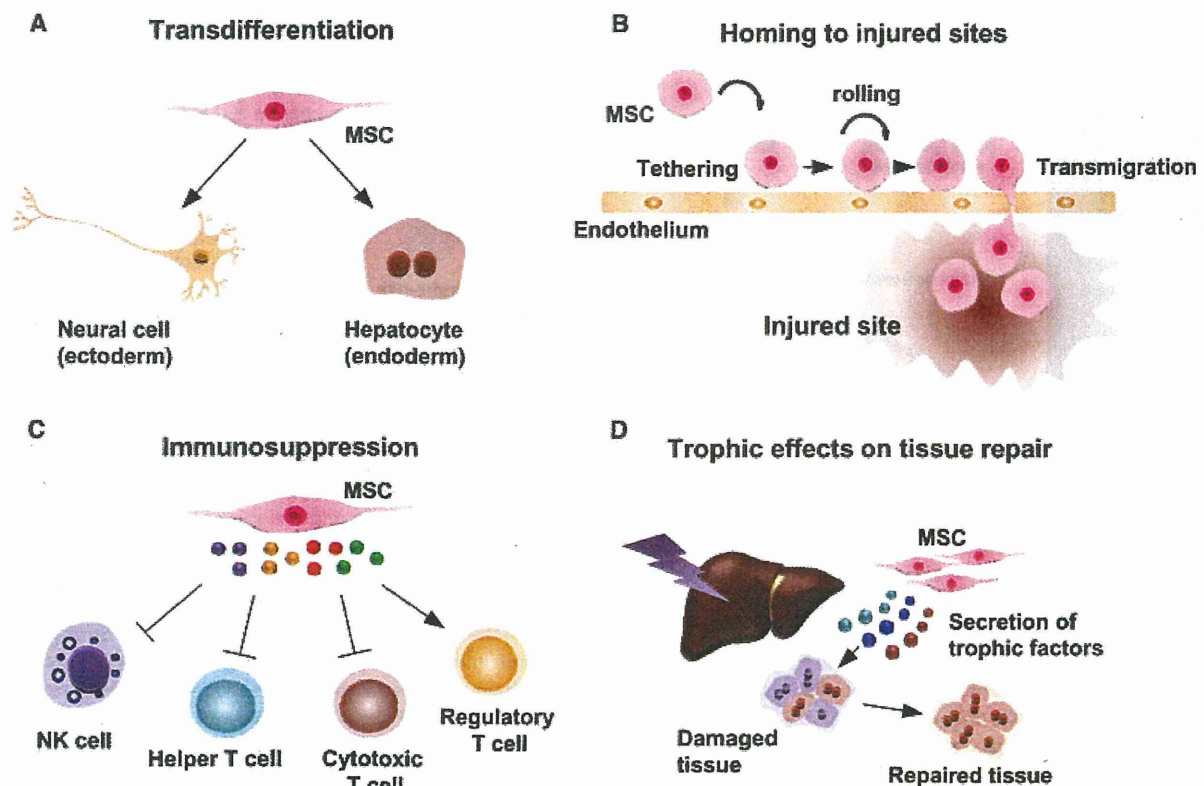
### 3.2 Multipotency of MSCs and their potential application in cell replacement therapy

A remarkable transition in MSC study occurred in the last decade when these cells were found to possess greater plasticity than that dictated by the established paradigms of embryonic development. MSCs have been shown to transdifferentiate into ectoderm including neuroectoderm cells [60], retinal pigment epithelial cells [61], and skin epithelial cells [62], and endoderm including hepatocytes [63], kidney tubular epithelial cells [64], and lung epithelial cells [65, 66]. In particular, a large number of papers have been published on the generation of neuroectoderm cells and hepatocytes.

Neuroectoderm differentiation was the first recognized transdifferentiation event in MSCs and has been extensively studied [60]. The brain has long been regarded as incapable of regeneration. Thus, the discovery of neural stem cells, which

are capable of undergoing expansion and differentiation into neurons, astrocytes, and oligodendrocytes, has generated intense interest [67]. However, the location of neural stem cell sources severely limits clinical utility because they are deep in the brain. Kopen et al. first demonstrated that MSCs that were injected into the CNS of newborn mice migrated throughout the brain and adopted the morphological and the phenotypic characteristics of astrocytes and neurons [68]. These results were supported by similar observations from several other groups [69–71]. In parallel, MSCs were also shown to transdifferentiate *in vitro* into neural ectoderm cells [72, 73]. Many researchers have since investigated methods for *in vitro* induction of MSCs to neurons using various soluble factors [60, 74]. Results from these studies have raised expectations for the development of a novel therapeutic approach for neurodegenerative diseases using MSCs.

Another cell type that researchers have attempted to generate using MSCs is hepatocytes, which are liver parenchymal cells. The only treatment for end-stage liver diseases is liver transplantation, but transplantation is limited due to a shortage of donors. Many alternative therapies for liver failure are currently being developed and studied, including hepatocyte transplantation [75], extracorporeal bioartificial liver support devices [76], and heterotopic transplantation of engineered liver tissue [77]. In these studies, mature hepatocytes have been the main cell source. However, the use of a large amount of mature hepatocytes is constrained by availability in a clinical setting. Many researchers have attempted to obtain functional hepatocytes from progenitor cells including ESCs [78–80], iPSCs [81–83], fetal liver cells [84–86], and adult liver stem cells [87–89]. However, the use of these cell types has been hampered by ethical concerns as well as poor availability and accessibility [90]. The possibility of hepatic differentiation of MSCs has opened up a new avenue for improving the availability of functional hepatocytes [63]. Chen's group and Lee's group were the first to each independently report that BM-MSCs and umbilical cord blood-derived MSCs can differentiate into hepatocyte-like cells expressing liver-specific markers *in vitro* [91, 92]. These results were further confirmed by other groups [93–95]. In



**Figure 2.** Various therapeutic effects of MSCs. (A) MSCs can transdifferentiate into cells of nonmesodermal origin including neurons (ectoderm) and hepatocytes (endoderm). (B) MSCs are capable of homing to injured tissues. The mechanism by which MSCs home to tissues and migrate across endothelium still remains unclear, but it is likely that injured tissue expresses specific receptors or ligands that facilitate trafficking, adhesion, and infiltration of MSCs to the site of injury in a manner similar to that in which leukocytes are recruited to sites of inflammation. (C) MSCs exhibit immunosuppressive activity through secretion of several cytokines that inhibit the activity of natural killer cells, helper T cells, and cytotoxic T cells while activating the generation of regulatory T cells. (D) MSCs produce trophic factors that promote repair of damaged tissue.

addition, hepatocyte-like cells were also generated from adipose tissue-derived MSCs (ADSCs) [96–99]. Our group reported that MACS-sorted CD105<sup>+</sup> fraction of ADSCs exhibited high hepatic differentiation ability in an adherent monoculture condition [98]. We further investigated the mechanism underlying ADSC plasticity by comparative analysis of the transcriptome and signal pathways and found that mesenchymal-to-epithelial transitions brought about the transdifferentiation of ADSCs into hepatocytes [100]. Furthermore, a clustering analysis revealed a striking similarity in gene clusters between ADSC-derived hepatocyte-like cells (ADSC-Hepa) and the whole liver, indicating that ADSC-Hepa were similar to mature hepatocytes.

### 3.3 The therapeutic significance of the secretory capacity of MSCs

The therapeutic potential of MSCs has been largely dependent on their secretory capacity rather than their differentia-

tion capacity. The multipotency of MSCs led researchers to examine whether these cells would contribute to the repair of injured tissue by replacing damaged cells through differentiation into functional cells. Our group examined whether MSCs would reveal therapeutic abilities for repairing injured liver by transplanting ADSC-Hepa into nude mice with acute liver failure. As expected, markers of liver injury, including alanine aminotransferase, aspartate aminotransferase, and ammonia, decreased after ADSC-Hepa transplantation [99]. To our surprise, however, transplantation of undifferentiated ADSCs resulted in higher levels of serological recovery and suppression of histopathological damage compared to those achieved by ADSC-Hepa (unpublished data). Further analyses suggested that the therapeutic capacity for liver disorders from undifferentiated ADSCs was a paracrine effect resulting from factors secreted by these cells, including interleukin (IL)-1RA, IL-6, IL-8, granulocyte-colony stimulating factor, granulocyte macrophage, monocyte chemoattractant protein-1, nerve growth factor, and hepatocyte growth factor [101]. These factors are known to be responsible for immunosuppression,

hepatocyte growth, and hematopoiesis. This finding is consistent with observations reported by other groups [102–104]. Similar observations have been made in the case of CNS injury. For example, researchers have reported the efficacy of the systemic administration of MSCs as treatment for experimental autoimmune encephalomyelitis, which is the animal model of human multiple sclerosis. Clinical efficacy of MSC treatment was sustained because of a significant reduction of demyelination and cellular infiltrates within the inflamed CNS and because of an impaired peripheral immune response against myelin antigens [105–108]. In addition to these beneficial effects, it was also observed that MSCs could engraft inside the CNS leading to reduced axonal loss [106, 107]. These observations show the significant anti-apoptotic effect of MSCs on neurons and lymphocytes, and they also show an antioxidant effect and the capacity to induce oligodendrogenesis and endogenous neurogenesis [109]. Collectively, these findings strongly suggest that given patients with a certain amount of potential for recovery, the secretory capacity of MSCs plays a more prominent role than MSC transdifferentiation in effecting tissue repair. However, in more serious cases where patients' organs neither maintain their own function nor have recovery potential, e.g. in the case of end-stage liver diseases, heterotopic transplantation of a tissue engineering-based auxiliary organ will be the better therapeutic option. In the latter case, the transdifferentiation capacity of MSCs has therapeutic potential. Thus, MSCs may provide options for both moderately and more serious patients.

Data from various studies suggest that MSCs can produce the immunomodulatory cytokines and associated trophic factors that correspond to a wide range of pathological conditions. MSCs can modulate both the innate and the adaptive immune systems [110]. MSC-mediated immunosuppression requires the previous activation of MSCs by immune cells, particularly secretion of the proinflammatory cytokine interferon (IFN)- $\gamma$  with tumor necrosis factor, IL-1- $\alpha$  or IL-1- $\beta$ . Upon stimulation of these proinflammatory cytokines, MSCs secrete immunosuppressive cytokines including prostaglandin E<sub>2</sub>, indoleamine 2,3-dioxygenase, transforming growth factor- $\beta$ 1, and IL-10 [110]. This activation pathway illustrates an important concept in MSC function. In addition to immunomodulatory effects, tissue repair by MSCs is not solely dependent on the rich mixture of soluble factors produced by MSCs in isolated cultures. Instead, MSCs are activated by cross-talk within the microenvironment that is generated by injured tissues. Activation results in the expression of factors that are most likely specific to the immediate needs of the tissue [111]. This idea is supported by various studies showing that MSCs exhibit therapeutic efficacy for not only specific disorders but also various types of disorders, including lung injury [112, 113], kidney disease [114], diabetes [115], myocardial infarction [116], and various neurological disorders [117].

In summary, it is widely argued that MSCs enable tissue repair in a wide range of disorders despite their low and/or tran-

sient levels of engraftment in vivo. Therefore, it is currently well accepted that MSCs contribute to tissue repair through secretion of immunomodulatory cytokines and trophic factors in response to pathological conditions without replacing the damaged cells.

## 4 Therapeutic potential of MSC-derived EVs

Collectively, the studies summarized above imply therapeutic potential for MSC-derived EVs. Research thus far has mostly focused on the secretion of cytokines and growth factors by MSCs. However, the fact that EVs can mirror the phenotype of their parent cell suggests that the therapeutic effects of MSC-derived factors are partly due to secreted EVs. In addition, the diversity of MSC-based therapeutic intervention implies the applicability of MSC-derived EVs to the treatment of various disorders. Furthermore, EVs may provide advantage over MSCs in that EVs can avoid the lung barrier, one of the major obstacles for systemic administration of MSCs [118].

In the last few years, several groups have investigated the therapeutic potential of MSC-derived EVs (Table 1). Their therapeutic effects have been observed in several different types of diseases, including kidney injury, cardiac injury, and brain injury. Although the number of reports are still limited, these findings strongly support the idea that MSC-derived EVs imitate the phenotype of parent MSCs and hold therapeutic potential for a wide range of diseases.

### 4.1 Kidney injury

The first study of the therapeutic effect of MSC-derived EVs focused on kidney injury. It is well recognized that MSCs contribute to repopulation of injured nephrons [64]. Repopulation is attributed to the transient recruitment of MSCs to the renal vasculature without direct incorporation of MSCs into regenerating tubules, suggesting that MSCs provide paracrine support to intrinsic repair mechanisms employed by the epithelial cells that survive injury. Indeed, MSCs were shown to protect the kidney from toxic injury by producing factors that limited apoptosis and enhanced proliferation of endogenous tubular cells. As a part of the paracrine effect, Camussi's group demonstrated that BM-MSC-derived EVs protected acute kidney injury (AKI), using a model mouse induced with glycerol [119]. The effect of EVs on the recovery of AKI was similar to that of MSCs. EVs induced proliferation and resistance to apoptosis in tubular epithelial cells. This effect was also confirmed using a lethal version of cisplatin-induced AKI [120]. Furthermore, the same group showed that a single administration of MSC-derived EVs immediately after ischemia-reperfusion injury protected against the development of both acute and chronic kidney injury [121].



**Table 1.** Reports on therapeutic potential of MSC-derived EVs

Species/MSC origin	Disease model	Mechanisms for the therapeutic effect	Molecules responsible for the therapeutic effect	References
Human/BM	Mouse model of acute kidney injury induced by glycerol	Induction of proliferation of surviving intrinsic epithelial cells	mRNAs (not specifically identified)	[119]
Human/BM	Mouse model of acute kidney injury induced by cisplatin	Induction of survival of tubular epithelial cells via anti-apoptotic effects	Not identified	[120]
Human/BM	Rat model of acute kidney injury induced by ischemia–reperfusion injury	Proliferative and anti-apoptotic effects on surviving intrinsic epithelial cells	Not identified	[121]
Mouse/BM	Rat model of 5/6 subtotal nephrectomy	Prevention of fibrosis, interstitial lymphocyte infiltrates, and absent tubular atrophy	Not identified	[122]
Human/ESC	Mouse model of myocardial ischemia/reperfusion injury	Not determined	Not identified	[128]
Human/fetus	Mouse model of myocardial ischemia/reperfusion injury	Not determined	Not identified	[129]
Rat/BM	Rat model of middle cerebral artery occlusion	Induction of neurite outgrowth of neural cells	miR-133b	[130]

They observed that EVs shuttle a specific subset of cellular mRNAs, such as those associated with the mesenchymal phenotype and with transcription control, proliferation, and immunoregulation [10, 119, 120]. In addition to these results, He et al. also described the potential renoprotective effect of MSC-derived EVs in the remnant kidney using a 5/6-nephrectomy mouse model [122].

#### 4.2 Cardiac injury

Recent studies from Lim's group suggest that the therapeutic effect of MSC-derived paracrine factors on cardiovascular disease is, in large part, due to EV fractions. MSC transplantation in animal models of acute myocardial injury has been reported to reduce infarct size, improve the left ventricular ejection fraction, and increase capillary density and myocardial perfusion [123]. As is the case for other organs, transplantation of MSCs to treat cardiac disease was predicated on the hypothesis that these cells would engraft, differentiate,

and replace damaged cardiac tissues [124]. However, it was observed that most transplanted MSCs remain in the lungs rather than the heart, which led to the hypothesis that their therapeutic effect is a result of paracrine effects [125, 126]. Consistent with this hypothesis, Lim's group demonstrated, using a porcine model of myocardial ischemia/reperfusion injury (MIR), that treatment with MSC-CM results in a reduction of myocardial infarct size [127]. Interestingly, in this report, they also showed that only the fraction of the CM containing products greater than 1000 kDa provided cardio-protection in a mouse model of MIR. They further demonstrated that the active component was enriched with 50–200 nm particles, which were identified as EVs. Further, the other fractions did not have protective effects on MIR [128, 129]. Currently, the specific molecules responsible for the protective effects remain unknown. EV-mediated injury protection may be dependent on cytokines and growth factors, similar to the case in MSC transplantation. Alternatively, miRNAs and/or mRNAs transferred from MSCs to damaged cardiac cells may provide therapeutic effects. More detailed

investigations are needed, but it is important to note that MSC-derived EVs mimic the phenotype of their parent cells and exhibit a protective effect on MIR.

### 4.3 Brain injury

MSC-derived EVs have been reported to contribute to tissue repair using a model of stroke in rats [130]. It has been widely shown that following intravenous administration, MSCs exhibit a therapeutic effect on stroke injury through secretion of neurotrophins and angiogenic growth factors [131]. In addition to these trophic proteins, Xin et al. recently found that EV-mediated secretion of miRNA contributes to the protective effect of MSCs on stroke [132]. It was previously demonstrated that miR-133b is specifically expressed in midbrain dopaminergic neurons and that it regulates the production of tyrosine hydroxylase and the dopamine transporter [132]. Xin et al. found that MSC treatment in rats that were subjected to middle cerebral artery occlusion (MCAo) had an increased miR-133b level in the ipsilateral hemisphere. The increase of miR-133b and subsequent induction of neurite outgrowth were shown to depend on EV-mediated miR-133b transfer from MSCs to neurons and astrocytes. Furthermore, Xin et al. found that exposure of MSCs to MCAo brain tissue extracts increased the miR-133b level in secreted EVs, suggesting that MSCs can increase the secretion level of therapeutic EVs in response to injury stimulus.

Recently, we have found a new therapeutic possibility for using MSCs-derived EVs against Alzheimer's disease (AD) [133]. One of the neuropathological hallmarks of AD is the accumulation of  $\beta$ -amyloid peptide ( $A\beta$ ) in the brain because of an imbalance between  $A\beta$  production and clearance. Recently, we have found that ADSCs, but not BM-MSCs, potentially contribute to  $A\beta$  clearance. Moreover, we have found ADSC-derived EVs reflect their parent cells, and also hold potential as a therapeutic tool for AD. Therefore ADSC-derived EVs warrant further investigation as a promising novel therapy for AD patients.

## 5 Comprehensive characterization of MSC-derived EVs toward their potential therapeutic applications

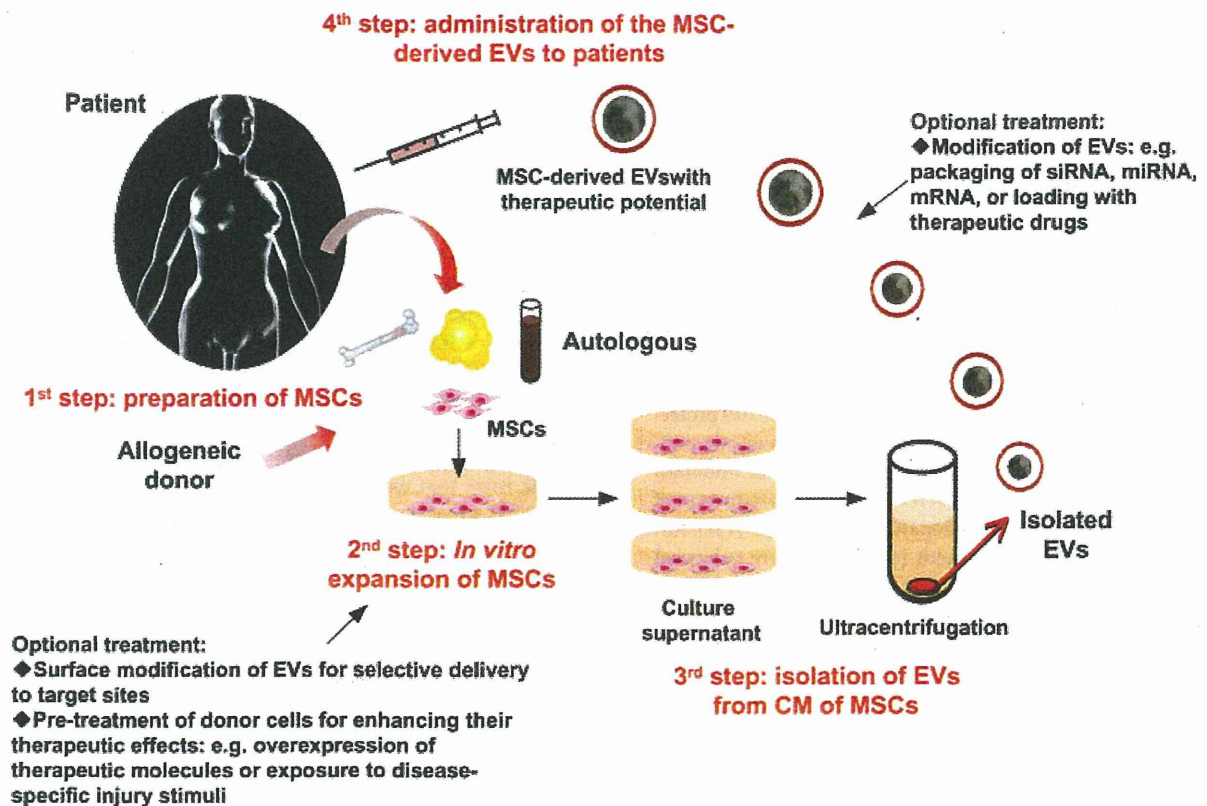
Comprehensive characterization of MSC-derived EVs will provide further insight into their potential benefits for clinical applications. In the preceding section, we summarized research that found that MSC-derived EVs were therapeutically effective for several disease types. The wide range of known MSC therapeutic effects suggests that the potential for their EVs in therapeutic applications is broader than those currently identified. To further explore the therapeutic potential of MSC-derived EVs, proteome and mRNA/miRNA microarray analyses may provide useful information.

### 5.1 Proteomic feature of MSC-derived EVs

Despite its potential utility, proteomic data for MSC-derived EVs are still lacking and require better characterization. Presently, only one report on a proteomic analysis of MSC-derived EVs is available [134]. Kim et al. performed LC-MS/MS analysis of BM-MSC-derived EVs and identified 730 EV proteins. These EV proteins exhibit characteristics of MSCs. The EVs contained positive MSC markers but no negative markers, and 122 of 730 EV proteins were shared by proteomes from BM-MSCs and UBC-MSCs. Furthermore, a functional enrichment analysis identified candidate EV proteins that are thought to be involved in the therapeutic effects of MSCs, including surface receptors, signaling molecules, cell adhesion, and MSC-associated antigens. Indeed, their data successfully predicted the therapeutic roles that were previously reported for some of the identified proteins, such as adhesion molecules including fibronectin-1 [135] and galectin-1 [136]. Regarding other identified adhesion molecules, such as EZR and IQGAP1, no functional association with the therapeutic effects of MSCs has been reported. However, EZR and IQGAP1 were shown to regulate EC proliferation and angiogenesis, implying that they have potential roles in MSC-derived EV-based tissue repair [137, 138]. Comprehensive exploration of the proteome from MSC-derived EVs and subsequent database-based functional analyses may predict new potential therapeutic roles for MSC EVs.

### 5.2 mRNA/miRNA profiling of MSC-derived EVs

mRNA/miRNA microarray analyses of MSC-derived EVs have also provided information for exploring their therapeutic potential. Lims' group was the first to perform a microarray analysis, and they found that MSC-CM contained RNAs of less than 300 nt encapsulated in EVs [139]. They observed that 45 of the 60 miRNAs identified in EVs were also present in MSCs, suggesting that released EVs echo the contents of their parent cell. In addition, they also defined miRNAs that were found only in EVs, suggesting that miRNA secretion is, at least in part, a selective and nonrandom process. Although the authors did not refer to the possible involvement of the EV-miRNAs with therapeutics, their data may provide insight into therapeutic applications of MSC-derived EV-miRNAs. For example, miR-124, one of the miRNAs present only in EVs, is a well-characterized brain-specific miRNA, which is involved in neurogenesis [140] and was recently reported to be involved in CNS disease pathogenesis [141]. Camussi's group performed microarray analysis of the miRNAs contained in EVs released from BM-MSCs and the liver resident stem cells that are termed as human liver stem cells (HLSCs) [142]. This group had previously reported that HLSCs shared phenotypes in part with MSCs [143] and that their EVs accelerated hepatic regeneration in hepatectomized rats [144]. Collino et al. compared miRNA profiles in EVs to matched parent



**Figure 3.** Schematic representation of the proposed strategy for clinical application of MSC-derived EVs. The first step is to prepare MSCs for production of EVs with therapeutic efficacy. MSCs can be obtained from a patient's own connective tissue such as BM, adipose tissue, and blood including peripheral and umbilical cord blood. In addition, the use of allogeneic MSCs is thought to be feasible because these cells evade and actively suppress the host immune response. The second step is to expand the prepared MSCs to obtain the required amount of EVs. At this step, optional treatments or gene engineering of the cultured MSCs may allow modification of EVs for selective delivery and/or enhancement of the therapeutic potential of the produced EVs. The third step is to harvest the EVs from the CM of MSCs. At this step, it will be possible to modify the harvested EVs by packaging siRNA, miRNA, and mRNA into the EVs, or loading the EVs with therapeutic drugs. The final 4<sup>th</sup> step is to administer MSC-derived EVs with therapeutic efficacy to the patient. For efficient delivery of the MSC-derived EVs, researchers are required to explore the best route of administration according to the target disease.

cells and identified miRNAs that were selectively enriched in EVs [142]. Gene ontology analysis implied that the selected miRNAs that were shuttled by EVs were associated with immune system regulation. This group also performed mRNA microarray analyses of BM-MSCs and HLSCs as well as their EVs and confirmed that mRNA profiles in EVs reflect their parent cell phenotypes [119, 144]. For example, BM-MSC-derived EVs shuttled a specific subset of cellular mRNAs, including those associated with differentiation into the mesenchymal phenotype and those associated with several cell functions involved in the control of transcription, proliferation, and cell immune regulation [119]. HLSC-derived EVs also shuttled a specific subset rather than a random sample of cellular mRNAs. They shuttled mRNAs that were related to several cell functions involved in the control of transcription, metabolism, and proliferation [144]. Out of the detected mRNAs, the CDK2 gene has been shown to be involved in

liver regeneration, highlighting the selective transport of this mRNA from HLSCs to recipient hepatocytes [145].

## 6 Discussion

In this review, we have summarized the functionality of EVs in a variety of biological events and highlighted the therapeutic potential of MSC-derived EVs. Despite promising results in animal studies, research on MSC-derived EVs is still in its infancy, and strategies for EV therapeutics need to be refined before their clinical application. Here, we will point to three issues that need to be addressed to realize clinical applications of MSC-derived EVs. We also provide some suggestions for future research. Finally, we will emphasize the importance of careful investigation of safety issues regarding the clinical application of MSC-derived EVs.

First, increasing the availability of MSC-derived EVs is of critical importance. Elucidation of the basic mechanisms of EV biogenesis and secretion is key to this issue. It is known that a p53-regulated gene product, TSAP6, enhances EV production in cells undergoing a p53 response to stress [146,147]. Several other intracellular proteins, such as diacylglycerol kinase- $\alpha$  [148] and brefeldin A-inhibited guanine-nucleotide exchange protein 2 [149], have also been proposed to have a role in the secretion of EVs. In addition, the pH of the microenvironment has been reported to regulate EV release in cancer cells [150]. Recent studies have found several molecules that play more general roles in EV biogenesis and secretion [19,20]. Modulating the expression of these genes or the pH of the microenvironment may result in increased EV production by MSCs. Care should be taken, however, because such treatment of MSCs may induce phenotypic changes in parent MSCs that may impair the therapeutic efficacy of any produced EVs. Thus, to boost the availability of MSC-derived EVs, it is important to increase MSC EV production while maintaining normal MSC phenotypes.

Second, specific delivery of MSC-derived EVs to target cells or organs is crucial. One possible approach to this issue is to modify the EV surface to target specific receptors on recipient cells. By fusing the EV membrane protein Lamp2b to peptides that bind to receptors expressed on specific cell types, Alvarez-Erviti et al. observed specific delivery of siRNAs to muscular and neural cells, respectively [151]. In addition, a better understanding of the mechanism for EV uptake may help us improve the efficiency of EV delivery. Considering the tropism of EVs for target cells, it seems possible to improve the efficiency of EV delivery as well as to circumvent nonspecific delivery to unwanted cells or organs. At this moment, however, EV uptake mechanisms other than those of the immune system remain unclear. Several studies have demonstrated that EVs can be taken up by immune cells through phagocytosis [152–154]. In contrast, the mechanisms underlying EV uptake in nonimmune cells are unclear. To improve EV delivery to specific tissues, future work could focus on elucidating EV uptake mechanisms.

Third, enhancing the therapeutic efficiency of MSC-derived EVs will increase the feasibility of their clinical application. One possible approach to this is to increase the amount of specific molecules such as mRNAs, miRNAs, and proteins in EVs through overexpressing them in parent MSCs using genetic modification. This idea is based on the fact that EV contents, at least in part, repeat those of parent cells. Indeed, our group showed that overexpression of a specific miRNA in parent cells leads to an increased secretion of the miRNA into the EVs [16]. Ciravolo et al. also observed that overexpression of human epidermal growth factor receptor2 protein in breast cancer cell lines leads to high expression of human epidermal growth factor receptor 2 in the released EVs [155]. Thus, in the case where the specific molecules necessary for a therapeutic effect are known, selective overexpression of those molecules in the parent MSCs may lead to an enhancement of the therapeutic efficiency of the EVs.

Another and more interesting possibility is global enhancement of the therapeutic potential of MSC-derived EVs. As described above, MSCs can respond to pathological stimuli and secrete various trophic factors required for tissue repair. Considering that EVs can contain many molecules that play certain biological roles, it is possible that in response to a pathological stimulus, MSCs can release EVs loaded with a set of molecules required for tissue repair. This suggests that exposure of MSCs to injury stimuli such as inflammatory cytokines may allow enrichment of a global set of trophic molecules in the released EVs. Furthermore, appropriate preconditioning of parent MSCs with injury stimuli related to a specific disease may allow the tailoring of EV contents to efficiently support repair of specific diseases. To this end, a recapitulation of injury stimuli based on a comprehensive understanding of the injury mechanism will be key to enhancement of the therapeutic efficiency of MSC EVs. On another front, it will be also important to extend the half-lives of EVs. Although information is currently lacking on local concentrations and half lives of EVs in tissues, it is possible that EVs are rapidly cleared by the mononuclear phagocytic system especially when the EVs involve exogenous modification. Recent advances in liposome technology will provide useful insights into this issue [156].

In summary, we propose a general strategy for clinical application of MSC-derived EVs (Fig. 3). The first step is to prepare MSCs by obtaining them from the patient himself/herself or from an allogeneic donor. The use of MSCs for clinical purposes takes advantage of their poor immunogenicity, which supports the possible use of MSCs obtained from allogeneic donors in the clinic [110]. The second step is to expand the prepared MSCs to obtain the required amount of EVs. During this step, optional treatments or gene engineering of the cultured MSCs may allow modification of EVs for selective delivery and/or enhancement of the therapeutic potential of the produced EVs. The third step is to harvest the EVs from the MSC-CM. Upon EV harvest, it will be possible to modify the harvested EVs by packaging siRNA, miRNA, and mRNA into the EVs [151], or loading the EVs with therapeutic drugs [157]. The final step is to administer MSC-derived EVs with therapeutic efficacy to the patient. For efficient delivery of the MSC-derived EVs, researchers are required to explore the best route of administration according to the target disease. Of note, recent reports suggest that EVs administered intravenously [151] or intranasally [157] cross the blood–brain barrier and result in successful delivery of the cargo directly into the brain.

Finally, we caution that researchers in this field should be mindful of safety issues regarding the clinical application of MSC-derived EVs. Despite increasing evidence for the therapeutic efficacy of MSC-derived EVs, this research field is still immature and much remains unclear. In particular, most of the molecules responsible for the therapeutic effects of MSC-derived EVs have yet to be characterized (Table 1). Toward realization of a clinical application for MSC-derived EVs, identification of the necessary molecules is required to

elucidate the mechanisms underlying MSC-EV-mediated therapeutics. Furthermore, it is especially important to carefully investigate whether MSC-derived EVs cause any side effects in animal experiments and preclinical tests. Therefore, future research must not only explore the novel therapeutic potential of MSC-derived EVs, but also carefully evaluate its safety and efficacy.

We thank Dr. Tomohiro Fujiwara for helping us prepare figures. This study was supported in part by a Grant-in-Aid for the Third-Term Comprehensive 10-Year Strategy for Cancer Control of Japan, a grant-in-aid for Scientific Research on Priority Areas Cancer from the Japanese Ministry of Education, Culture, Sports, Science, and Technology, a Grant-in-Aid for cancer research promotion from National Cancer Center of Japan, Grant-in-Aid for Japan Society for the Promotion of Science (JSPS) Fellows, the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NiBio) of Japan, Japan Science and Technology Agency (JST) CREST, and Grant-in-Aid for Scientific Research on Innovative Areas (“functional machinery for non-coding RNAs”) from the Japanese Ministry of Education, Culture, Sports, Science, and Technology.

The authors have declared no conflict of interest.

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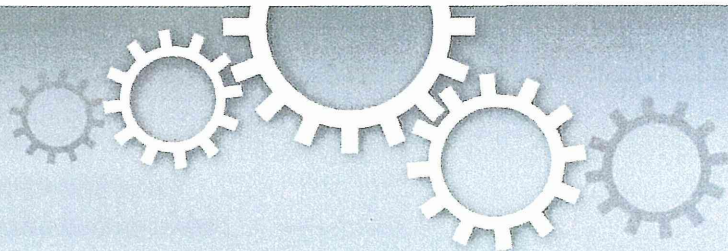
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## OPEN

## A novel platform to enable inhaled naked RNAi medicine for lung cancer

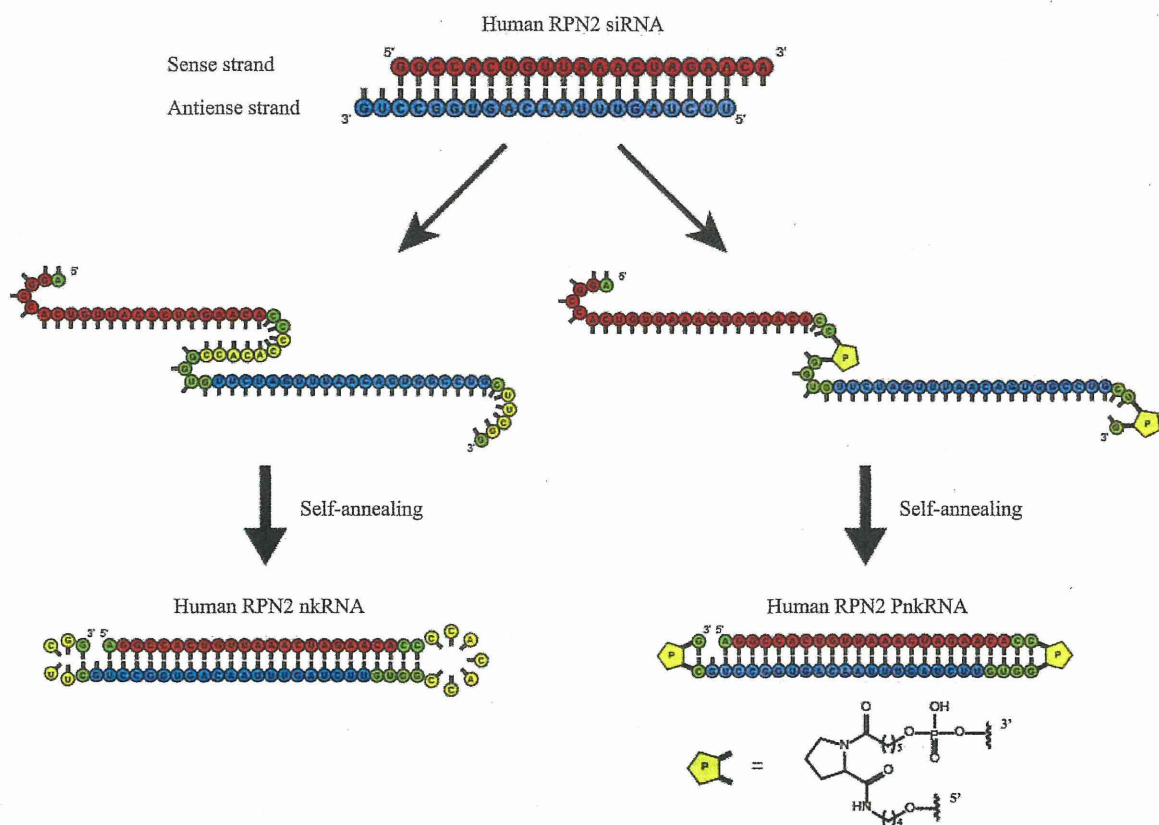
SUBJECT AREAS:  
DRUG DEVELOPMENT  
DRUG DELIVERY  
RNAI  
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Small interfering RNA (siRNA)-based therapeutics have been used in humans and offer distinct advantages over traditional therapies. However, previous investigations have shown that there are several technical obstacles that need to be overcome before routine clinical applications are used. Currently, we are launching a novel class of RNAi therapeutic agents (PnkRNA<sup>TM</sup>, nkRNA<sup>®</sup>) that show high resistance to degradation and are less immunogenic, less cytotoxic, and capable of efficient intracellular delivery. Here, we develop a novel platform to promote naked RNAi approaches administered through inhalation without sophisticated delivery technology in mice. Furthermore, a naked and unmodified novel RNAi agent, such as ribophorin II (RPN2-PnkRNA), which has been selected as a therapeutic target for lung cancer, resulted in efficient inhibition of tumor growth without any significant toxicity. Thus, this new technology using aerosol delivery could represent a safe, potentially RNAi-based strategy for clinical applications in lung cancer treatment without delivery vehicles.

RNA interference (RNAi) is a post-transcriptional gene-silencing mechanism. Small interfering RNA (siRNA) must be dissociated into their component single strands to act as a guide for the RNA-induced silencing complexes, which are the protein complexes that repress gene expression<sup>1</sup>. The development of siRNA technology has opened an avenue of opportunity to study gene function, as well as the possibility of novel forms of therapeutic intervention in several genetic diseases. In fact, siRNA-based therapy has enormous potential for the treatment of several diseases through either local or systemic administration of siRNAs that are being tested in experimental animal models or in clinical development<sup>2</sup>. Oncology is one of the medical fields that can benefit most from this powerful therapeutic strategy because this approach can modulate the expression of target genes involved in tumor initiation, growth, and metastasis<sup>3</sup>.

However, the clinical application of siRNAs has been impaired by problems related to their delivery, low biological stability, off-target gene silencing, and immunostimulatory effects<sup>4,5</sup>. Indeed, naked siRNAs are promptly degraded by nucleases in serum and extracellular fluids, and chemical modifications at specific positions or formulations with delivery vehicles have been shown to improve stability. However, these may attenuate the suppressive activity of siRNAs<sup>6</sup>. Furthermore, the cost of large-scale production is another obstacle to the clinical application of siRNAs<sup>7</sup>. For this reason, their translation to the clinical setting is dependent upon the development of an efficient delivery system that is able to improve the pharmacokinetic and biodistribution properties of siRNAs.

Recently, engineered designs, such as aptamer-siRNA chimeras and transferring-decorated nanoparticles, have continued to dramatically improve the precision of delivery for RNAi agents<sup>8</sup>. Advances in RNAi-based therapeutics may require new biochemical technologies to maximise drug potency while minimising off-target toxicity and immunogenicity. Meanwhile, we have already reported a novel class of RNAi therapeutic agents (PnkRNA, nkRNA) and evaluated their effectiveness<sup>9</sup>. We showed that PnkRNA and nkRNA directed against transforming growth factor (TGF)- $\beta$ 1 ameliorate outcomes in mouse models of acute lung injury and pulmonary fibrosis. This novel class of RNAi agents was synthesised on solid phase as single-stranded RNAs (ssRNAs) that self-anneal into a unique helical structure containing a central stem and two loops following synthesis (Fig. 1). The production of the novel RNAi agents is simple; because PnkRNA and nkRNA are synthesised as ssRNAs that spontaneously self-anneal, low-cost, large-scale production is possible. These novel RNAi agents have showed significant effectiveness in disease models and also superior resistance against nuclease degradation compared to



**Figure 1 | Structure of novel RNAi agents.** Both nkRNA and PnkRNA were prepared as single-stranded RNA oligomers that then self-anneal, as shown. Nucleotides in red indicate the sense strand of the target (RPN2); nucleotides in blue indicate the antisense strand; and nucleotides in green and yellow indicate the loop cassettes. P indicates a proline derivative.

canonical siRNAs. Additionally, by evaluating the induction of proinflammatory cytokines, our previous results suggest that none of the platforms were immunotoxic<sup>9</sup>. Thus, the novel RNAi therapeutic agents are safe and might be employed in clinical applications because they address several issues in siRNA-based therapy.

Lung cancer is the leading cause of cancer-related death in the world. Non-small cell lung cancer (NSCLC) accounts for approximately 85% of all lung cancers. Approximately 70% of all newly diagnosed patients present with local advanced or metastatic disease and require systemic chemotherapy<sup>10,11</sup>. Although NSCLC patients with epidermal growth factor receptor (EGFR) mutations initially respond to EGFR tyrosine kinase inhibitors<sup>12</sup>, most patients experience a relapse within 1 year. Despite the development of novel molecular therapies<sup>13</sup>, the prognosis of lung cancer is still poor and shows a median survival time of approximately 18 months in the operable stages. Hence, novel and more effective approaches are needed for the treatment of advanced lung cancer. Lung diseases in general are attractive targets for siRNA-based therapeutics because of their lethality and prevalence. In addition, the lung is anatomically accessible to therapeutic agents via the intrapulmonary route. Accessibility is a key requirement for successful RNAi-based *in vivo* and clinical studies, and this characteristic offers several important benefits over systemic delivery, including the use of lower doses of siRNAs, the reduction of undesirable systemic side effects, and improved siRNA stability due to the lower nuclease activity in the airways compared to the serum<sup>14</sup>. The direct administration of siRNAs into the target organs is a promising strategy for overcoming the problems of intravenous administration<sup>15</sup>. On the basis of these promising findings, we explored the effectiveness of this novel class of RNAi therapeutic agents in lung cancer. In the current study, we

prepared inhaled novel RNAi agents directed against luciferase and human-ribophorin II (RPN2) as candidate genes, and we compared their inhibitory activity to canonical siRNAs using *in vitro* assays and animal models of lung cancer. Furthermore, using this new technology, lung cancer xenograft studies showed that the aerosol delivery of a naked novel RNAi agent significantly inhibited tumor growth.

## Results

***In vitro* stability of novel RNAi agents.** The stability of the novel RNAi agents, PnkRNA and nkRNA, against degradation by ribonucleases was compared with that of siRNA. Each RNAi agent was incubated in the presence of ribonucleases, and the degree of degradation was followed overtime. siRNA was degraded after 5 min of incubation with ribonucleases, but both PnkRNA and nkRNA remained intact after 15 min of incubation (Supplementary Fig. 1). The novel RNAi platforms were more resistant to degradation than siRNA.

**Distribution of fluorescent siRNA after inhalation.** The inhaled administration of fluorescently labeled siRNA by MicroSprayer™ enabled the efficient intracellular distribution of lung cancer cells throughout the lung parenchyma. The efficient and homogeneous distribution of the RNAi agent to most parts of the lung tissue is a prerequisite for studying target-specific RNAi efficacy. The endotracheal application of suspended RNAi agents by a MicroSprayer™ can result in nanoparticle deposition in smaller airway and peripleural tumor cells (Fig. 2a,b).

**Monitoring luciferase inhibition of the inhaled novel RNAi agents' delivery system *in vivo*.** Mice with an intravenous injection of