Author's personal copy

1. Introduction

MicroRNAs (miRNAs) are small, non-coding RNAs that are approximately 22 nt in length [1]. Although more than 1500 mature human miRNA sequences are currently listed in the miRNA database [2], the function of many has not been clarified. The maturation process of miRNAs includes several post-transcriptional processing steps [1]. First, the primary miRNA transcripts (primary miRNAs; pri-miRNA) are generated from the genomic sequence. These long transcripts are mainly transcribed by RNA polymerase II. Second, these transcripts are processed to precursor miRNA (pre-miRNA) in 60–110 nt fragments in the nucleus by Drosha, an RNase III enzyme. Then the pre-miRNAs are transported to the cytoplasm by exportin-5. Dicer, another RNase III enzyme, processes the pre-miRNA into double-stranded RNA, which is a mature miRNA of approximately 22 bp. The mature miRNA is composed of an RNA-induced silencing complex (RISC) and binds to the complementary sequence in the 3′ untranslated region (3′ UTR) of target mRNAs, resulting in the degradation of the mRNA and/or inhibition of protein translation [3]. This multistep processing is regulated by many factors such as epigenetic modifications of the genomic sequence and by transcription factors, RNase and RNA-binding proteins. If there is a misregulation of one of these processing components, the miRNA expression will be dysregulated, leading to disease progression such as cancer malignancy [4]. Recently, it was reported that miRNAs do not exist only in the cell but are also secreted outside of cells [5,6]. In this review, we will discuss recent reports that indicate that exosomes carrying "secretory miRNAs" mediate various biological phenomena. In addition, we propose the usage of an exosome-delivered tumor-suppressive miRNA in cancer therapy.

2. Tumor-suppressive miRNAs are novel anti-cancer agents

It is known that the downregulation of miRNA expression leads to cancer development through various mechanisms such as genomic deletions, amplifications, mutations, epigenetic silencing, the dysregulation of transcription factors that target specific miRNAs, or the inappropriate processing of miRNA precursors [4]. These downregulated miRNAs are thought to act as tumor suppressors. There are various types of tumor-suppressive miRNAs; however, little is known regarding their precise mechanism of action (Table 1).

2.1. let-7

The expression of let-7 miRNA is significantly downregulated in lung cancer, and the overexpression of let-7 in a lung adenocarcinoma cell line suppressed lung cancer cell growth in vitro through the downregulation of KRAS and HMGA2 [7–10]. Interestingly, let-7 miRNA expression was markedly reduced in breast cancer tumor-initiating cells [11], which have self-renewal ability and resistance to chemotherapy and radiotherapy, and the expression of let-7 miRNA increased after differentiation [12]. The re-expression of let-7 in breast cancer tumor-initiating cells reduced proliferation, mammosphere formation and metastasis in a xenograft model through the reduction of KRAS and/or HMGA2. Many downregulation mechanisms of let-7 have been reported [13–19]. Importantly, disruption of the let-7 precursor processing by LIN28 and LIN28B, which are reported as overexpressed in primary human tumors, human cancer cell lines and pluripotent stem cells, is essential for controlling proper miRNA expression [14].

2.2. miR-16

MiR-15a and miR-16-1 are deleted or downregulated in the majority of chronic lymphocytic leukemia (CLL) cells [20–22], and re-expression of these miRNAs induced apoptosis through the downregulation of BCL2. In addition, the miR-15a and miR-16 levels significantly decrease in advanced prostate tumor cells [23]. Takeshita et al. reported that the injection of miR-16 with atelocollagen (a highly purified type I collagen that possesses low immunogenicity and is produced by treating calf dermis with pepsin) via the tail vein of mice significantly inhibited the growth of prostate bone metastases in a therapeutic bone-metastasis model [24].

2.3. miR-143

The suppression of miR-143 expression has been reported in several human cancers, including colorectal, prostate, cervical and ovarian [25–30]. Induction of miR-143 expression in those cancer cells resulted in the inhibition of cell proliferation or the induction of apoptosis through the suppression of its target genes such as KRAS and ERK5. Furthermore, Osaki et al. showed that miR-143 was the most downregulated miRNA in metastatic human osteosarcoma cell lines relative to the parental cell lines, and transfection of miR-143 into metastatic human osteosarcoma cell lines significantly decreased cell invasiveness but not proliferation [31]. In addition, intravenous injection into mice of miR-143 significantly suppressed the formation of lung metastases from metastatic human osteosarcoma cell lines. Moreover, cells positive for MMP13, a target of miR-143 in osteosarcoma cells, was found in lung metastasis-positive cases but not in at least three cases with higher miR-143 expression levels and without metastases.

2.4. miR-22

Xu et al. demonstrated that miR-22 expression is upregulated in senescent human fibroblasts and epithelial cells. In contrast, its expression is downregulated in various cancer cell lines, and the overexpression of miR-22 in those cases induces growth suppression

Table 1
The list of typical tumor suppressive miRNAs.

microRNA	Type of cancer	Target gene	Phenotype	References
let-7	Lung cancer	KRAS	Inhibition of cell proliferation	[7–19]
	Breast cancer	HMGA2		
miR-16	Chronic lymphocytic leukemia	BCL2	Induction of apoptosis	[20–24]
	Prostate cancer	CCND1	Inhibition of cell proliferation	
		WNT3A		
miR-143	Ovarian cancer			[25–31]
	Prostate cancer	ERK5	Induction of apoptosis	
	Cervical cancer	KRAS	Inhibition of metastasis	
	Osteosarcoma		Inhibition of cell proliferation	
miR-22	Colorectal cancer	Sp1		[32]
	Breast cancer	CDK6	Induction of growth suppression	
		SIRT1	Induction of senescent phenotype	

Representative cases are shown in the "type of cancer" and "target gene".

Author's personal copy

and a senescent phenotype in human normal and cancer cells through the downregulation of CDK6, SIRT1, and Sp1 genes [32]. In addition, in vivo injection of miR-22 inhibits tumor growth and metastasis through the induction of senescence in inoculated breast cancer cell lines, suggesting that miR-22 can be used as a senescence inducer, and this approach may be a novel cancer treatment method.

Taken together, these findings prompted the idea that delivery of tumor-suppressive miRNAs that are downregulated in cancer cells may provide a therapeutic option in combination with other cancer treatments such as chemotherapy or antibody therapy.

3. Exosomal miRNAs are novel humoral factors for cell–cell communication

As described above, the expression levels of tumor-suppressive miRNAs are known to be downregulated in cancer cells. Therefore, restoring the expression of these miRNAs might lead to the suppression of cancer progression. However, the nucleic acid delivery method is the most significant problem for nucleic acid therapy [33,34]. In 2007, Valadi et al. found that miRNAs are contained inside exosomes [35]. This study showed the possibility that miRNAs are not only intracellular gene regulators but are also humoral factors, suggesting that miRNAs could act as tools for cell–cell communication. Exosomes are lipoprotein complexes including small-membrane vesicles of endocytic origin (30–100 nm) [35]. Exosomes are formed through the inward budding of endosomal membranes that give rise to intracellular multivesicular bodies (MVBs) that later fuse with the plasma membrane, releasing the exosomes to the extracellular space [36–39].

3.1. The exosomal miRNAs are functional in recipient cells

Following the report from Valadi et al., three reports showed the functionality of exosomal miRNAs (Table 2). Pegtel et al. showed that miRNAs encoded by the EB virus are secreted from EBV-infected B cells through exosomes. These miRNAs repress the EBV target immunoregulatory genes in primary EBV-associated lymphomas [40]. Zhang et al. reported that exosomes from human monocyte/macrophage cell lines deliver miR-150 into human microvascular endothelial cells, and the expression levels of c-Myb were downregulated in microvascular endothelial cells and enhance their cell migration [41]. We demonstrated that secreted miR-146a, whose expression is known to be downregulated in prostate cancer, was transferred from miR-146a-overproducing HEK293 cells to cancer cells, where it suppressed its target gene and led to cell growth inhibition [42].

3.2. Immune cells use exosomal miRNAs for cell–cell communication

After the publication of these studies, many researchers reported the function of exosomal miRNAs in a variety of physiological and pathological phenomena (Table 2). The antigen-driven unidirectional transfer of exosomal miRNAs from the T cell to antigen-presenting cells has been observed, and this transfer results in the downregulation of target gene expression in recipient cells [43]. Furthermore, dendritic cells (DCs) release exosomal miRNAs, which then transfer to acceptor DCs [44]. Interestingly, DCs release exosomes with different miRNAs depending on the maturation of the DCs. Taken together, these two papers regarding immune cells describe a novel mechanism of complex immune cell communication mediated by exosomal miRNAs. However, the transfer of miRNAs between cells is not limited to immune cells.

3.3. The function of exosomal miRNAs in cancer development

The exosomal miR-223 from tumor-associated macrophages is transported to breast cancer cells, supporting the idea that macrophages regulate the invasiveness of breast cancer cells through exosome-mediated delivery of oncogenic miRNAs [45]. In addition to breast cancer cells, exosomal miRNAs derived from hepatocellular carcinoma cells can be taken up by other cells and target transforming growth factor β activated kinase-1, resulting in the enhancement of transformed cell growth in recipient cells [46].

Recently, we showed that proliferation of a prostate carcinoma cell line was inhibited by the addition of the exosome fraction isolated from a non-cancerous prostate epithelial cell line [47]. These observations suggest that exosomal miRNAs derived from non-cancerous cells were transferred to cancerous cells and inhibit proliferation. Indeed, some sets of tumor-suppressive miRNAs such as miR-16, miR-205, and miR-143 were downregulated in prostate cancer cell lines at the cellular and extracellular levels. This observation supports the idea that secretory tumor-suppressive miRNAs are transferred from non-cancerous to cancerous cells in accordance with the miRNA concentration gradient. To examine in depth the contribution of secretory tumor-suppressive miRNAs in cancer initiation, we generated miR-143 overproducing HEK293 cells. We found that a prostate cancer cell line showed an approximately 50% decrease in proliferation through the suppression of the miR-143 target gene KRAS after the addition of an exosome derived from the miR-143-overproducing HEK293 cells. Importantly, the decrease was reversed by the transfection of anti-miR-143 in the prostate cancer cell line. These data indicate that the cell growth inhibition is attributable to the secretory miR-143 contained in the exosome of miR-143-overexpressing HEK293 cells [47].

Table 2
The list of exosomal miRNA mediating cell–cell communication both in basic research and research of therapeutic purpose.

Type of small RNA	Donor cells	Recipient cells	Phenotype	Target gene	References
EBV-miRNAs	LCL (EBV-transformed lymphoblastoid B cells)	MoDC (monocyte-derived dendritic cells)		CXCL11 LMP1	[40]
miR-150	THP-1 (human monocyte/macrophage cell line)	HMEC-1 (human microvascular endothelial cell)	Promote cell migration	c-Myb	[41]
miR-146a	HEK293	PC-3M	Growth inhibition	ROCK1	[42]
miR-335	J77 (T-cell line)	Raji (B-cell line)		SOX-4	[43]
miR-451 miR-148a	Bone marrow derived DCs	DC2.4 (mouse dentritic cell line)		[a]	[44]
miR-223	SKBR3 (human breast cancer cell line)	IL-4-activated macrophages (human monocyte-derived macrophages)	Promote invasion	Mef2c	[45]
Hep3B enriched miRNAs[b]	Hep3B (hepatocellular carcinoma)	Hep3B	Reduction in cell viability	TAK1	[46]
miR-143	PNT-2 HEK293	PC-3M	Growth inhibition	KRAS ERK5	[47]
miR-133a	H9c2 (rat cardiomyoblasts)	293FT		[a]	[60]
siRNA	Bone marrow derived DCs	Mouse brain		GAPDH	[48]
siRNA	Huh-7 (human hepatoma cell line)	Mouse hepatocyte		CD81	[49]

[a] The sensor vector, which is complementary sequence of miRNA, was used in this study.
[b] The target gene TAK1 was predicted by various types of miRNAs that were highly expressed in the exosome isolated from Hep3B.

Author's personal copy

4. Exosomes can be used as a small RNA delivery system

As described previously, resolving the issue of miRNA delivery is essential for cancer treatment by tumor-suppressive miRNAs. Therefore, it is natural to examine exosomal tumor-suppressive miRNAs for cancer treatment.

4.1. Exosomes can be used for siRNA delivery

Recently, the exosomal delivery of siRNAs to the mouse brain was reported [48]. In this report, self-derived DCs, which express the exosomal membrane protein Lamp2b fused with the neuron-specific RVG peptide 3, was used for the reduction of immunogenicity. Exosomal siRNA against GAPDH, which was loaded by electroporation, was intravenously injected, and it was delivered specifically to neurons, microglia, and oligodendrocytes in the brain, resulting in specific gene knockdown. In addition, using human hepatoma cells producing the viral entry receptor CD81 siRNA, siRNA delivery was confirmed, causing suppression of CD81 expression in mouse hepatocytes in vivo [49].

4.2. Exosomes can be used for miRNA delivery

As shown above, siRNA can be delivered by exosomes. It is plausible that tumor-suppressive miRNAs can be similarly delivered to cancer cells in vivo. As we have previously shown [47], the loading mechanism of miRNA and siRNA into exosomes is the same; therefore, this technique might be used for miRNA-mediated therapy. To address this possibility, we injected conditioned medium obtained from miR-143-overproducing or parental HEK293 cells into nude mice implanted with prostate cancer cells. The tumor expansion was restrained for 8 days with intratumor administration of miR-143-enriched conditioned medium. Consequently, the tumor masses shrank by approximately 0.5 fold on day 8. In addition, the expression of miR-143 target genes such as KRAS and ERK5 decreased following miR-143-transduced conditioned medium injections.

In our report, exogenously-transduced miR-143 did not suppress the proliferation of non-cancerous cells, suggesting that excessive amounts of tumor-suppressive miRNAs did not provide an additional growth inhibitory effect on normal cells, in which the expression of tumor-suppressive miRNAs is maintained at physiological levels [48].

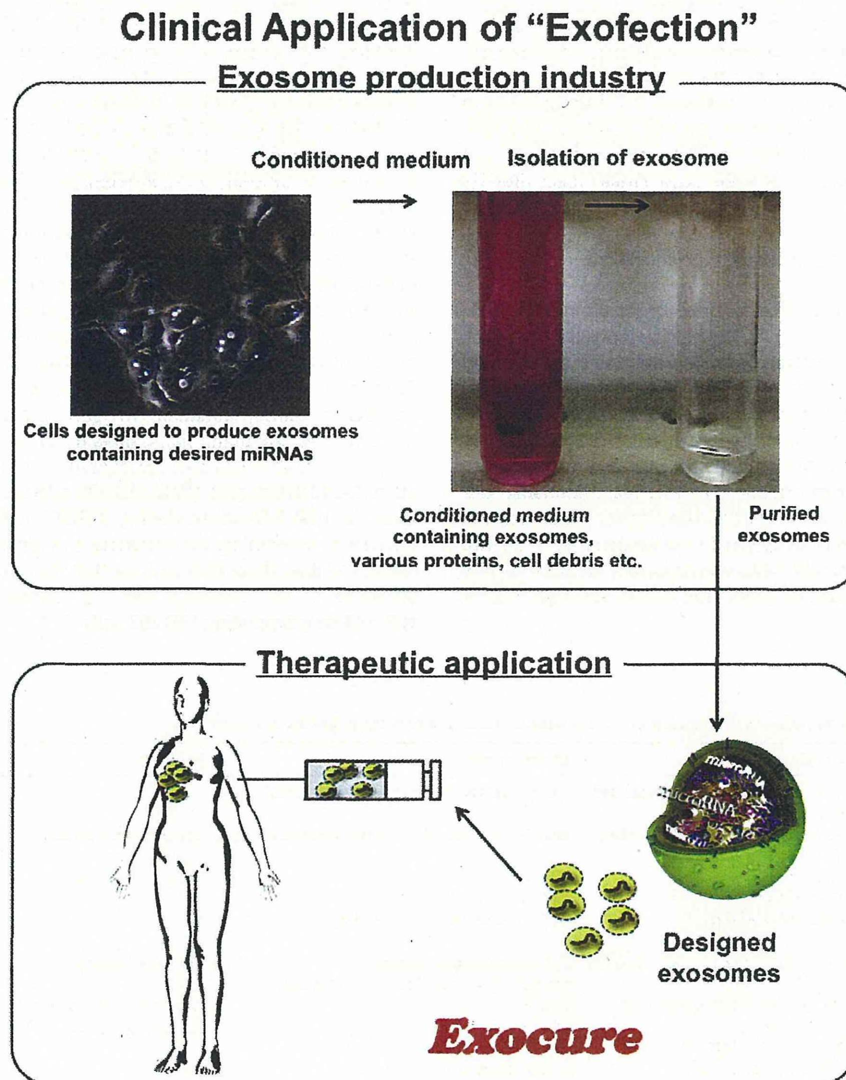

Fig. 1. A schematic explanation of "exocure". Exosome containing desired miRNAs, such as miR-16, miR-143 and so on, is produced by the "exosome production industry", and then these designed exosomes are delivered to cure the patient's disease. For this purpose, some of the issues which are showed in Fig. 2, need to be solved.

Author's personal copy

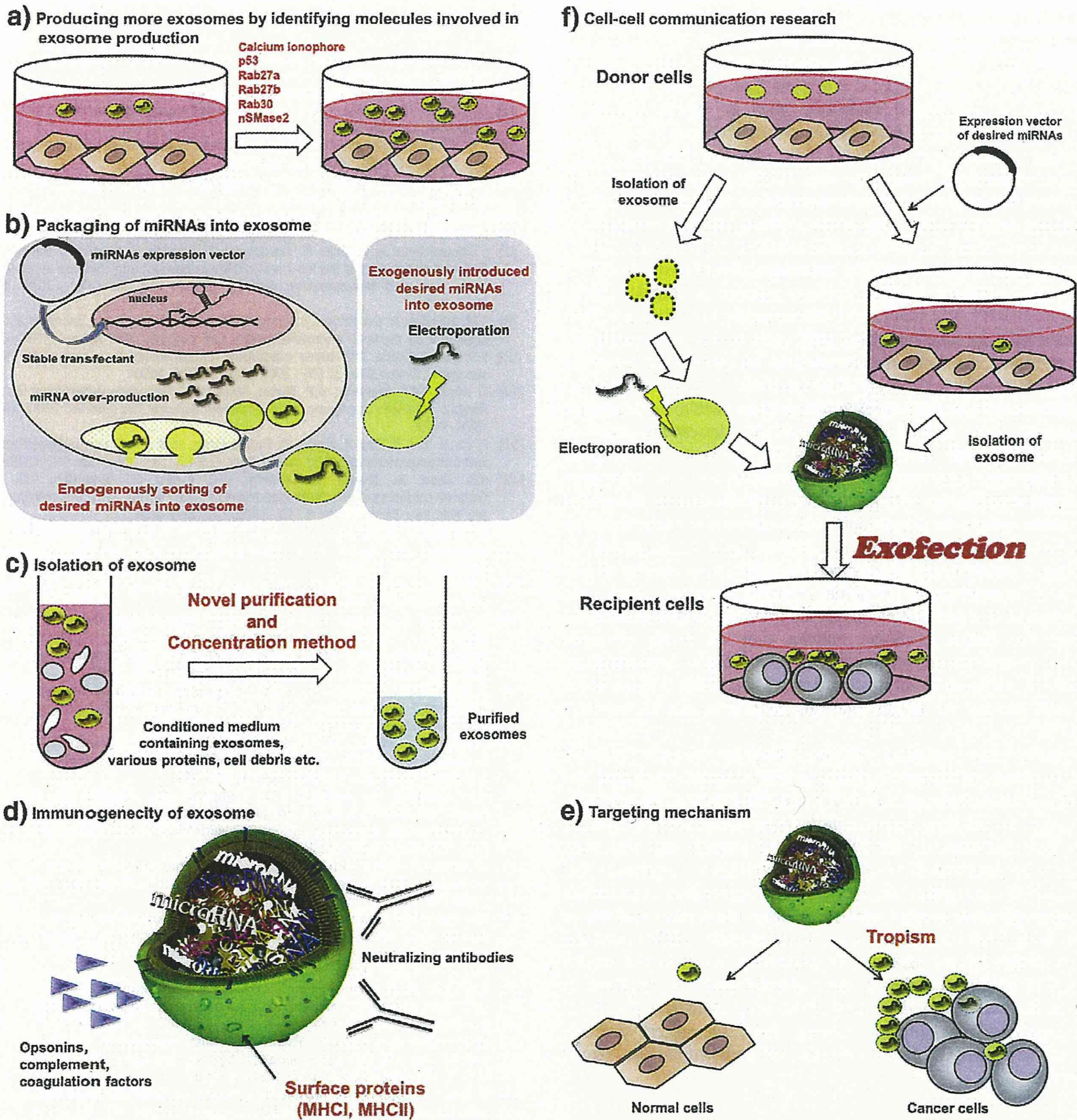

Fig. 2. Issues for the clinical use of "exocure". A schematic explanation of how the novel treatment "exofection" was established and the issues to be solved. To prepare exosomes containing tumor-suppressive miRNAs suitable for clinical usage, clarifying the mechanisms of exosome secretion is important. It is known that there are several molecules that regulate exosome secretion such as Rab27, Rab28, Rab30, nSMase2, p53 and calcium ionophore. Although the precise mechanism of exosome secretion has not yet been clarified, it is important to find the molecules that regulate exosome secretion (a). Currently, there are two kinds of methods to introduce desired miRNA into exosomes. One is to establish the cell line that stably overexpress desired miRNAs [47,49]. This enables us to obtain increased amount of desired miRNA in exosomes. The other one is exogenously introducing desired miRNAs using electroporation [48]. There are no knowledge about advantages and disadvantages in these two methods, moreover, the mechanism in which the tumor-suppressive miRNAs are sorted into exosomes is also unknown (b). These studies will enable us to obtain enough of the required exosomes for treatment. In addition, the establishment of exosome isolation methods is needed. The current, most popular isolation method, ultracentrifugation, is time-consuming and complicated, and its recovery rate is poor. Establishing more effective isolation methods is essential (c). Immunogenicity is an important factor to consider for the delivery of exosomes containing tumor-suppressive miRNAs. Exosomes are known to have reduced immunogenicity compared to other carriers such as viruses (d) [62]. Furthermore, the targeting mechanism of exosomes needs to be resolved (e). It has been known that exosomes have a tropism for target cells; however, the precise mechanism has not been found. Clarifying these five issues would enable us to establish the novel cancer treatment "exocure". The in vitro study of exosomal miRNAs might reveal many aspect of cross-kingdom research field, we suggest that this methods used for studying cell–cell communication be named "exofection" (f).

In addition, no overt side effects were observed in exosome-mediated gene delivery in vivo by dendritic cell-derived exosome [48]. Taken together, these reports suggest that exosomal tumor-suppressive miRNA therapy does not have serious side-effects [47,48].

5. Perspectives

In this review, we have summarized the knowledge regarding exosomal miRNAs for cancer therapy. We want to emphasize that

Author's personal copy

exosomal tumor-suppressive miRNAs are promising molecules for cancer therapy, (Fig. 1) although their use may have several difficulties (Fig. 2). First, the exosome must be abundant or highly enriched in order to utilize in therapy. It is known that exosome production was regulated by several molecules such as nSMase2, Rab27a, Rab27b, Rab35, p53 and calcium ionophore; however, the precise production mechanisms have not been clarified [50–59]. If the exosome production mechanism was clarified, we could develop "exosome-producing cells" by cell engineering. Second, the methods introducing desired tumor suppressive miRNAs into exosomes should be considered. There are two possible methods to introduce desired miRNAs into exosomes. One is the overexpression of desired miRNAs in the cells, resulting in the increased amount of miRNAs inside exosomes [42,47]. The other one is exogenously introducing miRNA into exosomes by electroporation [48]. Only few reports employed these methods and more studies need to be carried out for these methods to recognize more effective methods. Thirdly, methods for isolation of exosomes from conditioned medium need to be developed. The current, most popular isolation method, ultracentrifugation, is time-consuming and complicated, and its recovery rate is poor. Establishing more effective isolation methods is essential. Moreover, the exosome-producing cells need to be carefully chosen. Because of the tropism against the target cancer cells, we need to understand the mechanism of exosome uptake. In addition, the immunogenicity of exosomes is poorly understood. Resolving these issues may result in a *safe and cost-effective exosome delivery method*. *Last*, the functions and mechanisms of tumor-suppressive miRNAs need to be clarified. The choice of target miRNAs may aid in the decisions regarding cancer therapy such as radiotherapy and/or chemotherapy. Because the exosome is an ideal and promising delivery material for small RNA therapy, we suggest that this method be named "exocure" (Fig. 1).

In addition, this method can also be utilized for research of cell–cell communication. As shown in Table 2, these reports regarding the exosomal miRNAs open up a novel research field for the cell–cell communication. Surprisingly, recent reports showed that exogenous plant miRNAs can be found in the blood sera of animals and this plant miRNAs were considered to exist inside exosomes, suggesting that plant miRNAs can regulate the expression of target genes in mammals [61]. Although more studies need to be done about plant miRNAs in human body fluids, studying the exosomal miRNAs might unveil the mystery of this cross-kingdom gene regulation. To study the precise function of exosomal miRNAs, the methods that we proposed in Fig. 2 can be used. The in vitro study of exosomal miRNAs might reveal many aspect of cross-kingdom research field, we suggest that this methods used for studying cell–cell communication be named "exofection".

Acknowledgment

This work was supported in part by a Grant-in-Aid for the Third-Term Comprehensive 10-Year Strategy for Cancer Control, a Grant-in-Aid for Scientific Research on Priority Areas Cancer from the Ministry of Education, Culture, Sports, Science and Technology, and the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NiBio), and the Japan Society for the Promotion of Science (JSPS) through the "Funding Program for World-Leading Innovative R&D on Science and Technology (FIRST Program)" initiated by the Council for Science and Technology Policy (CSTP), a grant-in-aid for Project for Development of Innovative Research on Cancer Therapeutics (P-Direct), and Project for Development of Innovative Research on Cancer Therapeutics, 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.

References

[1] V.N. Kim, J. Han, M.C. Siomi, Biogenesis of small RNAs in animals, Nat. Rev. Mol. Cell Biol. 10 (2) (2009 Feb) 126–139.
[2] miRBase, Release 18, http://www.mirbase.org/.
[3] D.P. Bartel, MicroRNAs: target recognition and regulatory functions, Cell 136 (2) (2009 Jan 23) 215–233.
[4] C.M. Croce, Causes and consequences of microRNA dysregulation in cancer, Nat. Rev. Genet. 10 (10) (2009 Oct) 704–714.
[5] H.I. Suzuki, K. Miyazono, *Emerging complexity of microRNA generation cascades*, J. Biochem. 149 (1) (2011 Jan) 15–25.
[6] N. Kosaka, T. Ochiya, Unraveling the mystery of cancer by secretory microRNA: horizontal microRNA transfer between living cells, Front Genet. 2 (2011) 97.
[7] J. Takamizawa, H. Konishi, K. Yanagisawa, S. Tomida, H. Osada, H. Endoh, et al., Reduced expression of the let-7 microRNAs in human lung cancers in association with shortened postoperative survival, Cancer Res. 64 (11) (2004 Jun 1) 3753–3756.
[8] S.M. Johnson, H. Grosshans, J. Shingara, M. Byrom, R. Jarvis, A. Cheng, et al., RAS is regulated by the let-7 microRNA family, Cell 120 (5) (2005 Mar 11) 635–647.
[9] Y.S. Lee, A. Dutta, The tumor suppressor microRNA let-7 represses the HMGA2 oncogene, Genes Dev. 21 (9) (2007 May 1) 1025–1030.
[10] C. Mayr, M.T. Hemann, D.P. Bartel, Disrupting the pairing between let-7 and Hmga2 enhances oncogenic transformation, Science 315 (5818) (2007 Mar 16) 1576–1579.
[11] F. Yu, H. Yao, P. Zhu, X. Zhang, Q. Pan, C. Gong, et al., let-7 regulates self renewal and tumorigenicity of breast cancer cells, Cell 131 (6) (2007 Dec 14) 1109–1123.
[12] B.B. Zhou, H. Zhang, M. Damelin, K.G. Geles, J.C. Grindley, P.B. Dirks, Tumour-initiating cells: challenges and opportunities for anticancer drug discovery, Nat. Rev. Drug Discov. 8 (10) (2009 Oct) 806–823.
[13] S. Tokumaru, M. Suzuki, H. Yamada, M. Nagino, T. Takahashi, let-7 regulates Dicer expression and constitutes a negative feedback loop, Carcinogenesis 29 (11) (2008 Nov) 2073–2077.
[14] S.R. Viswanathan, G.Q. Daley, *Lin28: a microRNA regulator with a macro role*, Cell 140 (4) (2010 Feb 19) 445–449.
[15] T.C. Chang, L.R. Zeitels, H.W. Hwang, R.R. Chivukula, E.A. Wentzel, M. Dews, J. Jung, P. Gao, C.V. Dang, M.A. Beer, A. Thomas-Tikhonenko, J.T. Mendell, Lin-28B transactivation is necessary for Myc-mediated let-7 repression and proliferation, Proc. Natl. Acad. Sci. U. S. A. 106 (9) (2009 Mar 3) 3384–3389.
[16] D. Iliopoulos, H.A. Hirsch, K. Struhl, An epigenetic switch involving NF-kappaB, Lin28, Let-7 microRNA, and IL6 links inflammation to cell transformation, Cell 139 (4) (2009 Nov 13) 693–706.
[17] I. Heo, C. Joo, Y.K. Kim, M. Ha, M.J. Yoon, J. Cho, K.H. Yeom, J. Han, V.N. Kim, TUT4 in concert with Lin28 suppresses microRNA biogenesis through pre-microRNA uridylation, Cell 138 (4) (2009 Aug 21) 696–708.
[18] N.J. Lehrbach, J. Armisen, H.L. Lightfoot, K.J. Murfitt, A. Bugaut, S. Balasubramanian, E.A. Miska, LIN-28 and the poly(U) polymerase PUP-2 regulate let-7 microRNA processing in *Caenorhabditis elegans*, Nat. Struct. Mol. Biol. 16 (10) (2009 Oct) 1016–1020.
[19] J.P. Hagan, E. Piskounova, R.I. Gregory, Lin28 recruits the TUTase Zcchc11 to inhibit let-7 maturation in mouse embryonic stem cells, Nat. Struct. Mol. Biol. 16 (10) (2009 Oct) 1021–1025.
[20] G.A. Calin, C.D. Dumitru, M. Shimizu, R. Bichi, S. Zupo, E. Noch, et al., Frequent deletions and down-regulation of micro- RNA genes miR15 and miR16 at 13q14 in chronic lymphocytic leukemia, Proc. Natl. Acad. Sci. U. S. A. 99 (24) (2002 Nov 26) 15524–15529.
[21] G.A. Calin, M. Ferracin, A. Cimmino, G. Di Leva, M. Shimizu, S.E. Wojcik, et al., A microRNA signature associated with prognosis and progression in chronic lymphocytic leukemia, N. Engl. J. Med. 353 (17) (2005 Oct 27) 1793–1801.
[22] A. Cimmino, G.A. Calin, M. Fabbri, M.V. Iorio, M. Ferracin, M. Shimizu, et al., miR-15 and miR-16 induce apoptosis by targeting BCL2, Proc. Natl. Acad. Sci. U. S. A. 102 (39) (2005 Sep 27) 13944–13949.
[23] D. Bonci, V. Coppola, M. Musumeci, A. Addario, R. Giuffrida, L. Memeo, et al., The miR-15a-miR-16-1 cluster controls prostate cancer by targeting multiple oncogenic activities, Nat. Med. 14 (11) (2008 Nov) 1271–1277.
[24] F. Takeshita, L. Patrawala, M. Osaki, R.U. Takahashi, Y. Yamamoto, N. Kosaka, et al., *Systemic delivery of synthetic microRNA-16 inhibits the growth of metastatic prostate tumors via downregulation of multiple cell-cycle genes*, Mol. Ther. 18 (1) (2010 Jan) 181–187.
[25] S. Marchini, D. Cavalieri, R. Fruscio, E. Calura, D. Garavaglia, I.F. Nerini, C. Mangioni, G. Cattoretti, L. Clivio, L. Beltrame, D. Katsaros, L. Scarampi, G. Menato, P. Perego, G. Chiorino, A. Buda, C. Romualdi, M. D'Incalci, Association between miR-200c and the survival of patients with stage I epithelial ovarian cancer: a retrospective study of two independent tumour tissue collections, Lancet Oncol. 12 (3) (2011 Mar) 273–285.
[26] X. Chen, X. Guo, H. Zhang, Y. Xiang, J. Chen, Y. Yin, X. Cai, K. Wang, G. Wang, Y. Ba, L. Zhu, J. Wang, R. Yang, Y. Zhang, Z. Ren, K. Zen, J. Zhang, C.Y. Zhang, Role of miR-143 targeting KRAS in colorectal tumorigenesis, Oncogene 28 (10) (2009 Mar 12) 1385–1392.
[27] C. Clapé, V. Fritz, C. Henriquet, F. Apparailly, P.L. Fernandez, F. Iborra, C. Avancès, M. Villalba, S. Culine, L. Fajas, miR-143 interferes with ERK5 signaling, and abrogates prostate cancer progression in mice, PLoS One 4 (10) (2009 Oct 26) e7542.
[28] L. Liu, X. Yu, X. Guo, Z. Tian, M. Su, Y. Long, C. Huang, F. Zhou, M. Liu, X. Wu, X. Wang, miR-143 is downregulated in cervical cancer and promotes apoptosis and inhibits tumor formation by targeting Bcl-2, Mol. Med. Rep. 5 (3) (2012 Mar) 753–760.

Author's personal copy

[29] X. Wang, S. Tang, S.Y. Le, R. Lu, J.S. Rader, C. Meyers, Z.M. Zheng, Aberrant expression of oncogenic and tumor-suppressive microRNAs in cervical cancer is required for cancer cell growth, PLoS One 3 (7) (2008 Jul 2) e2557.

[30] W.O. Lui, N. Pourmand, B.K. Patterson, A. Fire, Patterns of known and novel small RNAs in human cervical cancer, Cancer Res. 67 (13) (2007 Jul 1) 6031–6043.

[31] M. Osaki, F. Takeshita, Y. Sugimoto, N. Kosaka, Y. Yamamoto, Y. Yoshioka, et al., *MicroRNA-143 regulates human osteosarcoma metastasis by regulating matrix metalloprotease-13 expression*, Mol. Ther. 19 (6) (2011 Jun) 1123–1130.

[32] D. Xu, F. Takeshita, Y. Hino, S. Fukunaga, Y. Kudo, A. Tamaki, et al., *miR-22 represses cancer progression by inducing cellular senescence*, J. Cell Biol. 93 (2) (2011 Apr 18) 409–424.

[33] D.H. Kim, J.J. Rossi, Strategies for silencing human disease using RNA interference, Nat. Rev. Genet. 8 (3) (2007 Mar) 173–184.

[34] K. Tiemann, J.J. Rossi, RNAi-based therapeutics-current status, challenges and prospects, EMBO Mol. Med. 1 (3) (2009 Jun) 142–151.

[35] H. Valadi, K. Ekstrom, A. Bossios, M. Sjostrand, J.J. Lee, J.O. Lotvall, Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells, Nat. Cell Biol. 9 (6) (2007 Jun) 654–659.

[36] M. Simons, G. Raposo, Exosomes—vesicular carriers for intercellular communication, Curr. Opin. Cell Biol. 21 (4) (2009 Aug) 575–581.

[37] C. Théry, M. Ostrowski, E. Segura, Membrane vesicles as conveyors of immune responses, Nat. Rev. Immunol. 9 (8) (2009 Aug) 581–593.

[38] N. Chaput, C. Théry, Exosomes: immune properties and potential clinical implementations, Semin. Immunopathol. 33 (5) (2011 Sep) 419–440.

[39] C. Théry, Exosomes: secreted vesicles and intercellular communications, Biol Rep. 3 (2011) 15 (F1000).

[40] D.M. Pegtel, K. Cosmopoulos, D.A. Thorley-Lawson, M.A. van Eijndhoven, E.S. Hopmans, J.L. Lindenberg, et al., *Functional delivery of viral miRNAs via exosomes*, Proc. Natl. Acad. Sci. U. S. A. 107 (14) (2010 Apr 6) 6328–6333.

[41] Y. Zhang, D. Liu, X. Chen, J. Li, L. Li, Z. Bian, et al., *Secreted monocytic miR-150 enhances targeted endothelial cell migration*, Mol. Cell 39 (1) (2010 Jul 9) 133–144.

[42] N. Kosaka, H. Iguchi, Y. Yoshioka, F. Takeshita, Y. Matsuki, T. Ochiya, *Secretory mechanisms and intercellular transfer of microRNAs in living cells*, J. Biol. Chem. 285 (23) (2010 Jun 4) 17442–17452.

[43] M. Mittelbrunn, C. Gutiérrez-Vázquez, C. Villarroya-Beltri, S. González, F. Sánchez-Cabo, M.Á. González, A. Bernad, F. Sánchez-Madrid, Unidirectional transfer of microRNA-loaded exosomes from T cells to antigen-presenting cells, Nat. Commun. 2 (2011) 282.

[44] A. Montecalvo, A.T. Larregina, W.J. Shufesky, D. Beer Stolz, M.L. Sullivan, J.M. Karlsson, C.J. Baty, G.A. Gibson, G. Erdos, Z. Wang, J. Milosevic, O.A. Tkacheva, S.J. Divito, R. Jordan, J. Lyons-Weiler, S.C. Watkins, A.E. Morelli, Mechanism of transfer of functional microRNAs between mouse dendritic cells via exosomes, Blood 119 (3) (2012 Jan 19) 756–766.

[45] M. Yang, J. Chen, F. Su, B. Yu, F. Su, L. Lin, Y. Liu, J.D. Huang, E. Song, Microvesicles secreted by macrophages shuttle invasion-potentiating microRNAs into breast cancer cells, Mol. Cancer 10 (2011 Sep 22) 117.

[46] T. Kogure, W.L. Lin, I.K. Yan, C. Braconi, T. Patel, Intercellular nanovesicle-mediated microRNA transfer: a mechanism of environmental modulation of hepatocellular cancer cell growth, Hepatology 54 (4) (2011 Oct) 1237–1248.

[47] N. Kosaka, H. Iguchi, Y. Yoshioka, K. Hagiwara, F. Takeshita, T. Ochiya, Competitive interactions of cancer cells and normal cells via secretory microRNAs, J. Biol. Chem. 287 (2) (2012 Jan 6) 1397–1405.

[48] L. Alvarez-Erviti, Y. Seow, H. Yin, C. Betts, S. Lakhal, M.J. Wood, Delivery of siRNA to the mouse brain by systemic injection of targeted exosomes, Nat. Biotechnol. 29 (4) (2011 Apr) 341–345.

[49] Q. Pan, V. Ramakrishnaiah, S. Henry, S. Fouraschen, P.E. de Ruiter, J. Kwekkeboom, H.W. Tilanus, H.L. Janssen, L.J. van der Laan, Hepatic cell-to-cell transmission of small silencing RNA can extend the therapeutic reach of RNA interference (RNAi), Gut (2011 Dec 23) (Epub ahead of print).

[50] K. Trajkovic, C. Hsu, S. Chiantia, L. Rajendran, D. Wenzel, F. Wieland, P. Schwille, B. Brügger, M. Simons, Ceramide triggers budding of exosome vesicles into multivesicular endosomes, Science 319 (5867) (2008 Feb 29) 1244–1247.

[51] M. Ostrowski, N.B. Carmo, S. Krumeich, I. Fanget, G. Raposo, A. Savina, C.F. Moita, K. Schauer, A.N. Hume, R.P. Freitas, B. Goud, P. Benaroch, N. Hacohen, M. Fukuda, C. Desnos, M.C. Seabra, F. Darchen, S. Amigorena, L.F. Moita, C. Thery, Rab27a and Rab27b control different steps of the exosome secretion pathway, Nat. Cell Biol. 12 (1) (2010 Jan) 19–30 (sup pp 1–13).

[52] C. Hsu, Y. Morohashi, S. Yoshimura, N. Manrique-Hoyos, S. Jung, M.A. Lauterbach, M. Bakhti, M. Grønborg, W. Möbius, J. Rhee, F.A. Barr, M. Simons, Regulation of exosome secretion by Rab35 and its GTPase-activating proteins TBC1D10A-C, J. Cell Biol. 189 (2) (2010 Apr 19) 223–232.

[53] N. Amzallag, B.J. Passer, D. Allanic, E. Segura, C. Théry, B. Goud, R. Amson, A. Telerman, TSAP6 facilitates the secretion of translationally controlled tumor protein/histamine-releasing factor via a nonclassical pathway, J. Biol. Chem. 279 (44) (2004 Oct 29) 46104–46112.

[54] X. Yu, S.L. Harris, A.J. Levine, The regulation of exosome secretion: a novel function of the p53 protein, Cancer Res. 66 (9) (2006 May 1) 4795–4801.

[55] A. Lespagnol, D. Duflaut, C. Beekman, L. Blanc, G. Fiucci, J.C. Marine, M. Vidal, R. Amson, A. Telerman, Exosome secretion, including the DNA damage-induced p53-dependent secretory pathway, is severely compromised in TSAP6/Steap3-null mice, Cell Death Differ. 15 (11) (2008 Nov) 1723–1733.

[56] B.D. Lehmann, M.S. Paine, A.M. Brooks, J.A. McCubrey, R.H. Renegar, R. Wang, D.M. Terrian, Senescence-associated exosome release from human prostate cancer cells, Cancer Res. 68 (19) (2008 Oct 1) 7864–7871.

[57] X. Yu, T. Riley, A.J. Levine, The regulation of the endosomal compartment by p53 the tumor suppressor gene, FEBS J. 276 (8) (2009 Apr) 2201–2212.

[58] A. Savina, M. Furlán, M. Vidal, M.I. Colombo, Exosome release is regulated by a calcium-dependent mechanism in K562 cells, J. Biol. Chem. 278 (22) (2003 May 30) 20083–20090.

[59] A. Savina, C.M. Fader, M.T. Damiani, M.I. Colombo, Rab11 promotes docking and fusion of multivesicular bodies in a calcium-dependent manner, Traffic 6 (2) (2005 Feb) 131–143.

[60] Y. Kuwabara, K. Ono, T. Horie, H. Nishi, K. Nagao, M. Kinoshita, S. Watanabe, O. Baba, Y. Kojima, S. Shizuta, M. Imai, T. Tamura, T. Kita, T. Kimura, Increased microRNA-1 and microRNA-133a levels in serum of patients with cardiovascular disease indicate myocardial damage, Circ. Cardiovasc. Genet. 4 (4) (2011 Aug 1) 446–454.

[61] L. Zhang, D. Hou, X. Chen, D. Li, L. Zhu, Y. Zhang, J. Li, Z. Bian, X. Liang, X. Cai, Y. Yin, C. Wang, T. Zhang, D. Zhu, D. Zhang, J. Xu, Q. Chen, Y. Ba, J. Liu, Q. Wang, J. Chen, J. Wang, M. Wang, Q. Zhang, J. Zhang, K. Zen, C.Y. Zhang, Exogenous plant MIR168a specifically targets mammalian LDLRAP1: evidence of cross-kingdom regulation by microRNA, Cell Res. 22 (1) (2012 Jan) 107–126.

[62] A. Clayton, C.L. Harris, J. Court, M.D. Mason, B.P. Morgan, Antigen-presenting cell exosomes are protected from complement-mediated lysis by expression of CD55 and CD59, Eur. J. Immunol. 33 (2) (2003 Feb) 522–531.

Proteomics 2013, *13*, 1637–1653 DOI 10.1002/pmic.201200373

REVIEW

The therapeutic potential of mesenchymal stem cell-derived extracellular vesicles

Takeshi Katsuda, Nobuyoshi Kosaka, Fumitaka Takeshita and Takahiro Ochiya

Division of Molecular and Cellular Medicine, National Cancer Center Research Institute, Tokyo, Japan

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 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

Correspondence: Dr. Takahiro Ochiya, Division of Molecular and Cellular Medicine, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan
E-mail: tochiya@ncc.go.jp
Fax: +81-3-5565-0727

Abbreviations: **Aβ**, β-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

© 2013 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

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 Extraceluar 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 multivesicular 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

© 2013 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

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 $CD8^+$ 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 effects 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

© 2013 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

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).

© 2013 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

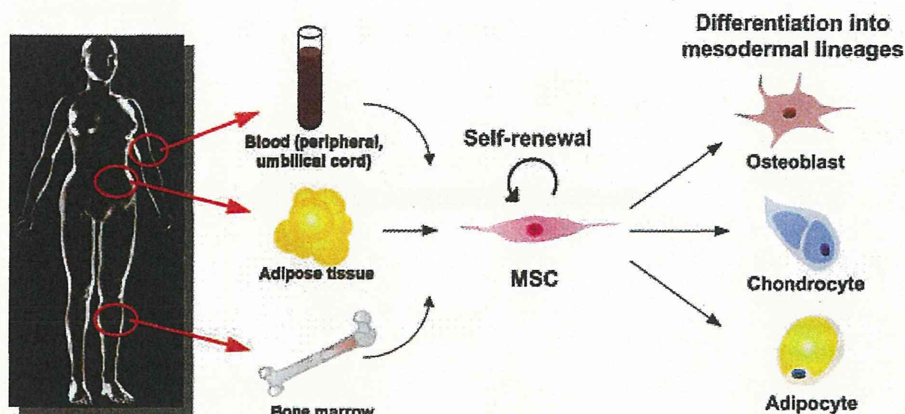

Figure 1. Origin and characteristics of mesenchymal stem cells. MSCs can be isolated from various tissues, including bone marrow, adipose tissue, and blood including peripheral and umbilical cord blood. MSCs have the capacity to self-renew and differentiate into multiple cell lineages including three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

© 2013 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

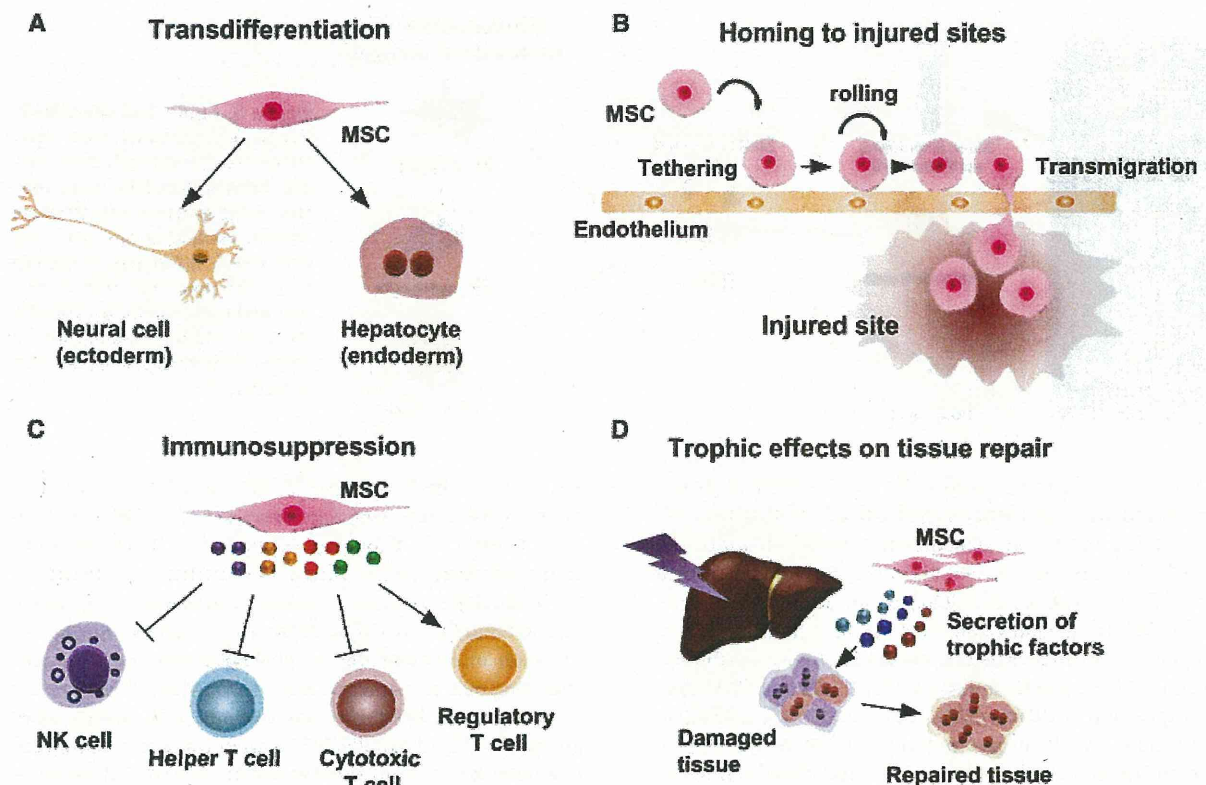

Figure 2. Various therapeutic effects of MSCs. (A) MSCs can transdifferentiate into cells of nonmesodermal origin including neurons (ectoderm) and hepatocytes (endoderm). (B) MSCs are capable of homing to injured tissues. The mechanism by which MSCs home to tissues and migrate across endothelium still 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 $CD105^+$ 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 differentiation 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 chemotactic protein-1, nerve growth factor, and hepatocyte growth factor [101]. These factors are known to be responsible for immunosuppression,

© 2013 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

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)-γ with tumor necrosis factor, IL-1-α or IL-1-β. Upon stimulation of these proinflammatory cytokines, MSCs secrete immunosuppressive cytokines including prostaglandin E2, indoleamine 2,3-dioxygenase, transforming growth factor-β1, 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 transient 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].

© 2013 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

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 cardioprotection 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

© 2013 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

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 β-amyloid peptide (Aβ) in the brain because of an imbalance between Aβ production and clearance. Recently, we have found that ADSCs, but not BM-MSCs, potentially contribute to Aβ 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

© 2013 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

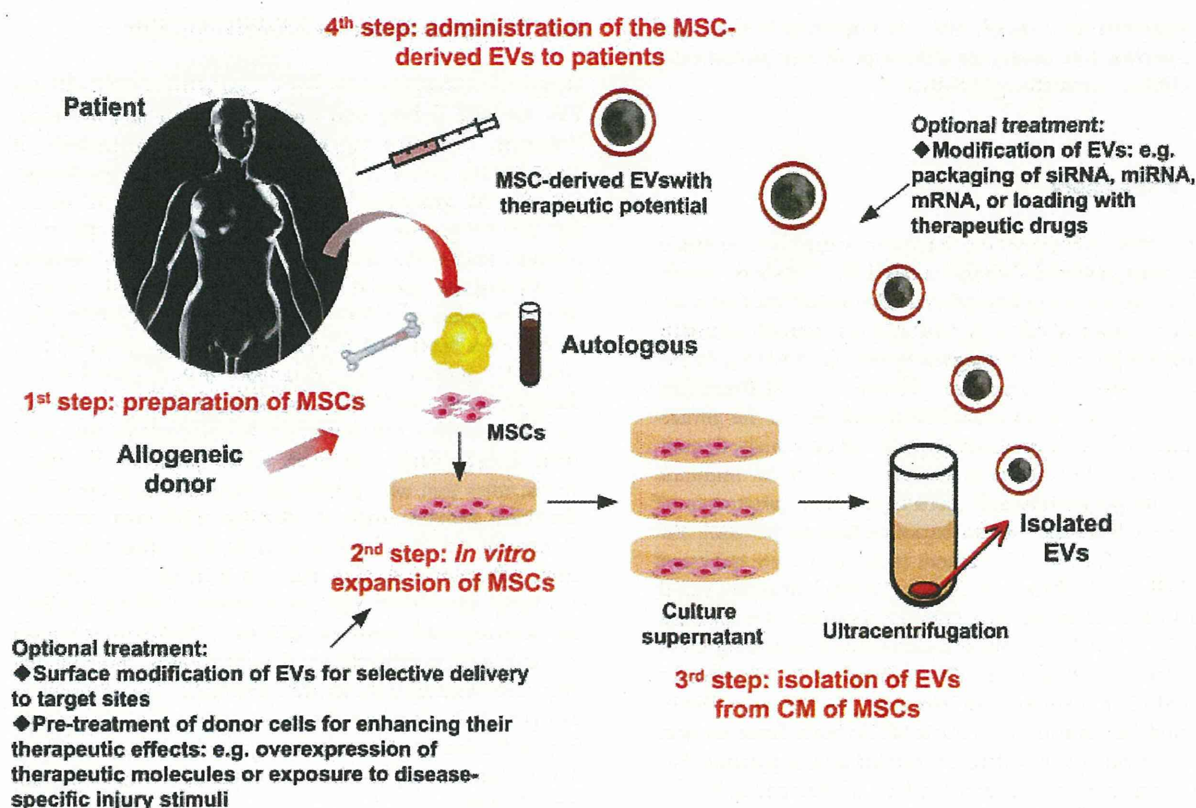

Figure 3. Schematic representation of the proposed strategy for clinical application of MSC-derived EVs. The first step is to prepare MSCs for production of EVs with therapeutic efficacy. MSCs can be obtained from a patient's own connective tissue such as BM, adipose tissue, and blood including peripheral 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 4th 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 ontogeny 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.

© 2013 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

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-α [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

© 2013 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

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.

7 References

[1] Behnke, O., An electron microscope study of the megacaryocyte of the rat bone marrow. I. The development of the demarcation membrane system and the platelet surface coat. *J. Ultrastruct. Res.* 1968, *24*, 412–433.

[2] Théry, C., Exosomes: secreted vesicles and intercellular communications. *F1000 Biol. Rep.* 2011, *3*, 15.

[3] Cocucci, E., Racchetti, G., Meldolesi, J., Shedding microvesicles: artefacts no more. *Trends Cell Biol.* 2009, *19*, 43–51.

[4] Harding, C., Heuser, J., Stahl, P., Receptor-mediated endocytosis of transferrin and recycling of the transferrin receptor in rat reticulocytes. *J. Cell Biol.* 1983, *97*, 329–339.

[5] Johnstone, R. M., Adam, M., Hammonds, J. R., Turbide, C., Vesicle formation during reticulocyte maturation. *J. Biol. Chem.* 1987, *262*, 9412–9420.

[6] Johnstone, R. M., Bianchini, A., Teng, K., Reticulocyte maturation and exosome release: transferrin receptor containing exosomes shows multiple plasma membrane functions. *Blood* 1989, *74*, 1844–1851.

[7] Johnstone, R. M., Mathew, A., Mason, A. B., Teng, K., Exosome formation during maturation of mammalian and avian reticulocytes: evidence that exosome release is amajorroute for externalization of obsolete membrane proteins. *J. Cell. Physiol.* 1991, *147*, 27–36.

[8] Johnstone, R. M., The Jeanne Manery-Fisher Memorial Lecture 1991. Maturation of reticulocytes: formation of exosomes as a mechanism for shedding membrane proteins. *Biochem. Cell Biol.* 1992, *70*, 179–190.

[9] Taylor, D. D., Homesley, H. D., Doellgast, G. J., Binding of specific peroxidase-labeled antibody to placental-type phosphatase on tumor-derived membrane fragments. *Cancer Res.* 1980 *40*, 4064–4069.

[10] Camussi, G., Deregibus, M. C., Bruno, S., Cantaluppi, V., Biancone, L., Exosomes/microvesicles as a mechanism of cell-to-cell communication. *Kidney Int.* 2010, *78*, 838–848.

[11] Raposo, G., Nijman, H. W., Stoorvogel, W., Liejendekker, R. et al., B lymphocytes secrete antigen-presenting vesicles. *J. Exp. Med.* 1996, *183*, 1161–1172.

[12] Ratajczak, J., Miekus, K., Kucia, M., Zhang, J. et al., Embryonic stem cell-derived microvesicles reprogram hematopoietic progenitors: evidence for horizontal transfer of mRNA and protein delivery. *Leukemia* 2006, *20*, 847–856.

[13] Valadi, H., Ekström, K., Bossios, A., Sjöstrand, M. et al., Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat. Cell Biol.* 2007, *9*, 654–659.

[14] Pegtel, D. M., Cosmopoulos, K., Thorley-Lawson, D. A, van Eijndhoven, M. A. et al., Functional delivery of viral miRNAs via exosomes. *Proc. Natl. Acad. Sci. USA* 2010, *107*, 6328–6333.

[15] Zhang, Y., Liu, D., Chen, X., Li, J. et al., Secreted monocytic miR-150 enhances targeted endothelial cell migration. *Mol. Cell.* 2010, *39*, 133–144.

[16] Kosaka, N., Iguchi, H., Yoshioka, Y., Takeshita, F. et al., Secretory mechanisms and intercellular transfer of microRNAs in living cells. *J. Biol. Chem.* 2010, *285*, 17442–17452.

[17] Théry, C., Zitvogel, L., Amigorena, S., Exosomes: composition, biogenesis and function. *Nat. Rev. Immunol.* 2002, *2*, 569–579.

[18] Savina, A., Furlán, M., Vidal, M., Colombo, M. I., Exosome release is regulated by a calcium-dependent mechanism in K562 cells. *J. Biol. Chem.* 2003, *278*, 20083–20090.

[19] Trajkovic, K., Hsu, C., Chiantia, S., Rajendran, L. et al., Ceramide triggers budding of exosome vesicles into multivesicular endosomes. *Science* 2008, *319*, 1244–1247.

[20] Ostrowski, M., Carmo, N. B., Krumeich, S., Fanget, I. et al., Rab27a and Rab27b control different steps of the exosome secretion pathway. *Nat. Cell Biol.* 2010, *12*, 19–30.

[21] Taylor, D. D., Gercel-Taylor, C., MicroRNA signatures of tumor-derived exosomes as diagnostic biomarkers of ovarian cancer. *Gynecol. Oncol.* 2008, *110*, 13–21.

[22] Sheldon, H., Heikamp, E., Turley, H., Dragovic, R. et al., New mechanism for Notch signaling to endothelium at a distance by Delta-like 4 incorporation into exosomes. *Blood* 2010, *116*: 2385–2394.

[23] Zitvogel, L., Regnault, A., Lozier, A., Wolfers, J. et al., Eradication of established murine tumors using a novel cell-free vaccine: dendritic cell derived exosomes. *Nat. Med.* 1998, *4*, 594–600.

[24] Skokos, D., Le Panse, S., Villa, I., Rousselle, J. C. et al., Mast cell-dependent B and T lymphocyte activation is mediated by the secretion of immunologically active exosomes. *J. Immunol.* 2001, *166*, 868–876.

© 2013 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

[25] Skokos, D., Botros, H. G., Demeure, C., Morin, J. et al., Mast cell-derived exosomes induce phenotypic and functional maturation of dendritic cells and elicit specific immune responses in vivo. *J. Immunol.* 2003, *170*, 3037–3045.

[26] Mittelbrunn, M., Gutiérrez-Vázquez, C., Villarroya-Beltri, C., González, S. et al., Unidirectional transfer of microRNA-loaded exosomes from T cells to antigen-presenting cells. *Nat. Commun.* 2011, *2*, 282.

[27] Dunn, G. P., Bruce, A. T., Ikeda, H., Old, L. J., Schreiber, R. D., Cancer immunoediting : from immuno-surveillance to tumor escape. *Nat. Immunol.* 2002, *3*, 991–998.

[28] Dunn, G. P., Old, L. J., Schreiber, R. D., The three Es of cancer immunoediting. *Annu. Rev. Immunol.* 2004, *22*, 329–360.

[29] Wolfers, J., Lozier, A., Raposo, G., Regnault, A. et al., Tumor-derived exosomes are a source of shared tumor rejection antigens for CTL cross-priming. *Nat. Med.* 2001, *7*, 297–303.

[30] Zeelenberg, I. S., Ostrowski, M., Krumeich, S., Bobrie, A. et al., Targeting tumor antigens to secreted membrane vesicles in vivo induces efficient antitumor immune responses. *Cancer Res.* 2008, *68*, 1228–1235.

[31] Gastpar, R., Gehrmann, M., Bausero, M. A., Asea, A. et al., Heat shock protein 70 surface-positive tumor exosomes stimulate migratory and cytolytic activity of natural killer cells. *Cancer Res.* 2005, *65*, 5238–5247.

[32] Andreola, G., Rivoltini, L., Castelli, C., Huber, V. et al., Induction of lymphocyte apoptosis by tumor cell secretion of FasL-bearing microvesicles. *J. Exp. Med.* 2002, *195*, 1303–1316.

[33] Abusamra, A. J., Zhong, Z., Zheng, X., Li, M. et al., Tumor exosomes expressing Fas ligand mediate CD8+ T-cell apoptosis. *Blood Cells Mol. Dis.* 2005, *35*, 169–173.

[34] Clayton, A., Mitchell, J. P., Court, J., Mason, M. D., Tabi, Z., Human tumor-derived exosomes selectively impair lymphocyte responses to interleukin-2. Responses to interleukin-2. *Cancer Res.* 2007, *67*, 7458–7466.

[35] Chalmin, F., Ladoire, S., Mignot, G., Vincent, J. et al., Membrane-associated Hsp72 from tumor-derived exosomes mediates STAT3-dependent immunosuppressive function of mouse and human myeloid-derived suppressor cells. *J. Clin. Invest.* 2010, *120*, 457–471.

[36] Gutwein, P., Stoeck, A., Riedle, S., Gast, D. et al., Cleavage of L1 in exosomes and apoptotic membrane vesicles released from ovarian carcinoma cells. *Clin. Cancer Res.* 2005, *11*, 2492–2501.

[37] Higginbotham, J. N., Demory Beckler, M., Gephart, J. D., Franklin, J. L. et al., Amphiregulin exosomes increase cancer cell invasion. *Curr. Biol.* 2011, *21*, 779–786.

[38] Biernat, W., Huang, H., Yokoo, H., Kleihues, P., Ohgaki, H., Predominant expression of mutant EGFR (EGFRvIII) is rare in primary glioblastomas. *Brain Pathol.* 2004, *14*, 131–136.

[39] Al-Nedawi, K., Meehan, B., Micallef, J., Lhotak, V. et al., Intercellular transfer of the oncogenic receptor EGFRvIII by microvesicles derived from tumour cells. *Nat. Cell Biol.* 2008, *10*, 619–624.

[40] Skog, J., Würdinger, T., van Rijn, S., Meijer, D. H. et al., Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. *Nat. Cell Biol.* 2008, *10*, 1470–1476.

[41] Gesierich, S., Berezovskiy, I., Ryschich, E., Zöller, M., Systemic induction of the angiogenesis switch by the tetraspanin D6.1A/CO-029. *Cancer Res.* 2006, *66*, 7083–7094.

[42] Nazarenko, I., Rana, S., Baumann, A., Mcalear, J. et al., Cell surface tetraspanin Tspan8 contributes to molecular pathways of exosome-induced endothelial cell activation. *Cancer Res.* 2010, *70*, 1668–1678.

[43] Al-Nedawi, K., Meehan, B., Kerbel, R. S., Allison, A. C., Rak, J., Endothelial expression of autocrine VEGF upon the uptake of tumor-derived microvesicles containing oncogenic EGFR. *Proc. Natl. Acad. Sci. USA* 2009, *106*, 3794–3799.

[44] Kaplan, R. N., Riba, R. D., Zacharoulis, S., Bramley, A. H. et al., VEGFR1-positive haematopoietic bone marrow progenitors initiate the pre-metastatic niche. *Nature* 2005, *438*, 820–827.

[45] Peinado, H., Alečković, M., Lavotshkin, S., Matei, I. et al., Melanoma exosomes educate bone marrow progenitor cells toward a pro-metastatic phenotype through MET. *Nat. Med.* 2012, *18*, 883–891.

[46] Jung, T., Castellana, D., Klingbeil, P., Cuesta Hernández, I. et al., CD44v6 dependence of premetastatic niche preparation by exosomes. *Neoplasia* 2009, *11*, 1093–1105.

[47] Deregibus, M. C., Cantaluppi, V., Calogero, R., Lo Iacono, M. et al., Endothelial progenitor cell derived microvesicles activate an angiogenic program in endothelial cells by a horizontal transfer of mRNA. *Blood* 2007, *110*, 2440–2448.

[48] Kosaka, N., Iguchi, H., Yoshioka, Y., Hagiwara, K. et al., Competitive interactions of cancer cells and normal cells via secretory microRNAs. *J. Biol. Chem.* 2011, *287*, 1397–1405.

[49] Katsuda, T., Sakai, Y., Ochiya, T., Induced pluripotent stem cell-derived hepatocytes as an alternative to human adult hepatocytes. *J. Stem Cells.* 2012, *7*, 1–17.

[50] Yamanaka, S., A fresh look at iPS cells. *Cell* 2009, *137*, 13–17.

[51] Fairchild, P. J., The challenge of immunogenicity in the quest for induced pluripotency. *Nat. Rev. Immunol.* 2010, *10*, 868–875.

[52] Chamberlain, G., Fox, J., Ashton, B., Middleton, J., Concise review: mesenchymal stem cells: their phenotype, differentiation capacity, immunological features, and potential for homing. *Stem Cells* 2007, *25*, 2739–2749.

[53] Djouad, F., Bouffi, C., Ghannam, S., Noël, D., Jorgensen, C., Mesenchymal stem cells: innovative therapeutic tools for rheumatic diseases. *Nat. Rev. Rheumatol.* 2009, *5*, 392–399.

[54] Salem, H. K., Thiemermann, C., Mesenchymal stromal cells: current understanding and clinical status. *Stem Cells* 2010, *28*, 585–596.

[55] Psaltis, P. J., Zannettino, A. C. W., Worthley, S. G., Gronthos, S., Concise review: mesenchymal stromal cells: potential for cardiovascular repair. *Stem Cells* 2008, *26*, 2201–2210.

© 2013 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

[56] Ochiya, T., Yamamoto, Y., Banas, A., Commitment of stem cells into functional hepatocytes. *Differentiation* 2010, *79*, 65–73.

[57] Friedenstein, A. J., Chailakhyan, R. K., Latsinik, N. V., Panasyuk, A. F., Keiliss-Borok, I. V., Stromal cells responsible for transferring the microenvironment of the hemopoietic tissues. Cloning in vitro and retransplantation in vivo. *Transplantation* 1974, *17*, 331–340.

[58] Friedenstein, A. J., Gorskaja, J. F., Kulagina, N. N., Fibroblast precursors in normal and irradiated mouse hematopoietic organs. *Exp. Hematol.* 1976, *4*, 267–274.

[59] Dominici, M., Le Blanc, K., Mueller, I., Slaper-Cortenbach, I. et al., Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 2006, *8*, 315–317.

[60] Phinney, D. G., Prockop, D. J., Concise review: mesenchymal stem/multipotent stromal cells: the state of transdifferentiation and modes of tissue repair–current views. *Stem Cells* 2007, *25*, 2896–2902.

[61] Zhang, Y., Wang, W., Effects of bone marrow mesenchymal stem cell transplantation on light-damaged retina. *Invest. Ophthalmol. Vis. Sci.* 2010, *51*, 3742–3748.

[62] Shi, C., Zhu, Y., Su, Y., Cheng, T., Stem cells and their applications in skin-cell therapy. *Trends Biotechnol.* 2006, *24*, 48–52.

[63] Banas, A., Yamamoto, Y., Teratani, T., Ochiya, T., Stem cell plasticity: learning from hepatogenic differentiation strategies. *Dev. Dyn.* 2007, *236*, 3228–3241.

[64] Humphreys, B. D., Bonventre, J. V., Mesenchymal stem cells in acute kidney injury. *Annu. Rev. Med.* 2008, *59*, 311–325.

[65] Kotton, D. N., Ma, B. Y., Cardoso, W. V., Sanderson, E. A. et al., Bone marrow-derived cells as progenitors of lung alveolar epithelium. *Development* 2001, *128*, 5181–5188.

[66] Ortiz, L.A., Gambelli, F., McBride, C., Gaupp, D. et al., Mesenchymal stem cell engraftment in lung is enhanced in response to bleomycin exposure and ameliorates its fibrotic effects. *Proc. Natl. Acad. Sci. USA* 2003, *100*, 8407–8411.

[67] Gage, F. H., Mammalian neural stem cells. *Science* 2000, *287*, 1433–1438.

[68] Kopen, G. C., Prockop, D. J., Phinney, D.G., Marrow stromal cells migrate throughout forebrain and cerebellum, and they differentiate into astrocytes after injection into neonatal mouse brains. *Proc. Natl. Acad. Sci. USA* 1999, *96*, 10711–10716.

[69] Chen, J., Li, Y., Wang, L., Lu, M. et al., Therapeutic benefit of intracerebral transplantation of bone marrow stromal cells after cerebral ischemia in rats. *J. Neurol. Sci.* 2001, *189*, 49–57.

[70] Zhao, L. R., Duan, W. M., Reyes, M., Keene, C. D. et al., Human bone marrow stem cells exhibit neural phenotypes and ameliorate neurological deficits after grafting into the ischemic brain of rats. *Exp. Neurol.* 2002, *174*, 11–20.

[71] Muñoz-Elias, G., Marcus, A. J., Coyne, T. M., Woodbury, D., Black, I. B., Adult bone marrow stromal cells in the embryonic brain: engraftment, migration, differentiation, and long-term survival. *J. Neurosci.* 2004, *24*, 4585–4895.

[72] Sanchez-Ramos, J., Song, S., Cardozo-Pelaez, F., Hazzi, C. et al., Adult bone marrow stromal cells differentiate into neural cells in vitro. *Exp. Neurol.* 2000, *164*, 247–256.

[73] Woodbury, D., Schwarz, E. J., Prockop, D. J., Black, I. B., Adult rat and human bone marrow stromal cells differentiate into neurons. *J. Neurosci. Res.* 2000, *61*, 364–370.

[74] Kitada M, Dezawa, M., Induction system of neural and muscle lineage cells from bone marrow stromal cells; a new strategy for tissue reconstruction in degenerative diseases. *Histol. Histopathol. [Internet]* 2009, *24*(5), 631–642.

[75] Fisher, R. A., Strom, S. C., Human hepatocyte transplantation: worldwide results. *Transplantation* 2006, *82*, 441–449.

[76] Carpentier, B., Gautier, A., Legallais, C., Artificial and bioartificial liver devices: present and future. *Gut.* 2009, *58*, 1690–1702.

[77] Sakai, Y., Nishikawa, M., Evenou, F., Hamon, M. et al., Engineering of implantable liver tissues. *Methods Mol. Biol.* 2012, *826*, 189–216.

[78] Teratani, T., Yamamoto, H., Aoyagi, K., Sasaki, H. et al., Direct hepatic fate specification from mouse embryonic stem cells. *Hepatology* 2005, *41*, 836–846.

[79] Soto-Gutiérrez, A., Kobayashi, N., Rivas-Carrillo, J. D., Navarro-Alvarez, N. et al., Reversal of mouse hepatic failure using an implanted liver-assist device containing ES cell-derived hepatocytes. *Nat. Biotechnol.* 2006, *24*, 1412–1419.

[80] Cai, J., Zhao, Y., Liu, Y., Ye, F. et al., Directed differentiation of human embryonic stem cells into functional hepatic cells. *Hepatology* 2007, *45*, 1229–1239.

[81] Si-Tayeb, K., Noto, F. K., Nagaoka, M., Li, J. et al., Highly efficient generation of human hepatocyte-like cells from induced pluripotent stem cells. *Hepatology* 2010, *51*, 297–305.

[82] Sullivan, G. J., Hay, D. C., Park, I. H., Fletcher, J. et al., Generation of functional human hepatic endoderm from human induced pluripotent stem cells. *Hepatology* 2010, *51*, 329–335.

[83] Touboul, T., Hannan, N. R. F., Corbineau, S., Martinez, A. et al., Generation of functional hepatocytes from human embryonic stem cells under chemically defined conditions that recapitulate liver development. *Hepatology* 2010, *51*, 1754–1765.

[84] Kamiya, A., Kojima, N., Kinoshita, T., Sakai, Y., Miyaijma, A., Maturation of fetal hepatocytes in vitro by extracellular matrices and oncostatin M: induction of tryptophan oxygenase. *Hepatology* 2002, *35*, 1351–1359.

[85] Hanada, S., Kojima, N., Sakai, Y., Soluble factor-dependent in vitro growth and maturation of rat fetal liver cells in a three-dimensional culture system. *Tissue Eng. Part A* 2008, *14*, 149–160.

[86] Katsuda, T., Teratani, T., Ochiya, T., Sakai, Y., Transplantation of a fetal liver cell-loaded hyaluronic acid sponge onto the mesentery recovers a Wilson's disease model rat. *J. Biochem.* 2010, *148*, 281–288.

© 2013 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim