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Karl L. Mettinger
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Exosomes, Stem Cells and MicroRNA

Aging, Cancer and Age Related Disorders

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Editorial

The Rise of Stem Cells, microRNAs and Microvesicles/Exosomes: New Frontiers in Age-Related Disorders and Cancer

We are currently at the cusp of a global revolution in medical research, where academic, biotechnology and pharmaceutical scientists are poised to work hand in hand to deliver new gene targets, cell therapy, microvesicles and tissue engineering products for an array of conditions, many of which lack treatment options.[1] Additionally, the development of new treatments may also replace current treatments that are associated with toxicity.

The early failures of gene therapy clinical trials provided avenues to develop therapies associated with stem cells. Scientists learnt the lessons of gene therapy as they embarked on future stem cell treatments. Between 1998 and 2013 more than 10,000 patients were enrolled in 200 clinical trials sponsored by 60 pharmaceutical companies or private stem cell clinics, more than half of them based in the US.[2] The majority of the listed trials used adult mesenchymal stem cells, progenitor cells or stroma vascular fractions (SVF). Most of them were exploratory Phase 1 studies, and few completed Phase 2 or 3 studies. Many of the studies were done outside the oversight of FDA regulation, in part due to lack or ambiguity of regulatory guidelines; this prompted a two day FDA hearing in Baltimore on September 12–13, 2016.[3]

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[2] SCSI – Fifteen years, 60 companies, 196 trials, Part II and III, Stem Cell Stock Index Report, 2013

[3] Hildreth C US FDA olds historic hearing, Bioinformant - Key Points of Agreement and Contention for Regulation of Stem Cells. http://www.bioinformant.com/fda-regulation-of-stem-cells/

Two parallel developments to stem cells are the revolutions in extracellular microRNA (miRNA) and macrovesicle/exosome research. First identified in late 1990s in *C.Elegans,* an early model species for aging research, miRNAs have been found to be key players in virtually all biological processes[4] and the number of publications grew exponentially from less than a dozen in 2001 to more than 500 in the next seven years[5] and a subsequent phenomenal explosion of annual output exceeding 2000 publications in 2009 and 6000 in 2013.[6] This prompted the initiation of NIH Extracellular RNA (ExRNA) Consortium in 2012 when NIH announced Request For Applications (RFAs) in five separate programs and committed to invest over 100M USD in multi-institutional extramural projects to address fundamental questions.[7,8]

Equally impressive was the number of publications involving extracellular vesicles (EV) including exosomes which exploded to more than 35,000 by 2015, spurring a revolution in liquid diagnostic tests and new prospects for extracellular carriers as new targeted therapeutics in a plethora of disease classes. This prompted the formation (2012 in Sweden) of International Society of Extracellular Vesicles (ISEV) and the International Journal of Extracellular Vesicles.

A particular promising area for current research involves the themes for this monograph, the involvement or miRNA and exosomes in stem cell biology and stem cell aging, as well as in cancer and a number of other age-related diseases. The number of publications of exosomes/stem cells have grown from less than 20/year in 2010 to more than 150/year in 2015. During the same period the number of publications involving exosomes/cancer increased to >160/year and exosomes/autoimmune or exosome/cardiovascular disorders to >100/year and exosomes/neurodegenerative disorders to >45/year (Figs. 1–3).[9]

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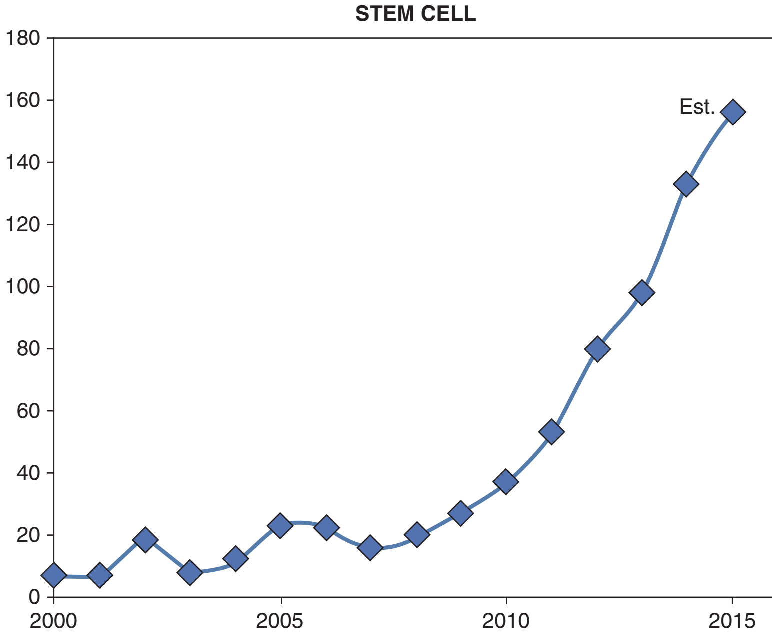

Fig. 1 Number of publications related to exosomes/stem cells. Source: Reproduced with permission from Oosta and Razvi (2015)

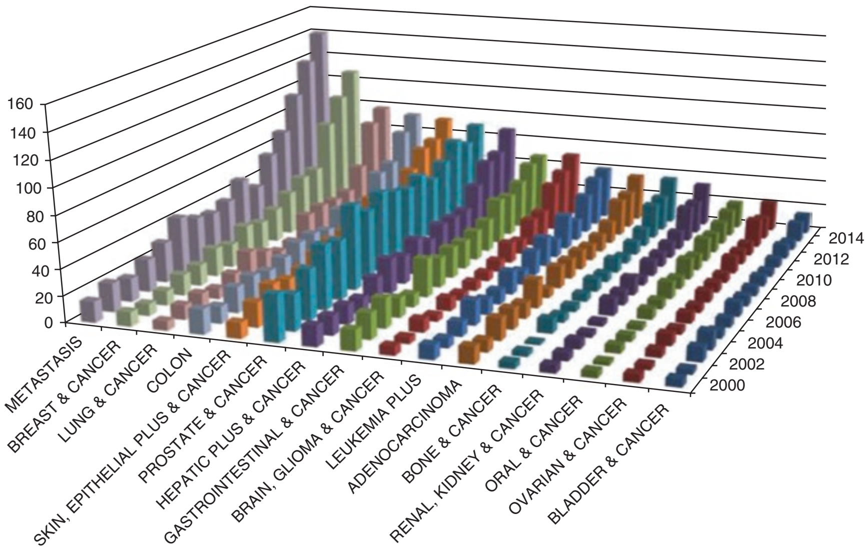

Fig. 2 Number of publications related to exosomes/cancer. Source: Reproduced with permission from Oosta and Razvi (2015)

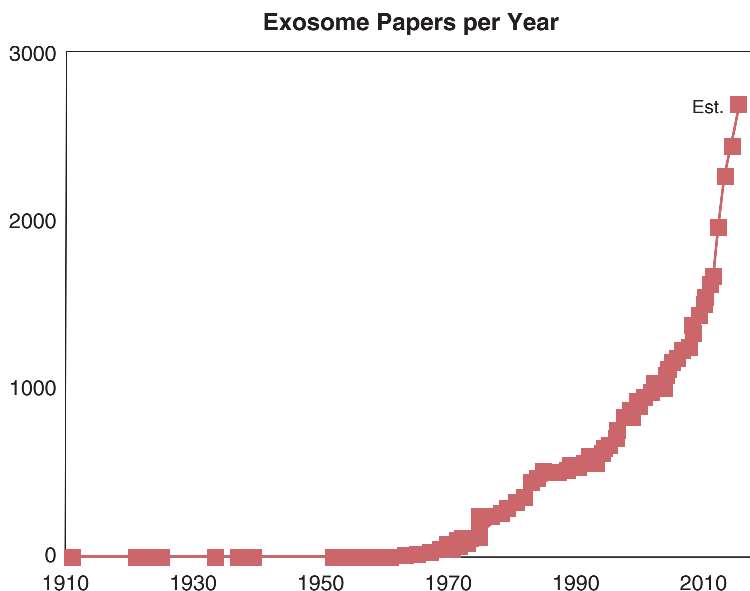

Fig. 3 Number of publications related to exosomes. Source: Reproduced with permission from Oosta and Razvi (2015)

Therefore, for the current volume we invited contributions from some leading experts to illuminate these cross-disciplinary developments towards understanding the role of exosomes/extracellular vesicles, miRNAs and stem cells in age-related disorders including CNS/cardiovascular disorders as well as hematological malignancies and cancer.

We thank our project leader Mr. Daniel Ignatius Jagadisan at Springer, now part of SpringerNature, a world leading publisher of cutting edge scientific literature. This book project aims to synthesize current understanding of the science and to stimulate new therapeutic developments. The volume may also serve as a helpful tool in education and inspire productive dialogue between students and teachers.

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Part I
The Role of miRNAs and Exosomes in Stem Cell Biology and Aging

Quesenberry P and Goldberg LG present an important view on the present paradigm of stem cells, discussing a continuum of hematopoietic stem cells (HSC). Although the chapter was focused on hematopoietic stem cells, the information could be extrapolated to other stem cells. In this regard, the information provided by these two authors should provide 'pause' when working with other stem cells. The chapter is relevant since treatment with exosomes as well as their use as vehicles for drug delivery to target cancer stem cell or other subsets must be aware that there could be a continuum of stem cells. The authors discussed actively cycling HSCs changing phenotype and function during cycle transition. These findings are highly relevant to the hierarchy of cancer cells, which are mostly based on the classical hierarchy of HSCs and hematopoietic progenitors. The novel model proposed by these authors must be considered by others who propose to use of microvesicles for therapeutic delivery to treat diseases such as cancer at metastatic sites. According to the authors, the miRNA profile and the contents of exosomes might vary as the cells change phenotype and function, while the cells are multipotent.

Dietrich C, Singh M, Kumar N and Singh SR expand on the issue of aging and miRNA by incorporating stem cells. In order to understand cancer, it is important to study stem cells since cancer could be considered as a stem cell disorder. The authors discussed the role of miRNAs in the regulation of translation/gene expression and link this to biological processes such as development, differentiation, cell death, stem cell proliferation and differentiation, immune response, aging and cancer. This chapter discusses the contribution of miRNAs in normal and aged stem cell function using different model systems.

Chapter 1
A New Stem Cell Biology: Transplantation and Baseline, Cell Cycle and Exosomes

Peter Quesenberry and Laura R. Goldberg

Abstract Hematopoietic stem cell biology has focused on stem cell purification and the definition of the regulation of purified stem cells in a hierarchical system. Work on the whole unpurified murine marrow cell population has indicated that a significant number of hematopoietic stem cells, rather than being dormant, are actively cycling, always changing phenotype and therefore resistant to purification efforts by current approaches. The bulk of cycling marrow stem cells are discarded with the standard lineage negative, stem cell marker positive separations. Therefore, the purified stem cells do not appear to be representative of the total hematopoietic stem cell population. In addition, baseline hematopoiesis does not appear to be determined by the transplantable stem cells but rather by many short-lived clones of varying differentiation potential. These systems appear to be impacted by tissue derived extracellular vesicles and a number of other variables. Thus hematopoietic stem cell biology is now at a fascinating new beginning with great promise.

Keywords Hematopoietic stem cells · Extracellular vesicles · Stem cell purification · Cell cycle · Stem/progenitor cells

1.1 Transplant Hematopoiesis

Classical hematopoietic stem cell biology has focused on studies of marrow cells repopulating irradiated murine hosts. In this scheme rare stem cells give rise to lymphoid, myeloid and erythroid lineages with a progressive loss of proliferative and lineage potential associated with differentiation [1–3]. The long-term repopulating stem cell has been characterized as non-cycling or dormant and has been separated based upon elimination of lineage positive cells with selection for

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remaining cells expressing various stem cell markers including c-Kit, Sca-1 and CD150 on their cell surface. This has formed the base for a large number of studies on the genetic make-up and regulation of stem cells and has led to the well-established hierarchical model in which rare predominantly dormant stem cells give rise to increasingly lineage restricted progeny in a relatively linear fashion until the production of end-stage differentiated progeny [4].

1.2 The Continuum Model

That this model may not be correct was suggested by early work from Ogawa and colleagues [5] on differentiation through one cell cycle transit. They showed that selected stem/progenitor cells on a single cell basis could give rise to totally different lineages in vitro; erythroid and myeloid being one example. This was not consistent with a simple one directional hierarchical differentiation model. Work over the past 10–11 years has suggested a different continuum model of stem cell hematopoiesis [6].

Studies of different stem/progenitor cell populations, lineage negative/rhodamine low/Hoechst low or lineage negative/Sca-1+ cells, stimulated to transit cell cycle by different cytokine combinations; interleukin 3 (IL-3), IL-6, IL-11 and steel factor or steel factor, thrombopoietin and FLT3-ligand, revealed reversibly changing phenotypes with cell cycle passage. Long-term multi-lineage engraftment, homing to marrow, differentiation into megakaryocytes and granulocytes, global gene expression, modulation by extracellular vesicles, shifts from stem cell to progenitor phenotype, and epitope expression all showed reversible changes with induced cycle transit. Work by Passegué et al. [7] on purified lineage negative/c-Kit+/Sca-1+/Thy1.1^{int}/Flk2- stem cells further separated into G0, G1 and S/G2/M fractions by exposure to Pyronin and Hoechst indicated that all long-term engraftment was found in the G0 fraction of their purified stem cells. This suggested that our work might represent an in vitro artifact. Accordingly, we carried out the similar stem cell separations and essentially confirmed their results on long-term engraftment. However, we noted that no one had carried these types of experiments out on unseparated murine marrow cells. We analyzed unseparated murine marrow cells separated by Pyronin and Hoechst into G0, G1 and S/G2/M fractions. We found that over 50% of the cells giving long-term engraftment were in S/G2/M**.** This was an instantaneous view of cycle status. We further confirmed these data using in vitro tritiated thymidine suicide, which selectively killed cells in S-phase over a 30 min incubation. In these experiments we found that 70–85% of long-term engrafting stem cells had passed through S phase during the incubation. In order to study the flux of lineage negative c-Kit+Sca-1+Flk2- cells through cycle we employed in vivo bromodeoxyuridine (BrdU) labeling over 48 h and isolated these cells for analysis of BrdU labeling. We found that up to 85% of the "quiescent" stem cells were labeled by 48 h [8]. In these studies, we ruled out the possibility that BrdU was triggering cells to enter cell cycle. These data indicated that long-term multi-lineage

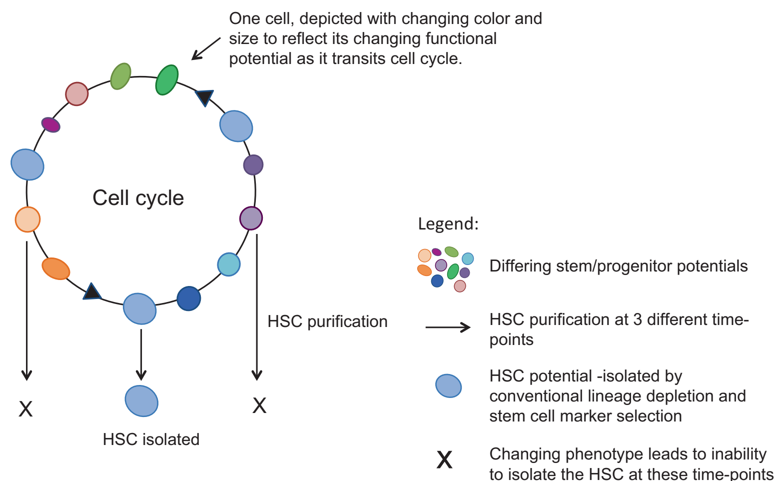

Fig. 1.1 This presents a picture of continual modification of the stem/progenitor phenotypes with passage through cell cycle

repopulating marrow stem cells were actively cycling and we postulate they are preferentially discarded with the purification. We have demonstrated this directly showing that a significant number of stem cells are actually in the lineage positive populations. These were not detected in the standard engraftment assays due to dilution by the large number of differentiated marrow cells present. Thus we have to reconsider stem cell biology in light of these results, which in fact, seem to call for a reevaluation of transplant stem cell biology. The continuum model, in which actively cycling stem cells are changing phenotype and functional potential as they transit cell cycle, is presented in Fig. 1.1.

1.3 Baseline Hematopoiesis; Short Term Clonal Mechanisms

In 1967, Patt [9] put forward the “myelocyte sink” theory, whereby myelocytes were responsible for the day-to-day production of granulocyte elements with stem cells being an emergency backup. This work attracted little attention over the years, but is quite consistent with more recent studies indicating that baseline hematopoiesis may be maintained by multiple relatively short-lived clones. These studies have involved irradiation marking [10, 11] and in vivo lentiviral tagging [12]. In addition, Sun et al. [13] have established an experimental model using bar coding where cells can be genetically labelled in situ to study native baseline hematopoiesis. In this model, classical long-term hematopoietic stem cells (LT-HSCs) appear to have limited contribution to blood production during most of adulthood. Rather it indicates the successive recruitment of thousands of lineage restricted and multipotent clones which account for steady-state hematopoiesis over at least 1 year. It appears that a

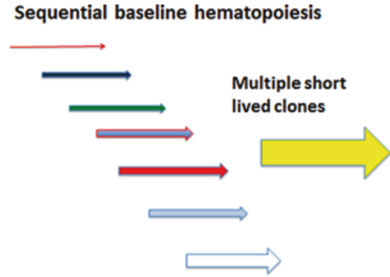

Fig. 1.2 Baseline clonal model of hematopoiesis. Each arrow represents a short lived clone with specific differentiation characteristics

large number of progenitors are specified in early post-natal life. This model is presented in Fig. 1.2.

Thus it would appear that we have two modes of stem cell biology; the transplant mode and the baseline mode. The latter is quite consistent with the continuum model described above.

1.4 Universally Modulated Hematopoiesis; Extracellular Vesicles

There is even more complexity to the hematopoietic regulatory systems. This relates to the phenotype lability of cells related to extracellular modulation of cell phenotype. Extracellular vesicles were initially felt to represent cell junk, largely from platelets and erythrocytes [14]. However, recently work has focused on the capacity of vesicle populations to alter the function and phenotype of various target cells. Our earlier work indicated that in the model systems under study when vesicles from pulmonary tissue were evaluated for their functional effects on murine marrow cells, the following were observed: (1) functional effects were enhanced with originator tissue injury (radiation), (2) entry into target cells was necessary for genomic changes to occur, (3) RNase treatment of vesicles had variable effects on outcomes, (4) vesicles were replete with protein, mRNA, non-coding RNAs including miRNA, DNA, and lipids, (5) vesicle-treated marrow cells showed marked elevations and depressions of both proteins and miRNAs, (6) vesicle entry and biologic effect varied with cell cycle status of target cells and injury status of the originator lung cells and (7) immediate genomic changes were mediated by transfer of originator mRNA and transcriptional activators to target marrow cells. Longer term changes were due to transcriptional modulation, a stable persistent epigenetic change in the target cells. Vesicles were noted to have specific effects on different marrow cell populations [15–17].

A number of specific effects on normal or injured tissues have been observed. We have shown that mesenchymal-derived stem cell vesicles could reverse monocrotaline-induced pulmonary hypertension in a mouse model while vesicles from monocrotaline injured mice could induce pulmonary hypertension [18, 19].

This suggested the "bad" versus "good" vesicle concept. If the vesicles derive from injured or diseased tissue they may make the situation worse, while if they evolve from normal or mesenchymal stem cells they act to repair or heal tissues. Similar results were found with vesicle effects on colorectal and prostate cancer cell lines; vesicles from mesenchymal stem cells reversed the malignant phenotype while vesicles from cancer cells increased the malignant phenotype [20, 21]. Recent studies showed that mesenchymal stem cell-derived vesicles could partially or completely reverse radiation damage to normal murine marrow stem/progenitor cells in vitro and in vivo [22]. These vesicles also stimulated normal marrow cell proliferation. Vesicle effects on marrow cells also varied with the cell cycle status of the marrow cells [23]. Lung vesicle entry into marrow granulocytes, erythrocytes, B cells and Lineage negative Sca-1+ progenitors varied between 15% and 29% [24]. Thus vesicle entry into and modulation of hematopoietic cell phenotypes adds another layer of complexity to their fate determination.

1.5 A Beautifully Complex and Variably Responsive System

Hematopoiesis has been felt to be the best-defined progenitor/stem cell system; a model for other stem cell systems. This does not appear to be the case. First the transplant stem cell models have in large part recently been studying only highly purified hematopoietic stem cells, disregarding the cycling stem cell population, and therefore not fully representative of the total hematopoietic stem cell population within marrow. The continuum model incorporates the cycling cells and indicates a continually changing population of cells, which makes sense when dealing with a critical life-and-death cell population. A hierarchy here makes no sense. It would be a setup for a catastrophic injury which could collapse the whole system.

The studies indicating that the baseline system is in fact determined by many short lived clones, positions the transplant system as a critical backup, which would act at times of marrow injury. However, the cycling nature of this population suggests that in fact it may be continually feeding the short-lived clones. Clearly more work is indicated to ascertain the relative role of each system in day-to-day hematopoiesis. The emergence of extracellular vesicles as a general modulator of cellular phenotypes adds an intriguing layer of complexity. It also provides what might be considered a moveable microenvironment capable of in vivo transplantation (Fig. 1.3).

Work from Liang et al. [25] indicates that stromal transplantation may now be a fertile field of investigation. Other variables impacting upon the hematopoietic system include considerations of cell density and the myriad of interactions which must be occurring in the densely packed bone marrow, circadian rhythms [26], aging phenomena, sex differences and neural regulation of marrow cells and niches. Hematopoietic stem cell niche biology has also been highlighted recently, but given the above, in particular the fact that most recent niche studies have been carried out using only purified stem cell populations and the possible role of extracellular vesicles as movable niche surrogates, much work remains in this area of investigation.

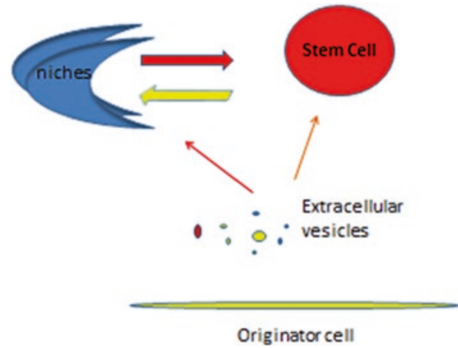

Fig. 1.3 Vesicle modulation of stem cells and niches

These considerations indicate that much progress has been made in the hematopoietic stem cell field and that with evolving insights, there is the promise of a much deeper appreciation of this fascinating biology.

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Chapter 2
The Emerging Roles of microRNAs in Stem Cell Aging

Catharine Dietrich, Manish Singh, Nishant Kumar, and Shree Ram Singh

Abstract Aging is the continuous loss of tissue and organ function over time. MicroRNAs (miRNAs) are thought to play a vital role in this process. miRNAs are endogenous small noncoding RNAs that control the expression of target mRNA. They are involved in many biological processes such as developmental timing, differentiation, cell death, stem cell proliferation and differentiation, immune response, aging and cancer. Accumulating studies in recent years suggest that miRNAs play crucial roles in stem cell division and differentiation. In the present chapter, we present a brief overview of these studies and discuss their contributions toward our understanding of the importance of miRNAs in normal and aged stem cell function in various model systems.

Keywords microRNAs · Stem cells · Cellular senescence · Aging

2.1 Introduction

Aging is linked with a gradual deterioration of tissues and organs that result in various age-related diseases. Accumulative evidence in recent years suggests that miRNAs are important regulators of cellular senescence and aging [1–3]. miRNAs are small, single stranded, non-coding RNAs (22–26 nucleotides) that play a key role in gene expression post-transcriptionally [4–7]. They bind to the 3′-UTR

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(untranslated region) of the target mRNAs and repress protein production by destabilizing the mRNA and silencing transcription. miRNAs' biogenesis consists of several key steps including processing by Drosha, DGCR8/Pasha, Exportin5, Dicer, RISC proteins, and P-bodies [8–12].

miRNAs work in a complex network in which each miRNA controls hundreds of distinct target genes, while the expression of a single coding gene can be regulated by multiple miRNAs. They are expressed in a tissue-specific and developmentally regulated way. The first miRNA gene, lin-4, and its target lin-14 were identified in a screening for genes that regulate developmental timing in *Caenorhabditis elegans* [9, 12]. Over several years and by employing molecular cloning and bioinformatic prediction strategies, hundreds of miRNAs have been identified in worms, *Drosophila*, mammals and plants. The human genome encodes over 1000 miRNAs and it is estimated that miRNAs target around 60% of human protein-encoding genes.

miRNAs are important mediators of embryonic development, neurogenesis, hematopoiesis, immune response, skeletal and cardiac muscle development, stress, metabolism, signal transduction, cellular differentiation, proliferation, apoptosis, stem cell fate, reprogramming, senescence and aging. Dysregulation of miRNAs pathway results in developmental defects, several human diseases, aging and cancer [13–25]. In addition, alterations in miRNAs have been shown in animal models and in humans with senescence or increasing age. This review is primarily focused on the involvement of miRNAs in the aging process of stem cells.

2.2 miRNAs in Stem Cell Division and Differentiation

Stem cells play a crucial role in tissue development and homeostasis. They are immature cells and have tremendous capacity for self-renewal and differentiation to form specialized cell types. Stem cells divide both symmetrically and asymmetrically. Asymmetric division of stem cells results in the formation of two daughter cells; one retains the stem cell characteristics and other one differentiates into specialized cell types (reviewed in [26, 27]).

Stem cells' self-renewal divisions are controlled by both intrinsic and extrinsic factors. Failure to maintain balance between self-renewal and differentiation of stem cells result in degenerative diseases (aging), while over-proliferation of stem cells results in tumor formation and cancer (reviewed in [27], Fig. 2.1). Accumulative studies suggest that stem cells can be used in regenerative medicine and cancer eradication (reviewed in [27]).

In recent years, miRNAs and their role in self-renewal and differentiation of stem cells in a variety of model systems have been adequately emphasized [4, 28–32]. miRNAs also function as a regulator of stem cell division. miRNAs can induce cellular differentiation by inhibiting cell cycle transition or epithelial to mesenchymal transition (EMT), and inhibiting "stemness" factors such as genetic (Sox2, Oct, and Nanog) or epigenetic (Bmi-1) [33–36].

Several miRNAs have very low level expression in stem cells, which increases upon differentiation [37]. Some miRNA can antagonize the effects of differentiation

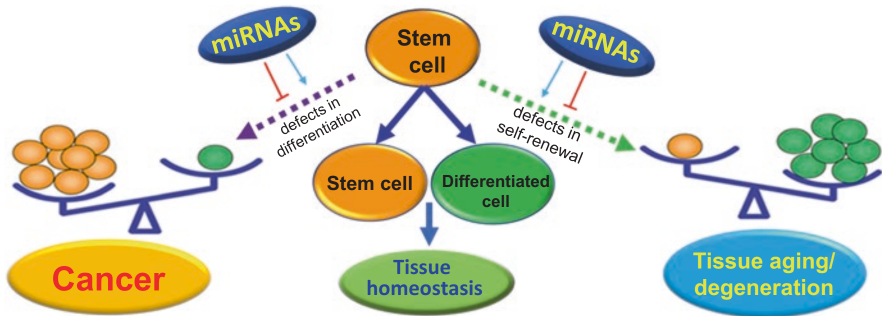

Fig. 2.1 Schematic diagram showing how disbalance between self-renewal and differentiation of stem cells result in aging and cancer and how miRNAs regulate this process

related miRNAs [38]. There are several miRNAs that express in different stem cells, such as mammary gland progenitor cells (miR-205, [39]), skin stem cell (miR-125b, [40]; miR-203, [41]), neuronal stem cell (miR-9, [42]; miR-124, [43]; miR-184, [44]; miR-371-3, [45]; miR-6b, miR-93, and miR-25, [46]), muscle satellite stem cells (miR-1 and miR-206, [47]), hematopoietic stem cells (miR-181, miR-223 and miR-142, [48]; miR-150, [49]; miR-125a, [50]), cardiomyocyte progenitor and stem cells (miR-499, miR-1, miR-10a, miR-6086, miR-6087, miR-199b and miR-495, [51–56]), osteogenic and chondrogenic differentiation of stem cells (miR-138, [57]; miRR-23b, [58]; miR335-5p, [59]) and play an important role in balancing their self-renewal and differentiation process.

2.3 miRNAs in Stem Cell Aging

Stem cells play an important role in replacing aged or damaged cells in the tissues and organs of organisms. As we age, the regenerative capacity of stem cells progressively declines, which results in tissue or organ dysfunction. In recent years, several miRNAs have been identified to play crucial role in defining the regenerative capacity of stem cells during aging (reviewed in [17, 18]). miRNAs that regulate the stem cell self-renewal and differentiation process are therefore important in the aging process (Fig. 2.1).

In the following section, we will explore these miRNAs in age associated changes to stem cell function in various model systems, including human.

2.3.1 C. elegans

C. elegans has been used as a powerful model system for investigating stem cell self-renewal, maintenance of pluripotency and reprogramming of differentiation [60, 61]. The first miRNA *lin-4*, and its target *lin-14* were identified in *C. elegans*

[9, 12]. Several miRNAs have been identified that regulate stem cell maintenance, proliferation and aging of germline and seam cells in *C. elegans* ([62–68], Table 2.1) as well. In *C. elegans*, life-span is regulated by signaling between the germline and the soma. miRNAs such as *lin-4* and its target *lin-14* has been shown to regulate aging in *C. elegans*. It has been demonstrated that mutation in *lin-4* resulted in a shortening of lifespan; on the other hand, mutation in its target gene, *lin-14* resulted in lifespan extension, which is mediated by its effector- DAF-16 [94]. Shen et al. [67] have demonstrated that removing germline stem cells (GSCs) from *miR-84;miR-241* gonads resulted in shortening of lifespan and upregulation of DAF-12 signaling. Further, they found that DAF-12 target miRNAs such as *miR-84; miR-241* are required for gonadal longevity through DAF-16 [67]. A study by Boulias et al. [62] shown that *miR-71* acts in neurons and is responsible for lifespan extension in GSC mutants by regulating DAF-16/FOXO. Recently, Wang et al. [68] reported that knockdown of *lin-28* extends lifespans and promotes the meiotic entry of GSCs. They further showed that *lin-28* is required for proper establishment of the GSC pool and acts in the germline to regulate GSC number because the mutant of *lin-28* shows smaller pool of GSC in young adult worms [68]. In addition, they reported that lin-28 exerts its effects on GSC number and lifespan though *let-7* and AKT-1/2 and requires DAF-16 to influence GSC number and longevity [68]. In addition to germline system, other studies have shown that some miRNAs regulate neuronal regeneration and seam stem cell function in older worms [63, 95]. Zou et al. [95] also reported that in older anterior ventral microtubule (AVM) axons, *let-7* inhibits their regeneration by downregulating *lin-41*. In the seam stem cells, miRNAs such as let-7 and lin-4 promote differentiation by inhibiting their self-renewal [63].

2.3.2 Drosophila

Drosophila have proven to be a best genetic model system for investigating aging related changes in stem cell function [69, 96, 97]. Several miRNAs have been identified that regulate self-renewal and differentiation and aging of germline and somatic stem cells in *Drosophila*. Recent studies demonstrated that miRNA pathways play an important role in the GSCs of *Drosophila* gonads [28, 69, 98–106]. Hatfield et al. [99] demonstrated that loss of function of *dicer*-1 results degeneration of developing egg chambers due to deficiency in germline cyst production. Toledano et al. [69] have shown that let-7 controls aging of *Drosophila* testis GSC and mediates age dependent decrease in the IGF-II messenger RNA binding protein (Imp), which in turn results in age-dependent decline of GSCs ([69], Table 2.1). Chen et al. [51] have reported that *lin-28* is required for adult intestinal stem cells (ISCs) expansion. They found persistent reduction of total numbers of ISCs in *lin-28* mutants with age. In miR-275 mutants, it has been shown that with age the proportion of ISC increases at the expense of more mature differentiated cells, which results in gut dysplasia and shorten life span ([70, 107], Table 2.1).

Table 2.1 miRNAs involved in stem cell aging and senescence

Stem cell type	miRNAs	Roles in	References
C. elegans GSCs	miR-84, miR-241, miR-71, LIN-28, let-7	Aging	[62, 67, 68]
Drosophila testis GSC	let-7	Aging	[69]
Drosophila ISC	Lin-28, miR-275-305	Aging	[51, 70]
Mouse NSC	let-7b	Aging	[20]
Human BM-MSC	let-7f, miR-29c, miR-369-5p, miR-371, miR-499	Senescence	[71]
Human BM-MSC	miR-17, miR-19a, miR-19b, miR-20a, miR-519d	Aging	[72]
Mouse/human BM-MSC	miR-543, miR-590-3p	Aging	[73]
Human BM-MSC	miR-335	Senescence/aging	[74]
Human BM-MSC	miR-29c-3p	Senescence	[75]
Human BM-MSC	miR-199b-5p	Aging	[76]
Mouse BM-MSC	miR-183-5p	Senescence	[14]
Mouse BM-MSC	miR-17	Aging	[77]
Human BM-MSC	miR-140, miR-146a/b, miR-195	Senescence	[78]
Rhesus monkey BM-MSC	let-7f, miR-23a, miR-125b, miR-199-3p, miR-222, miR-558, miR-766	Aging	[79]
Human UC-MSC	let-7a1, let-7d, let-7f1, miR-23a, miR-26a, miR-30a	Senescence	[80]
Human UC-MSC	miR-200c, miR-214	Senescence	[81]
Human UC-MSC	miR-141-3p	Aging	[25]
Mouse BM-HSC	miR-146a	Aging	[82]
Mouse BM-HSC	miR-125b	Aging	[24]
Mouse BM-HSC	miR-132, miR-212	Aging	[83]
Human ASC and BM-MSC	miR-122, miR-510, miR-452, miR-335, miR-935, miR-142-3p, miR-483-3p, miR-203, miR-153, miR-1277, miR-141	Aging	[84]
Human ASC	miR-27b, miR-106a, miR-199a, let-7	Aging	[85]
Rat ASC	miR-143, miR-204	Aging	[15]
Human ADSC	miR-17hg, miR-100hg	Senescence	[86]
Human SC (satellite)	let-7b, let-7e	Aging	[87]
Mouse and human satellite and myoblast cells	miR-143-3p	Aging	[88]
Porcine muscle stem cell	miR-1, miR-206, miR-24	Aging	[89]
Tendon stem/progenitor cell	miR-135a, miR-140-5p	Senescence	[90, 91]
Mouse cardiac progenitor cells	miR-675	Senescence	[92]
Human DPSC	miR-152	Senescence	[93]

2.3.3 *Mammalian System*

Several studies reported the important roles of miRNAs in self-renewal, pluripotency, proliferation, differentiation, senescence and aging of stem cells in various tissues and organs. We will be discussing studies involving miRNAs and aging on different stem cell system in the following subsections (Table 2.1).

2.3.3.1 Neural Stem Cells

Nishino et al. [20] have shown that loss of self-renewal potential in old neural stem cells is associated with age-dependent upregulation of let-7b that ultimately downregulates the expression of HMGA2, a repressor of the INK4a/ARF locus, which results in up-regulation of p16 and p19, which then results in the decline proliferation and self-renewal of neural stem cells (NSCs) [20].

2.3.3.2 Mesenchymal Stem Cells

Mesenchymal stem cells (MSC) are multipotent stem cells that can differentiate to form various specialized cell types. MSCs are isolated from several tissues including bone marrow (BM), umbilical cord blood (UCB), adipose tissues and muscle tissues. The regenerative capacity of MSCs provide great potential for regenerative medicine. Understanding the culture and differentiation of MSCs during the aging and senescence process has vital implications in clinics [18, 108]. Several miRNAs identified regulate age-associated alterations in MSCs [14, 15, 71–73, 75, 85, 86]. Wagner et al. [71] have addressed the impact of replicative senescence on human MSC cultures. They found upregulation of miR-371, miR-369-5p, miR-29c, miR-499 and let-7f is because of the passage effect, not because of replicative senescence. Upregulation of these miRNAs reduces the proliferative potential of MSCs, which results in loss of adipogenic differentiation potential [71]. Hackl et al. [72] have selected four replicative cell aging models (endothelial cells, renal proximal tubule epithelial cells, skin fibroblast cells, and $CD8^+$ T cells) and three organismal aging models (foreskin, MSCs, and $CD8^+$T cells form young and old donors). Hackl et al. [72] found that miR-17 was downregulated in all seven models, whereas miR-19b and miR-20a were downregulated in six models, and miR-106a was downregulated in five models. These results of this study identify miRNAs as novel markers of cell aging in humans [72].

To understand the cellular aging of human MSCs, Lee et al. [73] shown that AIMP3 (aminoacyl-tRNA synthetase-interacting multifunctional protein-3)/p18 regulates cellular aging in MSCs through miR-543 and miR-590-3p. Tomé et al. [74] have demonstrated that both aging and continuous propagation of MSCs induce a gradual increase in miR-335 expression, which is in turn associated with cell senescence alterations and results in loss of their therapeutic capacity, this is mediated by inhibition of activator protein 1 (AP-1) activity. Further, miR-29c-3p has

been identified to promotes the senescence of MSCs by targeting CNOT6 through p53-p21 and p16-pRB pathways [75]. They further found that both the p53-p21 and p16-pRB pathways were enhanced during the miR-29c-3p-induced senescence of MSCs. Peffers et al. [76] found the age-related increase of miR-199b-5p expression in MSCs, which results in age-related deterioration of MSC function through regulating SIRT1, TGFα and PODXL. Recently, Davis et al. [14] reported that aging and oxidative stress can dramatically increase the miR-183-5p cargo of extracellular vesicles in the bone marrow, which results in reduction in cell proliferation, osteogenic differentiation and the increased senescence of BM-MSCs mediated by reduction of heme oxygenase-1 (Hmox1) activity.

The senescence-associated secretory phenotype (SASP) has been found to be a novel mechanism that associates cellular senescence to tissue dysfunction. There is limited information are available to show the age-dependent alterations in the secretory behavior of stem cells. Hisamatsu et al. [77] identified growth differentiation factor 6 (Gdf6) as a regenerative factor secreted from young MSC, their expression was controlled by the miR-17, whose expression was downregulated with age. In addition, they found that miR-17 overexpression restores the differentiation potential of old MSCs, and the upregulation of Gdf6 ameliorates geriatric pathologies. Okada et al. [78] investigated the role of miRNAs in stem cell aging and their roles in cardiac repair. They reported that miR-195 upregulated in old MSCs induces stem cell senescence, resulting in a declining of their regenerative potential by deactivating telomerase reverse transcriptase (tert), and how downregulation of miR-195 can restore MSC aging, which suggests that rejuvenation of old MSCs by miR-195 inhibition could be used as a potential autologous strategy for cardiac repair in older patients [78]. Yu et al. [79] investigated the effect of aging on the properties of Rhesus Monkey bone marrow-MSC (rBMSC) and found decrease in proliferation and differentiation capacity of MSC with age. Their miRNA expression profiles identified an upregulation of miR-766 and miR-558 and downregulation of miR-let-7f, miR-125b, miR-222, miR-199-3p, miR-23a, and miR-221 in old MSCs compare to young MSCs.

In context of cellular senescence, which involves a decline in stem cell self-renewal and epigenetic regulation of gene expression, Lee et al. [80] demonstrated that the cellular senescence of human umbilical cord-derived MSCs (UCB-MSCs) caused by a decrease in histone deacetylases (HDACs) result in downregulation of high mobility group A2 (HMGA2) and increased expression of p16, p21 and p27. Further, they found that miR-23a, miR-26a and miR-30a inhibit HMGA2 to elevate cellular senescence in UCB-MSCs [80]. So et al. [81] further shown that DNMTs regulate cellular senescence of UCB-MSC by controlling the expression of p16 and p21. In addition, they found that the expression of miR-220c and miR-214 were upregulated in senescent UCB-MSCs. It has been reported that prelamin A accumulated in MSCs during cellular senescence, however the molecular mechanisms responsible for prelamin A accumulation in hMSCs was not known. Yu et al. [25] reported that ZMPSTE24, which is associated in the post-translational maturation of lamin A, is mainly responsible for the prelamin A accumulation, which results in cellular senescence in hMSCs. Their results provide a novel mechanism regulating

MSC aging, which has broad therapeutic implication in reducing age-associated MSC pool exhaustion [25].

2.3.3.3 Hematopoietic Stem Cells

Hematopoietic stem cells (HSCs) have enormous self-renewing and differentiation capacity; they can form all types of blood cells including immune cells. Several miRNAs are reported to regulate HSC numbers during stress, aging and contribute to age-related disorders such as acute myeloid leukemia (AML). In this context, Zhao et al. [82] reported that miR-146a regulates HSC numbers during chronic inflammatory stress such as miR-146a-deficiency. This deficiency results in progressive decline in the quality of long-term HSCs from young mice compared to wild type mice. This study has identified miR-146a to be a crucial regulator of HSC in mice during chronic inflammation [82]. Yalcin et al. [24] characterized the expression profiles of HSCs from young and old mice and mice treated with anti-aging interventions (such as calorie restriction and rapamycin) and found miR-125b as a critical regulator of HSC aging and that anti-aging interventions can employ their positive effects on HSC potential by regulating miR-125b expression [24]. Further, Mehta et al. [83] found that the miRNAs' 212/132 cluster is elevated in HSCs and upregulated during aging. This cluster also regulates HSCs self-renewal and survival during aging by targeting the transcription factor FOXO3. To understand the effect of biologic age-induced miRNA changes on MSCs, Pandey et al. [84] investigated miRNA profiles of MSCs derived from adipose tissue (ASCs) and bone marrow (BMSCs) from young and old human donors using an unbiased genome-wide approach. Their analysis showed significant differences in 45 miRNAs in BMSCs and 14 in ASCs. In addition, many miRNAs were downregulated in both ASCs and BMSCs in specimens from older donors as compared to younger donors. Their finding on miRNA profiling suggest that miRNAs play an important role MSC aging and ability to block inflammation and enhance cellular repair [84].

2.3.3.4 Muscle Stem Cells

Aging causes loss of skeletal muscle (sarcopenia), which results in falls and fractures. miRNAs are potential regulators of skeletal muscle mass and function. Studies in rodents and humans have also shown that aging reduces the satellite stem cell pool and their ability to proliferate and differentiate in humans [109, 110]. Drummond et al. [87] performed miRNA analysis on skeletal muscle biopsies of 36 young and older adults, using a miRNA array and confirmed that the expression of Let-7b and Let-7e was dramatically increased in older compared to younger subjects. In addition, they demonstrated that increased Let-7 expression is linked with a low number of satellite cells in older humans, where they found lower expression of PAX7 mRNA. These results suggest that low number of satellite cells can affect renewal and regeneration of muscle cells [87]. Redshaw et al. [89] measured the expression

of miR-1, miR-24 and miR-206 in the muscle stem cells that were isolated from two muscles: the diaphragm (DIA) and the semimembranosus (SM), from young and old pigs. They found that all three miRNAs are enriched in skeletal muscles. In addition, they showed older animals show low expression of miR-1 and miR-206, except for whereas, miR-24, which show higher expression [89]. Using satellite cells and primary myoblasts from mice and humans and an in vitro regeneration model, Soriano-Arroquia et al. [88] have shown that disrupted expression of miR-143-3p and its target gene, Igfbp5, plays crucial part in muscle regeneration in vitro because their expression is disrupted in satellite cells from older mice. In addition, they found miR-143 as a regulator of the insulin growth factor-binding protein 5 (Igfbp5) in primary myoblasts. Their findings suggest that dysregulation of miR-143-3p:Igfbp5 interactions in satellite cells with age could diminish the satellite cells' function [88]. Lee et al. [111] analyzed the miRNA expression profiles of myoblasts isolated from young and old mouse skeletal muscles and identified miR-431 as a novel age-associated miRNA which regulates SMAD4 expression and promotes differentiation and regeneration of old skeletal muscle. The low reprogramming efficiency in cells of older patients is a major challenge, in this context, Kondo et al. [112] demonstrated that blocking miR-195 expression could be helpful in reprogramming efficiency in old skeletal myoblasts.

2.3.3.5 Cardiac Progenitor Cells

Aging is the primary risk factor for cardiovascular diseases. It affects cardia progenitor/stem cells and suppresses their regenerative ability. miRNAs have emerged as important regulators of cardiovascular function and there are few miRNAs play crucial roles in cardiac aging [113, 114]. C-kit(+) cardiac progenitor cells (CPCs) have appeared as a good tool for the treatment of heart diseases [115]. However, the senescence of CPCs decrease their regenerative potential. Cai et al. [92] shown that melatonin antagonized premature senescence of CPCs via the H19/miR-675/USP10 pathway, which gives a novel mechanism by which melatonin inhibits CPCs senescence by promoting miR-675. Endothelial progenitor cells (EPCs) are known to contribute to the regeneration of endothelium. However, aging results in EPCs senescence, which leads to increased cardiac risk, reduced angiogenic capacity, and loss of cardiac repair function. Zhu et al. [116] provide the mechanism by which this EPCs senescence in aged mice. They found that miR-10A* and miR-21 control EPC senescence via suppression of Hmga2 expression, which suggests that modulating these two miRNAs could be a novel therapeutic intervention in ameliorating EPC-mediated angiogenesis and vascular repair.

2.3.3.6 Tendon Stem/Progenitor Cells

Aging of tendon stem/progenitor cells (TSPCs) may result in tissue degeneration and subsequent injury. Several studies have demonstrated that aging can affect the proliferation and differentiation capacity of TSPCs [117, 118], but the molecular

mechanism that regulates this process is still not clear. Recently, Chen et al. [90] investigated whether miRNAs modulate senescence of TSPCs. They found that miR-135a regulates senescence of TSPCs by targeting Rho-associated coiled-coil protein kinase 1 (ROCK1). miR-135a was dramatically downregulated in aged compared with young TSPCs. In addition, they reported that overexpression of miR-135a in young TSPCs inhibits senescence and restores their proliferation and differentiation capacity, while loss of miR-135a in aged TSPCs results in senescence of TSPCs. These studies suggest that miR-135a regulates TSPC senescence by repressing ROCK1 [90]. PIN1, a peptidyl-prolyl cis/trans isomerase, has been shown in age-related bone homeostasis and adipogenesis. Chen et al. [91] investigated the role of Pin1 in the aging of human TSPCs. They found a dramatic decrease in Pin1 expression during prolonged in vitro cultures of human TSPCs. Their loss-of-function and gain-of-function, studies show that overexpression of Pin1 delayed the progression of cellular senescence, while downregulation of Pin1 promoted senescence in TSPCs. In addition, they demonstrated that miR-140-5p regulates Pin1 expression at the translational level, which suggests miR-140-5p affects TSPC aging by targeting Pin1 [91].

2.3.3.7 Dental Pulp Stem Cells

Dental pulp stem cells (DPSCs) have emerged as a viable cell source for regenerative medicine in recent years [93, 119, 120]. Several miRNAs are known to control human DPSCs proliferation and differentiation [121, 122]. Recently, Gu et al. [93] studied the human DPSCs senescence and have shown that miR-152 is upregulated during HDPSC senescence. Further, they found that Sirtuin 7 (SIRT7), a target of miR-152, is downregulated in senescent HDPSCs; blocking miR-152 enhanced SIRT7, and blocking HDPSC senescence. In addition, overexpression of SIRT7 restored miR-152-induced senescence. Their results suggest that the miR-152/SIRT7 axis are crucial in the regulation of HDPSC senescence [93].

2.4 Conclusion

miRNAs are the novel regulatory molecules in various biological processes. The discovery of miRNAs has opened a new avenue in aging research, which will help researchers to obtain an extensive understanding of the molecular mechanism underlying this complex process. Altered expression of miRNAs resulted in developmental defects, loss of tissue homeostasis, cellular senescence, aging and cancer in various model organisms including humans. miRNAs play important roles in the stem cell self-renewal and differentiation processes, and regulate stem cell aging through multiple targets. Thus, miRNAs provide novel therapeutic options for the senescence and aging of stem cells in humans.

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Part II
Stem Cells and miRNAs- Therapeutic Challenges in Age-related Diseases

Brooks RW and Robbins PD discuss the method by which somatic stem cells could be used to treat age-related diseases such as those with severe and debilitating chronic illnesses such as cancer, diabetes, osteoarthritis, osteoporosis, neurodegenerative and cardiovascular disease. The authors proposed that the decline in stem cells could be key to the dysfunction of tissues and organs. They proposed that the loss of stem cells in the aging individuals results in the inability to of cells to restore damaged tissues. Thus, the chapter discusses the prospect that increased understanding of the aging adult stem cells may lead to new strategies in the development of novel therapeutics to prevent and reverse age-dependent stem cell decline. This and other chapters emphasize the importance of identifying new approaches to the multitude of clinical problems afflicting the vast aging population.

Nandi SS and Mishra PK discuss specific diseases that could be treated by target miRNA. They discussed diabetes mellitus since this condition is linked to multiorgan dysfunction such as cardiomyopathy, which is the leading cause of morbidity and mortality. The chapter discusses the authors' identification of miRNAs that are linked to the diabetic heart and the contribution to cardiomyopathy. The chapter expands on the cardioprotective roles of different miRNAs including those within exosomes. Overall, this chapter argues for miRNA as a potential therapeutic target for juvenile and adult diabetic cardiomyopathy.

Sugaya K and Vaidya M add neurodegenerative diseases among those that could be treated with stem cell. The authors proposed that embryonic stem cells and neural stem cells could be more efficient in the generation of neural cells. The authors cautioned the ethical and practical issues of using these sources of stem cells. Additional scientific issues include immune rejection since embryonic and neural stem cells will not be from autologous source. Other concern with embryonic stem cells include potential tumor formation. To overcome the limited developmental potential of adult stem cells, the authors discussed their technologies to increase the potency of somatic mesenchymal stem cells. Ectopic expression of the stem cell gene, Nanog, can increase the efficacy of the mesenchymal stem cells to generate neural cells. Other solutions to overcome the scientific and ethical issues of using embryonic and fetal stem cells include the identification of a small molecule to

increase the number of endogenous neural stem cells. The novel methods described in this chapter have implications for treatment of neurodegenerative therapies such as Alzheimer's disease. Since the pathology linked to Alzheimer's disease prevent neurogenesis from NSCs, the authors introduced the application of exosome as a tool to improve the modification of adult stem cells.

Chapter 3
Treating Age-Related Diseases with Somatic Stem Cells

Robert W. Brooks and Paul D. Robbins

Abstract Life expectancy in the developed world has advanced beyond the number of years in which healthy tissue homeostasis can be maintained, and as a result, the number of persons with severe and debilitating chronic illnesses, including cancer, diabetes, osteoarthritis, osteoporosis, neurodegenerative and cardiovascular disease has continued to rise. One of the key underlying causes for the loss in the ability to replenish damaged tissues is the qualitative and quantitative decline in somatic stem cell populations. A concerted effort to understand why aging adult stem cells fail to maintain "stem" potential while simultaneously developing new strategies and therapeutic interventions to prevent or reverse age-dependent stem cell decline is required to improve the overall healthspan of our rapidly aging population. This review focuses on what drives stem cell dysfunction with age, the contribution of stem cell dysfunction in driving aging and therapeutic approaches using stem cells to treat aging.

Keywords Stem cells · Senescence · Aging · Senolytics · Mesenchymal Stem Cells

3.1 Introduction

3.1.1 Aging

In 2014, according to the U.S. Department of Health and Human Services, the number of persons 65 years of age or greater in the US was calculated at 46.2 million. The survey revealed that more than 10,000 people/day turn 65 and estimates that the elderly population will nearly double by 2060. The troubling significance of these statistics is that more than 90% of individuals ≥65 years of age will be burdened with at least one chronic disease and up to 70% will be diagnosed with two or more

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[1]. These astonishing figures position age-related chronic illness as the most significant health care crisis of the twenty-first century. A concentrated effort is needed to develop innovative strategies to specifically target the underlying cause of age-related disease, rather than treating each disease associated with aging individually.

This focus on understanding the relationship between aging and age-related diseases and preventing/delaying disease by targeting fundamental mechanisms of aging is termed geroscience. The concept of geroscience, targeting the root cause of age related diseases instead of treating each age-related disease separately, would provide a far greater value in alleviating the significant economical and health care burden our afflicting our aging population than the current standard of care. Adding just 2.2 years of healthy life to patients suffering from age-related chronic illness would save an estimated $8 trillion in medical costs in the US alone [2]. Thus, it is imperative that we find an approach to therapeutically target the underlying cause of aging and to utilize this knowledge to reduce the period of functional decline associated with age.

3.1.2 Stem Cells and Aging

A characteristic of aging is the loss of regenerative capacity, leading to an impaired ability to respond to stress and therefore increased morbidity and mortality. This has led to the hypothesis that aging is caused, in part, by the loss of functional adult stem cells necessary for maintaining tissue homeostasis. For example, mice greater than 2 years of age have a significant reduction in the number and proliferative capacity of stem cells, for example, neuronal, adipose and muscle derived stem cells [3–5]. There are also age-related changes in bone marrow-derived mesenchymal stem cells (BM-MSCs) including loss of proliferation and differentiation potential, increase in senescence and loss of capacity to form bone in vivo [6]. Similarly, MSCs derived from the bone marrow of patients with Hutchinson-Gilford Progeroid Syndrome, a disease of accelerated aging, are defective in their ability to differentiate [7].

Adult stem cells maintain the capacity for multi-lineage differentiation, meaning a relatively small number of cells can give rise to all cells required for tissue generation. During the steady-state, in the absence of damage or stress, stem cells remain quiescent in specialized niches and upon stimulation, exit the niche and migrate to sites of damage where they can either differentiate to replenish lost tissue or secrete factors that contribute to repair of the damaged tissue. Immediately following this process, the stem cells can migrate back to their resident niche and reenter a quiescence state. This unique ability to remain in a dormant state allows stem cells to delay the stress of replication and maintenance of tissue homeostasis while simultaneously preserving stem cell self-renewal and differentiation potential throughout the lifetime of an organism. For example, treatment of mice with a duel CDK4/CDK6 inhibitor, preventing cycling of cells, results in protection of HSCs from chemotherapeutics [8]. However, the long-lived nature of these cells leaves them vulnerable to the accumulation of stress-induced damage, which can result in loss of self-renewal, reduced differentiation efficiency, cellular senescence and even apoptosis, all of which contribute to reduced functional capacity.

The stochastic way in which stem cells age, poor characterization of nascent tissue stem cells and limited knowledge of the age-dependent relationship between tissue stem cells and their respective niches makes defining an all-encompassing adult stem cell model of aging difficult. However, recent experiments demonstrating that systemic transplantation of only 1 million functional, young muscle derived stem cells was sufficient to extend healthspan and lifespan in two different progeroid mouse models clearly document a key role for young stem cells in maintaining tissue homeostasis [9]. Also, this result suggests that loss of stem cell function with age directly contributes to the aging process. As discussed below, the functional loss in stem cell capacity with age can be attributed to a combination of both intrinsic and extrinsic factors.

3.1.3 Cellular Senescence

An increase in genetic instability brought on by unrepaired DNA damage also can result in malignant transformation. Therefore, a critical anti-tumor response to these types of stress is for the cells to block cell division through permanent cell cycle arrest, a process termed cellular senescence. During this state, cells remain metabolically active, but functionally inert. Senescence can be induced by telomere shortening, accumulation of reactive oxygen species (ROS) and the accumulation of DNA damage. In addition, senescent cells can develop a senescence-associated secretory phenotype (SASP) involving an increase in secretion of pro-inflammatory cytokines and chemokines, tissue-damaging proteases, factors that can impact stem and progenitor cell function directly or through effects on the stem cell niche and growth factors [10]. Senescent cells also can have metabolic shifts such as increased reactive oxygen species generation that affect nearby cells. Like most types of cells, stem cells can undergo senescence due to the accrual of damage with aging, result in expression of their own form of SASP. The accumulation of senescent cells, including senescent stem cells, secreting inflammatory factors and proteases, disrupt normal tissue homeostasis both locally and systemically.

Although cellular senescence and SASP are essential for wound healing, chemoattraction for immune clearance of damaged or pre-malignant cells and for embryonic development [11–13], there is an exponential increase in senescence with age. Moreover, a decrease in the senescent cell burden associated with age improves both health and lifespan [14, 15]. Interestingly, it appears as if progenitor cells in fat, muscle and other tissues are the key senescent cell types important for driving aging. Also, reduction in the senescent cell burden improves the functional of multiple types of adult stem cells, whether cleared through genetic deletion in transgenic mice or treatment with agents that specifically kill senescent cells, termed senolytics [16, 17]. These studies demonstrate that targeting senescent cells that accumulate with age is therapeutic and can significantly improve both health and lifespan.

Recently, therapeutic approaches to specifically kill senescent cells, including senescent progenitor cells, with senolytic drugs have been developed that have the

ability to extend healthspan in mouse models of aging. For example, a combination of two drugs, dasatinib and quercetin (D + Q), which target several of these pro-survival pathways, induce death specifically in senescent murine and human cells in culture as well as enhance cardiovascular function in aged mice, decrease frailty, neurologic dysfunction and bone loss in progeroid mice [18]. Furthermore, treatment with D + Q reduce the senescent cell burden and improved improves lung function in the mouse model of idiopathic pulmonary fibrosis [19]. Interestingly, the natural compounds fisetin, a quercetin-related flavonoid, and piperlongumine also have senolytic activity in certain cell types in culture [20, 21]. Similarly, several inhibitors of Bcl-2 family members like navitoclax (ABT263), A1331852 and A1155463 are senolytic in some, but not all cell types [17, 22]. Navitoclax treatment of mice not only reduced senescent cell burden, but also alleviated radiation-induced hematopoietic stem cell dysfunction [17]. In addition, a FOXO4-interacting peptide that blocks the association with p53 recently was shown also to induce apoptosis in senescent cells and improve several aspects of aging in old mice [16]. Taken together, these results demonstrate that the accumulation of senescent cells with age contributes to driving age related pathologies and progenitor cell dysfunction.

3.1.4 Mechanisms for Decline of Stem Cell Function

The consequences of a functional decline in tissue stem cell homeostasis can be driven through multiple mechanisms. For example, adult hematopoietic stem cells (HSC) are not only required to regenerate the cells lost to tissue turnover, but also the renewal of cells responsible for the clearance of stressed or damaged cells such as the cells that comprise the immune system. Therefore, a significant decline in HSC function also will result in the accumulation of damaged, senescent cells through lack of clearance by functional immune cells.

Despite adult stem cells spending a significant portion of their existence in a quiescent state and expressing low levels of telomerase, the accumulation of stress induced damage related to age can be observed in numerous adult stem cell populations [23–28]. Whether the decline in stem cell function can be attributed to the direct effect of senescence within the stem cells themselves or the indirect effect conferred by differentiated senescent cells within individual tissues is still unclear. However, there is a clear correlation with age between dysfunction of adult stem cells, cellular senescence and loss of tissue homeostasis.

3.1.5 Targeting Aging in Stem Cells

Given that the increase in stem cell dysfunction with age contributes to driving aging through loss of function as well as a gain of function of expression of detrimental factors (e.g., SASP). In theory, both aspects can be targeting therapeutically,

either alone or in combination. Drug screening efforts have identified compounds that improve self-renewal and differentiation of aged stem cells. For example, rapamycin improves the function of aged muscle derived stem cells as well as HSCs [9, 29]. Given that rapamycin extends lifespan, at least in model systems of aging, it is possible that part of its life extension effects is conferred through improvement of endogenous stem cell function. Also, treatment of MDSCs from aged mice with inhibitors of IKK/NF-κB signaling, upregulated in dysfunctional stem cells, results in improved myogenesis [30]. In addition, stem cells isolated from aged mice heterozygous for the main subunit of NF-κB, p65/RelA, have improved function [31]. These studies demonstrating the dynamic relationship between cellular senescence and age-related deterioration suggest a novel direction for therapeutic intervention to extend healthspan. As described above, senolytic drugs have been identified, able to kill senescent cells specifically. However, it is unclear whether senolytics are directly targeting the dysfunctional, senescent stem cells or improving the stem cell niche. Each individual tissue stem cell presents with a unique phenotype in the context of aging, with some types of adult stem cells appearing refractory to senescence while others have elevated levels of senescent hallmarks. These varying phenotypes lend to the possibility that stem cells with low levels of senescence will not be affected by treatment with senotherapeutics. However, it is likely that stem cells with readily observable levels of senescence will be cleared by senolytic drug treatment. Overcoming the possible adverse effects conferred by extensive depletion of dysfunctional stem cells could be overcome through transplantation of healthy young stem cells or the molecules in which they secret (see below).

Functional adult stem cells are required to maintain tissue homeostasis and this loss of function is a key factor contributing to the severe debilitating disease associated with age. Thus, stem cells have significant therapeutic potential and are currently being utilized in both mouse and human clinical trials aimed at reducing or the severity of age related diseases. A few examples of the role of stem cell populations in disease and their use as therapeutics are presented below.

3.2 Mesenchymal Stem Cells

Among the adult stem cell populations with the most extensive potential to impact therapies targeting age-associated decline are mesenchymal stem cells (MSCs). MSCs maintain a broad spectrum of lineage potential and are responsible for repair of many mammalian tissues including osteoblasts (bone), chondrocytes (cartilage), myocytes (muscle), adipocytes (fat) and stromal cells (marrow). Ubiquitously located throughout the body, MSCs can act locally through chemotactic-induced migration from the perivascular niches in response to stress or injury as well as systemically through the secretion of various soluble factors such as chemokines, cytokines and exosomes. Tasked with maintaining the HSC niche through the regeneration of an extracellular matrix comprised of osteoblasts, adipocytes and endothelia cells, MSCs also maintain tissue homeostasis through modulating HSC function. MSCs also are

vital in maintaining blood vessel integrity through promotion of angiogenesis and thus are essential for systemic wound healing and tissue regeneration. Lastly, MSCs have a profound capacity to modulate the immune system, therefore modulating the immune response to stress and injury by regulating the pro-inflammatory response of macrophages and prohibiting lymphocyte proliferation [32, 33]. The large number of tissues maintained by MSCs makes their age-related decline a significant concern in terms of the onset and sustainability of chronic diseases.

Given that age negatively affects the tight regulation required to orchestrate the biological roles of MSCs, and consequently, abnormalities within the MSC and the microenvironment in which they reside result in an increased inflammatory milieu, reduced control of self-renewal and a dysfunctional or skewed differentiation potential. MSC dysfunction can be observed in the accumulation of adipocyte deposits in both the marrow and muscle, impaired wound healing, a high propensity towards apoptosis during stress/injury and an increase in immune dysfunction [4, 34]. These alterations culminate in the development of chronic illnesses such as osteoporosis, osteoarthritis, cardiovascular disease and chronic inflammation [35].

3.2.1 Osteoporosis

Osteoporosis remains one of the most prevalent musculoskeletal disorders affecting the elderly population today and while MSCs dysfunction drives the onset and supports the continuation of the disease state, repairing or replacing these cells is a promising therapeutic approach to alleviating this ailment and the associated burden. Osteoporosis results in the loss of bone density due to dysfunctional differentiation of MSCs resulting in enhanced adiopogenesis and decreased osteogenic output. Interestingly, the percentage of the MSCs with osteogenic potential is not affected by age, but the volume of adipocyte tissue within the bone marrow is significantly increased in aged/osteoporotic patients [36, 37]. Systemic injection of MSCs into mouse models of osteoporosis have shown significant protection in the loss of bone density [38]. These studies also revealed improvement of bone quality, turnover capacity and sustained micro-architectural competence [38]. These studies provide strong evidence that MSC transplantation can be utilized therapeutically to target the age-related complications associated with bone density loss.

3.2.2 Osteoarthritis (OA)

Another common ailment associated with age-related changes in MSC function is osteoarthritis. Osteoarthritis (OA) is a common age related pathology, leading to severe chronic pain in joints, major mobility issues and a significant decrease in quality of life. Almost half of individuals 65 years or older will develop symptoms related to OA in one of various joints of the body, which include hips, shoulders, and most commonly

knees [39]. The underlying mechanism can be attributed to the breakdown of cartilage within the joint as well a loss of cartilage repair by resident MSCs. In OA, MSCs exhibit both a qualitative and quantitative decline in functional capacity with a depleted local population of MSC at the site of disease [40–42]. The strong evidence supporting MSC functional decline as contributing to OA suggests that MSC transplantation should be therapeutic in reducing the complications associated with this chronic disease. Proof of principal clinical trials involving human MSCs as a treatment for the complications associated with OA have already been performed. Intra-articular injection of MSCs into an arthritic knee improved the overall function of the joint and promoted a sharp reduction in cartilage deterioration through the regeneration of hyaline articular cartilage [43]. Treating OA with MSCs also had the added benefit of reducing the pain associated with disease, potentially through their ability to modulate the immune response at the site of pathology [43]. The capacity to confer immunomodulation in combination with the ability to generate chondrocytes, either directly or indirectly, required to replace lost or damaged cartilage related to OA make MSCs a promising agent to combat the chronic pain and debilitation associated with this disease.

3.2.3 Inflammaging

Human aging coincides with a progressive increase in low grade inflammation, a condition known as inflammaging. This continuous inflammatory state is a significant contributing factor of both the onset and support of chronic disease and can eventually lead to complications resulting in death. In fact, many, if not all age-related chronic illnesses maintain a strong correlation to the inflammatory milieu of aged individuals. The chronic inflammatory microenvironment associated with age can be driven through several diverse mechanisms, which include a combination of immune regulatory dysfunction and SASP factors [44]. The capacity for MSCs to regulate the immune response could minimize the effects of the common inflammatory environment observed in the elderly. MSCs already have been shown to positively regulate immune responses in complete mismatched allogeneic stem cell transplants, where the severe immune reactions can result in the onset of graft vs. host disease and potentially death [45]. Transplantations studies utilizing MSCs to alleviated chronic inflammation are not currently published, however, the potential these cells maintain in immunomodulation make them a prime target for further investigations concerning inflammaging.

3.2.4 Other Therapeutic Applications of MSCs

As previously mentioned, MSCs are responsible for maintaining numerous tissues and the decline in their functional capacity can be observed in many chronic illnesses associated with age progression. Investigations and clinical trials involving

MSC transplantation as a targeted approach to minimalize the complications associated with age can be observed in numerous contexts. For example, autologous transplantation of MSCs to combat cardiovascular disease have shown significant promise in reducing adjudicated clinical cardiac events leading to improved outcomes in patient care [46, 47]. Transplanted MSCs have also shown promise as renotropic agents, with marked improvement in function and repair of acute renal failure mouse model study [48]. These findings were expanded upon in human kidney transplants, where significant improvement of renal function could be observed in patients co-transplanted with autologous MSCs with decreased acute rejection incidences, lowered risk of opportunistic infection and improved renal function 12-months after surgery [49]. MSCs also are being utilized to treat diseases ranging from diabetes, Alzheimer's and Parkinson's to ischemic stroke, inflammatory bowel disease and cancer [50–55]. The ability of MSC transplants to alleviate the multitude of chronic disease states associated with age in disease models suggests that they could be used clinically to extend healthspan.

3.3 Hematopoietic Stem Cells

Systemic aging of the immune system or "immunosenescence" can be characterized by any number of age-related perturbations within the hematopoietic compartment that can drive the development of autoimmune disorders, anemias and neoplasms as well as an increase vulnerability to infectious agents and reduced responsiveness to vaccinations. Dysfunction within an aging immune system can be attributed to the functional decline of both the adaptive and innate immune responses, which originate with alterations within the HSC pool [56]. The comprehensive characterization of these cells, both phenotypically and functionally, has made HSCs a focus for understanding the relationship between the decline in adult stem cell populations and age and as a strong candidate for therapeutic intervention of age-related pathologies.

HSCs are multi-potent stem cells with the capacity to self-renew, required to maintain all lineages of blood-derived cells throughout life. HSCs represent the quintessential model for studying stem cell function and homeostasis in both steady-state as well as diseased contexts. Analysis of naturally aged mice revealed a three to tenfold increase in the overall number of HSCs, depending on the model [57]. The significant expansion within the HSC compartment are matched with an equally significant decline in HSC function. The decline in function results in regulatory changes that reduce the ability of HSCs to control quiescence, self-renewal, migration/homing, engraftment and differentiation, culminating in severe alterations in cellular output and response to various stress/injuries states [28, 58–61]. Consequently, an aged hematopoietic system has both adaptive and innate immune dysfunctions characterized by limited B cell repertoire diversity, defective production of naïve T cells, and restricted macrophage and neutrophil activity, all hallmarks of immunoaging. Importantly, chronological aging plays a significant role in T cell decline due to thymic involution, therefore contributing further to the decline of both T and B cell functions.

The decline in hematopoietic function also can have a negative impact on the ability for immune cells to locate and clear damaged or senescent cells. An increase in the overall senescent burden creates a microenvironment that further drives the systemic decline of adult stem cells and culminates in the deterioration of numerous tissues.

The accumulation of senescent cells can also be attributed to a significant loss in innate immune function. The deterioration of the innate immune response culminates in the manifestations of numerous characteristics observed in elderly patients with decreased ability to fight infections, control inflammation and to clear senescent or damaged cells [62, 63]. The decline in the ability for the innate immune system to target and clear this cell type can be attributed to significant age-related changes in macrophages, neutrophils, monocytes and natural killer (NK) cells. NK cell immunosenescence can be characterized by changes in the NK cell pool organization, phenotypic makeup and overall function [64–67]. Similar decline in macrophage, monocytes and neutrophil populations can be observed with aging [68–70].

It is still not clear if HSC function is driven by cell-autonomous effects or by the microenvironment in which they reside. Like other replicating cells, HSCs are not resistant to the accumulation of intracellular genotoxicity associated with progressive division. Transplant studies demonstrate that HSCs will undergo senesce when driven to replicate, possibly due to multiple mechanisms. First, there is substantial evidence to support telomere attrition and genetic instability created through serial transplants as a significant contribution to HSC senescence [71, 72]. Second, the responses to the accumulation of DNA damage or DDR can activate premature differentiation in HSCs and drive their exhaustion [28, 73]. Finally, the accumulation of ROS can negatively affect the ability for HSC to regulate quiescence, self-renewal and differentiation [74, 75]. The aging of the microenvironment also contributes to a decline in HSC functional output. Here, heterochronic parabiosis studies, both in vitro and in vivo, have verified that an aged microenvironment can dysregulate HSC number and function [76]. Also, transplantation of young bone marrow into old recipients revealed a similar decline in functional HSC. Taken together, these data support a strong role for both cell-autonomous and non-autonomous mechanisms as a driving force for the decline in HSC function. HSCs have been successfully transplanted for treatment of numerous disease states and have shown great efficacy in improving the outcome of patients burdened with a myriad of chronic illnesses.

The potential of HSC transplantation as a therapeutic intervention has been documented through their use in cancer treatments [77–79] and for genetic and chemotherapeutically induced bone marrow failure and sickle cell anemia [80–82]. Furthermore, they have been investigated for their potential beneficial use in therapeutic treatment of autoimmune disorders, solid tumors, whole organ transplants and major immune deficiency disease states driven either by genetic mutations or viruses [83–87]. These HSC transplant (HSCT) clinical trials suggest they could be used to target age-related decline in not only the hematopoietic compartment, but possibly also for the systemic decline of all major adult stem cell populations.

HSCT based investigations into several age-associated diseases suggest they can be used therapeutically to limit the decline in healthspan of the elderly population. A sharp decline in immune function can lead to increased opportunistic infections

with reduced responsiveness to vaccines. A main underlying reason for the decrease in vaccine efficacy is the significant decline in naive T cells associated with thymic involution and time. Reconstitution of the thymus can be obtained via HSCT through de novo regeneration of thymic tissue by hematopoietic precursors [88]. De novo T cells from a newly regenerated thymus improves CD4+ T cell numbers and T cell repertoire diversity [88, 89]. Also, sex steroid ablation, interleukin 7 (IL-7) treatment, growth hormone and T cell precursors can augment thymic regeneration [90, 91]. Other branches of the adaptive immune system also respond favorably to HSCT regeneration. Transitional B cells in human blood after HSCT represent the bulk of circulating B cells and have shown to be vital intermediates for B cell maturation [92]. The implications for a recharged adaptive immunity are wide-ranging with suspected improvement in vaccine efficacy, the adaptive response to novel parasites and a revived ability to locate and clear senescent cells.

Reconstitution of the innate immune system via HSCT should be advantageous for improving age-related healthspan decline. First, NK cells have the fastest rebound time post HSCT, which can have beneficial repercussions in patient outcomes [93]. The reconstitution of the NK cell compartment carries potent advantages, especially in an allogeneic setting where graft vs. leukemia effect has shown to be highly adept to clearance of damaged cells. In fact, studies suggest that "uneducated" NK cells confer a stronger protective capacity than their "licensed" counterparts [94, 95]. Extending the improvement of innate immune function such as improving macrophage and neutrophils functions should also confer benefits. Reducing low grade inflammation, clearance of senescence and improved response to opportunistic infections are all associated with improved innate immune function.

3.4 Neural Stem Cells

Specific tissues such as the brain are emerging as key locations for age-related chronic decline. Neurodegeneration can be defined as a progressive and irreversible loss in the proliferation and function of neurons, which has a severe impact on cognition and memory. The prevalence of neurodegenerative disorders, including dementia, amyotrophic lateral sclerosis (ALS), Alzheimer's and Parkinson's disease, all significantly increase with age [96, 97].

Neuronal tissue turnover is robust and the process happens throughout early to mid-life [98, 99]. However, as we age, there is a significant decline in neurogenesis, which results in both a significant loss of neuronal stem/progenitor cells (NS/PCs) and the subsequent loss in neuronal tissue function. NS/PCs possess the capacity to differentiate into neuroblasts (neural precursors), astrocytes and oligodendrocytes, which give rise to hippocampal and subventricular zone tissues.

NS/PCs are being utilized in phase I/II clinical trials for amyotrophic lateral sclerosis (ALS) with notable improvement in MRC score and delayed progression of disease for up to 18 months after transplantation of hNSCs [100]. Importantly, the patients showed no adverse side effects to the transplanted cells and could maintain

disease stability on a significantly lower level immunosuppressant regiment. NS/PCs transplants also are showing promising results in alleviating the learning and memory loss associated with Alzheimer's disease, ischemic stroke, and potential Parkinson's disease [101–104]. A potential draw back to utilizing NS/PCs as a therapeutic tool to alleviated are-related neurodegeneration is complications with developing strategies for improved harvest and yield. Fortunately, induction of pluripotent neural stem cells from differentiated cells has shown promise in eventually overcoming this hurdle. These studies are showing that long-term survival as well as functional integration can be achieved with iNSCs in mouse models of neurodegeneration [105]. These studies and clinical trials reveal a promising future for NS/PCs transplantation efforts to alleviated neurodegenerative complications associated with age.

3.5 Summary

Adult stem cell populations play significant role maintain tissue homeostasis with aging through both direct and, in particular, indirect mechanism. Thus functional adult cell populations and factors secreted by the functional stem cells can be used as therapeutic agents targeting age-related chronic diseases as well as for improving both health and lifespan (Fig. 3.1).

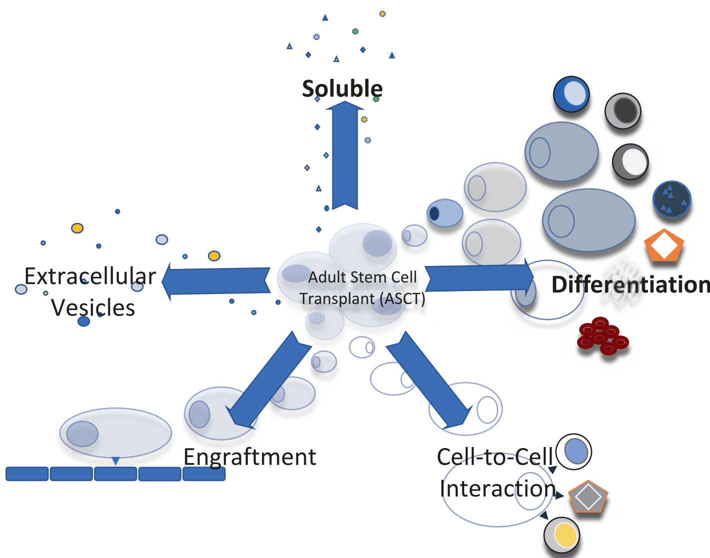

Fig. 3.1 The multifaceted potential of transplanted Adult Stem Cells aimed at targeting chronic disease. The capacity of adult stem cells to accomplish multiple tasks simultaneously makes their utility as therapeutic agents extremely valuable. These cells can act directly to replenish cells lost to disease states through differentiation, modulate cell signaling pathways through cell-to-cell interactions as well as through the release of soluble molecules and extracellular vesicles and engraft in the niche for long-term sustainability

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Chapter 4
Targeting miRNA for Therapy of Juvenile and Adult Diabetic Cardiomyopathy

Shyam Sundar Nandi and Paras Kumar Mishra

Abstract Prevalence of diabetes mellitus (DM), a multifactorial disease often diagnosed with high blood glucose levels, is rapidly increasing in the world. Association of DM with multi-organ dysfunction including cardiomyopathy makes it a leading cause of morbidity and mortality. There are two major types of DM: type 1 DM (T1D) and type 2 DM (T2D). T1D is diagnosed by reduced levels of insulin and high levels of glucose in the blood. It is caused due to pancreatic beta cell destruction/loss, and mostly found in juveniles (juvenile DM). T2D is diagnosed by increased levels of insulin and glucose in the blood. It is caused due to insulin receptor dysfunction, and mostly found in the adults (adult DM). Both T1D and T2D impair cardiac muscle function, which is referred to as diabetic cardiomyopathy. We and others have reported that miRNAs, a novel class of tiny non-coding regulatory RNAs, are differentially expressed in the diabetic heart and they contribute to diabetic cardiomyopathy. Here, we elaborated the biogenesis of miRNA, how miRNA regulates a gene, cardioprotective roles of different miRNAs including miRNAs present in exosomes, underlying molecular mechanisms by which miRNA ameliorates diabetic cardiomyopathy, and the role of miRNA as a potential therapeutic target for juvenile and adult diabetic cardiomyopathy.

Keywords Diabetic heart · T1D · T2D · miRNA · Exosome

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4.1 Introduction

Diabetes mellitus (DM) is a rapidly increasing menace throughout the world [1, 2]. It is associated with multi-organ disorders [3, 4], including cardiovascular disease [5, 6]. Despite current therapeutic strategies, prevalence of diabetes-mediated cardiomyopathy has markedly increased [7, 8]. Considering the increasing trend for prevalence of DM in the world [9], this number is projected to be higher in future. Therefore, a novel therapeutic strategy is warranted to ameliorate diabetic cardiomyopathy. Recent studies revealed that microRNA (miRNA) is a novel class of non-coding RNA, which is endogenously biosynthesized and regulates gene expression [10]. Empirical evidences demonstrate that miRNA has potential to mitigate/prevent pathological remodeling in various diseases [11–15], which makes it an attractive therapeutic target for various diseases. As per the Clinicaltrials.gov website, to date there are more than 300 clinical trials on miRNA for different diseases, including anti-miR-122 for Hepatitis C (clinicaltrails.gov # NCT01200420), and miR-34 mimic for primary liver cancer and solid tumors (clinicaltrials.gov #NCT01829971). miRNA is also emerging as a novel therapeutic target for cardiovascular diseases [16, 17], and diabetic cardiomyopathy [18–20]. It is reported that several miRNAs are differentially expressed in the diabetic heart [21–25]. Restoring the levels of specific miRNA in the diabetic heart may have therapeutic benefits. For example, miR-133a is downregulated in the diabetic heart [21, 26], and miR-133a mimic treatment to the diabetic heart improves contractility and ameliorates diabetic cardiomyopathy [27]. miRNAs are also present in an exosome, a small vesicle formed by inward folding of cell membrane. Exosomes are secreted from cardiomyocytes [28] and cardiac progenitor cells [29], and play a crucial role in cardiac remodeling [30–33]. This chapter embodies the role of miRNA in mitigating diabetic cardiomyopathy in juvenile and adult hearts.

4.2 Diabetes Mellitus

4.2.1 Background

DM is often diagnosed by an elevated blood glucose levels (fasting blood glucose level is higher than 120 mg/dL). The normal fasting blood glucose level in humans is 80 mg/dL. When the fasting blood glucose level ranges between >80 and <120 mg/dL, it is considered a pre-diabetic condition. In addition to increase in prevalence of DM population [1, 2], the number of pre-diabetic population is also rapidly increasing [34]. DM is a complex disease with metabolic disorder and multiple etiology [4, 35]. Despite insulin treatment to lower the blood glucose level especially in T1D, and metformin drug treatment to improve insulin sensitivity especially in T2D there are numerous incidence of morbidity and mortality in DM patients, which is corroborated in several clinical trials including Action to Control Cardiovascular Risk

in Diabetes (ACCORD), Action in Diabetes and Vascular Disease (ADVANCE), and Veterans' Administration Diabetes (VADT) [36]. Therefore, novel therapeutic strategies are warranted to ameliorate diabetic cardiomyopathy.

4.2.2 Types of Diabetes Mellitus

Based on insulin levels in the blood, DM is categorized into two major types: type 1 DM (T1D) and type 2 DM (T2D). In T1D the pancreatic beta cells, which biosynthesize and secretes insulin, are either less in number or non-functional that results in decreased insulin production and/or secretion in the blood. Reduced insulin levels in the blood compromise glucose uptake and metabolism that result in elevated blood glucose level. T1D is mostly prevalent in young individual and that is why they are also called juvenile DM. In T2D the pancreatic beta cells are functional and release insulin in the blood but circulating insulin is unable to enter a cell due to impaired insulin receptor function or insulin insensitivity. It results in accumulation of insulin in the blood. Therefore, in T2D both insulin and glucose levels are high in the blood. T2D are mostly prevalent in the adults. Besides T1D and T2D there is a third type of DM, which is present during gestation/pregnancy and it is called gestational DM. This DM is present only during gestation and after delivery of the baby blood glucose level is normalized. Pre-diabetes can be categorized as the fourth type of DM, however, it may progress to either T1D/T2D or revert to normal blood glucose levels [37].

4.2.3 Diabetic Cardiomyopathy

Diabetic cardiomyopathy (DCM) is a disease of heart muscle which leads to heart failure. It is defined as heart failure caused due to DM without any symptoms of hypertension, coronary artery disease, valvular disease, or ischemia [38]. DCM is categorized into three types: early stage where the changes are mostly at molecular levels such as altered calcium homeostasis, depleted glucose transporters GLUT1 and GLUT4, middle stage where the changes are also observed at structural levels such as increased size of left ventricle and cardiac fibrosis, and functional levels such as diastolic dysfunction, and late stage where besides molecular and structural changes both diastolic and systolic functions are compromised [39]. DM contributes to micro-, and macro-vascular complications [34, 40–42], and increases the chances of heart failure two to fourfolds as compared to age and gender matched non-diabetic individuals [43, 44]. The molecular mechanisms underlying DCM and the predictors and prevention of DCM at different stages are elaborated in one of our recent review articles [39].

Hyperglycemia leads to several changes in diabetic hearts. There is an increased production of reactive oxygen species (ROS) due to mitochondrial damage [45–47].

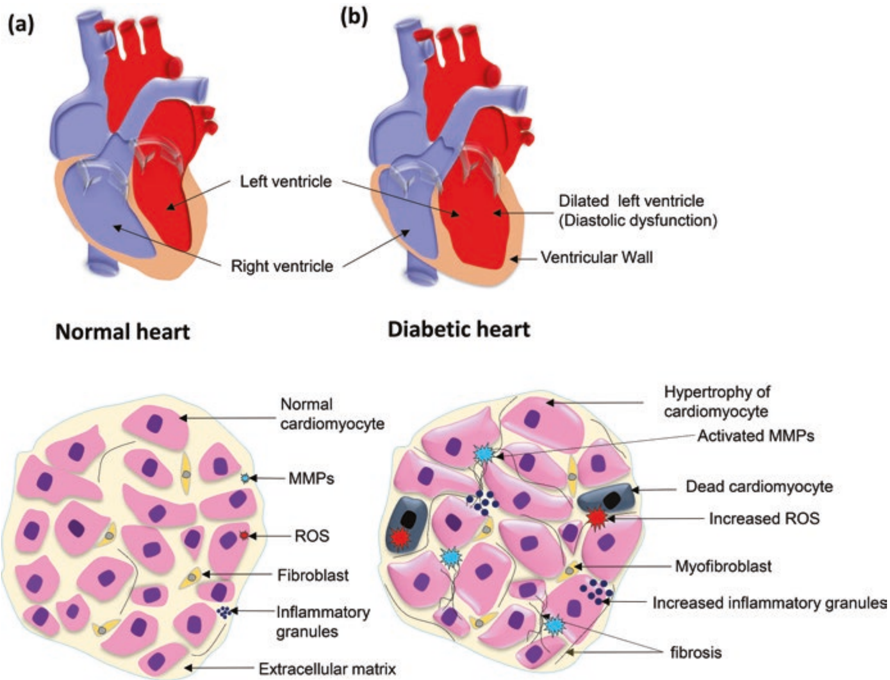

Fig. 4.1 Schematic representation for anatomical and histological features of diabetic cardiomyopathy. Top, schematic diagram showing (**a**) a normal heart and, (**b**) a diabetic heart. The dilated left ventricle is highlighted in the diabetic heart, which is a hallmark for diastolic dysfunction. Bottom, schematic diagram showing distinct histological features of a (**a**) normal and (**b**) diabetic hearts, such as cardiomyocytes in diabetic heart shows hypertrophic cardiomyocytes, increased cardiomyocyte death, presence of myofibroblasts that respond to inflammation, increased interstitial and perivascular fibrosis, and ROS production, inflammation, and MMPs activation

The ROS contributes to cardiac fibrosis by instigating matrix metalloproteinases (MMPs), especially MMP-2 and -9 which are collagenases that degrade extracellular matrix [48–52]. Cardiac fibrosis compromises cardiomyocyte contractility and cellular signaling leading to apoptosis. The remaining cardiomyocytes have more workload for contractility of the heart. As an adaptation, cardiomyocytes increase in size and hypertrophied. Hyperglycemia also increases the accumulation of inflammatory granules. Pro-inflammatory cytokines are increased in the diabetic heart and accumulate near hypertrophic cardiomyocytes [21]. Increased fibrosis of extracellular matrix compromises the compliance of the heart that leads to diastolic dysfunction. Further, the left ventricular wall becomes thin and the geometric shape of the heart changes and left ventricle become more round-shape [21, 51] (Fig. 4.1).

In DM hearts insulin signaling is compromised which causes altered metabolism, inflammation and apoptosis, mitochondrial damage and ROS generation, impaired calcium signaling, and cardiac hypertrophy and fibrosis [39, 41, 53]. Several miRNAs are involved in the regulation of these changes in the DM heart,

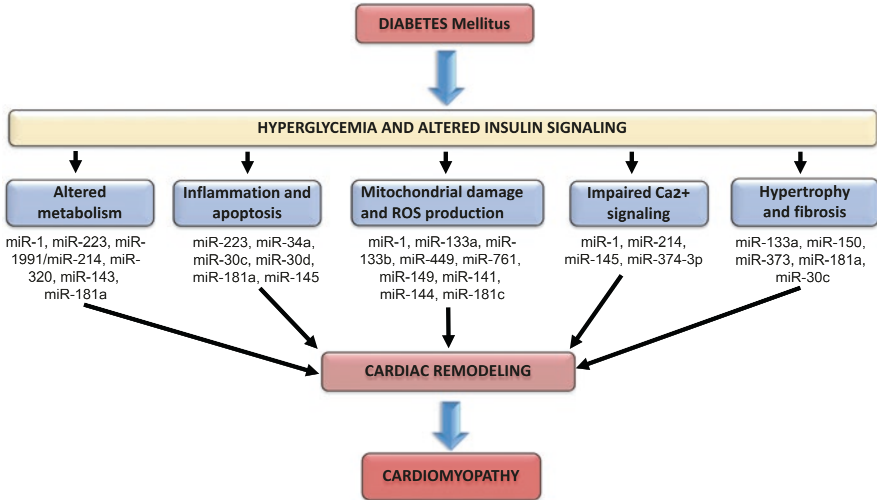

Fig. 4.2 Altered cellular pathways in diabetic hearts. MiRNAs associated with regulation of signaling genes in each pathways that lead to cardiac remodeling and diabetic cardiomyopathy

and differential expression of these miRNAs lead to pathological cardiac remodeling and cardiomyopathy [20, 27, 54, 55] (Fig. 4.2).

4.3 MicroRNA

4.3.1 Background

MicroRNAs (miRNAs) are a novel class of non-coding RNA that modulate gene expression either by mRNA degradation or translational repression [10]. They are an evolutionary conserved regulatory molecule which control almost all genes in biological processes. After the discovery of the first miRNA Lin-4 in 1993, miRNA field has progressed enormously within a decade and miRNA is emerging as a potential therapeutic target for several diseases [56]. There are more than 2000 miRNAs in humans [57], and each miRNA regulate more than one genes. Even one gene may be regulated by several miRNAs, which provides a layer of regulatory network for genes. Several members of the same family of miRNA may regulate a signaling cascade of a biological pathway or even one miRNA may regulate more than one genes in the same biological pathway [58]. This complex regulatory network is not completely understood, however, there is consensus that miRNAs are a crucial regulator for a gene and/or several genes in a signaling pathway. In the human heart, there are nearly 18 miRNA families that contribute to nearly 90% of the total miRNAs present in the heart, and several dozens of them are differentially expressed in the failing heart [59]. Overexpressing a downregulated miRNA or inhibiting an

upregulated miRNA are novel approaches for mitigating cardiac remodeling and improving cardiac function in the diabetic heart.

4.3.2 miRNA Biogenesis and Function

miRNA is transcribed as a primary miRNA (pri-miRNA) from intronic or intergenic region by RNA polymerase II/III as a single transcript or a polycistronic transcript [16]. The pri-miRNA is approximately 200 nucleotides long with a hairpin loop and contains 5′ cap and 3′ poly A tail. The Pri-miRNA is processed by Drosha and DiGeorge syndrome critical region gene 8 (DGRC8) to form a precursor miRNA (pre-miRNA), which is approximately 70 nucleotides long double stranded structure. The pre-miRNA assembles in exportin5 and RanGTP to make a complex, which is exported into cytoplasm. In the cytoplasm pre-miRNA is processed by dicer, a RNase III endonuclease, into a mature miRNA which is immediately loaded into RNA induced silencing complex (RISC). The two strands of pre-miRNA may form one or two mature miRNAs depending on degradation of passenger strand of miRNA (miRNA* or miR-5p) [19, 60] (Fig. 4.3).

Mature miRNA has a seed sequence which is 2–8 nucleotide from the 5′ end. The seed sequence of miRNA if binds perfectly to the 3′ untranslated region (UTR) of mRNA of a gene then it will degrade the mRNA but if the seed sequence match

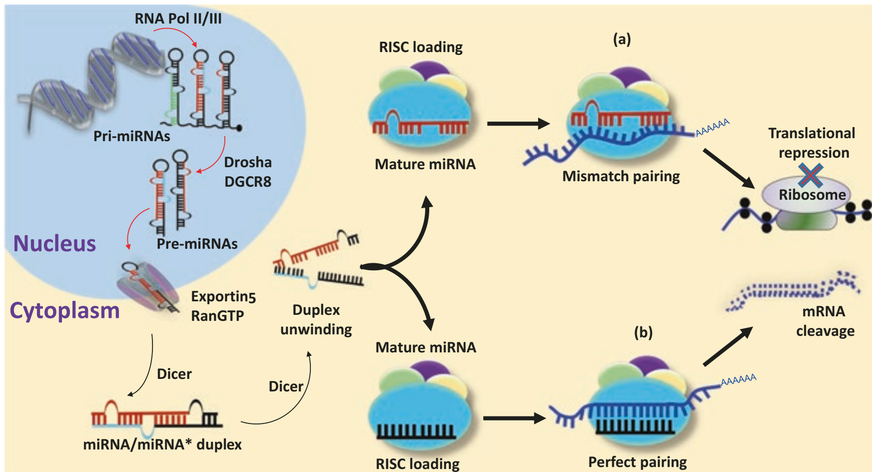

Fig. 4.3 MiRNA biogenesis and functions. Transcription of miRNA in the nucleus from non-coding DNA, their processing in the nucleus into primary miRNA (pri-miRNA) and precursor miRNA (pre-miRNA). Transport of pre-miRNA into cytoplasm and its processing by dicer into a mature miRNA, which is loaded into a RNA induced silencing complex (RISC). Mature miRNA can degrade mRNA when the seed sequence of miRNA perfectly match with the untranslated region of mRNA or inhibits translation of protein when the seed sequence imperfectly match with the untranslated region of mRNA

imperfectly with the 3′ UTR of mRNA then it will block the translational machinery and impair protein synthesis [16, 19] (Fig. 4.3).

4.3.3 miRNAs in Cardiac Regeneration

miRNAs are documented to regulate cardiac stem cell differentiation, which is pivotal for cardiac regeneration [61–64]. The extracellular matrix stiffness contributes to stem cell self-renewal [65], and survival and differentiation of cardiac stem cell [66, 67]. miR-1 and miR-133a promotes differentiation of embryonic stem cell into cardiac lineage by targeting histone deacetylase-4 and serum response factor, respectively [68]. miR-499 is also involved in regulation of embryonic stem cell into cardiomyocyte lineage [69].

miRNAs also regulate stem cell homing, differentiation and maturation, which is crucial for cardiac regeneration [70, 71]. Several recent reviews elaborate the potential roles of miRNA in cardiac regeneration [31, 72–78].

4.3.4 miRNAs in Exosome Improve Cardiac Functions of Diabetic Hearts

miRNAs are released into circulation after encapsulation into a membrane-bound vesicle called exosome [31]. These exosomes are also secreted from stem cell [79] and other cell types including cardiomyocytes [29], and plays a pivotal role in cardiac regeneration and regulation of cardiac functions [80–85]. In diabetic heart, cardiomyocytes-derived exosomes have elevated levels of miR-320, which is detrimental to the heart [86]. Therefore, exosome secretion inhibitor such as GW4869 could be a potential therapeutic strategy to mitigate exosome-mediated cardiac dysfunction in diabetic hearts [86–88].

4.4 MicroRNA as Therapeutic Target for Diabetic Cardiomyopathy

The expression of miRNA changes in the diabetic heart [21]. It is not necessary that all miRNAs showing altered expression in diabetic heart may have a crucial role in diabetic cardiomyopathy, however, empirical evidences based on loss-, and gain-of function studies on miRNA revealed several miRNAs that regulate diabetic cardiomyopathy [89]. For example, silencing of miR-195 [90] or upregulation of miR-30 [91] mitigates diabetic cardiomyopathy. miR-141 is upregulated in diabetic hearts and it decreases ATP production by suppressing ATP synthase activity [92].

Therefore, suppression of miR-141 can be a potential strategy to improve ATP production in the diabetic heart. miR-133a is downregulated in the diabetic heart [21, 55, 93], and overexpression of miR-133a by a mimic reduces cardiac hypertrophy [55], fibrosis [94], and cardiac contractility [27]. Therefore, targeting a particular miRNA involved in a specific signaling pathway in the diabetic heart may provide a therapeutic effect to ameliorate diabetic cardiomyopathy.

4.5 Conclusions

The two major types of diabetes, T1D and T2D, may have different pathophysiological adaptations due to different levels of insulin in the blood [95]. The miRNA profiling in diabetic hearts revealed several crucial miRNAs that contribute to diabetic cardiomyopathy. These miRNAs can be either present in the heart or are released into an exosome. Empirical evidences demonstrate that modulating the expression of miRNA by either a mimic or an inhibitor has potential to ameliorate diabetic cardiomyopathy. Although these are encouraging results for developing miRNA-based therapeutic strategy for diabetic cardiomyopathy, further investigations are required to understand the regulatory mechanisms by which miRNAs cross-talk to optimize the gene expression in diabetic hearts, considering the fact that one miRNA may have more than one target and one gene can be targeted by more than one miRNA. From therapeutic point of view, it is also important to investigate how miRNA should be delivered and which route of delivery is better. Overall, there is consensus that miRNA is emerging as a therapeutic target for diabetic cardiomyopathy and future studies will determine its clinical application.

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Chapter 5
Stem Cell Therapies for Neurodegenerative Diseases

Kiminobu Sugaya and Manjusha Vaidya

Abstract Stem cell therapies have been proposed as a treatment option for neurodegenerative diseases, but the best stem cell source and therapeutic efficacy for neuroregeneration remain uncertain. Embryonic stem cells (ESCs) and neural stem cells (NSCs), which can efficiently generate neural cells, could be good candidates but they pose ethical and practical issues. Not only difficult to find the good source of those cells but also they alway pose immunorejection problem since they may not be an autologous cells. Even if we overcome the immunorejection problem, it has also been reported that transplantation of ESCs develop teratoma. Although adult stem cells are more accessible, they have a limited developmental potential. We developed technologies to increase potency of mesenchymal stem cells, which allow them to develop into neural cells, by over expression of the ESC gene, nanog. We also developed a small molecule compound, which significantly increases endogenous NSCs by peripheral administration, eliminating even the necessity of stem cell injection to the brain. These novel technologies may offer neuroregenerative therapies for Alzheimers disease (AD). However, we found that AD pathological condition prevent neurogenesis from NSCs. This chapter discusses how to overcome the problem associated stem cell therapy under AD pathology and introduces exosome as a tool to improve the modification of adult stem cells. These new technologies may open a door for the new era for AD therapy.

Keywords Neural stem cells · Embryonic stem cells · Alzheimer's disease · Neurodegeneration · Parkinson's disease · Octamer 4 · Mesenchymal stem cell

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5.1 Introduction

More than five million Americans are living with Alzheimer's disease (AD) and it could become as high as 16 million. In 2017, AD and other dementias will cost $259 and these costs could rise as high as $1.1 trillion by 2050. Although pharmacological treatment for dementia and AD typically fails to improve cognition, several lines of evidence suggest that cell replacement therapy may reverse cognitive impairment. Advancement of stem cell technology shows very promising future for regeneration of tissues allowing us to replace many organs and body parts. However, development of stem cell therapy for neurodegenerative diseases, such as AD and Parkinson's disease (PD) requires to clear many huddles before getting in to the clinical use. The first supportive evidence for stem cell neurodegenerative therapy is that production and survival of new neurons is associated with cognition and is congruently impaired with aging [1, 2]. Secondly, adult neural stem/progenitor cell proliferation and neuronal differentiation is significantly diminished in AD and Down syndrome animal models both *in vitro* and *in vivo* [3–5]. Finally, cognitive performance improves through increasing neurogenesis from an enriched environment [6], various compounds [7], or following stem cell transplantation [8, 9]. Although the relationship between neurogenesis and cognition is not causative, as some studies fail to show a relationship in rodents [10, 11], the positive trend is supported by the literature. We demonstrated that transplantation using either human neural stem cells (NSCs).

Neural stem cell, committed to differentiate into neurons and glial cells, is a very good material to regenerate nerve tissue. In our previous study transplantation of human NSCs to the cognitively impaired aged rat significantly improved their cognition and new neurons were formed in the cortex and hippocampus [9]. This result indicated that NSCs transplantation can be used to treat age associated neurodegenerative diseases though there are the major problems associated with neuroregeneration therapies using NSCs. Beside the ethical issue, it may not enough source of the tissue available to treat. Since NSCs are only exist in sub-ventricular zone (SVZ) and granular layer of dentate gyrus in the hippocampus and proliferation ability of the adult stem cells is limited, it is very difficult to secure enough material. Another issue would be immunorejection. Although the brain is immune privilege tissue, the inflammatory conditions associate with neurodegeneration may cause rejection of the donor cells by the host. These complications of human NSC procurement and other practical issues led us to explore other stem cell options.

Embryonic stem cells (ESCs) which are pluripotent, capable of producing any kinds of somatic cells can be a good material to regenerate neural tissue. The use of ESCs to treat neurodegenerative diseases has been proposed, but concerns over ethics [12–14], immune response [15, 16], and tumor formation [17–19] have been major barriers for their clinical use. The production of ESCs involves distraction of human embryo, which rise the ethical problem. It may also have safety issues, such as formation of teratoma and immunorejection upon the transplantation. As we mentioned above immunorejection could happen under certain pathological

conditions. Also ESCs may not cause immunoresponse to the host when they were not differentiated but they may start produce antigens when they differentiated into the neural cells. Because of their pluripotency, ESCs can become all kinds of cells without instruction causing another problem, teratoma. To avoid these issue associated with ESCs, human cloning and extraction of NSCs from the cloned fetus could be considered though it is technically very difficult and again ethically problematic.

Utilization of adult stem cells could eliminate these issues because they can be harvested from a patient, modified, and autologously transplanted back to the patient. However, multipotent adult stem cells, such as bone marrow-derived mesenchymal stem cells (MSCs), may only develop along a few cell lineages. Although studies have claimed that MSCs can transdifferentiate into cells outside their restricted germ layer, the resultant transdifferentiation may have been from a very limited population of MSCs [20] or the result of cell fusion. Many clinical trials have been conducted in stroke cases using MSC infusion and some positive results were reported [21]. In these clinical trials, MSCs have been used to provide factors to protects neurons from stroke damage but not to regenerate neurons to replace the dead ones. Therefore, a strategy to increase the potency of adult stem cells is an attractive option for neuroregeneration.

Bone marrow MSCs can adopt characteristics of multiple cell types through cell fusion, albeit at low frequencies [22, 23]. Previous groups have taken advantage of this phenomenon to change characteristics of somatic cells. Cell fusion not only alters the properties of MSCs to adopt other characteristics, but it can also be a means of changing the potency of cells, allowing stem cells to develop into cells beyond their respective tissue lineage. Alternatively, the fusion of somatic cells to embryonic stem cells prompts expression of the embryonic stem cell gene Oct-4 [24, 25]. The expression of stem cell genes that regulate self-renewal and pluripotency, such as Oct-4, are likely to play an integral role in cellular reprogramming. We have found that such changes may be initiated by signaling within the host cell, particularly using nanog, similar to nuclear reprogramming following nuclear transfer [26]. Earlier work has indicated that the expression of certain stem cell genes are capable of maintaining ESCs in a pluripotent state. Nanog is a major regulator of ESCs and overrides signals to differentiate when over-expressed [27, 28]. The suppression of differentiation has been previously demonstrated with the over expression of other ESC cell genes including Pem [29] and Rex1 [30], although the presence of elevated levels of Oct-4 was insufficient to guard against ESC differentiation [31]. Thus, we took a different approach that developmental potency can be gained by changing the gene expression profile through the embryonic stem cell gene nanog, without the need for cell fusion. Modified cells can then be recommitted to develop along a neuronal lineage and serve as a means of treating neurodegenerative conditions, such as AD.

In this chapter, we will introduce exosome to increase efficacy of the dedifferentiation of adult stem cells and also pharmacological approach to increase the endogenous NSCs to regenerate neurons under AD pathological condition.

5.2 Embryonic Stem Cell Gene, Nanog, to Dedifferentiate Adult Stem Cells

This section demonstrates a novel method of dedifferentiating adult stem cells by expressing genes regulating pluripotency, with the end goal of facilitating neural transdifferentiation. Nanog transfection produced proliferative cells with morphological and gene expression similarities to ESCs. Yet the fact that the number of larger proliferative colonies that formed did not directly correspond to the number of transfected cells is likely to be the result of several factors. Namely, culturing with MSCs can support ESC expansion, but other culturing conditions may improve cell growth. It should be noted that a lack of sufficient feeder layer MSCs fails to support production of ESC-like cells, and growth inhibition resulting from overconfluence may inhibit transformation. Low ESC-like cell numbers were expected given that colonies had the propensity to differentiate when transferred, and thus colonies were lost with each passage. Continued passaging of cells has also been shown to have a negative effect on cell viability and differentiation potential. Continuous passaging of cells produces alterations and cell senescence, the proliferative limit of which is commonly referred to as Hayflick's limit [32, 33]. Changes to MSCs' ability to differentiate and rate of proliferation are evident by passage six [32]. Contributing to the differentiation of ESC-like cells may be due to changes in the underlying feeder MSCs. Although the proliferative limit for ESC is difficult to determine, given their heterogeneity, the accumulation of errors through multiple passages will favor the use of cells with low passage number. Yet the ratio between transient and stable transfected cells may be the most prominent factor in colony formation. Stable transfection is likely required for cellular conversion, and MSCs show extremely low rates of transfection [34]. Moreover, it is uncertain if all of MSCs in culture share an equal ability to undergo dedifferentiation. It remains to be seen if there is a sub-population of cells that are more responsive to dedifferentiation and are responsible for the cellular conversion we achieved.

We previously demonstrated the treatment with the nucleotide derivative BrdU allows for transdifferentiation [35], and other groups have demonstrated that fusion of stem cells and somatic cells can alter cell properties [23, 25, 36]. We developed a technology to produce pluripotent stem cells from MSCs, Our patent US8906683 has priority date of Oct 22, 2004 [37]. In this patent we have shown over expression of ESC gene nanog in MSCs with fully description of increased Oct-4 and Sox-2 genes expression after nanog over expression. We were able to show increased potency of these cells and produced neural cells in vitro and in vivo (Fig. 5.1).

One year later Yamanka group filed a patent, US8058065 priority date Dec 13, 2005, in which they transfect Oct-4, Sox-2, and KLF4 along with the oncogene cMyc to increase potency of the fibroblasts [38].

These results have been achieved in human cells independently using the same set of genes [39, 40] or with a combination of Nanog, Oct-4, Sox-2, and Lin28 [41]. Our approach shows that we can accomplish corresponding results in human stem cells using only nanog, although other required factors are likely present within the

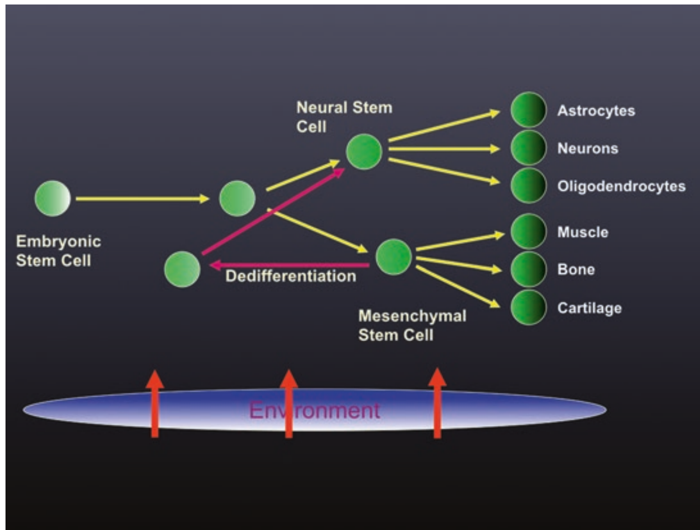

Fig. 5.1 Cell fate of ESC and adult stem cells. Potency of the adult stem cells can be increased by dedifferentiation with over expression of ESC genes

adult stem cells that gave rise to ESC-like cells. Our results run contrary to a previous report that failed to show any ESC-like cells using viral vectors for any single ESC gene in fibroblasts [42] nor Oct4, Sox2, Klf4 and Myc in adult MSCs [40]. This may be because that fibroblasts are terminally differentiated and it is difficult for the ESC genes to access the gene with the full DNA methylation. Also, it indicates the importance of nanog over other ES genes. The use of additional vectors for SV 40 T and the catalytic subunit for telomerase hTERT was able to produce a few colonies but showed cellular loss with expansion that was observed [40]. However these are the genes to use cell line form the primary cells and they are most likely a kind of tumor cells after these treatments.

Our technology clearly has merit as compare to the Yamanaka technology since it requires over expression of only one ES gene, nanog without any oncogene. This may be because different starting materials, we use adult stem cells, MSCs, which are not fully differentiated as fibroblasts, having much less DNA methylation and more potency. The difficulty in converting MSCs may be in part due to their sensitivity to culturing conditions and their propensity to undergo senescence after several passages [32]. Another explanation is the toxicity associated with viral transduction, particularly the use of the VSV-G vector in MSCs [43]. The negative results other groups observed are surprising since the combination of Oct4 and Sox2 up-regulate nanog [44, 45]. However, high levels of nanog may be the critical factor, since strong selection for nanog has yielded ESC-like colonies using the previously mentioned factors [46].

This method may be advantageous in developing neurons and glial cells without the generation of tetraploid hybrids or ESCs. The ability to generate human neural

cells form autologous adult stem cells will aid in the development of novel research and allow for the advancement of cell replacement therapies and the ability to create disease-specific cell lines from patients that can be used as a model for research and development.

5.3 Practical Use of Exosome to Deliver Biomaterials to the Adult Stem Cells

Exosome is a small, 30–100 nm, cell-derived vesicles originally found in the maturing process of erythrocyte, removing specific plasma membrane proteins. Thus, it has been though as a disposal mechanism of waste materials from the cells. Exosome is released to the extracellular space directory from the plasma membrane or fusion of the cells with multi-vesicular body. As the results, exosome contains various molecules derived from the cells including, protein, RNA and DNA. Findings of mRNA and miRNA as a cargo of exosome and fusion of the exosome to other cells, make us to believe the function of exosome as the intra-cellular transmission mechanism of the signaling molecule.

We investigated whether the human NSCs can be modified by having them accept exosomes from other cell type, such as HEK293 cells, that will eventually unload the cargo inside the cells.

HEK293 cells were cultured in T-75 cell culture treated flasks using media containing DMEM-1X containing 4.5 g/L Glucose, L-glutamine & sodium pyruvate. 10% each of L-glutamine (200 mM), non-essential amino acids, antibiotic/antimycotic and FBS. To avoid the interference of high amounts of bovine exosomes present in regular FBS, heat-inactivated exosome-depleted FBS media supplement from SBI (Catalogue # Exo- FBSHI-50A-1) was used.

At 75–80% confluency, the HEK cells were transfected with SBI vector XPack-RFP (XPack RFP Lentivector, SBI systems, Catalogue # XPAK531PA-1) using Lipofectamine® 2000 reagent (Invitrogen Catalogue # 11668-027) following the manufacturer's protocol and incubated at 37 °C with 5.0% CO_2.

Within 24 h of incubation, the transfected HEK cells were viewed under fluorescent microscope (Zeiss Axio imager) with a standard RFP filter. A cytoplasmic red florescence was observed in the cells, with bright intermittent fluorescent spots, a characteristic of exosome manufacturing cells (Fig. 5.2).

After 48 h of incubation, the cells culture media was collected and processed for harvesting exosomes. The media was centrifuged at 10,000 × *g* for half an hour in a centrifuge (Eppendorf Centrifuge 5804), maintaining the temperature at 4 °C to remove any cell debris that pallets. To the supernatant, 'total exosome isolation reagent from the cell culture media' (Invitrogen, Catalogue # 4478359) was added. After an overnight incubation at 4 °C, the exosomes were collected by centrifugation at 10,000 × *g* for an hour at 4 °C and the exosome pallet was re-suspended in 1 × PBS (pH 7.4).

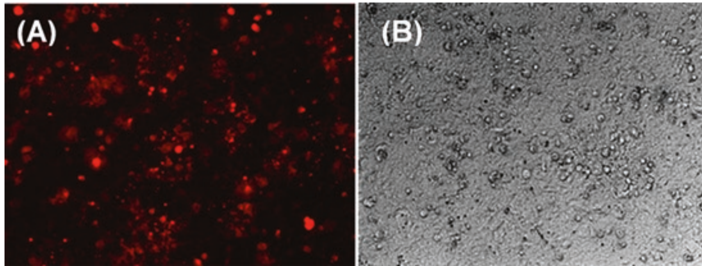

Fig. 5.2 (**a**) HEK293 cells transfected with XPack-RFP vector—Fluorescent and (**b**) Transmitted light (10×)

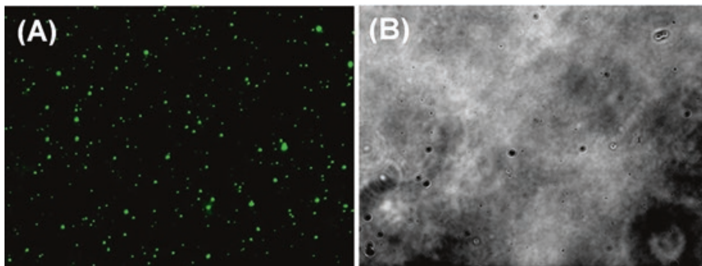

Fig. 5.3 (**a**) HEK cells derived exosomes dyed with DiO observed under Fluorescent and (**b**) Transmitted light (40×)

For a quick check, the exosomes were incubated at room temperature for an hour with a green fluorescent lipophilic dye DiO (Molecular Probes® Catalogue # D275) and imaged under the fluorescent microscope (Zeiss AXIO imager) using a standard GFP filter Z (Fig. 5.3).

Human NSCs in the form of neurospheres were cultured with culture media containing DMEM/F12, Heparin, EGF, bFGF and 2% B27 in 48 well suspension culture plate at 37 °C with 5.0% CO_2. Before adding the exosomes, the cell culture media was removed from the well without disturbing the cells and 50 μL of exosome suspension was added. The plate was incubated at 37 °C for 1 h and 150 μL of HNS-cell culture media was added to each well. The incubation under the same conditions was continued for 48 h.

The media (along with unattached, suspended exosomes) was removed and the neural stem cells were taken on a glass slide coated with Poly-L-Lysine. The samples were imaged under confocal microscope (Zeiss LSM710). As you see in Fig. 5.4 all the HNCs express RFP after optimal condition of the exosome treatment.

This study shows that exosome is a very efficient tool to deliver any type of cargo to the primary cells, which is crucial to modify of adult stem cells, to dedifferentiate and to transdifferentiate into target cells. This technology allow us to produce iPS cells mentioned above and differentiate using genetic manipulation (US patent# 9243226 Biasing of cells toward retinal, corneal or lens development, US

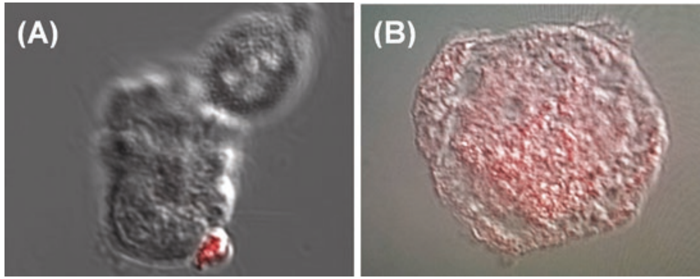

Fig. 5.4 HNS-cell treated with RFP packed exosomes (**a**) before optimized and (**b**) after optimized conditions

patent#9127291 Methods and products for biasing cellular development) without viral transfection.

5.4 Systemic Administration of Pyrrolopyrimidine Compound to Increase Endogenous NSCs

Pyrrolopyrimidine (MS-818) compound showed neurotropic properties and low toxicity [47–50]. In vivo, MS-818 was demonstrated to increase axonal outgrowths in acute injury models of rodent peripheral nerve regeneration [51]. Early research also provided evidence for the cell stimulating effects of MS-818 in non-neural contexts, including angiogenesis [52], gastric epithelia [53], muscle [54], and keratinocytes [55]. We've shown that MS-818 acts as a stem cell proliferation compound by increasing endogenous stem cell numbers as well as promoting migration within tissues [56, 57]. MS-818 treatment increased neuronal cell proliferation in the dentate gyrus (DG) of young and aged mice. After 4 weeks, a portion of these cells was shown to express NeuN [58]. Based on these results, the present study further characterizes the effects of MS-818 on stem cell populations.

As a method to increase stem cell proliferation, MS-818 could serve as a treatment for a number of diseases and conditions by allowing for the replacement of damaged cell types. Neurodegenerative disorders in particular have been noted to benefit from transplantation of neural stem cells [9, 59–63]. MS-818 treatment could have similar effects by increasing endogenous stem cell quantity. Parkinson's disease is a neurodegenerative disorder, characterized by a loss of dopamine-producing neurons in the substantia nigra (SN) region of the brain [64], subsequently, inducing such hallmark symptomology as involuntary tremors and muscular rigidity, as well as cognitive deficits [65]. Current dopamine replacement therapies only treat PD symptomology rather than target the root cause, while producing significant side effects and risks [66].

Endogenous NSCs could potentially serve as a source of dopaminergic neuron replacement in this context [67]. However, proliferation and neural differentiation

of NSCs may be impaired in patients with PD [68]. As such, some form of growth modulation may be necessary to utilize endogenous NSCs in PD treatment. Here, we investigate the potential for MS-818 to increase endogenous stem cell proliferation in this context. MS-818 Increased proliferation of stem cells, in vitro Human NSCs (hNSCs) showed increased metabolic activity based on the MS-818 concentration which indicates the proliferation of hNSCs. Metabolic activity and proliferation of human mesenchymal stem cells (hMSCs) were augmented in a dose dependent manner by MS-818. The EC50 of MS-818 for hMSCs was over 30 times greater than for hNSCs, indicating that the proliferative effect of MS-818 is selective for hNSCs. Immunostaining and unbiased stereology of aged-rat cortex indicated a significantly increased number of proliferating cells in MS-818 treatment groups. In the SVZ of control groups, reduced numbers of BrdU-positive cells were observed in the cognitively-impaired rats compared to the cognitively-unimpaired ones. In MS-818 -treated rats, we found increased BrdU-positive cells in both cognitively-impaired and cognitively- unimpaired aged animals. These BrdU-positive cells in the SVZ appeared to migrate extensively into white matter. Migrating cells in the cortex exhibited neural migration marker doublecortin (DCX) co-localized with BrdU. These results indicate that MS-818 not only increases NSC proliferation but also promotes a favorable environment for the migration of endogenous NSCs in aged animals. Immunohistochemical staining of brain sections (MPTP model mice) exhibited significant increases in BrdU signal in the SVZ and dentate gyrus (DG) in mice treated with MS-818. This elevated BrdU signaling is most likely due to an increase in the proliferation of endogenous stem cell populations. Neuronal migration marker doublecortin (DCX) signals were increased in MS-818 treatment groups compared to the control groups which indicate an increase in neuronal migration. These findings indicate that MS-818 may act as an enhancer of stem cell proliferation toward the generation of functional neuronal phenotypes in the brain. Both of these increases in proliferation were significant.

Immunohistochemical staining of brain section displayed increased levels of tyrosine hydroxylase (TH) in MS-818 treatment groups relative to MS-818 untreated MPTP model. This increased TH expression was present in both the SN and DG, indicating increases in dopamine production in sites local and distal to PD pathology. Additionally, BrdU signals are co-localized with TH production. Assessed in combination with data suggesting increased stem cell proliferation, these observations suggest that MS-818 treatment may serve to increase the production of dopaminergic neurons by stem cell proliferation and subsequent differentiation, or promote existing dopaminergic activity. This activity was observed in both the SN itself as well as the DG, suggesting that increased dopaminergic activity may occur outside the SN pathological area as a compensatory mechanism. MS-818 Treatment Enhances Rotarod Performance of MPTP Model Mice The rotarod behavioral test was employed to measure the effects of MS-818 treatment (3 mg/kg/day, i.p.) on sensory-motor performance in PD model mice. MPTP model/MS-818 treatment group mice had significantly longer rotarod running times than MPTP model/no treatment mice, indicating an increase in sensory-motor coordination. This increased performance was observed consistently at time points 1, 5, and 6 weeks after the

beginning of MS-818 treatment, demonstrating the continuity of functional improvement. The increased performance in the behavior was only observed in the tendency at the week 6. This could be due to recovery of the PD symptoms in MPTP models over time, as noted by multiple groups groups [69–71]. MPTP model/no treatment mice were significantly different from Controls at all time points. This stands in contrast to MPTP model/MS-818 treatment mice, which were not significantly different from Controls at any time point. The average performance of these MPTP/MS-818 treatment mice neared that of Controls in the final 2 weeks of testing, demonstrating the compounds convalescent effects. Non-MPTP model MS-818 treatment groups were not significantly different than control mice at any time point, indicating no notable sensorimotor detriments from MS-818 treatment. These results suggest that MS-818 treatment induces functional improvement in MPTP models and it may serve to reverse PD pathology. Pyrrolopyrimidine compound MS-818 is originally derived in the high throughput production of antimicrobial agents. Early characterization of this compound showed notable neurotropic properties [48, 49, 51]. However, despite pharmacological usefulness, the convalescent mechanism of MS-818 *in vivo* was never fully elucidated. Based on these initial findings, our group investigated the effects of MS-818 on hNSCs to elucidate a potential mechanism of convalescent action at the cellular level. Treatment of hNSCs with MS-818 was observed to increase metabolic activity in a dose dependent manner, suggesting increased cell proliferation in vitro Similar increases in metabolic activity were also noted in human mesenchymal stem cell lines. These results suggest that MS-818 may induce proliferation of multiple stem cell phenotypes, though the effect is more dramatic in hNSCs. Various disease states may benefit from generalized stem cell proliferation, potentially allowing for the replacement of damaged tissue utilizing endogenous stem cell populations. In light of these positive outcomes, our group attempted to study the proliferative effects of MS-818 in vivo. In our previous work, we have noted that injection of hNSCs into an aged rodent model improved cognitive performance [9]. As such, if proliferative effects of MS-818 were also seen *in vivo*, it may be possible to replicate the effect of exogenous NSC transplants by increased activity of endogenous NSC populations. Staining of the SVZ with BrdU in an aged rodent model indicated that MS-818 treatment increased the proliferation of endogenous NSCs. In addition to increases in the absolute number of proliferating NSCs, MS-818 treatment appears to have increased the migration of the cells into the white matter regions surrounding the SVZ. This migration is notable, as it highlights the potential for MS-818 treatment to induce convalescent effects by stem cell modulation in brain areas with reduced resident stem cell populations. Positive DCX staining was also increased in MS-818 treated animals, suggesting that NSCs not only proliferated, but also formed neural phenotypes with the capacity for migration within the brain tissue. Previous research has demonstrated that DCX expression indicates migratory potential and the formation of neuronal phenotypes from proliferating stem cells [72]. Toxicology testing was performed to ensure that MS-818 treatment had no adverse effects on liver and kidney protein levels in these in vivo treatments. These findings further suggest that MS-818 has no toxic effects at the employed dosages. In addition,

Ames testing was further confirmed the non-toxic nature of this drug, with no detectable mutagenic effects. The production of migrating, functional neural phenotypes from endogenous stem cell populations holds promise in the treatment of multiple neurological disorders. Parkinson's disease is an obvious candidate for treatment, as it is believed that a large degree of the pathology is caused by the loss of dopaminergic cells in SN. Replacement of this lost cell population could therefore potentially alleviate classical PD symptoms. As suggested by the in vivo experiments in aged model mice, treatment by MS-818 may serve to produce functional neurons to correct this cell deficit. In MPTP model mice, increased stem cell proliferation was observed by BrdU staining in the DG and SVZ of MS-818 treated animals. Proliferation in spite of the PD pathological condition provides further support for the robust nature of MS-818 's effects. Combined with our findings of increased proliferation in aged mice indicate the possible application in multiple neurological pathologies. Stereological analysis confirmed the significance of this increased proliferation in a quantitative manner. Importantly, increased DCX expression was observed in MS-818 treated animals, providing molecular indication of increased neuronal migration and formation of functional neuronal phenotypes. These observations provide evidence that MS-818 treatment promotes the proliferation of endogenous stem cells and the subsequent migration of functional neural phenotypes, suggesting a possible mechanism of any observed functional repair. In PD pathology, increase in the number of neural cells in the SN may not be sufficient and also need to improve the dopaminergic activity of the resident cells. In this study, staining of the DG showed increased levels of TH in MS-818 treated animals relative to a control, indicating increased dopaminergic activity. This TH production was colocalized to BrdU signaling, indicating that proliferating cells may be differentiating into dopaminergic phenotypes, or increasing the production of existing dopaminergic cells by stimulatory signaling. It is possible that increased dopaminergic production in the hippocampus may compensate in part for the pathological decreased production in the SN. Further supporting this hypothesis is evidenced in current dopamine replacement treatments, in which increased dopamine levels are capable of reducing symptomology even if delivered in a delocalized fashion [73, 74]. This observed increase in dopamine production could be considered a reversal of one component of PD pathology, providing further support for the potential therapeutic application of MS-818 in the treatment of PD. In addition to molecular data, evidence of functional improvement in MS-818 treated MPTP model mice in behavioral assessments provides support for the treatment's efficacy. In Rotarod testing, MS-818 treated MPTP model mice showed remarkable improvement over untreated MPTP model mice, with the trend increasing over the 6-week course of treatment and assessment. This difference was pronounced after only 1 week of treatment, suggesting that the convalescent effects of MS-818 are relatively fast acting. In the context of PD symptoms, such rapid pharmacokinetics may be beneficial in the treatment of late stage patients. Functional improvements in MPTP model mice are readily apparent in behavioral testing, providing direct evidence of the treatment's efficacy. Molecular data demonstrating the proliferation and migration of endogenous stem cells elucidate a possible

mechanism for this convalescent effect. Bridging the gap, observations of increased TH immunoreactivity suggests a mechanism of action by which PD pathology is reduced. As such, we believe based on these data that MS-818 is a promising candidate for treatment of PD. Stem cell proliferation and migration as promoted by MS-818 may also have the potential to apply to other neurological disorders. Acute brain trauma, as caused by stroke or blunt force is one such case. In rodent models of focal ischemia, treatment by MS-818 was demonstrated to decrease the size of the ischemic injury as well as reduce sensorimotor symptoms [75]. While delocalized neurological disorders provide a more challenging target for regenerative therapies, generalized promotion of endogenous NSCs proliferation and migration may nonetheless offer positive effects. Alzheimer's disease provides a model of a more distributed pathology for treatment with MS-818. Alzheimer's pathology leads to atrophy of neurons in a variety of brain regions [76] and changes in memory and behavior. Neural stem cell transplantation has improved the cognition of AD mice [59]. A similar increase in NSCs by increased endogenous proliferation may ameliorate AD pathology. In addition to neurological disorders, pathologies in other tissues related to deficient cell function or quantity may benefit from the promotion of endogenous stem cell proliferation and migration. As discussed above, mesenchymal stem cells reported increased metabolic activity following MS-818 treatment *in vitro*, suggesting that similar convalescent effects in other nuerodegenerative diseases. Proliferation of endogenous stem cells may provide a possible mechanism for the observed convalescent effects of MS-818 in non-neural contexts [50, 52–55]. MS-818 treatment not only increased NSC proliferation in the aged rats and PD mice model but also increased migrating neuronal phenotypes and neurogenesis. In behavioral tests of sensory-motor function significantly improved when compared to untreated mice, which indicates the formation of functional neuronal cells. Thus, MS-818 might be able to replace current major neurodegenerative therapeutic approaches, stem cell transplantation, and provides clinical studies in very near future since it does not have any ethical, immunological and technical issues always associate with the cell therapies.

5.5 Effect of Amyloid Precursor Protein on Stem Cell Biology

Genetic studies have showed that missense mutations in the APP and presenilins 1/2, encoded on chromosome 21, 14, and 1, respectively, cause early-onset familiar AD (FAD) [77–79]. Mutations of those genes enhance AD by upregulating the proteolytic process of APP, resulting in increase of Aβ peptide generation as well as extracellular senile plaque deposition.

APP is a type 1 single transmembrane protein, consisting of 695–770 amino acids. The APP gene is approximately 240 Kb, located on chromosome 21, and harbors 19 exons [80]. Depends on alternative splicing of exon 7, 8, and 15,

several different type of splice variants (APP695, APP751, and APP770) are generated in the cells with tissue specifically and each splice variants have distinctive characteristics. For example, APP770, containing exon 7, has a function in blood clotting since it contains Kunitz-type proteinase inhibitor (KPI) domain [81]. However, APP695, lacking KPI domain, is specifically expressed in neuron [82, 83].

The APP family consists of APP, APLP1, APLP2 [84–86], APL-1 in *C. elegans* [87], and APPL in *Drosophila* [88]. These APP molecules harbor several consensus motifs such as E1 and E2 and show higher sequence homology as well. Therefore, APP families share functional similarity and show functional redundancy in in-vivo AD studies.

Due to the cytotoxicity of Aβ peptides in the brain, mechanism of the proteolytic processing of APP has been intensively studied to understand the pathophysiology of AD. Generation of Aβ peptides result from various combination of the cleavage by β-, and γ-secretases. The major proteolytic cleavage of APP is performed by α-secretase (ex, ADAM10) [89], cleaves between residues Lys612 and Leu613 of APP, whereas β-secretase (ex, BACE1) cleaves APP after Met596 and Tyr606 [90–92]. Cleavage of α- and β-secretase produces soluble APPs (sAPPα and sAPPβ) as well as α- and β- carboxyl terminal of APP fragments (CTFs) of APP. Then, γ-secretase cleaves C-terminal of APP to liberate either amyloidogenic Aβ peptides or p3 in combination with BACE1 or ADAM10, respectively [93]. Furthermore, APP intracellular domain, functioning phosphoinositide-medidated calcium signaling [94], apoptosis [95] and transcription regulation [96], is generated by γ-secretase activity. Therefore, β- and γ-secretase has a critical role in Aβ generation and senile plaque deposition in AD (Fig. 5.5).

Despite the wealth of studies regarding the physiological function(s) of APP, there is little consensus between its function in vitro and in vivo. The main reason that physiological function(s) of APP is (are) still under debates is caused by the molecular complexity of APP. Due to its molecular complexity, various functions have been attributed in the same domain of APP in the CNS. Therefore, to investigate the physiological function of APP, the structural property of APP should be well understood. In general, functional domain of APP can be classified with E1 domain, central APP domain (CAPPD), E2 domain, and APP intracellular domain (AICD) [97]. E1 domain harbors growth-factor like domain (GFLD) (23-128 a.a) which has been know to have cysteine rich region and heparin binding site [97]. Since it contains highly positively charged surface, GFLD functions like a growth factor through protein-protein interaction with other counterpart molecules. Current studies have showed that GFLD is associated with cell adhesion, which contributes to neurite outgrowth and synaptogenesis [98]. Then, E1 domain is followed by the well-studied E2 domain. E2 domain contains RERMS sequence [99] and heparin-sulfate proteoglycan binding site [100], function as a growth promoting factor. AICDs are generated by subsequent cleavage of APP by γ-secretase/nicastrin complex. Since this domain has several important structural properties, AICD has been extensively studied and found that it is associated with multiple signaling pathways [94]. The structural properties of the cytoplasmic region of APP suggest

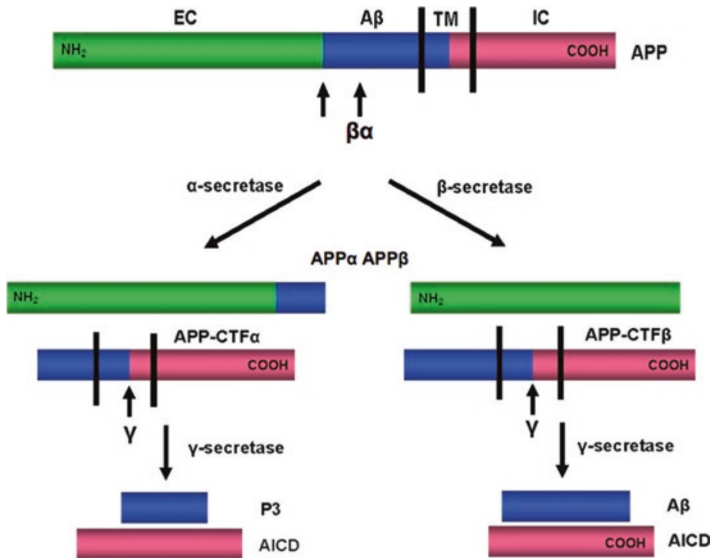

Fig. 5.5 Schematic diagram of sequential processing of APP by α-, β-, γ-secretases. *EC* extracellular, *IC* intracellular, *TM* transmembrane. Aβ domain is highlighted in blue

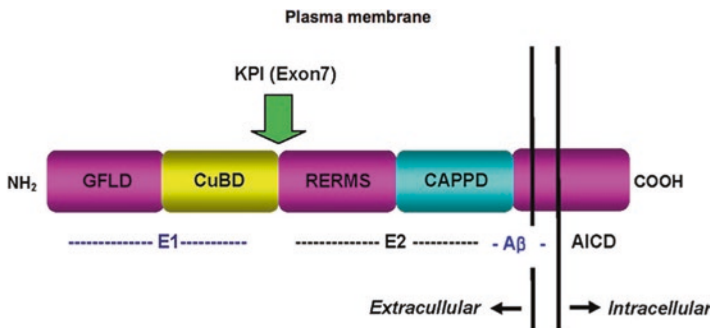

Fig. 5.6 Schematic diagram of domain organization of APP. E1 domain is composed with N-terminal growth factor like domain (GFLD) and copper-binding domain (CuBD). E1 is followed by E2/central APP domain (CAPPD) which is, so called, carbohydrate domain. Aβ indicates that Aβ domain. In APP751 and APP770, KPI domain is included by alternative splicing

that APP might have a role as a G-protein coupled receptor, via Go binding domain in AICD [101]. Above all, AICDs modulate signaling pathway by interacting with various partners, including Fe65, JIP, X11/Mint, and mDab1, via YENPTY motif [102] (Fig. 5.6).

To elucidate mechanism relevant to APP-induced glial differentiation, we examined the potential involvement of IL-6/gp130 and notch signaling pathway [103]. Since different members of the IL-6 cytokine have shown induction of distinct patterns of expression and phosphorylation status of signaling molecules involved in

IL-6/gp130 signaling pathway [104], treatment of APP may induce distinctive cellular responses. In our experimental condition, treatment of sAPP activates IL-6/gp130 signaling pathway via a physical interaction with gp130. Beside the result of immunoprecipitation, treatment of anti-gp130 antibodies, blocking a ligand binding site of gp130 [105], also showed that the interaction between APP and gp130 is crucial for activation of IL-6/gp130 signaling since the effect of sAPP was suppressed by anti-gp130 antibodies.

While a rapid phosphorylation of gp130 was induced in an early stage, phosphorylation of gp130 was quickly reduced back to basal level, presumably, by the internalization of ligand/IL-6R/gp130 complexes. In previous reports, when gp130 interacts with its ligands, it is targeted for degradation and gp130 should be synthesized before it appears in the membrane for preparing next cellular event [106]. Thus, treatment of sAPP significantly may promote the expression of gp130 from 60 min to maintain a certain amount of gp130 expression in the cell surface as a compensatory mechanism [107].

Our results have shown that treatment of sAPP promoted the expression of gp130, CNTF, and JAK1 in early son-set (<2 h) for glial differentiation. Thus, sAPP may also stimulate CNTF expression to activate the IL-6/gp130 signaling cascade. The expression of CNTF, a potent gliogenic factor [103], was increased in an early on-set of gliogenesis (<120 min) as well as up to 5 days in the presence of sAPP. However, since the application of siRNA of CNTF decreased GFAP expression, it reveals that CNTF may have the crucial function in APP-induced glial differentiation. Though further studies may be needed, treatment of sAPP may also induce glial differentiation by upregulating CNTF expression in NSCs.

JAK/STAT signaling pathway is an important regulatory system, implicated in GFAP expression, in glial differentiation [108]. Conformational changes of gp130 recruits non-receptor kinases such as JAKs and activated JAKs phosphorylate STAT3 molecules to form STAT3 homodimer complexes. Then, STAT3 complexes are translocated into nucleus to turn on GFAP expression. To examine whether signaling transducing molecules such as JAKs and STAT3 in APP-induced glial differentiation, we used siRNA and pharmacological inhibitor for suppressing the function of JAK1/2 or STAT3 molecules. Although treatment of sAPP enhanced phosphorylation of STAT3-p-Tyr705, siRNA of STAT3, JAK1 and JAK2 inhibitor (AG490) inhibited signal transduction, caused by APP, and diminished GFAP expression. Therefore, present results suggest that JAK/STAT molecules are crucial intracellular mediators of the sAPP-induced glial differentiation of NSCs.

The notch signaling pathway also has been known as an important glial differentiation mechanism of NSCs [109]. The treatment of sAPP promoted the generation of NICD and Hes1 gene expression, suggesting the activation of notch signaling in NT-2/D1 cells. However, since treatment of γ-secretase inhibitors (L-685,458) suppressed NICD generation, Hes1 expression, and GFAP expression in the presence of sAPP, our results indicate that sAPP can induce glial differentiation of neural progenitor cells via notch signaling pathway.

We examined mechanisms associated with APP-induced notch signaling activation to elucidate how sAPP stimulate notch signaling pathway. One possibility is

that sAPP may stimulate notch signaling cascade by increasing the expression of notch receptors and/or ligands such as Delta and Jagged. However, this possibility should be ruled out because mRNA expression level of Notch1, 2, Jagged, and Delta was not changed by treatment of sAPP. Instead, we found that APP can stimulate notch signaling cascade by physical interaction with notch receptors using immunoprecipitation. Several recent studies also have demonstrated a protein-protein interaction between APP and Notch in support of our findings [109–111]. Interestingly, N-terminal domain of APP (1-205) was enough to interact with Notch and promotes NICD generation as well as Hes1 expression. Also, APP (1-205) domain was sufficient to induce GFAP expression level in both protein and mRNA as well. It may be the reason that treatment of N-terminal recognizing antibodies (22C11) suppresses NICD generation as well as GFAP expression since a ligand/receptor binding is blocked by 22C11. Therefore, protein-protein interaction of APP with gp130 and notch receptor may be the crucial for the induction of glial differentiation.

Previously, Kamakura *et al.* demonstrated IL-6 signaling and notch can cross-talk and have a synergistic effect through interaction of Hes1 and JAK2 [112]. These complexes enhance the accessibility of STAT3 homodimers to promoter sites and potentiate the expression of target genes (ex, GFAP). Our results also indicate that treatment of γ-secretase inhibitor suppressed GFAP expression as well as STAT3 phosphorylation via cross-talking between IL-6/gp130 and notch signaling pathway. However, APP-induced glial differentiation may be not modulated by a synergistic way but induced by activation of both IL-6 and notch signaling for the glial differentiation. If APP-induced glial differentiation is occurred synergistically, although one signaling cascade (IL-6/gp130 or notch) is blocked by antibodies, siRNAs, or chemical inhibitors, we could observe certain level of GFAP expression, higher than control, because APP can still activate the other signaling pathway. However, when one signaling cascade is suppressed, overall GFAP expression level was decreased almost back to basal level. Therefore, our findings indicate that the activation of both two signaling cascade may be necessary for APP-induced glial differentiation (Fig. 5.7).

5.6 Conclusion

Still, consideration must be given to how transplanted cells will differentiate in the AD brain. Previous work by our lab has suggested that stem cells will have impaired neuronal differentiation following transplantation into an AD animal model [113, 114]. We observed both glial and neuronal differentiation in aged wild-type mice but it is uncertain if this will translate to efficient neuronal differentiation in a disease model [9, 35]. For example, transplanted NSCs to rodent models of AD differentiate into glial cells showing *in vivo* evidence that elevated levels of amyloid precursor protein induces glial differentiation [113, 114]. A closer examination *in vitro* demonstrated the dose-related effects of APP in regulating glial differentiation

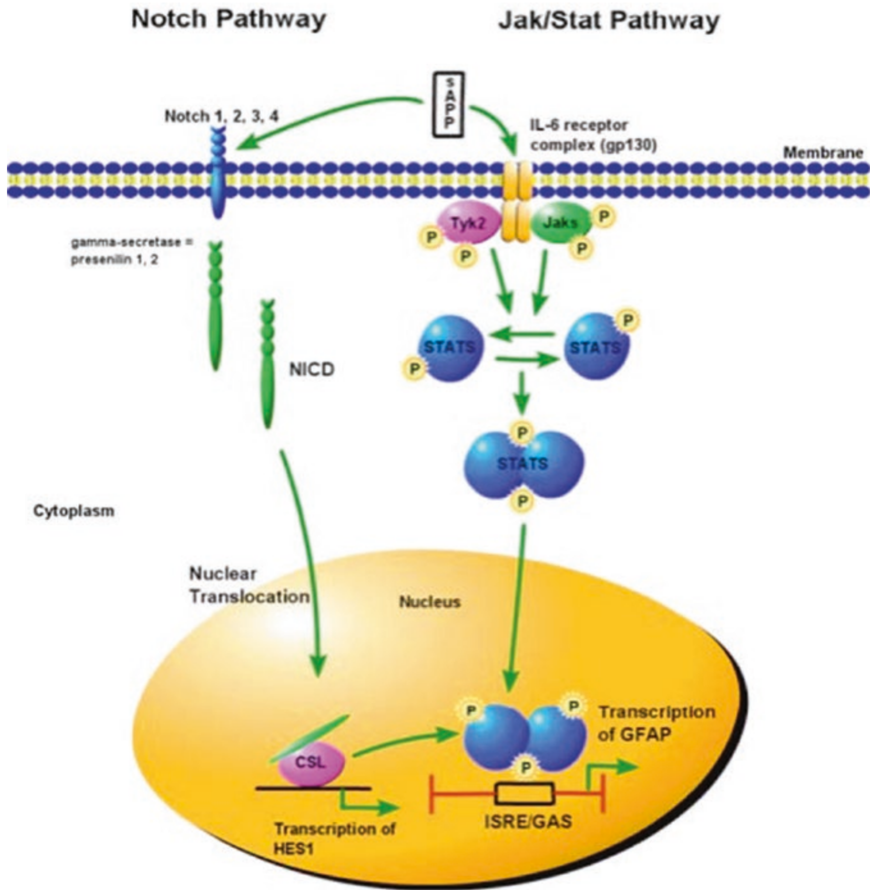

Fig. 5.7 Schematic diagram of sAPP-induced glial differentiation mechanism. Although the rate of neurogenesis of endogenous NSCs in the AD patient brain is slightly promoted in their early on-set, in the long run, pathologically-altered APP metabolism in AD or DS may cause a defect in neurogenesis and significantly destroy normal brain functions due to massive glial differentiation of endogenous NSCs. A gliogenic APP function could also prevent successful stem cell therapy for AD using NSC by influencing the differentiation of the transplanted cells into glial cells rather than into neurons. Thus, in order to use stem cell transplantation as a potential strategic intervention therapy for AD or DC, regulation of environmental APP levels and/or modifications of the APP signal pathways within the cells may need to be developed. Therefore, regulation of APP level could be a promising strategy to increase neurogenesis in AD brain

[113, 114]. Our lab has demonstrated that neuronal differentiation can be achieved by modifying the cellular environment to lower levels of amyloid precursor protein using a drug phenserine [115].

Induced pluripotent stem cell technology allow us to transplant patient's own cells to regenerate neural tissue. Exosome all us to eliminate the use of virus to accomplish this process and further to guid the differentiation of resulting iPS cells

to the target cells. We described a small molecule compound, which significantly increase NSCs by systemic administration. This compound, enhancing neurogenesis by not just increase proliferation but also increase the migration of the NSCs and neuroblasts, may be able to eliminate the transplantation of the cells. We further describe that effect of AD pathology, high concentration of APP, on NSC to increase glial differentiation of the cells and how to control the cell fate of NSCs by modifying the APP level. With all these technologies, we will be able to establish neurodegenerative diseases, including AD and PD, therapies based on the autologous stem cell transplantation and/or systemic administration of the drugs. We expect to have clinical studies based on these technologies in near future.

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Part III
Exosomes, miRNAs and Stem Cells in Cancer

Anfossi S, Fu X, Nagvekar R and Calin GA present a comprehensive review on how miRNAs regulate the biological functions of cells, including the signature phenotype of cancer cells, including drug resistance. The authors discuss how genetic and epigenetic changes in cancer cells lead to differences in the miRNA content of cancer cells. The authors showed how the changes in miRNA in cancer cells influence specific target genes. An intriguing section of the chapter focused on the role of the tumor microenvironment on miRNA expression with cytokines as mediators. Finally, the author showed the significance of miRNA changes by discussing how the miRNAs can mediate crosstalk between the microenvironment and the cancer cells.

Huang Y, Liu K, Li Q, Yao Y and Wang Y presented an overview that links this current era of immune therapy expansion with antibodies to the inhibitory receptors and chimeric antigen receptor. The authors presented an overview of exosomes as mediators between the tumor cells and the immune microenvironment. The authors described how this crosstalk affects the immune response to tumor. Based on the current literature, the authors proposed that the crosstalk between tumor cells and the surrounding microenvironment by exosomes contribute significantly to the tumor immune responses. The chapter discusses the roles of exosomes in promoting tumor cell survival, growth and chemoresistance. These functions are in addition to the exosomes regulating the immune response to benefit the tumor. The authors also proposed that exosomes could be employed as vehicles to deliver therapeutic targets for tumors.

Tickner JA, Richard DJ and O'Byrne KJ present a comprehensive chapter on microvesicles. The authors linked these vesicles to stem cells and cancer. The chapter discusses the role of extracellular vesicles (EV) in carcinogenesis. A broad view of the problem was addressed, including the role of EV on the immune system in the context of tumorigenesis. The chapter discusses the role of EVs in conditioning metastatic sites for tumors. Future challenges to identify how the contents of the MVs in carcinogenesis is addressed. Specifically, the MVs have individualized nucleic acid, protein, and lipid. Although these differences seem daunting, the authors remind the audience that the expression profiles of MVs often reflect the disease state. Hence, MVs might be diagnostic and prognostic. The chapter also expanded on other chapters to support a therapeutic role for MVs in drug delivery for cancer.

Sandiford OA, Moore C, Du J, Boulad M, Gergues M, Eltouky H and Rameshwar P describe the potential role of the aging microenvironment in cancer biology. The authors discussed how exosomes and microRNA (miRNA) as well as other non-coding RNA could mediate the changes in the environment, making cancer as a major risk. The authors introduced cancer stem cells and discussed the method by which the aging microenvironment within the lymphoid organ may propagate tumor progression as well as tumor dormancy. The authors suggested that age-associated cellular senescence could be a gradual process that began at middle age. MiRNAs, which are small non-coding RNA, can regulate cancer progression, recurrence and metastasis.

Chapter 6
MicroRNAs, Regulatory Messengers Inside and Outside Cancer Cells

Simone Anfossi, Xiao Fu, Rahul Nagvekar, and George A. Calin

Abstract MicroRNAs (miRNAs) are a class of short non-coding RNAs (ncRNAs) with typical sequence lengths of 19–25 nucleotides and extraordinary abilities to regulate gene expression. Because miRNAs regulate multiple important biological functions of the cell (proliferation, migration, invasion, apoptosis, differentiation, and drug resistance), their expression is highly controlled. Genetic and epigenetic alterations frequently found in cancer cells can cause aberrant expression of miRNAs and, consequently, of their target genes. The tumor microenvironment can also affect miRNA expression through soluble factors (e.g., cytokines and growth factors) secreted by either tumor cells or non-tumor cells (such as immune and stromal cells). Furthermore, like hormones, miRNAs can be secreted and regulate gene expression in recipient cells. Altered expression levels of miRNAs in cancer cells determine the acquisition of fundamental biological capabilities (hallmarks of cancer) responsible for the development and progression of the disease.

Keywords miRNAs · ncRNAs · Epigenetic · Genetic · Regulation · Exosomes · Cell-to-cell communication · Tumor microenvironment · Immunology

6.1 Introduction

The central dogma of molecular biology, which describes the flow of genetic information from DNA to proteins through the synthesis of RNA, can be summarized in a short sentence: "DNA transcribes RNA, which is then translated into proteins" [1]. However, protein-coding genes represent only 3% of the human genome. Our remaining genes code for RNAs that are not translated into proteins (ncRNAs). As their function was unknown for many years, these ncRNAs have been considered

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the dark matter of the human genome. Recently, there has been increasing evidence that ncRNAs actually play a critical role in the regulation of gene expression in both healthy and diseased cells [2, 3]. The family of ncRNAs can be divided into three major classes according to size: short, mid-length, and long ncRNAs. MiRNAs are short ncRNAs with an approximate length of 21 nucleotides, and due to their extraordinary abilities to regulate gene expression, they represent the most studied and characterized of all ncRNAs. Multiple cellular processes are controlled by miRNAs: proliferation, migration, invasion, apoptosis, differentiation, and drug resistance. Therefore, aberrant levels of miRNAs can alter these processes and lead to the development of cancer [4]. Genetic mutations (deletions, amplifications, mutation, translocations) and epigenetic alterations (methylation and histone modifications) are frequently observed in cancer cells and can be responsible for the dysregulated expression of miRNAs [5]. The tumor microenvironment can also regulate the expression levels of miRNAs of cancer cells. Soluble factors secreted in the tumor microenvironment, such as growth factors [e.g., epidermal growth factor (EGF)] and cytokines [e.g., transforming growth factor beta (TGF-β), IL-6], can affect miRNA expression [6]. Therefore, an altered tumor microenvironment, for example, during chronic inflammation, can have a great impact on tumor cell functions through the regulation of miRNA expression.

In the past few years, it has been discovered that miRNAs can also perform their regulatory functions outside the cells where they are expressed. Indeed, bioactive miRNA molecules can be secreted from their cells of origin into the extracellular space, be delivered to recipient cells (tumor and non-tumor cells), and regulate the recipient cells' gene expression in a hormone-like fashion [7]. In this way, tumor cells can regulate their surrounding microenvironments and create favorable conditions allowing them to survive, proliferate, escape from attacks by immune cells, and eventually disseminate to distant organs and generate metastases.

6.2 MiRNA Biogenesis

The past decade has seen an increasing interest in the roles of miRNAs in tumor cells, due to miRNAs' abilities to regulate the expression of genes controlling multiple cell processes frequently altered in cancer (e.g., cell cycle, proliferation, migration/invasion, differentiation, apoptosis) [4]. MiRNAs are some of the most abundant genes: there are 2588 miRNAs in humans, according to the latest miRNA database (miRBase). MiRNAs are short ncRNAs ~21 nucleotides (nts) in length, and they are encoded by sequences located within introns or exons of genes or in intergenic regions [8–11]. MiRNA genes are transcribed in the nucleus by RNA polymerase II (Pol II) as primary transcripts (pri-miRNAs), which are long several kilobases and contain characteristic hairpin structures. Following transcription, the pri-miRNA hairpin structure is recognized by a complex called Microprocessor—composed of the nuclear ribonuclease DROSHA (RNase III) and the essential cofactor DGCR8—which processes the stem-loop and generates a small hairpin-shaped RNA

(pre-miRNA) of ~65 nucleotides in length. Then, the pre-miRNA is exported to the cytoplasm, where it matures. The export step is mediated by a transport complex composed of the pre-miRNA, Exportin 5, and the GTP-binding nuclear protein Ran-GTP. The complex drives the pre-miRNA through the nuclear pores and into the cytoplasm where it is released following the disassembly of the transport complex. Next, the pre-miRNA is cleaved by a complex composed of DICER (RNase III-type endonuclease) and the transactivation-responsive RNA-binding protein (TRBP), forming a double-stranded miRNA molecule. During the final maturation step, this RNA duplex is incorporated into the RNA-induced silencing complex (RISC), whose primary component is the Argonaut protein 2 (AGO2). In the RISC, the double-stranded miRNA is unwound, generating two single miRNA strands: the mature (guide) and passenger strands [12]. In general, the passenger strand is quickly degraded, whereas the mature miRNA strand, which remains in the RISC, is guided to the target-site sequence of a messenger RNA (mRNA) to either inhibit mRNA's translation into protein or initiate its degradation. Particularly, miRNAs bind to specific binding sites in the 3′-untranslated regions (3′ UTRs) of mRNAs with different levels of complementarity, affecting the mechanism of translation inhibition. Perfect or near-perfect annealing between a miRNA and its binding site sequence induces the degradation of the target mRNA by RISC, whereas imperfect or partial annealing inhibits ribosomes' access to the target mRNA, blocking translation. The power of miRNAs in gene expression regulation is vast, as a single miRNA can target hundreds of different mRNAs. Therefore, altered expression of miRNAs can have significant impacts on many biological functions and pathways regulated by target mRNAs, leading to transformation of normal cells into tumor cells and the progression of cancer.

6.3 Regulation of miRNA Expression in Human Cancers

6.3.1 Genetic Alteration

Human miRNAs are frequently located at chromosomal fragile sites and in genomic regions involved in cancer [13]. These regions are associated with increased probabilities of various genetic alterations (such as deletions, insertions, amplifications, single point mutations, transitions, and transversions) that occur in different cancer types with different frequencies [5]. These genetic alterations have significant impacts on the cellular levels of miRNAs, leading to their aberrant expression and, accordingly, altered regulatory functions in several pathways involved in tumorigenesis and tumor progression. The first evidence of the correlation between genetic alterations and aberrant miRNA expression was found for the tumor suppressors miR-15a and miR-16-1, which are located at chromosome 13q14. Deletion of this region, which occurs in more than 65% of chronic lymphocytic leukemia (CLL) cases, as well as in 50% of mantle cell lymphomas, 16–40% of multiple myelomas,

and 60% of prostate cancers, causes a reduction in miR-15a and miR-16-1 levels [14, 15]. Other examples include the tumor suppressor miR-34a mapped on 11q23-q24, which is lost in breast and lung cancer; miR-123 located at 9q33, which is frequently deleted in non-small cell lung cancer (NSCLC); and miR-145 and miR-143 located at 5q32, which is frequently deleted in myelodysplastic syndrome [13]. On the other hand, genomic amplifications induce elevated levels of some miRNAs. The oncogenic miR-21 is located at 17q23 and is amplified in many tumors, including breast, colon, lung, pancreas, stomach, and prostate tumors [16]. The potent oncogenic miR-17-92 cluster is encoded by the 13q31-32 locus, which undergoes amplification in large B-cell lymphoma, follicular lymphoma, mantle cell lymphoma, and primary cutaneous B-cell lymphoma [13, 17] and is overexpressed in many cancer types, including lymphoma, colon, lung, breast, pancreas, and prostate cancer [16, 18, 19]. Elevated levels of miR-569 are associated with amplification of the 3q26.2 locus in ovarian and breast cancer [20]. Mutations at the DNA level can also affect the transcription of miRNAs or the maturation of pri- and pre-miRNAs, resulting in altered expression of mature miRNAs. Point mutations located in regions containing pri-miRNA recognition and processing motifs, which enhance pri-miRNA processing, determine reductions in mature miRNA levels. For instance, mutations in the basal UG and/or CNNC motifs affect the processing of pri-miR-16 and pri-miR-30a into their mature forms [21].

Mutations can also occur in the key regulators of miRNA biogenesis, DICER and DROSHA, greatly contributing to aberrant expression of miRNAs and cancer development. Indeed, mutations in DICER and DROSHA have been identified in diverse types of cancers and enhance cellular transformation and tumorigenesis [22–25].

6.3.2 Epigenetic Regulation

Besides the genetic alteration reported above, epigenetic regulation can also contribute to the aberrant expression of miRNAs in cancer. Indeed, DNA methylation and histone modification play important roles in the regulation of miRNA expression. Many miRNAs are embedded in CpG islands, and their promoter regions can undergo heavy methylation. DNA methylation and histone acetylation induce chromatin remodeling that regulates the transcription machinery's access to promoter regions, controlling miRNA expression [26]. The first evidence of alteration of miRNA expression by epigenetic changes was in breast and bladder cancer. Particularly, a rapid alteration of miRNA levels was measured in response to inhibitors of histone deacetylase (HDACi) [27] and DNA methylation [28]. Another important example of epigenetic regulation is represented by the miR-34 family, whose expression is repressed by hypermethylation in a variety of cancer types including breast, ovarian, esophageal, gastric, colon, renal, pancreatic, NSCLC, acute lymphocytic leukemia (ALL), and CLL. The downregulation of the tumor suppressor miR-34 is particularly relevant, as miR-34 cooperates with TP53 to suppress prostate cancer by regulating the stem cell compartment [29]. On the other

hand, hypomethylation can induce the overexpression of some miRNAs, such as the oncogenic miRNAs (oncomiRs) miR-21 and miR-29b. Hypomethylation determines high levels of these miRNAs in breast cancer and is associated with aggressive characteristics of tumor cells [30].

6.3.3 MiRNA Regulation by Oncogenes and Tumor Microenvironment

Other mechanisms can regulate the expression levels of miRNAs. Oncogenes and tumor suppressor genes can activate or repress the expression of miRNAs working as transcription factors or repressors. Therefore, either overexpression of oncogenes or downregulation of tumor suppressor genes can impact miRNA levels in cancer cells. For instance, TP53 directly transactivates miR-34 transcription by binding to the miR-34 promoter [31]. The oncogene *MYC* positively regulates the transcription of the miR-17-92 cluster and can also repress the expression of miR-34 [32].

The tumor microenvironment can also play an important role in regulating miRNA expression in cancer cells. A variety of cytokines produced in the tumor microenvironment are released directly by tumor cells or by tumor associated cells (e.g., immune and stromal cells) [33, 34] and can regulate the expression of miRNAs involved in tumor pathogenesis and progression. For example, the pro-inflammatory cytokine IL-6 induces the expression of *TWIST*, *SNAIL*, and *ZEB1*, key transcription factors that regulate the epithelial-mesenchymal transition (EMT). During EMT, tumor cells lose epithelial characteristics and acquire motility and invasive abilities [35, 36]. TWIST can bind directly to the putative promoter of miR-10b and induce its transcription [37], whereas SNAIL and ZEB1 bind to E-boxes in the miR-34a promoter, repressing miR-34a expression [38]. ZEB1 can also repress the expression of members of the miR-200 family by binding directly to their promoter sequences [39–41]. IL-6 can also suppress the expression of miR-34 through the activation of its signaling pathway mediator STAT3, which can bind to a conserved STAT3-binding site in the first intron of the miR-34 gene [42]. The TGF-β/Smad pathway regulates the expression of miR-155, and increased levels of miR-155 induce the acquisition of migration and invasion ability in breast cancer by targeting *RHOA* [43]. Additionally, growth factors can regulate the expression of miRNAs. For instance, EGF can modulate the expression of miR-30b, miR-30c, miR221, and miR-222, which play important roles in gefitinib-induced apoptosis and EMT in NSCLC [44].

Therefore, in addition to genetic alterations in tumor cells, soluble factors released in the tumor microenvironment play significant roles in the regulation of the expression of miRNAs in tumor cells. This may be particularly important under conditions of chronic inflammation [45, 46], both in the early stages of cancer formation and later during the progression of the disease, as cytokines released in the microenvironment can provide signals that regulate the expression of miRNAs

controlling tumor cell growth, differentiation, motility, angiogenesis, and resistance to treatment, as well as anti-tumor immune response [47–51].

6.4 miRNAs Regulate All Cancer Hallmarks

The hallmarks of cancer are the six fundamental biological capabilities acquired by tumor cells during the multistep process regulating the development of human tumors: self-sufficiency in growth signals, insensitivity to growth-inhibitory (anti-growth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis [52]. MiRNAs are involved in the regulation of all of these hallmarks. Accordingly, the aberrant expression of miRNAs significantly impacts the dysregulation of the stepwise cancer development. Some examples are reported below.

To move into an active proliferative state, normal cells require growth signaling provided by growth factors, extracellular matrix components, and cell-to-cell adhesion/interaction molecules. Whereas this dependence on growth signaling is a characteristic of normal cells, tumor cells show a greatly reduced dependence on exogenous growth stimulation. An example is the activation of the oncogene *RAS* that allows tumor cells to grow independently of external signals. It has been reported that all three *RAS* genes (*K-RAS*, *N-RAS*, and *H-RAS*) are directly regulated by the let-7 miRNA family [53].

Normal tissue is characterized by the presence of multiple antiproliferative signals that aim to maintain cellular quiescence and tissue homeostasis. The transcription factor *FOXO1* is a tumor suppressor that regulates cell cycle progression, proliferation, and apoptosis. *FOXO1* is the target of three miRNAs, miR-96, miR-182, and miR-183, and its repression by increased levels of these miRNAs leads to increased proliferation and reduced apoptosis [54].

Tumor cells' ability to expand in number is determined by the balance between the rates of cell proliferation and cell attrition. Apoptosis, also known as programmed cell death, represents a major source of attrition. Tumor cells are characterized by resistance to apoptosis. The tumor suppressor miR-34 induces cell cycle arrest and subsequent caspase-dependent apoptosis by targeting *BCL-2*, an important anti-apoptotic regulator [55].

Cells have a finite replicative potential, and after a certain number of cell divisions, they stop growing and enter into senescence. It was reported that during senescence, miR-29 and miR-30 are upregulated and target the oncogene *MYBL2*, inhibiting DNA synthesis [56].

Angiogenesis is an important process that supplies oxygen and nutrients for cell function and survival. Significant amounts of proangiogenic factors are produced and secreted in tumor tissue to promote neovascularization, which supports the growth of tumor cells. The vascular endothelial growth factor (VEGF) is the most important proangiogenic factor regulating neoangiogenesis and is highly expressed in most cancers. It is induced by low oxygen concentration (hypoxia) in the tumor

microenvironment. Hypoxia can modulate expression of several hypoxia-regulated microRNAs (HRMs), some of which, such as miR-210, miR-26, and miR-181, are directly controlled by hypoxia-inducible factor (HIF) [57]. It was found that miR-210 is involved in the regulation of endothelial cell chemotaxis and tubulogenesis [58]. Another HRM, miR-27a, targets the zinc finger gene *ZBTB10*, a negative regulator of the specific-protein (SP) transcription factors (such as Sp1, Sp3, and Sp4), resulting in the induction of Sp-dependent survival and angiogenic genes, including survivin (*BIRC5*), *VEGF*, and VEGF receptor 1 (*VEGFR1*) [59].

Metastasis is a complex process involving multiple steps that endow tumor cells with invasive properties (migration and invasion ability), intravasation (blood or lymphatic vasculature), blood circulation survival, extravasation, and growth at a new site [60]. It was reported that miR-10b can promote invasion and metastasis by targeting the transcription factor homeobox D10 (*HOXD10*) [37], which represses the expression of genes involved in cell migration and extracellular matrix remodeling: *RHOC*, α3 integrin (*ITGA3*), matrix metalloproteinase-14 (*MMP-14*), and urokinase-type plasminogen activator receptor (*UPAR*) [61].

Increasing evidence suggests that two additional hallmarks may be involved in the regulation of cancer development: reprogramming of energy metabolism and evasion of immune destruction. Tumor cells are able to adjust their metabolic pathways according to their energy requirements. The metabolism of glucose and glutamine represents the major source of energy for cells, and pathways using these two nutrients are often altered in cancer cells [62]. Oncogenes and miRNAs are involved in the regulation of these metabolic pathways. For instance, *MYC* modulates the metabolism of glutamine by repressing miR-23a/b, resulting in increased expression of their target protein, mitochondrial glutaminase [63]. Furthermore, glycolytic pathways are regulated by several miRNAs, including miR-378 [64] and miR-143 [65].

According to the immune surveillance hypothesis, the immune system plays an important role in recognizing and eliminating incipient and advanced stage tumors (metastasis). However, many tumors are able to evade immune cell attack using tumor immune escape mechanisms that include alterations in: tumor antigen processing and presentation by human leukocyte antigen (HLA) class I and II; signal transduction pathways; expression of co-stimulatory and co-inhibitory molecules; and secretion of immune-suppressive mediators [66]. Recently, the roles of miRNAs in regulating tumor immunogenicity and antitumor immune responses have been unveiled [67, 68]. For instance, miR-9 and miR-346 regulate the expression of MHC class I antigen processing machinery (APM) components and interferon (IFN)-induced genes [69, 70], and miR-148a and miR-181a target the expression of *HLA-C* and *HLA-A*, respectively [71, 72]. The major histocompatibility complex class I-related molecules (MICs) A and B are the ligands of the activating NK cell receptor NKG2D, which mediates NK cell-mediated cytotoxicity. The expression of MICA and MICB is controlled by miR-20a in breast cancer stem cells, resulting in reduced sensitivity to NK cell-mediated lysis and enhanced metastatic potential [73]. The B7 family includes both co-stimulatory and co-inhibitory molecules (CD80, CD86, CD28, CTLA-4, PD-1, PD-L1, PDL2, ICOSL) that play

important roles in immune responses [74]. It has been found that miRNAs can regulate the expression of B7 family members. For instance, the expression of *PD-L1* is controlled by miR-570 in gastric cancer [75], miR-34a in acute myeloid leukemia (AML) [76], miR-200 in NSCLC [77], and miR-138-5p in colorectal cancer (CRC) [78]. Recently, it was found that the expression of high levels of miR-124 could reverse the immunosuppressive phenotype of glioma cancer stem cells by targeting STAT3 signaling and reducing the generation of $FoxP3^{+}$ regulatory T cells (Treg) [79].

6.5 Circulating miRNAs as Tumor Biomarkers

Recently, it was observed that miRNAs can be released into the extracellular space [80] and can be detected in many biological fluids, such as serum, plasma, urine, saliva, and breast milk [81]. These circulating miRNAs can be actively secreted outside the cell either encapsulated within exosomes [82] or in an extracellular vesicle-free manner associated with the Ago2 protein [83, 84]. They can also be passively secreted into the blood circulation as a result of apoptotic [85] or necrotic cell death [86]. Importantly, aberrant levels of miRNAs can be detected not only in tumor cells but also in the biological fluids of cancer patients, possibly reflecting the expression patterns of the tumor tissues from which circulating miRNAs originate [87]. Due to their extraordinary stability in body fluids, resistance to storage handling, and the ease of assessment by quantitative PCR and miRNA microarrays [80, 88], circulating miRNAs are considered suitable biomarker molecules to differentiate normal from diseased states and monitor both progression of cancer and response to therapy (Table 6.1). Indeed, tumor-specific miRNAs were identified for the first time in the serum of patients with diffuse large B-cell lymphoma; in these patients, high levels of miR-21 were correlated with improved relapse-free survival [89]. Since then, many other studies have been published reporting the potential use of circulating miRNAs as tumor biomarkers in different types of cancer [7], such as miR-141 in prostate cancer [80], miR-486, miR-30d, miR-1, and miR-499 in NSCLC [95], miR-17-3p and miR-92 in CRC [96], miR-195 and let7-a in breast cancer [87], and miR-500 in liver cancer [97].

The potential use of tumor-specific miRNAs as diagnostic markers for cancer has been confirmed not only in serum and plasma but also in other body fluids, such as saliva and urine. For instance, levels of miR-125a and miR-200a were significantly lower in the saliva of cancer patients with oral squamous-cell carcinoma compared to healthy controls [100]. In another study, it was reported that miR-31 levels were higher in the saliva of patients with oral squamous-cell carcinoma compared to healthy controls, and a decrease in miR-31 levels was measured after tumor resection. The latter result was also demonstrated in plasma [101]. Finally, increased levels of miR-126, miR-152, and miR-182 were found in the urine of patients with bladder cancer, and the ratios of miR-126 to miR-152 and miR-182 to miR-152 could indicate the presence of bladder cancer with a specificity of 82% and a sensitivity of 72% [102].

Table 6.1 Circulating miRNAs in cancer

Type	Cancer	Deregulated miRNAs	Clinical uses	Ref
Serum/plasma	Diffuse large B-cell lymphoma	miR-21	Prognosis	[89]
	CLL	miR-125b and miR-532-3p	Rituximab response	[90]
	CLL	miR-155	Treatment response	[91]
	Myelodysplastic syndrome	let-7a and miR-16	Prognosis	[92]
	Prostate and breast cancer	miR-141	Diagnosis, prognosis	[80, 93]
	Prostate cancer	miR-21	Docetaxel-based chemotherapy response	[94]
	NSCLC	miR-486, miR-30d, miR-1, and miR-499	Prognosis	[95]
	Colorectal cancer	miR-17-3p and miR-92	Diagnosis	[96]
	Breast cancer	miR-195 and let7-a	Diagnosis	[87]
	Liver cancer	miR-500	Diagnosis	[97]
	Cervical squamous cell carcinoma	miR-1246, miR-20a, miR-2392, miR-3147, miR-3162-5p, and miR-4484	Lymph node metastasis	[98]
	Esophageal cancer	miR-200c	Prognosis and neoadjuvant chemotherapy response	[99]
Saliva	Oral squamous-cell carcinoma	miR-125a miR-200a, and miR-31	Diagnosis	[100, 101]
Urine	Bladder cancer	miR-126, miR-152, and miR-182	Diagnosis	[102]

miRNAs microRNAs, *NSCLC* non-small cell lung cancer, *CLL* chronic lymphocytic leukemia

Circulating miRNAs were also studied for their ability to predict prognosis and response to therapy. For instance, miR-125b and miR-532-3p predict the efficacy of rituximab-mediated lymphodepletion in chronic lymphocytic leukemia patients [90]. Six serum microRNAs can predict lymph node metastasis in cervical squamous cell carcinoma patients: miR-1246, miR-20a, miR-2392, miR-3147, miR-3162-5p, and miR-4484 [98]. In hormone-refractory prostate cancer, high serum miR-21 levels could identify patients who were resistant to docetaxel-based chemotherapy [94]. Circulating let-7a and miR-16 levels can predict progression-free survival and overall survival in patients with myelodysplastic syndrome [92]. Circulating miR-200c levels significantly predict prognosis and response to therapy in patients undergoing neoadjuvant chemotherapy for esophageal cancer [99]. Plasma levels of miR-155 can predict response to therapy in patients with chronic lymphocytic leukemia [91]. Finally, high levels of miR-141 in the serum of breast cancer patients were associated with shorter brain metastasis-free survival and were an independent predictor of both progression-free survival and overall survival [93].

6.6 Extracellular Vesicles Mediate Intercellular Communication in the Tumor Microenvironment

During the past few years, there has been increasing evidence to support the concept that miRNAs are able to mediate intercellular communication. This exchange of genetic information is mediated by extracellular vesicles (EVs), carrying miRNAs and other molecules, that are secreted by donor cells and taken up by recipient cells through several mechanisms [103] (Fig. 6.1). The first evidence of miRNA transfer was provided by Valadi et al. [82], who showed that functional RNA molecules (mRNAs and miRNAs) are transferred between mast cells through exosomes. Exosomes are extracellular vesicles of endosomal origin with diameters ranging from 30 to 100 nm [104]. The generation of exosomes is a highly controlled multi-step process [105] (Fig. 6.1). In the first step, the cell membrane buds inward, forming early endosomes in the endocytic pathway. Then, the early endosome membrane invaginates to generate multivesicular bodies (MVBs), each consisting of a large endosome containing exosomes of different sizes (called intraluminal vesicles or ILVs). During this second inward budding, the exosomes are loaded with different cellular components, including coding RNAs (mRNAs), short and long non-coding RNAs (miRNAs and lncRNAs), proteins, and DNA [82, 106–109]. In the final step, the fusion of MVBs with the plasma membrane allows the release of ILVs into the extracellular space as exosomes. The regulation of exosome formation, cargo

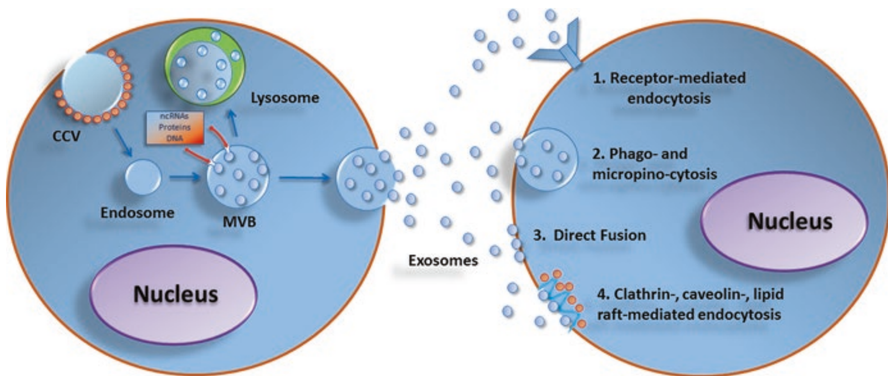

Fig. 6.1 Biogenesis of exosomes and cell-to-cell communication. Exosome biogenesis starts with the inward budding of the plasma membrane to form a clathrin-coated vesicle (CCV) and then an early endosome. Next, a second inward budding of the endosome membrane will generate a multivesicular body (MVB) containing exosomes. During the second inward budding, exosomes are loaded with their cargo (mRNAs, ncRNAs, proteins, and DNA fragments). The MVB can be directed either to the lysosome for degradation and recycling of MVB components or to the plasma membrane for secretion. Finally, the MVB fuses with the plasma membrane, and exosomes are released into the extracellular space. Secreted exosomes can be taken up by recipient cells through several mechanisms: (1) receptor-mediated endocytosis; (2) phago- and micropino-cytosis; (3) direct fusion with the recipient plasma membrane; (4) clathrin-, caveolin-, and lipid raft-mediated endocytosis

loading, and secretion involves several mechanisms [110]. Ceramide, synthesized by the neutral sphingomyelinase 2 (nSmase2), is involved in the budding of ILVs from MVBs and in exosome secretion [111, 112]. RAB proteins, such as RAB11, RAB27 and RAB35, participate in vesicle trafficking and exosome secretion [113]. The endosomal sorting complex required for transport (ESCRT) mainly regulates protein sorting into MVBs in a ubiquitin-dependent manner [114]; whereas miRNA loading into exosomes is regulated by ceramide and heterogeneous nuclear ribonucleoprotein A2B1 (hnRNPA2B1) [112, 115].

Exosomes are secreted from all types of cells and represent potent vehicles for cell-to-cell communication [116], as they can naturally deliver genetic and protein cargo to recipient cells and regulate these cells' biological functions. This intercellular communication mechanism is particularly important in cancer, as tumor cells produce significant amounts of EVs. Accordingly, the altered composition of cancer cell-derived exosomes' cargo can mediate dysregulated signaling. Furthermore, other components of the tumor microenvironment, such as mesenchymal stromal cells (MCS), cancer associated fibroblasts (CAFs), and immune cells (macrophages, dendritic cells, T cells, and NK cells), can participate in EV-mediated crosstalk with tumor cells and regulate their biological functions (Fig. 6.2). This generates a niche that facilitates tumor progression by regulating proliferation, differentiation, angiogenesis, metastasis, anti-tumor immune responses, and drug resistance.

Bone marrow mesenchymal stromal cells from the tumor microenvironment of multiple myeloma patients (MM BM-MSCs) support the growth of multiple

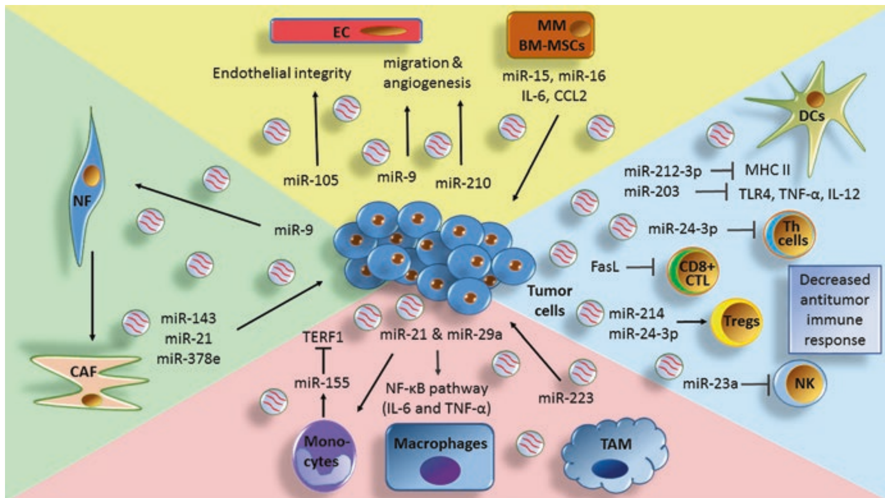

Fig. 6.2 Exosome-mediated cell-to-cell communication in the tumor microenvironment. Exosomes mediate intercellular communication between tumor cells and other cellular components of the tumor microenvironment. *EC* endothelial cells, *MM BM-MSCs* multiple myeloma bone marrow mesenchymal stromal cells, *DC* dendritic cells, *Th cells* T helper cells, *CD8+ CTL* CD8+ cytotoxic T lymphocytes, *Treg* T regulatory lymphocytes, *NK* natural killer cells, *TAM* tumor-associated macrophages, *CAFs* cancer-associated fibroblasts, *NF* normal fibroblasts

myeloma (MM) cells, whereas the bone marrow mesenchymal stromal cells from healthy donors (BM-MSCs) inhibit the growth of MM cells. Exosomes from MM BM-MSCs contain lower levels of the tumor suppressors miR-15 and miR-16 and higher levels of IL-6 and CCL2 compared with exosomes from BM-MSCs. The exosomes released from MM BM-MSCs can deliver their cargo to MM cells, playing a crucial role in MM pathogenesis, tumor growth, and disease progression [117]. The transfer of exosomal miRNAs to endothelial cells can promote angiogenesis and metastasis. It was found that exosomal miR-9 secreted by tumor cells induced endothelial cell migration and an in vivo increase in endothelial density, which promoted tumor growth [118]. Exosomes secreted by the leukemia cell line K562 carry miR-210, which increases tube formation by human umbilical vein endothelial cells [119]. Exosomal miR-210 can also be secreted by breast cancer cells and taken up by endothelial cells, promoting angiogenesis [112]. Exosomal miR-105 secreted from breast cancer cells can target cellular tight junctions and disrupt vascular endothelial barriers during early premetastatic niche formation [120].

Exosomes released by tumor cells can also contribute to the dissemination of malignant cells by remotely regulating a metastatic site. Indeed, exosomes from melanoma cells conditioned lymph node tissue and induced microanatomic niches that allowed metastasis of melanoma cells to lymph nodes [121]. Another study showed that exosomes released by renal cancer stem cells stimulated angiogenesis and the formation of a premetastatic niche in lung tissue [122]. Exosomes can also mediate communication between tumor and immune cells. Exosomes secreted from tumor associated macrophages (TAMs) can deliver miR-223 to breast cancer tumor cells, increasing their invasive abilities [123]. Cancer cell-derived exosomes containing miRNAs can also regulate the functions of immune cells. For instance, exosomal miR-21 and miR-29 released by mouse lung cancer cells can bind to Toll-like receptors (TLRs) 8 and 7 of mouse macrophages and activate the NF-κB pathway. This induces an inflammatory response mediated by TNF-α and IL-6, which promote tumor growth and metastasis [124]. An interesting miRNA-mediated bidirectional crosstalk between neuroblastoma (NBL) cells and monocytes was recently described. Particularly, exosomal miR-21 released by NBL cells can induce the expression of miR-155 in human monocytes. In turn, miR-155 is delivered from monocytes to NBL cells through exosomes and regulates resistance to cisplatin treatment [125].

Tumor cell-derived exosomes can also regulate the functions of immune cells present within the tumor microenvironment [126, 127]. Dendritic cells (DCs) play an important role in activation of anti-tumor immune responses, and their functions can be altered by tumor-derived-exosomes. For instance, exosomal miR-212-3p released from pancreatic tumor cells (PANC-1) can be transferred to DCs and affect their immune functions by inducing immune tolerance [128]. MiR-203 is expressed in PANC-1 cells, and its exosome-mediated delivery induced the downregulation of TLR4, TNF-α, and IL-12, resulting in impairment of immune response activation [129]. Exosomes from nasopharyngeal cancer cells containing miR-24-3p impair T cell proliferation and differentiation into Th1 and Th17 cells and promote the induction of T regulatory $CD4^+$ $CD25^{high}$ $Foxp3^+$ lymphocytes (Tregs) [130]. Tumor-

derived microvesicles can deliver miR-214 to $CD4^+$ T cells and promote the expansion of Tregs by targeting phosphatase and tensin homolog (*PTEN*), resulting in enhanced immune suppression [131]. High levels of miR-210 and miR-23a are present in hypoxic tumor-derived microvesicles and can be transferred to natural killer (NK) cells, leading to impairment of cytotoxicity against different tumor cells in vitro and in vivo [132]. Tumor-derived microvesicles inhibit proliferation and induce apoptosis of activated $CD8^+$ T cells [133].

Cancer-associated fibroblasts (CAFs) are the major components of tumor stroma and can participate in exosome-mediated crosstalk with tumor cells. Indeed, it was found that exosomes released by breast cancer CAFs had increased levels of miRs-21, -378e, and -143 compared to normal fibroblasts. Transfer of these exosomes to breast cancer cells induced a significantly increased capacity to form mammospheres, induced stem cell and EMT markers, and promoted anchorage-independent cell growth [134]. High levels of the pro-metastatic miR-9 are found in various breast cancer cell lines. Exosome-mediated delivery of miR-9 can modify the properties of human breast fibroblasts and promote in vivo tumor growth by enhancing the switch from the normal fibroblast (NF) state to the CAF state [135].

Exosomes can also play important roles in regulating drug resistance [136–138]. It was reported that exosomes from tamoxifen-resistant breast cancer cells ($MCF\text{-}7^{TamR}$) could transfer miR-221/222 and induce drug resistance in recipient breast cancer cells by targeting p27 (*CDKN1B*) and ERα (*ESR1*) [139]. In advanced renal cell carcinoma (RCC), the bioactive lncRNA named lncRNA Activated in RCC with Sunitinib Resistance (lncARSR) can be incorporated into exosomes. LncARSR can then transmit sunitinib resistance to sensitive cells by competitively binding miR-34/miR-449 to facilitate *AXL* and *MET* expression [140]. Exosomes can also perform their regulatory functions by interacting with drugs in the extracellular space. Indeed, it was found that exosomes released by the HER2-overexpressing tumor cell lines SKBR3 and BT474 express a full-length HER2 molecule, can bind to trastuzumab (anti-HER2 humanized monoclonal antibody), and accordingly reduce free molecules of trastuzumab. Exosomes with bound trastuzumab have been found in both HER2-positive tumor cell-conditioned supernatants and serum from breast cancer patients, which resulted in modulation of sensitivity to trastuzumab [141]. Drug-efflux pumps inserted in the exosome membrane can mediate drug sequestration from the cytoplasm. The presence of drug efflux transporters P-glycoprotein (P-gp) and ATP-Binding Cassette G2 (ABCG2) on EVs of breast cancer cells enabled the influx of drugs into the microvesicular compartment, resulting in an active sequestration of chemotherapeutic drugs from the cytoplasm [142, 143]. Then, chemotherapeutic drugs encapsulated inside EVs/exosomes can be expelled by active secretion [144, 145]. Interestingly, the transfer of EVs containing drug efflux pumps from drug-resistant to drug-sensitive tumor cells can contribute to the acquisition of multidrug resistance phenotypes by recipient cells [138].

6.7 Conclusions

MiRNAs play crucial roles in the regulation of physiological functions in normal cells; therefore, alterations in miRNA expression levels have significant impacts on cells' biology. Indeed, aberrant miRNA levels are associated with carcinogenesis and cancer progression. Dysregulation of the expression of miRNAs results from regulatory events at both the intracellular level (genetic and epigenetic modifications) and the extracellular level (signaling from the tumor microenvironment). Because a single miRNA has the potential to regulate the expression of up to 100–200 target genes, it is easy to understand how one or a few genetic alterations inside a cell or a disruption in homeostasis in the microenvironment can significantly impact many biological functions, as shown by the roles of miRNAs in regulating the hallmarks of cancer. Altered levels of circulating miRNAs can reflect a pathological status; therefore, these miRNAs can serve as predictive and prognostic biomarkers of cancer. Via secretion into the extracellular space, miRNAs can also perform their regulatory functions outside their cells of origin. Indeed, circulating miRNAs are important mediators of cell-to-cell communication, regulating cross-talk both locally, among different cellular components of the tumor microenvironment, and remotely, by regulating premetastatic niches.

A comprehensive understanding of miRNA functions at multiple levels will allow for the development of more precise and less toxic targeted treatments for cancer.

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Chapter 7
Exosomes Function in Tumor Immune Microenvironment

Yin Huang, Keli Liu, Qing Li, Yikun Yao, and Ying Wang

Abstract Immune cells and mesenchymal stem/stromal cells are the major cellular components in tumor microenvironment that actively migrate to tumor sites by sensing "signals" released from tumor cells. Together with other stromal cells, they form the soil for malignant cell progression. In the crosstalk between tumor cells and its surrounded microenvironment, exosomes exert multiple functions in shaping tumor immune responses. In tumor cells, their exosomes can lead to pro-tumor immune responses, whereas in immune cells, their derived exosomes can operate on tumor cells and regulate their ability to growth, metastasis, even reaction to chemotherapy. Employing exosomes as vehicles for the delivery products to initiate anti-tumor immune responses has striking therapeutic effects on tumor progression. Thus, exosomes are potential therapeutic targets in tumor-related clinical conditions. Here we discuss the role of exosomes in regulating tumor immune microenvironment and future indications for the clinical application of exosomes.

Keywords Exosome · Innate immune responses · Adaptive immune responses · Tumor microenvironment · Mesenchymal stem/stromal cells

Tumor immune microenvironment is one of the hallmarks of tumor growth, progression and therapeutics, always characterized as tumor-promoting inflammation and invalid immune surveillance for tumor cells [1]. The potential link between inflammation and tumors was first discovered by Rudolf Virchow in nineteenth century, when he observed the presence of leukocytes in tumors. In last decades, detailed analysis on the immune cells in neoplastic lesion clearly demonstrated that distinct cell types of the immune system, including T lymphocytes, B lymphocytes,

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macrophages, myeloid derived suppressor cells, mast cells, dendritic cells and neutrophils, were involved in tumorigenesis and progression [2, 3].

These distinct types of immune cells control tumor progression by functioning pro-tumor immunity or anti-tumor immune responses [4, 5]. For example, by employing mice genetically engineered to be deficient for certain subtype of immune cells or blocking their infiltration in tumor, $CD8^+$ T cells, nature killer cells, $CD4^+$ helper T (Th) cells were found to contribute significantly for immune surveillance. Avoiding immune destruction by these immune cells promoted tumor progression. Also, macrophages, myeloid derived suppressor cells, and neutrophils were found to be indispensable in constructing the pro-tumor immune microenvironments and dictating tumorigenesis and progression. Consistent infiltrations of these cells tightly relate to wound healing and chronic inflammation. Indeed, chronic inflammation, such as obesity induced inflammation, environmental exposure associated inflammation, damaged cells and senescence cells-induced inflammation can build up the pro-tumor inflammatory environment and enhance the risk for tumor. Meanwhile, pro-tumor inflammatory environment can be induced by malignancy cells. Investigations have demonstrated that some oncogenes mutation in stromal cells, like *myc*, *ras* and *p53* family member, can help to construct tumor immune microenvironments through recruitment of immune cells, production of various cytokines and chemokines, as well as inhibition of anti-tumor immune responses. Tumor promoting inflammation and anti-tumor immunity coexist during tumorigenesis and progression, while their balance in tumor was controlled by microenvironmental conditions. Likewise, the same type of immune cells can exert anti-tumor immunity in one tumor and tumor-promoting inflammation in another, relying on their cytokine profiles and functions in shaping tumor progression.

The formation of tumor immune microenvironment is shaped by the communication of diverse immune cells, more importantly, controlled by tumor cells and their surrounding stroma cells, including mesenchymal stem/stromal cells (MSCs), endothelial cells, and fibroblasts [2]. These tumor cells and tumor stromal cells talk with immune cells by means of direct contact or cytokine and chemokine production in an autocrine and paracrine manner.

Besides cytokines and chemokines, exosomes released by tumor cells and tumor stromal cells is found to be pivotal in shaping tumor immune microenvironment (Fig. 7.1) [6–9]. Exosomes, as one of the most imperative extracellular vesicles and microvesicles, generate inside multivesicular bodies, or can be formed and released by budding from plasma membrane [10]. Exosome contains plenty of DNA, mRNAs, miRNAs, as well as enzymes that are known to exert an assortment of functions to shape tumor immune microenvironment and control tumor progression. The measurement of exosomes in body fluid can indicate the risk of tumorigenesis and the prognosis of the established tumors or predict the therapeutic effect in various kinds of cancers, including gastric cancer, lung cancer and prostate cancer [11–13]. Notably, exosome-derived from tumor immune cells can act on tumor cells for their growth and metastasis. In this chapter, we will decipher the role of exosomes in mediating the crosstalk between tumor cells and tumor immune microenvironment.

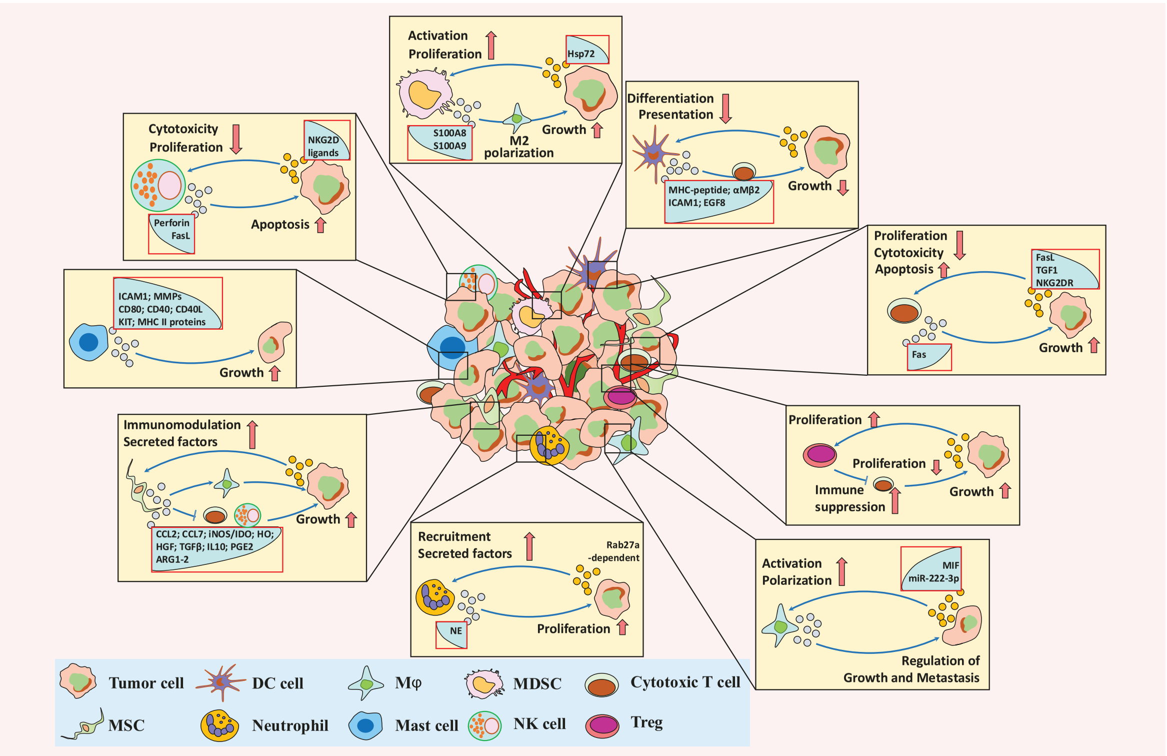

Fig. 7.1 Exosomes function in tumor immune microenvironment. In the tumor microenvironment, exosomes are released by various cell types, including tumor cells, stromal cells and immune cells. Tumor-derived exosomes participate in the recruitment, activation, differentiation, and proliferation of multiple immune cells and stromal cells. Meanwhile, exosomes produced by immune cells and tumor stromal cells regulate tumor progression and metastasis through modulating tumor microenvironment. Exosomes derived from NK cells and DCs are shown to promote tumor cell apoptosis directly or through inducing cytotoxic T cells, however, exosomes from mast cells, neutrophils, macrophages, MDSCs and T cells are shown to exert pro-tumor effects. MSCs serve as an essential member in tumor microenvironment, could also produce exosomes and promote tumor growth through blocking cytotoxicity responses of T cells and NK cells and inducing macrophages recruitment

7.1 Tumor Cell-Derived Exosomes Shape Innate Immune Responses in Tumor Microenvironment

7.1.1 Nature Killer Cells (NK cells)

NK cells are well-known in exerting immune surveillance and anti-tumor immunity by directly killing tumor cells or producing cytotoxic cytokines [14]. Those cytotoxic cytokines, such as perforin, were identified in NK cell-derived exosomes, and exerted cytotoxic activity against tumor cells [15, 16]. The dysfunction of NK cells in tumorigenesis and progression can be trained by tumor derived-exosomes. Exosomes produced by human prostate cancer cells contain ligands for natural killer group 2D (NKG2D). These exosomes downregulated the NKG2D expression on NK cells and impaired the cytotoxicity of NK cells [17, 18]. Detailed analysis on the ligands for NKG2D in tumor derived-exosomes found that MICA, MICB and ULBP1/2 are the major components in suppressing the cytotoxicity of NK cells [19]. Additionally, tumor derived-exosomes can inhibit the cytotoxicity of NK cells through suppressing perforin production [20]. However, exosome derived from tumor cells does not always destroy the cytotoxicity of NK cells. In cancer treatment, exosomes produced by hepatocellular carcinoma cells were found to contain plenty of heat shock proteins (HSPs). Consistent with studies revealing the inhibitory effects of tumor derived exosomes on NK cells, these HSP-bearing exosomes upregulated the expression of inhibitory receptor CD94, and decreased the expression of activating receptors CD69, NKG2D and NKp44. However, those exosome derived from tumor cells with chemotherapy treatment efficiently stimulated the production of granzyme B by NK cells, hence promoted the tumoradical function of NK cells [21]. Therefore, the effects of tumor derived exosomes on NK cells are still controversial and remain to be further investigated.

7.1.2 Dendritic Cells (DCs)

DCs are critical for antigen presentation and activation of adaptive anti-tumor immune response, as well as for cytokine production and immunosuppression in tumor progression [22]. DCs can process tumor antigen and present them by bound to MHC molecules on cell surface to CD4 and CD8 T cells, leading to the activation of T cells. Cytotoxic T cell activation can also be induced by exosomes released by mature DCs which harbored MHC-peptide complexes, thereby inhibiting tumor growth and eliminating established tumors [23–25]. Additionally, other membrane and immune-associated molecules were found in exosomes derived from DCs, including integrin α and β-chains (αMβ2), Ig family member ICAM-1and milk fat globule EGF factor 8, which are involved in the recruitment and activation of immune cells in the tumor microenvironment. More importantly, those membrane

associated molecules are responsible for the endocytosis of exosomes into target cells so that components in the exosomes could exert their effects efficiently [25–27]. The status of DCs in tumor microenvironment can be influenced by tumor cell-derived exosomes. In vitro experiments suggested that exosome products by TS/A mammary tumor cells could inhibit the process of DC differentiation from myeloid cells [28]. These exosomes were found to target on $CD11b^{+}$ myeloid precursors and induce interleukin (IL) -6 productions and its downstream signaling-STAT3, resulting in the blockade of DC differentiation. Additionally, tumor derived exosomes were link to impair the function of cytotoxic T cells through downregulating the expressions of CD11c and costimulatory receptors. Taken together, a strong relationship between tumor cells and DCs was functioned by exosomes.

7.1.3 Macrophages

Macrophages are the major cellular components of tumor immune arena. By classifying as type 1 macrophages (M1), they act anti-tumor immune responses by functioning as antigen presenting cells and producing type 1 IFN, IL-12, and nitric oxide. In contrast, type 2 macrophages (M2) are the common phenotype of tumor associated macrophages and form pro-tumor immunity. These cells always characterize with downregulated expression of MHC class II and IL-12, enhanced production of anti-inflammatory cytokines, such as IL-10, arginase, transforming growth factor β (TGFβ), as well as plenty of growth factors, and angiogenic factors [2]. The status of M2 was associated with the tumor progression and poor patient prognosis. In comparison of exosomes released by M1 and M2, study found that exosomes secreted from M1, but not M2, could enhance activity of lipid calcium phosphage nanoparticle-encapsulated Trp2 vaccine by enhancing antigen-specific cytotoxic T cell responses [29]. However, exosomes derived from M2 promoted breast cancer cell growth and invasion by transferring miR-223 [30].

The phenotype of macrophages can be fine-tuned by a wide range of stimuli and their production of mediators is specifically regulated by the signals received. In this process, exosomes released by tumor cells were verified as one of stimuli to regulate the status of macrophages. By isolating exosomes from epithelial ovarian cancer, microRNA-222-3p (miR-222-3p) was enriched and found to modulate the polarization of tumor macrophages to type 2 macrophages by targeting SOCS3 signaling pathway [31]. Moreover, studies found that exosomes derived from pancreatic ductal adenocarcinomas (PDACs) can stimulate kupffer cells and induce plenty of TGFβ production, subsequently, to form liver pre-metastatic niche by induction of fibronectin deposition and macrophage recruitment. Macrophage migration inhibitory factor (MIF) was assessed as the key component in these PDAC-derived exosomes in mediating the formation of liver inflammation and pre-metastatic niche [32].

7.1.4 Neutrophils

Neutrophils are one of the key participants in innate immune system and play both tumor-promoting and tumoricidal functions through productions of cytokines, proteases, and reactive oxygen species (ROS), as well as direct cytotoxicity and regulation of $CD8^+$ cytotoxic T cells (CTLs) responses respectively [2]. Some cytokines and mediators were found to be loaded by exosomes released by neutrophils, and promote tumor development. Neutrophil elastase (NE) was discovered in exosomes released by neutrophils and promoted the proliferation of epithelial lung cancer cells through the hydrolysis of insulin receptor substrate 1 (IRS-1) [33]. In turn, the status of neutrophils can be regulated and polarized by the stimuli in the tumor microenvironment, consequently, to shape tumor immune responses and modulate tumor progression. During the investigations on the key role of Rab27a in exosome production by breast cancer cells, exosomes were found to facilitate tumor progression by inducing systemic mobilization of neutrophils [34]. Thus, neutrophil related exosomes exert dual roles in tumor immune responses.

7.1.5 Myeloid-Derived Suppressor Cells (MDSCs)

MDSCs have been identified in tumor microenvironment as a population of immature myeloid cells with the ability to suppress T cell activation. In molding tumor immune microenvironments, exosomes released by MDSCs, containing S100A8 and S100A9, were shown to mediate the chemotaxis of granulocytes and induce the switch of macrophages toward a type 2 macrophage phenotype [35]. Notably, MDSC accumulation in tumor microenvironment can be induced by tumor cell-derived exosomes. During this process, Hsp72 enriched in tumor-derived exosomes was demonstrated to trigger STAT3 activation in MDSCs in a TLR2/MyD88-depedent manner through autocrine production of IL-6 [36].

7.1.6 Mast Cells

The recognition on mast cell function does not limit to their responsibility for allergic reactions and removal of pathogens. The accumulation of mast cells in tumor sites accounts for the construction of tumor immune microenvironment. It has been reported that mediators released by mast cells can promote tumor growth and angiogenesis, such as matrix-degrading enzymes (MMPs), vascular endothelial growth factor (VEGF), proteases (chymase), and inhibit tumor progression, such as inflammatory cytokines [37]. In 2001, the observation that mast cells could produce exosomes was firstly reported [38]. Exosomes delivered the regulatory signals released by mast cells to T and B cells, DCs, even tumor cells. A recent study demonstrated

that mast cell line HMC-derived exosomes transferred KIT protein to lung adenocarcinoma cells, consequently, to promote tumor growth by activating SCF signaling [39]. Other components carried by exosomes released by mast cells were also related to tumor progression, such as MHC II proteins, co-stimulatory (CD86, CD40, and CD40L), adhesion-related molecules (ICAM-1), as well as matrix metalloproteinase (MMP-2, MMP-9) [40]. However, further investigations should decipher the components of exosomes released by mast cells and their detailed functions in molding tumor microenvironment and dictating tumor progression.

7.2 Exosomes Mediate the Crosstalk Between Tumor Cells and Adaptive Immune Cells

Tumor antigens processed and presented as peptide complexes with MHC class molecule by antigen presenting cells initiate T cell mediated immunity [41]. Based on their functions and cytokine productions, T cells are classified as $CD8^+$ cytotoxic T cells and $CD4^+$ Th cells, which further divide into Th1, Th2, Th17 and regulatory T cells (Tregs) [42]. These T cells enriched in the tumor microenvironment can perform both tumoricidal effects and tumor-promoting effects, relying on their functions in lysis of tumor cells and production of cytotoxic cytokines, or in construction of immunosuppressive microenvironments [2]. Accumulating evidence showed that exosome is one of major mediators for intercellular communications among adaptive immune cells, tumor cells. Exosomes can deliver many biological molecules, including proteins, lipids and nucleic acids, to modulate the function of T cell subsets.

7.2.1 Effector T Cells

Cytotoxic T cells and Th1 cells are the major warriors in T cell-mediated immune surveillance and anti-tumor immune responses. In vivo experiments demonstrated that genetically deficiency in T cells or blockade of their cytotoxic molecules can promote tumorigenesis and progression. Indeed, their high expression in tumor-bearing host was correlated with the better survival of some cancers, such as colon cancer. Insufficient T cell-mediated anti-tumor immunity always accompanies in the tumorigenesis, progression and therapeutics. The reasons for impairing T cell function can be related to tumor derived exosomes. In vitro studies showed that exosomes released by tumor cells can suppress antigen-specific $CD8^+$ T cells through inducing their apoptosis [43, 44]. Studies found that the apoptosis of T cells in tumor microenvironment can be related to the high expression of FasL on the surface of exosome derived from tumor cells [45, 46]. Additionally, tumor derived exosomes could promote T cell apoptosis through regulation of PI3K/Akt signaling

pathway [47], both intrinsic and extrinsic pathways in induction of apoptosis [48], as well as STAT activity [8]. Also, two functional receptors for T cell activation, T cell receptor and IL-2 receptor, can be negatively modulated by tumor derived exosomes, leading to the inhibition of T cell proliferation [6, 49]. By assessing the influence of tumor-derived exosomes in lymphocyte responses, membrane-associated TGFβ1 in these exosomes was found to exert the inhibition on T cell activation, as well as the promotion on the suppressive function of Tregs [50].

7.2.2 Tregs

Tregs play a tumor-promoting effect through inhibition of anti-tumor immune responses and promotion of tumor angiogenesis, while they may exert suppression of pro-tumor inflammation under certain condition. The amount of Tregs in tumor-bearing host, especially those with breast [51], gastric [52], and ovarian cancer [53, 54], is indicative of poor prognosis [55]. Therefore, elimination of the appearance of Tregs in tumor microenvironment holds the promise in enhancement anti-tumor immunity and therapeutic outcomes. Distinct from effector T cells, Tregs are resistant to apoptosis induced by tumor-derived exosomes. A close relationship between tumor-derived exosomes and Treg induction was disclosed. TGF-β and IL-10 in exosomes mediated the conversion of $CD4^+CD25^-$ T cells into $CD4^+CD25^+Foxp3^+$ Tregs [56]. Those Tregs were with increased expressions of FasL, IL-10, TGF-β1, CTLA-4 [57, 58]. Similarly, after co-culture with tumor-derived exosomes, $CD4^+CD39^+$ Tregs showed higher levels of cyclooxygenase-2 (COX-2) and IL-10 [6]. These Tregs exerted their suppressive functions to limit T cell proliferation [59].

In turn, exosomes can be released by T cells with TCR signaling activation [60]. Theses exosomes led to the invasion of melanoma and lung cancer cell through secretion of MMP9, a critical enzyme in degradation of extracellular matrix component [61]. Also, exosomes released by Tregs is the mediator in construction of pro-tumor immune microenvironment. As demonstrated in B16 melanoma, exosome-derived from Tregs can inhibit T cell proliferation and IFN-γ production, as well as the cytotoxicity of $CD8^+$T cells, resulting in the destruction of anti-tumor immunity [62, 63].

7.3 Towards a Broader Understanding of Exosomes in Tumor Immune Microenvironment

Besides various types of immune cells, other cellular components in tumor microenvironment, including MSCs, fibroblasts, as well as endothelial cells, have active roles in tumor initiation, promotion, progression and metastasis. Among them, MSCs are enriched in tumor sites [64]. By sensing signals released by tumor, MSCs

were found to actively migrate to tumor sites and orchestrated the tumor immune microenvironments, together with immune cells [65]. After arriving at tumor sites, MSCs licensed by inflammatory cytokine, tumor necrosis factor α (TNFα), will change into tumor MSCs. These tumor MSCs can build up the pro-tumor immunity by facilitating the accumulation of monocytes, macrophages, and neutrophils in tumor microenvironments, with the capability to promote tumor growth and metastasis. Interestingly, tumor MSCs can endow normal MSCs with the similar potential in forming pro-tumor immunity [66]. Yet, the function of exosomes in their communication remains unclear. Detail analysis on the ménage-à-trois among tumor cells, MSCs and immune cells during the tumor growth found that tumor cell-derived exosome could educate normal MSCs with a pro-tumor phenotype. In this process, exosomes can be uptaken by MSCs and promote the enriched production of CCR2 ligands (CCL2 and CCL7), which are responsible for macrophage recruitment [67].

Multiple suppressive factors expressed by MSCs are reported to mediate their immunosuppression, including indoleamine 2, 3 dioxygenase (IDO), inducible nitric oxidase synthase (iNOS), hemeoxygenase (HO), arginase 1 and 2, hepatocyte growth factor (HGF), TGF-β, IL10 and prostaglandin E2 (PGE2) [68]. Exosomes isolated from human MSCs was also demonstrated to exert an inhibitory effect on T cell activation and IFN-γ production [69, 70]. Similar to the license function of inflammation on MSC immunosuppression, exosome-derived from MSCs with inflammatory cytokine stimulation contained multiple mediators to suppress the proliferation of T cells, B cells and NK cells, as well as the differentiation of plasma cells and antibody production [71, 72], and to induce Tregs [73]. Other stromal cells, such as cancer-associated fibroblasts and endothelial cells are critical in regulation of tumor growth, angiogenesis and metastasis. Exosomes derived from cancer-associated fibroblasts or endothelial cells can transfer the "signals" to tumor cells and promote tumor progression, yet their roles in building up tumor immune microenvironments need further investigation.

7.4 The Application of Exosomes in Tumor Immunotherapy

Not limited to pro-tumor immunity, exosomes were found to enhance anti-tumor immunity based on the diversity of their cargos, indicating their potentials in tumor treatment [74]. HSP, known to function as an endogenous signal that can increase the immunogenicity of tumors, were found in exosomes and promote the cytotoxicity of T cells and NK cells [75–78]. By employing the carrier function of exosomes, strategies were developed by modifying exosomes with high levels of tumor antigens or certain chemokines. These antigen anchored or chemokine carrying exosomes can efficiently recruit anti-tumor immune cells to the tumor sites and induce tumor-specific cytotoxicity, thereby resulting in more obvious inhibition on tumor growth [79, 80]. The optimized strategies were employed by isolating exosomes from TLR agonist activated DCs. These exosomes can induce robust activation of

tumor specific lymphocytes and promote the recruitment of cytotoxic immune cells (T cells, NK cells, and NK T cells) to the tumor site, leading to the significant suppression on tumor growth [81]. In 2008, a phase I clinical trial showed that administration of DC-derived exosomes and GM-CSF can ameliorate colorectal cancer progression through induction of tumor specific cytotoxicity by T cells [82]. Therefore, exosomes can be modified to express tumor antigens or mediators to enhance anti-tumor immunity. These armed exosomes hold the great promise in tumor treatment.

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Chapter 8
EV, Microvesicles/MicroRNAs and Stem Cells in Cancer

Jacob A. Tickner, Derek J. Richard, and Kenneth J. O'Byrne

Abstract The role of extracellular vesicles (EV) in carcinogenesis has become the focus of much research. These microscopic messengers have been found to regulate immune system function, particularly in tumorigenesis, as well as conditioning future metastatic sites for the attachment and growth of tumor tissue. Through an interaction with a range of host tissues, EVs are able to generate a pro-tumor environment that is essential for tumorigenesis. These small nanovesicles are an ideal candidate for a non-invasive indicator of pathogenesis and/or disease progression as they can display individualized nucleic acid, protein, and lipid expression profiles that are often reflective of disease state, and can be easily detected in bodily fluids, even after extended cryo-storage. Furthermore, the ability of EVs to securely transport signaling molecules and localize to distant tissues suggests these particles may greatly improve the delivery of therapeutic treatments, particularly in cancer. In this chapter, we discuss the role of EV in the identification of new diagnostic and prognostic cancer biomarkers, as well as the development of novel EV-based cancer therapies.

Keywords Extracellular vesicles · Non-coding RNA · Cancer · Mesenchymal stem cell · Exosomes · miRNA

8.1 EV as Novel Cancer Biomarkers

The need for novel cancer biomarkers is fundamental in improving patient outcomes. This search has resulted in the emergence of EV as new predictive, diagnostic, and prognostic factors in cancer. EV can be obtained from virtually any body fluid or tissue, by safe and minimally invasive or non-invasive methods.

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Additionally, the intrinsic nature of EV protects the internalized (and external, to a degree) contents from host and environmental degradation, allowing easier EV isolation and storage. As EV are often released in higher concentration from tumor tissue, and the expression profile often mimics/reflects host cell expression profiles, they can be used as a liquid biopsy of the cancer tissue, even tissue that is unreachable via conventional methods [1–4]. EV may also be used as a future indicator of disease in healthy populations, leading to improved health planning and patient outcomes. This aids in determining the most effective treatment options, resulting in decreased economic burden and fewer unwanted side effects in patients.

Two major challenges exist in the development of EV diagnostics and prognostics in cancer. The first challenge is our limited understanding of the spectrum of signaling options that are available due to the complexity of EV surface expression. The sometimes-low concentrations of certain EVs, as well as the diversity and heterogeneity of EV type and expression profile also hamper development [5–7]. This will be improved with biobanking of both healthy and diseased tissue for adequate comparative analyses [7]. This problem is common in emerging diagnostics/prognostics and requires substantial resources and investment to generate a reliable and affordable repository. The second challenge is the development of economical methods of isolating and analyzing EV from samples. Though the liquid biopsy is a safe and effective method, high-sensitivity methods of isolating and characterizing EV are only beginning to be established [7, 8].

EV contain a varied assortment of factors, that present significant diagnostic and prognostic potential in cancer treatment. For these purposes, EV are most often obtained from patient serum, though plasma and urine are also easily utilized [9, 10]. Factors isolated from EV not only discern healthy from diseased patients but can also be effective in staging disease. Many studies have identified EV nucleic acid, particularly miRNA, as an effective cancer biomarker [11–17]. These studies identified many indicative miRNA species in a vast array of cancers, often using quantitative PCR and/or sequencing for RNA detection [18, 19]. Undoubtedly many studies utilizing serum miRNA as diagnostic and prognostic disease markers have accidentally harvested exosomal miRNA. In fact, exosomal miRNA may represent a significant fraction of commonly isolated miRNA in some studies. Other nucleic acids that have been identified as demonstrating biomarker potential are mRNA, DNA (containing oncogenic mutations), short non-coding RNA, and circular RNA [20–25]. Much like EV miRNA, many studies have utilized mass spectrometry techniques to identify an array of proteins that are highly indicative of disease state [10, 26–28]. Protein markers have thus far demonstrated significant potential, with a recent study identifying a marker that displayed unprecedented accuracy in diagnosing and staging disease state in pancreatic cancer patients [29]. Analysis of the lipid composition of EV has shown lipid expression profiles may also be a potential cancer biomarker [30].

8.2 EV Biomarker Technology in Cancer

The future of EV as diagnostic and prognostic markers in cancer relies on the development of systems that rapidly capture and identify markers of disease. Common methods for isolating EV for biomarker analyses include standard isolation techniques based on filtration combined with ultracentrifugation, and immunoaffinity capture methods [6, 7]. Though effective, the cost of these technologies is currently prohibitive for large scale implementation [31]. Thus, new technologies are being developed to utilize the vast content of EV for therapeutic purposes. Recent developments in the modification of existing technologies used in liquid biopsy analysis have already provided new diagnostic methods [8, 31]. These include several effective immunoaffinity capture methods, including the *ExoChip*, *ExoScreen* and *ExoSearch* technologies, that allow rapid identification of specific EV markers associated with oncogenesis [32–34]. Fortunately, EV factors can be identified using a range of methods including PCR, mass spectrometry, nuclear magnetic resonance, and immunofluorescence [26, 35–41]. Two diagnostic EV technologies are currently available that identify RNA signatures in the urine of prostate cancer patients and the serum of lung cancer patients (www.exosomedx.com) [42, 43]. These markers help diagnose disease and determine treatment options. Although only two methods are currently available, many clinical trials utilizing EV-based technologies in cancer diagnostics are under investigation.

8.3 Novel Role of EV in Cancer Therapy

The burgeoning area of EV function in cellular communication derives from their ability to protect and transport a range of cargoes to a wide array of tissues [3, 19, 44–48]. This ability is being utilized in the development of novel therapies in the treatment of many diseases, particularly cancer [8, 49–51]. Most EV-based therapies utilized natively-derived (obtained from patients) or semi-synthetic/bioengineered EV (mimetics) that deliver compounds which either activate/enhance antitumoral immune responses (cancer vaccines) or deliver antiproliferative agents directly to the tumor tissue (therapy delivery) [52–69]. Apart from the aforementioned vaccination and therapy delivery, the removal of EV or inhibition of EV production to reduce cancer growth and/or pre-metastatic niche formation is also evaluated [70–74]. This has been investigated via the reduction of Rab27a protein expression, as well as the removal of circulating EV via filtration or immunoaffinity capture [6, 31, 75–78].

EV make excellent delivery vehicles due to their bioavailability and lack of unwanted immunogenicity. When compared with the delivery of soluble factors alone, EV-internalized or associated factors often display increased efficacy with minimal off-target/side effects [56, 79–81]. The complexity and hence similarity of

exosomal surface expression to host cells both increases the effectiveness of EV as delivery systems, as opposed to synthetic vehicles, and reduces unwanted immune responses due to their syngeneic nature [8, 80, 82]. This can result in increased uptake of exosomal contents by host cells compared to synthetic particles, such as liposomes [49, 50]. This is advantageous in the delivery of certain compounds, such as chemotherapeutics, where tumor uptake is enhanced (increased tumor cytotoxicity) while unwanted drug deposition is reduced (reduced side effects). This complexity also permits the encapsulation of multiple compounds that could target several cell types or targets.

However, there are also disadvantages to using biological EVs as therapeutic vehicles [8, 51]. Sometimes generalized increased uptake is not required, but more limited and specific uptake in certain sites or tissues. Although synthetic EV can have unwanted toxicity and immunogenicity, enhanced immunogenicity may be required to maximize antitumor effects. These issues require a modified delivery system that does not necessarily prevent uptake of the nanovesicle, but prevents content release unless the desired inter/intracellular conditions are met. With current technology, synthetic particles have been advantageous in this respect, as the regulation of surface expression is far easier, and the particle structure can be easily modified to prevent release at unwanted sites, such as low or neutral pH [83–86]. Thus, the two main advantages of synthetic and semi-synthetic EV delivery systems are that the manufacturing process limits unwanted variability/heterogeneity (an issue when utilizing current biological systems for EV generation), and that synthetic EV can be generated on large scale, suitable for drug delivery or vaccination. Future therapies will most likely rely on a combination of these methods, as well as the generation of EV mimetics, a type of EV of biological origin, generated via non-biological mechanisms [67, 68, 87, 88].

8.4 Generation and Modulation of EV for Cancer Therapy

As of 2016, there were no commercial EV-based therapies available for the treatment of cancer. Although synthetic nanovesicle delivery systems have been established in the treatment of array of diseases, the potential of EV to deliver therapeutic compounds is beginning to be elicited [8, 51]. The generation of EV to be used in cancer treatment relies, fundamentally, on two methods; the isolation of EV from the patient, tissue, or cell culture, followed by modification (drug, protein, nucleic acid, lipid) and reintroduction to the patient as treatment; or the large-scale isolation/fabrication of EV from cell culture, bioreactor or animal body fluid, again, followed by modification and introduction to the patient. *Ex vivo* modification of EV is often required to regulate antigen presentation or surface expression in order to modulate immunostimulatory potential and enhance selective uptake and delivery of EV contents [80, 85, 89, 90]. These contents can be internalized utilizing a range of methods. The cells used to generate the EV can be treated with factors that

regulate EV expression of protein and nucleic acid, and to produce exosomes that contain said factor [60, 85]. EV themselves can also be treated to incorporate specific contents. Simple incubation can facilitate uptake of certain compounds, while more complex methods, such as electroporation or enzymatic poration can also be used [49, 51, 62].

Though EV can be isolated from nearly all cell types and bodily fluids, exosome production for cancer therapy is limited. This includes primarily dendritic cells, cancer cells, and stem cells, each having distinct advantages and disadvantages. The first study to demonstrate the effectiveness of EVs as a mechanism for delivery showed that EV could deliver siRNA while effectively crossing the blood blain barrier [19, 57]. Though not a cancer treatment, the use of the host's EV for therapy propogated widespread interest in this method. In this study, dendritic cells were harvested and modified before reintroduction into the host, but these are not the only cell types that can be used in the production of therapeutic EV [19]. Regardless of the method utilized, substantial data indicates the necessity for diligent selection of the cell type to be used due to unwanted side-effects. These effects are intrinsic due to the heterogeneity in surface expression of EV.

Besides the significant changes in yield between and within these methods, the most important consideration is the surface expressed factors that dictate uptake, as complex EV expression profiles can obscure other functions [91]. The use of EVs as therapy requires the utmost stringency in the selection, isolation, and preservation to ensure patient safety. Exosomes derived from cancer cells tend to express higher levels (sometimes only) of MHC class I and a diverse array of growth factors, while EV from dendritic cells tends to express higher levels of MHC class II and lower amounts of growth mediators [8, 81, 92–97]. EV from mesenchymal stem cells (MSC) have been shown to be anti-inflammatory but can both enhance and inhibit tumor growth in different contexts [98]. Depending on whether the chosen method is to engage the immune system or directly kill tumor tissue, certain complications are inherent to EV-producing cell types and may have both positive and negative effects for the development of novel treatments. For example; aiming to generate an immune response that engages and destroys tumor tissue may have indirect proliferative effects on tumor tissue, while directly targeting tissue with EV cytotoxic drugs may compromise anti-tumor immune responses. Thus, modification of surface expressed factors is often required to elicit effectiveness, by improving immunogenicity or cytotoxicity.

Of the cell types discussed, MSC have shown the most potential, due to their low immunogenicity and ability to generate substantial quantities of EV [99, 100]. They are also relatively easy to obtain from patients allowing for personalized treatment. Recently, the use of bioreactors to culture adipose-derived MSC was shown to increase EV yield approximately 100-fold compared to conventional culturing methods [101]. Other methods for the large-scale purification of EV include harvesting from bovine milk, or the generation of EV mimetics, generated via serial extrusion [67, 68, 87, 88, 102]. This process generates nanovesicles of identical biological composition to EV, opening their potential for use in therapy.

8.5 Therapeutic Contents of EV in Cancer Therapy

Therapeutic contents of EV utilized in the treatment of cancer consist primarily of RNA or chemotherapeutics. Several studies have investigated the delivery of compounds via modified EV derived primarily from MSC. MSC-derived EV containing miRNA and anti-miRNA could increase sensitivity or re-sensitize tumor tissue to chemotherapeutics, and inhibit tumor growth [53, 64–66] . The efficacy of these methods can be improved my modifying the expression profile of the EV, resulting in greater uptake by target cells. The use of therapeutic siRNA is also being investigated, where preliminary studies have shown significant increases in mRNA depletion, leading to substantial decreases in cancer cell proliferation and viability [54, 56, 103]. EV, particularly from MSC, have also been used to enhance the effect of chemotherapeutics [58, 59, 61]. MSC treated with chemotherapeutics release large quantities of drug-containing EV. These EV can be more effectively used to deliver compounds to target cells [60]. Off-target effects can be further minimized by delivering modified EV that contain enzymes which activate prodrugs in tumor tissue [54]. Prodrug accumulation in other tissues is insignificant as the negligible levels of EV uptake by non-cancerous cells minimize drug activation. Currently, only two trials have investigated EV as method for drug delivery in cancer treatment, both utilizing plant-derived EV to either enhance the delivery of chemotherapeutics to tumor tissue (NCT01294072) or minimize side-effects of standard therapy (NCT01668849).

EV can also be utilized to deliver cargo that activates or enhances anti-tumor immune responses, producing a retroactive cancer vaccine [80, 95, 97, 104–106]. EV from tumor cells, and particularly dendritic cells, can contain be induced/modified to express/contain increased levels of MHC complexes for antigen presentation, as well as immunostimulatory components, such as heat shock proteins, interferon, and granulocyte macrophage colony stimulating factor [8, 81, 92–97]. These EV serve to enhance cytotoxic T-cell and Natural Killer cell responses against tumor tissue. Thus far, trials have investigated EV as an anti-cancer vaccine in lung (NCT01159288) and colorectal cancer, as well as malignant glioma (NCT01550523, NCT02507583). Studies investigating malignant glioma utilized a novel method for EV delivery. Rather than systemic delivery of EV, modified glioma cells captured within diffusion chambers were surgically inserted in the patient. As the glioma cells undergo apoptosis due to prior *ex vivo* modification, they release a range of vesicles, in particular EV, that serve to stimulate glioma-specific anti-tumor immune responses [107]. Although showing great promise, EV-based therapies for cancer have yet to make it to market.

8.6 Summary

EV are intriguing and present a new paradigm in our understanding of the dynamics of cancer pathology and treatment. Though the function of exocytosis in oncogenesis is not fully understood, many studies have demonstrated the capabilities of EV

in many aspects of cancer diagnostics and treatment. Though EV-based cancer treatments are still in clinical trials, EV-based biomarkers have recently become available for cancer diagnosis. With an increased understanding of the complex signaling potential of EV, combined with rapid and sensitive analysis methods, these nano-sized particles will undoubtedly provide a range of new options in cancer treatment.

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Chapter 9
Human Aging and Cancer: Role of miRNA in Tumor Microenvironment

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Abstract Human aging is an inevitable and complex phenomenon characterized by a progressive, gradual degradation of physiological and cellular processes that leads from vulnerability to death. Mammalian somatic cells display limited proliferative properties *in vitro* that results in a process of permanent cell cycle arrest commonly known as senescence. Events leading to cellular senescence are complex but may be due to the increase in tumor suppressor genes, caused by lifetime somatic mutations. Cumulative mutation leaves an imprint on the genome of the cell, an important risk factor for the occurrence of cancer. Adults over the age of 65+ are vulnerable to age related diseases such as cancers but such changes may begin at middle age. MicroRNAs (miRNAs), which are small non-coding RNA, can regulate cancer progression, recurrence and metastasis. This chapter discusses the role of miRNA in tumor microenvironment, consequent to aging.

Keywords miRNA · Breast cancer · Aging · Hematopoiesis · Microenvironment · Bone marrow

9.1 Introduction

In cellular terms, aging can be seen as a gradual loss of cell function through a complex process such DNA damage, telomere shortening, oxidative stress, and chromatin structural rearrangements. These age-related changes have all been implicated to cause the loss of the cells' functional integrity [1]. A well-studied system is the hematopoietic source organ, which have been proved to have diminished potential as the individual age, resulting in increased incidence of hematological malignancies

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in the aging individuals. More importantly, the decrease in cell function has been shown to be partially at the level of the hematopoietic stem cells (HSCs) [2]. In this regard, the hematopoietic system could serve as a prototype organ to study age-related diseases.

The aged HSCs have decreased proliferative potential and increased apoptosis [2]. Age can cause enhanced inflammation of the bone marrow microenvironment, B-cell dysfunction with regard to the avidity of their released antibody and, a shift in lymphoid subsets. The latter has been well-studied with the literature reporting the HSC niche showing significant age-related changes such as genomic damage and myeloid shift. The change in the HSCs has been demonstrated with decrease efficiency with a reduced capacity to support HSC engraftment in transplantation [3].

Age-related dysfunction in the emerging immune system is not limited to the bone marrow. Secondary lymphoid tissues demonstrate this same effect, with the aged thymus producing fewer T-cells [3]. The effects of aging has also been reported for adipose tissue consequent to oxidative stress, DNA damage, and telomere shortening. The aging adipose tissues have an accumulation of macrophages and activation of the tumor suppressor p53, which has been suggested to inhibit stem cell renewal and, to promote obesity-related disease processes such as insulin resistance [4]. The overall changes caused by aging can be linked to many disease processes. This article plans to focus on the aging microenvironment and cancer development, and to discuss how small non-coding RNA (miRNA) mediate the crosstalk between cancer cells and the tumor niche. The chapter discusses how miRNA and exosomes could be linked to the mechanisms of age-related cancer.

9.2 Cancer as an Aging Disorder

The process of cellular aging/senescence was first described by Leonard Hayflick and his colleagues in 1961 when they discovered that there is an intrinsic limit to the number of cell divisions of cultured human fibroblasts, even if the cells are given abundant nutrients and sufficient growth area [5]. This phenomenon demonstrated that normal human cells have limited capacity to divide and is referred to as the 'Hayflick limit'. Cellular senescence, as well as cell proliferation, differentiation, and apoptosis, are basic aspects of cell function. During the lifetime of a cell, somatic mutations occur continuously. Every mutation leaves an imprint on the genome of the cell. Cumulative mutations may lead to aging which is known to be an important risk factor for the occurrence of cancer. The dominant proliferation and differentiation abilities of cancer cells make these cells more likely to resist aging. As the incidence of some kinds of tumors increases with age, understanding the relationship between cancer and aging has an important clinical significance.

Although senescent cells retain some basic metabolic properties, many fundamental changes have taken place in either cell morphology or cell function that render them senescent. There are many opinions about the exact mechanism of cell senescence and many factors are involved in this process, including genomic insta-

bility, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, and stem cell exhaustion [6]. Currently, the most widely accepted processes are two overlapping signaling pathways: $p19^{ARF}$-p53 and $p16^{INK4a}$-Rb pathways [7, 8]. These pathways can be classified as stress or abnormal signal-induced senescence pathways. Bypassing the aging pathway is required for carcinogenesis. Hence, the study of cell senescence is a possible clinical research direction for tumor treatment.

On the other hand, senescent cells continuously accumulate various cytokines, inflammatory factors, and epithelial growth factors, resulting in damage to the cellular microenvironment which can indirectly damage normal cells and induce malignant proliferation of cells in the microenvironment. Additional studies are required to balance the contradictory challenges in our understanding of senescent cells.

As commonly reported, the change of gene expression level determines the decline of cells, tissues, and organs during aging and is also related to the tumor formation, recurrence, and metastasis. MicroRNA (miRNA or miR) which is a type of small (~22 nucleotides) non-coding, highly conserved, single stranded RNA plays pivotal role in expression of approximately 60% of all human genes [9]. Over the last 10 years, the world has seen a burst of research on the differential expression of miRNAs in the aging and tumorigenesis process, and further in-depth research of its role in the relationship of aging and tumor. This extensive research has confirmed that miRNAs could influence the balance of aging and tumorigenesis specifically through a variety of ways.

9.3 Role of miRNA in Cancer Biology

miRs are instrumental in intercellular communication including processes involved in tissue regeneration, tumor progression and inflammatory response [10–12]. miRs have the potential to influence a broad spectrum of gene pathways, making them imperative for modulating cell senescence, inflammation and tumorigenesis. miRs are also shown to mediate the aging process and, thus, can be classified as Oncogenic-miRs (Onco-miRs), Inflamma-miRs, and Senescence Associated-miRs (SA-miRs).

9.3.1 Onco-miRs

miRs that have the potential to modulate oncogenic activity are known as oncogenic miRs or onco-miRs. These miRs target oncogenes and consequently support tumor growth. Table 9.1 lists representative onco-miRs with known oncogenic links.

Table 9.1 Onco-miRs, Inflamma-miRs and Senescence Associated-miRs and associated target genes

miRNAs	mRNA targets	References
Onco-miRs		
miR-9	CDH1	[89–91]
miR-15	Bcl-2, CCNE1	[92, 93]
miR-19a	NPEPL1, IMPDH,	[94]
miR-21	PDCD4, TPM1, TIMP3, PTEN	[91, 95]
miR-124	SphK1	[96]
miR-125b	Ets1, EPO	[97]
miR-145	MUC1, CDH2, Oct4	[91, 93]
miR-146a	UHRF1	[91, 98]
miR-155	STAT3, SOCS1, CASP3, CTLA-4	[91, 92, 99]
miR-181a	CFIM25	[100]
miR-200 family	ZEB1, ZEB2, ERBB3, Sec23a, SIRT1	[91]
miR-214	EZH2, CTNNB1	[96]
miR-221	SNAI2 (Slug)	[101, 102]
miR-290	ARID4b	[103]
miR-335	SOX4, BRCA1, ESR1, IGF1, Sp1	[91]
Inflamma-miRs		
Let-7 family	H-ras, HMGA2, APP, NRAS	[91, 93, 104, 105]
miR-19a	Fra-1	[91]
miR-29	T-bet, EOMES	[12, 106]
miR-181b	TLR4	[12, 107]
miR-126	SDF-1a	[108]
miR-155	FOXO3a, IFNGR1, c-Maf	[12, 109–112]
Senscence Associated -miRs		
miR-26b	TAF12, PTP4A1, CPSF7, ALS2CR2	[113–115]
miR-181a	ATG5	[116]
miR-210	HIF-1a	[117]
miR-326	Ets1	[118]
miR-424	Smad3	[113, 119]
miR-519	ELAVL1 (HuR)	[120]

CDH1 E-cadherin, ***Bcl-2*** BCL2 Apoptosis Regulator, ***CCNE1*** Cyclin E1, ***NPEPL1*** Aminopeptidase-Like 1, ***IMPDH*** Inosine Monophosphate Dehydrogenase, ***PDCD4*** Programmed Cell Death 4, ***TPM1*** Tropomysin 1 (Alpha), ***TIMP3*** TIMP Metallopeptidase Inhibitor 3, ***SphK1*** Sphingosine Kinase 1, ***Ets1*** ETS Proto-Oncogene 1, ***EPO*** Erythropoietin, ***MUC1*** Mucin 1, ***CDH2*** Cadherin 2, ***Oct4*** Octamer-Binding Transcription 4, ***UHRF1*** Ubiquitin Like with PHD and Ring Finger Domains 1, ***STAT3*** Signal Transducer And Activator Of Transcription 3, ***SOCS1*** Suppressor Of Cytokine Signaling 1, ***CASP3*** Caspase3, ***CTLA-4*** Cytotoxic T-Lymphocyte Associated Protein 4, ***CFIM25*** Cleavage Factor Im 25, ***ZEB1*** Zinc Finger E-Box Binding Homeobox 1, ***ZEB2*** Zinc Finger E-Box Binding Homeobox 2, ***ERBB3*** also known as HER3, Receptor tyrosine-protein kinase erbB-3, ***Sec23a*** Sec23 Homolog A, Coat Complex II component, ***SIRT1*** Sirtuin 1, ***EZH2*** Enhancer of Zeste 2 Polycomb Repressive Complex 2 Subunit, ***CTNNB1*** Catenin Beta 1, ***SNAI2*** Snail Family Transcriptional Repressor 2, ***ARID4b*** AT-Rich Interaction Domain 4B, ***SOX4*** SRY-Box 4, ***BRCA1*** DNA repair associated, ***ESR1*** Estrogen Receptor 1, ***IGF1*** Insulin Like Growth

(continued)

Table 9.1 (continued)

Factor 1, ***Sp1*** Sp1 Transcription Factor, ***H-ras*** Hras Proto-Oncogene, GTPase, ***HMGA2*** High Mobility Group AT-Hook 2, ***APP*** Amyloid Beta Precursor Protein, ***NRAS*** NRAS proto-Oncogene, GTPase, ***Fra-1*** FOS like 1, ***T-bet*** T-Cell Specific T-Box Transcription Factor, ***EOMES*** Eomesodermin, ***SDF-1a*** Stromal Cell-Derived Factor 1, ***FOXO3a*** Forkhead Box O3, *S* ***IFNGR1*** Interferon Gamma Receptor 1, ***c-Maf*** C-Maf Inducing Protein, ***TAF12*** TATA-Box Binding Protein Associated Factor 1, ***PTP4A1*** Protein Tyrosine Phosphatase Type IVA, Member 1, ***CPSF7*** Cleavage And Polyadenylation Specific Factor 7, ***ALS2CR2*** Amyotrophic Lateral Sclerosis 2 (Juvenile) Chromosome Region, Candidate 2, ***ATG5*** Autophagy Related 5, ***HIF-1a*** Hypoxia Inducible Factor 1 Alpha Subunit, ***Ets1*** ETS Proto-Oncogene, Transcription Factor, ***SMAD3*** SMAD Family Member 3, ***ELAVL1*** *(HuR)* ELAV Like RNA Binding Protein 1

9.3.2 Inflamma-miRs

Age-related changes in the T-cell compartment caused immune dysfunction and this is referred as immunosenescence. Low level or chronic systemic inflammation in the absence of infection, called “Inflamm-aging”, is characterized by the upregulation of inflammatory responses that occurs with age. Inflamm-aging results in a systemic pro-inflammatory state and this has been linked to cancer. The pro-inflammation caused by the process of inflamm-aging occurs through the NFκB pathways, which are influenced by miRs such as miR-155, miR181b, miR-29 (Table 9.1) [12].

9.3.3 Senescence Associated (SA)-miRs

As discussed above, senescence is a process through which cells undergo growth arrest, which can be initiated by the RB/p16 and/or p53/p21 tumor suppressor pathways. Senescent phenotype can be characterized by gene expression which is often targeted via SA-miRs shown in Table 9.1.

9.4 miRNA Role in the Tumor Microenvironment

The primary tumor microenvironment consists of several miR-mediated functions, resulting in metastasis to secondary sites. In this section, we will look at the interaction of cancer cells and the TME through miRs in the support of primary tumor growth, cell transformation through epithelial-mesenchymal transition (EMT), angiogenesis, establishment of the pre-metastatic niche, and metastasis.

9.4.1 Primary Tumor Site

The dynamics and interactions of cells within the primary tumor microenvironment (TME) are crucial for the maintenance of cancer cells, and the regulation of cell transformation, growth, and metastasis [13]. Tumor cells and the TME can interact through signaling by cytokines, growth factors, hormones, direct cell-cell interactions, and microvesicles such as exosomes [14–18]. miRs are known pleiotropic regulatory molecules that can affect the TME and act intracellularly as an oncogene or tumor suppressor, or extracellularly through exosomes and direct cell-cell interactions [18–20]. miRs have been shown to be key players in the survival of tumor cells through metastasis to distant tissues in a variety of cancers including those originating in the breast (miR-200 family), colorectal (miR-9), and prostate (miR-141) [21].

The maintenance of the primary tumor depends on various factors, such as cell growth, cell proliferation, invasion of tissues, immune evasion, supporting stromal cells, extracellular matrix (ECM), angiogenesis (with EMT), and maintenance of stem cell character. These processes are subject to some level of epigenetic regulation through miRs originating from the tumor cells [22].

It is reported that tumor progression is affected by miR-21 upregulation and/or miR-34 downregulation [23–25].

Upregulation of miR-21, a prominent biomarker in a variety of cancers directly regulates and inhibits the tumor suppressor protein phosphatase and tensin homolog (PTEN), leading to tumor growth. In relation to the TME, miR-21 has been found to induce the transition of fibroblasts into cancer-associated fibroblasts (CAFs) by inhibiting Smad7. This increases the effects of TGF-β1 in carcinomas, colorectal cancers, and pancreatic cancers. Once induced into CAFs, these stromal support cells have the capacity to promote initiation, tumor growth, progression, and metastasis. Other mechanisms include releasing growth factors and enzymes such as matrix metalloproteinases (MMP) that degrade the ECM or cause fibrosis and hypoxia, allowing cancer cell invasion and metastasis [26–28]. CAF-associated mechanisms also resort to the use of specific miRs themselves, including miRs-23, -24, -26, -27, -103, -107, -181, -210, and -213 [29, 30]. Differential miR expressions between CAFs and normal stromal cells are extensive, with at least a dozen differences in a single TME. In the case of CAFs isolated from endometrial cancer, 11 onco-miRs are upregulated and tumor suppressor genes are downregulated in CAFs compared to normal fibroblasts. Other miRs, such as miR-149, have been shown to mediate communication between cancer cells and CAFs via an IL-6 and prostaglandin E2 dependent manner [31].

A compromised and restructured microenvironment is a first step in EMT. miR-21 is known to be a major regulator of the epithelial-mesenchymal transition and, as previously mentioned, is implicated in increased tissue invasion through the upregulation of MMPs. The induction of TGF-β from upregulated miR-21 can result in TGF-β-induced EMT by repressing the translation of TIAM1 and PTEN [31, 32].

The most explored EMT-regulating miRs belong to the miR-200 family: miR-200a, miR-200b, miR-200c, miR-141, and miR-429. These miRs prevent EMT and are downregulated in various cancers, as they silence the transcripts for ZEB and TGF-β and indirectly regulate the p53 transcription factor [31]. On the other hand, miR-9 positively regulates EMT by inhibiting E-cadherin, resulting in overexpression of E-cadherin's inhibitory target, VEGF-A [22]. More specific to the primary TME, selective pressures like hypoxia can result in the expression of regulatory miRs. As previously discussed, hypoxia can result in the recruitment of many miRs.

It is important to note that there is evidence to support the release of soluble factors and miR-containing extracellular vesicles (e.g. exosomes) from primary tumors into the circulation, and this could contribute to conditioning of future, distant metastatic sites [33–35]. Also, these cells in the "pre-metastatic niche" release similar factors that aid in the establishment of metastases [33]. These reciprocal interactions underpin the role of the microenvironment in cancer progression.

9.4.2 Secondary Tumor Site

According to the seed and soil hypothesis proposed by Paget, different organs have the capacity to support or limit the survival and growth of micrometastases [36]. Metastatic breast cancer (BC) cells (BCCs) preferentially disseminate from the primary tumor to the bone marrow through the circulation [37, 38]. When BCCs extravasate into the bone marrow, they first enter the perivascular niche and then move through the highly-cellular central marrow toward the endosteal niche, the hypoxic region situated adjacent to the long bone [39]. During this journey across the cavity, BCCs leverage the local microenvironment to survive and persist [10, 40–42]. As such, presence of BCCs in the bone marrow is predictive of poor clinical prognosis [38, 43–45]. This section dissects the role of miRs in regulating aspects of BC persistence in the endosteal niche of the marrow.

The entry of BCCs into bone marrow leads to colonization, which may result in overt metastasis with bone invasion or the cancer cells adapting dormancy. Recently, the miR-200 family (which includes miR-200a, miR-200b, miR-200c, miR-141, and miR-429) has been shown to promote colonization of the metastatic site by simultaneously regulating intrinsic and extrinsic BCC functions. For instance, miR-200 family members are strongly implicated as inhibitors of EMT, cell migration, and BCC dissemination intrinsically through the Zeb-E-cadherin axis [46–48]. Additionally, it has been shown that silencing miR-200 via miR-22 promotes BC development and metastasis [49]. Extrinsically, miR-200 family members influence the cancer secretome via the Sec23a-mediated transport pathway, manipulating signaling to nearby normal and cancerous cells, affecting collective behavior during colonization [46]. Thus, miR-200 family members leverage the metastatic microenvironment to support the survival of invading cancer cells.

During colonization of the bone marrow, BCCs have been shown to affect normal bone remodeling by increasing bone resorption, or osteolysis [50–52]. Osteolytic cancers release various factors from the mineralized matrix during bone resorption (i.e. TGF-ß, IGF-1, Ca^{2+}) that stimulate osteoblasts to secrete RANKL, which promotes osteoclast development and subsequent bone resorption [53]. TGF-ß plays a well-known role in enhancing BCC malignancy while simultaneously encouraging further bone breakdown [54–57]. Importantly, miRs that are downregulated during osteoclastogenesis act as potent inhibitors of BC-related osteolytic metastases while the upregulation of miR-16 and miR-378 serves as a biomarker for this event [53, 58].

Transition into cellular dormancy is a mechanism utilized by BCCs to survive and persist for extended periods in the bone marrow [59]. Dormant BCCs are in a cycling quiescent and chemoresistant state that renders them impervious to conventional cancer treatment regimens [42, 60]. The transition of BCCs into dormancy has been shown to be facilitated by bone marrow stroma [10, 61]. For instance, miR-127, miR-197, miR-222, and miR-223 can be transported from stroma to BCCs through gap junctions and facilitate dormancy [10]. The aforementioned miRs directly target CXCL12 in BCCs, reducing its levels and, subsequently, decreasing cell proliferation. Also, to a lesser extent, it has been shown that bone marrow resident mesenchymal stem cells (MSCs) release exosomes containing miR-222 and miR-223 that have been shown to contribute to BCC quiescence [61]. Likewise, high levels of miR-23b have been identified within MSC-derived exosomes and shown to promote dormancy in metastatic BCCs [62]. Hence, these miRs decrease BCC cycling which allows dormant BCCs to evade chemotherapy and radiation treatments aimed at proliferating cells [60].

In a related mechanism, maintenance of the cancer stem cell (CSC) population is required for long-term persistence of BC. The CSCs are a subset of the heterogeneous BCC population that possess characteristics of both cancer cells and stem cells. The common properties include long doubling times, active DNA repair, resistance to apoptosis, drug resistance, and tumor-initiating capacity. The CSCs can survive at distant organs, eventually leading to cancer resurgence and relapse [42, 63–66]. Dormant BCCs found at the bone marrow endosteum have been shown to exhibit a CSC phenotype [42, 61]. The multipotency of CSCs is linked to miR let-7 and miR-200c. Let-7 is suggested to inhibit stem cell self-renewal in normal breast cells and BCCs. Breast CSCs isolated from patients after receiving chemotherapy did not express let-7, but strongly upregulated let-7 after plating these cells under conditions that favored differentiation [67]. On the other hand, miR-200c links stemness and metastasis through inhibition of BMI1, a gene that promotes self-renewal, and ZEB1/2, which are genes that drive EMT [68]. In addition to intrinsic regulation of CSCs, the hypoxic nature of the BM endosteal niche enhances the expression of hypoxia-induced factors (HIFs) implicated in promoting BC survival and maintaining CSC phenotype [69–71]. Several miRs have been reported to regulate the HIFs including, but not limited to: miR-100, miR-210, and miR-21 [72–79].

9.4.3 Therapeutics Uses of miRNAs

miRs provide a new and potentially powerful contender for therapeutic intervention against various diseases [80]. Although our knowledge of miRs has evolved significantly, this field is still in its infancy with much remaining as 'black boxes'. The unknown information about miRNA include, but is not limited to the mechanisms of miR gene regulation, and their targets. Regardless, miRs are already being considered to be serious candidates for drug therapies.

Previously, we discussed possible ways that altered expression of miRNAs can be implicated in the drive and maintenance of several diseases including metabolic disease, cancer, and even some infection. During these events, miRs are either over- or under-expressed giving rise to a signature miRNA pattern in disease. Thus, the logical next step is to take advantage of these discoveries by developing miR-targeted therapeutic agents. The latest studies in animals and humans present data that reinforces the possibility of miRs to establish a novel class of drugs [81, 82]. miRs possess many attractive qualities in drug development, including their small size, known sequences, and conservation across species. To target these miRs, labs are using antisense technology to develop an effective oligonucleotide known as anti-miR that has a high affinity and specificity to the miR of interest.

In detecting varied levels of signature miRs, it is possible to manipulate miR expression by introducing miRs in a way similar to the use of antisense mRNA and RNAi [83]. By injecting artificial antisense miRs, these specially designed molecules can be employed to target inhibit the dangerously over-expressed onco-miRs. However, there is a vital qualification that must be available for this method to have proper effects in reducing cancer growth. Signature miRs must be identified and their mechanisms of action and possible downstream effects in the cell must be understood. Once these specific miRs have been thoroughly vetted, the process of miR therapeutics will follow similar steps to other drug discovery and development.

There are several possible strategies to manipulating miRs, which depend on whether the targeted miR expression is aberrantly down or upregulated. Onco-miRs, the miR equivalent to oncogenes, reciprocally inhibit tumor suppressor genes. For example, miR-21 targets the tumor suppressor PTEN and is upregulated in lung cancer [84, 85]. Oncomirs can be targeted by complementary sequences called antisense oligonucleotides (AMO), or antagomirs [81]. The newest strategy to regulating high levels of signature miRs is a "miRNA sponge". miRNA sponges have multiple complementary sites and thereby downregulates the miR of interest [86].

On the other hand, expression of some miRs may be reduced in cancer cells to allow for tumor growth. If miRs are endogenously downregulated in cancer cells, this calls for methods that can restore the appropriate levels of miRNA to prevent tumor growth. For example, by over-expressing the otherwise decreased miR-26a in hepatocellular carcinoma (HCC) in mouse liver, cancer proliferation was inhibited [87]. Additionally, an increase of atypically downregulated miR-34a by delivering artificial miR-34a with NOV340 liposome in an orthotopic model of HCC [88]. This resulted in overall reduced tumor size and prolonged survival of the mice.

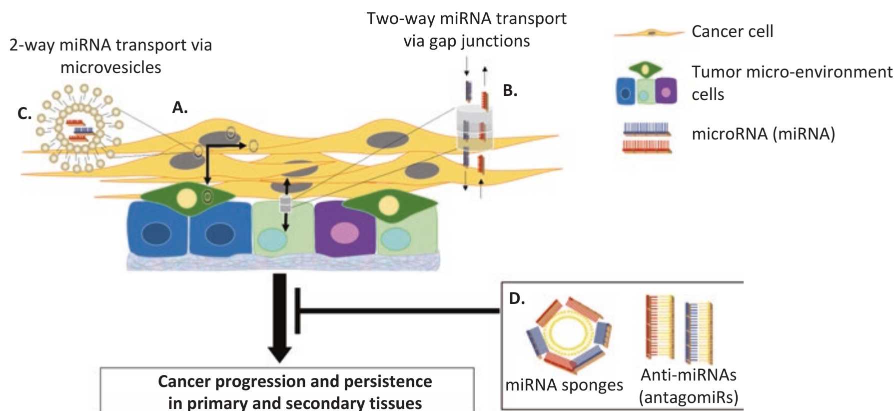

Fig. 9.1 Breast Cancer-Micronenvironment axis mediated by miRNA-containing exosomes. (**a**) Shown are the spatial location of cancer cells with cells of the tumor microenvironment (TME). (**b**) Communication between TME cells and cancer cells can occur by gap junctional intracellular communication (GJIC). (**c**) Intercellular communication is shown to occur by contact-independent manner in which exosomes and their miRNA cargo can be exchanged. (**d**) These miRNA can be targeted by representative methods shown in the diagram

The functionality of miRs in controlling diverse gene expression in cancer and various other important diseases makes miRNA an ideal candidate for therapeutic applications. Research shows that miR expression is altered in various human diseases and its selective modulation through antisense inhibition or replacement has potential to notably improve prognosis. The continued advancement to improve the administration and uptake of small non-coding RNA, it is expected that several miR targets may be identified to treat clinical diseases.

Figure 9.1 summarizes two major methods by which cancer cells interact with cells within the microenvironment. Shown are microvesicles such as exosomes and GJIC forming a 2-way communication and GJIC. After understanding how the miRNA sustain the survival of cancer cells, methods can be developed to target the process. Although several methods could be developed to block the action of miRNA, the cartoon shows the utility of miRNA sponges such as circular RNA and antago-miRs or anti-miRNAs. Ongoing studies in several laboratories are involved in optimizing how stem cells can be used to deliver antagomiRs to tumor cells. These latter methods are not discussed in this chapter.

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