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Review

Gap junctional shuttling of miRNA — A novel pathway of intercellular gene regulation and its prospects in clinical application

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ABSTRACT

The gap junctional exchange of small molecules between adjacent cells is crucial for maintaining tissue homeostasis and for a large number of cellular processes, including differentiation and proliferation. miRNAs represent a novel class of signalling molecules capable of crossing gap junction (GJ) channels, thereby directly affecting gene expression in the recipient cell. Here, we give an overview about the current knowledge on the biological significance of miRNA shuttling in different cell types (e.g. stem cells, cardiac cells, macrophages), which indicates the GJ-dependent transfer of miRNA as a general mechanism for intercellular gene regulation. Notably, shuttling via GJs is superior to exosome-mediated intercellular transfer regarding specificity and efficiency. We further elucidate this mechanism as a promising approach for miRNA delivery in clinical applications. Using a cell-based gap junctional dependent system, in vivo delivery of therapeutic miRNAs might become more efficient compared to systemic delivery methods. We will discuss the advantages of such a delivery system and the challenges that have to be overcome for its successful application in miRNA therapy.

Contents

1. Introduction

The interaction between cells is crucial for a number of complex cellular processes. A number of these interactions are mediated by gap junctions (GJs). GJs are specialized protein structures in the plasma membrane that contain clusters of channels for direct communication between adjacent cells (Fig. 1). They allow passive, diffusional exchange of small molecules up to a molecular mass of ~1 kD–1.5 kD, including metabolites, ions or second messengers [1]. Gap junctional intercellular communication (GJIC) plays a critical role during embryogenesis, as cell-cell coupling in the developing embryo enables the formation of communication compartments and developmentally important

Abbreviations: Cx, connexin; CD40, cluster of differentiation 40; FRAP, fluorescence recovery after photobleaching; GFP, green fluorescent protein; GJ, gap junction; GJIC, gap junctional intercellular communication; mRNA, messenger RNA; miRNA, microRNA; MSC, mesenchymal stem cells; pre-miRNA, precursor microRNA; pri-miRNA, primary microRNA; RISC, RNA-induced silencing complex; shRNA, small hairpin RNA.

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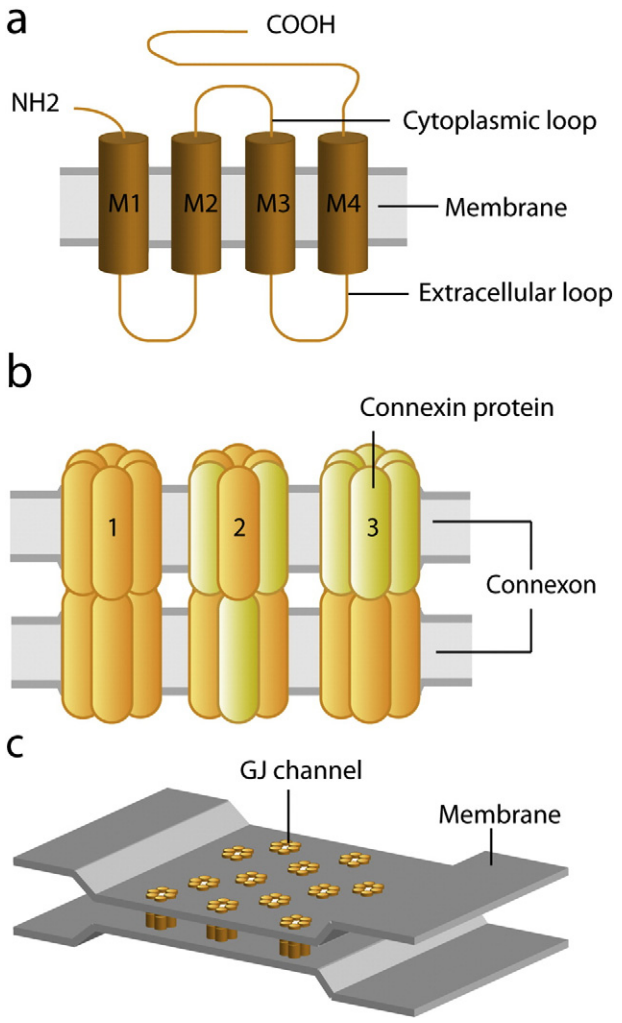

Fig. 1. Molecular organization and topology of a GJ plaque. (a) Cx proteins consist of 4 membrane spanning domains (M1–M4), connected by 1 cytoplasmic and 2 extracellular loops that are crucial for docking. Cx proteins vary mainly in their C- and N-terminal region. (b) HCs, formed by 6 Cx subunits, can dock to each other and establish a GJ channel. Three different types of GJs are known, depending on their molecular composition. 1) homomeric/homotypic, 2) heteromeric and 3) heterotypic. Homotypic and heterotypic GJs are composed of two identical or two different HCs. Homomeric or heteromeric HCs comprise one or more Cx isotypes. (c) Gap junction channels are organized as GJ-plaques in the plasma membrane.

domains [2–4]. These different compartments ensure the establishment of morphogen gradients as well as electrical and metabolic synchronization, which is important for proper embryo patterning and differentiation [5]. At adult stages, GJs maintain homeostasis and control cell and tissue functions. In particular, a pronounced role for GJs was demonstrated in neurons, where GJs as electrical synapses mediate synchronization and transduction of electrical signals. Likewise, GJs control heart functions by regulating intercellular calcium signalling and transmission of action potentials [6,7]. There are a several excellent reviews giving a broad overview about the role of GJIC in development and function of different tissues, including heart and brain [1,8–10]. More than 30 years after Loewenstein first described GJs as mediators for intercellular communication, several molecules have been identified, capable to cross GJ channels. Lately, small RNAs have been found to be transferred between adjacent cells via gap junctional cell-cell contacts [11]. This finding raises serious questions about the biological impact of miRNA shuttling. Here, we will discuss this novel cell signalling mechanism of intercellular gene regulation and its involvement in cellular physiology. We will further cover new possibilities using this pathway for clinical application by the establishment of a GJ-dependent, cell-based delivery system for therapeutic miRNAs.

2. The structure of gap junctions

GJ channels consist of 2 connexons (hemichannels), each provided by one of the neighbouring cell. A single connexon is composed of 6 Connexin (Cx) subunits, belonging to a family of structurally related transmembrane proteins. All Cxs share a similar structural topology that is characterized by 4 alpha helical transmembrane domains (M1-4, Fig. 1a), connected by 2 extracellular loops, 1 intracellular loop linking M2, and M3 and a cytoplasmic N-and C-terminal region (Fig. 1a). To date, 20 members of the Cx gene family have been identified in the mouse and 21 in the human genome [12]. The expression of different Cx isotypes results in a great variance of GJ channel composition, which directly affects the specific permeability. Connexons can consist of either one single Cx isoform leading to homomeric connexons, or they contain different Cx types and form heteromeric structures [13]. Once a single GJ channel is formed, clustering leads to the establishment of large GJ plaques (Fig. 1c) that can contain up to many thousands of GJs with several micrometres in size [14,15].

The channel-dependent function of Cx is not exclusively restricted to its role as a GJ-forming protein. As hemichannels in the plasma membrane, Cxs allow the release or uptake of molecules and ions between the intracellular and extracellular environment, indicating its physiological importance in signalling functions and cellular homeostasis [16]. Their permeability characteristics are similar to that of GJs. However, in contrast to GJs, which remain mostly in an open state, hemichannels were found to have a low open probability under resting conditions that can be increased by certain stimuli [17].

3. Gap junctions as a passage way for miRNA

miRNAs are small 20–25 nucleotide non-coding RNAs that bind to mRNAs to regulate gene expression [18,19]. The canonical miRNA biogenesis pathway starts with the expression of long double-stranded primary transcripts (pri-miRNA) that are processed by the RNase Drosha into 70–100 nucleotides long hairpin precursors (pre-miRNA). Following Exportin-mediated translocation into the cytoplasm, pre-miRNAs are cleaved into ~22-mer miRNA duplexes by Dicer and incorporated in the RNA-induced silencing complex (RISC) that mediates miRNA-mRNA interaction [20] (Fig. 2). Upon binding to their targets, miRNAs induce blockage of translation or trigger the degradation of mRNA [21]. In contrast, it was shown that a miRNA can up-regulate the expression of its target genes [22]. One miRNA can target many mRNAs which in turn can influence different cellular functions, indicating the importance of these small molecules for a wide range of biological processes [23].

Due to the large number of nucleotides (~70) and the stem-loop structure, GJ-dependent shuttling of double-stranded precursor miRNAs is impossible [24]. Thus, only mature miRNA duplexes are able to pass GJs. This was firstly shown by Valiunas and colleagues who investigated the permeability of synthetic oligonucleotides that have a similar structure as miRNAs [11]. They demonstrated that morpholinos with a molecular weight of 2–4 kD, minor diameters of ~1 nm and lengths up to 7.6 nm (24-mer) are capable to diffuse through GJs. Interestingly, the efficiency of transfer was found to be dependent on the Cx-subtype. While Cx43-formed GJs showed strong permeability to tested oligonucleotides, Cx32 and Cx26-containing GJs were impermeable. Nevertheless, due to the diversity of Cx proteins, additional data is needed to confirm the hypothesis about a Cx-subtype specific shuttling of miRNAs. In addition to Cx composition, GJ permeability is also tightly regulated by posttranslational modifications of Cx proteins, by speed and efficiency of Cx delivery to the plasma membrane, by growth factors and by intracellular conditions like voltage or

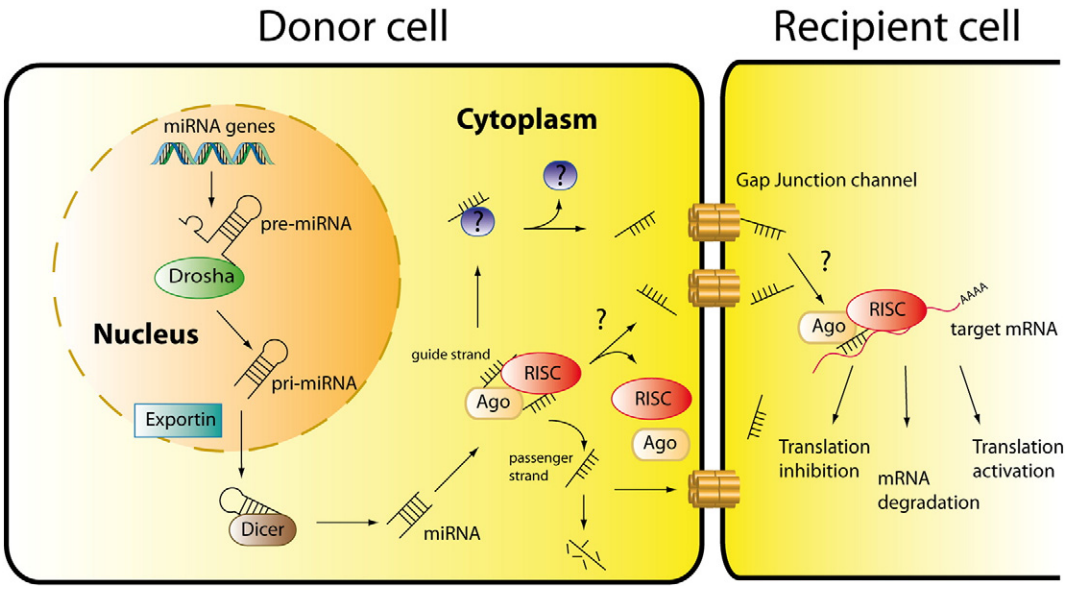

Fig. 2. Schematic model of the GJ-mediated shuttling of miRNA. Pri-miRNA is processed by the RNase Drosha. Following Exportin-dependent transport to the cytoplasm, the pri-miRNA is further cleaved by Dicer leading to ~22 nucleotide long, double stranded miRNA. The miRNA duplex is loaded into the RISC/Ago complex, unwinded and split into the guide strand and the passenger strand. The latter one is released from the RISC/Ago complex and degraded or subjected to GJ transport. However, additional unknown miRNA binding proteins might be involved that promote miRNA stability and intercellular shuttling (question mark). To allow gap junctional transfer, the miRNA is released from protein complexes. Upon reaching the adjacent cell, the miRNA is incorporated into the RISC complex to impair mRNA translation or stability.

calcium concentration [25–29]. However, it is uncertain whether these regulatory events specifically affect the transfer of small RNAs. Moreover, it remains to be investigated whether miRNAs are actively transported to GJs or just passively diffuse within the cytoplasm and into the recipient cells. The large molecular size of miRNA-Protein complexes cannot cross GJ channels and it needs further studies to clarify the mechanisms and molecular compounds that mediate the intercellular shuttling (Fig. 2). As miRNA molecules play a critical role in cell differentiation and development, the gap junctional exchange might represent an important signalling mechanism in cellular physiology [30–32]. This is supported by the fact that the protein machinery for miRNA synthesis and function as well as Cx proteins are highly conserved in several types of tissue und throughout different species [12,30]. Using GJs for intercellular shuttling, miRNAs can act not only in the cell in which they are produced, but also in neighbouring cells [33,34]. Next, some physiological and pathological cellular processes will be introduced that are regulated by the GJ-dependent exchange of miRNA.

4. The physiological role of miRNA shuttling

The first studies suggesting an involvement of GJs in the cell to cell transfer of miRNA were performed using exogenously introduced small RNAs. Using a GFP/shRNA reporter construct, Wolvetang and colleagues showed that shRNA is transferred between Cx43 expressing human embryonic stem cells leading to a reduction of GFP fluorescence in the recipient cell [35]. Chemical blockage of GJIC by α-glycyrrhetinic acid prevented the decrease of the GFP signal. Similar results were obtained in co-culture experiments with glioma cells, showing that miRNA transfer silenced the activity of a luciferase reporter [36].

One of the first studies that determined a physiological significance of GJ-mediated miRNA transport was performed by Lim and colleagues [37]. Using a co-culture system, they observed that the GJ-dependent import of miR-127, 197, 222 and 223 from bone marrow stromal cells affects the proliferation of breast cancer cells. Mechanistic analysis revealed that these specific miRNAs impair expression of C-X-C motif chemokine 12, leading to cell cycle quiescence and reduced cancer cell growth. In contrast to the reports by Wolvetang and Katakowski [35,36], Lim et al. also addresses the impact of miRNA transport via exosomes, which, however, was less important for cell cycle disturbance. These data indicate that the gap junctional transfer of miRNA might be important for the dormancy of metastases and the elimination of cancer cells. This is in line with a report that demonstrates the GJ-dependent delivery of miR-124 and miR-145 mimics from mesenchymal stromal cells to glioma cells. As a result, cancer cells showed a decreased cell migration and impaired self-renewal abilities [38]. Likewise, macrophages were found to transfer miRNA to hepatic carcinoma cells in a cell-cell contact and GJ-dependent manner [33]. Interestingly, the authors showed that only specific miRNAs are exchanged between cells, indicating a selective permeability of GJs for certain miRNAs. Similar to the report of Lim et al. [37], these results suggest miRNA transfer as a mechanism of the immune system to repress cancer cell growth. Consistently, gap junctional coupling was attributed to the anti-tumour activity of miR-124 in glioma cells [39]. While stimulation of GJ coupling promoted the miR-124 mediated anti-proliferative effect, functional inhibition of GJs by Cx knockdown or chemical compounds eliminated the anti-cancer effect. Reversely, cancer cells can also affect surrounded stromal cells to increase invasiveness by direct transfer of miRNA. GJ-mediated miRNA signalling between glioma cells and brain tissue was recently shown to enhance the invasive ability of tumour cells [40]. Two specific miRNAs, miR-5096 and miR-4519, were identified, which were transferred from glioma cells to astrocytes to promote glioma growth.

As mentioned above, miRNAs are crucial regulators of cell fate determination. The intercellular transfer of developmentally important miRNAs from donor cells to stem or progenitor cells can alter their differentiation status and change tissue composition. This process was found to be involved in the regeneration of cardiac tissue after myocardial infarction [34]. In vitro co-culture experiments revealed a GJ-dependent import of miR-499 from cardiomyocytes to cardiac stem cells, leading to the repression of Sox6 and Rod1 accompanied by the induction of cardiac differentiation. Hence, it was assumed that adjacent cardiomyocytes trigger the transformation of progenitor cells into functionally competent cardiomyocytes by miR-499 shuttling after tissue degeneration. Although the authors demonstrated the promoting effect of miR-499 on cardiac differentiation in cultivated stem cells, no data was presented showing the gap junctional exchange of miR-499 in vivo. However, the study provided evidence that the control of

stem cell development is not only intrinsic but also mediated by an intercellular, gap junctional import of miRNAs from the cellular environment.

As the reader can appreciate, the intercellular delivery of miRNA is not only restricted to a certain cell type, but occurs in a variety of cells and tissue (Table 1). It is likely that researchers will elucidate more cell types capable of shuttling miRNAs for intercellular communication in the near future.

5. Alternative pathways for the intercellular transfer of miRNA

Cell-cell contact dependent transfer of genetic material can also occur via tunnelling nanotubes [41]. These intercellular structures are long, actin-based cell extensions that mediate the transport of organelles, endosomes and lysosomes [41]. Using time lapse microscopy and fluorescent labelled miRNA, Thayanithy provided visual evidence of miRNA transfer through nanotubes in cancer cells [42]. Another possibility for intercellular shuttling of miRNA was found in immune cells that can form an immunological synapse for intercellular communication [43]. miRNA, encapsulated in exosomes, were shown to be transported between T-cells and antigen presenting cells, and become functional in the recipient cells [43].

Since miRNAs have been found in blood and other body fluids, cell-cell contact independent mechanisms have been described that enable the secretion of miRNA containing exosomes into the extracellular space [44–46]. For example, miRNA containing exosomes originated from mesenchymal stem cells (MSC) are transferred to target cells to induce gene regulatory effects [46]. Additionally, apoptotic bodies can carry miRNAs serving as alarm signals to provoke regenerative processes [47]. Moreover, miRNA can also be exchanged by a non-cell derived vesicle mechanism. A study by Vickers and colleagues provided evidence that miRNAs bound to high density lipoprotein (HDL) are able to enter hepatocytes to target their specific mRNA [48]. However, it is unclear when and where these HDL-miRNA complexes are formed and no additional reports have demonstrated HDL as a carrier for miRNA.

The capability of cells to transfer miRNA by exosomes or HDL specifically to a target cell is limited. Once released into the extracellular milieu, vesicles cannot be guided to the recipient cell, but rather find their target, randomly. The dilution effect decreases the local concentration of exosomes with distance from the releasing cell, suggesting that physiological relevant effects, triggered by miRNA, might mainly be induced in cells that are in close proximity to the donor cell [49–51]. Additionally, exocytotic and endocytotic processes are very common to many different cell types, which also impede a targeted delivery of miRNA [52–55]. However, cell specific internalization of vesicles was demonstrated in dendritic cells that preferentially bind exosomes originated from B-cells [56]. Although the detailed molecular mechanisms of exosome uptake are not well investigated, the involvement of individual surface proteins on both the exosome and the recipient cell might mediate a more specific cargo delivery [55,57]. Several studies also provide evidence that the incorporation of miRNA into exosomes is an active process and only a subset of miRNAs is selectively enriched in exosomes [55,58,59]. Similarly, a selective transport of miRNA via GJs was also observed in macrophages [33].

Moreover, it is unclear how many miRNA molecules can be delivered or up-taken via exosomes in order to provoke significant effects on gene expression. Even given that all delivered miRNA copies are functional, exosome-mediated transfer must increase the level of miRNA to more than 1000 molecules per cell, which was estimated the median level of miRNA in normal tissue [60,61]. The concentration of miRNA differs widely, depending on the cell type and the physiological and pathological conditions. For example, mir-21, which is used as a tumour biomarker, was detected at a level of approximately 12,000 copies per cell in MCF-7 breast cancer cells [62]. The number of miR-155 molecules in B cell lymphomas is increased by 10–30 fold, compared to normal circulating B cells that contain 150 miR-155 copies per cell [63].

To estimate the efficiency of exosome-mediated miRNA shuttling, it is important to quantify the number of miRNA molecules being transported from cell to cell. Exosomes with an average diameter of 20-100 nm possess an internal volume of 0.03–0,38 al (10e-18 l) that is 6.2 × 10e-6% of the entire cell volume (average cellular diameter of 10 μm) [64]. Taking into account that a single cell can release 1900–16,000 vesicles within 24 h, the total exchange of cellular volume is about 0.1% [65]. Indeed, based on patch clamp analyses, Brink et al. roughly calculated that the average cellular volume transferred by exocytosis is about 0.7% within 24 h, suggesting a possible transfer of many thousand miRNA molecules [66]. However, exosomes contain a large number of different molecules such as lipids, adhesion or cytoskeletal proteins, enzymes and mRNA and the capacity for miRNA cargo seems to be low [56]. In a recently published study, the amount of miRNA was quantified in exosomes isolated from human plasma, seminal fluid and in vitro sources, including dendritic cells, mast cells, and ovarian cancer cells [67]. By comparison of the total number of exosomes (1e + 8.2e + 9/μl) and the absolute concentration of miRNA (4e + 5-10e + 7/μl), the authors found that even abundant miRNAs, like mir-126 or let-7b are present at less than one molecule per exosome in all samples. This was confirmed by Asby and co-workers [68] who analysed exosomal fractions from human serum and calculated copy numbers of several individual miRNAs (e.g. mir-375, mir-122, let-7a) at 3e + 6 molecules per μl. Similar low concentrations were detected for mir-186 in human plasma and for mir-486 in serum [69,70].

Table 1
Gap junctional exchange of miRNA in different co-culture systems.

Co-culture system		miRNA	Target/effect	Ref
Donor cell	Recipient cell			
Mesenchymal stem cells	Breast cancer	miR-127 miR-197 miR-222 miR-223	CXCL2; Reduced proliferation of cancer cells	[37]
Mesenchymal stem cells	Glioma cells	miR-124 miR-144	Laminin γ1 and integrin β1 SCP-1 or CDK6; Reduced migration and self-renewal of cancer cells	[38]
Macrophages	Hepato carcinoma cells	miR-142 miR-223	Stathmin-1 IGF-1R; Reduced proliferation in cancer cells	[33]
Glioma cells	Glioma cells	mir-124	Cyclin dependent kinase 6; Reduced proliferation	[39]
Glioma cells	Astrocytes	miR-5096 miR-4519	Increased glioma cell invasion	[40]
Cardiomyocytes	cardiac progenitor cells	miR-499	Sox6, Red1; Promote differentiation of cardiac progenitor cells	[34]
Mesenchymal stem cells	Cardiomyocytes	miR-210	Caspase8-associated protein-2; pro survival effect on cardiomyocytes	[99]

According to these results, two different modes of exosomal transport of miRNA are possible: (1) Only a small fraction of exosomes carries a low number of miRNA copies, which in turn needs an accumulation of hundreds of vesicles inside the target cell to reach a significant level of at least ~1000 miRNA molecules. Depending on the cell type, the uptake of such a large quantity of exosomes could take several hours [71,72]. (2) Few individual exosomes contain many miRNA copies. The probability that one single exosome will reach the recipient cell is quite low due to the lack of specificity of exosome guidance. Further, the incorporation of delivered exosomes/vesicles into lysosomes and subsequent degradation also decreases transfer efficiency [73].

Compared to exosome-mediated miRNA delivery, gap junctional shuttling allows a more targeted delivery of miRNA molecules as no extracellular pathways are involved. miRNAs can be immediately transported into the adjacent cell without further processing and packaging into vesicles, which also promotes transport speed. Valiunas et al. showed that labelled siRNA molecules are exchanged between two cells within minutes [11,74]. The transfer rate of 22 nucleotide long oligos by GJs is about 1–2% within 40 min, which would result in an equal concentration of miRNA in donor and recipient cell after 24 h [11]. A detailed characterization of the gap junctional shuttling of miRNA has not been obtained so far and diffusion coefficients or specific permeabilities are yet unknown. For intracellular movement, diffusion coefficients for miRNA might be similar to folded proteins ($D = 10^{-7}$ cm^2/s) [75]. Based on dye flux studies, the diffusion coefficients of fluorescent tracers are lowered by 90% when crossing GJs [76]. Accordingly, the intercellular diffusion coefficient of miRNA would be $D = 0.09$ μm^2/s. Thus, a large number of active GJ channels could facilitate the intercellular flux of thousands of miRNA molecules within few hours, which is enough to influence the gene expression of the recipient cell. However, the number of molecules passing a GJ channel per second is mainly driven by diffusion, and therefore depends on the intracellular concentration of miRNA in the donor cell [60,61]. Further, channel size and other GJ-regulatory events, like Cx phosphorylation, affect channel gating and additional studies are required to investigate how miRNA shuttling is regulated by these parameters [9,25,77].

Taken together, there is no doubt that GJs as well as exosomes provide a pathway for the intercellular delivery of miRNAs. Both mechanisms have been shown to effectively modulate gene expression in the recipient cell [37,38,74,78]. Nevertheless, the direct intercellular exchange of miRNA via GJs is most likely superior regarding transport specificity and efficiency.

6. Clinical applications for GJ-mediated miRNA delivery

As gene regulatory molecules, miRNAs possess a strong diagnostic and therapeutic potential [79]. Secreted miRNAs can be used as biomarkers for certain pathological processes or can be directly delivered to the cell to influence gene expression [79,80]. However, the effective delivery of therapeutic miRNA to the target tissue is one of the major challenges for clinical application [81,82]. To overcome these problems, various viral and non-viral based strategies for gene delivery have been developed. Lentiviral, adenoviral and adenoassociated vectors expressing miRNA mimics or antagonists are effective delivery systems due to their high transfection efficiency [83–85]. However, the risk of an acute immune response, the possibility of integration of viral DNA into the host genome and the difficulties in virus production have led researchers to find safer and cheaper alternatives including physical approaches (needle injection, electroporation) and chemical vectors (liposome, polymers) [86–89]. Besides the lack of specificity, these delivery systems use an extracellular pathway leading to a many-fold dilution of miRNA and strongly decrease the efficiency of delivery. Likewise, the uptake by endocytosis often results in the incorporation of miRNAs into endosomes and subsequent degradation, which also hindered the gene silencing effect [81,90].

Cell based systems might represent an alternative approach for the safe and efficient in vivo delivery of miRNA. Pre-loading the cell vector with miRNA would increase the retention of miRNA in the host tissue and reduce the concentration of miRNA that is necessary to alter cellular functions as no dilution effects will occur. For clinical translation, the miRNA delivering cell system requires a certain expression level of Cx proteins that are incorporated into the plasma membrane to allow the establishment of functional GJs with adjacent cells. Further, high permeability of GJs to miRNAs is needed to mediate sufficient intercellular transfer [11]. As an increased immune response can affect miRNA delivery, the use of immunologically inert vectors is necessary to avoid undesired side effects [90].

6.1. A cell based, GJ-dependent delivery system for miRNAs

Although not GJ-mediated, several cell-based delivery systems have been developed as drug carriers or for gene therapy to carry therapeutic agents to target tissue, including tumour cells, stem cells, dendritic cells or Sertoli cells [91–93]. Emerging data indicate that MSCs are a suitable cell vehicle for in vivo delivery of miRNA. These multipotent stromal cells can be readily isolated from human donors in large quantities and subsequently used for autologous transplantation which minimizes the risk of an immune response and inflammatory events [94–96]. MSCs express only low levels of human leukocyte antigen major histocompatibility complex I and II and do not express the co-stimulatory molecules CD40 and B7 [97]. This unique immunophenotype allows to inhibit innate and adaptive immune cells and to suppress inflammatory effects. In addition, MSCs can be easily manipulated in order to introduce miRNA or other therapeutic factors [92,98,99]. Another strong advantage is their homing capacity that promotes specific targeting of injured tissue [100]. Due to these benefits, the number of clinical trials using MSCs has been strongly increased for the last 10 years. For instance, MSCs were used in the treatment of liver cirrhosis, osteoarthritis or for immune disorder therapies [96]. Moreover, Cx43 expression and GJIC was demonstrated for MSCs by several researchers [101–104]. Taking into account that Cx43 composed GJs are the least selective channels, MSCs have the ability to deliver sufficient amounts of miRNAs into the tissue of interest [11]. In fact, MSCs were utilized to deliver the pro-survival miR-210 via GJs to ischemic myocardium, thereby protecting hypoxic cardiomyocytes from apoptosis [99]. In vivo data showed that the infarction size was decreased and cardiac function was recovered after transplantation of miR-210 containing MSCs. Fluorescence in situ hybridization analysis of recipient heart sections revealed extensive presence of miR-210 in cardiomyocytes. The involvement of GJs in this cytoprotective effect was confirmed by pharmacological inhibition in co-cultures of MSCs and cardiomyocytes. In a recently published study, MSCs were used as a vehicle to deliver functional siRNA into synovial fibroblasts to induce gene silencing [105]. Knockdown of Cx43 in MSCs verified that the exchange of siRNA was, at least in part, a GJ-mediated process. Likewise, GJ-dependent shuttling of labelled miRNA was also observed between MSCs and glioblastoma cells [78]. Although other mechanisms of miRNA transfer exist, these studies substantiate the hypothesis that MSC based gap junctional delivery of miRNAs is a promising strategy for repairing damaged tissue by targeting gene expression.

6.2. GJ-mediated miRNA delivery in regenerative medicine and cancer therapy

Since miRNAs were shown to trigger direct reprogramming of cells, the efficient delivery of miRNA is desirable for new therapies in regenerative medicine to recover the function of injured tissue [106]. After introduction of different miRNAs, cardiac fibroblasts were directly converted into functional cardiomyocytes in vivo and in vitro, resulting in an improvement of cardiac function [107–109]. In vivo delivery of miRNAs was realized by lentiviral application, which is efficient but

unsafe for clinical application. It is tempting to speculate that non-viral GJ-mediated, cell based delivery systems can be used to introduce miRNAs into infarcted myocardium, thereby triggering the reprogramming of cells in order to recover tissue function. The widespread expression of Cxs in heart tissue would allow gap junctional crosstalk between the used cell vector and cardiac cells, including cardiomyocytes and fibroblasts [110,111]. Besides cardiac cells, reprogramming of pancreatic cells holds great potential in the treatment of diabetes. Pancreatic cells also express Cxs such as Cx43, Cx36 and Cx26 and demonstrate intense cell-cell interaction via GJs [112–114]. However, miRNA based lineage conversion of these cells has not been achieved and it needs further investigations to identify specific small RNAs as new reprogramming factors for the generation of new β-cells.

miRNA delivery by a gap junctional, cell based system for reprogramming might also be considered for the central nervous system. Neurodegenerative diseases and CNS traumata are one of the leading causes of death and disability. The activation or reprogramming of endogenous neural progenitors or astroglia by miRNA delivery can represent a new therapeutic approach. Several in vitro studies demonstrated that astrocytes have the capacity to be converted into functional neurons and miR-9 and miR-124 were found to be important regulators in this reprogramming process [115]. In vivo reprogramming of neural cells was achieved by the introduction of transcription factors like Sox2 or Ascl1 [116,117]. Although it is established that miRNAs play a crucial role in neuronal cell fate determination it is uncertain whether miRNAs can induce similar effects when delivered by gap junctional shuttling [118]. According to several cell therapy studies, MSCs might serve as suitable cell vectors for miRNA delivery into neural cells [119,120]. Importantly, human MSCs increased axonal sprouting and density in brain injured Wistar rats after transplantation, indicating that MSCs can survive when engrafted into brain tissue [121,122].

Various types of cancer are associated with changes of the intracellular miRNA level, showing the therapeutic value of miRNA in cancer therapy. For example, deregulated expression of miR-21 was reported to be involved in breast cancer, leukaemia or liver metastasis [123,124]. Similarly, miR-34, miR-145 and mir-204 demonstrated a reduced expression in several cancer cells, while reconstitution of these miRNAs impaired tumour growth [125–127]. Due to their capability to migrate to tumour sites, stem cells may become useful as a vehicle to deliver therapeutic miRNAs into cancer cells via a GJ-dependent pathway [100]. Genetically modified MSCs have already been shown to transport anti-tumour proteins such as Interleukin-12, interferons or anti angiogenic factors into mouse tumours with beneficial effects [128]. Likewise, MSCs could be modified to express anti-cancer miRNAs that translocate into the tumour cell. Indeed, previous reports already demonstrated the potential of MSCs to deliver miRNAs into breast cancer and glioma cells in vitro leading to a decrease of tumour cell growth [37,38,78].

6.3. Challenges for cell based miRNA delivery

Since the establishment of GJs between vector cell and target cell requires a certain period of time, the retention of transplanted cells is a significant challenge for cell based delivery of miRNAs. The amount of retained cells, especially in the heart, is low independent of the delivery method. For example, direct injection of human MSCs into injured rat, porcine or human heart results in a loss of 90–99% of the transplanted cells after 24 h [129–132]. The use of extracellular matrix mimicking biomaterials such as alginate hydrogel or collagen patches can strongly promote cell retention as well as cell survival, compared to cells delivered in saline or media alone [130]. With chitosan hydrogels as carrier, the amount of injected stem cells was 3 times higher than observed for cells diluted in phosphate buffered saline [133]. Recently, Ban et al. demonstrated that encapsulation of HL-1 cardiomyocytes into a new developed nanomatrix gel increased cell retention by 3-fold when transplanted into mouse myocardium [134]. Moreover, immunostaining of Cx43 revealed that injected cells were able to establish GJs with cardiomyocytes of the host tissue, suggesting a possible intercellular exchange of molecules [134].

Although stem cells, like MSCs, have the tendency to home to injured tissue, the application of cell based vectors requires a targeting strategy that ensures specific navigation of cells in vivo. Tukmachev and co-workers utilized magnetic nanoparticles to guide stem cells towards the damaged tissue in a spinal injury mouse model [135]. Likewise, the use of magnetic nanoparticle based transfection reagents allows both introduction of therapeutic miRNA into the cell vector and guidance of cells in vivo [86]. However, although the number of observed cytotoxic effects is limited, magnetic nanoparticles were found to produce free radicals when excessively released which can provoke oxidative stress [136]. Other reports showed magnetic nanoparticle-induced toxic effects on the cardiovascular and central nervous system [137]. Therefore, additional experimental in vivo studies are required to clarify the composition and concentration of nanoparticles that ensure the safe use for clinical applications.

To date, a large pool of miRNAs have been identified but their function in cellular physiology is not fully understood [138–140]. As miRNAs can have hundreds of mRNA targets, functional screenings are needed to determine their precise role in regulating cellular processes and to evaluate their therapeutic value for clinical application [141]. The functional analysis of miRNAs also ensures that the loading of miRNA will not alter the properties of the cell vector, including proliferation, differentiation, cell adhesion or Cx expression, a prerequisite that is especially important when delivering miRNAs for in vivo reprogramming. Importantly, as several types of cancer are characterized by alterations of miRNA expression, it is essential to exclude that introduction of exogenous miRNAs into the cell vector promotes cancer development when applied in vivo [124].

7. Conclusion and future directions

There is no doubt about the fundamental role of GJIC in tissue development and function. To understand the mechanisms controlling proliferation and differentiation, it is necessary to identify the compounds that are exchanged within cellular tissues. miRNAs represent a novel class of molecules which are able to cross GJ channels [37]. The number of cells and tissues capable of forming GJs strongly suggests miRNA shuttling as a general signalling mechanism in the regulation of physiological processes by altering gene expression of the recipient cell. In contrast to vesicle-mediated export/import, GJ-dependent transfer is faster, directed and more efficient as it does not involve the extracellular space. Although a number of reports showed that GJs are permeable for miRNAs, the functional consequences remain elusive. Only few data were obtained showing the physiological and pathological significance of cell-cell shuttling of small RNAs [34,38]. Regarding the role of miRNAs as cell fate determinants, further investigations will reveal to what extent miRNA transfer contributes to cell differentiation and tissue development. Moreover, there is a lack of reports showing shuttling of endogenously expressed miRNAs and its effect on cellular processes in vivo. Various studies demonstrated the involvement of GJIC in intercellular miRNA shuttling by application of pharmacological blockers. Since these agents act non-specifically with Cxs and can also affect other signalling mechanisms, data interpretation has to be done very carefully [17]. Thus, pharmacological blockage in combination with selective knockdown approaches is recommended. However, alterations in Cx expression have been shown to affect the expression of other genes [17].

As miRNAs accumulate with several proteins, which mediate their gene regulatory function, it remains to be investigated what kind of intracellular machinery is involved in the transport process (Fig. 2). The architecture of GJs only allows crossing of naked miRNA, indicating that release from miRNA binding proteins is mandatory before transport into the recipient cell. Conversely, after transfer from the

donor cell, miRNAs have to be re-integrated into the RISC complex to successfully silence gene expression and to prevent them from degradation [21](Fig. 2). Moreover, when using GJs as a route for cell-cell communication, controlled intracellular trafficking of miRNA would strongly promote the efficient delivery to neighbouring cells. Since the cytoskeleton mediates the rapid and directed intracellular movement of mRNA [142,143], it seems possible that actin filaments or microtubules are used in a similar way to transport miRNA cargos to the cell periphery (Fig. 2). This would augment the local concentration of miRNA molecules in the area of gap junctional cell-cell contacts, which in turn could increase the probability of channel crossing. Some data already indicated an interaction of miRNA binding proteins with cytoskeletal elements [144,145].

A deeper understanding of these molecular mechanisms that regulate gap junctional transfer is crucial to establish a cell based delivery system as a new approach for therapeutic miRNA delivery. Successful clinical application will require knowledge about their intercellular distribution within target cell populations in order to adjust and optimize miRNA-delivery conditions. Compared to viral approaches, cell based delivery systems bear less safety concerns and therefore have the potential to become a powerful tool in regenerative medicine, in which cell transplantation and engraftment are a major focus [146–148]. In particular, autologous MSCs have been widely used for transplantation studies [97,102,149,150]. Indeed, MSCs seem to fulfil the prerequisites of suitable miRNA delivering cell vectors. While a number of reports have been shown the possibility of GJ-dependent, cell based miRNA delivery, additional studies in small and large animal model systems are needed to elucidate whether miRNA can effectively be delivered in a way sufficient to induce therapeutic effects in vivo.

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