Extracellular miRNA: A new era of miRNA research

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Abstract
MicroRNAs are a class of endogenously initiated, small non coding RNAs typically regulates the expression of mRNAs in post transcriptional level either via translational repression or mRNA degradation. Recently, it has been revealed that miRNAs are not only present in cellular environment but also in extracellular milieu specially in different bio-fluids including blood plasma, follicular fluid and even in cell culture media. Such circulating miRNAs are remarkably stable in the extracellular harsh environment with the presence of high RNAse activity. Although the precise cellular release mechanisms of miRNAs remain largely unknown, the first studies revealed that these extracellular miRNAs may be delivered to recipient cells, where they can regulate translation of target genes. In this review, we will discuss the nature of the stability of extracellular miRNAs that present in the bloodstream and other bio fluids and their release mechanisms. Furthermore, based on available evidences we will shed lights on the possible role of these circulating miRNAs in distant cell-to-cell communication.
Key words: Genetics; miRNA; Extracellular miRNA; Cell-cell Communication

Hücre dışı miRNA'lar: miRNA araştırmalarında yeni bir dönem

Özet
Mikro RNA'lar, mRNA ifadesini post-transkripsiyonel düzeyde translasyonel baskılama veya mRNA'nın degredasyonu yoluyla düzenleyen küçük kodlamayan RNA'lardır. Yakın zamanda miRNA'ların sadece hücre içeresinde olmayıp, hücre dışında kan plazması, folikül sıvısı ve hücre kültürü ortamı gibi farklı biyo-sıvılarda da bulunduğu gözlenmiştir. Bu sıvılarda bulunan ve dolaşan RNA'ların hücre dışındaki zorlu ortamda yüksek RNAaz aktivitesi sayesinde stabil biçimde bulunduğu gözlenmiştir. Dolaşan miRNA'ların rolü tam olarak bilinmemekle birlikte miRNA'ların hedef hücreye girdiği ve hedef hücrede bazı genlerin translasyonunu regüle ettiği bildirilmiştir. Bu derlemede dolaşan miRNA'ların stabilitesinin doğasını ve biyo-sıvılara nasıl aktarıldığını tartışacağız. Ayrıca, literatürde bulunan kaynaklar dahilinde dolaşan mikro RNA'ların hücre-hücre iletişiminde ki rolü de incelenecektir.

Anahtar kelimeler: Genetik; miRNA; Dolaşan miRNA; Hücre-hücre iletişimi

Extra-cellular miRNAs

While the majority of miRNAs detected intracellularly, a considerable number of miRNAs, commonly known as circulating miRNA or extra-cellular miRNA, have been also detected outside cells, mainly in various bio-fluids (Gilad et al. 2008; Hunter et al. 2008; Mitchell et al. 2008; Weber et al. 2010). Recent studies have shown that miRNAs are not only present in serum (Chen et al. 2008; Chin and Slack 2008; Mitchell et al. 2008) or plasma (Arroyo et al. 2011;

Chim et al. 2008; Shen et al. 2010) but also different extra-cellular body fluids including saliva urine, tears, seminal fluid, breast milk, colostrum, peritoneal fluid, bronchial lavage and cerebrospinal fluid (Weber et al. 2010). Moreover, the expression profile of extra-cellular miRNAs from different types of body fluids in relation to different physiological / pathological conditions shows a specific pattern which indicating that extra-cellular miRNAs are not only passively released from the necrotic or injured cells but also selectively released from the cells (Pigati et al., 2010).

Characteristics of extra-cellular miRNAs

In contrast to circulating miRNAs, when synthetic miRNAs spiked into human plasma, it shows rapid degradation (within few minutes). While denaturing solution inactivated RNase activity in plasma, the exogenous miRNAs get released from degradation (Mitchell et al. 2008). Thus, synthetic miRNA species are vulnerable and susceptible to quick degradation in plasma, where as circulating miRNAs are protected and more resistant to high endogenous RNase activity, indicating that circulating miRNAs are likely wrapped with some protective manner to bypass high RNase activity in the extra-cellular environment. Moreover, studies have been demonstrated that circulating miRNAs in body fluid remain stable even they subjected to harsh conditions like boiling, high or low pH, prolonged storage time and multiple freeze-thaw cycles while most of cellular RNAs were degraded quickly (Gilad et al. 2008; Taylor and Gercel-Taylor 2008). In addition, recent studies have been demonstrated that miRNAs in serum gives specific expression pattern in quantitative PCR (qPCR) after being subjected to incubation at room temperature for 24 h (Mitchell et al. 2008) and maximum 10 freeze-thaw cycle (Chen et al. 2008). Chen et al. found that isolated serum miRNAs can survive the treatment of RNase A, compared to other endogenous RNAs such as 18s rRNA, 28s rRNA, GAPDH, β-actin and U6 (Chen et al. 2008). Most serum miRNAs maintain considerable expression levels after 3 hours or overnight RNase-A treatment; however large RNAs were degraded following 3 hours of RNase-A treatment (Chen et al. 2008).

The mechanism underlying the remarkable stability of circulating miRNAs in the RNase-rich environment of blood is not well understood. Many hypotheses have been proposed to explain the possible mechanisms through which RNAs and miRNAs are released and protected from endogenous RNase activity in circulation. One of the earliest theory suggested that RNAs might conjugated with protein which would later protected them from both DNase and RNase activity (Sisco 2001). However, later on it has been showed that RNA species present in plasma are protected from degradation probably due to inclusion in lipid or lipoprotein complexes, not by binding with DNA (El-Hefnawy et al. 2004). Another hypotheses is that miRNAs are wrapped with microvesicles (exosomes, microparticles and apoptotic bodies), which shaded miRNAs in circulation and protect from RNase activity (Valadi et al. 2007). On the other hand some other studies have shown that, after isolation of microvesicles using high-speed ultra centrifugation from culture media (Turchinovich et al. 2011; Wang et al. 2010c) or plasma, a handful miRNAs are still detectable in the microvesicles free fraction, suggested that the presence of non-vesicle associated miRNA (might be miRNA-protein or miRNA-lipid/lipoprotein complexes) in extra-cellular fluid .

Possible pathways to release miRNAs

There are lots of arguments regarding how these miRNAs are released in extracellular body fluids and serves cell-cell communications. Numbers of studies have demonstrated that the transfer of protein, mRNA and miRNA in different body fluids can be mediated via exosomes, microvesicles apoptotic bodies and RNA-protein or RNA-lipoprotein complexes that are released from a variety of cell types to modulate angiogenesis, cell proliferation/ apoptosis, tumor cell invasion and cell-cell communication.

Transport of circulating miRNA through Exosomes

Exosomes are homologous small vesicles (50-90 nm) and have an endosomal origin (Camussi et al. 2010). Exosomes are formed by invagination of the membrane of endosomes to produce intraluminal vesicles, thus rendering these organelles multivesicular bodies (Urbe et al. 2003). In response to cell stimulation, budding of endosomes occurs, a process dependent on calcium influx, calpain and cytoskeleton reorganization (Johnstone 2006). Currently accumulating evidence suggests that these secretory vesicles can function as intercellular transmitters to convey their contents, in particular, microRNA (miRNA) (Valadi et al. 2007; Rechavi et al. 2009). A total of 121 miRNAs were identified in exosomes from mast cells ad the expression pattern of certain miRNAs was higher in microvesicles than in the donor cells (Valadi et al. 2007). Valadi and colleague reported that exosomes that released from human and murine bone marrow-derived mast cells contain mRNA and miRNA, which are transferrable to other human or mouse mast cells. When exosomes from mouse mast cells transferred to human mast cells, they produce new mouse protein in recipient cells, indicating that the exosomal mRNAs are functional and they can be translated after entering into another cell (Valadi et al. 2007). Furthermore, it has been shown that embryonic stem cell derived microvesicles are miRNA enriched and they can transfer a subset of miRNAs to mouse embryonic fibroblasts in culture, suggesting that gene expression of neighbouring cells might affected by exosomal miRNA that released by embryonic stem cells (Yuan et al. 2009). It has been demonstrated that miRNA released in exosomes by Epstein-Barr virus (EBV)-infected cells can be taken up by peripheral blood mononuclear cells and these EBV-miRNAs repressed confirmed EBV target genes (Pegtel et al. 2010).

Transport of circulating miRNA through microparticles

Microparticles are lipid vesicles that are <1 μm in diameter and are secreted into extracellula environment by different type of cells and platelets (Chironi et al. 2009). Many cell types are known to secrete microparticles and these include some cancer cells (Castellana et al. 2009), neurons and many of the vascular and hematopoietic cell types such as endothelial cells, dendritic cells and B cells (Shet 2008). Microparticles are larger than exosomes and form through plasma membrane budding and also contain miRNA (Yuan et al. 2009). Initially microparticles were considered as cell debris but experimental evidence suggests that microparticles influences diverse biological functions for example cardiovascular disorders, including atherogogenesis and thrombosis (Mack et al. 2000; Mause and Weber 2010; Shantsila et al. 2010). The presence of miRNAs in microvesicles has now been reported from different cells including mesenchymal stem cell (Chen et al. 2009), mast cells (Valadi et al. 2007), cancer cells (Taylor and Gercel-Taylor 2008), platelets (Hunter et al. 2008) and endothelial cells (Skog et al. 2008). Recently it

has been shown that chemically modified miR-143 entrapped by microvesicles was significantly secreted from miR-143-transfected human monocytic leukemia THP-1 cells during incubation in serum-free medium (Akao et al. 2011). These findings highly support that at least some extracellular miRNAs are used for cell-cell communication via microparticles but still thorough investigation need to understand the mechanisms how miRNAs are selected for secretion, recognized for uptake, and what information can be transmitted.

Transport of miRNA through apoptotic bodies

Apoptotic bodies (Abs) are small membranous particles released during programmed cell death (Hasselmann et al. 2001). The formation of ABs is a final outcome of apoptotic cell death. Budding of microparticles occurs mainly during early apoptosis, whereas ABs are formed in the late stages of this death process. ABs are larger than MPs and represent the compacted or condensed remnants of the shrinking apoptotic cells (Beyer and Pisetsky 2009). Recently it has been reported that ABs plays an important role in the repair of injured cells. In vitro experiment shows that endothelial progenitor cells (EPCs) engulf ABs released from endothelial cells (ECs) which trigger the secretion of cytokines and/or growth factors indicating that it may facilitate the repair of injured endothelial cells or somatic cells. A microarray result shows that the presence of a panel of miRNAs in ABs derived from ECs whereas mir-126 is the most abundant one (Zernecke et al. 2009). Sprouty-related protein, SPRED1, and phosphoinositol-3 kinase regulatory subunit 2 (PIK3R2) are the negative regulators/ intracellular inhibitor of the vascular endothelial growth factor (VEGF) signalling pathway. Through repressing the expression of these negative regulators in ECs mir-126 facilitates the pro-angiogenic actions of VEGF and fibroblast growth factor (FGF) and promotes blood vessel formation (Fish et al. 2008; Wang et al. 2008). Moreover, mir-126 knockout in mice results leaky vessels, and partial embryonic lethality, because of a loss of vascular integrity, defects in endothelial cell proliferation and angiogenesis (Wang et al. 2008). In damaged tissue, the CXC chemokine CXCL12 and its receptor CXCR4 counteract apoptosis and recruit progenitor cells. In case of atherosclerosis, endothelial cell-derived ABs are generated and convey paracrine signals to recipient vascular cells that trigger the production of CXCL12 in a mir-126 dependent manner (Zernecke et al. 2009). Though, these observations confirms that ABs may serve as a carrier of circulating miRNA, more studies need to determine how specific miRNA secreted, recognized for up-take via this process.

Transport of circulating miRNA through protein complex

Recent studies have shown that a handful number of extracellular miRNA can be delivered to the extracellular environment through protein complex (Arroyo et al. 2011). One of the major components of miRNA silencing complex is Ago2. Ago2-miRNA complexes were detected in different cell culture media and western blot immunoassay shows that extracellular miRNA ultrafiltrated together with the Ago2 protein, a part of RNA-induced silencing complex, not associated with microvesicles (Turchinovich et al. 2011). Furthermore, it has been demonstrated that only 10% cell-free miRNAs released in plasma through micro vesicles whereas potentially 90% of the miRNAs in the circulation cofractionated with ribonucleo-protein complexes (Arroyo et al. 2011). Size-exclusion chromatography has been used to exclude the micro vesicle contamination from protein complexes and shows that most of the miRNA co-purified with non-

vesicle-associated ribonucleo-protein complexes, only few miRNA, such as miR-16 and miR-92a associated predominantly with micro vesicles (Arroyo et al. 2011). However, Ago2-miRNA is not only the protein complex that released in cell culture supernatant, mass spectrometry results reviled that a total of 197 proteins present in human fibroblast conditioned medium 2 h after serum starvation, of which 12 were known RNA-binding proteins (Wang et al. 2010). Although, the role of other RNA-binding protein complexes, except Ago2, is presently unclear, collectively these results indicate that Ago2-protein complexes might be involved with the deliver of miRNA from donor cell to recipient cells and facilitate cell-cell communications.

Transport of circulatory miRNA through high density lipoprotein

Recently, Vickers et al. reported that purified fractions of HDL from healthy human plasma contain a number of miRNAs (Vickers et al. 2011). Highly purified HDL that is negative for exosomal marker proteins is rich in small RNA molecules that are 15 to 30 nucleotides in length but devoid of long mRNAs (Vickers et al. 2011). Total RNA extracted from HDL and exosomes isolated from the plasma of healthy individuals revealed that their miRNA profile is distinct. A specific miRNA signature of HDL-miRNA complexes was identified in patients with familial hypercholesterolemia, including miR-22, miR-105, and miR-106a (Vickers et al. 2011). The authors further showed that direct delivery of miRNAs to recipient cells can also occur by HDL in a ceramide signaling pathway dependent manner. Thus, native HDL is associated with miRNAs in a way that resembles artificial gene delivery vehicles, acting as a carrier or depot for circulating miRNAs in plasma and facilitating their transport and delivery to recipient cells. Finally, the study by Vickers et al. provides evidence that the miRNAs within HDL alter the cellular miRNA pool and functionally downregulate corresponding miRNA targets (Vickers et al. 2011), suggesting that the miRNA content of HDL is biologically relevant. Collectively, these results indicate that besides its classical role as a delivery vehicle for excess cellular cholesterol, HDL may also function as a transporter of endogenous miRNAs.

Potential role of extra-cellular miRNAs

Whether the extra-cellular forms of miRNAs are simply waste products from cells or have a biological function, such as participating in intercellular communication is not yet clear. There are reports showing increased level of miRNAs in blood upon organ toxicity (Laterza et al., 2009), and this could of course represent waste products. Nevertheless, since the various forms of extracellular miRNAs are probably products of distinct cellular processes, they might play different roles, and therefore it is important to distinguish between them. Apoptotic bodies are by definition formed during apoptosis. miRNA bound to Ago2 may be released from cells upon apoptosis or necrosis (Turchinovich et al. 2011), but it is not known if miRNA-Ago2 complexes also can be transported out of viable cells. This means that miRNAs bound to Ago2 proteins and miRNAs incorporated into apoptotic bodies might solely be by-products from dying cells or represent a way for dying cells to communicate with neighboring cells. They could represent a signal warning the organism about cellular dysfunction.

Shedding vesicles and exosomes are thought to be released by viable cells, though it is not ruled out whether these vesicles also are released by dying cells. Therefore, these vesicles have to a greater extent been suggested to play a role in intercellular signaling (Hunter et al. 2008; Valadi et al. 2007). Indeed, it has been shown that miRNAs can be transferred by exosomes from one

cell to another in vitro and result in downregulation of target genes in the recipient cell (Kogure et al., 2011). These findings indicate a role in intercellular communication which could have a huge impact. However, this remains to be shown in vivo conditions. Interestingly, it has been reported that injection of exosomes loaded with siRNA into mice can result in specific gene knockdown in certain cells. It has been questioned whether the concentration of exosomes in biological fluids is high enough to play a role in intercellular communication, but this does not exclude a role in autocrine or paracrine signaling (Turchinovich et al. 2011). Exosomes probably exert their effect on neighboring cells, and thereby participate in creating a specific microenvironment. In this scenario, the exosomes found in body fluids would only be residual amounts, representing a secondary effect.

In addition to their conventional role in post-transcriptional gene regulation, a new role for miRNAs as signaling molecules has recently been described by two independent groups. Interestingly, extra-cellular let-7 was shown to activate Toll-like receptor 7 in neurons and induce neurodegeneration (Lehmann et al., 2012). By another group, exosomal miR-21 and miR-29a was shown to activate TLR 7 and 8 in immune cells, triggering a prometastatic inflammatory response that may lead to tumor growth and metastasis (Fabbri et al., 2012). Thus, extra-cellular miRNAs could be important regulators of tumor microenvironment as well as exacerbate CNS damage, through agonistic effect on TLR 7 and 8.

Another possible role for miRNAs in exosomes and microvesicles is that they might function together with the RNAi machinery. RISC proteins have been shown to be associated with MVBs and exosomes (Gibbings et al. 2009). Moreover, blocking MVB formation by depletion of ESCRT (endosomal sorting complex required for transport) components has been reported to result in impaired miRNA silencing, indicating a role in RNAi dynamics (Gibbings et al., 2009).

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