Functional Roles for Exosomal MicroRNAs in the Tumour Microenvironment

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ABSTRACT

Extracellular microRNAs are released from cells both passively and actively. The presence of these microRNAs in the tumour microenvironment (TME) can significantly impact on the plasticity of cancer cells leading to the promotion of metastatic and angiogenic processes. These extracellular microRNAs can act not only on other cancer cells, but also cells present in the TME, such as immune cells, endothelial cells, fibroblasts, and others acting to subvert the host immune system and drive tumour progression. In this review we highlight the current understanding of both the mechanisms by which microRNAs are released from tumour cells and the downstream functional effects that extracellular microRNAs have on recipient cells.

1. Introduction

Cancer research has traditionally focused on tumour intra-cellular gene expression and signalling pathway activation. This view postulates that cancer cells proliferate due to genetic mutations that activate growth signalling pathways. While these mechanisms are necessary and important events in tumour initiation and progression, they do not account for the complexity of the microenvironment in which the tumour sits. Tumours are complex systems composed of not only tumour cells, but stroma containing blood and lymph vessels, fibroblasts, endothelial cells, and immune cells among others [1]. This TME plays an essential role in the initiation, growth, and metastatic spread of cancer. Additionally, while genetic mutations initiate tumourigenesis, numerous post-transcriptional and post-translational mechanisms are at play within tumour cells and other cells within the TME that ultimately contribute to tumour progression. A large proportion of the human genome is made up of non-coding RNAs, including two of the most well studied classes of non-coding RNAs; long noncoding RNA (lncRNA) which are ~200 or more nucleotides in length and microRNAs, which are small (17–27 nucleotide) non-coding RNAs that regulate approximately 30–60% of all protein-coding genes through post-transcriptional mechanisms [2,3]. MicroRNAs regulate gene expression by binding to the 3′UTR of target mRNAs, resulting in translation repression or RNA

degradation [4]. Approximately 50% of microRNAs are located in regions of chromosomal abnormalities that are associated with cancer [5], meaning that in cancer cells with genetic abnormalities significant changes in specific microRNA clusters are likely. Particular microRNAs are known to act as both tumour suppressors and oncogenes in the development of tumours. For example, the miR-17-92 family of microRNAs is one of the most well characterised oncomiRs and has been shown to exert anti-apoptotic effects through its ability to down-regulate Bim and PTEN tumour suppressors [6]. MiR-21 and miR-155 are also well characterised oncomiRs that promote both tumour growth and metastasis by targeting numerous mRNAs. In contrast, miR-15a, miR-16-1, miR-34a, and the let-7 family of microRNAs have been shown to suppress tumour growth and metastasis by inducing apoptosis, cell cycle arrest and senescence (reviewed in [4]). In addition to their functional effects on tumour cell signalling pathways, microRNAs have been shown to exhibit tissue specific expression patterns [7], suggesting that they have potential utility as clinical biomarkers [8].

Cell-free microRNAs are found in the circulation and since their discovery have become promising diagnostic, prognostic, and therapeutic response biomarkers for cancer. Indeed, circulating microRNA profiles can be used to identify disease types, with elevated circulating microRNAs being significantly associated with disease-associated genetic variants [9]. These circulating microRNAs have been found to be present in several different body fluid types [10,11] and are incredibly stable, being able to withstand room temperature for extended periods of time and numerous freeze–thaw cycles [11]. The stability of endogenous microRNAs is in direct contrast to synthetic exogenous microRNAs spike-ins (e.g. cel-miR-39-3p) which when added to serum or media are rapidly degraded

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[11,12]. Based on this evidence, circulating microRNAs have been hypothesised to be protected by being enclosed in extracellular vesicles or bound to proteins [11–13]. Given their prevalence and stability in biofluids it is not surprising that recent evidence points to extracellular microRNAs playing functional roles as autocrine, paracrine, and endocrine signalling molecules.

Here we review the mechanisms governing cellular release of microRNAs and the evidence for extracellular microRNA activity in cell-cell communication with a focus on how cell-free microRNAs have been shown to have functional roles influencing tumour progression and metastasis.

2. Extracellular Vesicles

The term extracellular vesicle encompasses exosomes, microsomes and apoptotic bodies [14,15]. Exosomes are 30–100 nm in size and are formed from inward budding of the endosomal membrane [16]. This forms a multivesicular body (MVB) which contains several exosomes. The MVB then fuses with the plasma membrane and releases the exosomes from the cell [17,18]. The resulting vesicles contain cytosol and can be characterised by the presence of tetraspanin proteins CD9, CD63, CD81, and CD82 [15]. Other vesicles in the circulation include microsomes which are produced from the disruption of the plasma membrane and are of a larger size than exosomes 100–1000 nm and apoptotic bodies which are released from apoptotic cells and are 1 μm–5 μm in size [19]. Therefore, studies looking at the selective release of microRNAs have focused on the exosomal fraction of extracellular vesicles. It must be noted that it is difficult to achieve pure isolates of exosomes experimentally, meaning that most studies report RNA data from enriched populations of exosomes which still contain protein bound microRNAs or other extracellular vesicles [14,15]. Studies have sought to examine if circulating microRNAs are mainly protein bound or enclosed in extracellular vesicles. Turnchovich et al. [12] found that the majority of circulating microRNAs are associated with AGO2 protein and only a small proportion of circulating microRNAs are enclosed in extracellular vesicles. They hypothesised that microRNA/AGO2 complexes are released by cells during apoptosis [12]. A global profiling study has suggested that AGO2 may play a role in a selective pathway, which targets microRNAs into extracellular vesicles [20]. This study compared different cell lines and identified a subset of microRNAs that are released in extracellular vesicles through a common mechanism for sorting microRNA [20]. These microRNAs are not processed through the canonical pathway by Dicer, but through an AGO2 mediated maturation pathway, which leads to preferential release in extracellular vesicles [20].

3. Exosomal RNA Function

Several types of RNA have been found in exosomal fractions, with microRNAs and mRNAs being the most abundant species. These microRNAs and mRNAs were found to be enclosed inside exosomes, as opposed to being attached to the outside. Exosomes isolated from cultures of glioblastoma cells were found to fuse with the membranes of host cells and release the contents into the host cell [21]. The exosomal contents were found to be fully functional in recipient cells, with microRNAs promoting the downregulation of their targets [21] and exosomal mRNAs being translated into protein in the host cell [22]. In contrast, a study by Chevillet et al. [23] questions the physiological relevance of exosomal microRNA, as stoichiometric analysis of the absolute copy number of microRNAs and the number of exosomes indicates that even for abundant cancer biomarkers (in this case prostate cancer) there is less than one microRNA copy per exosome. Therefore, this study brings into question whether or not exosomal microRNAs are in sufficient abundance in exosomes to have an effect on the recipient cells. Potentially, a high level of exosome uptake or non-canonical microRNA activation would be required to elicit exosomal microRNA-mediated effects [23]. However, given that numerous exosomal microRNAs have been shown to have functional effects (discussed below), more research is needed to ascertain the stoichiometric requirements for exosomal microRNA function.

4. Passive Release of microRNAs from Cells

Several studies have found that the bulk of microRNA released in exosomes reflects the cellular microRNA expression profile [24,25], and the majority of microRNAs (~66%) are released from cells passively by mass action. miR-16 was identified as a microRNA that represents this passive exosomal microRNA release, and the amount of miR-16 in the cell matches the amount found in exosomes [25]. In agreement with this idea, Squadrito et al. [26] demonstrated that the microRNA content of exosomes is determined by the levels of the microRNA target mRNA in the cell. For example, if there is an abundance of the mRNA target of the microRNA, the microRNA is bound and this decreases the amount of that specific microRNA in the exosomes. If there is a high abundance of the microRNA relative to its targets, then an increased level of the microRNA is observed in the exosomes, leading to the suggestion that the exosome pathway is a mechanism to maintain microRNA:mRNA homeostasis in the cell [26]. In contrast to this passive release mechanism, a subset of microRNAs are overrepresented in exosomes, meaning that they are enriched in comparison to levels observed in the cell, indicating a selective release mechanism [25].

5. Selective Release of microRNAs from Cells

Investigation of the mechanistic requirements governing microRNA inclusion in to exosomes found that the neutral sphingomyelinase 2 (nSMase2) is required for microRNA exosomal release [27]. The nSMase2 enzyme catalyses the rate limiting step in ceramide synthesis. Ceramide is required to promote budding in the endosomal compartment. Therefore, inhibition of nSMase2 inhibits the formation of exosomes [27,28]. The active selection of microRNAs for packaging into exosomes has been suggested to be sequence specific [29], with the selection of microRNAs for inclusion into exosomes being determined through the binding of chaperone proteins. The protein hnRNPA2B1 has been shown to play a key role in targeting a subset of microRNAs with a specific motif into exosomes [30]. In addition, 3′ modifications of microRNAs have been suggested to determine whether a microRNA is retained in the cell or exported in exosomes. Indeed, non-templated additions of 1,2 or 3 bases of uridine or adenosine to the 3′ end of certain microRNAs has been found to influence microRNA release into exosomes, with poly-adenylated microRNAs being more likely to be retained in the cell and poly-uridynilated microRNAs packaged into exosomes [31].

Alterations in cancer-associated cell signalling pathways also alter the microRNA profile of exosomes [32,33]. For instance, p53 has been shown to govern the release of exosomes from cells. On activation by DNA damage, p53 transcriptionally up-regulates the expression of tumour suppressor activated pathway 6 (TSAP6), which has been found to be essential for p53-mediated exosome release [34]. Although a direct mechanism linking changes in microRNA content in these exosomes has not yet been described, it illustrates the influence that cell signalling pathways have on exosomal release. In colorectal cancer cells, exosomal loading has been shown to be dependent on KRAS mutational status, with mutant KRAS cells releasing higher levels of miR-100 and wild-type KRAS cells releasing higher levels of miR-10 into the exosomal fractions [26,35], through a mechanism requiring nSMAse. Additionally, KRAS-dependent activation of MEK-ERK signalling inhibits sorting of AGO2 bound microRNAs into exosomes [36]. Although it is clear that oncogenic signalling pathways influence microRNA content in exosomes, the exact mechanisms by which these microRNAs are sorted into exosomes and the contribution of passive versus active sorting