Review

Emerging roles of extracellular vesicle-associated non-coding RNAs in hypoxia: Insights from cancer, myocardial infarction and ischemic stroke

Dirk M. Hermann[1][*][✉], Wenqiang Xin[2][*], Mathias Bähr[2], Bernd Giebel[3], Thorsten R. Doeppner[2,4,5,6][✉]

1. Department of Neurology, University Hospital Essen, University of Duisburg-Essen, Essen, Germany
2. Department of Neurology, University of Göttingen Medical School, Göttingen, Germany
3. Institute of Transfusion Medicine, University Hospital Essen, University of Duisburg-Essen, Essen, Germany
4. Research Institute for Health Sciences and Technologies (SABITA), Medipol University, Istanbul, Turkey
5. Department of Anatomy and Cell Biology, Medical University of Varna, Varna, Bulgaria
6. Department of Neurology, University of Giessen Medical School, Giessen, Germany

*Equal contribution.

✉ Corresponding authors: Prof. Thorsten R. Doeppner, MD, MSc, MHBA. Department of Neurology, University of Giessen Medical School, Klinikstr. 33, 35392 Giessen, Germany. Email: thorsten.doeppner@neuro.med.uni-giessen.de; Phone: +49-641-985-45301; Fax: +49-641-985-45309. Prof. Dirk M. Hermann, MD. Department of Neurology, University Hospital Essen, Hufelandstr 55, 45122 Essen, Germany. Email: dirk.hermann@uk-essen.de; Phone: +49-202-723-2180; Fax: +49-201-723-5534

Abstract

Hypoxia is a central pathophysiological component in cancer, myocardial infarction and ischemic stroke, which represent the most common medical conditions resulting in long-term disability and death. Recent evidence suggests common signaling pathways in these diverse settings mediated by non-coding RNAs (ncRNAs), which are packaged in extracellular vesicles (EVs) protecting ncRNAs from degradation. EVs are a heterogeneous group of lipid bilayer-covered vesicles released from virtually all cells, which have important roles in intercellular communication. Recent studies pointed out that ncRNAs including long non-coding RNAs (lncRNAs) and microRNAs (miRNAs) are selectively sorted into EVs, modulating specific aspects of cancer development, namely cell proliferation, migration, invasion, angiogenesis, immune tolerance or drug resistance, under conditions of hypoxia in recipient cells. In myocardial infarction and stroke, ncRNAs shuttled via EVs have been shown to control tissue survival and remodeling post-hypoxia by regulating cell injury, inflammatory responses, angiogenesis, neurogenesis or neuronal plasticity. This review discusses recent evidence on EV-associated ncRNAs in hypoxic cancer, myocardial infarction and stroke, discussing their cellular origin, biological function and disease significance. The emerging concept of lncRNA-circular RNA/ miRNA/ mRNA networks is outlined, upon which ncRNAs synergistically respond to hypoxia in order to modify disease responses. Particular notion is given to ncRNAs participating in at least two of the three conditions, which revealed a large degree of overlaps across pathophysiological conditions. Possible roles of EV-ncRNAs as therapeutic products or theranostic markers are defined.

Introduction

Hypoxia, a pathophysiological condition characterized by reduced tissue oxygen content, is a hallmark of a variety of pathophysiological conditions [1, 2]. Among these conditions, cancer, myocardial infarction and ischemic stroke play an eminent role, since they represent the most prevalent causes of long-term disability and death in medicine [3]. Hypoxia profoundly influences transcriptional responses in the affected cells, e.g., by the transcription factor hypoxia-inducible factor-1α

(HIF1α), which is degraded under normoxic conditions by the von Hippel Lindau protein-mediated ubiquitin protease pathway but which is stabilized upon hypoxia [4], regulating a large variety of genes controlling cell proliferation, metabolism, survival and differentiation [5, 6]. In cancer, HIF1α-dependent gene expression promotes the development of aggressive tumor phenotypes [1, 7, 8]. In myocardial infarction and stroke, persistent hypoxia and ischemia compromise tissue remodeling and recovery [2, 9]. The pathophysiological mechanisms underlying hypoxia in cancer, myocardial infarction and stroke are very different. The main cause of hypoxia in cancer tissue are proliferating tumor cells growing out from blood vessels, which often have irregular network characteristics with compromised blood supply [1, 8]. On the contrary, the primary cause of hypoxia in myocardial infarction and stroke is reduced blood flow associated with vascular occlusions and atherosclerosis [2, 10]. Despite obvious pathophysiological differences, the gene responses to hypoxia in these three pathologies exhibit a high degree of similarities. Similarities relate to protein-encoding RNAs and non-encoding RNAs (ncRNAs).

Resulting from gene expression changes, cellular biology and communication are fundamentally altered under conditions of hypoxia. To coordinate tissue responses, extracellular vesicles (EVs) are released from hypoxic cells [11, 12]. Based on their size and cellular origin, EVs are regularly classified as exosomes (60 to 150 nm), microvesicles (100 to 1000 nm) and apoptotic bodies (typically larger than 500 nm) [11, 13]. Exosomes originate from the late endosomal compartment, whereas microvesicles and apoptotic bodies are derived from plasma membrane. EV secretion is considered as an evolutionarily conserved process, which plays important roles in intercellular communication [13]. For this purpose, EVs transfer a large variety of cargos, including proteins, RNA, DNA, bioactive metabolites and lipids. It is broadly assumed that all these molecule species can be delivered to recipient cells. The heterogenous group of ncRNAs, which are widely found in EV preparations, has received great interest in EV-mediated cell signaling, since ncRNAs profoundly regulate gene responses at the transcriptional, post-transcriptional or epigenetic levels by interacting with DNAs, RNAs or proteins [14, 15]. ncRNAs are categorized by the number of nucleotides constituting RNAs. ncRNAs with less than 200 nucleotides are defined as small ncRNAs, which include microRNAs (miRNAs), small interfering RNAs and piwi-interacting RNAs, whereas long ncRNAs (lncRNAs), which also comprise circular RNAs (circRNAs), are composed of more than 200 nucleotides [14, 16]. Although the precise subcellular source of these EVs is still a matter of discussion – some EV-ncRNAs arise from nucleus – [17], shuttled ncRNA transport via EVs has been proposed to regulate tissue responses to hypoxia [18]. In light of their roles in disease processes, EV-derived ncRNAs might be promising disease biomarkers and even be considered as therapeutic tools [19-21].

The present review summarizes the latest literature regarding the role of EV-ncRNA contents in the progression of hypoxic tumors, myocardial infarction and stroke. Aspects regarding the cellular and subcellular source of EV-associated ncRNAs, their cellular targets and biological functions are evaluated. The possible utility of EV-associated ncRNAs as therapeutic products and theranostic biomarkers is discussed.

1. EVs and ncRNAs

1.1 The characteristics and cellular origins of EVs

EVs are constantly released from eukaryotic cells, archaea and bacteria. They consist of a phospholipid bilayer and are abundantly present in various body fluids including blood, cerebrospinal fluid, urine, breast milk, and lacrima [22, 23]. EVs can be classified into subcategories based on their biogenesis, size and physicochemical properties. Exosomes with a size of 60-150 nm and a density of about 1.10-1.21 g/ml [24, 25] are formed as intraluminal vesicles (ILVs) by membrane budding of late endosomes, which are released into the extracellular space as multivesicular bodies (MVBs) by MVB fusion with the plasma membrane [26, 27]. Microvesicles, which are 100-1000 nm in diameter with a density of about 1.04-1.07 g/ml [25], are released from the plasma membrane by plasma membrane budding [26, 28]. Apoptotic bodies, which are typically larger than 500 nm in diameter, are formed during programmed cell death by plasma membrane budding. In cancer, extremely large vesicles called oncosomes are produced, which are considered to transfer oncogenic messages. Under pathophysiological conditions, apoptotic bodies or oncosomes may be more abundant in certain fluids than exosomes or microvesicles and thus confound EV analyses [29, 30]. Whereas exosomes and microvesicles are suggested as 'safe containers' for cargos mediating cell-to-cell communication, apoptotic bodies are released for degradation during the disassembly of dying cells.

Importantly, not all EVs released from live cells are involved in cell communication. Live cells may

also release vesicles through a cellular excretion machinery that do not aim at transmitting biological signals but are meant for remote degradation especially in the liver [15, 17]. Apparently, such garbage vesicles may contain ncRNA cargos that lack cell signaling roles. Besides, apoptotic cells not only fragment into the larger apoptotic bodies, but also generate many EVs in the size of exosomes. When preparing EVs in the size range of exosomes, combinations of exosomes, small microvesicles, small apoptotic vesicles and other small EVs, including nuclear EVs, are enriched [31]. To harmonize the nomenclature, the *International Society of Extracellular Vesicles* recommended to define all prepared vesicles independent of their origin as EVs; if they are all in the size of exosomes, the EVs might be termed more specifically as small EVs [32]. Unfortunately, not all scientists and especially researchers from the industry adopted the nomenclature and use the term exosomes as a synonym for small EVs. The issue is further complicated by the fact that hardly any method allows isolation of EVs; regularly concentrated EVs contain a number of non-EV associated byproducts, e.g. blood-derived EV samples typically contain a high load of lipoproteins and urine samples frequently aggregates of Tamm-Horsfall protein also named uromodulin [33, 34]. Furthermore, in addition to potentially contributing to functional impacts, byproducts in EV preparations may significantly influence EV quantification [35, 36]. The reader should be aware that at least in most of the studies EVs were not isolated but rather enriched likely to contain a panel of different non-EV-associated byproducts.

1.2 The characteristics and associated functions of ncRNAs

Among the different EV cargos, ncRNAs are most systematically studied [37, 38]. Despite their heterogeneity of origin and diversity of biological function, there is meanwhile broad evidence supporting a role of ncRNAs in coordinating tissue responses to injuries. Although more than 20,000 proteins are encoded by the human genome, they only account for approximately 20% of the whole genome [39]. Emerging evidence demonstrates that both short ncRNAs and lncRNAs play a crucial role in the regulation of gene expression in numerous pathophysiological states [40].

miRNAs are single-strand RNAs, which typically are 21-23 nucleotides in size and belong to the family of short ncRNAs. Released from the nucleus as single-strand pre-miRNA hairpins, pre-miRNAs are processed to mature miRNAs in the cytosol via cleavage by the endonuclease Dicer [41].

Together with Dicer and associated proteins, miRNAs form the RNA-induced silencing complex (RISC) [42]. As part of the RISC, miRNAs interact with complementary gene sequences in the 3' untranslated region of target mRNA sequences, repressing gene expression by argonaute (AGO)-mediated mRNA cleavage, by mRNA poly(A) tail shortening that destabilizes the mRNA, or by interference with mRNA-ribosome interactions [43-45]. The human genome contains >600 genes with robust evidence of miRNA functions [46]. These miRNAs target >60% of all human genes [47]. Thus, single miRNAs can have hundreds, sometimes >1000 mRNA targets [47]. In many cases, these miRNAs moderately influence mRNA expression levels. Due to their multiple mRNA targets, the biological consequences of this action are profound.

In contrast to miRNAs, siRNAs are formed as double-strand RNAs in the nucleus which are typically longer than pre-miRNAs [48, 49]. Following cleavage by Dicer, 21-24 nucleotide siRNAs result, which dissociate to single-strand siRNAs upon interaction with the RISC. As part of the RISC, these siRNAs scan complementary mRNA sequences [50]. Unlike miRNAs, siRNAs have tight target specificity [48, 50]. siRNA binding induces cleavage of these target mRNAs.

Among small ncRNAs, piwi-interacting RNAs (piRNAs) are the largest in size. Their length varies between animal species, it typically ranges from 26 to 31 nucleotides [51]. piRNAs form complexes with piwi-AGO proteins capable of binding mRNAs and cleaving them [51].

lncRNAs are transcripts with more than 200 nucleotides that are not translated into protein [39]. lncRNAs include intergenic and intronic ncRNAs, and may involve sense and antisense RNA sequences. Although the biological role has so far been shown only for a small lncRNA proportion, they control transcription and translation in multiple ways, namely as transcription coregulators, ligands to nuclear transcription repressors, activators of transcription factors, regulators of epigenetic modifications, assistants in DNA double-strand break repair, as well as mRNA processing, splicing, transport, translation, and degradation [52, 53]. The recently described circRNAs display a circular covalently bonded structure associated with a higher tolerance to exonucleases [54]. They serve as scaffolds for chromatin-modifying complexes, regulate gene transcription and mRNA splicing, and act as miRNA sponges [55, 56].

1.3 ncRNA loading into EVs

Although ncRNAs inside EVs originate from the

transcriptome of their source cells, the composition of these ncRNAs differs from their source cell ncRNAs [57]. Among ncRNAs enriched in EVs, miRNAs are most abundant [58, 59]. Several studies analyzed the loading and sorting processes of ncRNAs into EVs, for which numerous signaling pathways have been shown to be involved [60-62]. Recent work demonstrated that miRNA sorting into EVs is not a random but a highly regulated process [63, 64]. miRNAs are characterized by a uridine or adenine residue at their 3′-end, which is important for their recognition by AGO2. miRNAs with an adenylated 3′-end are predominantly found in cells, whereas miRNAs with a uridylated 3′-end are sorted in EVs, as shown in RNA sequencing studies on human B cells and their EVs [63]. These results suggest that posttranscriptional miRNA modifications, notably, 3′-end adenylation and uridylation, might play a pivotal role in EV packaging.

There is increasing evidence that ncRNA sorting into EVs critically depends on membrane lipid and, more specifically, sphingolipid metabolism. The lipid composition of the EV membrane resembles that of membrane microdomains, which are characterized by a high content of cholesterol, phosphatidyl choline, sphingomyelin and ceramide [65]. Ceramide formation is controlled by neutral sphingomye-linase-2 and acid sphingomyelinase, which are localized in the cytosolic and luminal membrane leaflets, respectively, and hydrolytically cleave sphingomyelin to ceramide [66-68]. Ceramide has unique biophysical properties, as it can self-associate through hydrogen bonding, providing the driving force that results in the coalescence of microscopic microdomains to a large-scale macrodomains [67] and the budding of ILVs from MVBs [65]. It has been proposed that membrane microdomains act as platforms for MVB sorting and that ncRNAs integrating into these platforms exhibit specific nucleotide motifs differentially predisposing these ncRNAs for microdomain membrane interaction [62]. Indeed, RNAs binding to microdomains possess a specific secondary structure that differs from other RNAs [69]. Randomly structured RNA sequences revealed 20-fold lower affinity to the microdomain domains. In addition, specific nucleotide sequences appear to be required for enhanced affinity to phospholipid bilayers, and domains with membrane affinity have not been observed in random RNA sequences [61, 70]. Specific exosome-sorting RNA motifs have been shown for both miRNAs (called EXOmotifs) and mRNAs [62].

Intracellular transfer of ncRNAs from the nucleus to other subcellular compartments involves RNA-binding proteins, from which larger ribonucleoprotein particles are formed ensuring traveling along the cytoskeleton [71]. To date, more than 500 RNA-binding proteins have been reported, which consist of approximately 25% of the protein content of EVs [57, 72]. Emerging evidence demonstrates that ncRNAs can be transmitted into MVBs for exosome packaging or to the plasma membrane for extracellular secretion. This transmission takes place in association with RNA binding proteins like heterogeneous nuclear ribonucleoproteins A2/B1 (hnRNPA2B1), YBX1, SYNCRIP, AGO2 and others [57]. Villarroya et al. revealed that in T cells, hnRNPA2B1 can target a specific motif of numerous miRNAs and transmit them into the ILVs [73]. SYNCRIP selectively loads hepatocyte miRNAs with a 4-nucleotide motif near the 3′-end [74], whereas YBX1 selectively carries miR-223 into HEK-293T cell EVs [75]. Deletion of AGO2 in HEK293T cells significantly decreased miR-142-3p and miR-451 miRNAs in EVs [76, 77]. Although a first picture of the mechanisms underlying ncRNA sorting is currently evolving, the extent to which these pathways are specific to certain ncRNA species in defined EV subsets is elusive. Future studies will have to evaluate how hypoxia regulates these packaging mechanisms.

1.4 Uptake of ncRNA-loaded EVs by recipient cells

To transmit ncRNAs into recipient cells, EVs may merge directly with the recipient cell by direct membrane fusion or be internalized through clathrin-mediated or caveolin-mediated endocytosis, phagocytosis or macropinocytosis [78]. EV docking can be assimilated by recipient cells through directly targeting corresponding receptors on the plasma membrane, which in turn activates or inhibits associated signaling pathways [79]. The receptors can be manipulated on the cell surface to increase EV uptake. For instance, enzymatic depletion or pharmacological inhibition of the extracellular matrix heparan sulfate proteoglycans on the plasma membrane has been found to promote the uptake of tumor-derived EVs by endocytosis [80]. In the latter study, EV uptake was specifically inhibited by free heparan sulfate chains, whereas closely related chondroitin sulfate had no effect [80]. Several integrin receptors have been shown to modulate EV uptake in a variety of cancers [57]. As such, integrin receptors were shown to be enriched in cancer EVs compared to EVs obtained from benign epithelial cells [81]. The total EV integrin levels, including the quantity of integrins α6, αv, and β1, correlated with tumor stage across a variety of epithelial cancers, while integrin α6 was prominently expressed on breast and ovarian

progenitor cells [81], suggesting a role of these integrins in cellular EV uptake and the utility of EV integrins as potential theranostic markers. A crucial role in EV uptake might be related to phosphatidylserine, which is highly abundant on the surface of apoptotic cells but also present on a subpopulation of EVs [82], where phosphatidylserine considerably acts as 'eat me' signal by phagocytes [83]. In mouse macrophages, the EV uptake is mediated via interaction of phosphatidylserine with T cell immunoglobulin and the mucin domain-containing protein 4 (Tim4) [83]. Indeed, the delivery of anti-Tim4 antibody prevented EV uptake by thymic macrophages [83]. Despite these emerging data, our current understanding of EV uptake mechanisms is still preliminary. Open questions remain about the endosomal escape of internalized EVs, which is required to deliver luminal EV cargo including ncRNAs into the cytosol of the EV recipient cells. In this context, it is interesting to note that even after successful EV uptake the generation of functional proteins from EV-derived mRNAs was negligible in recipient prostate cancer cells [57, 84]. Presumably, these mRNAs could not be released from the endosomal system and thus were unable to reach the ribosomes. Hence, dissection of potential endosomal escape mechanisms is vital for understanding whether ncRNA mediate biological functions after EV uptake. It needs to be considered that EVs containing ncRNAs are taken up as cell nutrition. After EV internalization residual parts are degraded in the lysosome or excreted for digestion in remote cells or tissues, including the liver. Indeed, EV biodistribution studies detect the liver as very prominently labeled organ [85]. A brief overview of mechanisms of communication between cells by EVs is shown in **Figure 1.**

1.5 Possible ncRNA artifacts associated with EV isolation and purification

In a variety of well-defined settings, authors could not confirm that ncRNAs and more specifically miRNAs are sorted into EVs [57]. Certainly, results strongly depend on the EV preparation method. Originally and still often today, EVs are isolated by differential ultracentrifugation [86], which as stated before, also results in the preparation of many lipoprotein particles [87]. Indeed, lipoproteins
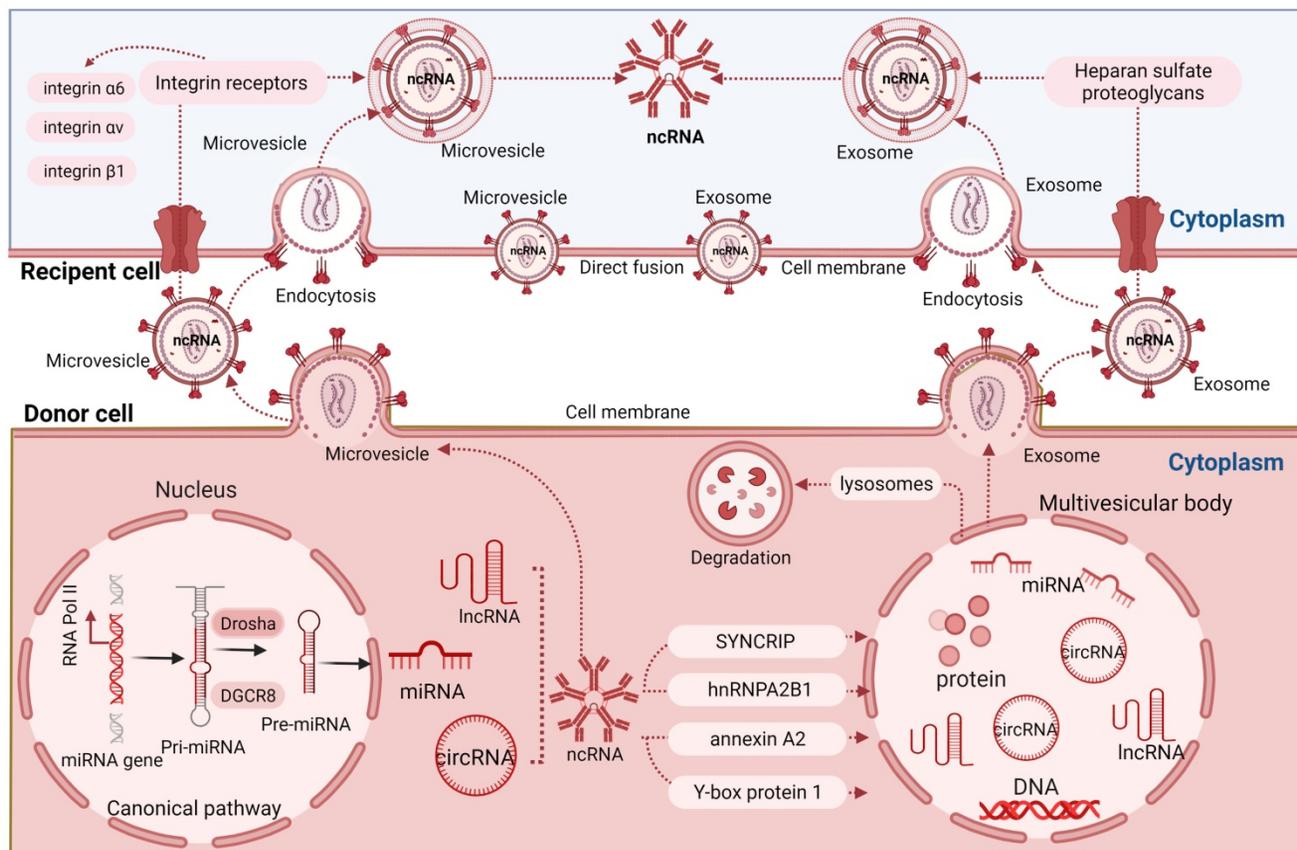

Figure 1. Brief overview of mechanisms of cellular communication by EVs. miRNAs are formed in the nucleus as pre-miRNAs that are processed to pre-miRNAs and mature miRNAs that are released into the cytosol. ncRNAs containing different RNA motifs are loaded into microvesicles and multivesicular bodies (MVBs) via different RNA-binding proteins. ILVs are formed within MVBs which are released into the extracellular space as exosomes. Recipient cells can take up EV-associated ncRNAs by direct fusion or endocytosis, both of which may be controlled by integrin receptor signaling.

frequently contain miRNA-binding AGO proteins and thus can protect extracellular miRNAs [88]. Bead capturing experiments using EVs obtained from mesenchymal stromal cells (MSCs) revealed that EVs recovered by cholera toxin b, a GM1 ganglioside ligand and membrane microdomain marker, contained many exosome markers but hardly any RNAs [17]. In contrast, EVs captured by the globotriaosylceramide ligand shiga toxin b were abundant in nuclear markers and contained large amounts of RNAs [17]. Interestingly, among the many studies using proteomic methods to analyse the composition of EVs, only one has reported the presence of AGO2 and none has detected Dicer [57]. Apparently, the cellular source of EVs and physiological condition in which cells are raised decisively influence EV-ncRNA cargos. In the interpretation of ncRNA findings, possible artifacts related to EV isolation and purification carefully need to be considered.

2. Roles of EV-ncRNAs in the hypoxic tumor microenvironment

Hypoxia is a key feature of solid tumors [89].

Highly proliferating tumor cells outgrow the existing local blood supply, forming irregular vessel networks that cannot compensate for tissue oxygen needs [90, 91]. Hence, tissue oxygen levels can drop below 2% in tumor masses, which has profound effects on the release and ncRNA contents of EVs [92]. ncRNAs released from hypoxic tumor cells via EVs play an important role in creating a microenvironment that supports tumor growth [93-101]. Notably, a large number of ncRNAs are increased in EV preparations obtained from hypoxic tumors. Several of these ncRNAs have been attributed to signal pathways associated with cell survival and proliferation, such as the FoxO pathway, the proteoglycans in cancer pathway, the HIF-1 signaling pathway or the mitogen-activated protein kinase (MAPK) pathway [11]. The involvement of EV-derived ncRNAs in regulating tumor cell proliferation, angiogenesis, immunosuppression, drug resistance has been studied extensively in specific cancers under conditions of hypoxia [93-136], as outlined in **Table 1** and **Figure 2**. The main findings are summarized in the following.

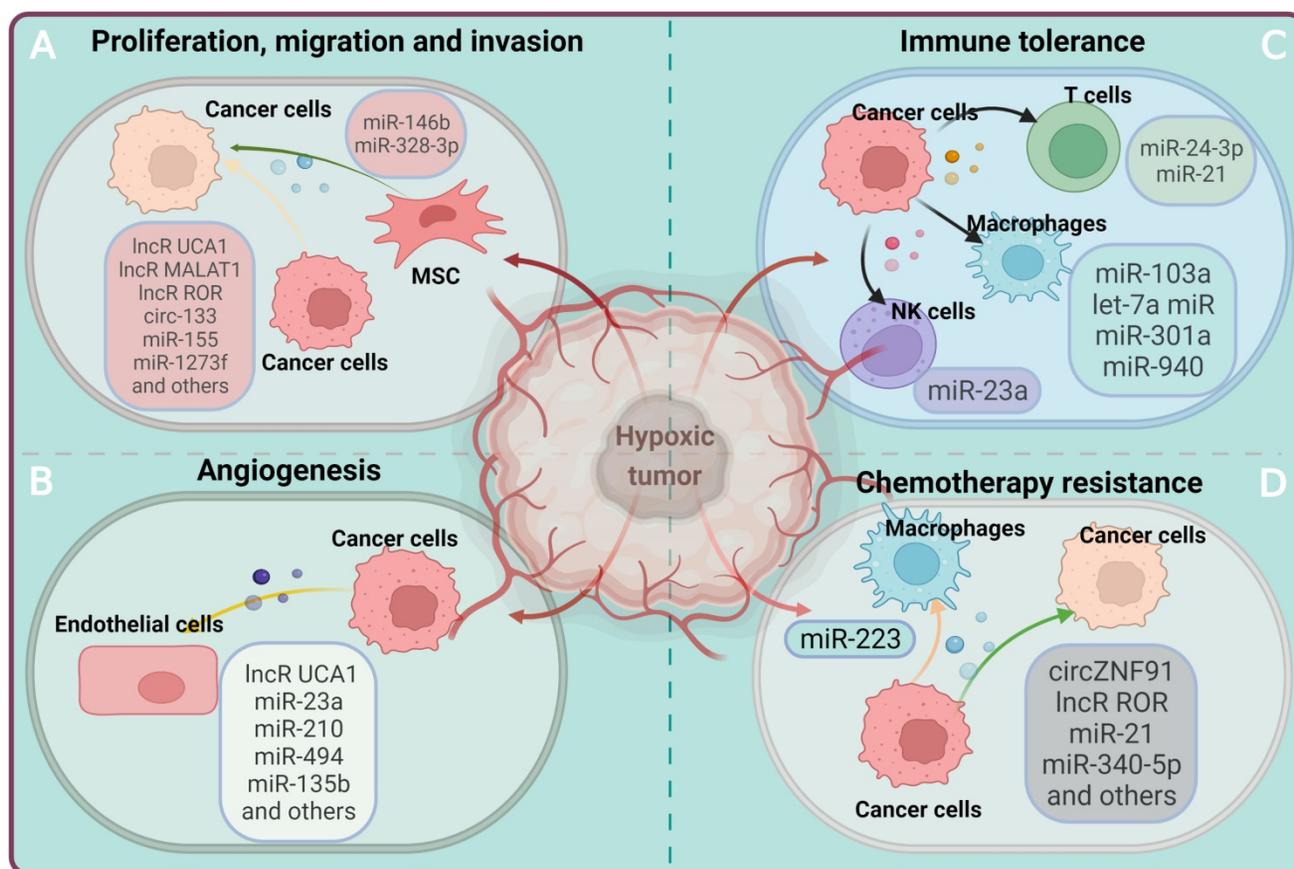**Figure 2. The involvement of EV-ncRNAs derived from the hypoxic tumor in the regulation of the tumor microenvironment.** Hypoxic cancer cells can affect recipient cells by transferring ncRNAs via EVs, which, in turn, are taken up by recipient cells and modulate various biological processes including cell proliferation, immune tolerance, angiogenesis and drug resistance, thus facilitating tumor growth and progression.

Table 1. Preclinical studies assessing the effect of ncRNAs transferred via EVs in the hypoxic tumor microenvironment.

Authors [reference]	Cancer type	ncRNAs	EV provenance	Recipient cell	Primary action	Mechanism of action
Xue et al. [102]	Bladder carcinoma	lncR UCA1	5637 cancer cells	UMUC2 cancer cells	Tumor cell proliferation	Promotion of epithelial-mesenchymal transition
Zhang et al. [123]	Lung carcinoma	lncR MALAT1	Patient serum	A549 and H1299 cancer cells	Tumor cell proliferation, migration and survival	Not determined
Rong et al. [124]	Lung carcinoma	lncR MALAT1	Patient serum, A549 and H1299 cancer cells	A549 and H1299 cancer cells	Tumor cell proliferation, invasion and survival	miR-515 sponging, EEF2 upregulation,
Wang et al. [125]	Lung carcinoma	lncR MALAT1	A549 and H1299 cancer cells	A549 and H1299 cancer cells	Tumor cell proliferation, colony formation and glycolysis	miR-613 sponging, COMMD8 upregulation
Zhou et al. [122]	Breast carcinoma	lncR NEAT1	Patient serum	MCF-7 and MDA-MB-231 cancer cells	Tumor cell proliferation, migration, invasion and metastasis	miR-141-3p sponging, KLF12 upregulation
Takahashi et al. [120]	Hepatocellular carcinoma	lncR ROR	HepG2 cancer cells	HepG2 cancer cells	Tumor cell proliferation	miR-145 downregulation, HIF1α stabilization
Chen et al. [95]	Ovarian carcinoma	miR-21–3p/ miR-125 b-5p/ miR-181d-5p	SKOV3 cancer cells	SKOV3 cancer cells/ macrophages	Tumor cell proliferation/ immune tolerance	HIF-1α/ HIF-2α stabilization, M2 macrophage polarization
Hu et al. [129]	Hepatoblastoma	miR-126	huH6 and HepG2 cancer cells	huH6 and HepG2 cancer cells, MSCs	Tumor cell proliferation, tumor growth	MSC differentiation into cancer cells
Katakowski et al. [128]	Glioma	miR-146b	MSCs	Glioma cells	Tumor cell proliferation/ tumor growth	Not determined
Meng et al. [104]	Renal carcinoma	miR-155	786-O and Caki-1 cancer cells	786-O and Caki-1 cancer cells	Tumor cell proliferation	FOXO3 downregulation
Liu et al. [105]	Lung carcinoma	miR-328-3p	MSCs	A549 and H125 cancer cells	Tumor cell proliferation, migration, invasion/ tumor growth	Promotion of epithelial-mesanchymal transition; NF2 downregulation, inhibition of Hippo pathway activation
Li et al. [103]	Colorectal carcinoma	miR-361-3p	CRC cancer cells	HCT116 and HT29 cancer cells	Tumor cell proliferation, survival; tumor growth	TRAF3 downregulation, NF-κB activation
Yu et al. [98]	Hepatocellular carcinoma	miR-1273f	Huh7 and 97H cancer cells	Huh7 and 97H cancer cells	Tumor cell proliferation	LHX6 downregulation
Yang et al. [136]	Colorectal carcinoma	circ-133	Patient serum, SW 480 and HCT 116 cancer cells	SW 480 and HCT 116 cancer cells	Tumor cell migration/ metastasis	miR-133a sponging, GEF-H1 and RhoA elevation
Li et al. [114]	Oral squamous cell carcinoma	miR-21	Patient serum	SCC-9 and CAL-27 cancer cells	Tumor cell migration/ invasion	HIF1α/HIF2α stabilization
Liu et al. [133]	Hepatocellular carcinoma	miR-25-5p	HuH-7 and HCCLM3 c cancer ells	HuH-7 and HCCLM3 cancer cells	Tumor cell migration/ invasion	Not determined
Yu et al. [121]	Lung carcinoma	miR-31-5p	A549 and H1299 cancer cells	A549 and H1299 cancer cells	Tumor cell migration/ invasion	SATB2-revered epithelial-mesenchymal transition, ERK1/2 activation
Zhang et al. [97]	Lung carcinoma	miR-193a-3p/ miR-210-3p/ miR-5100	MSCs	A549, H358, H460 and LLC cancer cells	Tumor cell migration/ invasion	STAT3 activation, epithelial-mesenchymal transition
Li et al. [130]	Lung carcinoma	miR-126	Patient serum	A549 and H460 cancer cells	Inhibition of tumor cell proliferation, colony formation, migration, invasion and survival	ITGA6 downregulation
Guo et al. [106]	Pancreatic carcinoma	lncR UCA1	MIA PaCa-2 cancer cells	Endothelial cells	Angiogenesis	miR-96-5p sponging, AMOTL2 repression reversal
Hsu et al. [108]	Lung carcinoma	miR-23a	CL1-5 cancer cells	Endothelial cells	Angiogenesis	PHD1/ PHD2 downregulation, HIF1α stabilization
Zeng et al. [134]	Colorectal carcinoma	miR-25-3p	Patient serum and CRC cancer cells	Endothelial cells	Angiogenesis, vascular permeability, metastasis	KLF2 and KLF4 downregulation
Taverna et al. [131]	Chronic myeloid leukemia	miR-126	LAMA84 cancer cells	Endothelial cells	Angiogenesis	CXCL12 and VCAM1 downregulation
Umezu et al. [101]	Multiple myeloma	miR-135b	RPMI8226 cancer cells	Endothelial cells	Angiogenesis	FIH downregulation, HIF1α stabilization
Tadokoro et al. [107]	Leukemia	miR-210	K562 cancer cells	Endothelial cells	Angiogenesis	EFNA3 downregulation
Mao et al. [109]	Lung carcinoma	miR-494	A549 cancer cells	Endothelial cells	Angiogenesis, tumor growth	PTEN downregulation, Akt/ eNOS activation
Li et al. [119]	Oral squamous cell carcinoma	miR-21	Cal-27 and SCC9 cancer cells	γδ T cells	Immune tolerance	γδ T cell deactivation through PTEN/PD-L1 axis regulation
Berchem et al. [111]	Different cancers, including lung carcinoma	miR-23a	IGR-Heu and K562 cancer cells	NK cells	Immune tolerance	NK cell deactivation through CD107a downregulation
Ye et al. [110]	Nasopharyngeal carcinoma	miR-24-3p	TW03, C666 and CNE2 cancer cells	T cells	Immune tolerance	T cell deactivation through FGF11 downregulation, ERK1/2 and STAT1/3 activation and STAT5 deactivation
Guo et al. [93]	Glioma	miR-10a/ miR-21	P3 and GL261 cancer cells	MDSCs	Immune tolerance	MDSC expansion, RORA and PTEN downregulation
Yang et al. [126]	Melanoma	lncR NEAT1	MSCs	Macrophages	Immune tolerance	M2 macrophage polarization through miR-374 sponging, LGR4-dependent IQGAP1 upregulation

Authors [reference]	Cancer type	ncRNAs	EV provenance	Recipient cell	Primary action	Mechanism of action
Hsu et al. [116]	Lung carcinoma	miR-103a	CL1-5 cancer cells	Macrophages	Immune tolerance	M2 macrophage polarization through PTEN downregulation, Akt/ STAT3 activation
Wang et al. [117]	Pancreatic carcinoma	miR-301a	PANC-1 cancer cells	Macrophages	Immune tolerance	M2 macrophage polarization through PTEN downregulation, PI3Kγ activation
Chen et al. [118]	Ovarian carcinoma	miR-940	SKOV3 cancer cells	Macrophages	Immune tolerance	M2 macrophage polarization
Park et al. [100]	Melanoma	let-7a miR	B16 melanoma cells	Macrophages	Immune tolerance	M2 macrophage polarization through inhibition of insulin/ Akt/ mTOR signaling
Yang et al. [127]	Glioma	lncR MALAT1	Glioma stem cells	Microglia	Proinflammatory response	miR-129-5p sponging, HMGB1 upregulation, IL6, IL8 and TNFα release in response to lipopolysaccharide exposure increased
Zeng et al. [112]	Pancreatic carcinoma	circZNF91	BxPC-3 and SW1990 cancer cells	BxPC-3 cancer cells	Chemotherapy resistance	miR-23b-3p sponging, SIRT1 upregulation, HIF1α stabilization
Wang et al. [135]	Colorectal carcinoma	ciRS-122	SW480 and L-OHP cancer cells	SW480 cancer cells	Chemotherapy resistance, glycolysis promotion	miR-122 sponging, PKM2 upregulation
Takahashi et al. [99]	Hepatocellular carcinoma	lncR ROR	HepG2 cancer cells	HepG2 cancer cells	Chemotherapy resistance	CD133+ cell formation
Dong et al. [94]	Lung carcinoma	miR-21	A549 cancer cells	A549 cancer cells	Chemotherapy resistance	PTEN downregulation
Guo et al. [132]	Ovarian carcinoma	miR-98-5p	Cancer-associated fibroblasts	A2780 cancer cells	Chemotherapy resistance	CDKN1A downregulation
Zhu et al. [113]	Ovarian carcinoma	miR-223	Macrophages	SKOV3 cancer cells	Chemotherapy resistance	PTEN downregulation, PI3K/Akt activation
Chen et al. [115]	Oral squamous cell carcinoma	miR-340-5p	Te13, Te1 and Eca109 cancer cells	Te13, Te1 and Eca109 cancer cells	Radiotherapy resistance	KLF1 downregulation

Abbreviations: EVs, extracellular vesicles; lncR, long non-coding RNA; MDSCs, myeloid-derived suppressor cells; MSCs, mesenchymal stromal cells.

2.1 Tumor cell proliferation, migration and invasion

Excessive cellular proliferation is a fundamental characteristic of cancer, which results from the activation of oncogenic signals that overrule the physiological inhibition of cell growth [137]. EV-derived ncRNAs contribute to tumor cell proliferation, as has been brought into the spotlight recently. In EVs obtained from bladder carcinoma cells raised under hypoxic conditions, the content of urothelial carcinoma associated-1 (UCA1), a hypoxia-responsive lncRNA, was found to be enriched compared with EVs obtained from normoxic bladder carcinoma cells [102]. UCA1 transfer via EVs obtained from hypoxic bladder carcinoma cells promoted tumor cell proliferation, migration, and invasion in recipient cells via mechanisms that involved epithelial-mesenchymal transition, a process relevant for cancer progression [102]. The level of lncR-UCA1 in human serum-derived EVs of bladder carcinoma patients was higher than that in healthy control patients [102]. Similarly, in hepatocellular carcinoma, lncRNA ROR accumulated in hypoxic tumor cell EVs was found to promote cancer growth by miR-145 downregulation and HIF1α stabilization [120]. In non-small cell lung adenocarcinoma, lncRNA metastasis-associated lung adenocarcinoma transcript-1 (MALAT1) significantly increased tumor cell proliferation, migration, invasion, colony formation and glycolysis via mechanisms involving miR-515 and miR-613 sponging, followed by EEF2 and COMMD8 upregulation [123-125]. In breast carcinoma, lncRNA nuclear enriched abundant transcript-1 (NEAT1) promoted tumor cell proliferation, migration, invasion and metastasis via miR-141-3p sponging and KLF12 upregulation [122]. In colorectal carcinoma, hypoxic EV-associated circ-133 promoted tumor cell migration and metastasis via miR-133 sponging, GEF-H1 and RhoA elevation [136].

Besides lncRNAs, EV miRNAs have been involved in the regulation of tumor cell proliferation in hypoxia. In EVs derived from colorectal carcinoma cells, miR-361-3p was described to be enriched, when cells were cultivated under hypoxic compared with normoxic conditions [103]. EV-mediated miR-361-3p delivery from hypoxic cells promoted tumor growth and suppressed tumor cell apoptosis in recipient cells by interaction with TRAF3 resulting in the activation of the NFκB pathway [103]. Likewise, in EVs from hepatocellular carcinoma cells, miR-1273f was enriched when cells were raised under hypoxic compared with normoxic conditions [98]. miR-1273f delivery via EVs obtained from hypoxic hepato-cellular carcinoma cells was reported to increase miR-1273f levels in normoxic target cells, enhancing their proliferation by downregulating LHX6 expression [98]. Studies in renal cell carcinoma and lung carcinoma noted roles of miR-155 and miR328-3p in hypoxic cancer proliferation via mechanisms involving inhibition of FOXO3 expression and activation [104] or inhibition of tumor suppressor NF2 targeted Hippo pathway activation [105],

respectively. In oral squamous cell carcinoma and ovarian carcinoma, a role of EV miR-21 has been shown in hypoxic cancer cell proliferation, migration and invasion via HIF1α and HIF2α stabilization [95, 114], and in lung carcinoma, roles of miR-31-5p, miR-193-3p, miR-201-3p and miR-5100 were found via SATB2-revered epithelial-mesenchymal transition, MAPK/ERK1/2 activation and STAT3 activation [97, 121]. In the latter studies, roles in the regulation of tumor cell proliferation, migration and invasion were shown for miRNAs associated with cancer cell EVs and MSC EVs.

2.2 Tumor-associated angiogenesis

Tumor growth vitally depends on tumor-associated angiogenesis, which involves a plethora of events such as the basal membrane degradation, endothelial proliferation, migration, tube formation and branching [138]. EV-derived ncRNAs are claimed to facilitate tumor-associated angiogenesis under conditions of hypoxia. In this process, the lncRNA UCA1 again seems to play a prominent role. UCA1 was elevated in EVs obtained from hypoxic compared with normoxic pancreatic carcinoma cells and in serum-derived EVs from pancreatic carcinoma patients [106]. In serum EVs of pancreatic carcinoma patients, UCA1 levels were associated with poor patient survival [106]. Exposure of HUVECs to UCA1 enriched EVs promoted angiogenesis *in vitro* and *in vivo* by acting as a sponge for miR-96-5p that relieved the repressive effects of miR-96-5p on its target gene AMOTL2 [106]. In leukemia, miR-210 was upregulated in hypoxic compared with normoxic tumor cells and their EVs, inducing angiogenesis by downregulating EFNA3 expression [107]. Angiogenesis was also facilitated by miR-23a-enriched EVs from hypoxic lung carcinoma cells, which induced tight junction breakdown, vascular permeability, transendothelial tumor cell migration and tumor growth [108]. As underlying mechanism, the suppression of the miR-23a targets prolyl hydroxylase 1 and 2 (PHD1 and 2) that promoted HIF1α accumulation was identified [108]. In lung carcinoma, the delivery of miR-494 enriched EVs derived from hypoxic tumor cells enhanced angiogenesis via PTEN, Akt and eNOS signaling [109]. Studies in hypoxia-resistant multiple myeloma revealed that miR-135b enrichment in EVs enhanced angiogenesis under conditions of hypoxia via suppression of its target factor inhibiting HIF1 (FIH) [101]. In colorectal carcinoma, tumor EV miR-25-3p promoted angiogenesis, vascular permeability and metastasis by downregulating KLF2 and KLF4 [134].

2.3 Immune tolerance

Immune surveillance plays a central role in controlling tumor growth. To support its own growth, tumors can induce immune tolerance. In this process, ncRNAs transmitted via EVs are thought to be involved [18, 139]. In nasopharyngeal carcinoma, tumor cells found to be enriched in miR-24-3p were shown to inhibit T cell proliferation and Th1 and Th17 differentiation by downregulating its target FGF11, thus increasing MAPK extracellular kinase (ERK)-1/2 activity, increasing signal transducer and activator of transcription (STAT)-1 and STAT3 activity and reducing STAT5 activity [110]. In a study comparing a variety of tumor cells models including lung carcinoma cells, EVs from hypoxic tumor cells displayed elevated miR-210 and miR-23a abundance [111]. The authors showed that miR-23a in hypoxic EVs downregulated CD107a expression in NK cells and thus lowered their antitumor response [111]. In oral squamous cell carcinoma, elevated miR-21 in EVs of hypoxic tumor cells inhibited γδ T cell activation by regulating PTEN and PD-L1 [119]. Myeloid-derived suppressor cells (MDSCs) modulate the immunosuppressive tumor microenvironment by inhibiting T cell activation. A sequencing analysis of miRNAs from both hypoxic and normoxic glioma-derived EVs found that miR-10a and miR-21 induced upon hypoxia promoted MDSC expansion and activation by targeting RAR-related orphan receptor alpha (RORA) and phosphatase and tensin homolog (PTEN) [93]. In lung carcinoma, melanoma, pancreatic carcinoma and ovarian carcinoma, NEAT1, miR-103a, miR-301a, miR-940 and let-7a were shown to regulate macrophage polarization towards an immunotolerant M2 phenotype via mechanisms involving the PTEN, PI3K/ Akt, STAT3 or IQGAP1 pathways, besides others [100, 116-118, 126]. The induction of immune tolerance facilitates tumor growth.

2.4 Chemotherapy resistance

Acquired chemotherapy resistance in response repeated drug exposure is an essential factor that contributes to poor prognosis in cancer. Unraveling the underlying mechanisms is a prerequisite for developing novel cancer treatments. ncRNAs shuttled via EVs have recently been shown to contribute to chemotherapy resistance development. Microarray analysis of EV-derived circRNAs from hypoxic and normoxic pancreatic carcinoma cells displayed that circZNF91 was increased in EVs obtained from hypoxic pancreatic carcinoma cells [112]. Overexpression of circZNF91 induced chemotherapy resistance in normoxic PC cells, while circZNF91 knockdown attenuated chemotherapy resistance by competitively targeting miR-23b-3p [112]. In

colorectal carcinoma, EV-associated circRNA ciRS-122 promoted glycolysis and induced oxaliplatin chemotherapy resistance through miR-122 sponging and PKM2 upregulation [135]. In hepatocellular carcinoma, the hypoxia-responsive lncRNA ROR was highly abundant in tumor cells and their EVs [99]. Incubation of tumor cells with lncRNA ROR-rich EVs induced chemotherapy-resistance to sorafenib [99]. Interestingly, sorafenib similarly increased lncRNA ROR levels in tumor cells and their EVs, whereas siRNA-mediated lncRNA ROR knockdown restored chemotherapy responsiveness via mechanisms involving diminished CD133+ cells in response to transforming growth factor-β (TGFβ), a known stimulant inducing chemotherapy resistance [99]. In non-small cell lung carcinoma cells, hypoxia increased miR-21 levels in tumor cells and their EVs, promoting cisplatin chemotherapy resistance of recipient cells by downregulating PTEN [94]. Notably, high miR-21 levels in non-small cell lung carcinoma samples was associated with short survival in patients receiving chemotherapy, but not in patients not receiving chemotherapy [94]. In hypoxic macrophages, elevated miR-223 levels were noted in EVs conferring chemoresistance in ovarian carcinoma cells; and miR-223 was shown to mediate this action via mechanisms involving PTEN downregulation and phosphatidylinositol-3 kinase (PI3K)/ Akt overactivation [113]. The role of EV-shuttled ncRNAs supports a role as markers in diagnostics or theranostics in cancer.

3. Roles of EV-ncRNAs in myocardial infarction

The occlusion of a coronary artery in myocardial infarction results in a series of pathological events, among which necrosis, apoptosis, autophagy and inflammatory damage may ultimately lead to heart failure and death [2]. Unlike in cancer, in which tumor development occurs progressively, myocardial infarction is an acute disorder characterized by an abrupt interruption of blood flow. Owing perhaps to the acute nature and severity of injury associated with myocardial infarction, several ncRNAs are downregulated in hypoxic-ischemic heart tissue in response to myocardial infarction, as previously shown for lncRNA HCP5, miR-21, miR-24, miR-30e, miR-98-5p, miR-150p, miR-185 and miR-212-5p [140-147], whereas other ncRNAs, such as lncRNA NEAT1 and miR-328-3p, are increased [148, 149]. Delivery of EVs derived from bone marrow-derived mesenchymal stromal cells (MSCs) or patient blood seem to restore reduced ncRNA levels in ischemic heart tissue [140, 141, 143, 144, 147, 150]. Suggestively, EV-ncRNAs play important roles in coordinating responses to myocardial injury, i.e., by modulating cardiomyocyte survival, inflammatory responses, angiogenesis and cardiac functional recovery [59, 151, 152]. While EV-ncRNAs in hypoxic tumors have been widely studied in patient-derived cancer tissue, similar information from tissue obtained from patients with myocardial infarction is scarce due to the lack of access to histological tissue samples. Unlike in cancer, previous studies on myocardial infarction have mainly been conducted using tissues or cells without preexisting injury, which were experimentally exposed to hypoxia or ischemia [140-150, 153-180], as summarized in **Table 2** and **Figure 3** and further specified in the following.

Table 2. Preclinical studies assessing the effects of ncRNAs transferred via EVs in models of myocardial infarction.

Authors [reference]	ncRNAs	EV provenance	Recipient cell	Ischemia model	Primary action	Mechanism of action
Diao et al. [153]	lncR UCA1	MSCs	Cardiomyocytes	H/R, LAD ligation	Cell survival, autophagy inhibition	miR-143 sponging/ Bcl2 elevation
Shyu et al. [154]	lncR MALAT1	Cardiomyocytes	Cardiomyocytes, endothelial cells	H/R, LAD ligation	Cell survival	miR-92a sponging, KLF2 and CD31 elevation
Kenneweg et al. [149]	lncR NEAT1	Cardiomyocytes	Cardiomyocytes, fibroblasts	LAD ligation	Cell survival, cardiac function recovery, antifibrosis	NEAT1 transcriptionally upregulated in large EVs by hypoxia through HIF2α
Chen et al. [155]	lncR KLF3-AS1	Cardiomyocytes	Cardiomyocytes, BMSCs	LAD ligation	Cell survival	miR-23c sponging, STAT5B upregulation
Mao et al. [156]	lncR KLF3-AS1	MSCs	Cardiomyocytes	H/R, LAD ligation	Cell survival, Pyroptosis inhibition	miR-138-5p sponging, SIRT1 upregulation
Li et al. [140]	lncR HCP5	MSCs	Cardiomyocytes	H/R, LAD ligation	Cell survival	miR-497 sponging, IGF1 upregulation, PI3K/Akt activation
Gu et al. [141]	miR-21	Patient serum	Cardiomyocytes	OGD/R, LAD ligation	Cell survival	PDCD4 downregulation
Song et al. [157]	miR-21	HEK293T	Cardiomyocytes, endothelial cells	H2O2, LAD ligation	Cell survival	PDCD4 downregulation
Zhang et al. [147]	miR-24	MSCs	Cardiomyocytes	LAD ligation	Cell survival, cardiac function recovery	Bax, caspase-3 and activated caspase-3 reduction
Peng et al. [181]	miR-25	MSCs	Cardiomyocytes	OGD/R, LAD ligation	Cell survival	FASL and PTEN downregulation, EZH2 and H3K27me3 reduction elevating eNOS and SOCS3
Pu et al. [142]	miR-30e	MSCs	Cardiomyocytes	H/R, LAD ligation	Cell survival, cardiac function recovery	LOX1 downregulation, NF-κB p65 and caspase-9 deactivation

Authors [reference]	ncRNAs	EV provenance	Recipient cell	Ischemia model	Primary action	Mechanism of action
Zhang et al. [143]	miR-98-5p	MSCs	Cardiomyocytes	LAD ligation	Cell survival, cardiac function recovery, antiinflammation	TLR4 downregulation, PI3K/ Akt activation, reduced macrophage infiltration
Zhu et al. [159]	miR-125b	MSCs	Cardiomyocytes	H/R, LAD ligation	Cell survival, cardiac function recovery	p53/BAK1 downregulation
Chen et al. [176]	miR-126	Endothelial cells	Cardiomyocytes	MCAO	Cell survival, cardiac function recovery	Vascular cell adhesion protein-1 and monocyte chemotactic protein-1 reduction
Luo et al. [160]	miR-126	MSCs	Cardiomyocytes, endothelial cells	LAD ligation	Cell survival, antiinflammation, antifibrosis, angiogenesis	Reduced proinflammatory cytokine formation
Zheng et al. [158]	miR-129	Endothelial cells	Cardiomyocytes	OGD/R, LAD ligation	Cell survival, antiinflammation	TLR4 downregulation, NF-κB and NLRP3 inflammasome deactivation
Wang et al. [161]	miR-129-5p	MSCs	Cardiomyocytes	Coronary artery ligation	Cell survival, antiinflammation, antifibrosis	HMGB1 downregulation
Zhu et al. [162]	miR-133a-3p	MSCs	Cardiomyocytes	H/SD, LAD ligation	Cell survival, cardiac function recovery	Akt activation
Pan et al. [163]	miR-146a	MSCs	Cardiomyocytes	H/R, LAD ligation	Cell survival, antiinflammation, antifibrosis	EGR1 downregulation, TLR4/ NFκB deactivation
Wu et al. [144]	miR-150-5p	MSCs	Cardiomyocytes	LAD ligation	Cell survival, cardiac function recovery	Bax downregulation
Li et al. [145]	miR-185	MSCs	Cardiomyocytes	Coronary artery ligation	Cell survival	SOCS2 downregulation
Barile et al. [177]	miR-210	Cardiac progenitor cells	Cardiomyocytes	LAD ligation	Cell survival	Ephrin-A3 and PTP1b downregulation
Cheng et al. [178]	miR-210	MSCs	Cardiomyocytes	LAD ligation	Cell survival	AIFM3 downregulation
Wu et al. [146]	miR-212-5p	MSCs	Cardiomyocytes	LAD ligation	Cell survival, antifibrosis	NLRC5 downregulation, VEGF/ TGFβ1/ SMAD deactivation
Ke at al. [164]	miR-218-5p/ miR-363-3p	Endothelial progenitor cells	Cardiomyocytes	LAD ligation	Cell survival, antifibrosis, angiogenesis	p53/ JMY downregulation
Fu et al. [165]	miR-338	MSCs	Cardiomyocytes	H₂O₂, LAD ligation	Cell survival, cardiac function recovery	MAP3K downregulation, JNK/Bax reduction, Bcl2 elevation
Wang et al. [150]	miR-671	ASCs	Cardiomyocytes	OGD/R, LAD ligation	Cell survival, antiinflammation, antifibrosis	TGFBR2 downregulation, SMAD2 deactivation
Sanchez-Sanchez et al. [166]	miR-4732-3p	MSCs	Cardiomyocytes, endothelial cells	OGD/R, LAD ligation	Cell survival, cardiac function recovery, antifibrosis, angiogenesis	Not determined
Lin et al. [167]	lncR HCG15	Patient serum	Cardiomyocytes	H/R, LAD ligation	Cell death/ apoptosis, proinflammation	NF-κB/ p65 and p38 activation, IL1, IL6 and TNFα upregulation
Ning et al. [168]	miR-153-3p	MSCs	Cardiomyocytes, endothelial cells	OGD/R	Cell death	ANGPT1 downregulation, VEGF/ VEGFR2/ PI3K/ Akt/ eNOS deactivation
Huang et al. [148]	miR-328-3p	Cardiomyocytes	Cardiomyocytes	LAD ligation	Cell death/ apoptosis	Caspase-3 activation
He et al. [169]	miR-21-5p	Patient serum	Endothelial cells	Orthotopic xenograft model	Angiogenesis, vascular permeability	KRIT1 downregulation, β-catenin activation, VEGF/ CCND1 elevation
Zhu et al. [170]	miR-31	MSCs	Endothelial cells	HLI, LAD ligation	Angiogenesis, cardiac function recovery	FIH1 downregulation, HIF1α elevation
Wang et al. [179]	miR-210	MSCs	Endothelial cells	LAD ligation	Angiogenesis, cardiac function recovery	EFNA3 downregulation
Yang et al. [180]	miR-223	MSCs	Endothelial cells	H₂O₂, LAD ligation	Angiogenesis, antiinflammation, antifibrosis	P53 downregulation, S100A9 reduction
Youn et al. [171]	miR-322	Cardiac progenitor cells	Endothelial cells	LAD ligation	Angiogenesis	NOX2 and reactive oxygen species (ROS) elevation
Li et al. [172]	miR-486-5p	MSCs	Endothelial cells, cardiomyocytes	LAD ligation	Angiogenesis, cardiac function recovery	MMP19 downregulation, VEGFA elevation due to reduced cleavage
Liu et al. [173]	miR-494-3p	Dendritic cells	Endothelial cells	H/R, LCA ligation	Angiogenesis	VEGF elevation
Climent et al. [174]	miR-143/ miR-145	Smooth muscle cells	Endothelial cells	NA	Inhibition of angiogenesis, inhibition of endothelial proliferation	HKII and integrin-β8 downregulation, respectively
Jiang et al. [175]	miR-2p8b	Patient plasma	Endothelial cells	NA	Inhibition of angiogenesis, promotion of endothelial death	CDKN1A, *FAK*, *RAF1*, *MAPK1* and *Bax* upregulation, Bcl2 downregulation

Abbreviations: EVs, extracellular vesicles; lncR, long non-coding RNA; MSC, mesenchymal stromal cells; H/R: hypoxia-reoxygenation; LAD, left anterior descending artery; OGD/R, oxygen-glucose deprivation and reoxygenation/ recultivation; MCAO, middle cerebral artery occlusion; H/SD, hypoxia and serum deprivation; HLI, hindlimb ischemia; LCA, left coronary artery; NA, not available.

3.1 Cell survival and injury

Studies in experimental models mimicking myocardial infarction imply that EV-associated lncRNAs and miRNAs can promote ischemic cardiomyocyte survival and cardiac function recovery. As such, UCA1, MALAT1, NEAT1, KLF3-AS1 and HCP5 lncRNAs shuttled via EVs from cardiomyocytes or MSCs were found to promote cardiomyocyte survival, inhibit cardiomyocyte autophagy and promote cardiac function recovery by mechanisms including miR-143, miR-92a, miR-23c, miR138-5p and miR-497 sponging [140, 149, 153-156]. miRNA sponging potently increased the miRNA targets KLF2, STAT5B, SIRT1 and IGF1. Cardioprotective effects were demonstrated for several EV-miRNAs, namely miR-21, miR-25, miR-30e, miR-125b, miR-126, miR-146a, miR-185, miR-210, miR-212-5p, miR-338 and miR-671, which, collected from MSCs, cardiac progenitor cells, endothelial cells, endothelial

progenitor cells or patient serum, promoted cardiomyocyte survival by downregulating miRNA targets including PDCD4, FASL, PTEN, LOX1, p53, BAK or SOCS2 [141, 145-147, 150, 159, 162, 164, 165, 176-178, 181]. As a consequence of EV administration, intrinsic and extrinsic cell death pathways were inhibited. Importantly, not all ncRNAs contained in EV samples protect against ischemic damage. Hence,

EV-associated HCG15 lncRNA, miR-153-3p and miR-328-3p were found to exacerbate ischemic injury in myocardial infarction models via mechanisms involving NFκB/ p65 and p38 activation, PI3K/ Akt deactivation and caspase-3 activation, when obtained from ischemic cardiomyocytes, patient serum or MSCs [148, 167, 168].
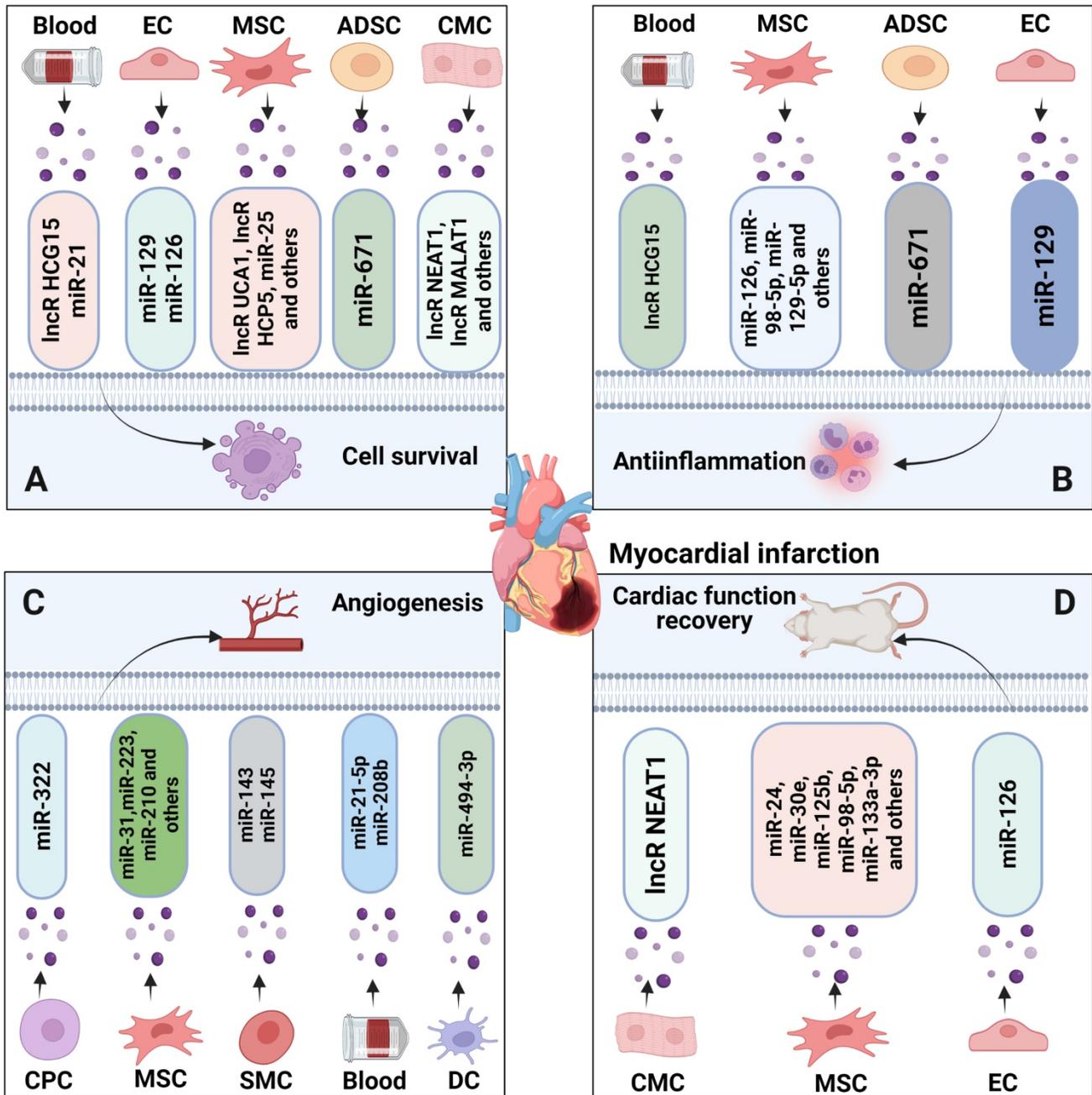

Figure 3. The involvement of ncRNAs shuttled via EVs in myocardial infarction. Different donor cells can impact recipient cells by transmitting ncRNAs via EVs, which can be taken up by recipient cells and alter various biological responses including cell survival, autophagy, inflammation and angiogenesis, thus regulating myocardial infarction progression and recovery. ADSC, adipose tissue-derived mesenchymal stromal cell; CMC, cardiomyocyte; CPC, cardiomyocyte precursor cell; DC, dendritic cell; EC, endothelial cell; SMC, smooth muscle cell.

3.2 Inflammation

The activation of pattern recognition receptors (PRRs) via damage-associated molecular patterns (DAMPs) may exacerbate ischemic cardiomyocyte injury via activation of the inflammasome, a multiprotein complex capable of cleaving and releasing proinflammatory IL1β levels, resulting in the activation of a proinflammatory type of programmed cell death called pyroptosis. EV-ncRNAs modulate inflammatory responses via a variety of miRNA targets. Hence, miR-98-5p and miR-129 transferred via endothelial cell EVs or MSC EVs reduced inflammasome activation in the ischemic myocardium by downregulating their miRNA target, the PRR TLR4, which in turn inhibited NFκB and the inflammasome component NLRP3 [143, 158]. Likewise, miR-146a and miR-671 shuttled via MSC EVs reduced TLR4, NFκB and SMAD2 signaling responses by downregulating their targets EGR1 and TGFBR2 [150, 163]. Patient serum-derived EV lncRNA HCG15, conversely, promoted IL1, IL6 and TNFα formation in ischemic myocardium via NFκB activation [167].

3.3 Angiogenesis

Vascular protective and angiogenic effects were shown for a variety of EV associated ncRNAs derived from cardiomyocytes, MSCs, cardiac progenitor cells, dendritic cells or patient serum, namely MALAT1 lncRNA, miR-21, miR-31, miR-126, miR-210, miR-223, miR-322, miR486-5p, miR-494-3p and miR-4732-3p in models of myocardial infarction [154, 157, 166, 168, 169, 171-173, 179, 180, 182]. These ncRNAs were found to promote endothelial survival, proliferation, migration and tube formation by downregulating the miRNA targets including Krev interaction trapped protein-1 (KRIT1), matrix metalloproteinase-19 (MMP19), and FIH, thus inducing β-catenin activation, VEGF, CCND1, NOX2 or HIF1α elevation, respectively [154, 157, 166, 168, 169, 171-173, 180, 182]. As a consequence of the enhanced tissue vascularization, fibrotic scar formation was reduced and cardiac function was enhanced [166, 170, 172, 180]. In contrast, miR-153-3p delivered by MSC EVs compromised endothelial survival by downregulating its target angiopoietin-1 (ANGPT1) resulting in β-catenin activation, VEGFR2, PI3K/ Akt and eNOS deactivation [168], whereas miR-143 and miR-145 delivered by smooth muscle cell EVs reduced angiogenesis by downregulating their targets hexokinase-II (HKII) and integrin-β8 [174]. The cardioprotective, anti-inflammatory and angiogenic roles of EV-ncRNAs support a possible role of ncRNAs as diagnostic/theranostic markers and therapeutic targets in myocardial infarction.

4. Roles of EV-ncRNAs in ischemic stroke

The occlusion of a cerebral artery affects the survival of brain neurons, glial cells and vascular cells. Among these different cells, the vulnerability of brain neurons is highest. Neuronal viability, structural connectivity and functional responses are vital for the recovery of lost neurological functions [183, 184]. Yet, neurological recovery post-stroke critically depends on the successful restitution of vascular and glial functions. In the process of brain tissue remodeling, neurons, glial cells and vascular cells tightly interact with each other, preparing the stage so that successful functional recovery can occur [183]. Similar to myocardial infarction, a variety of ncRNAs, namely circSCMH1, miR-124-3p, miR-126, miR-132, miR-221-3p and miR542-3p, are reduced in the ischemic brain and blood [185-191], whereas others, namely miR-98 and miR-494, are increased at defined time-points [192-194]. Delivery of MSC-derived EVs can boost ncRNA levels in ischemic brain tissue [195-198]. Although ischemic stroke and myocardial infarction have distinct pathophysiological features, they thus share common signaling pathways. Hence, EV-ncRNAs have vital roles in coordinating tissue responses to ischemic stroke in the acute and post-acute stroke setting, in which ncRNAs modulate neuronal survival, inflammatory responses, angiogenesis, neurogenesis and neuronal plasticity [151, 199]. Similar to myocardial infarction, most studies on ischemic stroke have previously been performed using tissues or cells, which were experimentally exposed to hypoxia or ischemia [185-192, 195-198, 200-224], as summarized in **Table 3** and **Figure 4** and outlined in the following.

4.1 Cell survival and injury

Experimental stroke studies revealed that EV-ncRNAs can promote ischemic brain tissue survival in the acute stroke phase, reduce the development of brain atrophy in the chronic phase and enhance neurological recovery. Thus, EV samples obtained from hypoxic astrocytes that contained circSHOC2 lncRNA promoted neuronal survival via mechanisms involving miR-7670-3p sponging, resulting in SIRT1 overexpression [200]. In HT22 neuronal cells, lncRNA MALAT1 shuttled via MSC EVs promoted neuronal survival via mechanisms including the recruitment of the serine-arginine-rich splice factor-2 (SRSF2), resulting in alternative splicing of protein kinase CδII (PKCδII) and Bcl2 elevation [225].

Table 3. Preclinical studies assessing the effects of ncRNAs transferred via EVs in ischemic stroke models.

Authors [reference]	ncRNAs	EV provenance	Recipient cell	Ischemia model	Primary action	Mechanism of action
Chen et al. [200]	circSHOC2	Primary astrocytes	Primary neurons	OGD, MCAO	Cell survival, autophagy inhibition	miR-7670-3p sponging/ SIRT1 elevation
El Bassit et al. [225]	lncR MALAT1	MSCs	HT22 neuronal cells	Oxidative stress	Cell survival and proliferation	SRSF2 recruitment, alternative PKCδII splicing, Bcl2 elevation
Zhang et al. [201]	miR-22-3p	MSCs	Primary neurons	OGD, MCAO	Cell survival	KDM6B downregulation, BMP2/ BMF deactivation
Kuang et al. [202]	miR-25	MSCs	Primary neurons	OGD, MCAO	Cell survival, autophagy inhibition	p53 downregulation, BNIP3 deactivation, reduced LC3-II abundance
Hou et al. [203]	miR-26a	MSCs	Primary neurons	OGD, MCAO	Cell survival	KLF9 downregulation, TRAF2 and KLF2 elevation
Li et al. [197]	miR-26b-5p	MSCs	SH-SY5Y, PC12, primary microglia	OGD, MCAO	Cell survival, antiinflammation	CH25H downregulation, TLR4 deactivation, inhibition of M1 microglia polarization
Lv et al. [204]	miR-31	MSCs	Primary neurons	OGD, MCAO	Cell survival, functional neurological recovery	TRAF6 downregulation, IRF5 elevation, Bax/ activated caspase-3 reduction
Wu et al. [205]	miR-34c	Astrocytes	N2a neuronal cells	OGD, MCAO	Cell survival	TLR7 downregulation, NFκB/MAPK deactivation
Xu et al. [206]	miR-92b-3p	Primary astrocytes	Primary neurons	OGD	Cell survival	Not determined
Yang et al. [192]	miR-98	Primary neurons	Primary microglia	OGD, MCAO	Cell survival, antiinflammation	PAFR downregulation, inhibition of microglia phagocytosis
Li et al. [207]	miR-124	M2 BV2 microglia	Primary astrocytes	OGD, MCAO	Cell survival, inhibition astrocytic activation, proliferation and scar formation, functional neurological recovery, antiinflammation	STAT3 downregulation, GFAP reduction, nestin elevation
Qi et al. [189]	miR-124-3p	Patient serum	BV2 microglia	AIS patients	Cell survival, antiinflammation	ERK1/2, PI3K/ Akt and p38 MAPK deactivation
Cui et al. [208]	miR-126	Patient serum	SH-SY5Y neuronal cells	RIPC	Cell survival	DNMT3B downregulation
Geng et al. [190]	miR-126	MSCs	Neurons, endothelial cells, BV2 microglia	OGD, MCAO	Cell survival, functional neurological recovery, anti-inflammation, neurogenesis, angiogenesis	Reduced microglial activation
Feng et al. [185]	miR-132	MSCs	Primary neurons	OGD, MCAO	Cell survival	ACVR2B downregulation, SMAD2/ c-Jun inhibition
Xiao et al. [209]	miR-134	MSCs	Primary oligodendrocytes	OGD	Cell survival	Caspase-8 deactivation
Liu et al. [210]	miR-135a-5p	M2 microglia	HT-22 neuronal cells	OGD, MCAO	Cell survival, antiinflammation, autophagy inhibition	TXNIP downregulation, NLRP3 deactivation, reduced IL1β and IL18 formation
Zhang et al. [211]	miR-137	M2 microglia	Primary neurons	OGD, MCAO	Cell survival, functional neurological recovery	NOTCH1 downregulation
Deng et al. [212]	miR-138-5p	MSCs	Primary astrocytes	OGD, MCAO	Cell survival, antiinflammation	LCN2 downregulation, IL1β, IL6 and TNFα reduction, Bcl2 elevation, Bax reduction
Zhang et al. [213]	miR-146a-5p	MSCs	BV2 microglia	OGD, MCAO	Cell survival, antiinflammation, functional neurological recovery	IRAK1/ TRAF6 deactivation, reduced microglial activation
Song et al. [214]	miR-181c-3p	Primary neurons	Primary astrocytes	OGD, MCAO	Cell survival, antiinflammation	CXCL1 downregulation, reduced astrocyte activation
Zhong et al. [215]	miR-206/ miR-1-3p	MSCs	Primary neurons	OGD	Cell survival	RMRP downregulation, PI3K/ Akt/ mTOR deactivation, eNOS elevation
Ai et al. [186]	miR-221-3p	MSCs	Primary neurons	OGD, MCAO	Cell survival, antiinflammation	ATF3 downregulation
Zhao et al. [216]	miR-223-3p	MSCs	BV2 microglia	OGD, MCAO	Cell survival, antiinflammation, functional neurological recovery	CysLT2R downregulation, M2 microglia polarization
Bu et al. [217]	miR-361	Primary astrocytes	PC12 neuronal cells	OGD, MCAO	Cell survival	CTSB downregulation, AMPK/ mTOR deactivation
Cai et al. [188]	miR-542-3p	MSCs	HA1800 astrocytes	OGD, MCAO	Cell survival, antiinflammation	TLR4 downregulation, ROS, IL6, TNFα and MCP1 reduction
Yue et al. [218]	miR-1290	Endothelial cells	Primary neurons	OGD, MCAO	Cell survival	Neuronal EV uptake caveolin-1 dependent, increased by hypoxia-ischemia
Ye et al. [219]	miR-27-3p	Patient serum	BV2 microglia	MCAO	Cell death, inflammation, compromised neurological recovery	PPARγ downregulation, microglial overactivation, proinflammatory cytokine formation
Yang et al. [220]	miR-181b	MSCs	Brain microvascular endothelial cells	OGD, MCAO	Angiogenesis	TRPM7 downregulation, HIF1α and VEGF elevation, TIMP3 reduction
Zhang et al. [221]	miR-210	MSCs	Brain microvascular endothelial cells	MCAO	Angiogenesis	Integrin-β3, VEGF and CD34 elevation
Gregorius et al. [198]	-	MSCs	Brain microvascular endothelial cells	OGD, MCAO	Angiogenesis	Hypoxic MSC preconditioning induces angiogenic activity. miR-126-3p, miR-140-5p, let-7c-5p upregulated, miR-186-5p, miR-370-3p, miR-409-3p downregulated in endothelial cells in response to hypoxic but not normoxic MSC EVs
Ling et al. [223]	miR-26a	Urine-derived stem cells	Neural stem cells	OGD, MCAO	Neurogenesis	HDAC6 inhibition
Yang et al. [224]	miR-124	MSCs	Neural progenitor cells	Focal cortical ischemia	Neurogenesis	Not determined

Authors [reference]	ncRNAs	EV provenance	Recipient cell	Ischemia model	Primary action	Mechanism of action
Yang et al. [191]	circSCMH1	Genetically engineered HEK293T cells	Neurons, glial cells, leukocytes	Photothrombosis	Neuronal (=dendritic and synaptic) plasticity, functional neurological recovery, antiinflammation	Release of MeCP2 transcription repression, microglial activation reduced, IL1β, TNFα and IL6 formation reduced
Xin et al. [222]	miR-17-92	MSCs	Neurons, glial cells	MCAO	Neuronal (=axonal, dendritic and synaptic) plasticity, neurogenesis, functional neurological recovery, myelin remodeling	PTEN downregulation, PI3K/ Akt/ mTOR activation, GSK3β deactivation
Venkat et al. [187]	miR-126	Endothelial cells	Neurons, endothelial cells, oligodendrocytes, microglia	Photothrombosis	Neuronal (=axonal) plasticity, functional neurological recovery, myelin remodeling, angiogenesis	M2 macrophage polarization
Xin et al. [195, 196]	miR-133b	MSCs	Neurons, astrocytes	MCAO	Neuronal (=axonal) plasticity, functional neurological recovery	CTGF and RhoA downregulation

Abbreviations: EVs, extracellular vesicles; MSCs, mesenchymal stromal cells; OGD, oxygen-glucose deprivation; MCAO, middle cerebral artery occlusion; AIS, acute ischemic stroke; RIPC, remote ischemic preconditioning.
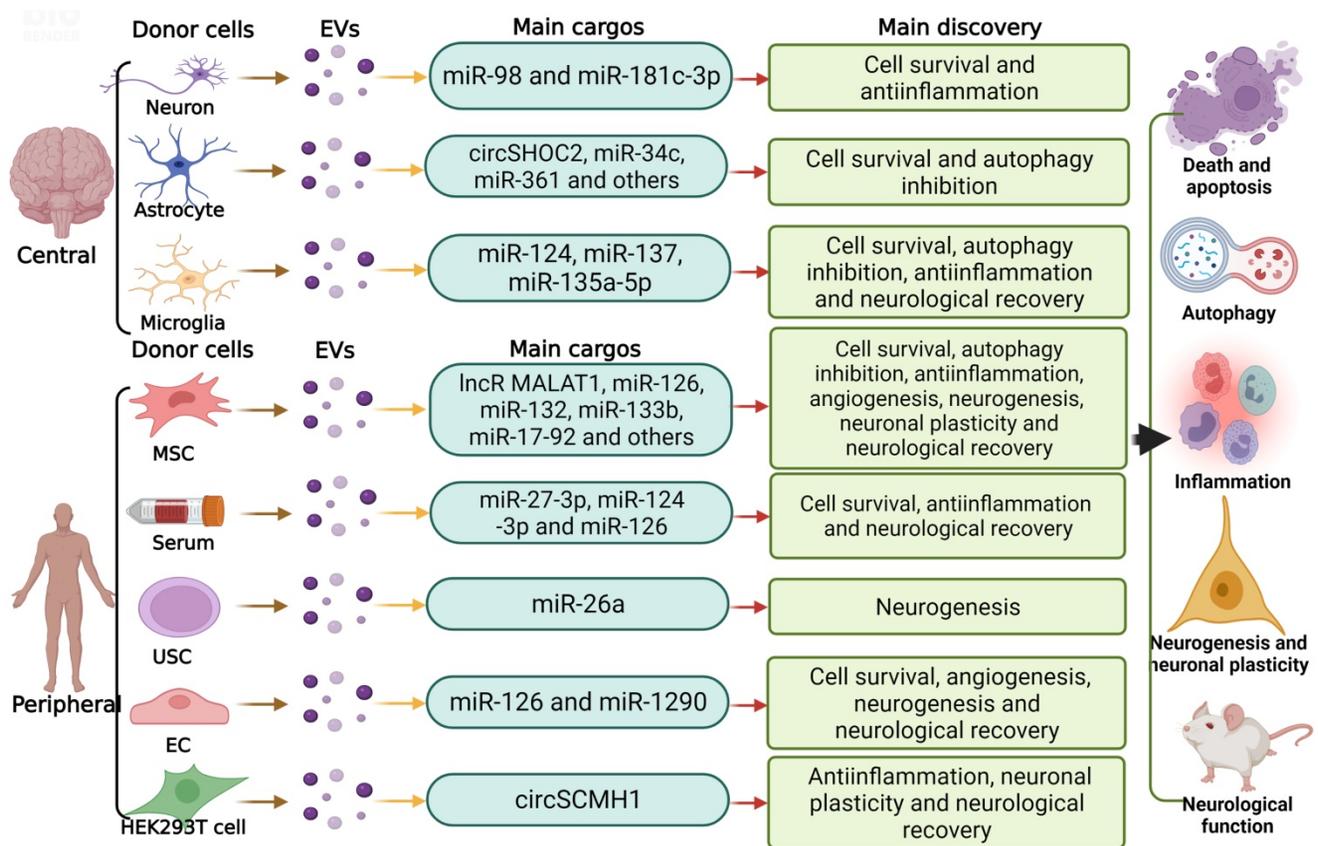

Figure 4. The involvement of ncRNAs shuttled via EVs in ischemic stroke. In the central and peripheral nervous system, different donor cells including neurons, microglia, astrocytes and MSCs can regulate recipient cells by transferring various EV-ncRNAs, modulating biological behaviors including neuronal survival, autophagy, inflammation, angiogenesis, neurogenesis and neuronal plasticity, thus modifying ischemic stroke progression and recovery. USC, umbilical cord-derived mesenchymal stromal cell; EC, endothelial cell

A large set of MSC EV-associated miRNAs, including miR-22-3p, miR-25, miR-26, miR-31, miR-126, miR-138-5p, miR-146a-5p and miR-223-3p, were found to promote neuronal, astrocytic, oligodendrocytic and microglial survival by downregulating target genes including KDM6B, p53, KLF9, CH25H, TRAF6, ACVR2B, LCN2 or CysLT2R [185, 186, 188, 190, 197, 201-204, 209, 212, 213, 215, 216]. Besides, miR-34c, miR-92b-3p and miR-361 transferred with EVs from normoxic or ischemic astrocytes increased neuronal survival by downregulating the targets TLR7 and cathepsin-B

(CTSB) [205, 206, 217]. Likewise, M2 microglial EV miR-124, miR-135a-5p and miR-137 increased neuronal survival and reduced astrocytic activation, proliferation and scar formation via mechanisms involving STAT3, thioredoxin interacting protein (TXNIP) and NOTCH1 downregulation [207, 210, 211]. Neuroprotective effects were furthermore reported for miR-1290 derived from endothelial EVs [218]. In the latter study, EV uptake by neurons occurred calveolin-1 dependently, and this uptake was increased by hypoxia-ischemia [218]. Survival-promoting effects of patient serum-derived EV

miR-124-3p and miR-126 via mechanisms involving DNA methylase-3b (DNMT3B) were noted in neurons and microglial cells [189, 208], whereas patient serum-derived miR-27-3p increased neuronal death via mechanisms involving PPARγ downregulation and microglial overactivation [219]. The combined evidence of these studies demonstrates that various types of brain cells mutually influence responses to stroke via EV-associated ncRNAs.

A peculiar mechanism associated with ncRNA-induced neuroprotection appears to be the inhibition of autophagy in recipient cells. Autophagy is an evolutionarily conserved mechanism, which maintains cellular homeostasis by degrading misfolded or nonfunctional proteins or damaged organelles [226, 227]. Upon severe cellular stress, excessive autophagy may result in cellular accumulation of toxic metabolites or cellular self-degradation, ultimately resulting in cell death [228-230]. Recent studies evaluating effects of MSC EVs showed that EV miR-25-3p protected primary neurons exposed to oxygen-glucose deprivation against injury via autophagy inhibition [202]. On the molecular level, p53 expression was downregulated by miR-25-3p, resulting in the inhibition of BNIP3 activity and reduced autophagic flux examined by LC3-II levels. Application of a miR-25-3p oligonucleotide mimic promoted neuronal survival, whereas an miR-25-3p anti-oligonucleotide increased autophagic flux and cell death by mechanisms involving p53 overexpression and BNIP3 overactivation [202]. Inhibitory effects on autophagy associated with neuronal survival were also described for circSHOC2 and miR-135a-5p released in astrocytic and M2 microglial EVs [200, 210]. Hence, autophagy inhibition might represent a more general, hitherto underexplored mechanism via which EV-ncRNAs protect ischemic neurons.

4.2 Inflammation

Similar to the heart, proinflammatory cytokines, namely IL-1β, contribute to ischemic brain injury via pyroptosis. In the brain, proinflammatory cytokines, such as IL-6, TNF-α and IL-1β, are released from a variety of cells, including M1 microglial cells, astrocytes and neurons. ncRNAs transferred via EVs seem to regulate these inflammatory responses, as has been observed for EVs originating from a number of cell sources. Hence, miR-26b-5p, miR-126, miR-138-5p, miR-138-5p, miR-221-3, miR-223-3p and miR-542-3p released via MSC EVs inhibited inflammatory responses of neurons, microglial cells and astrocytes via CH25H, LCN2, RMRP, ATF3, CysLT2R and TLR4 downregulation, resulting in the inhibition of the TLR4, SMAD2, IRAK1/ TRAF6 and

PI3K/ Akt/ mTOR pathways [186, 188, 190, 197, 212, 213, 216]. Via this mechanism, neuronal survival was enhanced, microglial cells adopted a restorative M2 phenotype, and astrocytic inflammatory responses were inhibited. Likewise, miR-135a-5p derived from M2 microglial EVs reduced inflammatory responses of neurons via TXNIP downregulation, NLRP3 deactivation and decreased IL1β and IL18 formation [210], while neuronal EV miR-98 and miR-181c-3p promoted microglia survival and inhibited microglia phagocytosis or inhibited astrocyte inflammatory responses by PAFR or CXCL1 downregulation, respectively [192, 214]. Patient serum-derived EV miR-124-3p inhibited microglial inflammatory responses by mechanisms involving ERK1/2, PI3K/ Akt and p38 MAPK deactivation [189], whereas patient serum-derived EV miR-27-3p promoted M1-like microglial activation via mechanisms involving PPARγ downregulation, resulting in increased cytokine formation and cell death [219].

4.3 Angiogenesis and neurogenesis

In models of cerebral ischemia, angiogenic effects have been shown for miR-181b and miR210 transferred via MSC EVs and for miR-126 transferred via endothelial EVs via mechanisms involving TRPM7 downregulation, HIF1α and VEGF elevation and TIMP3 reduction [187, 220, 221]. When cultured under conditions of hypoxia, MSC EVs that were otherwise non-angiogenic adopted a recovery-promoting phenotype that reproducibly induced cerebral microvascular endothelial proliferation, migration and tube formation across a wide range of MSC sources [198]. Compared with EVs from normoxic MSCs, hypoxic MSC EVs significantly increased miR-126-3p, miR-140-5p and let-7c-5p and reduced miR-186-5p, miR-370-3p and miR-409-3p in recipient endothelial cells [198]. The delivery of these hypoxic MSC EVs *in vivo* to ischemic mice exposed to middle cerebral artery occlusion enhanced microvascular remodeling, increased microvascular densities, increased microvascular length and increased branching point densities, as revealed by 3D lightsheet fluorescence microscopy in the periinfarct rim [198]. Newly formed microvessels act as guidance sheaths for neural progenitor cells migrating from progenitor cell niches to the stroke lesion. Delivery of miR-17-92, miR-26a and miR-124 shuttled via EVs from MSCs or urine-derived stem cells promoted post-ischemic neurogenesis via mechanisms involving histone deacetylase-6 (HDAC6) inhibition [222-224].

4.4 Neuronal plasticity

In response to stroke, axons and dendrites in the

vicinity and at distance to the evolving brain infarct sprout, forming new synaptic connections [183]. Cell-based therapeutics, including exogenously administered neural progenitor cells or MSCs, promote neuronal plasticity [231, 232]. Within this process, EVs and their ncRNAs may play significant roles. Thus, the EV-derived lncRNA circSCMH1 was shown to increase dendritic length, dendritic branches and synaptic spines of ischemic cultured neurons in vitro and of periinfarct cortical neurons of rats exposed to photothrombotic stroke in vivo, as revealed by morphological Golgi-Cox staining analysis [191]. EV circSCMH1 improved functional neurological recovery of ischemic rats, reduced microglial activation and reduced the formation of the proinflammatory cytokines IL1β, TNFα and IL6 [191]. The effect of circSCMH1 was mediated by binding methyl-CpG binding protein-2 (MeCP2), a nuclear transcription factor directly binding methylated DNA, as revealed by proteomic assays, RNA sequencing and transcriptional profiling studies [191]. By MeCP2 binding, MeCP2 target gene transcription repression was released. In rat and mouse models of middle cerebral artery occlusion, neuronal plasticity and neurological recovery promoting effects were reported for MSC EV miR-17-92 and miR-133b and for endothelial cell EV miR-126 [187, 195, 196, 222]. Via mechanisms involving downregulation of PTEN, connective tissue growth factor (CTGF) or RhoA, the three miRNAs were found to increase axonal, dendritic and synaptic sprouting in the periinfarct tissue, as revealed by anterograde tract tracing analysis using biotinylated dextran amine combined with immunohistochemical stainings. In case of miR-17-92, the plasticity-promoting effects were associated with PI3K/ Akt/ mTOR activation and GSK3β deactivation [222]. In case of miR-126, which was evaluated in a type-II diabetes stroke model, the neurorestorative effects were linked to a shift of macrophage polarization towards the M2 phenotype [187].

5. Overarching roles of EV-ncRNAs across pathophysiological conditions

lncRNAs, circRNAs, miRNAs and mRNAs form complex RNA networks that synergistically respond to stressors [233]. As outlined in sections 2-4, several of these networks are highly active or inactive under conditions of hypoxia and ischemia, representing master regulators of gene expression. We are just starting to understand the complex biology behind these ncRNA networks. miRNAs recognize response elements on RNAs that mediate their interaction and binding. lncRNAs and circRNAs serve as competing endogenous RNAs to miRNAs, and thus act as miRNA sponges. mRNA binding of miRNA induces translational repression or instability, thus regulating protein translation [233]. Importantly, lncRNAs do not only interact with miRNAs but can also target DNA transcription and mRNAs directly [234]. Thus, EV-circSCMH1, which is decreased in plasma of stroke patients and periinfarct cortex of stroke mice, was found to induce post-ischemic dendritic and synaptic plasticity, antiinflammation and neurological recovery by binding the nuclear transcription factor MeCP2, resulting in release of MeCP2 mediated transcription repression [191] (see also section 4 and **Table 3**). Using antisense oligonucleotide studies and RNA immunoprecipitation assays on HT22 neuronal cells, lncRNA MALAT1, which is highly abundant in MSC EVs, was shown to promote neuronal survival and proliferation by mechanisms involving SRSF2 recruitment, alternative PKCδII splicing and Bcl2 elevation [225] (**Table 3**). For further insights into lncRNA-circRNA/ miRNA/ mRNA networks, the reader is referred to references [235-237]. In view of their highly integrated mode of action, ncRNAs profoundly modify disease responses.

5.1 ncRNAs involved in more than one of three hypoxic conditions exhibit a large degree of overlaps regarding modes of action

From the above EV-ncRNA intervention studies, a total of 19 ncRNAs, including 3 lncRNAs and 16 miRNAs, have meanwhile been identified for which robust evidence suggests their involvement in more than one of the three hypoxic pathophysiological conditions. The modes of action of these ncRNAs have been summarized in **Table 4**. Notably, 8 of these 19 EV-ncRNAs have been shown to be involved in all three pathophysiological conditions. Including studies evaluating effects of ncRNAs irrelevant whether ncRNAs were associated with EVs, joint evidence in all three pathophysiological conditions exists for 16 of the 19 ncRNAs. Strikingly, the modes of action reveal a high degree of overlaps between the three pathophysiological states. Hence, studies describing promotion of cell survival, proliferation, migration or angiogenesis in one pathophysiological condition usually had corresponding actions in the two other conditions, as shown for lncRNA MALAT1, miR-21, miR-25, miR-31, miR-135, miR-146 and miR-210. Similarly, ncRNAs with roles in immune tolerance, antiinflammation or chemotherapy resistance in one condition also revealed related actions in the two other conditions, as shown for miR-98 and miR-223. Hence, corresponding actions in all three conditions have been reported for 9 of the 16 ncRNAs. Importantly, diverging actions have been reported for 3 ncRNAs. Perhaps due to the different

nature of hypoxia, opposing actions were described for cancer compared to myocardial infarction and stroke in case of two ncRNAs. Hence, miR-133a, which was found to be abundant in cancer EVs at low concentration, reduced tumor cell proliferation, survival, migration and metastasis in gastric carcinoma and colorectal carcinoma [136, 238], whereas miR-133a-3p and miR-133b promoted cardiomyocyte survival and neuronal plasticity in myocardial infarction and stroke, respectively, and enhanced functional tissue recovery [162, 195, 196]. Likewise, miR-328b-3p promoted tumor cell proliferation, migration, invasion and tumor growth in lung carcinoma [105], whereas miR-328b-3p augmented cardiomyocyte death and apoptosis in myocardial infarction [148] and augmented neuronal death and neuroinflammation in ischemic stroke [239]. Besides, EV-associated miR-361 promoted tumor cell proliferation and survival in colorectal carcinoma [103] and neuronal survival in ischemic stroke [217], whereas cardiac-specific miR-361 overexpression reduced cardiomyocyte survival by increasing mitochondrial fission in myocardial infarction [240]. Differences in the actions of EV-associated miR-361 and genetically overexpressed miR-361 may explain diverging findings in the two ischemia studies. Important for potential clinical translation, therapeutic miRNA modification may have opposite roles within the same disease category via distinct modes of actions. Hence, EV-associated miR-126 promoted tumor cell proliferation, angiogenesis and growth in hepatoblastoma and chronic myeloid leukemia via mechanisms involving CXCL12 and VCAM1 downregulation [129, 131], but inhibited tumor cell proliferation, colony formation, migration, invasion and survival in lung carcinoma via mechanisms including ITGA6 downregulation [130]. NEAT1 overexpression promoted neuronal survival in ischemic stroke by regulating the MFN2/ SIRT3 pathway [241], whereas NEAT1 knockdown increased neuronal survival by inhibiting M1 microglia polarization via the Akt/ STAT3 pathway [242]. Again in ischemic stroke, miR-494 agomir (that is, mimic) promoted neuronal survival, axonal plasticity and neurological recovery via HDAC3 downregulation [193], similar as miR-494 antagomir (that is, inhibitor), which increased neuronal survival and neurological recovery by reducing the Th1 helper cell shift and decreasing post-ischemic brain neutrophil infiltrates via HDAC2 upregulation [194, 243]. In view of the multifaceted roles of ncRNAs, their therapeutic modulation is particularly prone to ambiguous actions in different types of cells. In case of therapeutic interventions, careful actions are needed in order to avoid contrary results of therapeutic

interventions, i.e., therapeutic benefits in one cell type (e.g., in neuron) or via one pathway (e.g., MFN2/ SIRT3) vs. harmful actions in another cell type (e.g., brain invading T cells) or pathway (e.g., Akt/ STAT3). The genetic overexpression or knockdown and the delivery of miRNA agomirs or antagomirs are gross interventions that affect miRNA levels in a non-targeted way. In comparison, the delivery of miRNA-loaded EVs is more fine-tuned and allows targeting distinct types of cells.

6. EV-associated ncRNAs as therapeutic products or theranostic biomarkers

From studies in cancer, myocardial infarction and ischemic stroke, there is meanwhile solid evidence that EV-associated ncRNAs regulate gene responses in target tissues under conditions associated with hypoxia, modifying cell survival, proliferation, migration and differentiation and influencing disease outcomes in clinically relevant ways. In most pathophysiological conditions, we still lack of detailed information about the precise subcellular origin of therapeutically active EVs. Of note, the association of ncRNAs with EVs does not imply that ncRNAs are exosome constituents [15], and concerns have been raised whether ncRNAs are released from cells as part of the EVs [17]. EVs are widely isolated by differential ultracentrifugation [86], which enriches non-EV constituents including lipoproteins [87] that contain large amounts of ncRNAs [88]. Bead-capturing experiments revealed that EVs captured by GM1 binding cholera toxin b were largely devoid of RNAs (see also section 1.5). EV contents may differ depending on pathophysiological conditions. Besides exosomes, microvesicles might contain ncRNAs. Irrespective of the precise ncRNA and EV origin and overriding open methodological questions, intervention studies in models of cancer, myocardial infarction and stroke consistently revealed therapeutic actions of EV preparations that were associated with ncRNAs. The observation raises the question about the utility of EV-associated ncRNAs as therapeutics or theranostics in human patients.

6.1 ncRNA-loaded EVs as therapeutic products

Representing instable single-strand RNA molecules, miRNAs are rapidly degraded in the blood by RNAses, unless specifically protected. EVs are abundant in virtually all body fluids, protecting ncRNAs from degradation. Representing nanoparticles covered by lipid bilayer membrane, EVs are capable of transmitting complex biological information to defined target cells. The presence of multiple signals in a single EV allows inducing

synergistic cellular responses. When evaluating EV actions, we must consider that not all EVs transmit biological information and that some EVs eliminate waste products, including RNAs, from hypoxic cells (see section 1.1). The proper definition of cells or tissues of origin is decisive in the development of EV products [244]. In the development of EV-based therapeutics, we have to be aware that EV contents, including ncRNAs, may greatly differ between EV preparations, even when these preparations are performed from the same source cells [198, 245]. This raises the need of potency assays evaluating the efficacy of each EV preparation before this individual preparation is administered to human patients [246]. When considering ncRNAs as therapeutic EV contents, it must be taken into account that the biological effect of a given ncRNA may differ between pathophysiological settings and disease-relevant target cell types. As example, miR-494 was shown to promote post-ischemic neuronal survival, axonal plasticity and neurological recovery via its target HDAC3 in one setting [193], while it had opposite effects by modulating Th1 helper cell shifts and brain neutrophil infiltrates via HDAC2 in two other studies [194, 243] (see also **Table 4**). Thorough studies in animal models would be required elucidating various modes of action before clinical proof-of-concept studies are performed. Considering their biological properties that resemble cellular therapeutics, but are more easy to handle and lack intrinsic risks of cellular therapeutics (such as malignant transformation) [244], the administration of EVs is an elegant strategy to boost hypoxic tissues. EVs might potentially be loaded with ncRNAs that prevent disease progression or improve disease outcome, or be loaded with inhibitors or siRNAs for ncRNAs that promote disease progression or deteriorate disease outcome. EV ncRNA contents might possibly be modified by transgenic techniques. Following transfection of cancer cells with plasmid DNA encoding for wild-type p53 and miR-125b, Trivedi et al. observed that the ncRNA profile of EVs was altered, shifting the polarization of recipient macrophages towards the M1 phenotype [247].

6.2 EV-ncRNAs as theranostic biomarkers

EVs carry surface markers specifying their cellular origin and their source cell's activation state. Cell type by cell type, tissue responses can be tracked in remote body fluids. In multiple dimensions, detailed information can be obtained about hypoxic or ischemic tissue states. For this type of biomarker analysis, the term liquid biopsy has been coined. Deregulated EV-ncRNA levels may be used as diagnostic or prognostic markers. As such, Que et al.

reported that EV miR-17-5p and miR-21 were elevated 3.2-fold and 5.9-fold, respectively, in the serum of patients with pancreatic adenocarcinoma, and serum miR-21 furthermore differentiated pancreatic adenocarcinoma from chronic pancreatitis [248]. In myocardial infarction, serum EV-derived miR-1915-3p, miR-457, and miR-3656 were significantly less abundant compared with patients with stable coronary artery disease, and the miR-3656 level was positively correlated with left ventricular ejection fraction [249]. Indeed, by comparing EV-ncRNAs abundancies at baseline, before and after treatment with subsequent correlation with clinical variables, such biomarkers offer an elegant possibility to predict disease outcomes and therapy responses. For instance, in pancreatic adenocarcinoma, the level of serum EV circPDE8A was positively correlated with lymphatic invasion, TNM stage and poor survival rate [250]. On the contrary, miR-134 – known as a brain-specific miRNA – has been associated with neuronal injury under conditions of ischemic stroke [251]. Abundances of EV-derived miR-134 were significantly increased in stroke patients, where they were positively correlated with neurological deficits, stroke volume and functional outcome [252]. A detailed and comprehensive analysis of the utility of miRNAs as diagnostic markers is beyond the scope of this review. For more details, the reader is referred to Table 5 [102, 108, 110, 113, 114, 248-250, 252-266]. Specifically, the here-presented data on the role of EV-ncRNAs in the development of chemotherapy resistance (summarized in section 2.4) supports their role in treatment monitoring. In a variety of cancers, including pancreatic carcinoma, colorectal carcinoma, ovarian carcinoma, hepatocellular carcinoma and lung carcinoma, pathophysiologically grounded evidence was collected supporting a role of defined ncRNAs in chemotherapy resistance development (summarized in section 2.4 and **Figure 2**). With the identification of concentration cutoffs, these EV-ncRNAs could now be used for chemotherapy monitoring. As theranostics, ncRNA-EVs should provide clinically significant information, whether a given drug is still likely to have preserved its actions or whether it should be exchanged due to drug resistance development. For example, hypoxia-associated miR-21 abundance has revealed its utility as theranostic marker in non-small cell lung carcinoma, where high miR-21 abundance was associated with short survival in patients receiving chemotherapy with cisplatin, but not in patients not receiving chemotherapy [94]. The predictive value of EV-ncRNAs may further be enhanced by evaluating EV-ncRNA combinations or EV-ncRNA combinations with classical biomarkers. For instance, serum α-fetoprotein was found to have

an insufficient sensitivity and specificity in hepatocellular carcinoma patients, resulting in an unacceptably high false-negative detection rate [267]. Combining α-fetoprotein with serum EV ENSG00000258332.1 and LINC00635 markedly increased α-fetoprotein's sensitivity and specificity. With the new biomarker, a higher percentage of patients could now be classified correctly [267].

Table 4. ncRNAs transferred via EVs that have been reported to participate in more than one of the three hypoxic conditions.

ncRNAs	Modes of action in cancer	Modes of action in myocardial infarction	Modes of action in ischemic stroke
lncR UCA1	Promotes tumor cell proliferation and tumor-associated angiogenesis [102, 106]	Promotes cardiomyocyte survival and inhibits autophagy [153]	Not assessed
lncR MALAT1	Promotes tumor cell proliferation, migration, invasion, colony formation and glycolysis; promotes inflammation [123-125, 127]	Promotes cardiomyocyte and endothelial cell survival [154]	Promotes neuronal survival [225]
lncR NEAT1	Promotes tumor cell proliferation, migration, invasion, metastasis, immune tolerance and chemoresistance [122, 126, 269]	Promotes cardiomyocyte and fibroblast survival; inhibits fibrosis [149]	[NEAT1 overexpression promotes neuronal survival via MFN2/ SIRT3 pathway [241]; NEAT1 knockdown promotes neuronal survival by inhibiting M1 microglia polarization via Akt/ STAT3 pathway [242]
miR-21	Promotes tumor cell proliferation, migration and invasion; induces immune tolerance via M2 macrophage polarization, γδ T cell deactivation and myeloid-derived suppressor cell expansion; induces chemoresistance [93-95, 114, 119]	Promotes cardiomyocyte and endothelial cell survival; promotes periinfarct angiogenesis and vascular permeability [141, 157, 169]	[miR-21 agomir promotes neuronal survival [270]
miR-24	Induces immune tolerance by T cell deactivation [110]	Promotes cardiomyocyte survival and cardiac function recovery [147]	[miR-24 agomir promotes neuronal survival [271]
miR-25	Promotes tumor cell migration, invasion, angiogenesis and metastasis [133, 134]	Promotes cardiomyocyte survival [181]	Promotes neuronal survival and inhibits autophagy [202]
miR-31	Promotes tumor cell migration and invasion [121]	Promotes periinfarct angiogenesis and cardiac function recovery [170]	Promotes neuronal survival and functional neurological recovery [204]
miR-98	Promotes chemotherapy resistance [132]	Promotes cardiomyocyte survival; induces cardiac function recovery; induces antiinflammation (reduced macrophage infiltration) [143]	Promotes neuronal survival and antiinflammation (reduced microglial phagocytosis) [192]
miR-125	Promotes tumor cell proliferation; induces immune tolerance via M2 macrophage polarization [95]	Promotes cardiomyocyte survival and cardiac function recovery [159]	Not assessed
miR-126	Promotes tumor cell proliferation, angiogenesis and growth in some tumors [129, 131]; opposite effects in other tumors [130]	Promotes cardiomyocyte survival and cardiac function recovery; induces antiinflammation (proinflammatory cytokines reduced) and antifibrosis; promotes periinfarct angiogenesis [160, 176]	Promotes neuronal survival and functional neurological recovery; induces anti-inflammation (reduced microglial activation); induces periinfarct neurogenesis and angiogenesis; induces neuronal (=axonal) plasticity and myelin remodeling [187, 190, 198]
miR-133	[miR-133a agomir reduces tumor cell proliferation, survival, migration and epithelial-mesenchymal transition; miR-133a antagomir promotes tumor cell survival and migration [238]; EV-associated circ-133, a miR-133a sponge, promotes tumor cell migration and metastasis [136]	Promotes cardiomyocyte survival and cardiac function recovery [162]	Promotes neuronal (=axonal) plasticity and functional neurological recovery [195, 196]
miR-135	Promotes tumor-associated angiogenesis [101]	[miR-135a overexpression promotes cardiomyocyte survival and cardiac function recovery and induces antiinflammation via TLR4 downregulation [272]	Promotes neuronal survival; induces antiinflammation (reduced proinflammatory cytokines); inhibits autophagy [210]
miR-146	Promotes tumor cell proliferation and tumor growth [128]	Promotes cardiomyocyte survival; induces antiinflammation (reduced leukocyte infiltration) and antifibrosis [163]	Promotes neuronal survival; induces antiinflammation (reduced microglial activation); promotes functional neurological recovery [213]
miR-181	Promotes tumor cell proliferation; induces immune tolerance via M2 macrophage polarization [95]	Not assessed	Promotes astrocyte survival; inhibits astrocytic inflammatory response; promotes periinfarct angiogenesis [214, 220]
miR-210	Promotes tumor cell migration and invasion; increases tumor-associated angiogenesis [97, 107]	Promotes cardiomyocyte survival, cardiac function recovery and angiogenesis [177-179]	Promotes angiogenesis and animal survival [221]
miR-223	Induces chemotherapy resistance [113]	Promotes angiogenesis; induces antiinflammation and antifibrosis [180]	Promotes neuronal survival; induces antiinflammation (M2 microglia polarization); promotes functional neurological recovery [216]
miR-328	Promotes tumor cell proliferation, migration, invasion and epithelial – mesenchymal transition; promotes tumor growth [105]	Augments cardiomyocyte death and apoptosis [148]	[miR-328-3p agomir augments neuronal death, neurological deficits, brain neutrophil invasion and proinflammatory cytokine levels [239]
miR-361	Promotes tumor cell proliferation and survival; promotes tumor growth [103]	[Cardiac-specific miR-361 overexpression reduces cardiomyocyte survival and increases mitochondrial fission; miR361 has knockdown with opposite effects [240]	Promotes neuronal survival [217]
miR-494	Promotes tumor-associated angiogenesis and tumor growth [109]	Promotes periinfarct angiogenesis [173]	[miR-494 agomir promotes neuronal survival, axonal plasticity and neurological recovery via HDAC3 downregulation [193]; miR-494 antagomir promotes neuronal survival and neurological recovery by reducing Th1 helper cell shift and decreasing brain neutrophil infiltrates via HDAC2 upregulation [194, 243]

For ncRNAs without studies examining the role of EV-associated ncRNAs, data from ncRNA agomir, antagomir, overexpression or knockdown studies are shown in

brackets in the table.
Abbreviations: EVs, extracellular vesicles; lncR, long non-coding RNA; miR, microRNA.

Table 5. EV-associated non-coding RNAs as biomarkers in the three hypoxic conditions.

Author, year, reference	Disease	EV provenance	Non-coding RNA	Abundance	Clinical significance
Bjornetro et al., 2019 [253]	Locally advanced rectal carcinoma	Plasma	miR-486-5p	Downregulated	Associated with tumor invasion and lymph node metastasis
Bjornetro et al., 2019 [253]	Locally advanced rectal carcinoma	Plasma	miR-181a-5p	Downregulated	Associated with tumor invasion and lymph node metastasis
Bjornetro et al., 2019 [253]	Locally advanced rectal carcinoma	Plasma	miR-30d-5p	Upregulated	Associated with tumor metastasis
Zhang et al., 2019 [254]	Non-small cell lung carcinoma	Serum	lncR MALAT-1	Upregulated	Associated with cell proliferation and migration
Rong et al., 2020 [255]	Non-small cell lung carcinoma	Serum	lncR MALAT-1	Upregulated	Associated with cancer pathology
Wang et al., 2018 [256]	Pancreatic carcinoma	Serum	miR-301a	Upregulated	Associated with cancer metastasis
Qeu et al. 2013 [248]	Pancreatic carcinoma	Serum	miR-17-5p	Upregulated	Associated with cancer pathology
Qeu et al. 2013 [248]	Pancreatic carcinoma	Serum	miR-21	Upregulated	Associated with cancer pathology
Li et al. 2018 [250]	Pancreatic ductal adenocarcinoma	Plasma	circPDE8A	Upregulated	Associated with lymphatic invasion, TNM stage and poor survival rate
Zhu et al., 2019 [113]	Ovarian carcinoma	Serum	miR-223	Upregulated	Associated with cancer recurrence
Ye et al., 2016 [110]	Nasopharyngeal carcinoma	Plasma	miR-24-3p	Upregulated	Associated with disease-free survival
Hsu et al., 2017 [108]	Lung carcinoma	Serum	miR-23a	Upregulated	Associated with cancer pathology
Li et al., 2016 [114]	Oral squamous cell carcinoma	Serum	miR-21	Upregulated	Associated with T stage and N stage
Xue et al., 2017 [102]	Bladder carcinoma	Serum	lncR UCA1	Upregulated	Associated with cancer pathology
Zhou et al., 2021 [257]	Breast carcinoma	Serum	lncR NEAT1	Upregulated	Associated with cancer pathology
Zheng et al., 2020 [258]	Myocardial infarction	Plasma	lncR ENST00000556899.1	Upregulated	Associated with myocardial infarction pathology
Zheng et al., 2020 [258]	Myocardial infarction	Plasma	lncR ENST00000575985.1	Upregulated	Associated with myocardial infarction pathology, inflammatory markers, disease severity and prognosis
Sun et al., 2020 [259]	Myocardial infarction	Plasma	lncR UCA1	Upregulated	Associated with myocardial infarction pathology
Chen et al., 2020 [260]	Myocardial infarction	Serum	lncR NEAT1	Upregulated	Associated with myocardial infarction pathology
Chen et al., 2020 [260]	Myocardial infarction	Serum	miR-204	Downregulated	Associated with myocardial infarction pathology
Ling et al., 2020 [261]	Myocardial infarction	Serum	miR-122-5p	Upregulated	Associated with myocardial infarction pathology
Ling et al., 2020 [262]	Myocardial infarction	Serum	miR-126	Upregulated	Associated with myocardial infarction pathology and disease severity
Ling et al., 2020 [262]	Myocardial infarction	Serum	miR-21	Upregulated	Associated with myocardial infarction pathology
Su et al., 2019 [249]	Myocardial infarction	Serum	miR-1915-3p, miR-4507 and miR-3656	Downregulated	Associated with myocardial infarction pathology
Zhou et al., 2018 [252]	Ischemic stroke	Serum	miR-134	Upregulated	Associated with ischemic stroke pathology, severity and prognosis
Wang et al., 2018 [263]	Ischemic stroke	Plasma	miR-21-5p	Upregulated	Associated with ischemic stroke pathology
Wang et al., 2018 [263]	Ischemic stroke	Plasma	miR-30a-5p	Upregulated	Associated with ischemic stroke pathology
Chen et al., 2017 [264]	Ischemic stroke	Serum	miR-223	Upregulated	Associated with ischemic stroke pathology, severity, and short-term outcome
Li et al., 2017 [265]	Ischemic stroke	Plasma	miR-422a	Downregulated	Associated with ischemic stroke pathology
Li et al., 2017 [265]	Ischemic stroke	Plasma	miR-125b-2-3p	Downregulated	Associated with ischemic stroke pathology
Ji et al., 2016 [266]	Ischemic stroke	Serum	miR-9	Upregulated	Associated with ischemic stroke pathology and severity
Ji et al., 2016 [266]	Ischemic stroke	Serum	miR-124	Upregulated	Associated with ischemic stroke pathology and severity

7. Conclusion and perspectives

We herein showed that cancer, myocardial infarction and ischemic stroke, which represent the most prevalent medical conditions resulting in disability and death [3], profoundly regulate EV-associated ncRNAs, controlling physiological responses including cell survival and proliferation, migration, drug resistance, angiogenesis and neuronal plasticity. As underlying mechanism, hypoxia, which decisively influences tissue fate in all three conditions [1, 2], was found to control endogenous ncRNA responses, some ncRNAs being upregulated and others downregulated upon hypoxia. In several studies, the delivery of

ncRNA-loaded EVs harvested from healthy human blood, perilesional tissue, progenitor cells (including MSCs) or antiinflammatory immune cell subsets (such as M2 microglia) allowed attenuating disease progression and restoring functional tissue recovery. Remarkably, EV-ncRNAs exhibited a large degree of overlaps regarding their modes of action across pathophysiological states. Thus, EV-ncRNAs open fascinating perspectives as theranostic biomarkers and besides this, but more challenging, perhaps also as therapeutic products. With respect to clinical translation, major challenges remain with respect to the large-scale isolation and preparation of EVs, the reproducibility of EV preparations under defined culturing conditions as well as the development of potency assays, which ensure that EV activity characteristics are maintained in a given EV preparation before this individual preparation is administered to human patients [245, 246, 268]. Only with such skills, we can ensure the success of clinical proof-of-concept trials. Currently, there are 23 clinical trials on EV-enveloped ncRNAs registered at https://clinicaltrials.gov/ in diverse medical conditions. Among these studies, currently eight trials are active and recruiting, four are active but not recruiting, four are not recruiting and seven are completed. The results of these ongoing studies are envisaged with great interest. Meanwhile, ongoing preclinical efforts will sharpen our understanding of the clinical potential of EV-ncRNA technologies.

Competing Interests

D.M.H., B.G. and T.R.D. hold a patent on extracellular vesicles for the treatment of inflammatory conditions (US9877989B2).

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