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miRNAs Modulate the Purinergic Signalling Network

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Corresponding Author:	Davide Ferrari, Ph.D. Ferrara, ITALY
First Author:	Davide Ferrari, Ph.D.
Order of Authors:	Davide Ferrari, Ph.D.
	Nicoletta Bianchi, Dr.
	Holger K. Eltzschig, Dr.
	Roberto Gambari, Prof.
Abstract:	MicroRNAs are small non-coding RNA molecules endowed of the ability of silencing messenger RNA targets to regulate gene expression. Dysregulation in miRNAs have been implicated in cancer development, heart and neurological diseases, loss of immune tolerance, lipid metabolism. Therefore, miRNAs are gaining increasing attention as novel disease biomarkers and therapeutic targets. Recent studies showed that purinergic receptors i.e. the plasma membrane receptors activated by extracellular nucleotides (ATP, ADP, UTP, UDP) and nucleosides such as adenosine are subjected to miRNA regulation. This opens a new and previously unrecognized opportunity to modulate the purinergic network thus avoiding abnormal activation of specific receptor subtypes. miRNA technology will hopefully contribute to prevent purinergic-mediated tissue damage underlying neurodegeneration, atherosclerosis, graft rejection and even neoplasia.
Suggested Reviewers:	Sean P. Colgan, Dr. PhD. Principal investigator, University of Colorado (U.S.A.) sean.colgan@ucdenver.edu Leading scientist in the field of inflammation and purinergic mediated responses.
	Simon C. Robson, M.D. Principal Investigator, Harward University (U.S.A.) srobson@bidmc.harvard.edu Outstanding scientist in the field of extracellular ATP metabolism.
	Sen K. Chandan K., PhD. Professor and Associate Dean, The Ohio State University, Columbus, Ohio, USA chandan.sen@osumc.edu
Opposed Reviewers:	

To the Editor Dr. Catarina Sacristán

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Dear Dr. Sacristán

As previously discussed, we have submitted to Trends in Molecular Medicine our review on microRNAs and purinergic signalling. We followed your useful suggestions by highlighting the health and disease angle and providing examples promising to treat various pathologies. Three figures and one table have also been included.

For competing interests I would exclude Francesco DI VIRGILIO as a REVIEWER.

We look forward to hearing from you at your earliest convenience,

Kind Regards

Davide Ferrari PhD,

Nicoletta Bianchi PhD,

Holger K. Eltzschig M.D., PhD.

Roberto Gambari PhD.

Davide Ferrari PhD

Department of Life Science and Biotechnology
Biotechnology Centre
University of Ferrara
via Fossato di Mortara 64 b
I-44100 Ferrara
Italy

Tel. +39-0532-455547
e-mail dfr@unife.it
ORCID link http://orcid.org/0000-0002-5727-9204

miRNAs Modulate the Purinergic Signalling Network

Davide Ferrari,[1,*] Nicoletta Bianchi,[1] Holger K. Eltzschig,[2] Roberto Gambari[1]

[1]Department of Life Science and Biotechnology, University of Ferrara, I-44100 Ferrara, Italy.

[2]Organ Protection Program, Department of Anesthesiology, University of Colorado School of Medicine, Aurora, CO 80045, USA.

[*]Correspondence: davide.ferrari@unife.it (D. Ferrari)

Keywords

P1 receptors, P2 receptors, extracellular nucleotides, miRNAs

Abbreviations

ADA, adenosine deaminase; ALS, amyotrophic lateral sclerosis; CD73, ecto-5'-nucleotidase; ICAM-1, Intercellular Adhesion Molecule 1; GVHD, graft-versus host disease; MPM, malignant pleural mesothelioma; NTPDases, ectonucleoside triphosphate diphosphohydrolases; PDCD4, programmed cell death 4; PTEN, tissue inhibitor of metalloproteinase 3, phosphatase and tensin homolog; ROIs, reactive oxygen intermediates; Th, T helper lymphocyte.

Abstract

MicroRNAs are small non-coding RNA molecules endowed of the ability of silencing messenger RNA targets to regulate gene expression. Dysregulation in miRNAs have been implicated in cancer development, heart and neurological diseases, loss of immune tolerance, lipid metabolism. Therefore, miRNAs are gaining increasing attention as novel disease biomarkers and therapeutic targets. Recent studies showed that purinergic receptors i.e. the plasma membrane receptors activated by extracellular nucleotides (ATP, ADP, UTP, UDP) and nucleosides such as adenosine are subjected to miRNA regulation. This opens a new and previously unrecognized opportunity to modulate the purinergic network thus avoiding abnormal activation of specific receptor subtypes. miRNA technology will hopefully contribute to prevent purinergic-mediated tissue damage underlying neurodegeneration, atherosclerosis, graft rejection and even neoplasia.

Introduction

The purinergic signalling network

Nucleotides (ADP, ATP, UDP, UTP, GTP) play fundamental roles in living cells as monomers for nucleic acids building, energy carriers in metabolic pathways and components of coenzymes [1]. However, nucleotides and nucleotides such as adenosine have completely different functions in the extracellular space where they can be released and behave as mediators of cell-to-cell communication by binding and activating specialized plasma membrane receptors expressed by virtually all tissues [2,3]. Receptors for extracellular nucleotides are named P2 receptors and divided into two subgroups: P2Y and P2X receptors [4-6]; in contrast, adenosine receptors are referred to as P1 receptors and can be divided into four subtypes known as A_1, A_{2A}, A_{2B} and A_3 receptors [7]. Extracellular ADO is generated by the sequential activation of plasma membrane ectonucleotidases known as ectonucleoside triphosphate diphosphohydrolase (NTPDase or CD39), ecto-5'-nucleotidase (CD73), ectonucleotide pyrophosphatase/phosphodiesterase and alkaline phosphatases [8,9]. If ADO does not bind P1 receptors can either be transported back into the cytoplasm or degraded extracellularly to inosine by the enzyme adenosine deaminase (ADA) or aminohydrolase (**Fig. 1**).

Purinergic receptors modulate many biological functions ranging for heart rate, vascular tone, neuron excitation, tissue repair, and immune response [10-14] (**Fig. 1**). Specialized plasma membrane molecules (connexin hemichannels, pannexin channels, the P2X7 receptor, ABC transporters, ATP conducting anion channels) allow release of ATP into the extracellular *mileu* [15-17], while extracellular adenosine is generated by the sequential activation of plasma membrane ectonucleotidases known as ectonucleoside triphosphate diphosphohydrolase (NTPDase or CD39), ecto-5'-nucleotidase (CD73), ectonucleotide pyrophosphatase/phosphodiesterase and alkaline phosphatases [8,9]. Uncontrolled ATP liberation such as in the case of plasma membrane

mechanical stress, trauma, allergen inhalation, infection, causes over-activation of P2X and P2Y receptors with excessive production of inflammatory mediators (prostaglandins, ROIs, pro-inflammatory cytokines) and massive recruitment of immune cells thus favouring the establishment of a background of chronic inflammation [18-20].

Different pathologic states including neuronal degeneration, chronic pain, metabolic dysfunctions, atherosclerosis, thrombosis, allergy, asthma and even cancer have been linked to dysregulation in the purinergic signalling network [21-26]. Although stimulation or inhibition of specific P1 or P2 receptors has become a new opportunity to treat different pathologic states [27] such as in the case of P2Y12 receptor antagonists clopidogrel and ticagrelor which are successfully used for antithrombotic therapy in patients [28,29], many other human diseases have no real cure and await for therapies able to normalize purinergic signaling dysregulation. The recent acquisition that purinergic receptors and ecto-nucleotidases are under the control of miRNAs opens the possibility to modulate their transcription by miRNA applied technologies. In the next sections we will summarize recent knowledge on modulation of the purinergic signaling network by miRNAs.

MicroRNAs: promising tools for new therapies

MicroRNAs (miRNAs) are widely present in *Metazoa* and endowed of the ability of silencing messenger RNA targets to regulate gene expression during organism development, cell proliferation and death, hematopoiesis, immune mediated functions [30-34]. miRNAs are a family of small (19 to 25 nucleotides in length) noncoding single-stranded RNAs that post-transcriptionally regulate gene expression by sequence-selective targeting of messenger RNAs (mRNAs), leading to translational repression or mRNA degradation, depending on the degree of complementarity between miRNAs and target mRNA sequence [35-37]. Due to their importance as regulatory molecules and to the availability of new and effective tools to screen for the presence of single or pathway-associated

miRNAs have been made available [34] increasing attention is given to them and the number of mRNA sequences deposited in the miRBase databases is rapidly growing [35,36]. Although miRNA working principles are quite simple as low miRNA expression normally determines high expression of targets mRNAs and conversely, high expression of miRNAs induces low expression of the target mRNAs. It has to be considered that the situation is much more complicated since a single miRNA can target several mRNAs and a single mRNA might contain in the 3'UTR region several target sites for miRNA recognition. Moreover, it is calculated that at least 10-40% of human mRNAs are targets for miRNAs [37] opening previously unsuspected opportunities for modulating eukaryotic gene transduction of structural and regulatory proteins thus controlling fundamental biological functions such as cell proliferation, differentiation and death [38].

Many *in vivo* studies with different animal models have highlighted the possibility to identify miRNA dysregulation as diagnostic marker for numerous pathologies. Moreover, efficacy of miRNAs as potent modulators of transduction of genes involved in different diseases have been pointed out. Among candidate pathologies to be treated with miRNAs, cancer holds a prominent position, nevertheless a growing number of diseases are going to be included; among them neuronal dysfunctions caused by viral infection and chronic inflammatory diseases [39-42]. In the last years, identification of miRNAs playing a role in specific pathologic contexts has been paralleled by an in deep investigation on miRNA delivery approaches. Among various utilized, erythrocyte ghosts, cationic lipoplexes, neutral lipid emulsion, nanoparticles [43-46].

miRNAs: an effective way to modulate purinergic signalling

Purinergic signalling modulation

Molecular components of the purinergic signalling network can be either activated or inhibited in different ways [47-49]. As an example, large spectrum or specific P1 and P2 receptors agonists and

antagonists have been made available and allow wide, restricted or punctual modulation of purinergic receptors [50-52]. The knockout mouse model has also been a valuable tool for discerning the role of single purinergic receptors and ectonucleotidases [53-54]. Lack of expression of single receptor or nucleotide degrading enzyme genes has provided insight into their physiological role in mice and allow to speculate on function in humans [55,56].

Moreover, an indirect way often used both in *in vitro* and *in vivo* experiments to reduce or block purinergic receptor activation consists in depleting the extracellular *milieu* of purinergic agonists (ATP, ADP, adenosine) by their enzymatic degradation. Apyrase is an ATP diphosphohydrolase catalyzing ATP hydrolysis to AMP and inorganic phosphate. Application of this strategy has great potential for clinical applications in different pathologic states [57,58].

MicroRNAs involved in the modulate the purinergic signalling network might also be considered in the future a suitable target for therapeutics intervention. Strategies for modulating purigenic signalling network are based on miRNA targeting and/or miRNA mimicking. Hence, inhibition of miRNA activity can be readily achieved by the use of: i) oligomer miRNA-inhibitors (miRNA anti-sense therapy), ii) small molecule inhibitors (LNAs, PNAs, morpholinos, miRNA sponges, mowers) or through, iii) miRNA masking, that is inhibition of miRNA function by covering miRNA binding sites with a modified single-stranded RNA complementary to the target sequence [52]. On the contrary, increase of miRNA function (miRNA replacement therapy) can be achieved by the use of modified miRNA mimetics, either synthetic, or produced by plasmid or lentiviral vectors. miRNAs are emerging as effective translational regulators of P1 and P2 receptors, particularly for A_{2A}, A_{2B} and P2X7 receptors, moreover crucial enzymes of the purinergic network such as CD39 and ADA are also regulated by miRNAs **(Fig. 2)**. Recent data show that miRNAs modulate purinergic receptors both in normal and transformed cells and a list of recent publications on this issue is reported in **Table I**.

P1 receptors are modulated by mRNAs

The A_{2A} receptor has become an attractive target for medicine and pharmacology due the multiplicity of functions played, ranging from cancer to inflammatory, Parkinson's and cardiovascular diseases [59]. Recent data showed that the A_{2A} receptor is under the control of different miRNAs: miR-34b, miR-214, miR-15 and miR-16 have been identified [60,62]. Of particular interest is the report showing that endogenous A_{2A} receptor protein levels increased when miR-34b was blocked by using a specific anti-miR-34b [60]. Moreover, a **luciferase** reporter assay with point mutations in a miR-34b predicted binding site within the 3'UTR region of A_{2A} mRNA abolished the effect of the miRNA using a miR-34b mimic molecule. These findings are potentially very important considering that A_{2A} receptor expression increases in Parkinson's disease patients both in putamen and peripheral blood cells and this correlates with severity of the disease [63-66]

Besides neurodegenerative disorders, the A_{2A} receptor plays an important role in inflammatory tissue damage and fibrosis. At least in the mouse model, a mutual influence between miR-214 and A_{2A} has been shown [62]. As both molecules are involved in inducing fibrotic changes in different organs [67,68], a deeper investigation is needed to clarify the specific pathways and down-modulating expression of these molecules in pathologic states where fibrosis plays a major role.

It has been shown that the A_{2B} receptor subtype is an important regulator of the immune response and blood vessel physiology [69] and very recently mir-15 and miR-16 have been associated to maturation of NK cells and inhibition of TGFβ-pathway in the heart [70,71]. This will hopefully open the possibility to target this subtype to treat immune-mediated pathological states and circulation dysfunctions. A role for the A_{2B} receptor has also been hypothesized in colitis as this subtype is under the control of the cytokine TNF-alpha and is upregulated in mice models of colitis and human bowel disease. Interestingly, A_{2B} receptor protects animals from colitis [72,73]. It is thus relevant the recent acquisition that at least in the mouse model, the A_{2B} mRNA shows four putative miRNA target sites,

i.e. miR27a, miR27b, miR128a, miR128b and that miR27b and miR128a expression levels are greatly reduced by the pro-inflammatory cytokine TNF-alpha [74]. The intimate relationship between A_{2B} receptor and miRNAs has potentially important consequences as this P1 subtype favors progression of the human oral cancer [75] and microRNA-128b has recently been identified as a miRNA promoting apoptosis of cancer cells and thus suppressing gastric cancer growth by targeting the A_{2B} receptor [76]. Therefore, the important intimate relationship between miRNAs modulating the P1 receptors and different pathologic states fosters further investigative efforts to favor a therapeutic application of their modulation.

P2 receptors are modulated by miRNAs

The purinergic P2X7 receptor subtype is activated by extracellular ATP and mediates a variety of cell responses, either positive or negative, depending on cell type, level of expression of the receptor and duration of the stimulation. Therefore, P2X7 has been the focus of increasing interest in disparate pathophysiological conditions as its activation has been linked to persistent inflammatory states, diabetes induced retinal damage, neuronal death, seizures during **epilepsy**, Alzheimer's disease, tumor growth and metastatization, and even virus infection [77-81]. It is thus remarkable for the wide range of possible implications, the acquisition that P2X7 receptor expression is regulated by miRNAs. Hence, recent reports have shown that P2X7 receptor is under control of different miRNAs. Different lines of experimental studies showed that the messenger RNA of P2X7 has a 3'UTR region recognized by different miRNAs, including miR-216b, miR-150, miR-186, miR-22 and miR-9 [82-84]. A role for miR-150 and miR-186 in tumor progression has been shown in different cancers and expression of these miRNAs has been linked to inhibition of tumor growth and metastatization [85-86]

Using human embryonic kidney-293 cells expressing a full-length 3'-UTR-P2X7 luciferase reporter, it was nicely shown that miR-186 and miR-150 inhibitors increased luciferase activity, whereas on the contrary, miR-186 and miR-150 mimics decreased luciferase activity after **actinomycin D** (**act D**) (inhibitor of gene transcription) treatment, confirming that P2X7 is down-regulated by miR-150 and miR-186 through activation of sites of instability at the 3'-untrasleted region of the P2X7 messenger RNA [83]. Moreover, normal epithelial cells express higher mRNA and protein level of P2X7 compared to pre-cancerous and cancerous epithelial cells and this is associated to induction of apoptosis. Since defective regulation of apoptotic cell death have been associated to cancer, the author hypothesize that a diminished P2X7 expression could be linked to growth of uterine cancers [83,84].

Anti-cancer drugs may have an effect on miRNA expression, such as in the recently identified case of carboplatin, a chemotherapic drug suppressing miR-21 and inhibiting *in vitro* TGFβ receptor signaling which is at the basis of non-small cell lung cancer (NSCLC) cell invasion [85]. To this purpose, it is very important the recent acquisition of the interplay between P2X7 receptor and miR-21. Hence, in NSCLC cells, up-regulation of miR-21 corresponds to a low P2X7 expression and a decreased survival of NSCLC patients [86]. Another recent report showed that although expression of the P2X7 subtype is regulated by miRNAs, activation of the receptor is in turn able to modulate different miRNAs among which miR-21. This P2X7-mediated activity was linked to the expression of vascular endothelial growth factor (VEGF) and IL-6, and has been hypothesized to play a role in psoriatic lesions [87]. Amyotrophic lateral sclerosis (**ALS**) is a brain and spinal cord disease in which motoneurons progressively degenerate and dye. miRNA dysregulation and subsequent neuroinflammation have been indicated among the mechanisms involved in ALS pathogenesis [88,89]. To this purpose, it has recently been compared the miRNA transcription profile of control mice microglial cells and ALS microglia in resting conditions and upon P2X7 receptor stimulation

with the agonist BzATP, finding that miR-22, miR-125b, miR-146b and miR-155 were upregulated [90]. They also proved that miR-365 and miR-125b interfere with transcription of IL-6 and STAT3 pathway increasing the transcription of a pro-inflammatory cytokine related to ALS neuro-inflammation, i.e. TNF-α [90]. Involvement of the P2 receptors in mediating and maintaining nociceptive sensitivity, neuropathic and inflammatory pain has been ascertained in different studies [91]. At present there is one interesting study linking P2X7 receptor-mediated signaling and its modulation by miR-9 to pain sensation in a diabetes rat model [92] opening future scenario also in human pathologic nociception.

The micro RNA miR-22 has recently emerged as an important regulator of the adhesion protein ICAM-1 which is expressed by endothelial cells and involved in adhesion and trans-endothelial migration of immune cells to tissues. It has been recently shown that UTP and ATP reduce endothelial inflammation by miR-22-mediated ICAM-1 Inhibition. [93]. This finding is potentially very important for all pathologic states in which excessive adhesion of leukocyte to the endothelium causes damage to endotheliocytes and modify sub-endothelial tissue composition, such as in atherosclerotic lesions.

Are ecto-nucleotidases and adenosine deaminase modulation by miRNAs?

miR-155 plays relevant roles as an immune-modulator by regulating responses mediated by IL-10 in mast cells or by T helper(Th)17 lymphocytes [94, 95]. Ecto-nucleotidases (CD39 and CD73) play a fundamental role in regulating nucleotide and nucleoside concentration in the extracellular *milieu*. Therefore, agonist availability and P1 and P2 receptors activation is strictly dependent on their activity. A recent report showed that miR-155, a miRNA involved in immunomodulation, is up-regulated in patients with sepsis and in a murine model of sepsis and this is accompanied by an increase in the number of $CD39^+$ T regulatory lymphocytes which decreases in mice transfection

with miR-155 inhibitor [96]. Although a mechanistic explanation still lacks, the information is clinically relevant as elevated number of $CD39^+$ Tregs correlates with a poor prognosis for sepsis patients [96]. Another finding suggesting a link between miR-155 and molecular component of the purinergic network is the fact that miR-155 deficiency in mouse dendritic cells is associated with a reduced chemotactic response, defective and IL-1beta secretion upon stimulation with ATP [97] and this is accompanied by a reduced graft-versus host disease (GVHD) [98]. Modulation of the other membrane nucleotidase, CD73 by miRNA have been shown in mouse retina. A recent study on differentiation of retina precursor cells and $CD73^+$ has shown that [99] opening the way for future investigation on miRNA expression in human retina. Another relevant point concerns adenosine deaminase regulation by miRNAs. The enzyme is expressed in two isoforms (ADA1 and ADA2) in humans and it is endowed of the ability to degrade ADO to inosine which is inactive at P1 receptors, thus causing termination of P1-mediated signaling network **(Fig. 1)**. Since extracellular adenosine plays an anti-inflammatory role in different contexts its concentration increase favors anti-inflammatory responses and tissue protection from damage, while on the contrary its degradation determines the establishment of a pro-inflammatory background and increases the risk of cell and tissue damage.

Adenosine deaminases (ADA1 and ADA2) represent a fundamental part of the purinergic signaling network **(Fig. 1)** as they are endowed of the ability of inactivating adenosine thus terminating P1 signalling. Since extracellular adenosine play an anti-inflammatory role, its degradation determines the establishment of a pro-inflammatory background, while on the contrary a concentration increase favors anti-inflammatory responses and tissue protection from damage. Recent data show that miRNA-146b-3p is involved in modulation of retinal inflammation at it decreases ADA2 expression and in vitro TNF-alpha secretion [100]. Therefore, the finding of an intimate relationship

between miRNAs and nucleotide/nucleoside metabolizing enzymes assume a great importance in the optic of blocking or reducing retinal inflammation.

Conclusions and future perspectives

Signaling through purinergic receptors has attracted increasing interest for the multiplicity of responses elicited in virtually every tissue. Dysregulation of the purinergic network has been detected in pathologic states ranging from neurodegeneration to allergy, graft rejection, diabetes, osteoporosis, stroke and cancer; therefore, it is urgently needed to find effective and safe ways to transiently or stably block purinergic signaling in specific body districts to prevent deleterious consequences of aberrant activation. An exciting advancement in purinergic signalling biology is the recent acquisition that miRNAs modulate expression of molecular components of the purinergic network (**Table 1** and **Fig. 3**). This fosters the development of new therapeutic approaches and encourages research efforts on impact of each miRNA in post-transcriptional regulation. This is a mandatory issue, since several miRNAs can cooperate in regulating the activity of a single mRNA target. Strategies based on mimicking miRNAs activity or targeting of miRNAs, also known as "miRNA replacement therapy" or "therapeutic miRNA targeting" have been the object of great interest with respect to multiple future therapeutic developments. Although the number of clinical trials is at present very limited (www.clinicaltrials.com) and mostly restricted to Phase I studies for the treatment of cancer [101], it has to be noticed that one of these, performed in patients failing to repond to standard therapy (MesomiR 1: A Phase I Study of TargomiRs as 2nd or 3rd line treatment for patients with recurrent malignant pleural mesothelioma (MPM) and NSCLC) is based on miR-16 mimic (TargomiRs). Since miR-16 is also involved in controlling A_{2A} expression [53] it can be envisaged that future application of miRNA technology would be used for example to decrease A_{2A} receptor expression in pathologic conditions, such as in tumour cells or in specific brain regions during the

course of Parkinson's disease [60]. Another potentially very useful application of miRNAs technology in the treatment of neurological diseases could take advantage from A_1-mediated neuronal protection, by inducing for example an increased expression of this subtype in neuronal cells in those pathological states characterized by neurodegeneration due to accumulation of protein aggregates such as for example in Alzheimer's disease.

Encouraging results of application of miRNA technology comes from experiments performed by *in vivo* injection of microRNA-22 mimics that have been shown to be able to transiently suppress spontaneous seizures in mice [102], suggesting that miRNA-22 might represent a potentially very useful therapeutic target for prevention of inflammatory brain conditions and subsequent development of secondary epileptogenic focuses. Similarly, miRNA-related applications may be useful to avoid noxious effects dependent on $P2X_7$ receptor activation in ALS [103] or to diminish adenosine degradation in the retina [99].

Expression of specific miRNA or pre-miRNA has been considering of important both for evolution and prognosis of very different diseases [104-106], among which cancer has a prominent role. To this purpose, an exciting and potentially very relevant finding is the demonstration up-regulation of miR-150 appears to be inversely associated with P2X7 and that at least in breast cancer, miR-150 over-expression promotes growth and clonogenicity of tumor cells while it reduces their apoptosis, thus favoring tumor mass increase [107,108]. Different studies have shown that a high level of expression of miR-21 is associated with downregulation of tumor suppressor genes among which the ras homolog gene family member B, maspin, programmed cell death 4 (PDCD4), tissue inhibitor of metalloproteinase 3, phosphatase and tensin homolog (PTEN), tropomyosin 1. Accordingly, miR-21 silencing causes cell cycle arrest and increased chemosensitivity to anticancer drugs [109]. Due to the relevance of both miR-21 and P2X7 in tumor biology, the interplay between them should be

examined in detail. Another point deserving an in deep investigation concerns the ability of purinergic receptors to regulate miRNA expression.

Therefore, therapies based on modulation of specific miRNAs will hopefully be introduced for acute or chronic pathologic states such as septic shock, allergy, colitis, cancer and neurological diseases in which purinergic signaling plays a crucial role [72,79,80,110]. Although we are well aware of the fact that important improvements in methods for the sustained release of miRNA or anti-miRNA are still needed, we think that miRNA modulation of the purinergic signaling network will give a concrete chance to treat many human diseases.

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We apologize to all whose work we could not cite due to limited space required by the journal.

Conflict of interest

Authors declare no conflicts of interest.

Glossary

ALS: Amyotrophic lateral sclerosis or Lou Gehrig's disease characterized by the gradual degeneration and death of motor neurons of the brain and spinal cord. It is characterized by a rapid progression and fatal outcome. Sporadic and familial forms of the disease have been identified. Although a treatment slowing progression of the disease in some subjects exist, a real cure halting or reversing the disease is not yet available.

Actinomycin D: known also as actinomycin C1, actinomycin IV or dactinomycin is an antibiotic produced by *Streptomyces sp.* It interferes with the elongation of the RNA chain by binding to premelted DNA.

Epilepsy: chronic neurological disorder characterized by seizures, periods of strange unusual behavior, strange sensations sometimes culminating in loss of consciousness. Main manifestations of epilepsy are partial or generalized seizures due to an excess of electrical activity in the brain.

Luciferase: a family of enzymes present in different organisms and sharing the ability to emit light. Luminescence consists in photon emission as a result of a chemical reaction occurring without heat production. Bioluminescence assays are widely used to measure a variety of biological parameters and functions.

microRNA sponge strategy: is a molecular biology approach used to generate RNAs containing miRNAs binding sites.

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Figure Legends

Figure 1. Nucleotide and nucleoside signaling at the plasma membrane. Nucleotides (ATP, ADP, UTP, UDP) and nucleosides (ADO) are released by the cell in the extracellular *milieu* where they can be degraded or bind to specific purinergic receptors named P1 and P2 receptors. P1 receptors are activated by adenosine (ADO), while P2 receptors are activated by ATP (P2X) and/or ADP, UTP, etc. (P2Y). The fate of ATP and ADP depends on expression of plasma membrane ecto-nucleotidases (CD39 and CD73) that sequentially transform ATP/ADP to AMP and ADO. ADO is irreversibly inactivated to inosine by adenosine deaminase (ADA).

Figure 2. Representation of the human 3'UTR sequences of A2A (A), A2B (B), P2X7 (C), receptors and CD39 (D) and ADA2 (E) enzymes. Putative miRNA target sequences resulted from analysis performed with *http://www.microrna.org/*. Among all proposed sequences, for the A2A receptor have been considered the following: NM_000675, AK289871, AK301420, AK312946, CR611621, S46950, X68486. For the A2B receptor: NM_000676. For the P2X7 receptor: NM_002562, AY847300, AY847302, AY847304. For CD39: NM_001776, NM_001098175, NM_001164178, NM_001164179. For ADA2: NM_017424, NM_177405, AK304818, AK314321. In red, target sites of conserved miRNAs with good mirSVR scores; in blue, target sites of conserved miRNAs with all mirSVR scores; in brown, target sites of all miRNAs with good mirSVR scores.

Figure 3. Relationship between miRNAs involved in and different pathologic conditions and modulating purinergic receptors. Circles include miRNAs mainly implicated in cancer, inflammation, neurologic diseases and fibrotic states. MiRNAs are indicated in black if modulate the A_{2A} receptor, red for A_{2B} modulation and white for those involved in P2X7 modulation.

Trends Box

- Nucleotides and nucleosides play multiple functions within the cell. However, they behave as signaling molecules once released extracellularly, where they bind to specific cell membrane purinergic receptors. ATP, ADP, UTP, UDP, UDP-glucose activate P2 receptors while adenosine activate P1 receptors.

- Plasma membrane ectonucleotidases CD39 and CD73 convert ATP/ADP to AMP, and AMP to adenosine, respectively, thus regulating the concentration of P2 and P1 receptor agonists and consequent responses. Adenosine deaminase (ADA) terminates P1-mediated signaling by inactivating adenosine.

- MicroRNA (miRNA) are small RNA molecules regulating gene expression by silencing messenger RNA targets. They show enormous regulatory potential and act during organism development, cell proliferation and death, hematopoiesis, immune mediated functions. miRNA dysregulation has been found in many pathologic states including cancer, neurological and muscular diseases, metabolic disorders.

- miRNAs modulate expression of P1 and P2 purinergic receptors as well as that of CD39 and ADA2. Therefore, they can affect the global outcome of the purinergic response, influencing many physiological and pathological responses mediated by extracellular nucleotides/nucleosides and consequently, final tissue fate.

Box 1. Outstanding questions

- Is it possible to target pro-inflammatory molecular components of the purinergic network to treat inflammatory diseases in humans?
- Would it be possible to use miRNAs to treat neurological diseases such as Alzheimer, epilepsy and amyotrophic lateral sclerosis?
- Are miRNAs controlling P2X7 receptor expression useful to block tumor progression?
- Are P2Y receptors under the control of miRNAs? How this affects cell specific functions and differentiation?
- Do molecular variants of purinergic receptors have a different miRNA regulatory pathway? Do they induce a different panel of miRNAs and can this impact disease states?
- Is it CD73 modulated by miRNAs?

Figure 1

Fig. 1

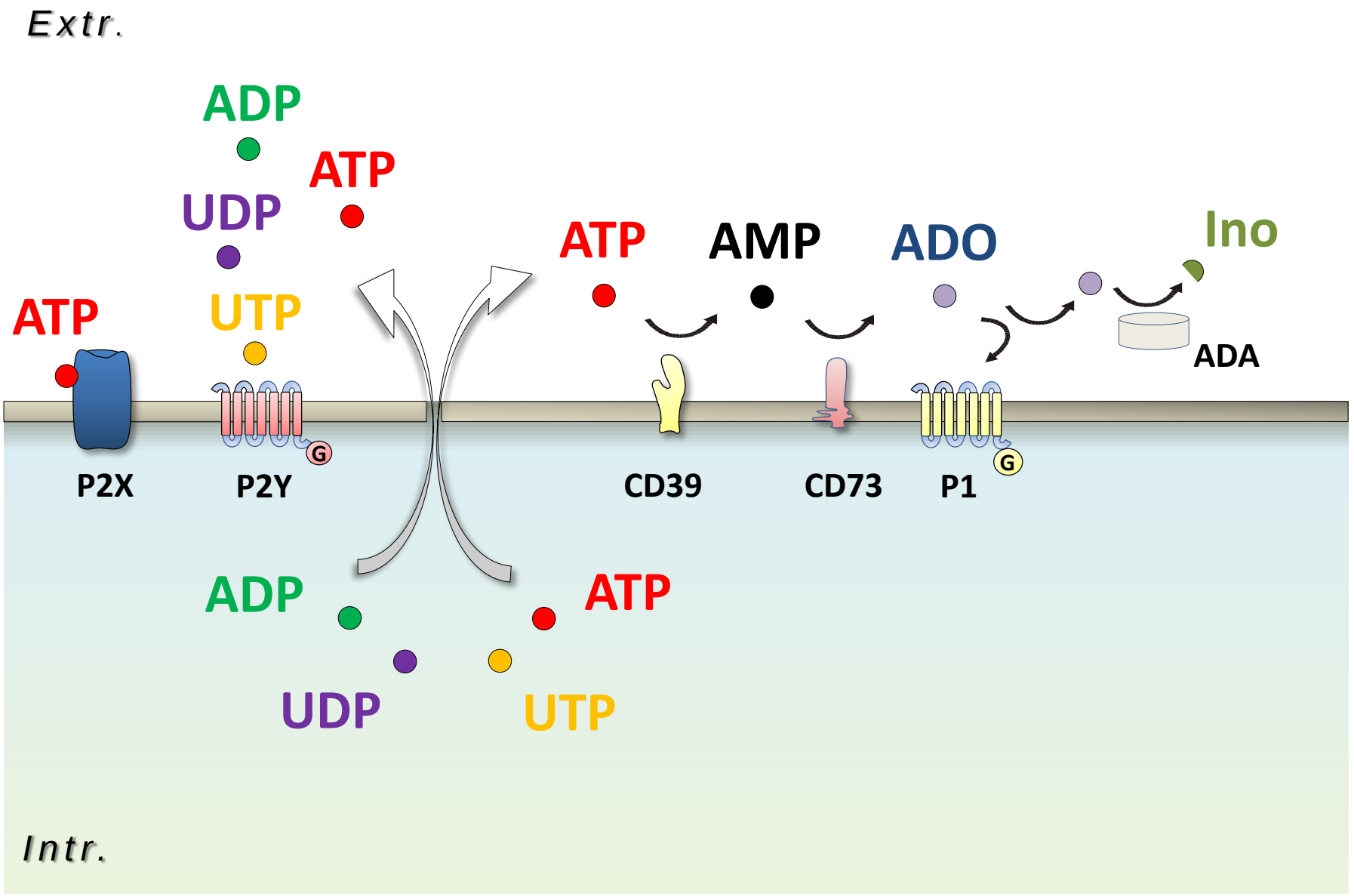


Figure 2

Fig. 2

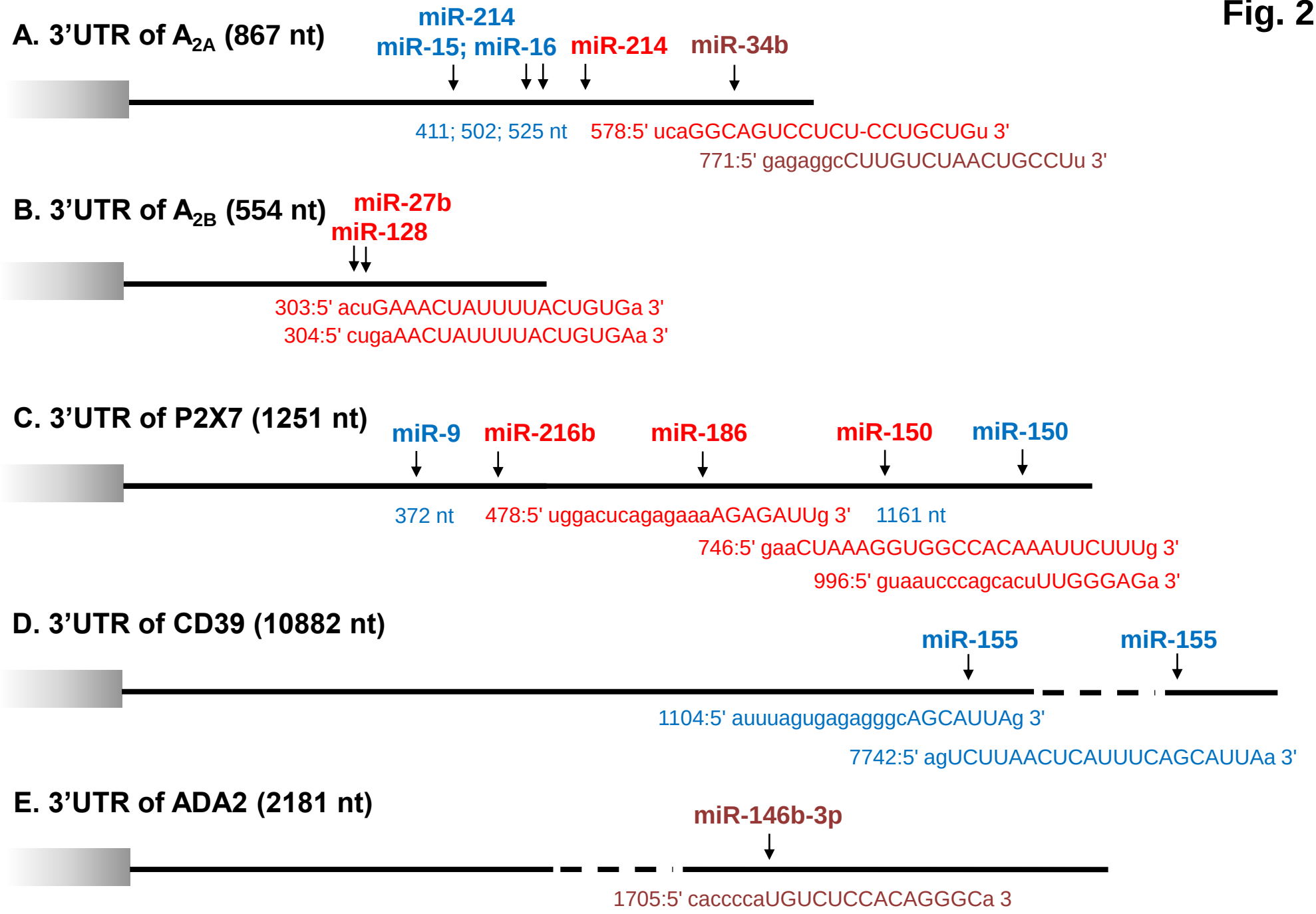
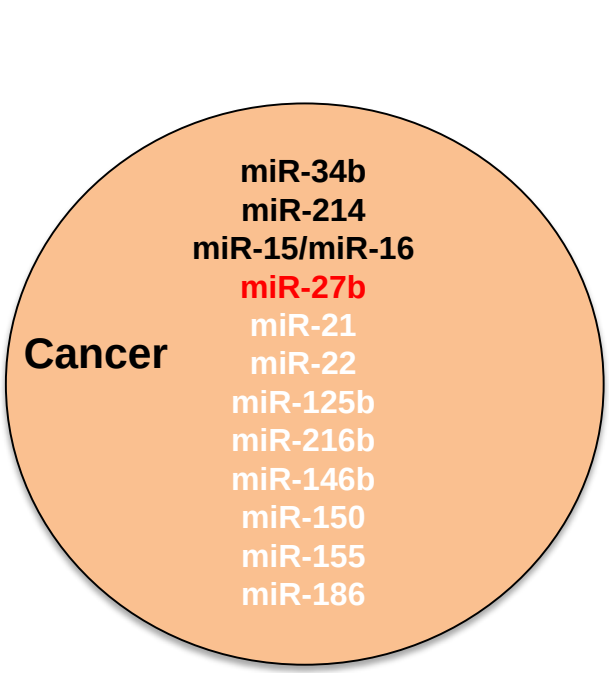
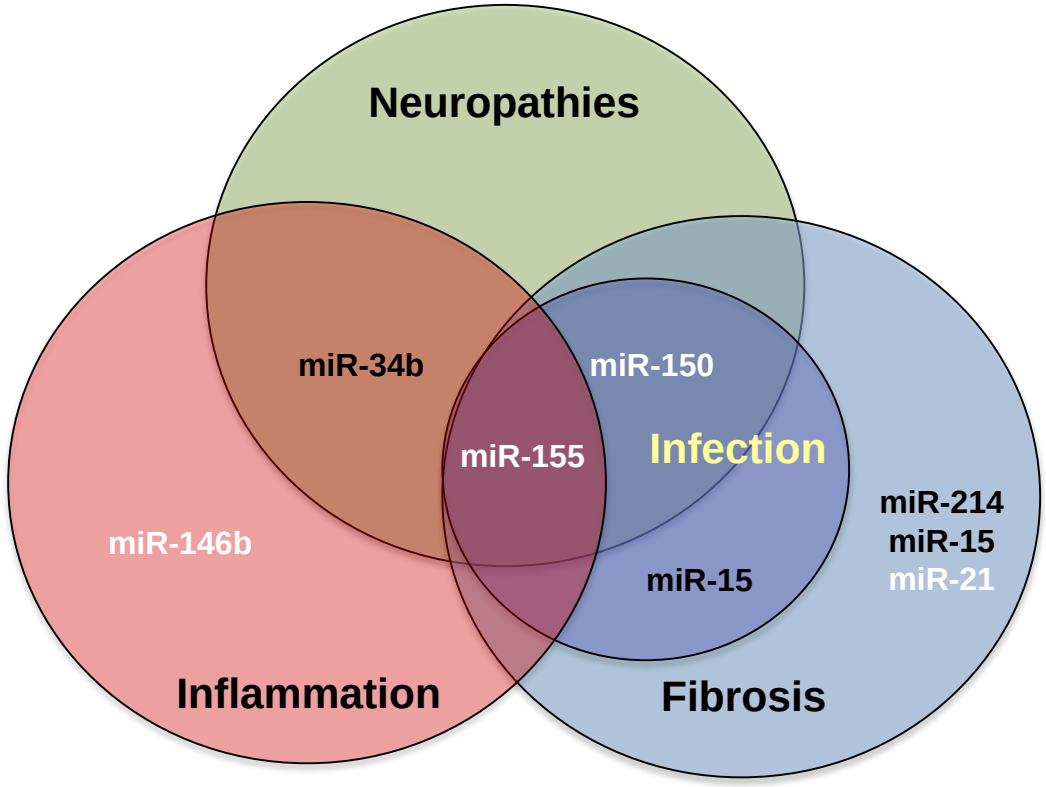


Figure 3

Fig. 3





A2A
A2B
P2X7

Table 1. Molecular components of the purinergic network modulated by miRNAs.

Purinergic receptors	Regulatory miRNAs	Description of experimental evidence	Refs
A_{2A}	miR-34b	A point mutations in a miR-34b predicted binding site within the 3'UTR region of the A_{2A} mRNA abolished the effect of the miRNA using a miR-34b mimic (luciferase reporter assay). A_{2A} protein levels increased when miR-34b function was blocked by a specific anti-miR-34b.	[60]
A_{2A}	miR-214 miR-15 miR-16	Bioinformatic analyses and reporter gene assays revealed that A_{2A} expression was controlled by miRNA-214, miRNA-15, and miRNA-16.	[61,62]
A_{2B}	miR-27b miR-128a mir-128b	Binding of miRNAs to the 3'-untranslated region (UTR) of the A_{2B} mRNA was examined by cloning 3'-UTR sequence downstream the luciferase gene in a pMIR-REPORT.	[74]
P2X7	miR-9	Lentivirus encoding pre-miR-9 transduced into neurons. CALHM1 short hairpin RNA was subcloned into the pLB vector to generate CALHM1 shRNA-expressing lentiviral vectors.	[92]
P2X7	miR-150 miR-186	HEK-293 cells heterologously expressing the full-length 3'-UTR-P2X7 luciferase reporter. miR-186 and miR-150 inhibitors increased luciferase activity, whereas miR-186 and miR-150 mimics decreased luciferase activity after **act D** treatment.	[83]
P2X7	miR-150	A **microRNA sponge strategy** was used to inhibit miR-150 in *vitro*. P2X7 3'UTR region has a highly conserved miR-150-binding motif and its direct interaction with miR-150 down-regulated P2X7 protein.	[107]
P2X7	miR-216b	Using bioinformatic analysis and 3'UTR luciferase reporter assay, it was found that P2X7 was targeted by miR-216b down-regulating P2X7 mRNA and protein levels. Down-regulation of miR-216b in breast cancer was inversely associated with P2X7 expression.	[108]
P2X7	miR-22	Analysis of RISC-loaded microRNAs using a high-throughput platform, as well as functional assays, suggested that P2X7 was a target of microRNA-22. Accordingly, its inhibition increased P2X7 expression and cytokine levels in the hippocampus.	[102]
P2X7	miR-21	Quantification of P2X7 mRNA and mature Let-7 g, miR-21, and miR-205 expression in 96 NSCLC patients using quantitative reverse transcription PCR. Up-regulation of miR-21 corresponded to low P2X7 expression and to a lower survival of non-small cell lung cancer (NSCLC) patients.	[86]
P2X7	miR-22, miR-125b, miR-146b and miR-155	miRNA transcription profile of control mice microglial cells and ALS microglia in resting conditions and upon P2X7 stimulation with the agonist BzATP. miR-22, miR-125b, miR-146b and miR-155 were upregulated upon stimulation. miR-365 and miR-125b interfered with transcription of IL-6 and STAT3 pathway increasing TNF-α transcription.	[90]
CD39	miR-155	miR-155 was up-regulated in patients with sepsis and in a murine model of sepsis. This was accompanied by a $CD39^{+}$ Treg lymphocyte increase. Their number decreased in mice transfected with a miR-155 inhibitor.	[96]
ADA2	miRNA-146b-3p	miRNA-146b-3p decreased ADA2 expression and *in vitro* TNF-alpha secretion.	[100]