

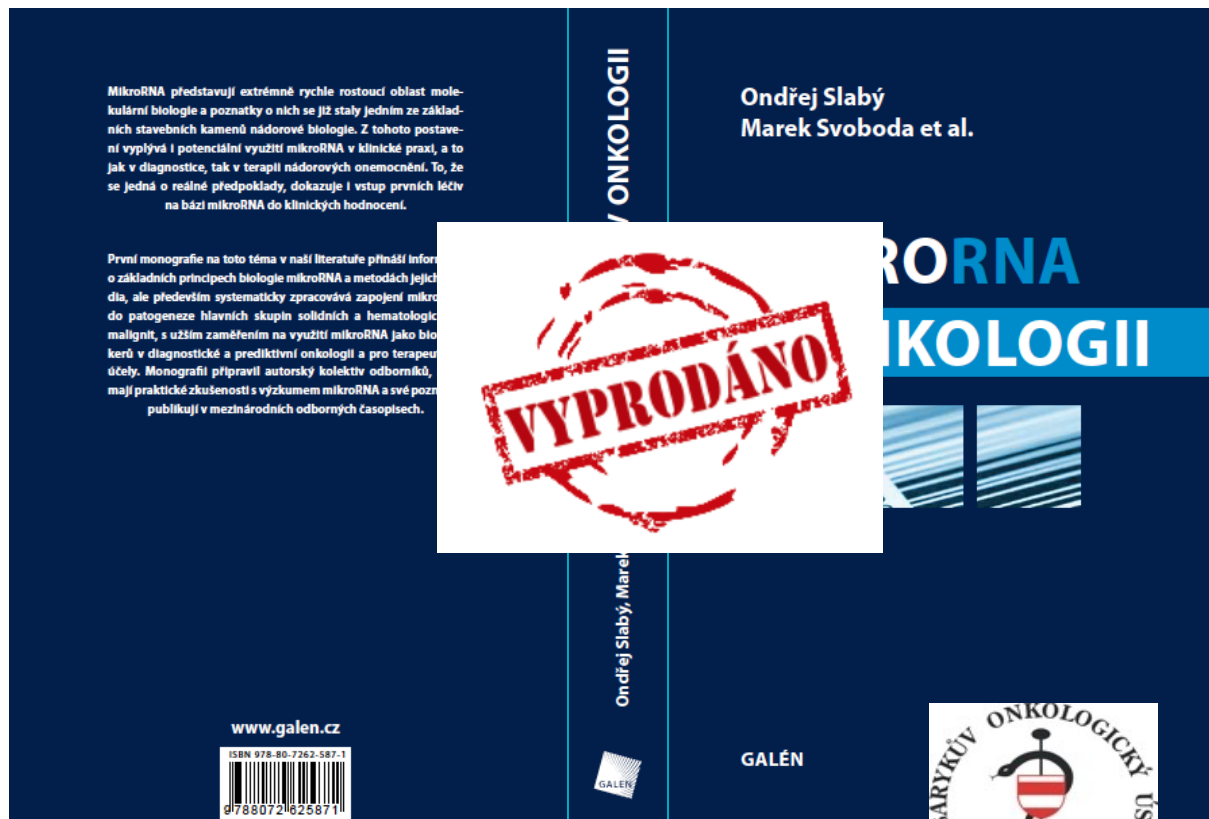
Nové trendy ve výzkumu mikroRNA

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Molecular Oncology II – Solid Cancer Laboratory



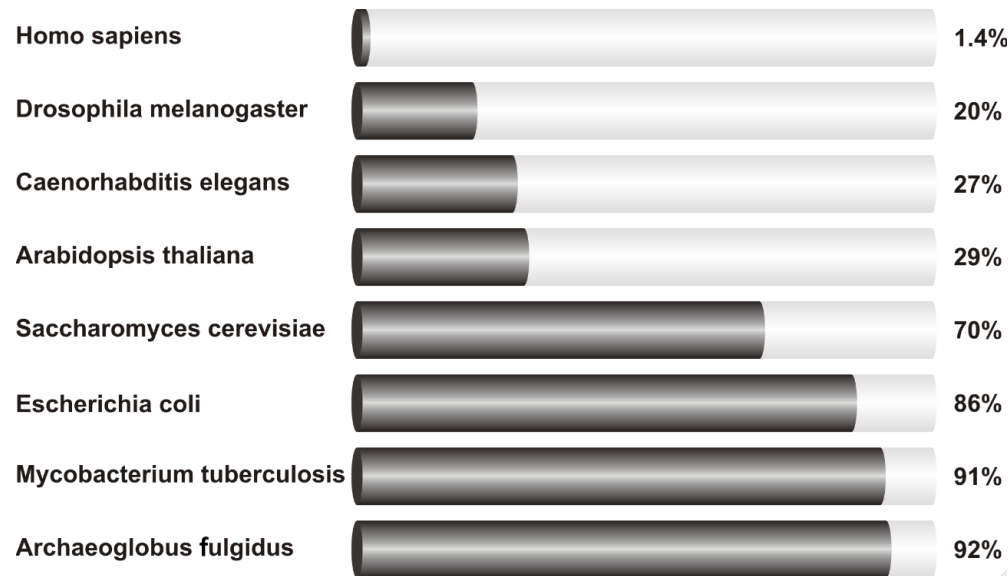
Svět nekódujících RNA

kódující geny představují méně než 2% celkové sekvence genomu

vs.

minimálně 90% lidského genomu je aktivně transkribováno

čím více je organizmus komplexní, tím více obsahuje nekódujících RNA



Recent evidence suggests that the non-coding RNAs (ncRNAs) may play major biological roles in cellular development, physiology and pathologies. NcRNAs could be grouped into two major classes based on the transcript size: small ncRNAs and long ncRNAs.



REVIEW

Open Access

Novel classes of non-coding RNAs and cancer

Jiri Sana^{1,2}, Petra Faltejskova^{1,2}, Marek Svoboda¹ and Ondrej Slaby^{1,2,3*}

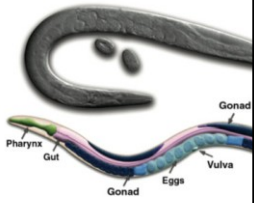
Nové třídy nekódujících RNA

Table 1: Types of recently discovered human non-coding RNAs.

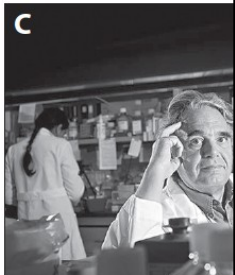
	Class	Symbol	Characteristic	Disease / biological function associations
Small	MicroRNAs	miRNAs	18-25 nt; account 1-2% of the human genome; control the 50% of protein-coding genes; guide suppression of translation; Drosha and Dicer dependent small ncRNAs	Initiation of various disorders including many, if not all, cancers / regulation of proliferation, differentiation, and apoptosis; involved in human development
	all interfering RNAs	siRNAs	19-23 nt; made by Dicer processing; guide sequence specific degradation of target mRNA;	great potential in diseases treatment / posttranscriptional gene silencing mainly through RISC degradation mechanism; defence against pathogenic nucleic acids
	si-interacting RNAs	piRNAs	26-30 nt; bind Piwi proteins; Dicer independent; exist in genome clusters; principally restricted to the germline and somatic cells bordering the germline	relationship between piRNAs and diseases has not yet been discovered / involved in germ cell development, stem self-renewal, and retrotransposon silencing
	sall nucleolar RNAs	snoRNAs	60-300 nt; enriched in the nucleolus; in vertebrate are excised from pre-mRNA introns; bind snoRNP proteins	association with development of some cancers / important function in the maturation of other non-coding RNAs, above all, rRNAs and srRNAs; miRNA-like snoRNAs regulate mRNAs
	oter-associated small RNAs	PASRs	20-200 nt; modified 5' (capped) ends; coincide with the transcriptional start sites of protein- and non-coding genes; made from transcription of short capped transcripts	relationship with diseases has not yet been discovered / involved in the regulation of the transcription of protein-coding genes by targeting epigenetic silencing complexes
	Transcription Initiation RNAs	tRNAs	- 18 nt; have the highest density just downstream of transcriptional start sites; show patterns of positional conservation; preferentially located in GC-rich promoters	
	Centromere repeat associated small interfering RNAs	crasiRNAs	34-42 nt; processed from long dsRNAs;	relationship between crasiRNAs and diseases has not yet been discovered / involved in the recruitment of heterochromatin and/or centromeric proteins
	Telomere-specific small RNAs	tel-sRNAs	- 24 nt; Dicer independent; 2'-O-methylated at the 3' terminus; evolutionarily conserved from protozoa to mammals; have not been described in human up to now	relationship between tel-sRNAs and diseases has not yet been discovered / epigenetic regulation
	Pyknonas		subset of patterns of variable length; form mosaics in untranslated and protein-coding regions; more frequently in 3' UTR	expected association with cancer biology / possible link with posttranscriptional silencing of genes, mainly involved in cell communication, regulation of transcription, signaling, transport, etc.
	Long non-coding RNAs	Long intergenic noncoding RNAs	lincRNAs	ranging from several hundreds to tens of thousands nts; lie within the genomic intervals between two genes; transcriptional cis-regulation of neighbouring genes
Long intronic noncoding RNAs			lie within the introns; evolutionary conserved; tissue and subcellular expression specified	aberrantly expressed in human cancers / possible link with posttranscriptional gene silencing
Telomere-associated noRNAs		TERRAs	100 bp - >9 kb; conserved among eukaryotes; synthesized from C-rich strand; polyadenylated; form intermolecular G-quadruplex structure with single-stranded telomeric DNA	possible impact on telomere-associated diseases including many cancers / neovative regulation of telomere length and activity through inhibition of telomerase
Long noRNAs with dual functions			both protein-coding and functionally regulatory RNA capacity	deregulation has been described in breast and ovarian tumors / modulate gene expression through diverse mechanisms
Pseudogene RNAs			code for a protein; potential to regulate their protein-coding cousin; made through retrotrans-position; tissue specific	and cancer progression / regulation of tumor suppressors and oncogenes by acting as microRNA decoys
	Transcribed-ultraconserved regions	T-UCRs	longer than 200 bp; absolutely conserved between orthologous regions of human, rat, and mouse; located in both intra- and intergenic regions	expression is often altered in some cancers; possible involvement in tumorigenesis / antisense inhibitors for protein-coding genes or other ncRNAs

HISTORIE

1993 Ambros, Ruvkun – objev miRNA lin-4



Cell, 1993

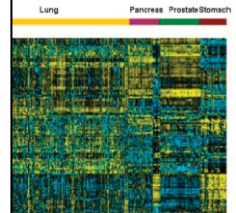


1998 Fire, Mello – popis RNA interference

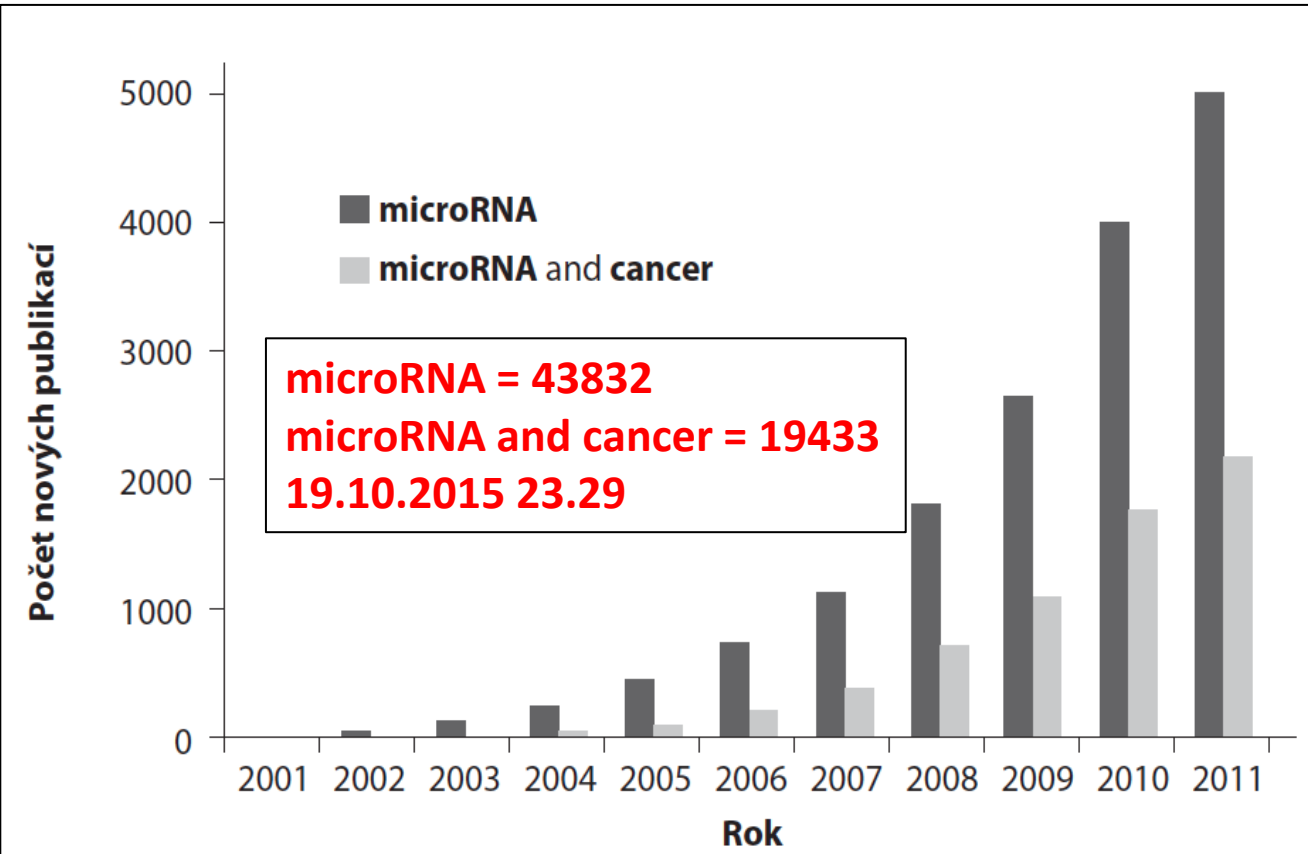


... winners
... Craig Mello for
... of the RNAi

Nature, 1998



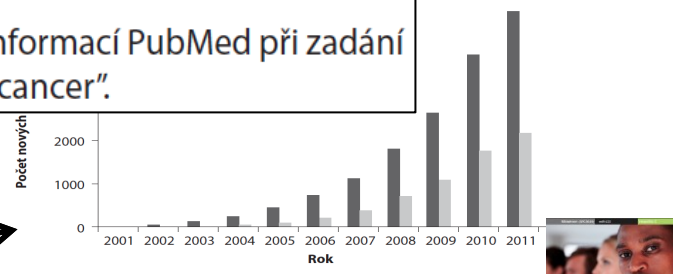
PNAS, 2004



microRNA = 43832
microRNA and cancer = 19433
19.10.2015 23.29

Obr. 1.2. Počet nových publikací v databázi medicínských informací PubMed při zadání klíčového slova „microRNA“ nebo kombinace „microRNA“ a „cancer“.

2007 První originální práce na téma miRNA v onkologii v ČR 😊



Obr. 1.2. Počet nových publikací v databázi medicínských informací PubMed při zadání klíčového slova „microRNA“ nebo kombinace „microRNA“ a „cancer“.





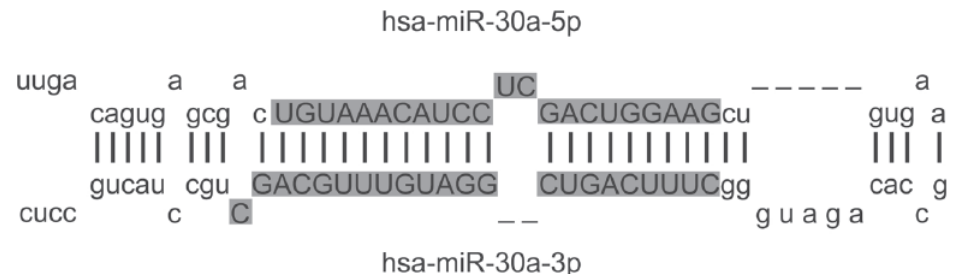
Victor Ambros
2012, CSHL

Definice mikroRNA

MikroRNA je definována jako krátká, 21–23 nukleotidů dlouhá jednořetězcová RNA, která vzniká z dlouhého primárního transkriptu (pri-miRNA) a vlásenkové prekurzorové struktury (pre-miRNA) účinkem ribonukleáz v jádře a cytoplazmě a jejíž hlavní funkcí je post-transkripční regulace genové exprese.

Kritéria:

- 1) krátká RNA musí být jednoznačně detekovatelná pomocí Northern blotu, RT-PCR nebo jiné standardní metody umožňující detekci RNA,
- 2) krátká RNA se musí vyskytovat v kmenové části vlásenkové, asi 70 nukleotidů dlouhé prekurzorové struktury,
- 3) sekvence krátké RNA a jejího prekurzoru musí být fylogeneticky konzervovaná (s výjimkou druhově specifických miRNA),
- 4) inhibice klíčových ribonukleáz v biogenezi miRNA musí vést k poklesu hladin krátké RNA a k akumulaci její prekurzorové struktury.



Obr. 1.5. Ukázka prekurzorové vlásenkové struktury mikroRNA.

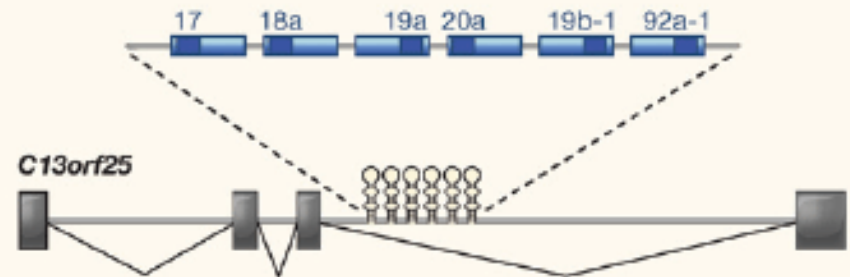
Základní fakta o mikroRNA

- Přibližně 3% predikovaných lidských genů jsou geny pro miRNA
- Jsou fylogeneticky vysoce konzervované
- Geny pro miRNA jsou rozmístěny na všech lidských chromozomech s výjimkou Y
- V databázi miRBase je anotováno 1390 mikroRNA* (1000 -2000 predikovaných)
- Maturovaná miRNA je dlouhá 20-25 nukleotidů
- MiRNA mají potenciál regulovat více než polovinu lidských kódujících genů
- Jedna miRNA může regulovat desítky až stovky cílových mRNA
- miRNA často tvoří rodiny nebo jsou kódovány v klastrech (eg. cluster miR-17)

miRNA family members can be very similar eg let-7 family:

```
mmu-let-7a:  UGAGGUAGUAGGUUGUAUAGUU
mmu-let-7b:  UGAGGUAGUAGGUUGUGUGGUU
mmu-let-7c:  UGAGGUAGUAGGUUGUAUGGUU
mmu-let-7d:  AGAGGUAGUAGGUUGCAUAGUU
mmu-let-7e:  UGAGGUAGGAGGUUGUAUAGUU
mmu-let-7f:  UGAGGUAGUAGAUUGUAUAGUU
mmu-let-7g:  UGAGGUAGUAGUUUGUACAGUU
mmu-let-7i:  UGAGGUAGUAGUUUGUCUGUU
```

miR-17-92
(13q31.3)

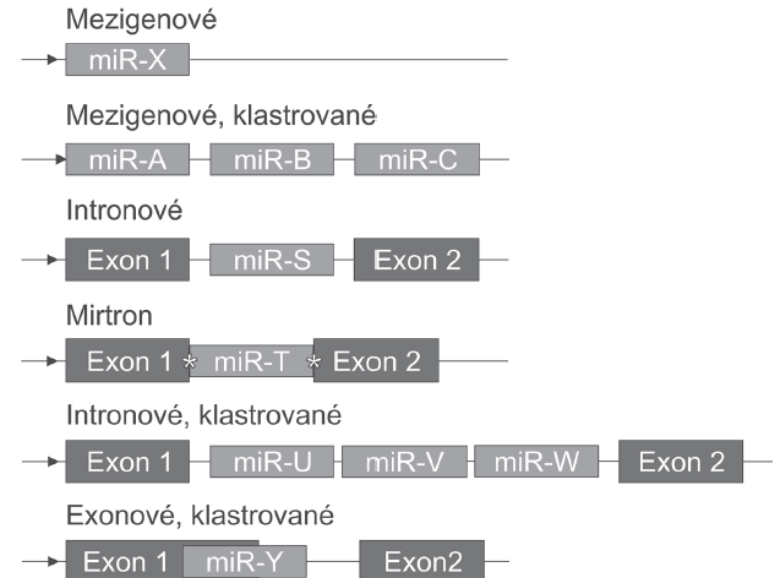


Struktura a distribuce genů pro mikroRNA v lidském genomu

- Geny pro miRNA jsou rozmístěny na všech lidských chromozomech s výjimkou chromozomu Y.
- Přibližně 50 % miRNA se nachází v podobě klastrů (u člověka 55 klastrů), které jsou přepisovány jako polycistronní primární transkripty.

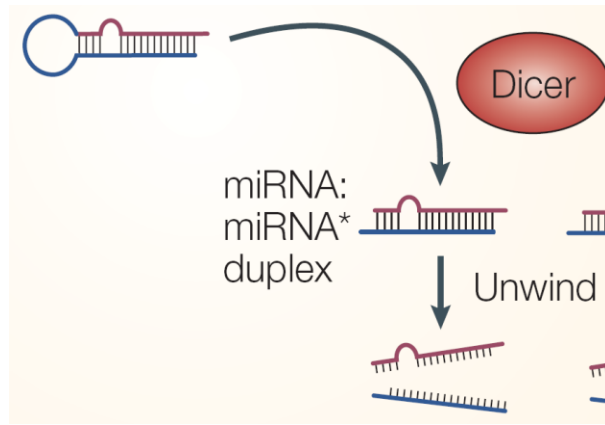
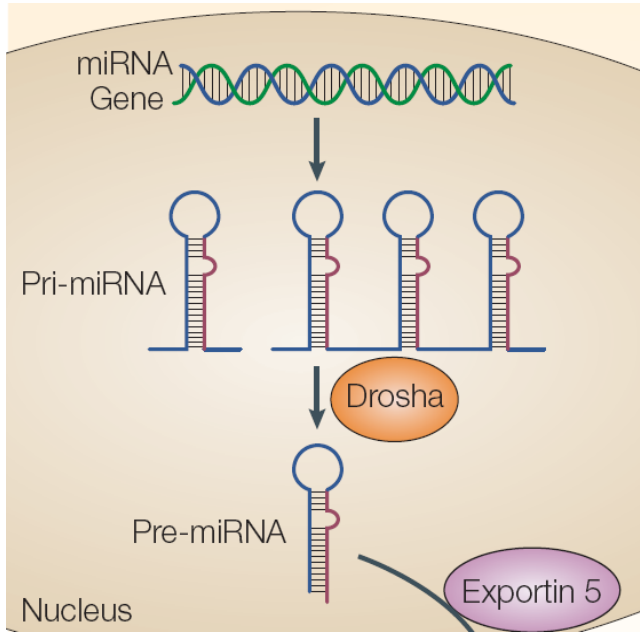
Dle vztahu ke známým a definovaným transkripčním jednotkám dělíme na

- Mezigénové miRNA (přibližně 50 %)
- Intronové miRNA (přibližně 40 %)
- miRtrony
- Exonové miRNA



Obr. 1.3. Genomová lokalizace genů pro mikroRNA.

Biogeneze a funkce mikroRNA

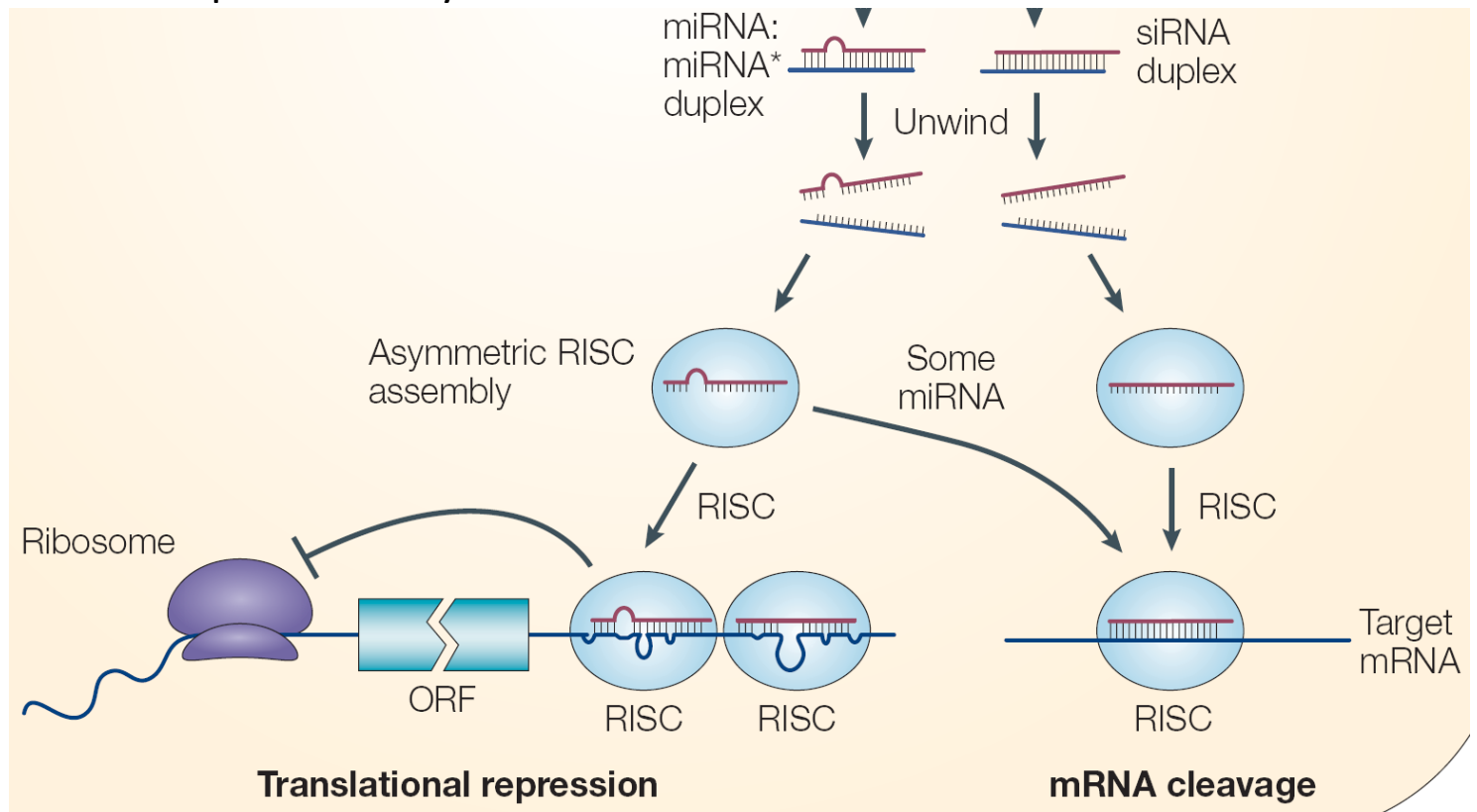


1. Transkripce miRNA genu
2. pri-miRNA jsou zpracovány pomocí RNáz Drosha a Pasha
3. pre-miRNA exportovány pomocí Exportinu 5 do cytoplazmy
4. Zpracování pomocí RNázy Dicer

microRNA: licensed to kill messenger

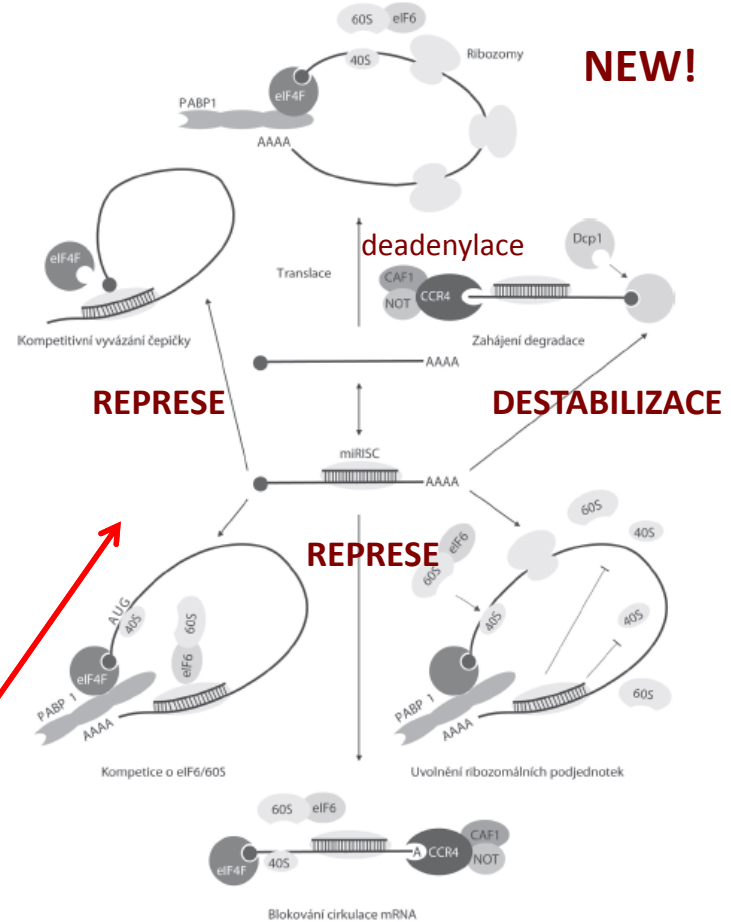
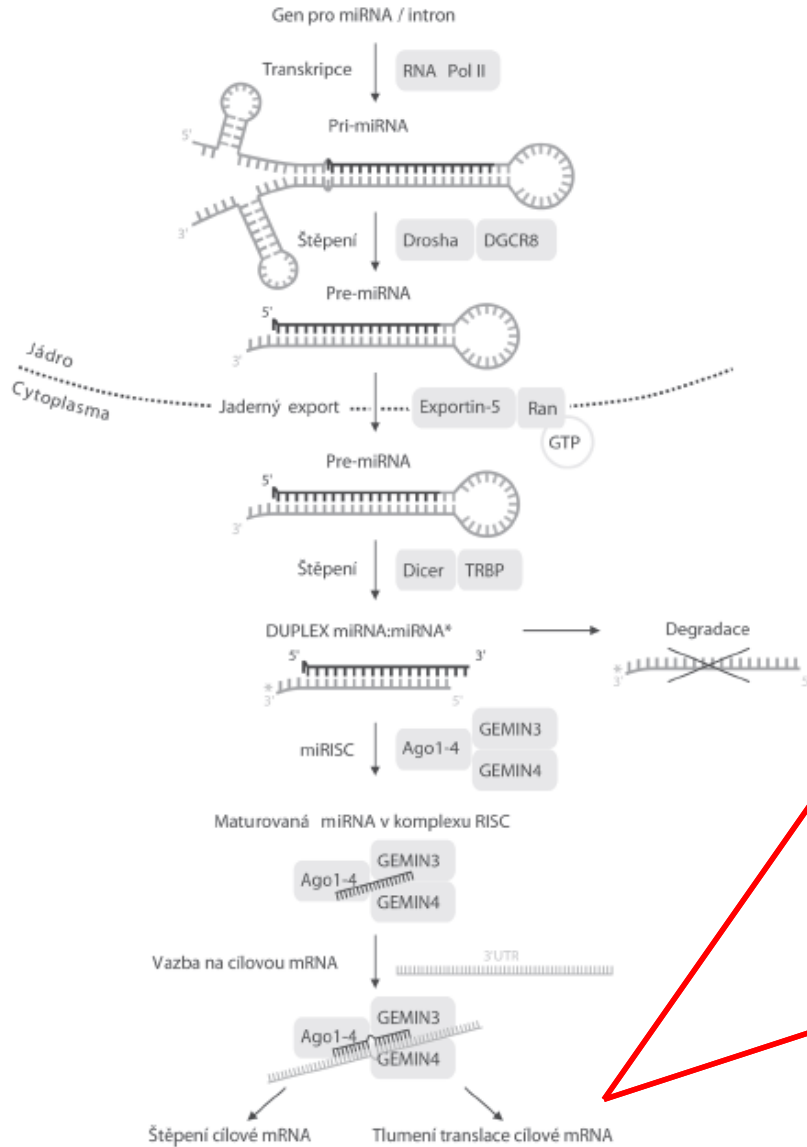
5. Aktivní vlákno je inkorporováno do komplexu RISC
6. Represe translace nebo degradace mRNA v závislosti na míře komplementarity

Biogeneze a funkce mikroRNA



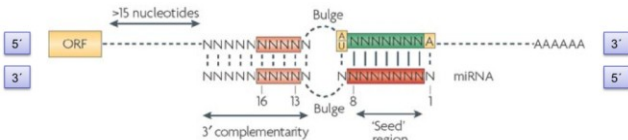
Kanonický model biogeneze a funkce mikroRNA

NEW!



DESTABILIZACE mRNA!!!

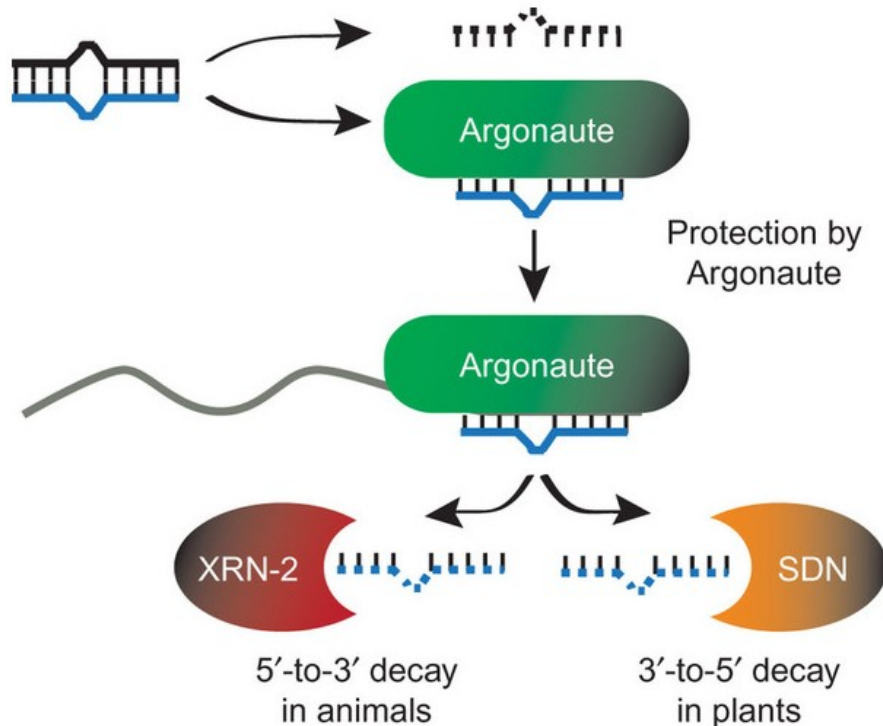
Pokles hladiny proteinových produktů cílových genů je ve více než 80% způsoben poklesem hladin mRNA v důsledku jejich destabilizace a nikoliv translační represí. (využití RPF)



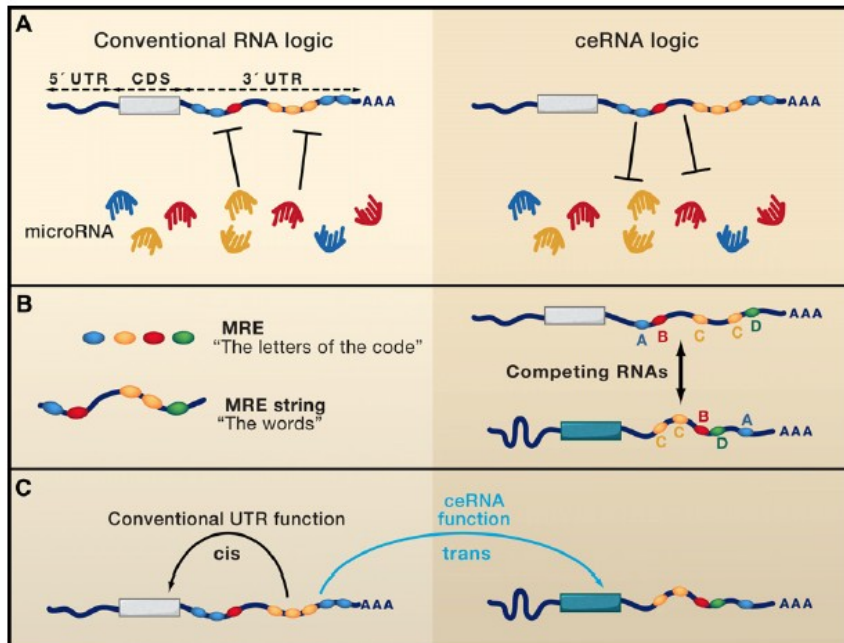
Biologický poločas a rozpad mikroRNA

MiRNA obecně představují vysoce stabilní molekuly, poločas miRNA v buněčných liniích nebo orgánech dosahuje mnoho hodin nebo dokonce i dnů!

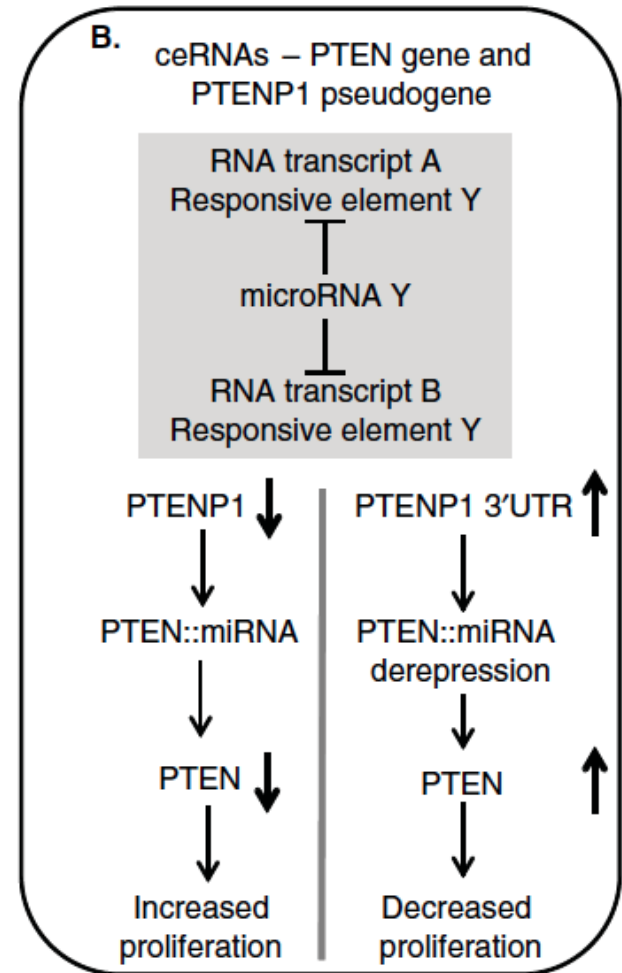
MiRNA mohou být regulované například blokováním jejich vazebných míst na mRNA pomocí RNA-vazebných proteinů. Volné miRNA jsou méně stabilní



Opačné vnímání regulačního mechanismu miRNA → mRNA, kdy tzv. „competing endogenous RNA“ (ceRNA) regulují hladinu daného transkriptu tak, že soutěží o vazbu miRNA, která má schopnost daný transkript post-transkripčně regulovat. Všechny transkripty, které mají vazebná místa pro jednu miRNA, a tak možnost soutěžit její vazbu a vzájemně ko-regulovat svou expresi, potom vytvářejí komplexní síť (ceRNA network).



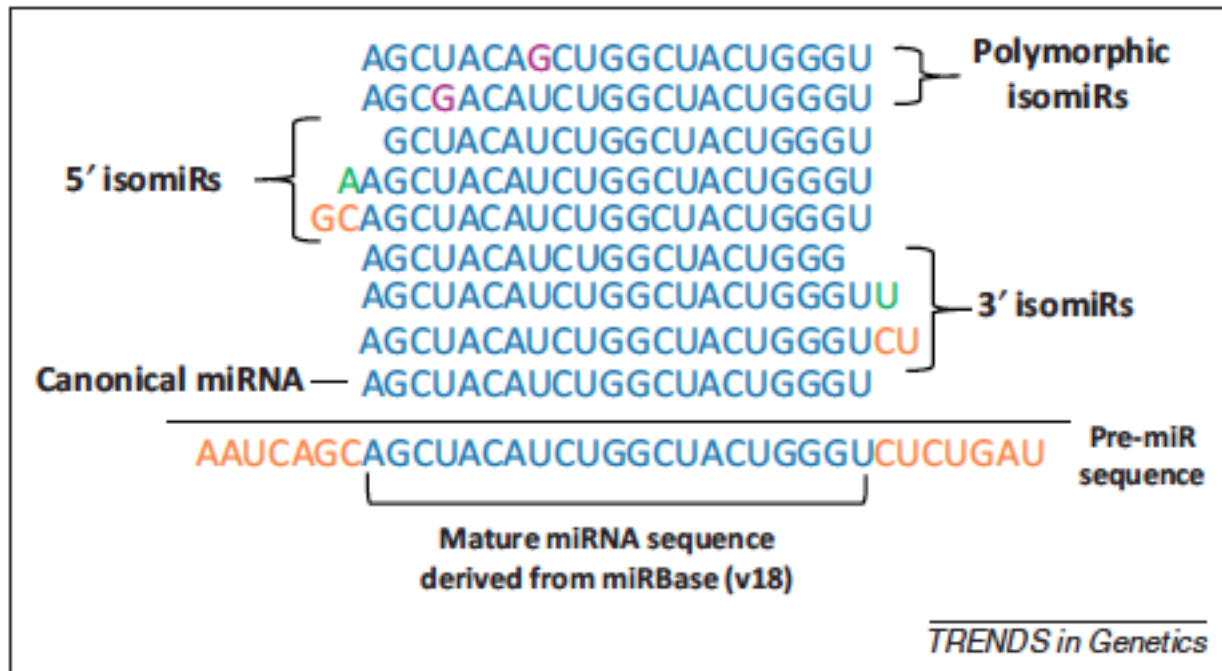
Salmena, Pandolfi, Cell, 2011



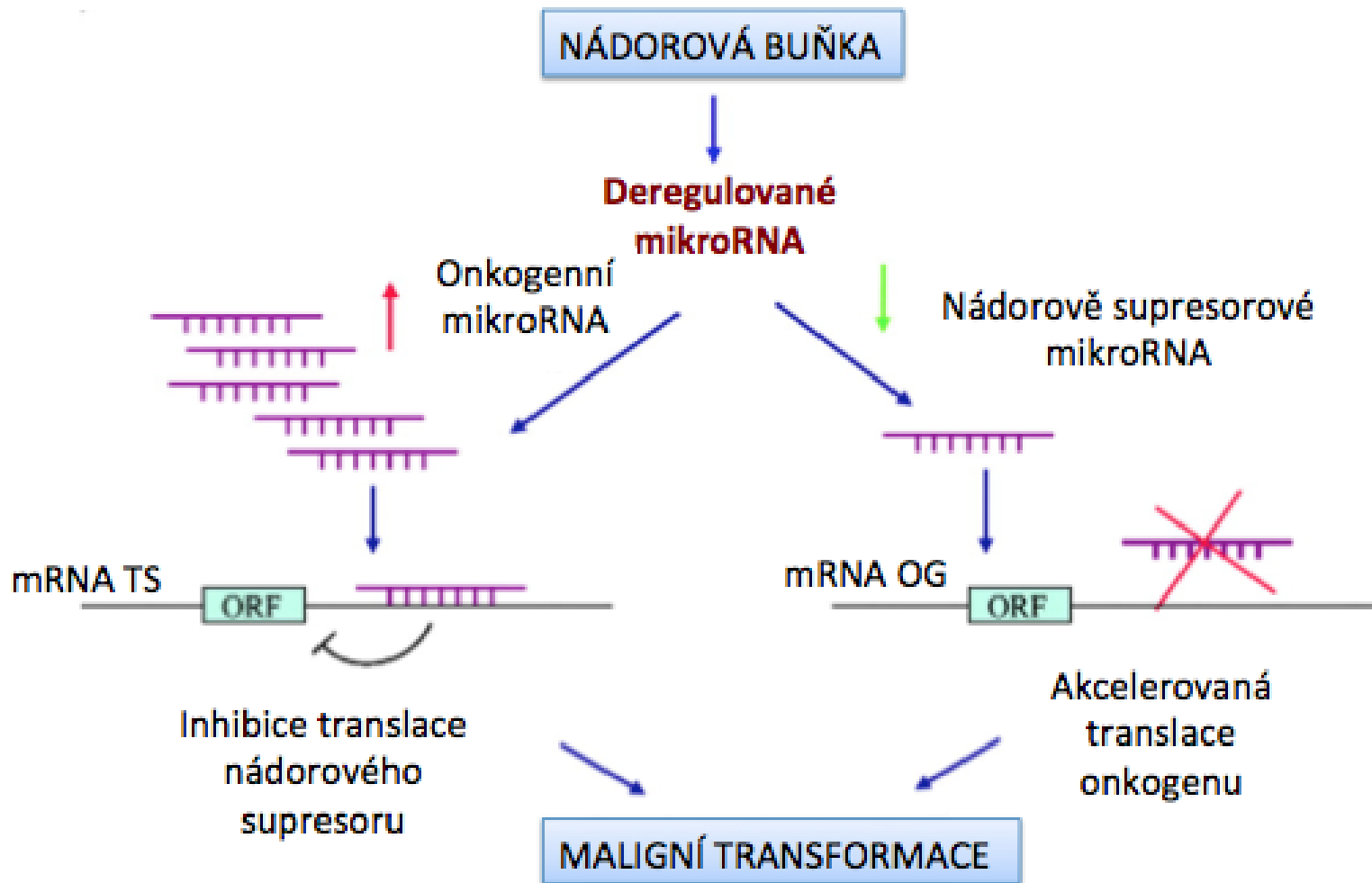
Alemeida, Calin, Expert Opin. Biol. Ther., 2012

- Poprvé popsány v roce 2008 na základě výsledků sekvenačních studií (Morin et al., 2008, Genome Res)
- Jedna oblast kódující miRNA může vést ke vzniku mnoha rozdílných izomiRs.
- Zastoupení jednotlivých izomiRs je tkáňově specifické a může se měnit v rámci odpovědi na různé biologické signály.
- Vznik 5' a 3' izomiRs je podmíněn štěpením Droshou a Dicerem nebo účinkem transferáz.
- Polymorfní izomiRs mohou vznikat v důsledku SNP (vzácné) nebo editací mikroRNA (A-I).

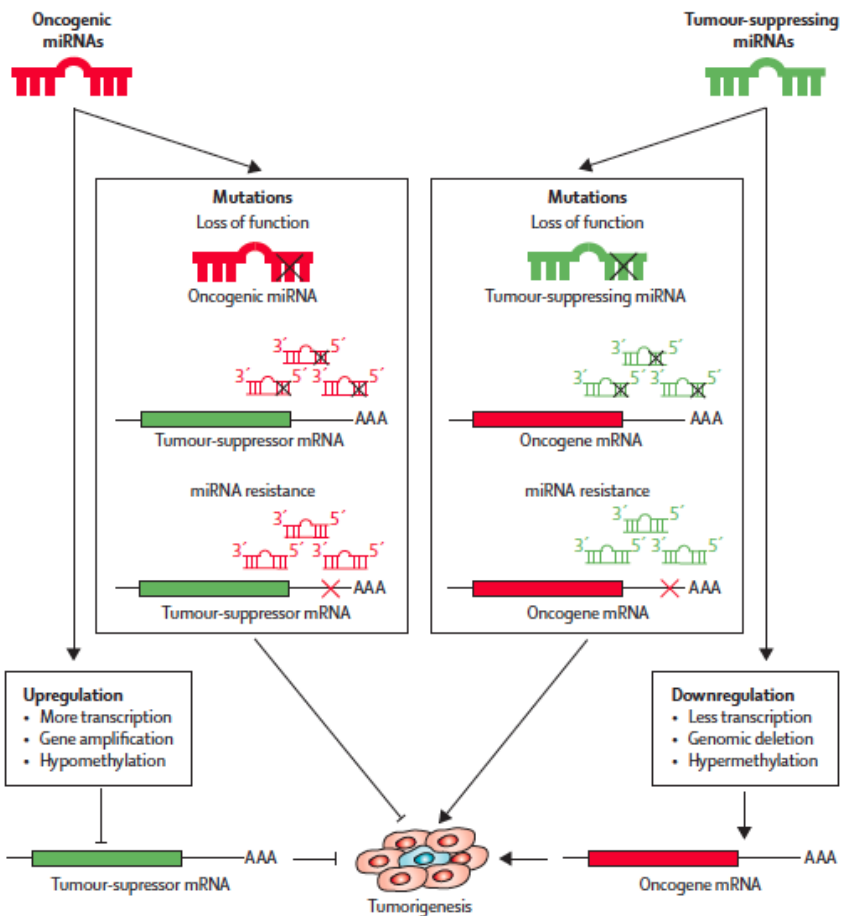
Funkční dopady: specificita pro cílové mRNA, Ago loading, biologický poločas



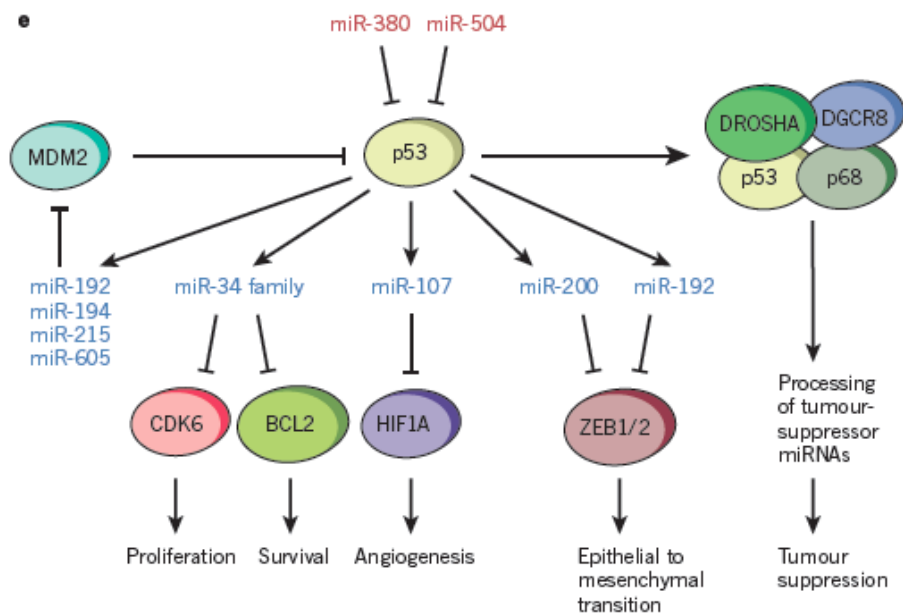
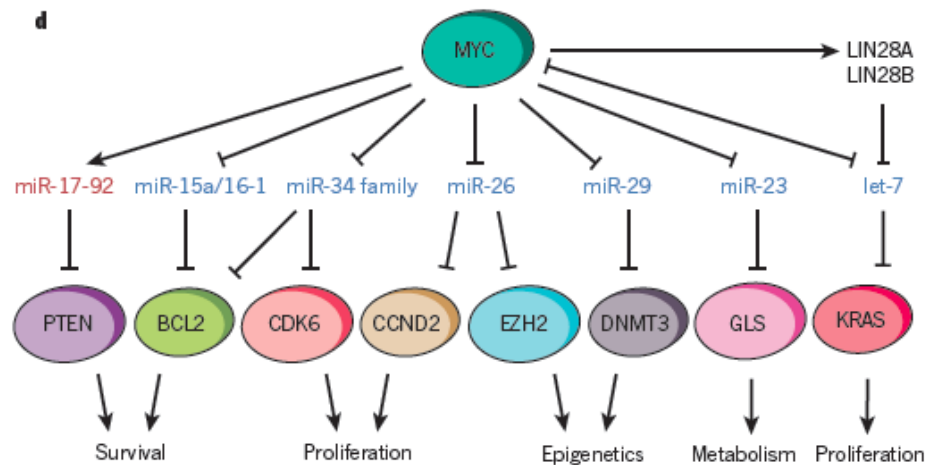
MikroRNA jako onkogeny nebo nádorové supresory



MikroRNA jako onkogeny nebo nádorové supresory

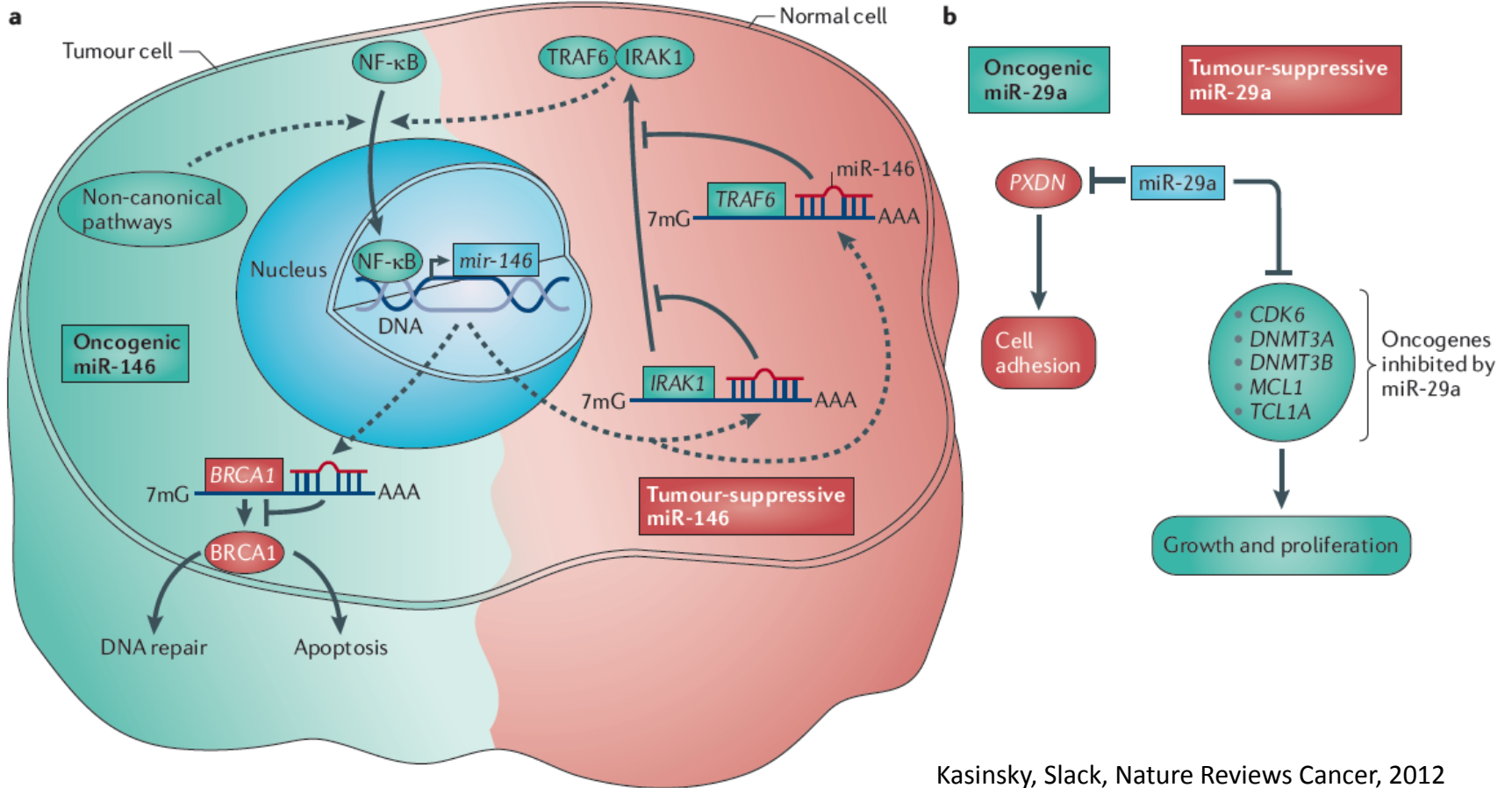


Kong et al, Lancet Oncology 2012



Lujambio, Nature 2012

MikroRNA jako onkogeny nebo TS v závislosti na kontextu



MikroRNA v patogenezi nádorových onemocnění

MikroRNA jsou zapojeny do regulace všech šesti klasických a čtyř nových znaků maligního nádoru dle Weinberga a Hanahana. (Hallmarks of Cancer: The Next Generation, Cell, 2011)

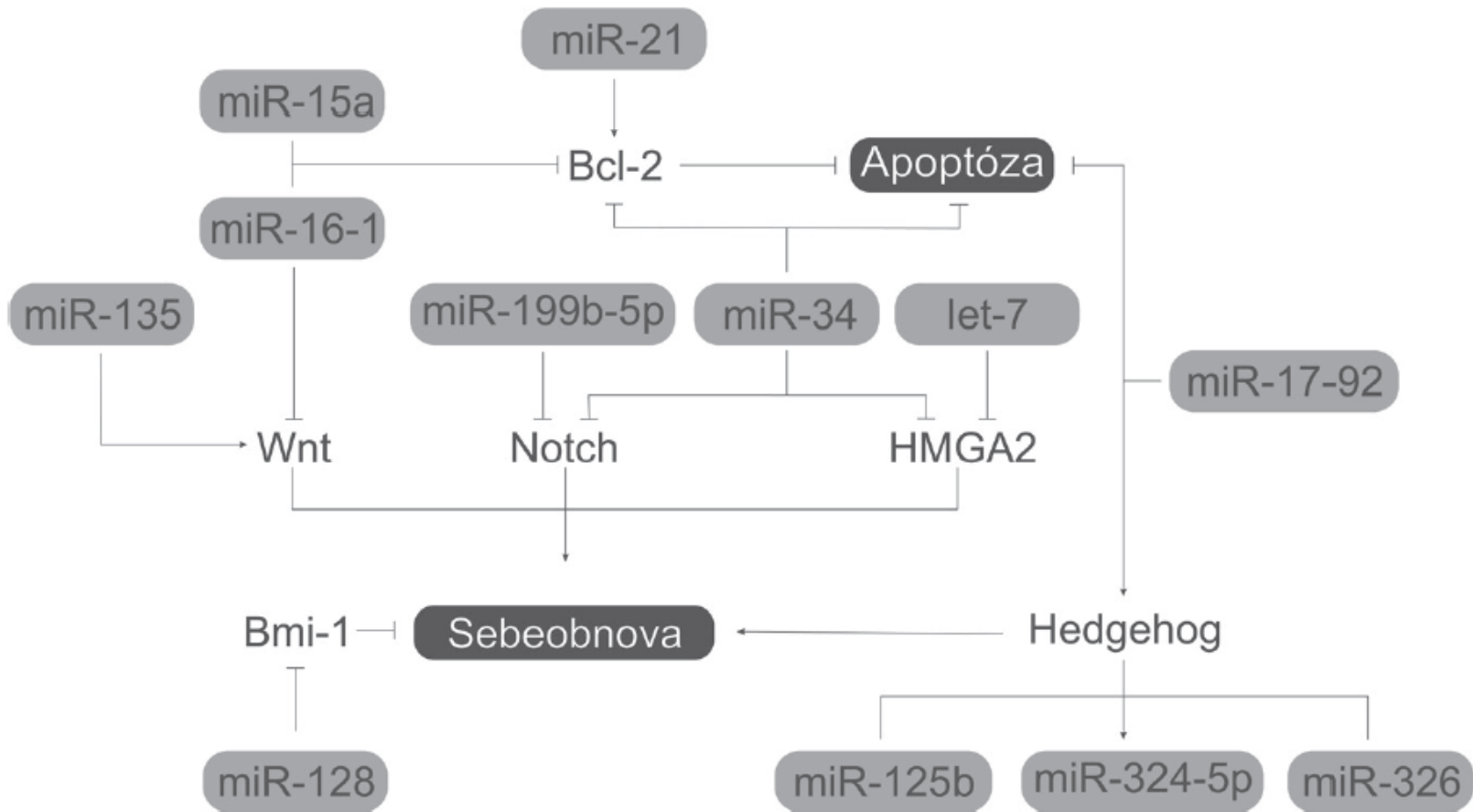
Znaky maligní transformace	MikroRNA
soběstačnost v produkci růstových signálů	let-7, miR-7, miR-21, miR-34b/c, miR-125a/b, miR-126, miR-128, miR-143, miR-145, miR-199, miR-331
necitlivost k signálům zastavujícím buněčný cyklus	klastr miR-17-92 a miR-106b-25, miR-34a, miR-221, miR-222
poškozená apoptóza	miR-15, miR-16, miR-21, miR-29, rodina miR-34, miR-125b, miR-133, miR-145, miR-221, miR-222, miR-605
neomezený replikační potenciál	rodina miR-34, miR-138, miR-290, miR-372, miR-373
angiogeneze	miR-23, miR-24, miR-26, miR-27, miR-103, miR-107, miR-126, miR-181, miR-210, miR-213, miR-221, miR-222, miR-296
invazivita a metastazování	rodina miR-9, miR-10b, miR-21, miR-31, miR-122, miR-146a, miR-148a/b, miR-155, rodina miR-200, miR-210, miR-373, miR-520c
narušený energetický metabolismus	miR-23a/b, miR-122, miR-375
únik imunitnímu systému	miR-17-5p, miR-20a, miR-93, miR-106b, miR-372, miR-373 a miR-520c, miR-155, hcmv-miR-UL112
genomová nestabilita	klastr miR-17-92, miR-15, miR-16, let-7
nádorový zánět	miR-9, klastr miR-17-92, miR-21, miR-101, miR-146a, miR-192

MikroRNA v patogenezi nádorových onemocnění

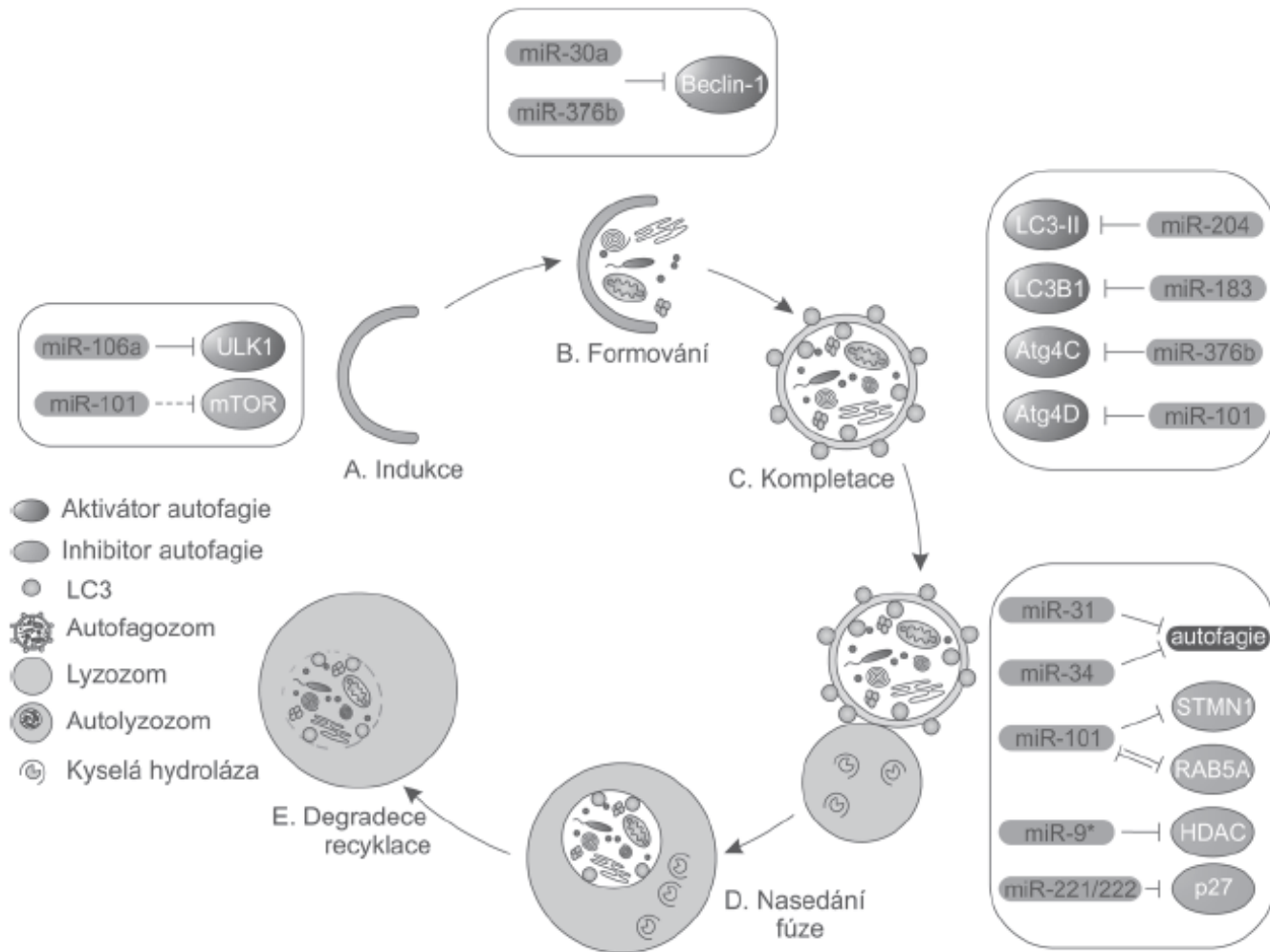
NEW!

MikroRNA jsou zapojeny do regulace kmenové vlastnosti buněk.

ch signálních drah regulujících



MikroRNA se podílejí na regulaci různých procesů autofagie, jako jsou signalizace mTOR, skládání komplexu ULK1, interakce proteinu Beclin-1 nebo ATG4 signalizace.



Milestones of circulating nucleic acid biomarkers

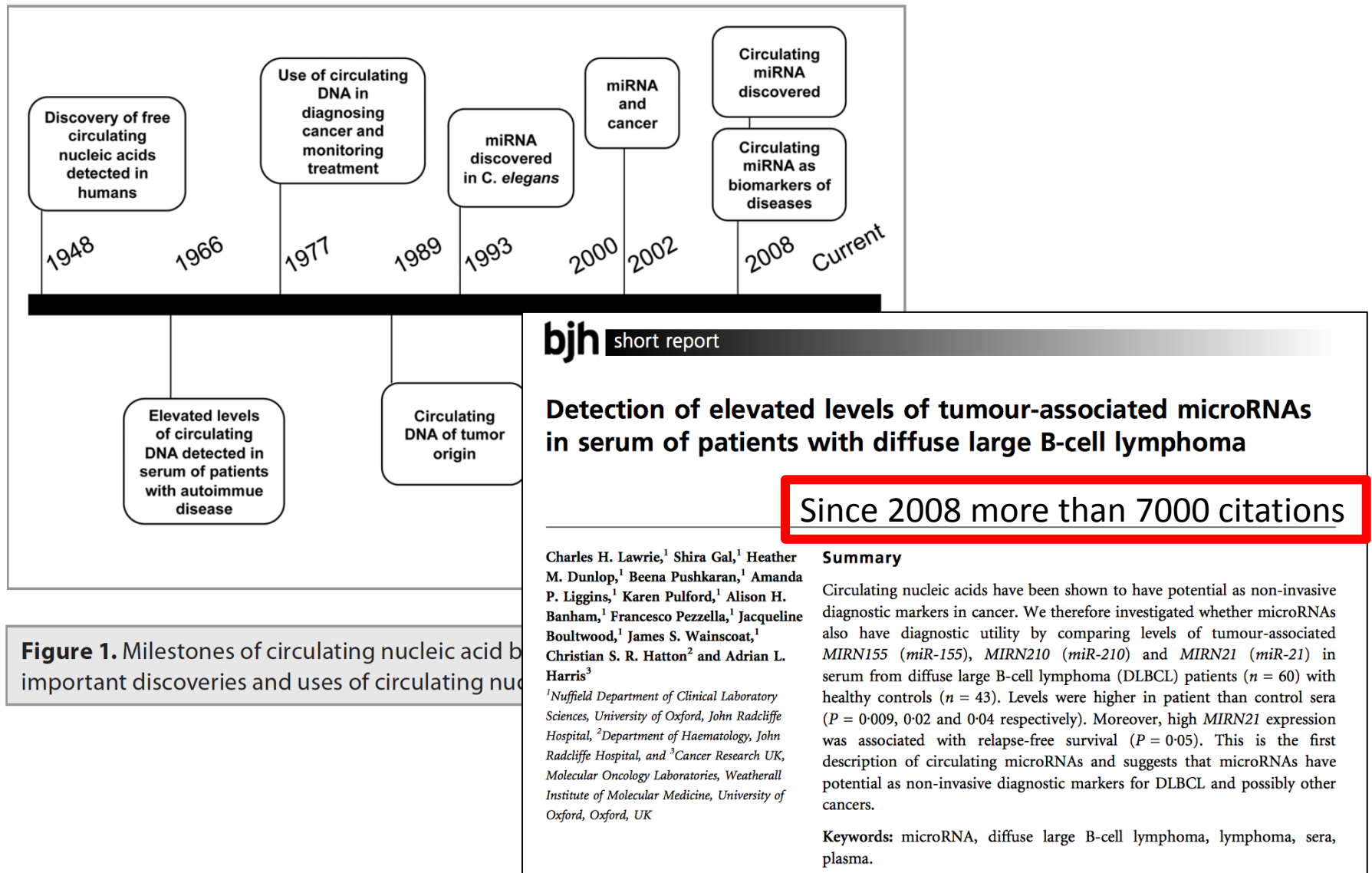
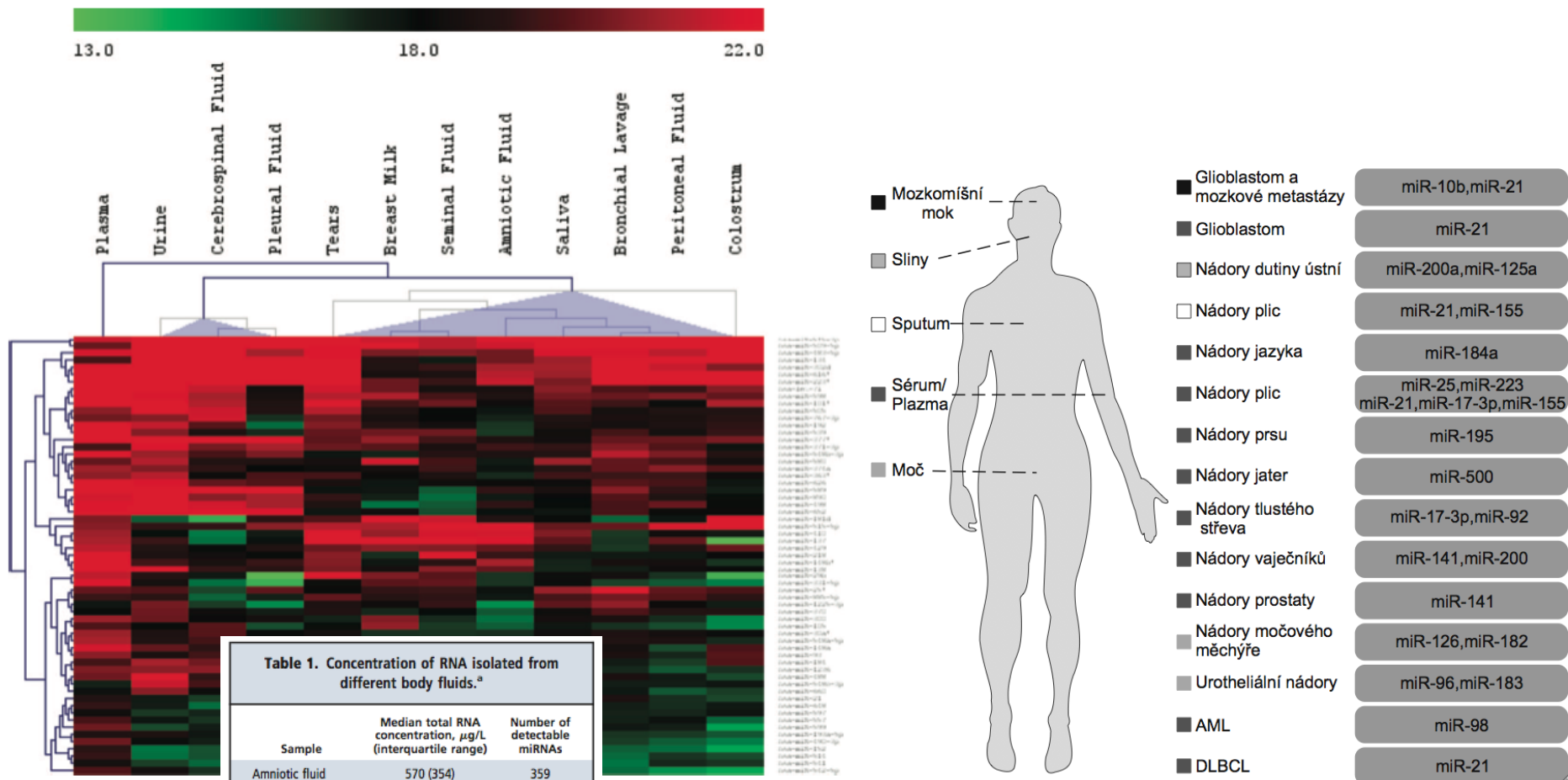


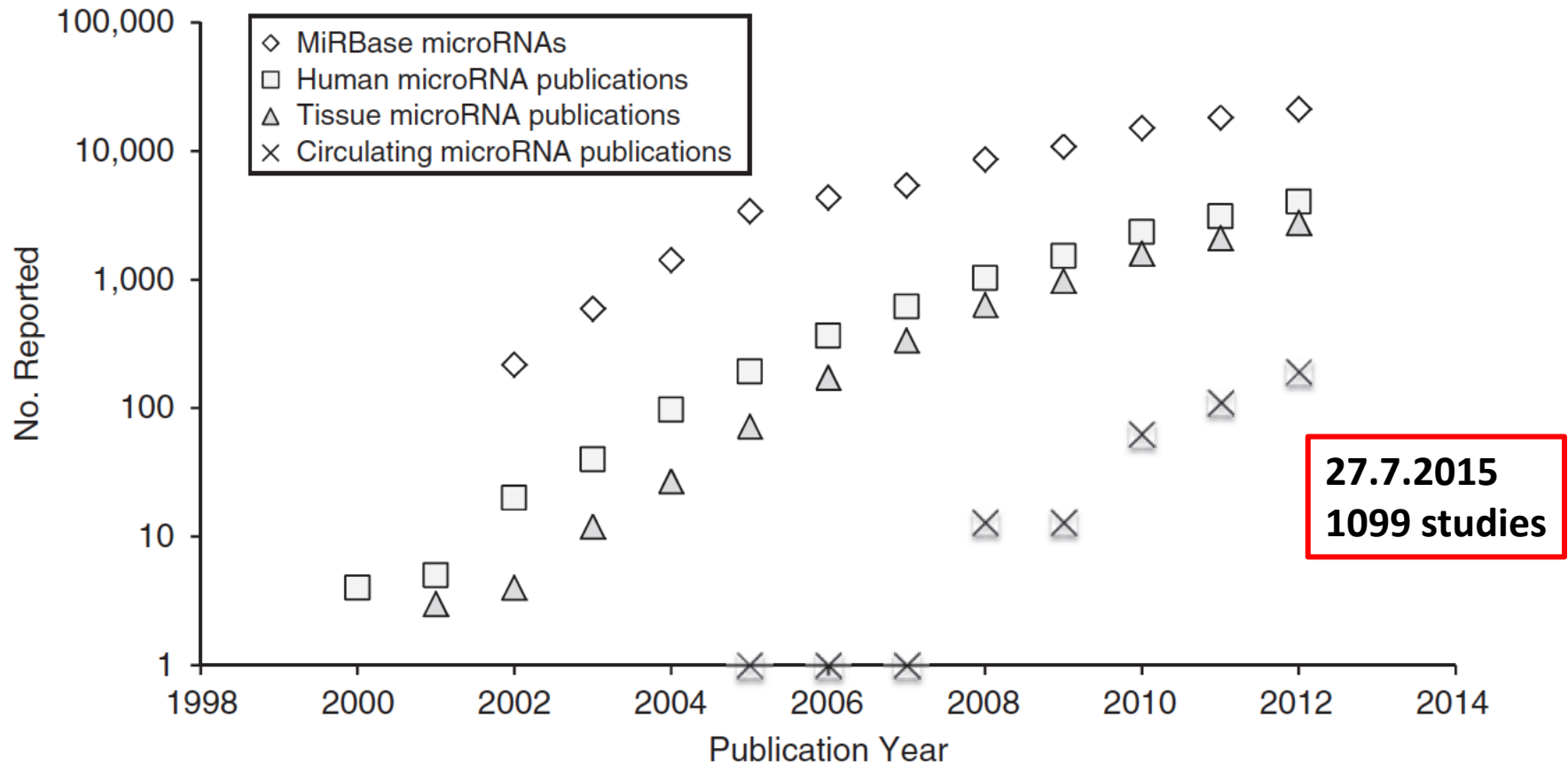
Figure 1. Milestones of circulating nucleic acid biomarkers: important discoveries and uses of circulating nucleic acids

Cirkulující mikroRNA jsou přítomny ve dvanácti typech tělních tekutin

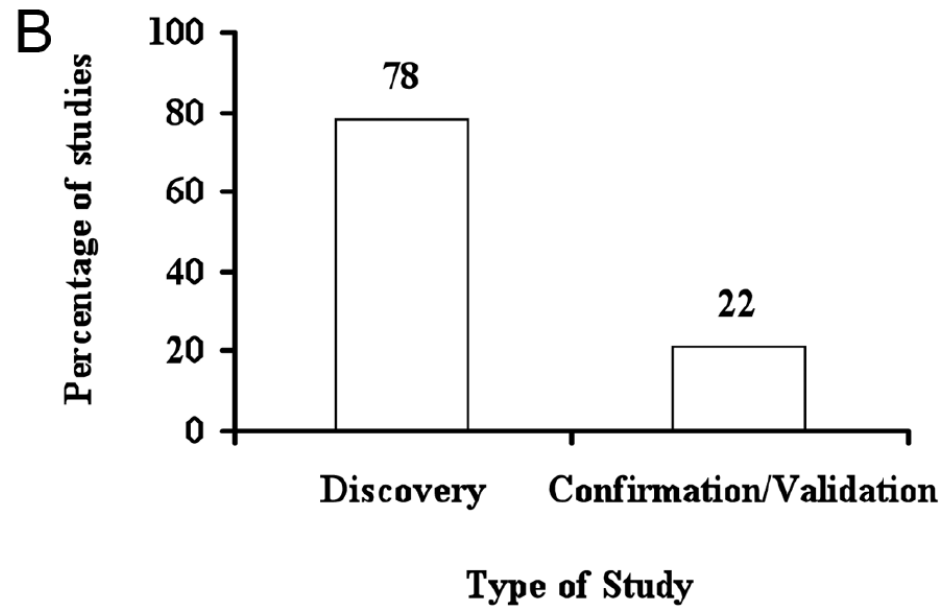
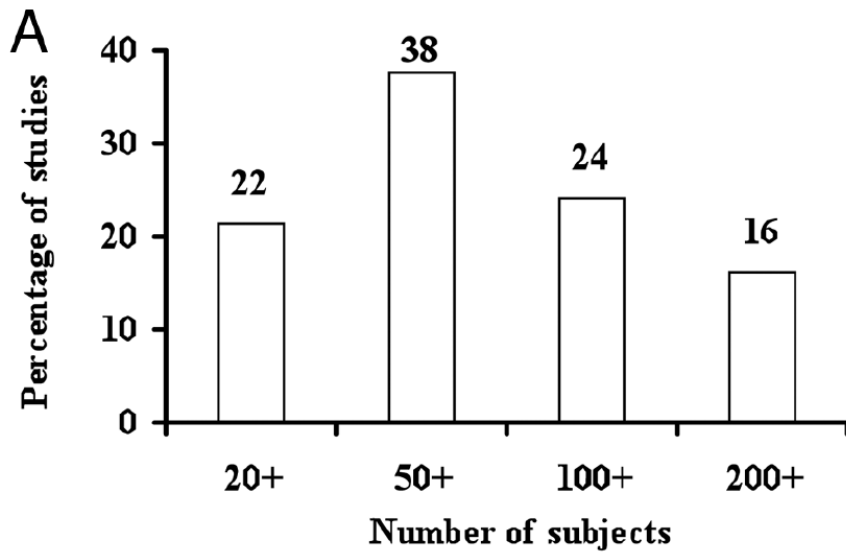


Weber et al., Clin Chem, 2010

Number of publications focusing tissue/circulating microRNAs



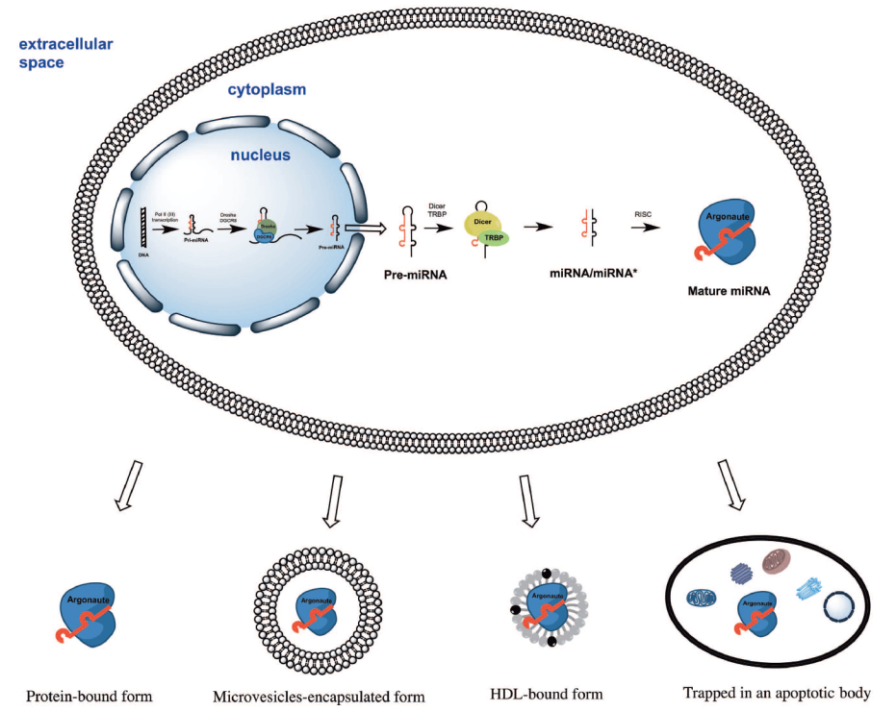
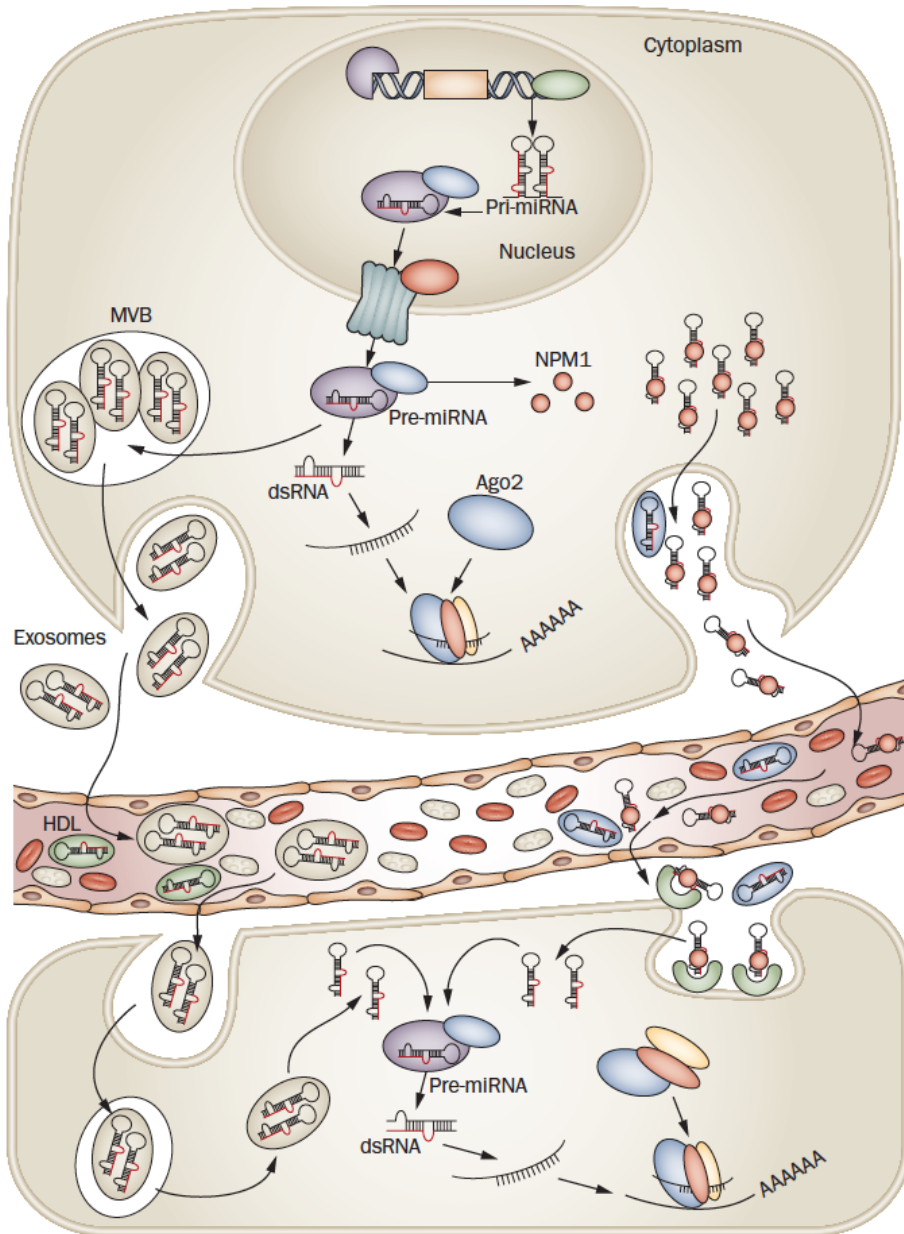
Survey of circulating microRNA study design (Jan 2012)



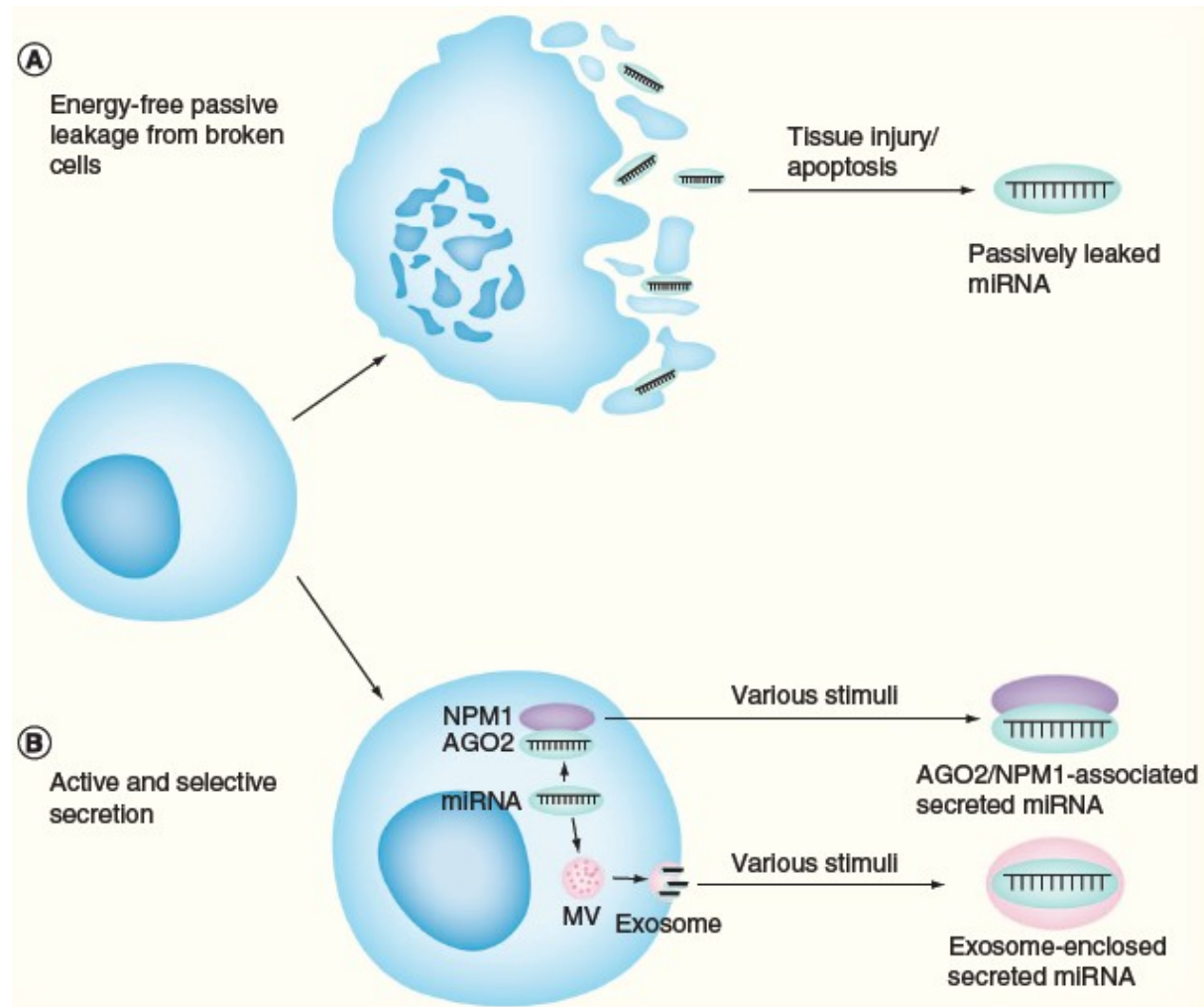
**MAJORITY OF THE STUDIES SUFFER WITH
LOW NUMBER OF SUBJECTS!
NO INDEPENDENT VALIDATION!**

Weiland et al, RNA Biology 2012

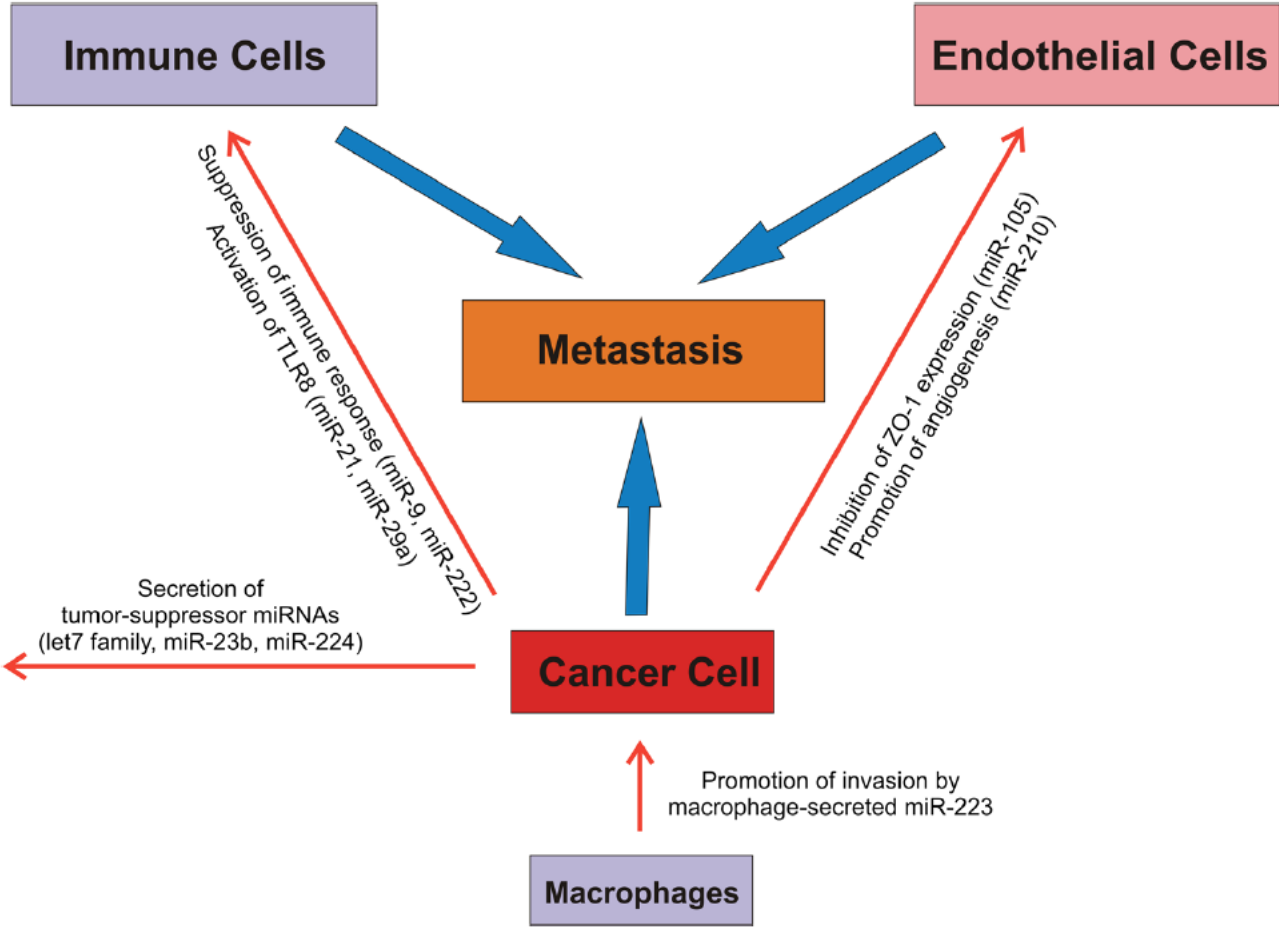
Biogenesis and mechanism of action of circulating microRNAs



Biogenesis and mechanism of action of circulating microRNAs



Tumor cell-derived exosomes to recipient cells



Reports to demonstrate cell-cell transfer of extracellular miRNAs

Donor cells	Acceptor cells	miRNA	Impact	References
MC/9, HMC-1	MC/9 and HMC-1	Multiple miRNAs	Not investigated	Valadi et al., 2007
THP-1	HMEC-1 cells	Overexpressed miR-150	Reduction of miR-150 target c-Myb Increase in HMEC-1 cells migration	Zhang et al., 2010b
EBV-infected B-cells	Dendritic cells, HeLa	Mature EBV-encoded miRNAs	Repression of EBV-miRNAs target CXCL11	Pegtel et al., 2010
COS-7, HEK 293	COS-7, HEK 293	Overexpressed luciferase siRNA	Repression of luciferase reporter gene	Kosaka et al., 2010a
COS-7	PC-3M (metastatic prostate cancer cells)	Overexpressed miR-146a	Decrease in proliferation Repression of miR-146a target ROCK1	Kosaka et al., 2010a
Mesenchymal stem cells	Tubular epithelial cells (mTEC)	Multiple miRNAs	Repression of PTEN, cyclin D1, Bcl-2 proteins	Collino et al., 2010
Macrophages	SKBR3 and MDAMB-231 breast cancer cells	Endogenous miR-223	Reduction of miR-223 targeted Mef2c mRNA Increased migration of SKBR3 and MDA-MB-231 cells	Yang et al., 2011
J77 T-cells, primary T-cells	Raji B cells (antigen presenting cells)	Overexpressed miR-335, endogenous miR-335, miR-92	Repression of miR-335 targeted 3'-UTR of SOX4 gene	Mittelbrunn et al., 2011
Hep3B	Hep3B	Endogenous miRNAs enriched in exosomes	Repression of putative miRNAs target TAK1	Kogure et al., 2011
Primary mesenchymal stromal cells	Primary astrocytes and neurons	Endogenous miR-133b	Increase in neurite outgrowth	Xin et al., 2012
Primary dendritic cells	Primary dendritic cells	Endogenous miR-451 and miR-148a	Repression of luciferase reporter	Montecalvo et al., 2012
HUVEC	Aortic smooth muscle cell	Endogenous miR-143/145	Repression of multiple miR-143/145 targets Protection against atherosclerotic lesion formation	Hergenreider et al., 2012

MiRNAs are present in 12 body fluids

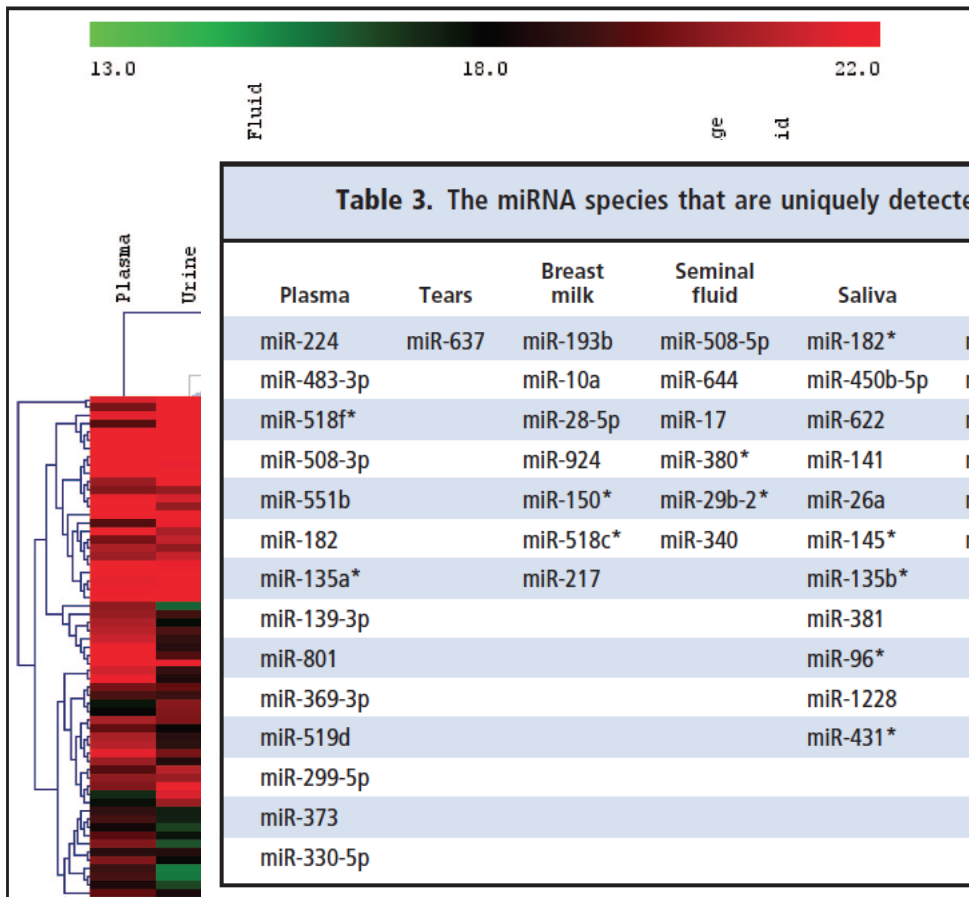


Fig. 1. The body fluid types can be grouped into 2 major clusters based on the profile of commonly expressed miRNAs (unsupervised hierarchical clustering). Plasma is separate from the 2 major clusters.

Table 3. The miRNA species that are uniquely detected in each of the different body fluid types.

Plasma	Tears	Breast milk	Seminal fluid	Saliva	Amniotic fluid	Cerebrospinal fluid	Peritoneal fluid	Colostrum
miR-224	miR-637	miR-193b	miR-508-5p	miR-182*	miR-636	miR-577	miR-129*	miR-18a*
miR-483-3p		miR-10a	miR-644	miR-450b-5p	miR-92a-1*		miR-583	miR-513a-5p
miR-518f*		miR-28-5p	miR-17	miR-622	miR-376b		miR-223	miR-10b*
miR-508-3p		miR-924	miR-380*	miR-141	miR-26b		miR-627	miR-192*
miR-551b		miR-150*	miR-29b-2*	miR-26a	miR-556-5p		miR-29b-1*	miR-193b*
miR-182		miR-518c*	miR-340	miR-145*	miR-593*			miR-130a*
miR-135a*		miR-217		miR-135b*				
miR-139-3p				miR-381				
miR-801				miR-96*				
miR-369-3p				miR-1228				
miR-519d				miR-431*				
miR-299-5p								
miR-373								
miR-330-5p								

Table 4. Concentration of RNA isolated from

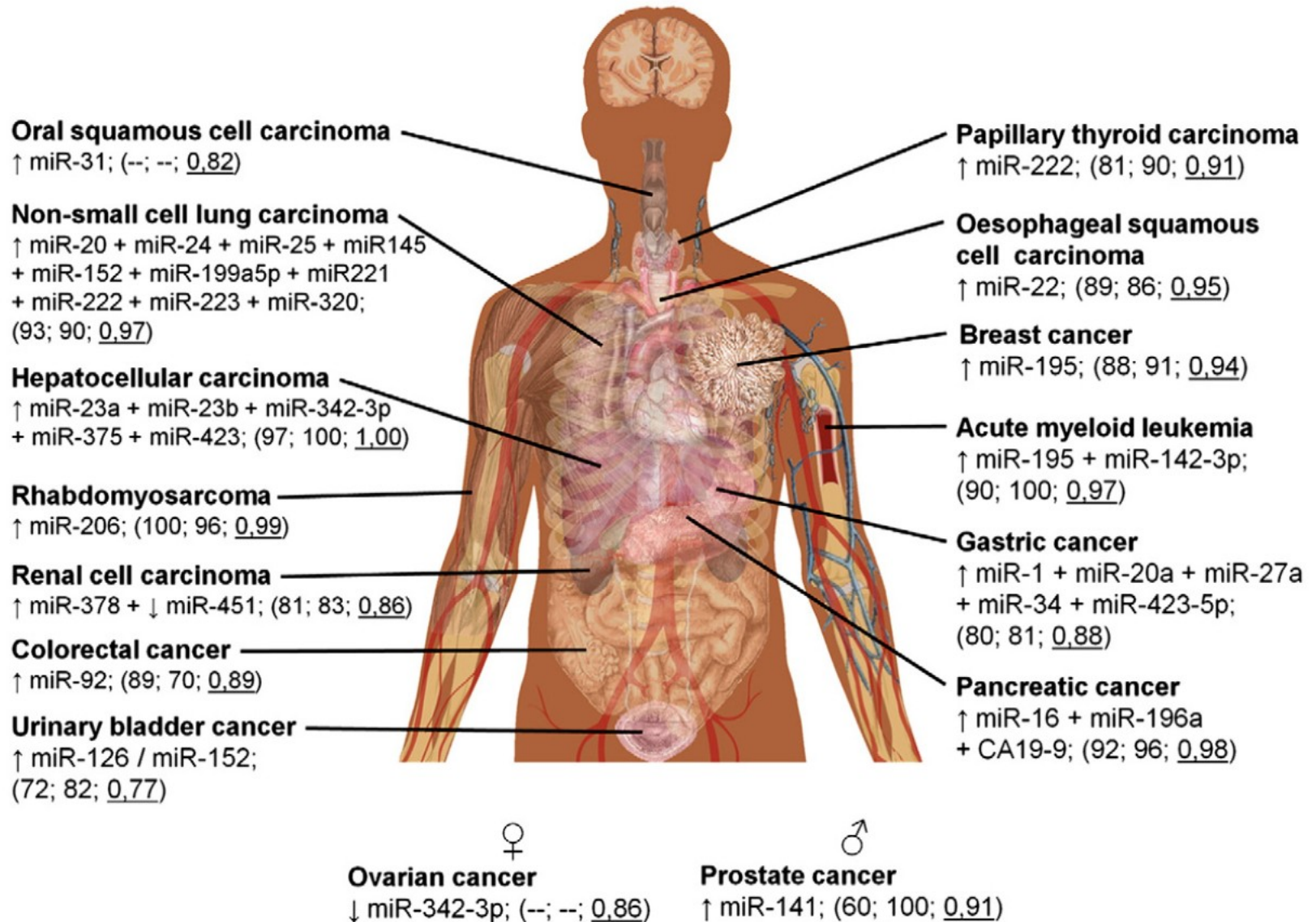
number of detectable miRNAs

359
429
260
212
386
397
349
210
458
436
320
204

RNA 6000 Picoles except co- is based on

the number of miRNA species with a level of >80% of the global mean.

There are specific profiles of circulating microRNAs in cancer



Circulating microRNAs are associated with wide range of pathologies

Acute pulmonary embolism

↑ miR-134; (--; --; 0,83)

Active tuberculosis

↑ miR-29; (83; 80; 0,83)

Hepatitis B virus

↑ miR-375 + miR-10a + miR-223;
(99; 99; 1,00)

Cirrhosis

↓ miR-122 + ↑ miR-192 + ↑ miR-21
+ ↓ miR-223 + ↓ miR-26a
+ ↓ miR-27a + ↑ miR-801;
(75; 91; 0,88)

Hepatitis C virus

↑ miR-92a + miR-423; (98; 99; 1,00)

Relapsing-remitting multiple sclerosis

↑ Hsa-miR-145; (90; 90; 0,96)

Acute cerebral ischemia

↓ miR-210; (88; 41; 0,66)

Traumatic brain injury

↓ miR-16 + ↓ miR-92a
+ ↑ miR-765; (100; 100; 1,00)

Heart failure

↑ miR-423-5p; (--; --; 0,91)

Acute myocardial infarction

↑ miR-208b; (100; 100; 1,00)

Crohn's disease

↑ miR-16; (74; 100; 0,90)

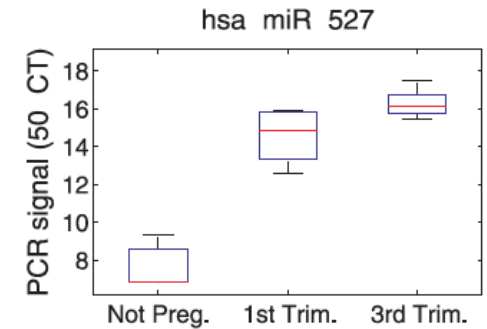
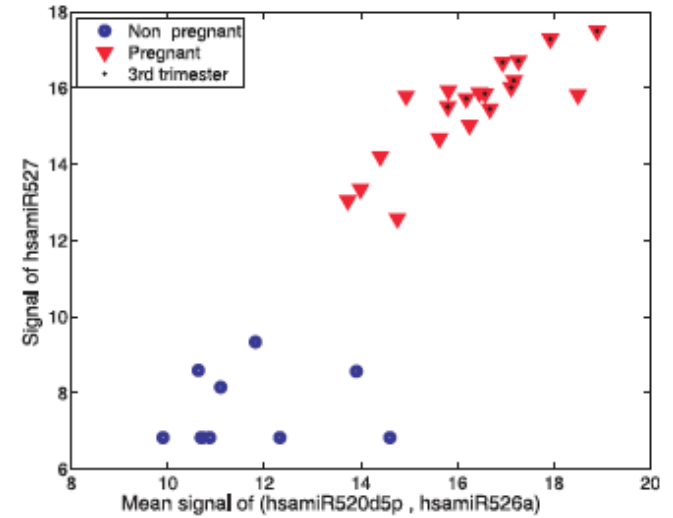
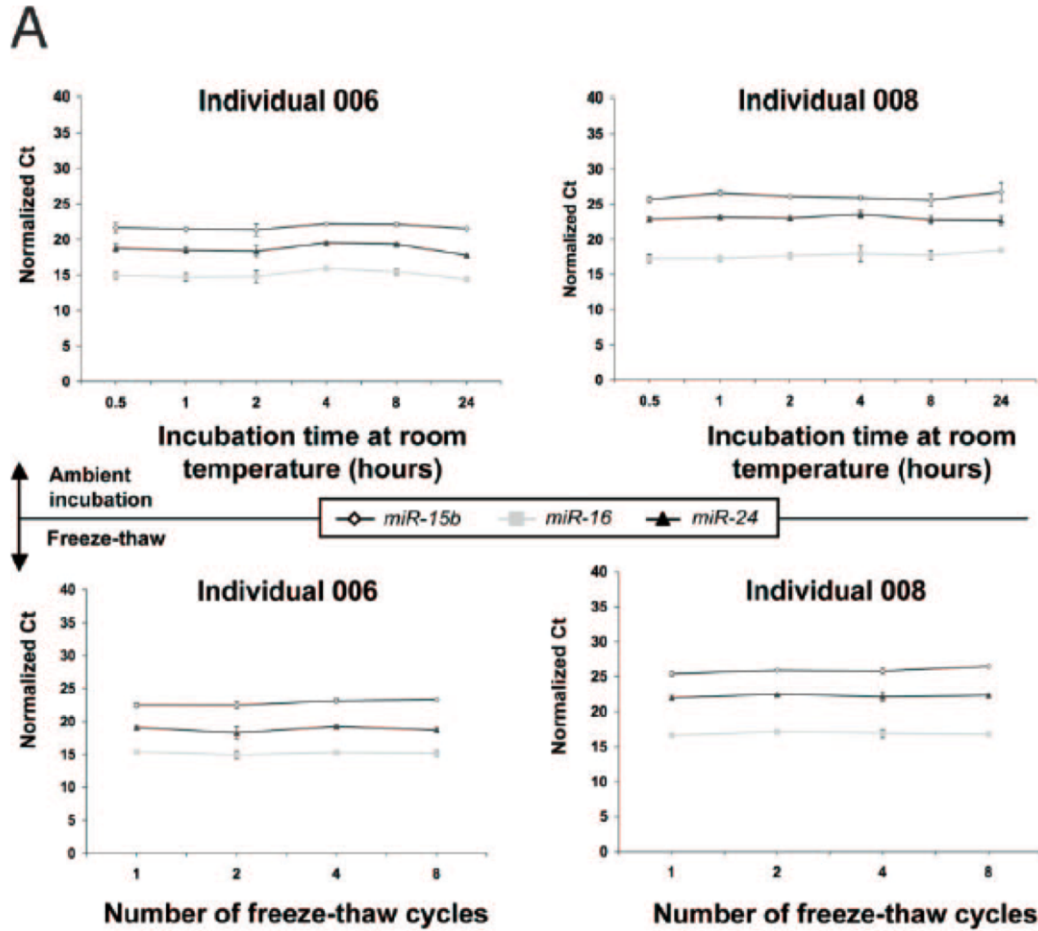
Sepsis

↓ miR-223; (80; 100; 0,86)

Hand-foot-and-mouth disease

↑ miR-148a + miR-143
+ miR-324-3p + miR-628-3p
+ miR-140-5p + miR-362-3p;
(97; 93; 0,99)

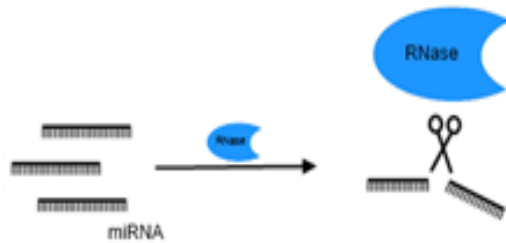
Stabilita cirkulujících mikroRNA



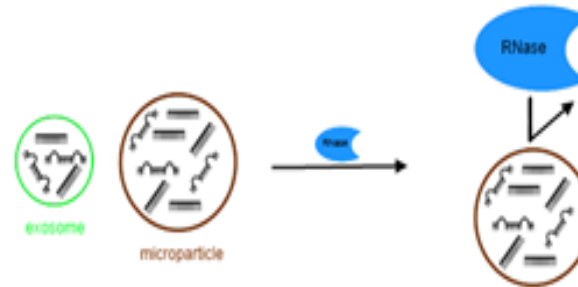
Gilad et al., 2008

Příčina stability cirkulujících mikroRNA

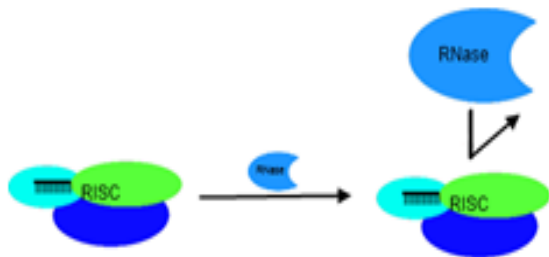
A. Naked miRNAs



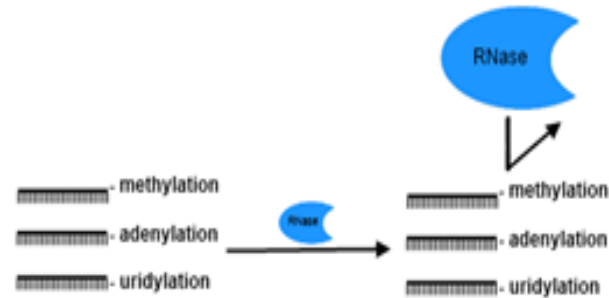
B. MiRNAs in microvesicles



C. MiRNAs as a part of RISC



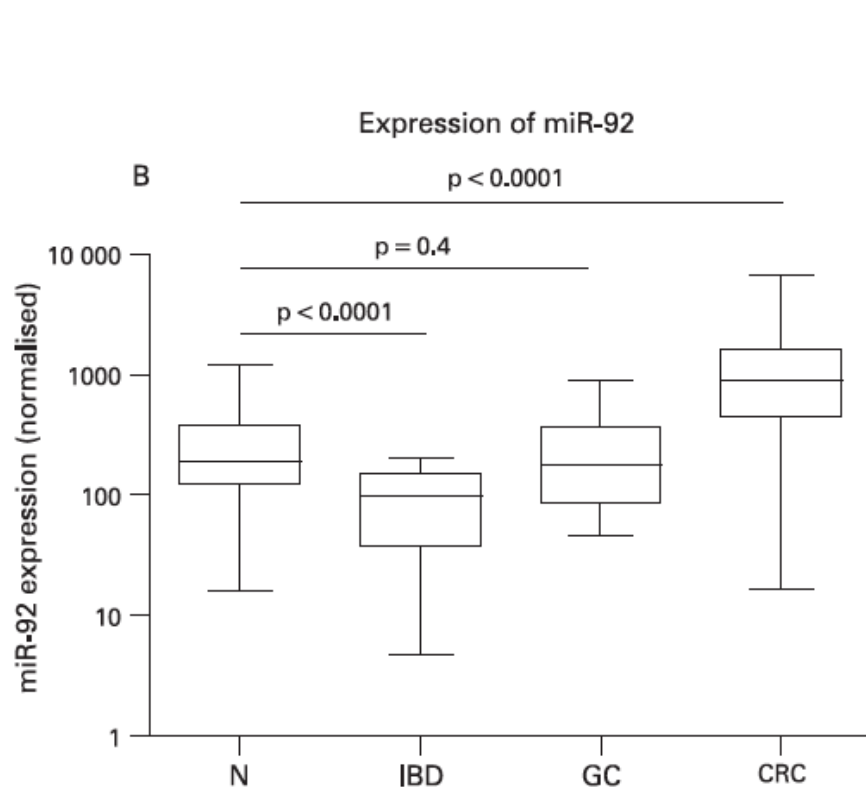
D. Modifications of MiRNAs (methylation, adenylation, uridylation)



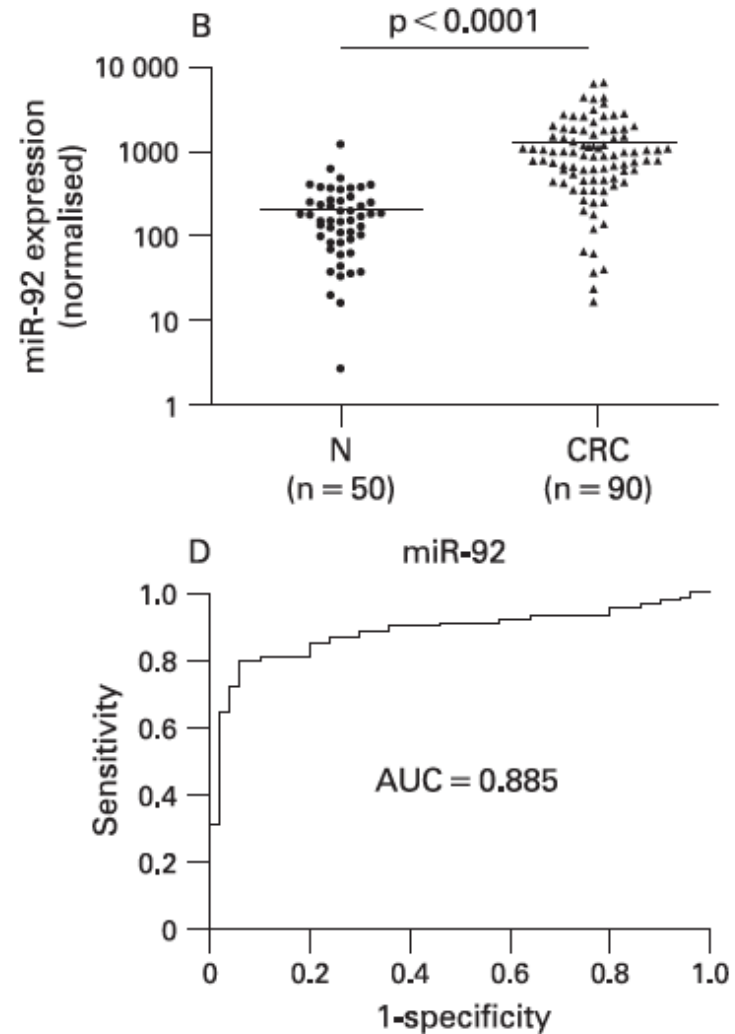
Pre-analytical variables affecting analysis of circulating microRNAs

- **Specimen collection and processing**
(collection tubes, additives (serum-clot activators, plasma-anticoagulants EDTA, heparin-heparinase), centrifugation, clotting time, time to processing,...)
- **Storage and stability**
Circulating microRNAs should be stable when stored at -20 and -80°C and for a short time at RT or 4°C
There are conflicting data about the effect of freeze/thaw cycles
- **Blood cell count**
Different blood cell count significantly change the microRNA expression profile
- **Haemolysis**
miRNAs present in RBC could falsely elevate levels in haemolyzed samples
- **Platelet contamination**
MiRNA present in platelets could significantly contaminate serum profiles
- **Plasma volume/components**
optimal volume is necessary because due to presence of enzyme inhibitors
- **Pre-amplification of microRNAs**
- Other common preanalytical variables:
gender, age, smoking, fasting, dietetic miRNAs

Sérové mikroRNA jako diagnostické biomarkery u CRC



89% senzitivita
70% specificita

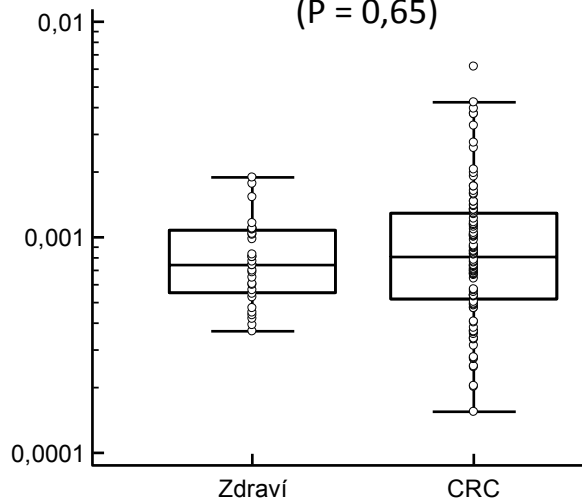


ALE.....

Sérové mikroRNA jako diagnostické biomarkery u CRC

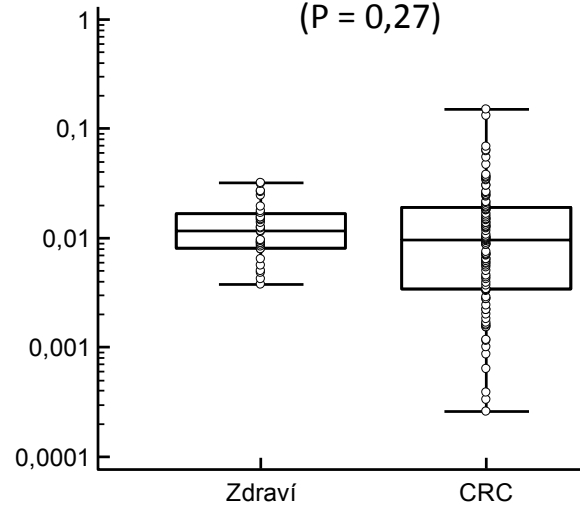
miR-17-3p

(P = 0,65)



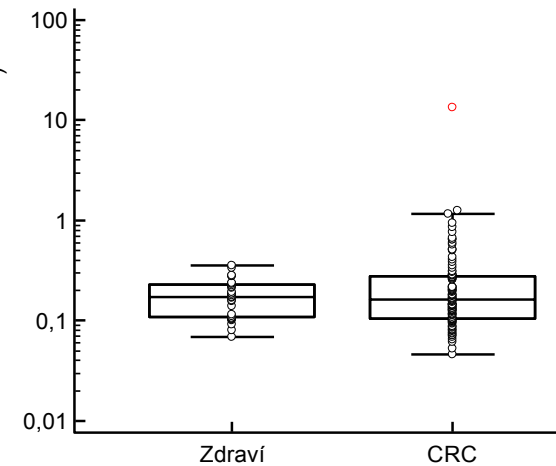
miR-29a

(P = 0,27)



miR-92a

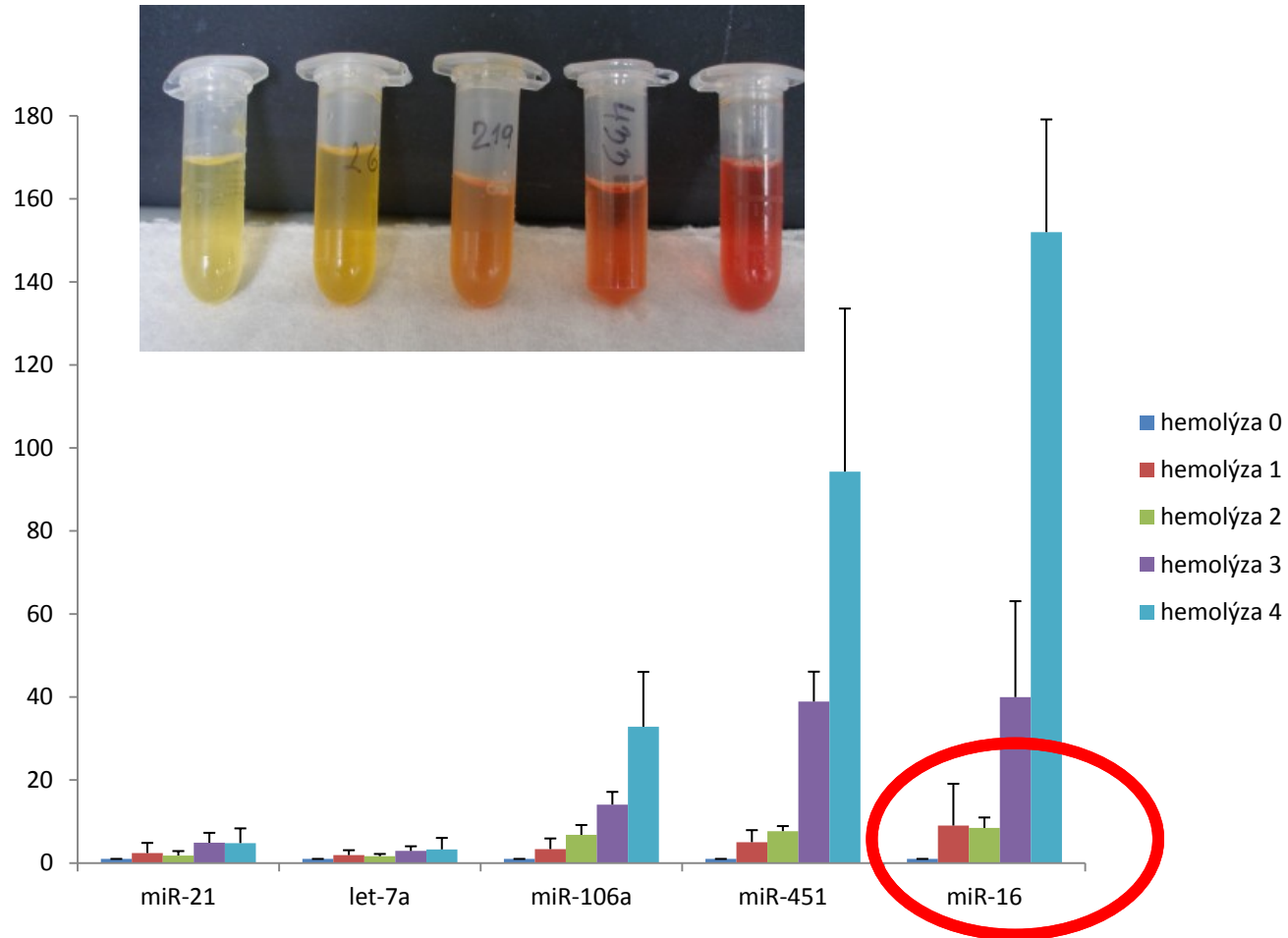
(P = 0,90)



- miR-17-3p, miR-29a, miR-92a, miR-135b
- endogenní kontrola: miR-16
- 100 pacientů, 30 zdravých dárců

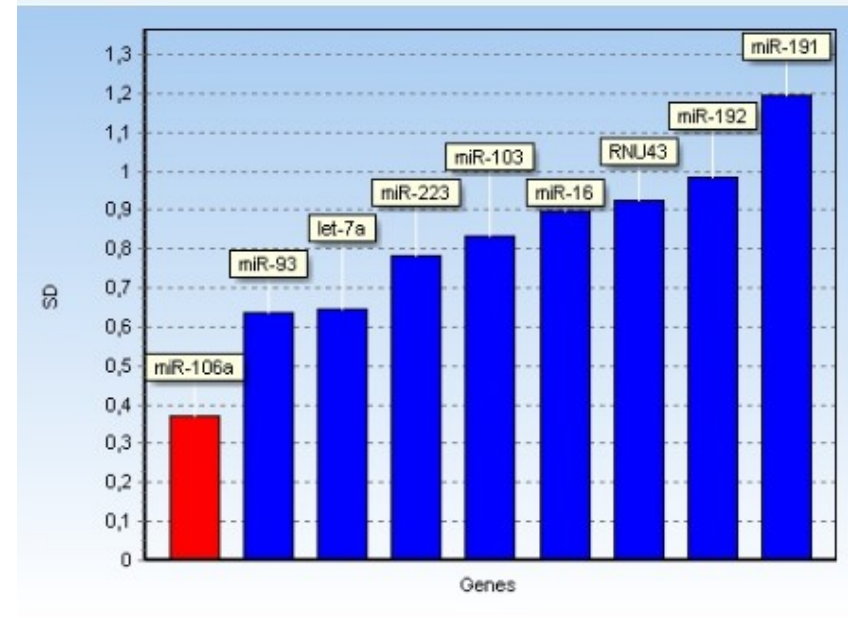
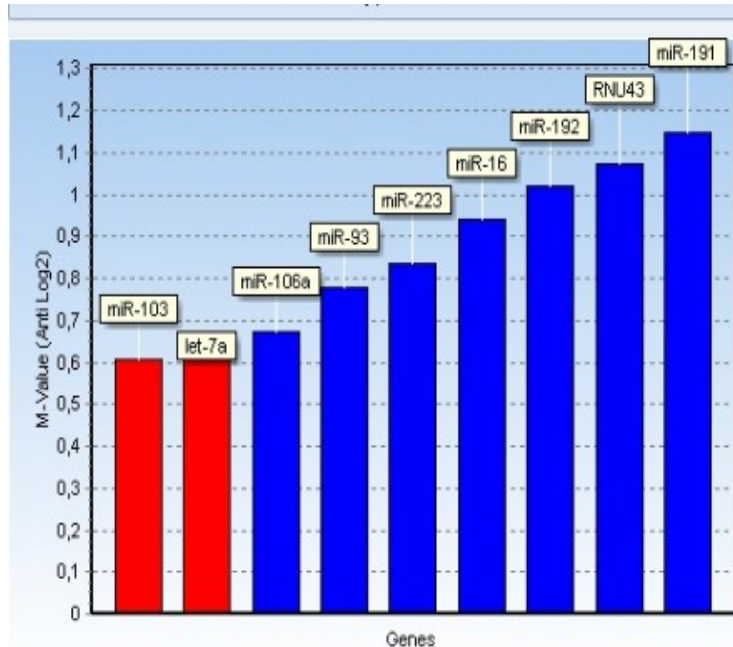
ČESKÁ REPUBLIKA

Vliv hemolýzy na hladiny vybraných sérových mikroRNA



Unpublished data, 2014

Vliv normalizace dat na hladiny sérových mikroRNA



30 zdravých dárců, 30 pacientů s CRC, 30 pacientů s RCC

9 genů/miRNA nejčastěji používaných k normalizaci exprese miRNA v tělních tekutinách:

let-7a, miR-16, miR-93, miR-103, miR-106a, miR-191, miR-192, miR-223 and RNU43

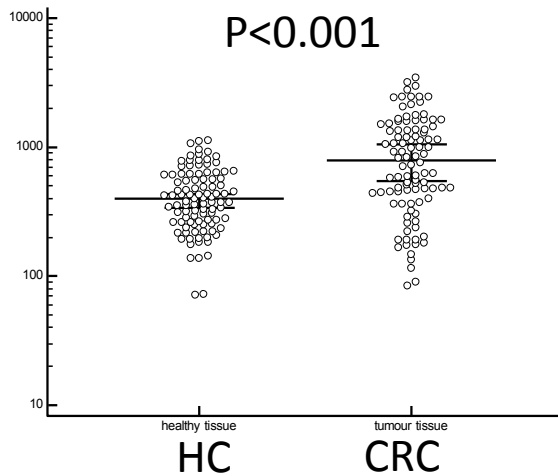
Identifikace nejvíce vhodných referenčních genů užitím NormFinderu a GeneNormu

Vliv normalizace dat na hladiny sérových mikroRNA

Užití rozdílných přístupů k normalizaci exprese cirkulujících mikroRNA může vést ke zcela rozdílných výsledkům!

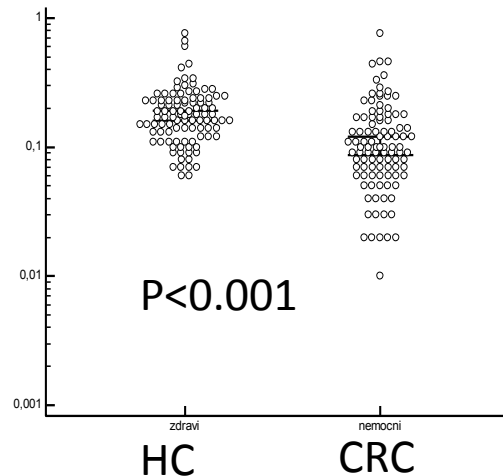
ZVÝŠENÁ HLADINA

Absolute quantification



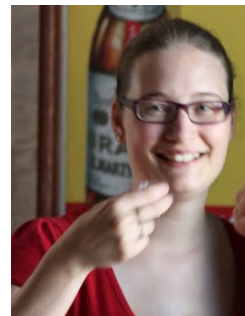
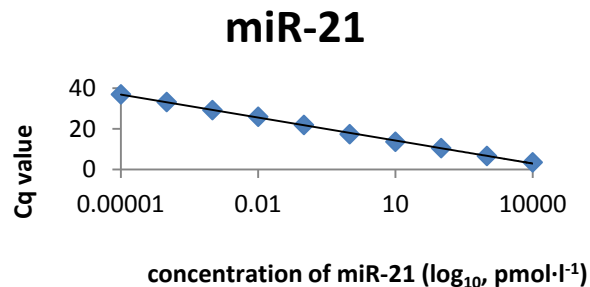
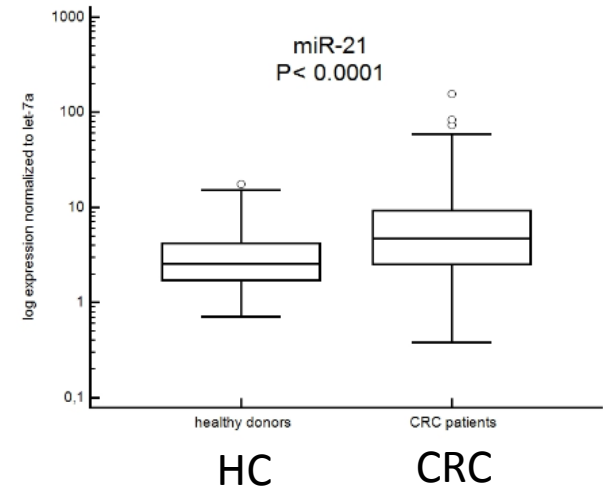
SNÍŽENÁ HLADINA

miR-16 as reference gene



ZVÝŠENÁ HLADINA

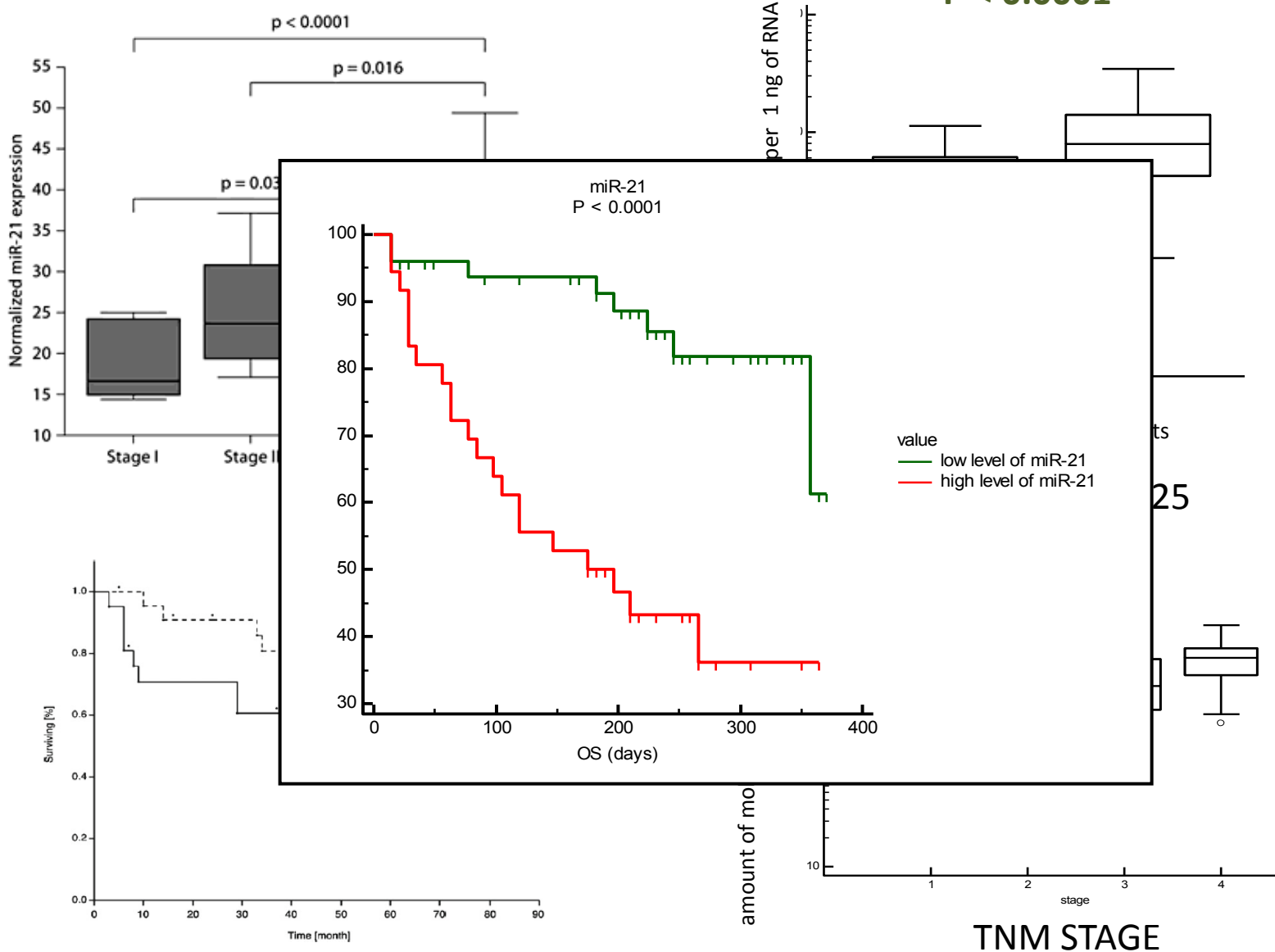
let-7a as reference gene



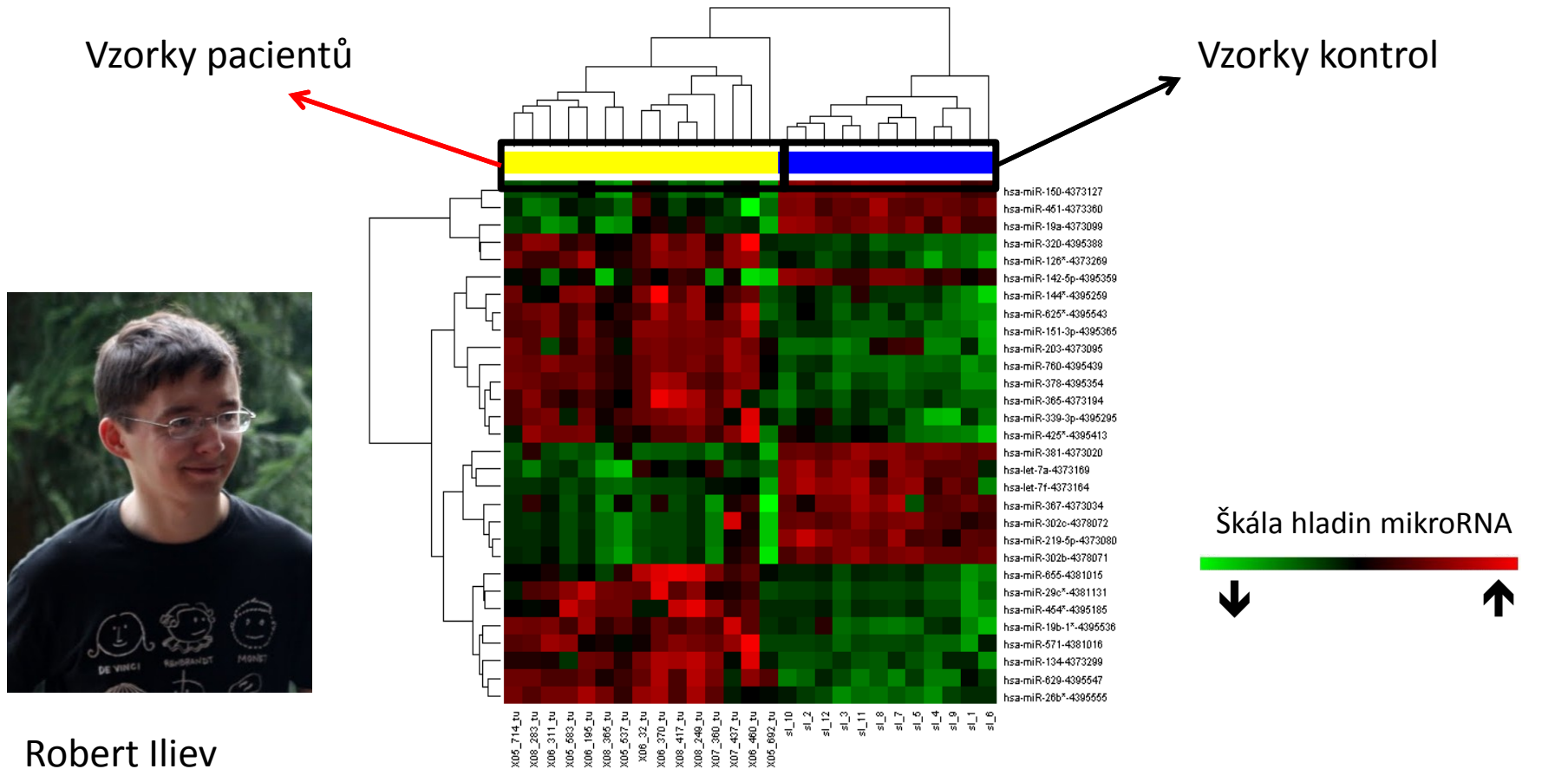
Petra Faltejsková

MiR-21 jako biomarker u kolorektálního karcinomu

P < 0.0001

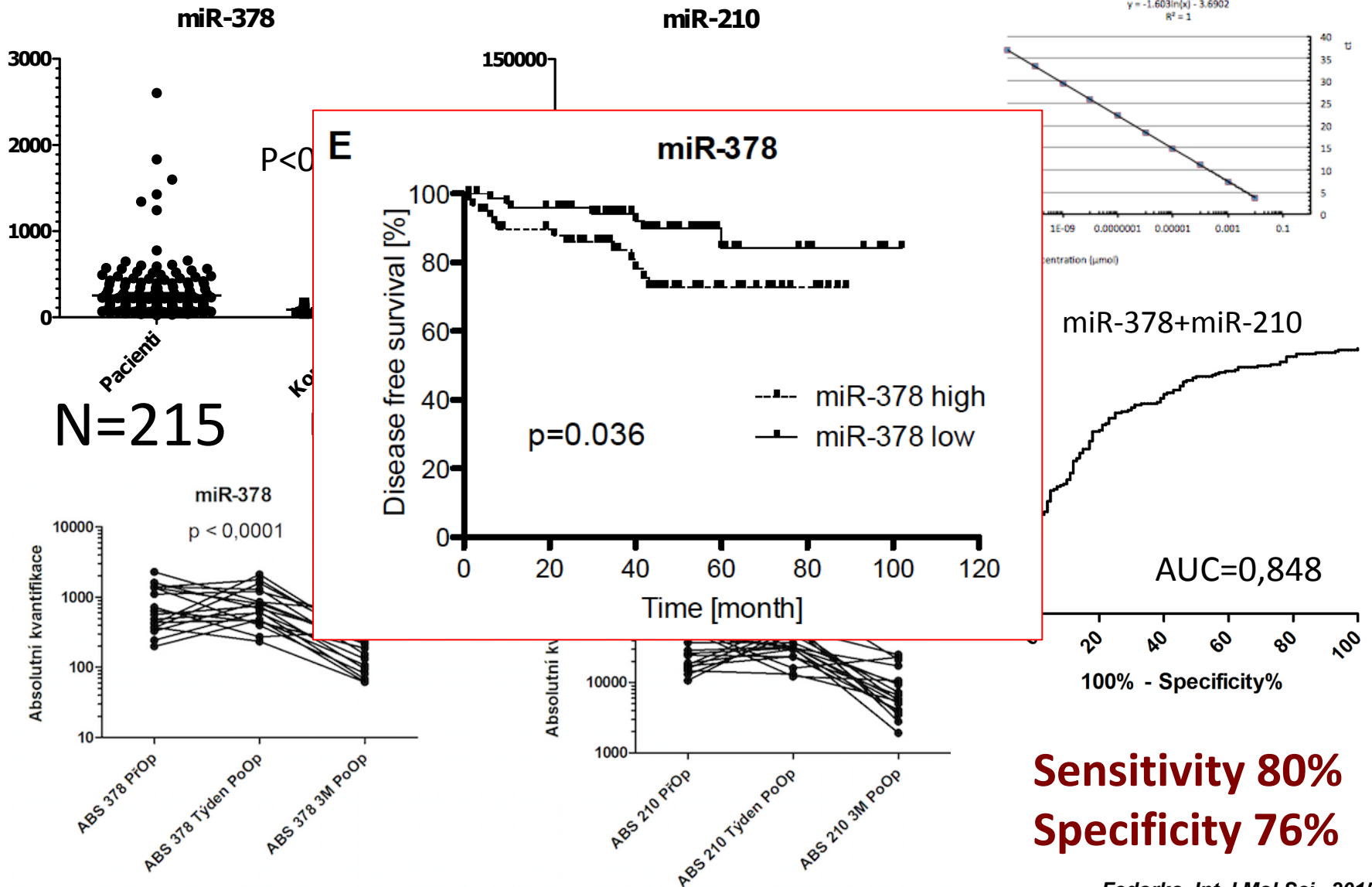


Cirkukující sérové mikroRNA v diagnostice renálního karcinomu



Vzorky krevního séra rozdělené do skupin na základě rozdílné exprese miRNA (žlutá barva znamená vzorky pacientů s renálním karcinomem, modrá pak vzorky kontrolních jedinců, vyhodnoceno metodou LIMMA, $p < 0001$)

Cirkukující sérové mikroRNA v diagnostice renálního karcinomu

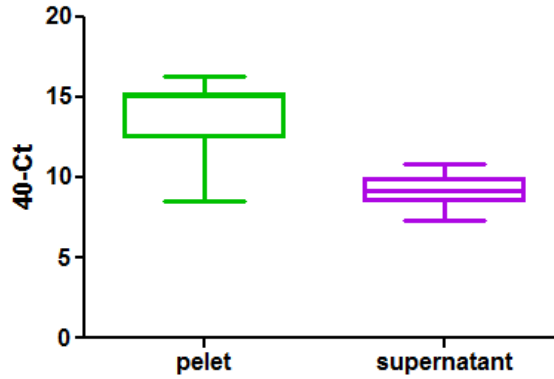


Močové mikroRNA jako diagnostické biomarkery uroteliálního karcinomu

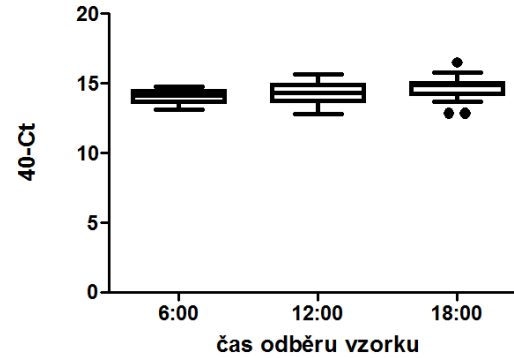


Hanka
Mičochová

4 zdraví jedinci, 5 dní (miR-21)



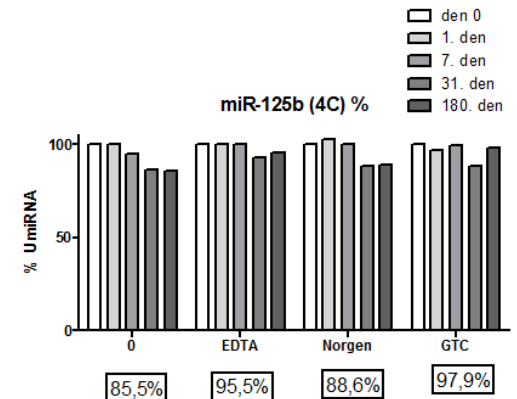
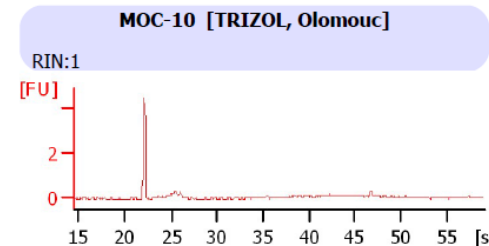
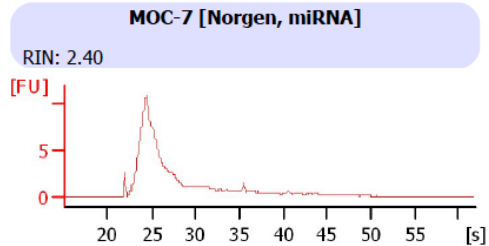
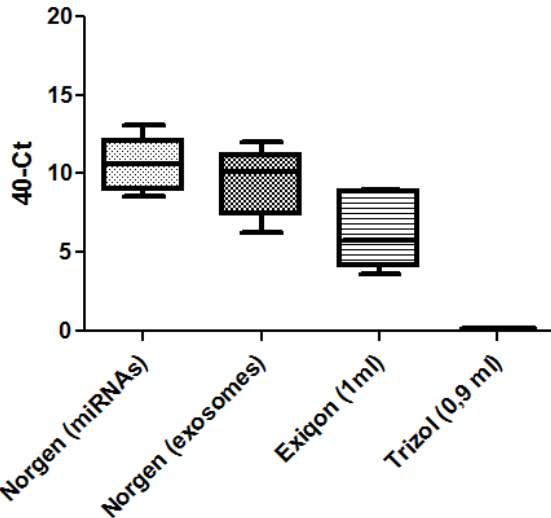
miR-21



Jarda Juráček

4 zdraví dobrovolníci
odběry 3x denně po dobu 5 dnů

miR-21

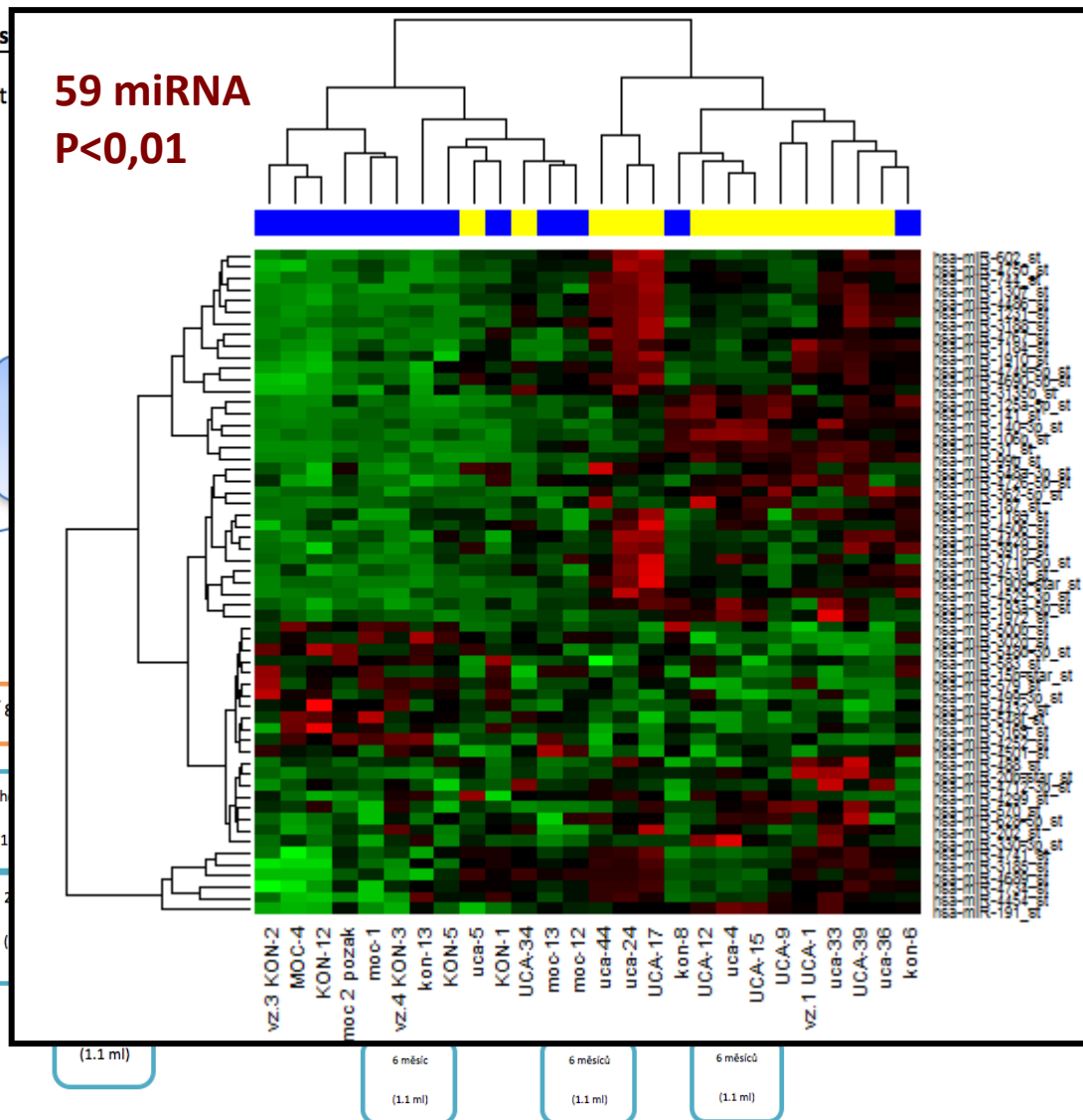


Močové mikroRNA jako diagnostické biomarkery uroteliálního karcinomu

A, základní experiment (s

P – bude uschována pelet

59 miRNA
P < 0,01



Pokojevá teplota (6 ml)

Centrifugovat těsně před IZOLACÍ

4 hod. (1.1 ml)

6 měsíců (1.1 ml)

24 hod. (1.1 ml)

1 měsíc (1.1 ml)

1 týden (1.1 ml)

(1.1 ml)

6 měsíc (1.1 ml)

6 měsíců (1.1 ml)

6 měsíců (1.1 ml)

24 ml moče

(0.85g guanidium thiocyanate)

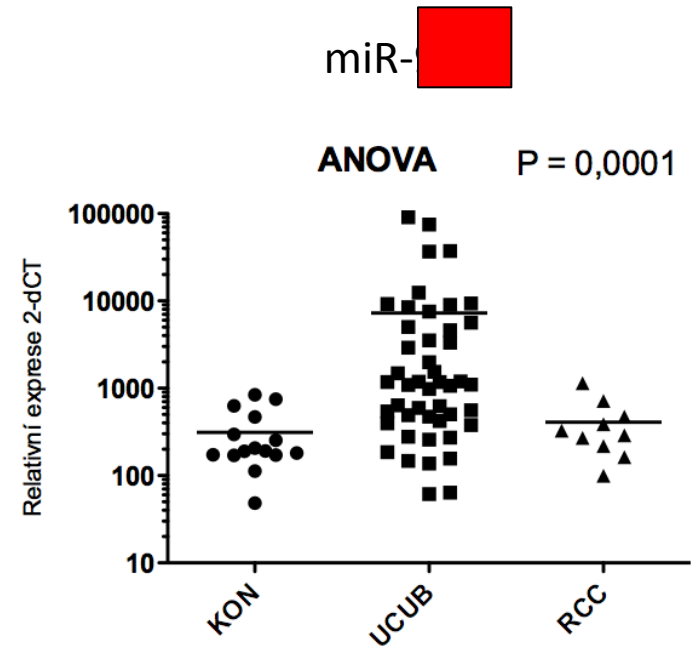
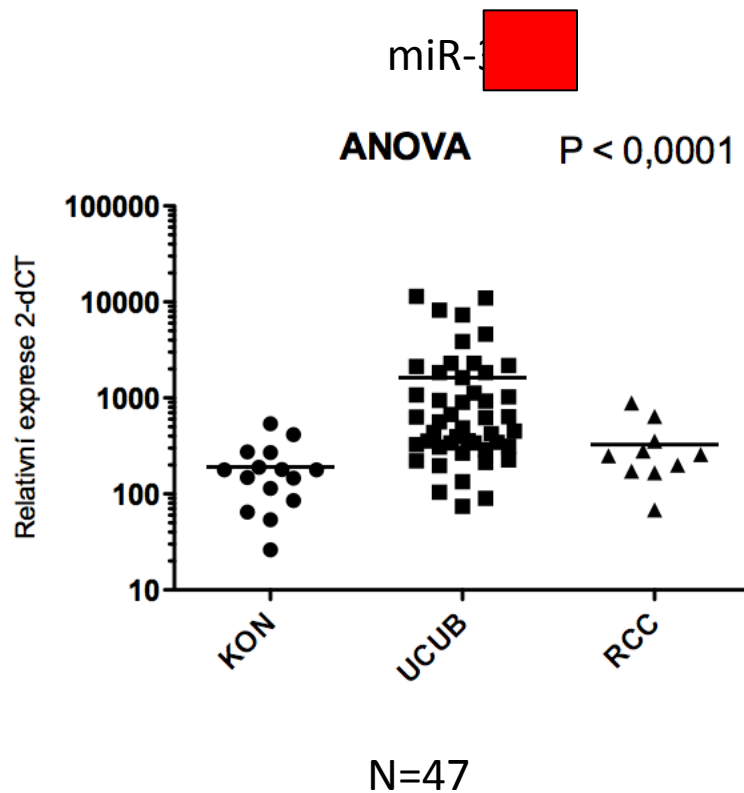
-20°C

Pokojevá teplota

+4°C

20°C

Močové mikroRNA jako biomarkery karcinomu močového měchýře



Two examples of successful validation

Second phase of validation, patent

Small RNA sequencing in blood serum of colorectal cancer patients

Pooled samples

Only spectrophotometrically evaluated non-heamolyzed samples were included

1 pool = blood serum of 12 patients (200ul serum, one pool=2,4 ml)

- 5 pools of healthy donors = 60 healthy donors
- 12 pools of CRC patients based on serum samples of 144 colorectal cancer patients (36 CRC patients of each clinical stage I-IV)

RNA purification - miRNeasy Serum/plasma Kit (Qiagen) (glycogen as carrier)

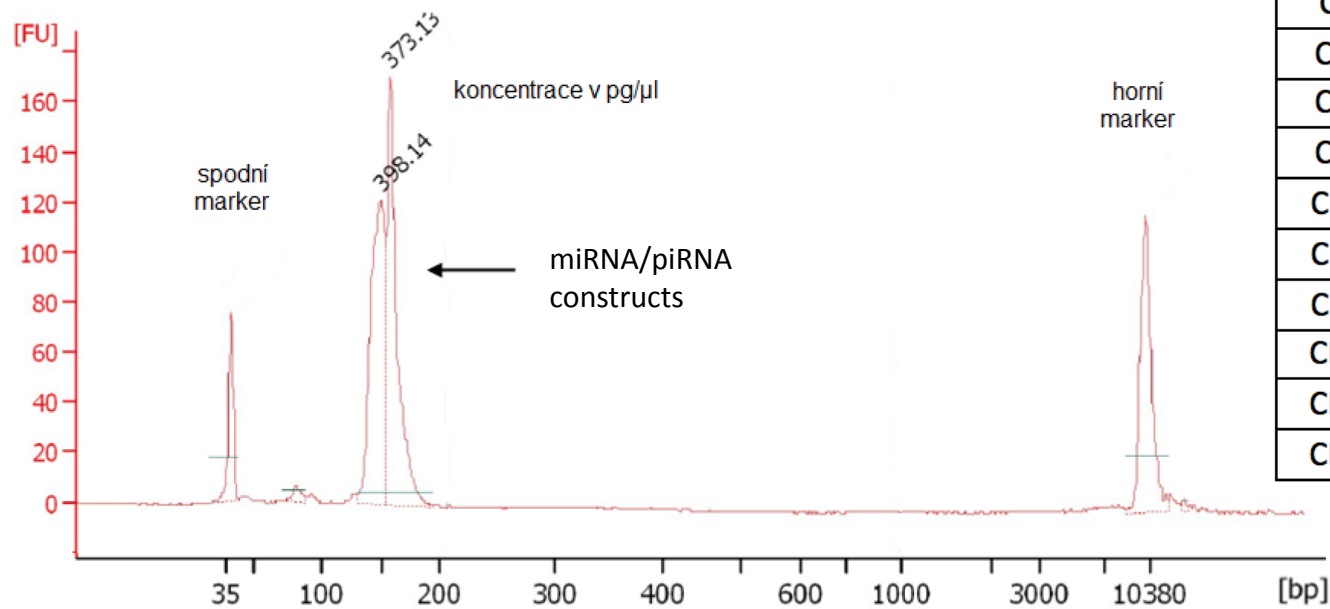
RNA yield 70-140 ng

Illumina MiSeq, TruSeq Small RNA kit

2 pools in one sequencing run (7,5M per pool)



High Sensitivity DNA Bioanalyzer assay as checkpoint for correct size selection during library preparation

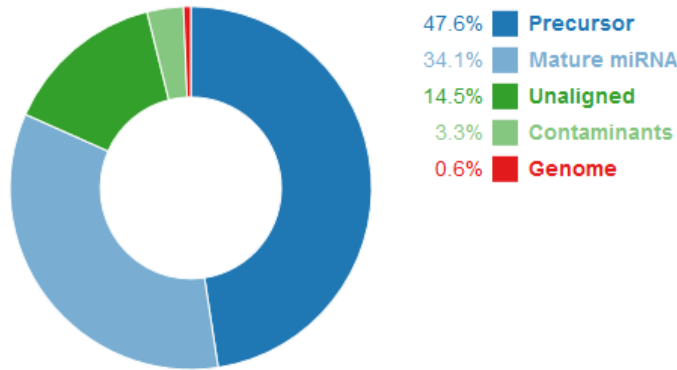


Peak Size

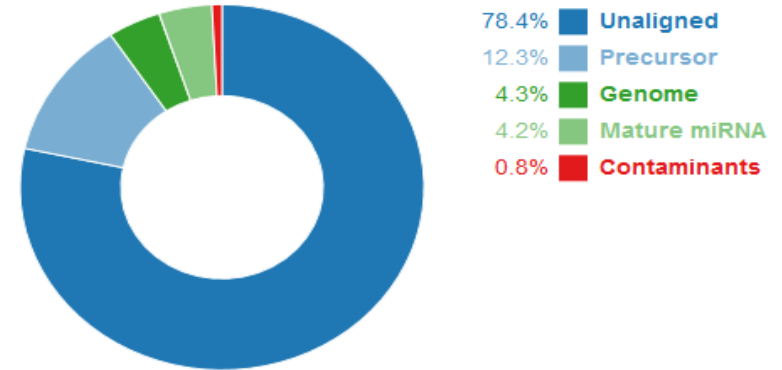
ID	Length (bp)
Z 1	145, 152
Z 2	145, 151
Z 3	145, 152
Z 4	145, 151
Z 5	145, 153
CRC I 1	137, 159
CRC I 2	139
CRC I 3	138, 152
CRC II 1	137, 143
CRC II 2	139, 152
CRC II 3	145, 153
CRC III 1	145
CRC III 2	140
CRC III 3	137, 142
CRC IV 1	138, 145
CRC IV 2	134
CRC IV 3	136

The proportions of annotated and non-annotated reads

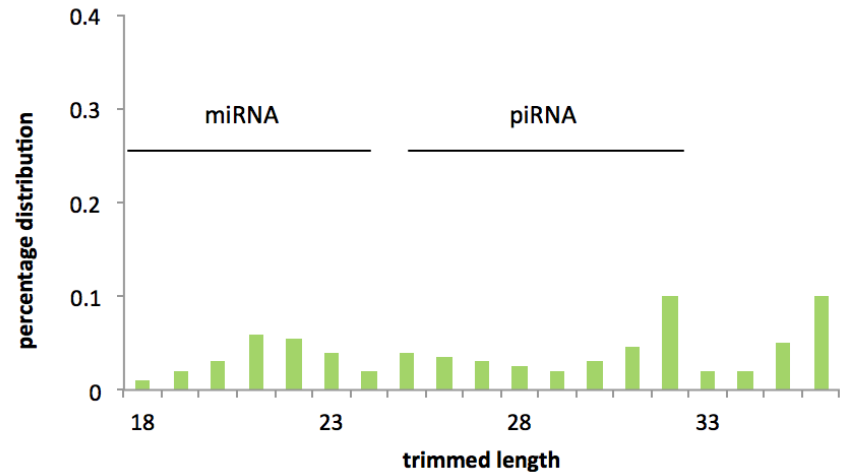
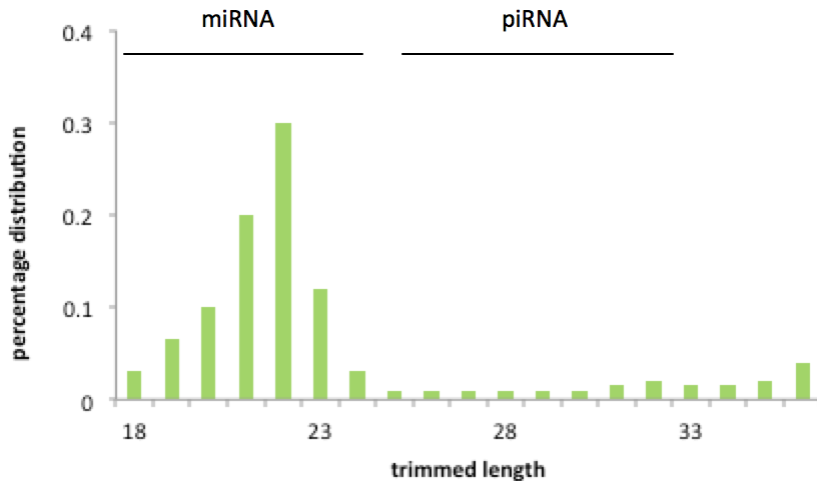
TUMOR TISSUE



BLOOD SERUM

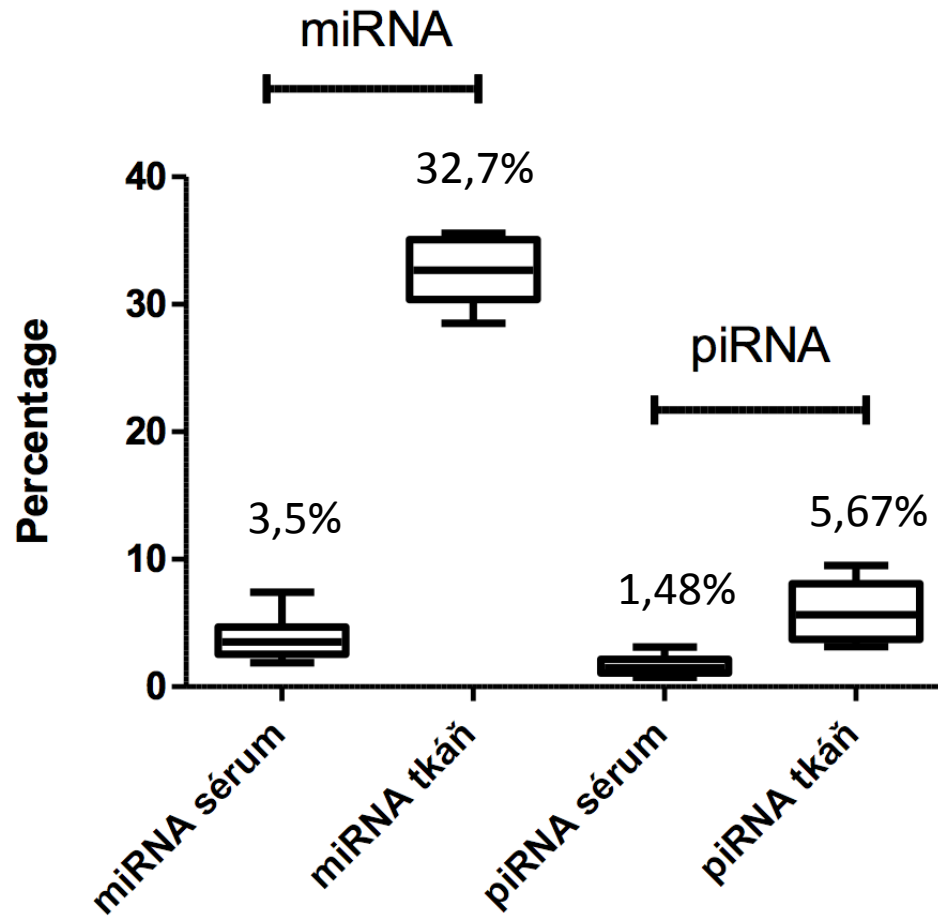


34% vs 4% mature miRNAs



More than 95% of all reads showed an average Q score >30.

Percentage of mapped and annotated miRNA/piRNA reads compared to the total number of sequences



Ten most abundant microRNAs among all blood serum samples analyzed

miRNA	Average readcounts
mir-486	322467,4
mir-16	17470,71
mir-191	16941,29
mir-26a	7471,647
mir-30d	5864,294
mir-423	5229,412
mir-10b	5225,882
mir-451a	4974
mir-181a	4561,941
mir-10a	4445,176

4 of 10 most abundant miRNAs
in serum originated from RBC

isomiRs in circulation and tissue

miR-92 in colon tissue

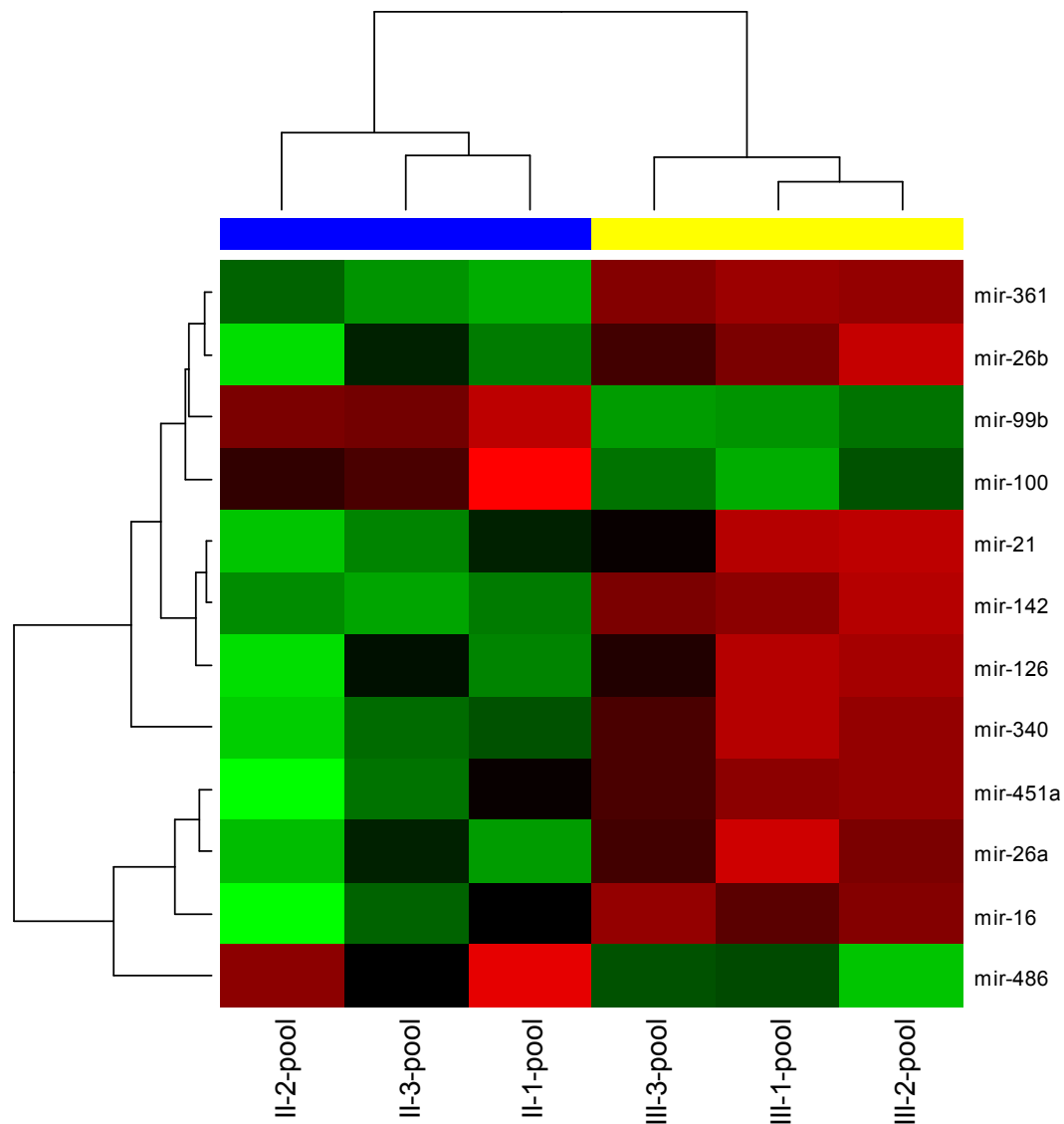
Sequence miRNA 5' 3'	Délka (nt)	Readcounts	Type of izomiR	Mismatch		
AGGTTGGGATCGGTTGCAATGCT	23	10	Mature	0		
TATTGCACTTGTCGCCGCCTGT	22	4600	Mature	0		
izomir						
TATTGCACTTGTCGCCGCCTGTTT	24	514	Mature Super variant	1		
TATTGCACTTGTCGCCGCCTGTT	23	7	Mature Super	0		
TATTGCACTTGTCGCCGCCTGTTAT	25	75	Precursor variant	2		
TATTGCACTTGTCGCCGCCTGTTA	24	63	Sekvence miRNA 5' - 3'	Délka (nt)		
TATTGCACTTGTCGCCGCCTGTTT	25	41			AGGTTGGGATCGGTTGCAATGCT	23
TATTGCACTTGTCGCCGCCTGTAA	25	3	TATTGCACTTGTCGCCGCCTGT	22		
TATTGCACTTGTCGCCGCCTGAT	23	52	izomirny			
ATTGCACTTGTCGCCGCCTGTTT	23	11	TATTGCACTTGTCGCCGCCTGTTT	24	9605	Mature 3' super variant
TATTGCACTTGTCGCCGCCTGTTC	24	7	TATTGCACTTGTCGCCGCCTGTT	23	8047	Mature 3' super
ATTGCACTTGTCGCCGCCTGTT	22	160	TATTGCACTTGTCGCCGCCTGTTAT	25	3077	Precursor variant
TATTGCACTTGTCGCCGCCTGTGA	24	2	TATTGCACTTGTCGCCGCCTGTTA	24	2024	Mature 3' super variant
TATTGCACTTGTCGCCGCCTGTTA	25	5	TATTGCACTTGTCGCCGCCTGTTTT	25	1325	Precursor variant
			TATTGCACTTGTCGCCGCCTGTTAA	25	529	Precursor variant
			TATTGCACTTGTCGCCGCCTGAT	23	263	Mature 3' super variant
			ATTGCACTTGTCGCCGCCTGTTT	23	149	Mature 3' sub/super variant
			TATTGCACTTGTCGCCGCCTGTTT	24	144	Mature 3' super variant
			ATTGCACTTGTCGCCGCCTGTT	22	108	Mature 3' sub/super
			TATTGCACTTGTCGCCGCCTGTGA	24	107	Mature 3' super variant
			TATTGCACTTGTCGCCGCCTGTTA	25	101	Precursor variant

miR-92 in blood serum of healthy donor

IsomiRs with additional bases are increased in blood serum !!!

MicroRNA differentially expressed in serum of lymph node-negative and positive CRC cases

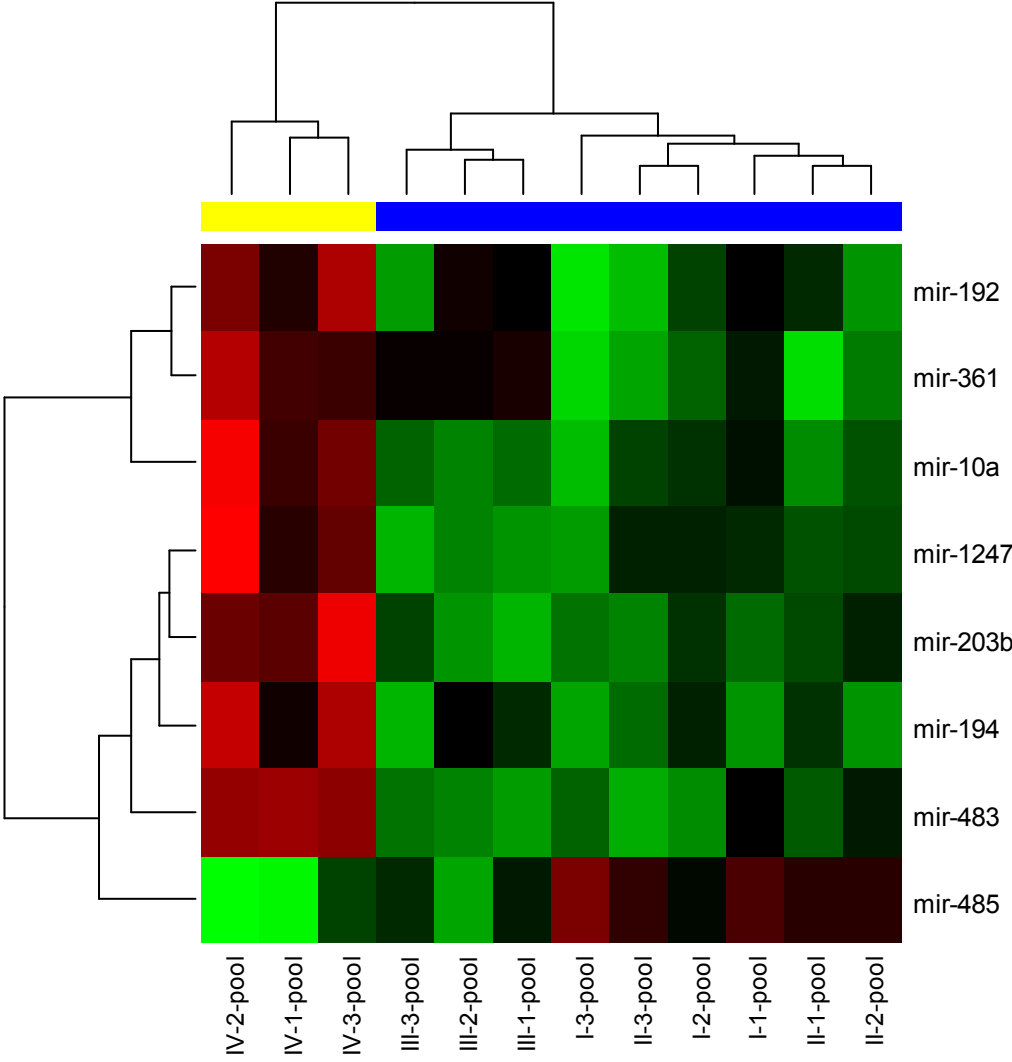
12 miRNAs with $p < 0.00001$



MicroRNA differentially expressed in serum of CRC cases with and without distant metastasis

8 miRNAs

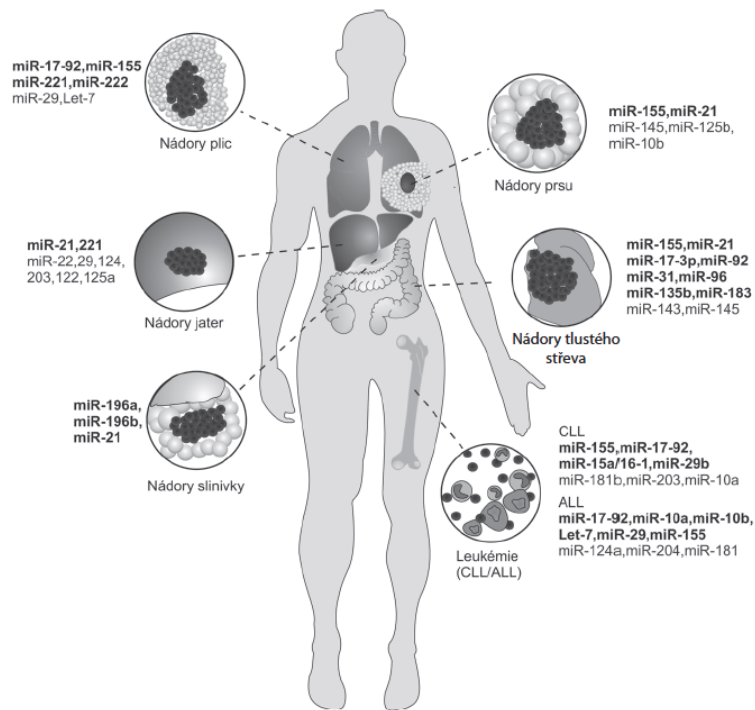
with $p < 0.001$



CIRCULATING microRNAs as biomarkers in clinical trials

MicroRNA	Trial reference	Disease	Trial
miR-34a	NCT01829971	Liver cancer and liver metastases	Interventional phase I multicenter study to investigate the safety, pharmacokinetics and pharmacodynamics of MRX34
Numerous	NCT01964508	Thyroid cancer	Observational study of microRNA expression in fine needle aspirates
Circulating	NCT01722851	Breast cancer	Observational studies of microRNA expression levels as biomarkers of response to treatment in the tumors and circulation
Numerous	NCT01220427	High-risk prostate cancer	Observational studies of microRNA expression levels as biomarkers of response to treatment in the tumors
miR-10b	NCT01849952	Glioma	Observational studies of miR-10b expression levels as biomarkers of tumor grade, survival, and genotypic variation
miR-29b	NCT02009852	Oral squamous cell carcinoma	Observational study to explore the prognostic value of miR-29b in tissue, blood, and saliva
Numerous	NCT02127073	Breast cancer	Interventional study employing intranasal oxytocin to increase the volume of nipple aspirate fluid for biomarker identification and subsequent microRNA profiling of this aspirate
Circulating	NCT01595139	Low-grade glioma	Observational study of the circulating microRNA expression patterns in low grade glioma as early predictors of cancer and as a marker of response to therapy
Numerous	NCT01828918	Colorectal carcinoma	Observational study identifying biomarkers for patient stratification in tissue samples
Numerous	NCT01119573	Endometrial cancer	Observational study of association of microRNA expression and lymph node metastasis in endometrial cancer
Circulating	NCT01595126	Central nervous system cancer	Observational study of microRNA expression in the blood, cerebrospinal fluid and urine of patients through the course of their treatment
Numerous	NCT01827854	Head and neck squamous cell carcinoma	Observational study to investigate the role of microRNA in T1-T4-mediated cancer metastasis
Numerous	NCT01453465	Rhabdoid tumors	Observational study to identify microRNA expression patterns between rhabdoid tumors of the kidney and atypical teratoid rhabdoid tumors
Circulating	NCT01391351	Ovarian cancer	Observational studies of biomarker of response to treatment
Circulating	NCT01505699	B-cell acute lymphocytic leukemia	Observational studies of biomarkers of clinical outcomes
Numerous	NCT01957332	Breast cancer	Observational study correlating microRNA expression patterns with imaging and clinical data
Circulating	NCT01556178	Pediatric brain cancer	Observational study of microRNAs in the blood and cerebrospinal fluid as biomarkers
Numerous	NCT01050296	Pediatric solid tumors	Observational study of microRNA expression profiles in different tumor types
Numerous	NCT00864266	Non-small-cell lung cancer	Interventional study to identify a signature of response to chemotherapy
Numerous	NCT01108159	Hematologic cancer	Observational studies of biomarker of expression profiles in initiation, progression and treatment response

MikroRNA jako tkáňové biomarkery spojené se známými histologickými a molekulárními podtypy nádorových onemocnění



Tab. 4.1. Příklady mikroRNA asociovaných se známými histologickými a molekulárními podtypy nádorových onemocnění. Doplněno podle [1].

Nádorové onemocnění	MikroRNA	Literatura
Mamární karcinom		
ER status	miR-26a/b, rodina miR-30, miR-29b, miR-155, miR-342, miR-206, miR-191	[12–14]
PR status	let-7c, miR-29b, miR-26a, rodina miR-30, miR-520g	[15,16]
HER2/neu status	miR-520d, miR-181c, miR-302c, miR-376b, miR-30e	[12,15]
Karcinom plic		
dlaždicobuněčný vs. adenokarcinom	miR-205	[17]
malobuněčný vs. nemalobuněčný karcinom	miR-17-5p, miR-22, miR-24, miR-31	[18]
mutace EGFR/KRAS	miR-155, miR-25, miR-495, let-7g	[19]
Kolorektální karcinom		
mutace KRAS	miR-127-3p, miR-92a, miR-486-3p, miR-378	[24]
Karcinom žaludku		
difuzní vs. intestinální karcinom	miR-29b/c, rodina miR-30, miR-135a/b	[20]
Karcinom endometria		
endometroidní vs. serózní papilární karcinom	miR-19a/b, miR-30e-5p, miR-101, miR-452, miR-382, miR-15a, miR-29c	[21]
Rendlní karcinom		
světlobuněčný vs. papilární karcinom	miR-424, miR-203, miR-31, miR-126	[22]
onkocytom vs. chromofobní karcinom	miR-200c, miR-139-5p	[22]
Maligní melanom		
s mutací BRAF V600E	miR-193a, miR-338, miR-565	[23]
Akutní myeloidní leukemie		
t(15;17)	miR-382, miR-134, miR-376a, miR-127, miR-299-5p, miR-323	[25]
t(8;21) nebo inv(16)	let-7b/c, miR-127	[25]
mutace NPM1b	miR-10a/b, let-7, miR-29, miR-204, miR-128a, miR-196a/b	[25]
s FLT3 ITD	miR-15	[25]
Chronická lymfatická leukemie		
hladiny ZAP-70 a status IgVH	miR-15a, miR-195, miR-221, miR-155, miR-23b	[26,27]

MikroRNA jako tkáňové biomarkery

mikroRNA nepodléhají degradačním procesům během formalínové fixace a archivace v parafinových blocích jako je tomu u mRNA

→ ARCHIVY FFPE MATERIÁLU NA PATOLOGÍCH JE TAK MOŽNÉ VYUŽÍVAT PRO ROZSÁHLE RETROSPEKTIVNÍ STUDIE

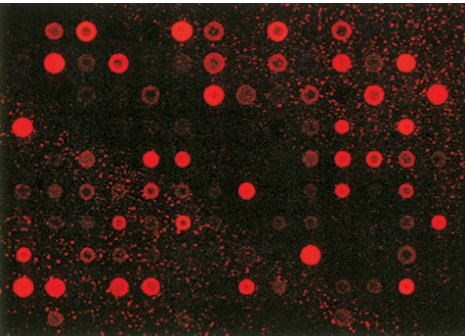
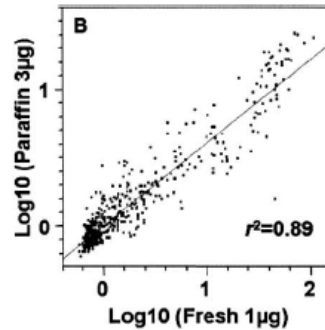
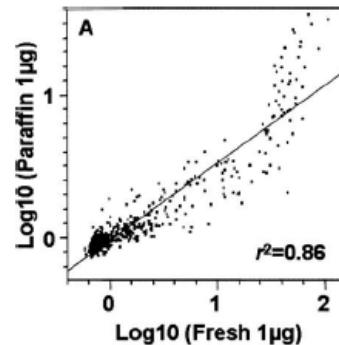


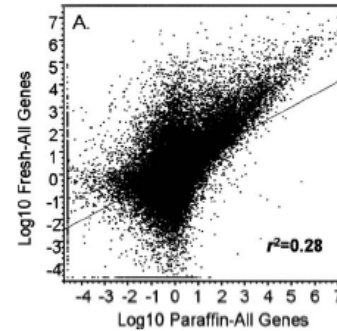
FIGURE 7. Representative miRNA array image from a 10-yr-old FFPE colon cancer sample.



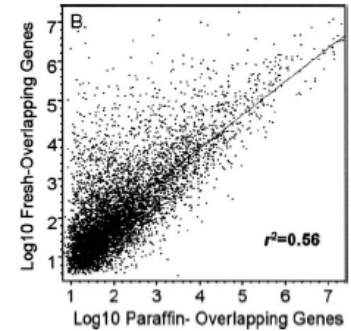
mikroRNA z FFPE



versus



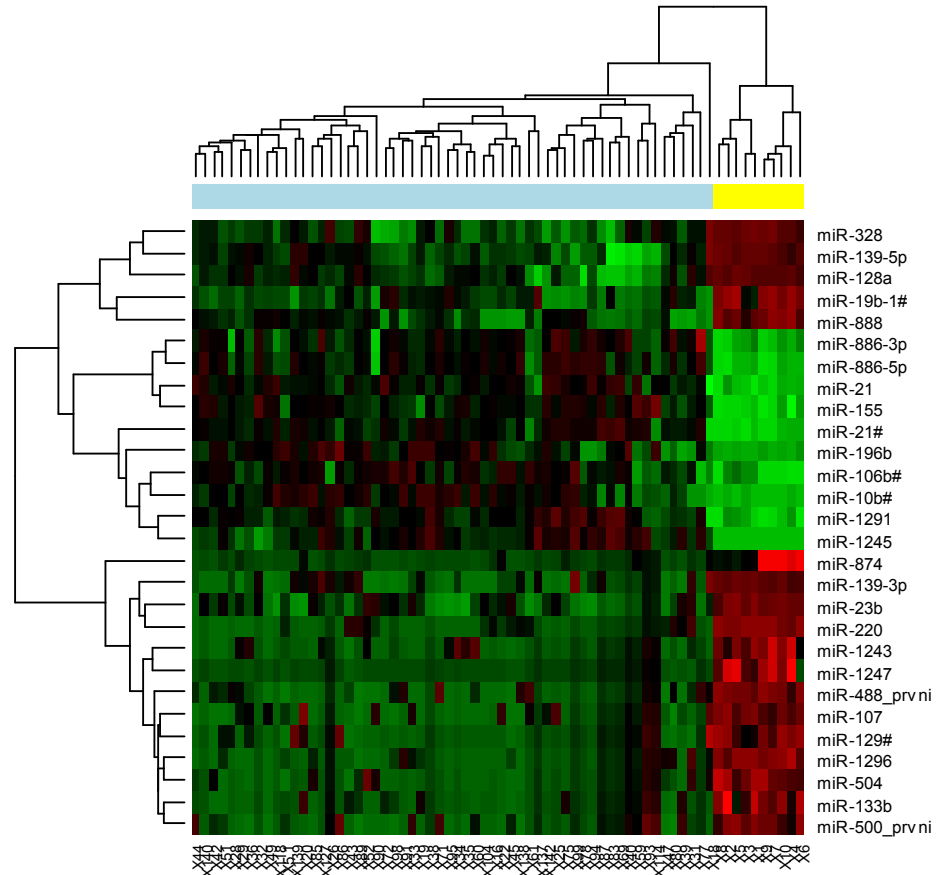
mRNA z FFPE



Yaguang Xi, RNA, 2007, 13:1-7

MikroRNA rozdílně vyjádřené v nádorové tkáni glioblastomu

	n = 58	%
Age		
Median (ranges)	56 (24 - 71)	
≤50	18	31
>50	40	69
Gender		
Male	34	59
Female	24	41
Resection		
Total	9	15
Subtotal	41	71
Parcial	8	14
Performance status		
0	4	7
1	48	83
2	6	10
Adjuvant TMZ		
Yes	31	53
No	27	47



$p < 0.00001$

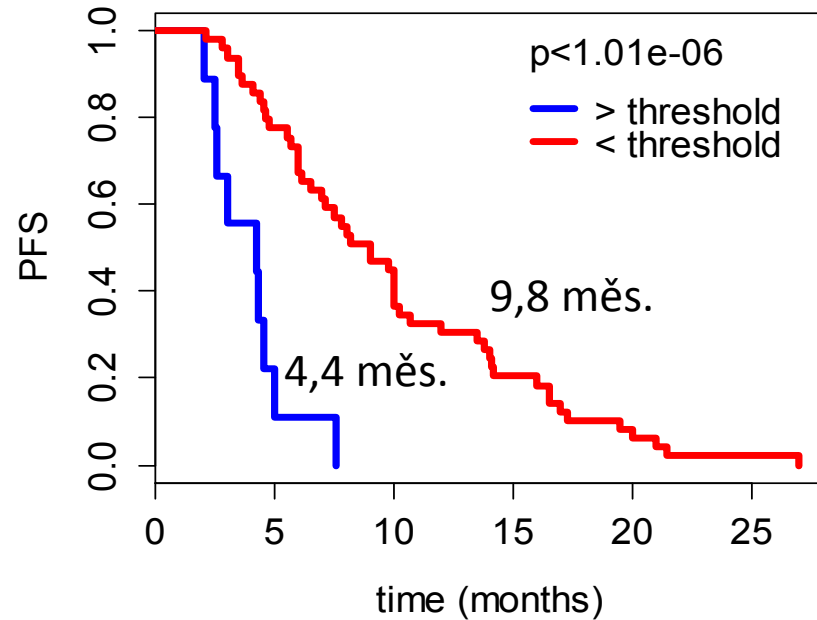
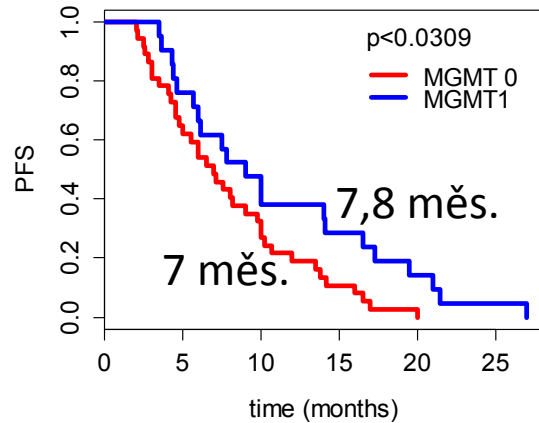


Jiří Šána



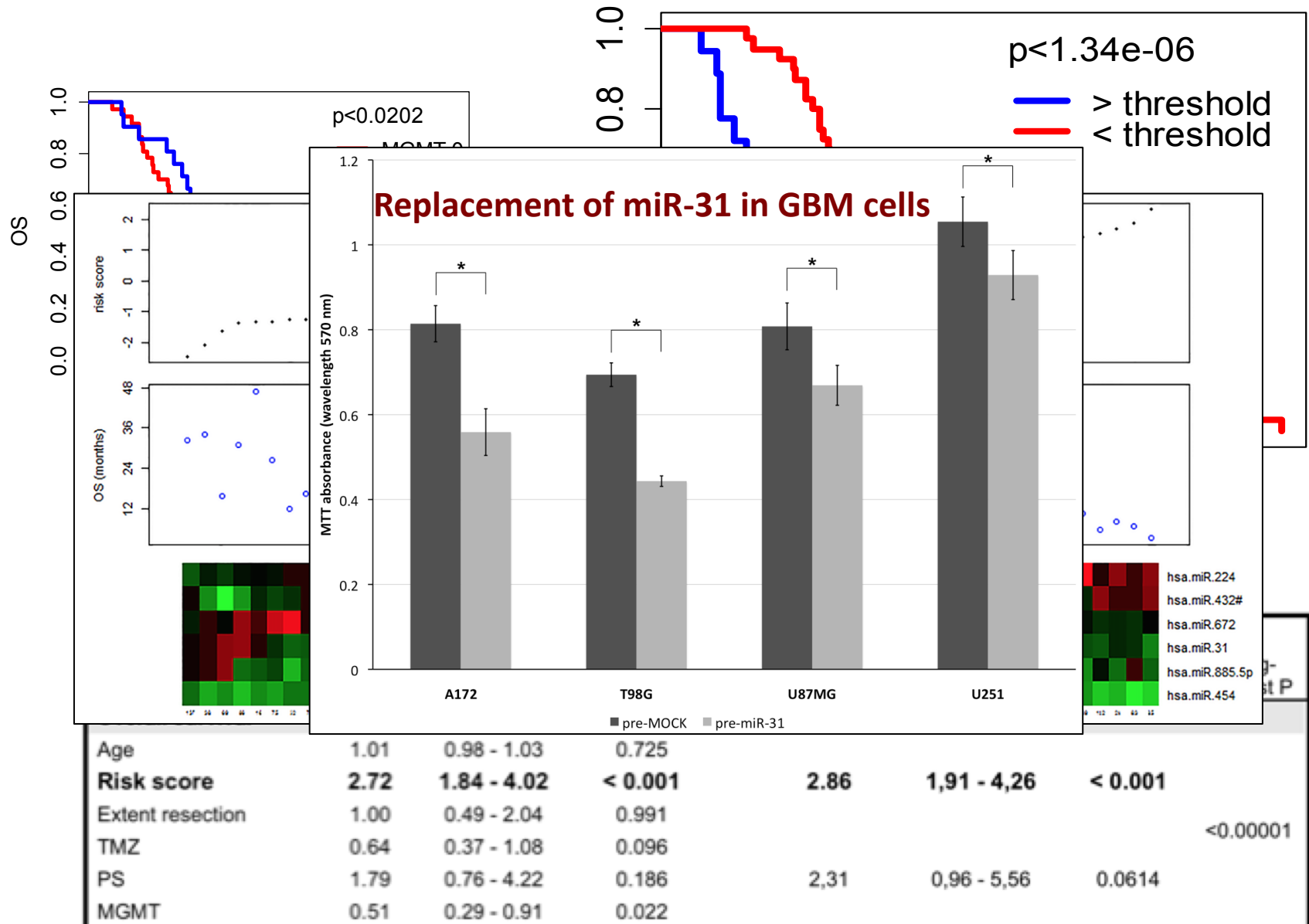
Andrej Bešše

Prognostická sada 6-miRNA u pacientů s glioblastomem

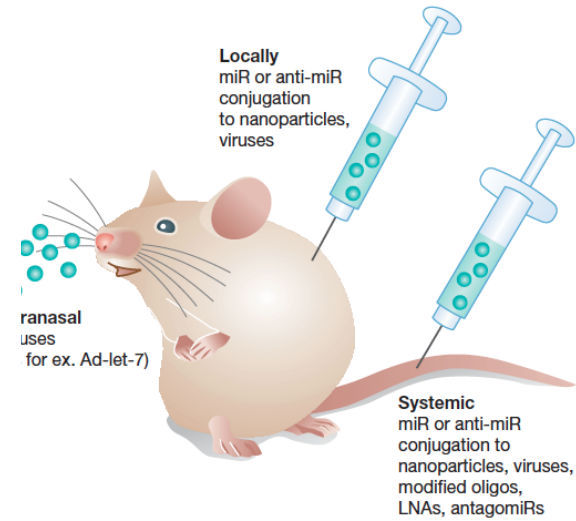
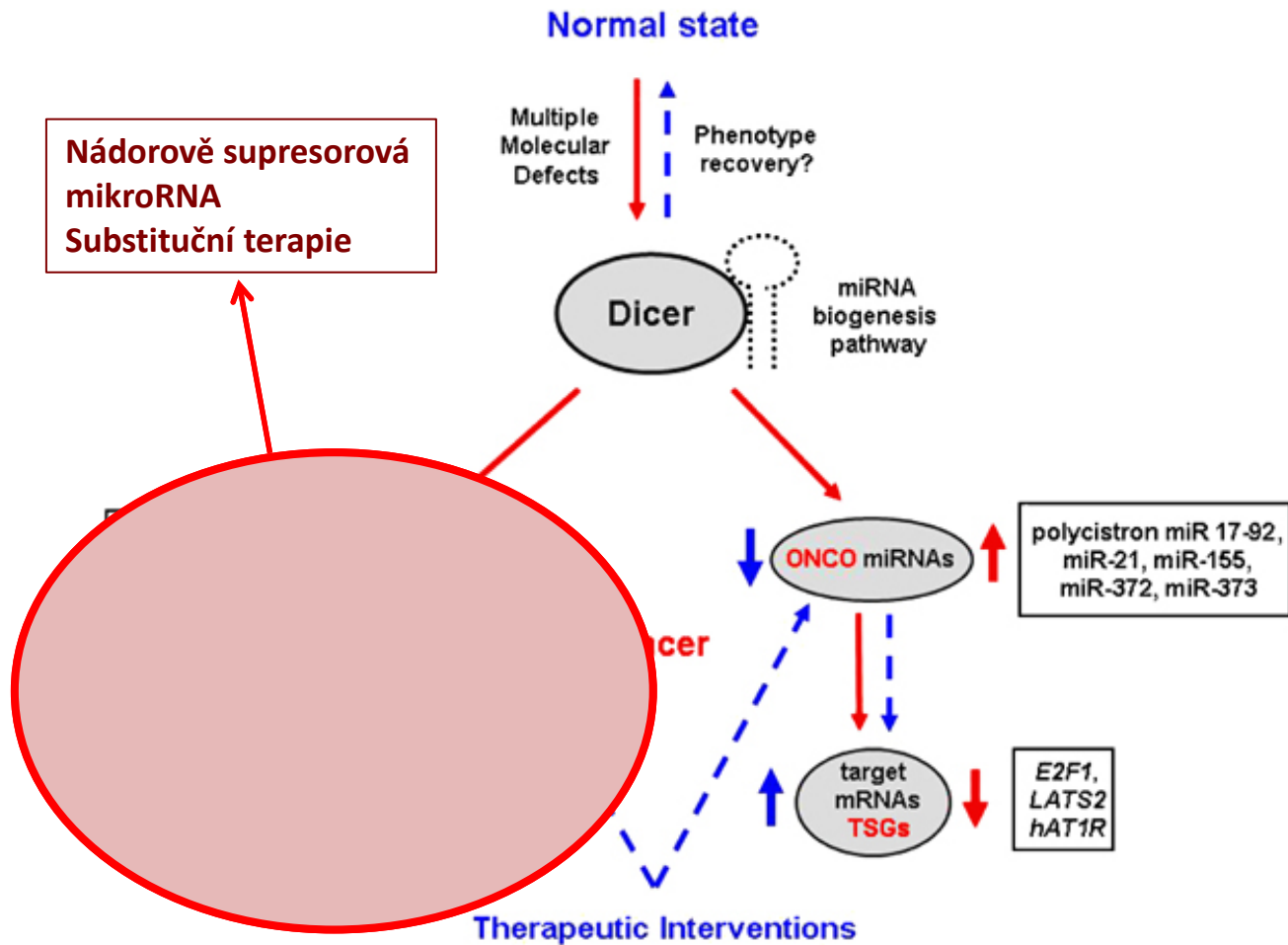


Factors	Univariate Cox Regression			Multivariate Cox Regression			Long-rank test P
	HR	95% CI	P value	HR	95% CI	P value	
Progression free survival							
Age	1.02	0.99 - 1.05	0.222				
Risk score	2.72	1.67 - 4.42	<0.001	1.97	1.33 - 2.94	< 0.001	
Extent resection	1.52	0.74 - 3.13	0.260				<0.00001
TMZ	0.48	0.28 - 0.82	0.007	0.56	0.32 - 0.97	0.04	
KPS	1.69	0.70 - 4.04	0.242	2.16	0.88 - 5.30	0.09	
MGMT	0.54	0.23 - 0.96	0.035				

Prognostická sada 6-miRNA u pacientů s glioblastomem



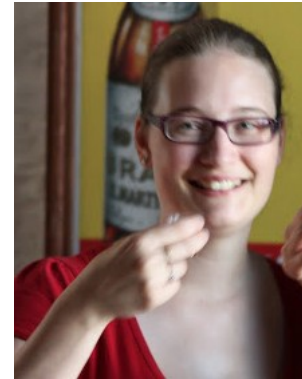
MikroRNA jako terapeutické cíle



Iorio et al., EMBO Mol Med, 2012

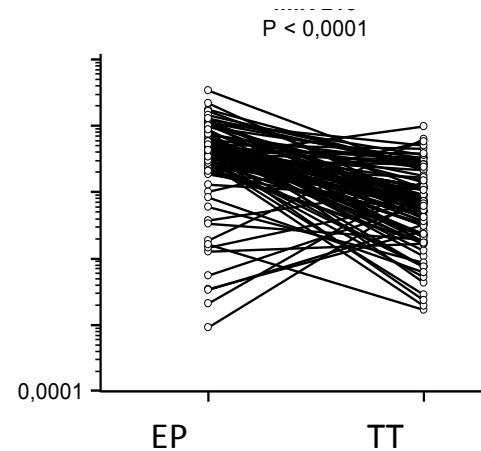
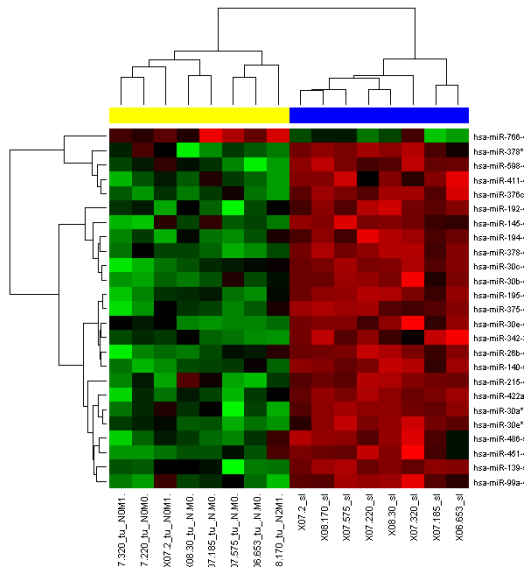
Wurdinger et al., Pharmacogenomics J, 2008

MiR-215 jako nádorový supresor u kolorektálního karcinomu



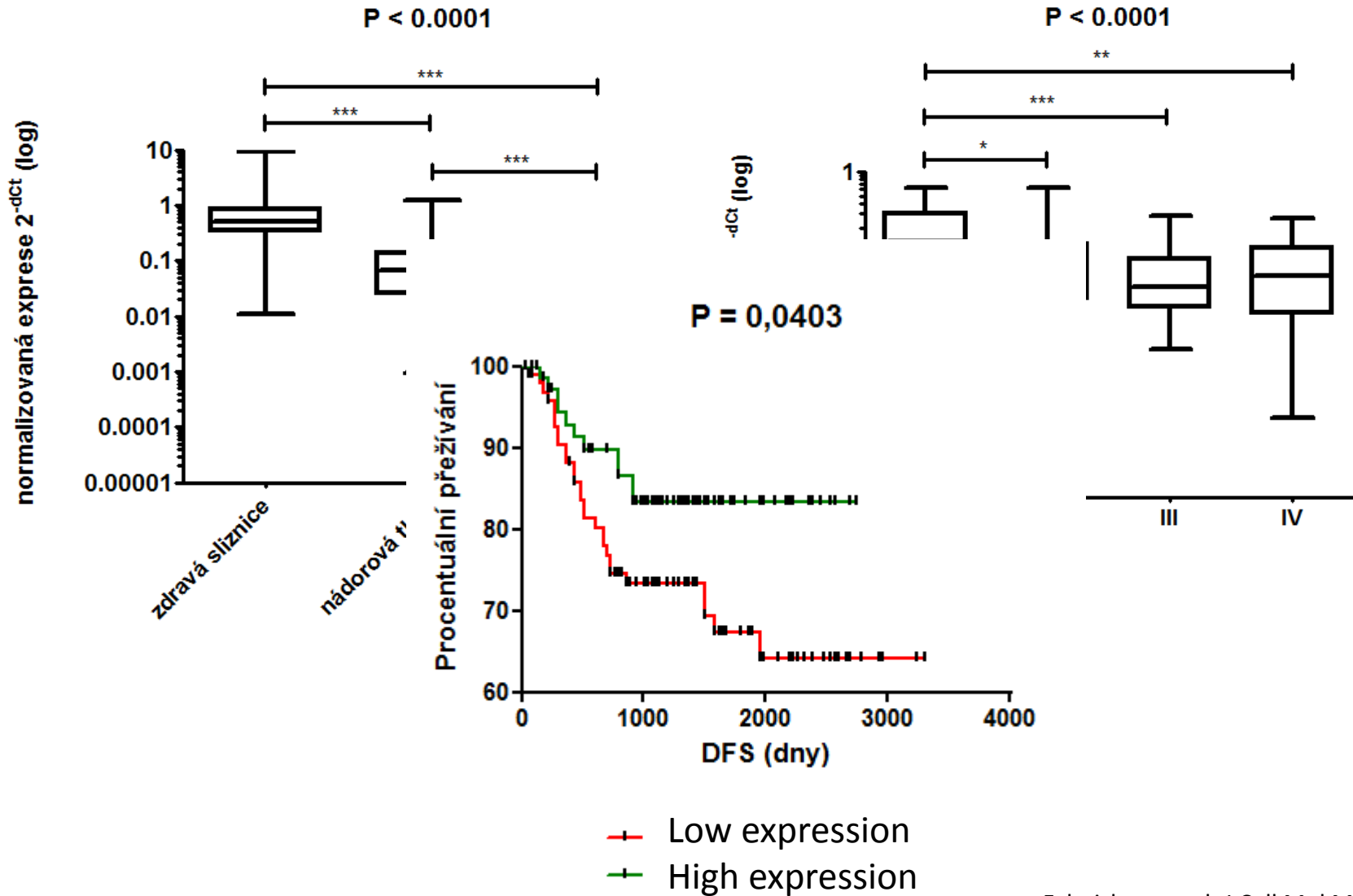
Petra Faltejsková

miR-215

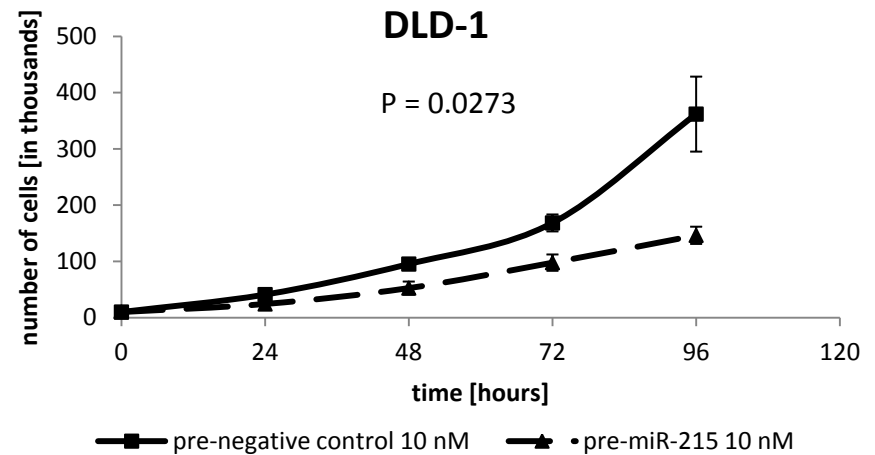
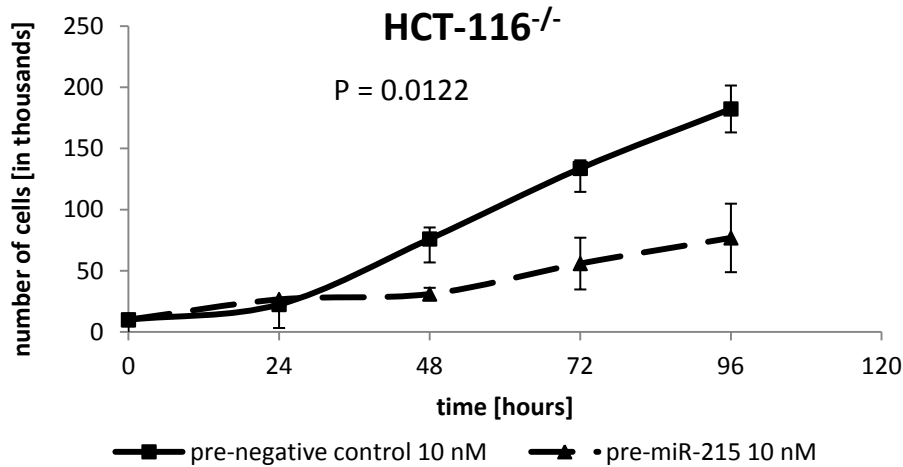
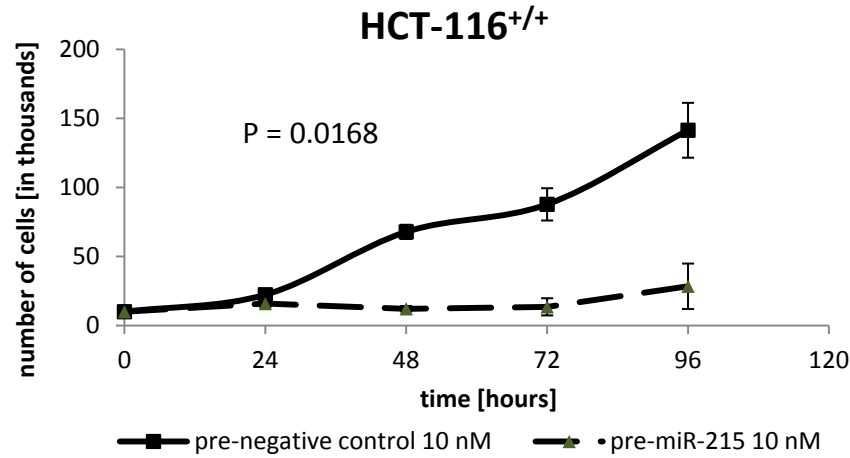


N=240 párových vzorků (tumor vs. non-tumoral colon tissue)

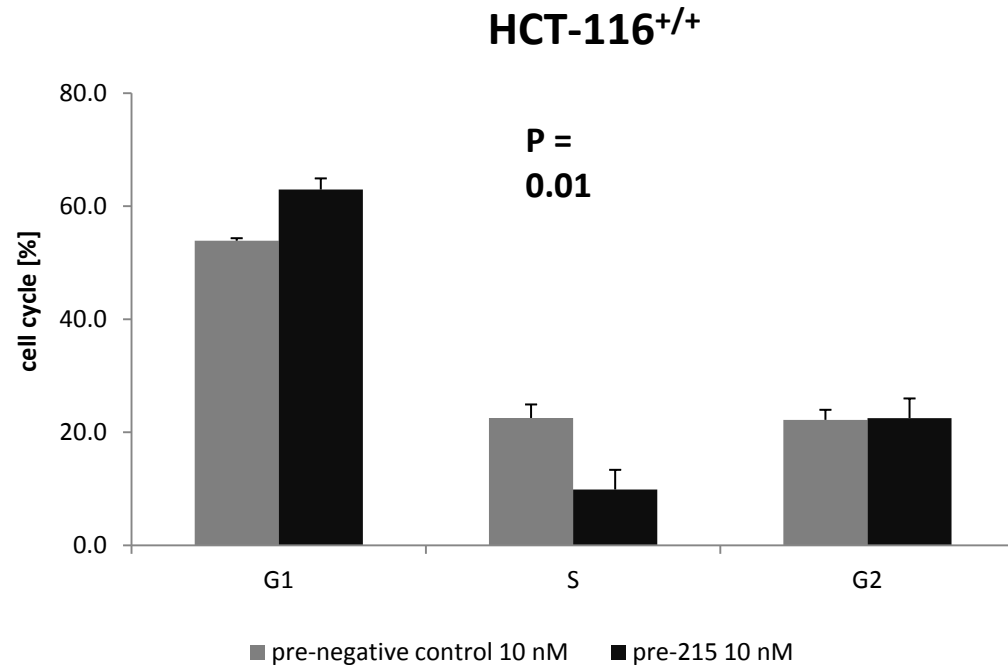
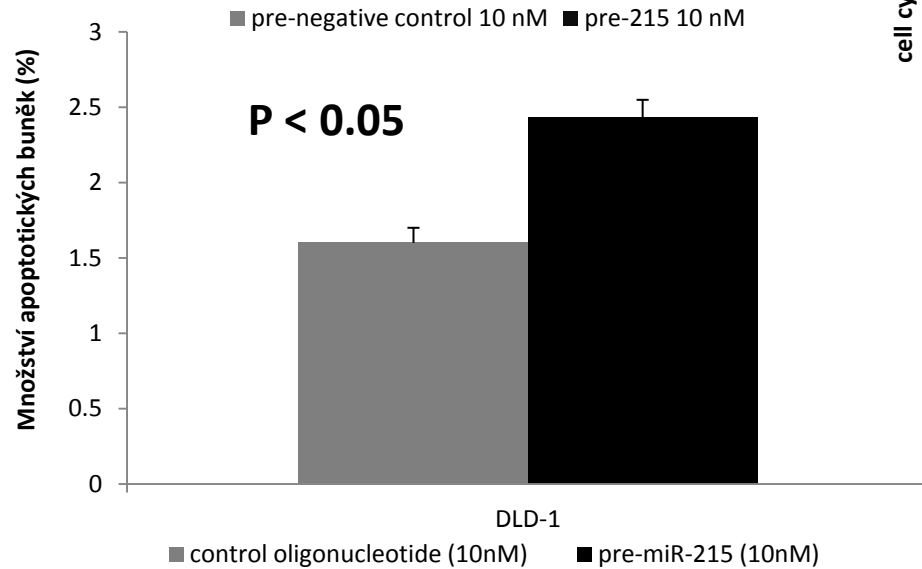
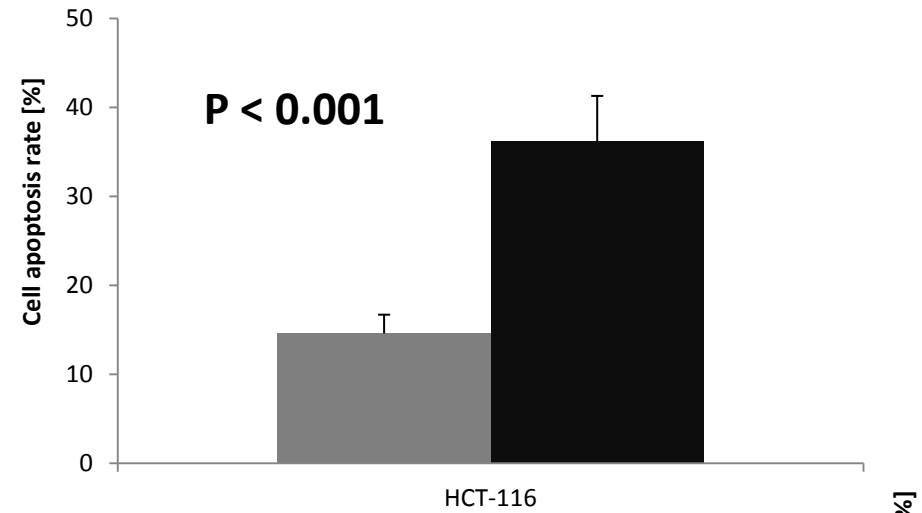
MiR-215 koreluje s klinickým stádiem a bezpříznakovým přežíváním u pacientů s CRC



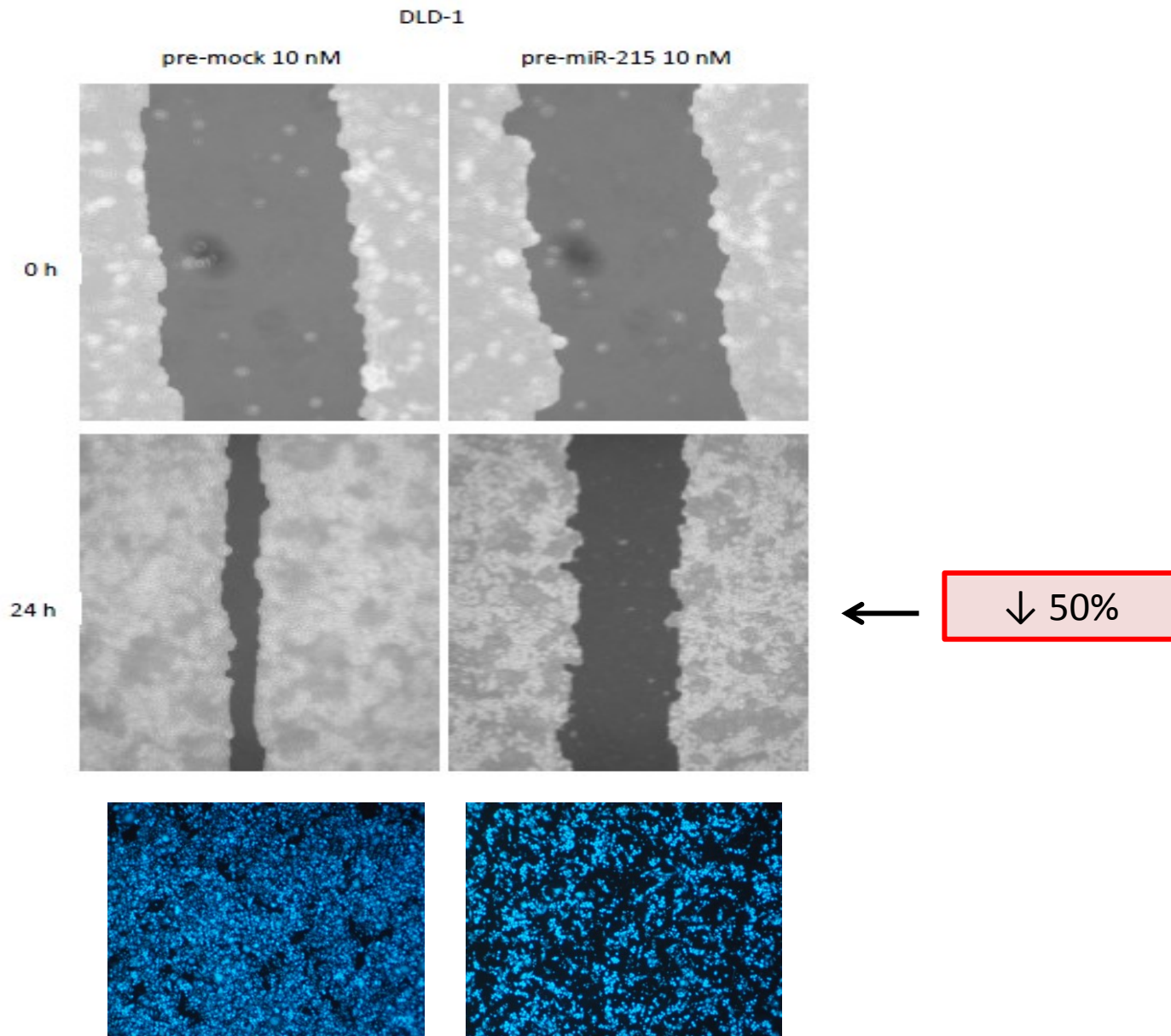
Navýšení hladin miR-215 tlumí proliferaci buněk odvozených od CRC



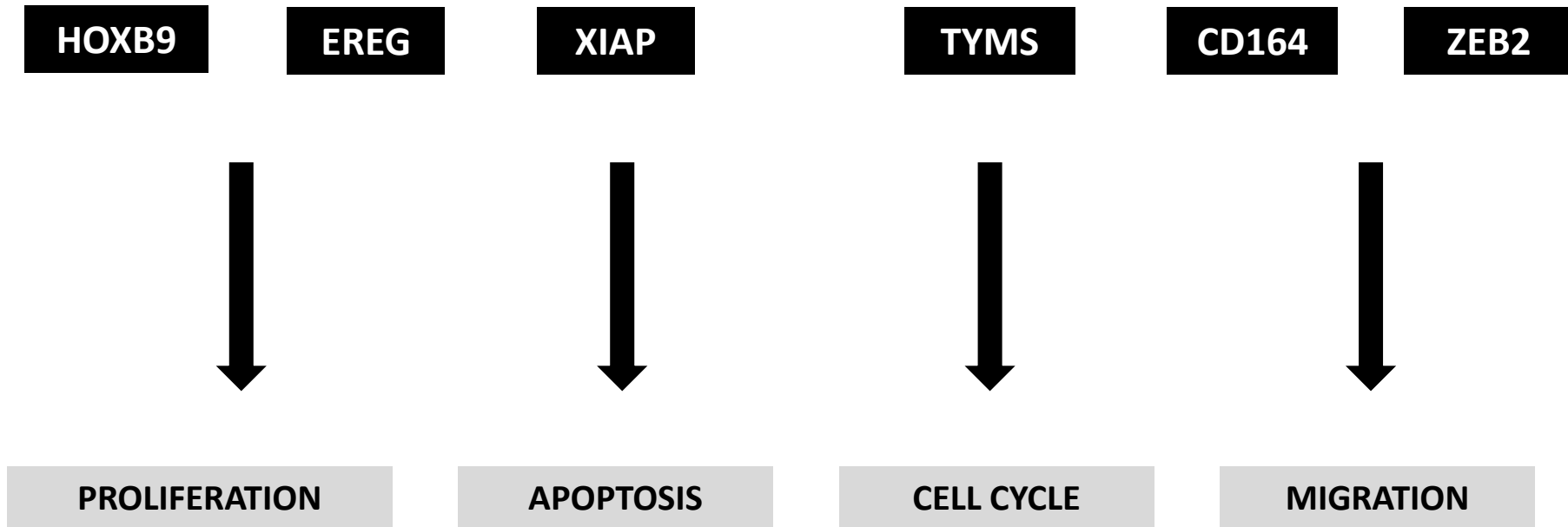
MiR-215 indukuje apoptózu a zástavu buněčného cyklu v G1 fázi



MiR-215 snižuje migrační kapacitu nádorových buněk

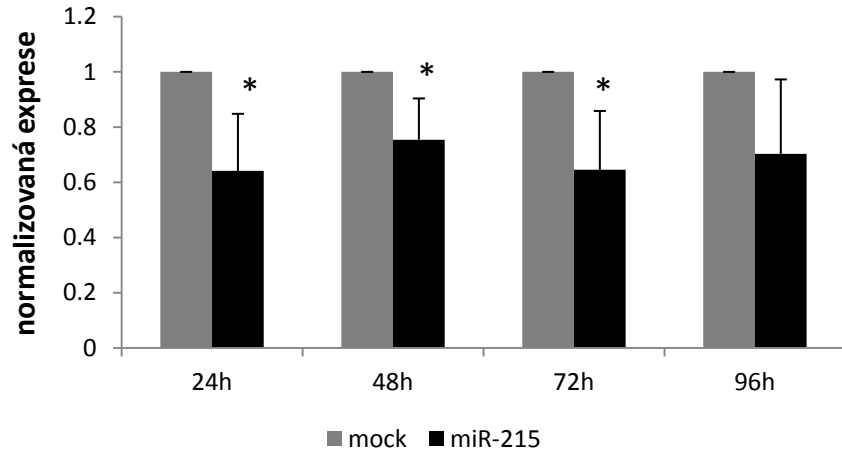


Jaké mRNA jsou pomocí miR-215 regulovány???

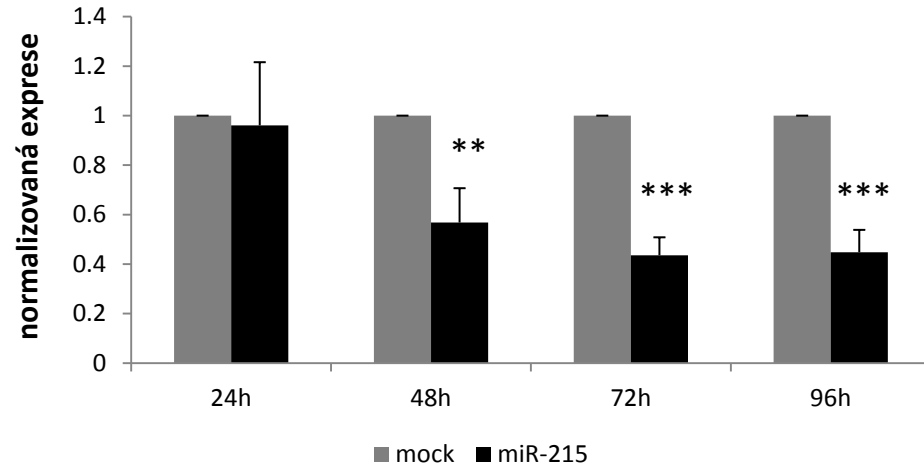


Jaké mRNA jsou pomocí miR-215 regulovány???

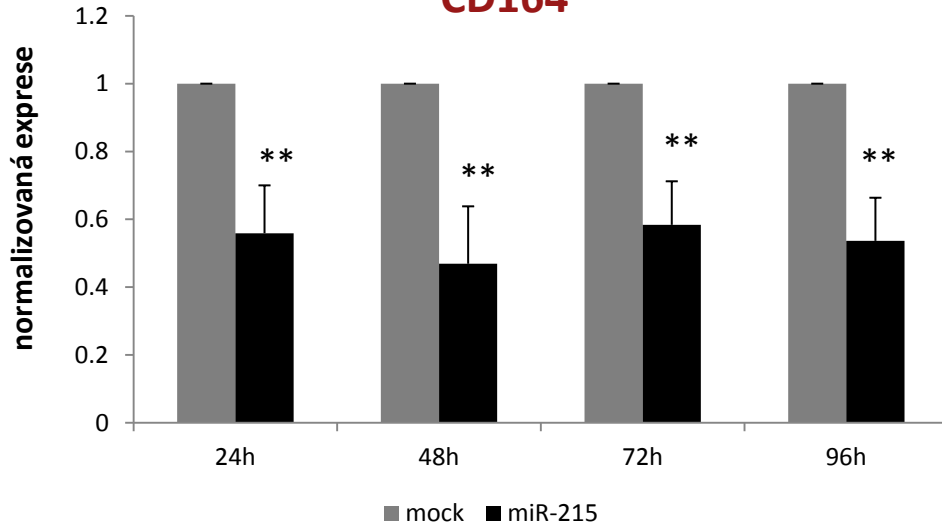
XIAP



TYMS

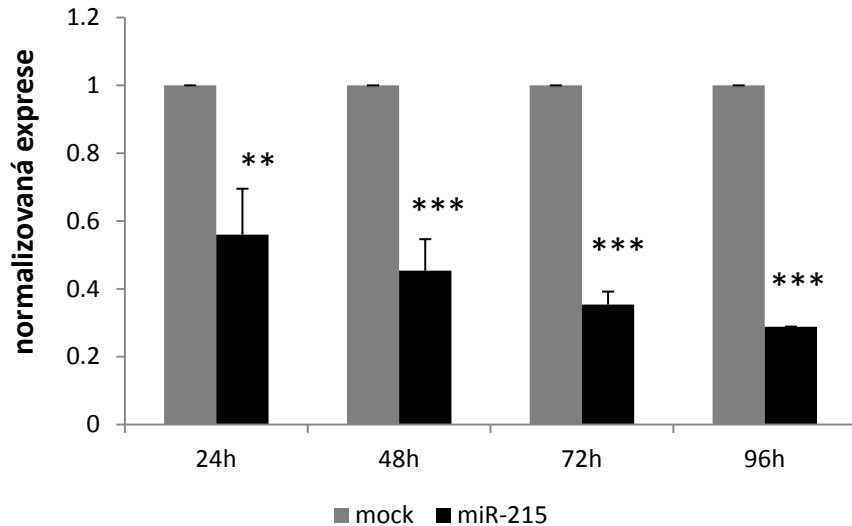


CD164

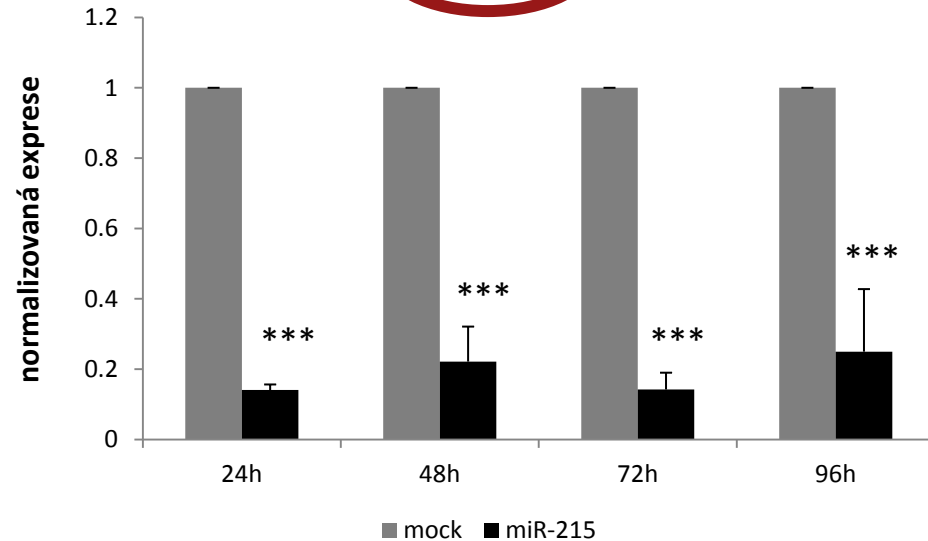


Jaké mRNA jsou pomocí miR-215 regulovány???

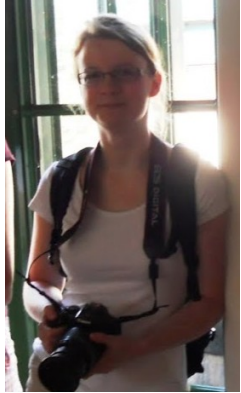
HOXB9



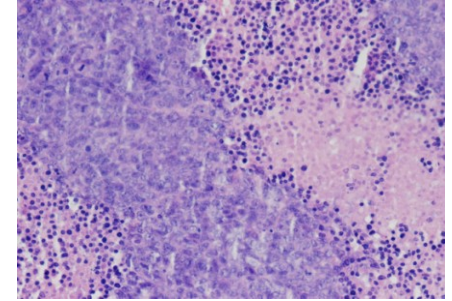
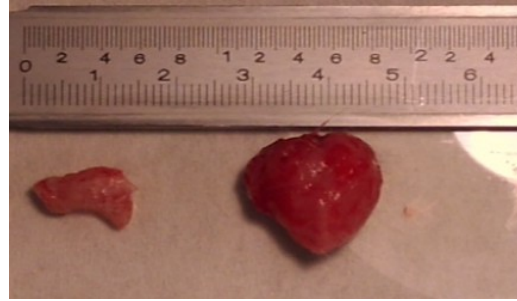
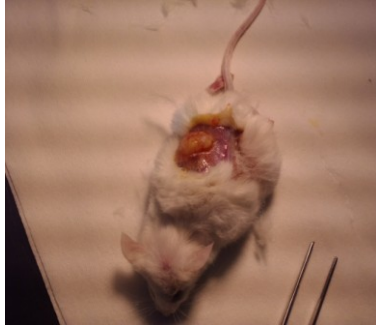
EREG



MiR-215 ovlivňuje růst nádoru in vivo

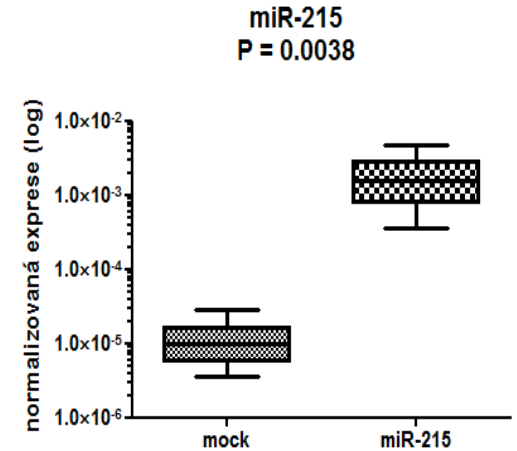
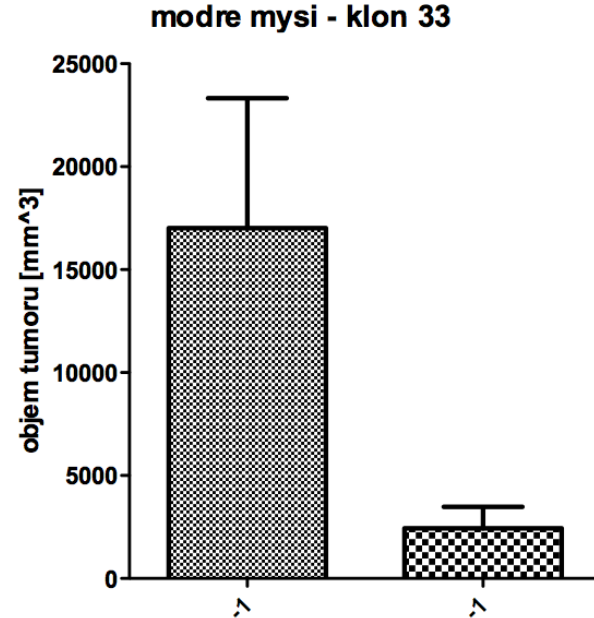


Jana
Merhautová



HCT-116^{+/+} (clone 33)

N=5



MikroRNA jako terapeutické cíle

Seznam mikroRNA které jsou testovány jako potenciální terapeutické cíle

MikroRNA	Indikace	Společnost	Validace cíle	Optimalizace vůdčí struktury	Pre-klinické testování	Fáze 1	Fáze 2
Inhibiční strategie							
miR-122*	hepatitida typu C	Santaris Pharma	X	X	X	X	X
miR-21	multiformní glioblastom	Regulus Therapeutics	X	X	X		
miR-21	hepatocelulární karcinom	Regulus Therapeutics	X	X	X		
miR-21	renální fibróza	Regulus Therapeutics	X	X	X		
miR-33a/b	ateroskleróza	Regulus Therapeutics	X	X	X		
miR-208/miR-499	chronické srdeční selhání	miRagen Therapeutics	X	X	X		
miR-15/miR-195	remodelace po infarktu myokardu	miRagen Therapeutics	X	X	X		
miR-451	polycythemia vera	miRagen Therapeutics	X	X	X		
Substituční strategie							
miR-34	hepatocelulární karcinom fáze 1 – první čtvrtletí 2013	miRNA therapeutics	X	X	X	X	
miR-34	nemalobuněčný karcinom plic	miRNA therapeutics	X	X	X		
miR-34	karcinom prostaty	miRNA therapeutics	X	X	X		
let-7	nemalobuněčný karcinom plic	miRNA therapeutics	X	X	X		
miR-29	srdeční fibróza	mirage Therapeutics	X	X			

MikroRNA ja ko terapeutické cíle

Miravirsen, anti-miR-122

Léčivou látkou je nový LNA-antimiR™ oligonukleotid s přiřazeným mezinárodním nechráněným názvem (INN) miravirsen sodium. Léčivá látka je vyráběna jako sodná sůl. Miravirsen sodium je hygroskopický, bílý až lehce nažloutlý prášek rozpustný ve vodě. Sumární vzorec a molekulová hmotnost jsou uvedeny níže.

Sumární vzorec (protonovaná forma): $C_{156}H_{181}N_{49}Na_{14}O_{83}P_{14}S_{14}$
($C_{156}H_{195}N_{49}O_{83}P_{14}S_{14}$)

Molekulová hmotnost (protonovaná forma): 5,274 8 g/mol (4,967 g/mol)

Název molekuly: SPC3649

INN: miravirsen sodium

Miravirsen je 15nukleotidový (15-mer) fosforothioatový oligonukleotid, tvořený -D-oxy-LNA a DNA monomery. Molekula miravirsenu obsahuje osm nukleotidů s LNA modifikací a sedm DNA nukleotidů uspořádaných v následující sekvenci:

5'- mC c A t t G T c a mC am C t mC mC -3'

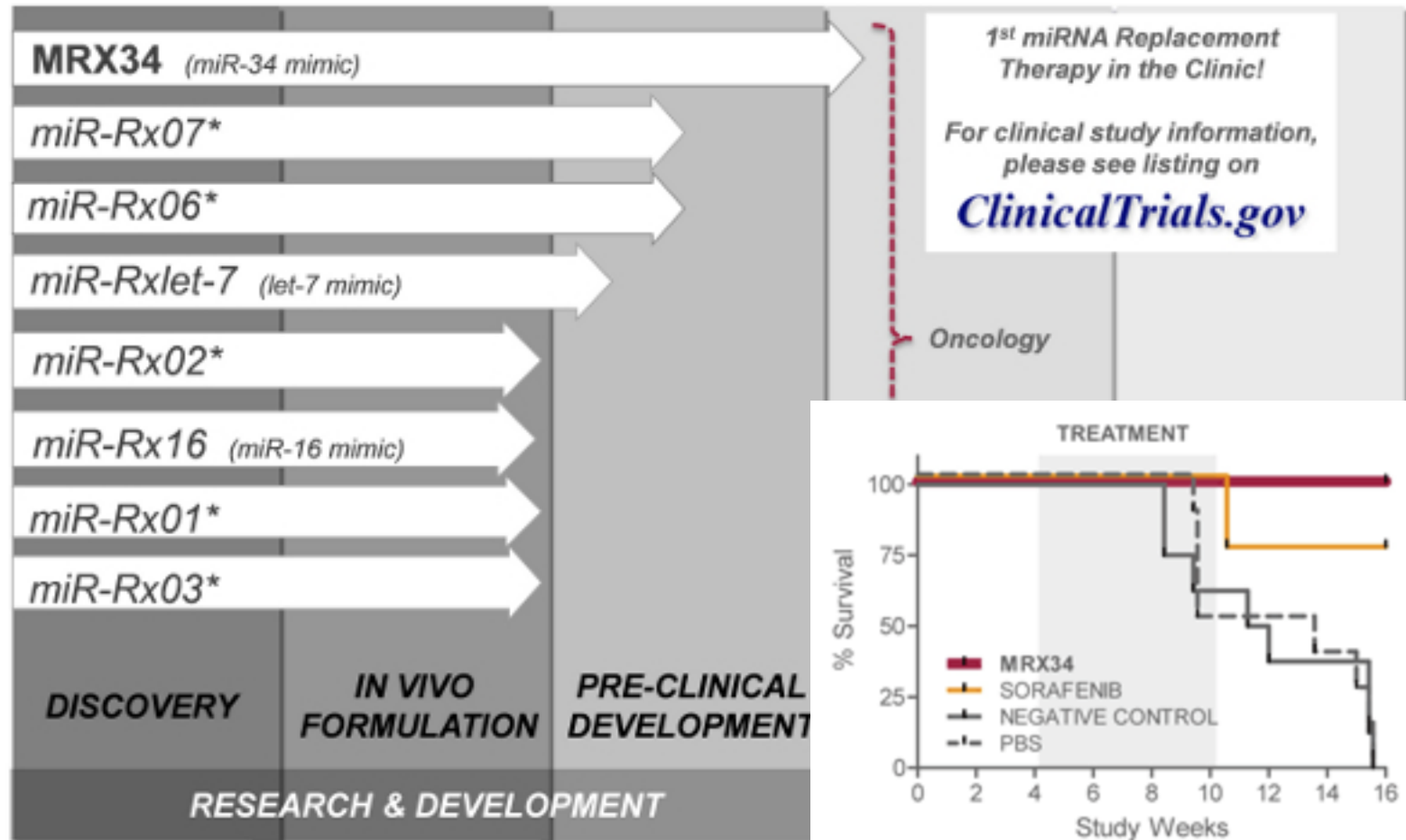
Velká písmena označují nukleotidy s LNA modifikací (mC značí LNA-5-methylcytidin) a malými písmeny jsou označeny nukleotidy DNA. LNA modifikace významně zvyšuje vazebnou afinitu molekuly k cílové miR-122 a také její biologický poločas. Všechny vazby mezi nukleotidy u miravirsenu, celkem 14, mají charakter fosforotioátu, což zlepšuje farmakokinetické vlastnosti oligonukleotidu.

Miravirsen (SPC3649)
A New Treatment Targeting Hepatitis C

Delivering on the promise of RNA medicines today

MikroRNA jako terapeutické cíle: from bench to bedside

MRX34 – hepatocelulární karcinom a jaterní metastázy



* miRNA mimics undisclosed

Poděkování CEITEC, Skupina Molekulární onkologie II



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Prof. MUDr. R.
Doc. MUDr. J.
MUDr. Radek L.

FN Brno

prof. MUDr. Z.

MUDr. Jan Hlavsa, Ph.D.,..... FNUSA BRNO – prof. MUDr. Marketa Hermanova, Ph.D., doc. MUDr. Radim Jancaček, Ph.D.