1° Meeting Italiano Internazionale Congiunto
IPOHA Onlus e Fop Italia Onlus

1st International Italian Joint Meeting on POH and FOP

20-21 marzo 2009
Cerignola (FG)

Renata Bocciardi
S.C. Genetica Molecolare e Citogenetica
Laboratorio di Genetica Molecolare
Istituto G. Gaslini
Genova

Summary of the clinical features and molecular analysis of the presented FOP patients set.

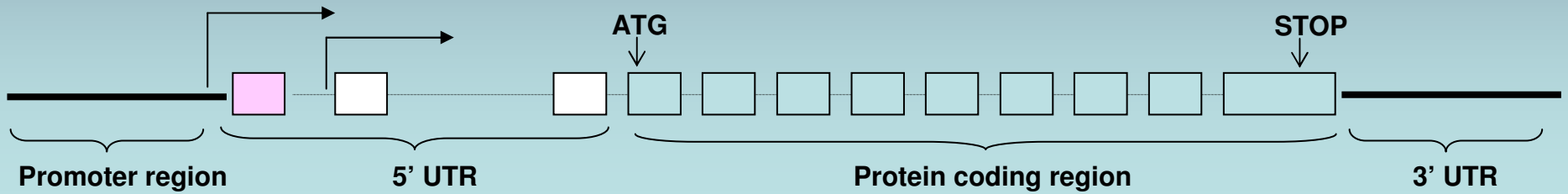
	Pt	Age	Sex	Age Ossification onset	Great toe malf.	Mutation	Other
1	FOP1	33 y	M	na	y	c.617G>A, nd	
2	FOP2	5 y	M	15 months	y	c.617G>A, *de novo*	Hydrocephalus secondary to a posterior bulbar expansive lesion of unknown origin (surgical correction)
3	FOP3	10 y	F	4 y	y	c.617G>A, *de novo*	
4	FOP4	5 y	F	20 months	y	c.617G>A, *de novo*	
5	FOP5	n.a.	M	8 y	y	c.617G>A, nd	
6	FOP6	29 y	F	6 y	y	c.617G>A, nd	
7	FOP7	28 y	F	11 y	y	c.617G>A, nd	
8	FOP8	50 y	F	11 y	y	c.617G>A, nd	
9	FOP9	45 y	M	3 y	y	c.617G>A, nd	
10	FOP10	36 y	M	14 y	y	c.617G>A, nd	
11	FOP12	9 y	M	6 months	y	c.617G>A, nd	
12	FOP13	22 y	F	4 y	No	c.774G>C, *de novo,* R258S	c.44C>G dbSNP rs13406336, inherited
13	FOP14	49 y	M	5 y	y	c.617G>A, nd	Episodic seizures
14	FOP15	37 y	M	4	y	c.617G>A, nd	
15	FOP17	9 y	M	6 y	y	c.617G>A, nd	
16	FOP18	17 y	F	na	Short hallux	c.617G>A, nd	Fusion of C4-C5, osseous dysplasia
17	FOP20	44 y	F	14 y	No	c.774G>C, *de novo,* R258S	Vitamin D deficiency, thyroid goiter
18	FOP22	38 y	F	9 months	y	c.617G>A, nd	
19	FOP23	11 y	M	5 y	hypoplasic	c.617G>A, nd	Neck stiffness, NOGGIN neg
20	FOP24	13 y	M	13 y	y	c.619C>G, *de novo,* Q207E	Malformed shortened thumbs

Pt, patient code; nd, mutation origin not determined; na, not available; 26 patients have been screened, 20 with confimation of the clinical diagnosis, see table, 6 with exclusion of FOP

Study of the regulation of *ACVR1* expression

Characterization of the structure and composition of the *ACVR1* transcripts.

Definition and characterization of genomic regions playing a role in the regulation of *ACVR1* expression, such as gene promoter/s and/or other sequences with regulatory activity.



Transcriptional regulation
- ✓**Promoter**
- ✓**Enhancer/silencer**

transcription

mRNA 1
mRNA 2

translation

Post-transcriptional regulation
- ➢**Splicing**
- ➢**mRNA stability/availability**
- ➢**Small RNA**
- ➢**Effect on translation**

protein

✓Characterization of the structure and composition of the *ACVR1* transcripts.

Definition and characterization of sequences playing a role in the regulation of *ACVR1* expression.

- **5'*UTR* sequences**
- Promoter region
- **3'*UTR* region**

Characterization of the structure and composition of the *ACVR1* transcripts
How many are ACVR1 transcripts?

```
tgttgctgcc tggttaatca cagctccaga cactcattgg ccgagcgctc  158441139
cgcagccact tcctcctcct cctcctcccg ctcctcccgc tccggctcct  158441089
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AACTTTTCTA CTCTCTGTGA CGGTCTCCCC CGCCGCCCCC GGGGGCAAGC  158440989
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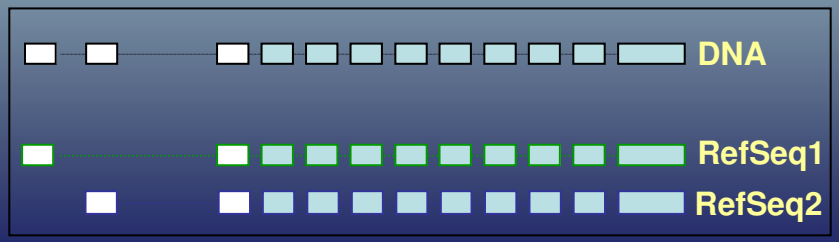
EST
CN481300

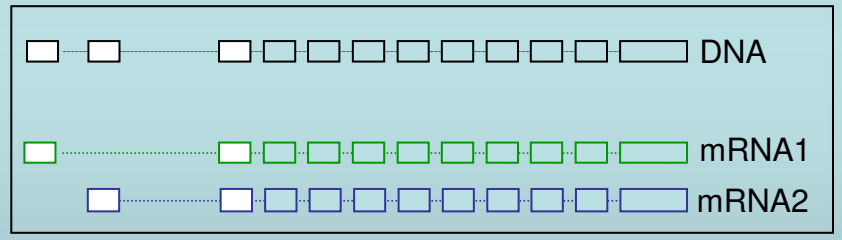
RefSeq 1
NM_001111067.1

RefSeq 2
NM_001105.4

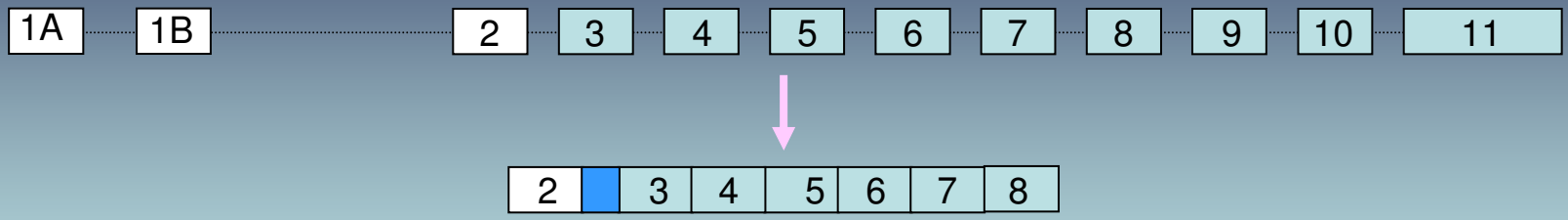


Two main *ACVR1* transcripts are detectable in cells expressing the gene and are present in Gene Bank.

These transcripts share an untranslated exon 2 and all the protein coding sequences, but show an alternative exon 1 probably due to the presence of two different transcription start sites.



However, we have identified a new transcript isoform showing an insertion of 116 bp between the untranslated exon 2 and first protein coding exon.

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CAGATGTGAC CAAGAGCCTG CATTAAGgta gctgagtaag ctttacccag 158383120
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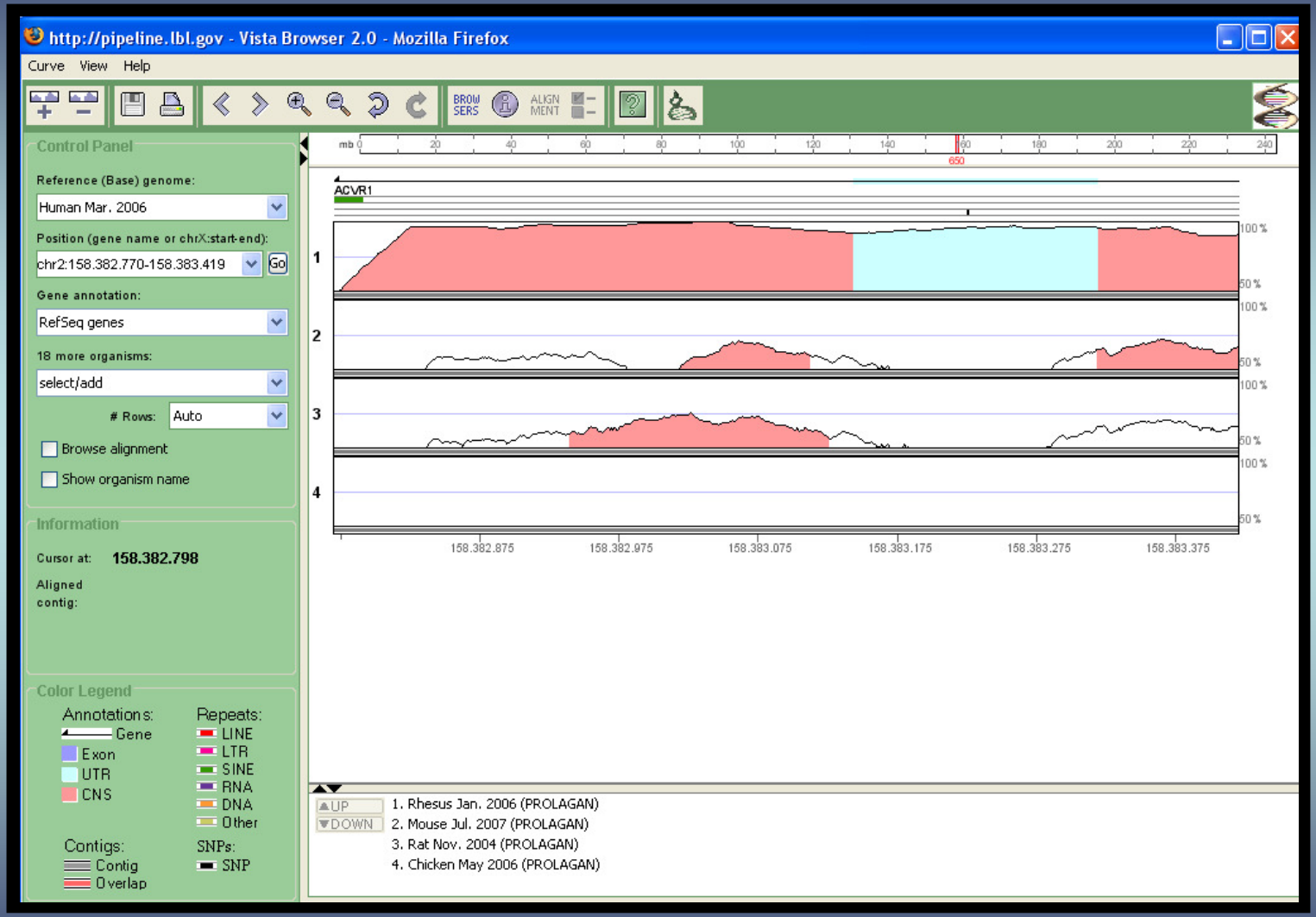
cactcttttt ataataaata tttggtgatt attgactatt ttgaaggaaa  158364039
```

This isoform is due to an alternative splicing leading to the incorporation of 116 bp of the 5’end of intron 3 in the mRNA.

The inserted sequence is highly conserved among species and is even more conserved than the adjacent exon.



This sequence can have a role at post-transcriptional level and we are currently investigating in this direction.

Characterization of the structure and composition of the *ACVR1* transcripts.

✓**Definition and characterization of sequences playing a role in the regulation of *ACVR1* expression.**

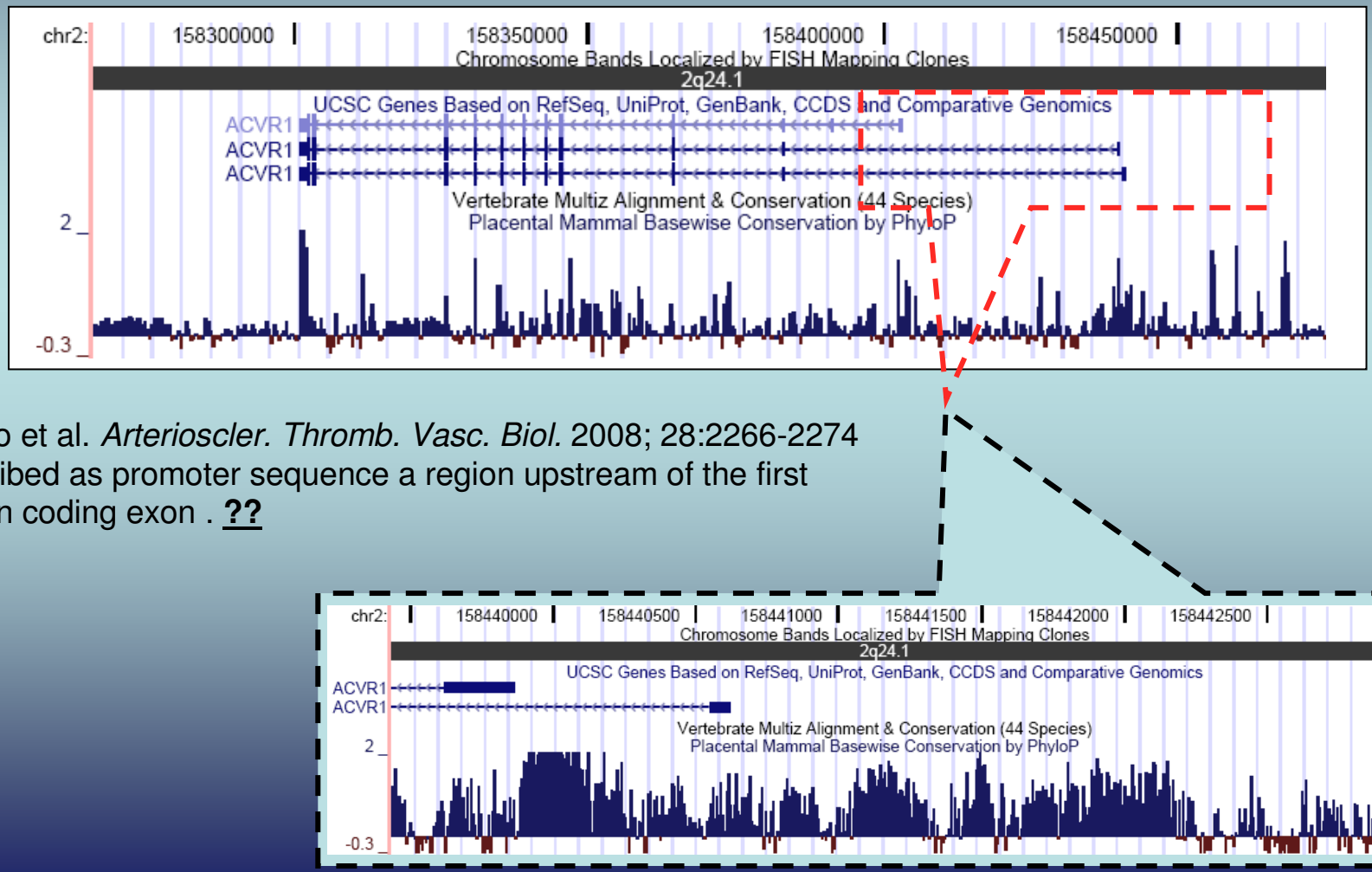
➢5'*UTR* sequences

➢**Promoter region**

✓**3'*UTR* region**

Identification and characterization of *ACVR1* promoter region

We are currently focusing on a region of 3400 bp upstream the *ACVR1* gene and spanning the two alternative first exons



Y. Yao et al. *Arterioscler. Thromb. Vasc. Biol.* 2008; 28:2266-2274
Described as promoter sequence a region upstream of the first protein coding exon . **??**

Characterization of the structure and composition of the *ACVR1* transcripts.

Definition and characterization of sequences playing a role in the regulation of *ACVR1* expression.

- 5'*UTR* sequences
- Promoter region

✓3'*UTR* region

Analysis of the *ACVR1* 3'*UTR*

```
CATTTTCATA GTGTCAAGAA GGAAGATTTG ACGTTGTTGT CATTGTCCAG   50
CTGGGACCTA ATGCTGGCCT GACTGGTTGT CAGAATGGAA TCCATCTGTC  100
TCCCTCCCCA AATGGCTGCT TTGACAAGGC AGACGTCGTA CCCAGCCATG  150
TGTTGGGGAG ACATCAAAAC CACCCTAACC TCGCTCGATG ACTGTGAACT  200
GGGCATTTCA CGAACTGTTC ACACTGCAGA GACTAATGTT GGACAGACAC  250
TGTTGCAAAG GTAGGGACTG GAGGAACACA GAGAAATCCT AAAAGAGATC  300
TGGGCATTAA GTCAGTGGCT TTGCATAGCT TTCACAAGTC TCCTAGACAC  350
TCCCCACGGG AAACTCAAGG AGGTGGTGAA TTTTTAATCA GCAATATTGC  400
CTGTGCTTCT CTTCTTTATT GCACTAGGAA TTCTTTGCAT TCCTTACTTG  450
CACTGTTACT CTTAATTTTA AAGACCCAAC TTGCCAAAAT GTTGGCTGCG  500
TACTCCACTG GTCTGTCTTT GGATAATAGG AATTCAATTT GGCAAAACAA  550
AATGTAATGT CAGACTTTGC TGCATTTTAC ACATGTGCTG ATGTTTACAA  600
TGATGCCGAA CATTAGGAAT TGTTTATACA CAACTTTGCA AATTATTTAT  650
TACTTGTGCA CTTAGTAGTT TTTACAAAAC TGCTTTGTGC ATATGTTAAA  700
GCTTATTTTT ATGTGGTCTT ATGATTTTAT TACAGAAATG TTTTTAACAC  750
TATACTCTAA AATGGACATT TTCTTTTATT ATCAGTTAAA ATCACATTTT  800
AAGTGCTTCA CATTTGTATG TGTGTAGACT GTAACTTTTT TTCAGTTCAT  850
ATGCAGAACG TATTTAGCCA TTACCCACGT GACACCACCG AATATATTAC  900
TGATTTAGAA GCAAAGATTT CAGTAGAATT TTAGTCCTGA ACGCTACGGG  950
GAAAATGCAT TTTCTTCAGA ATTATCCATT ACGTGCATTT AAACTCTGCC 1000
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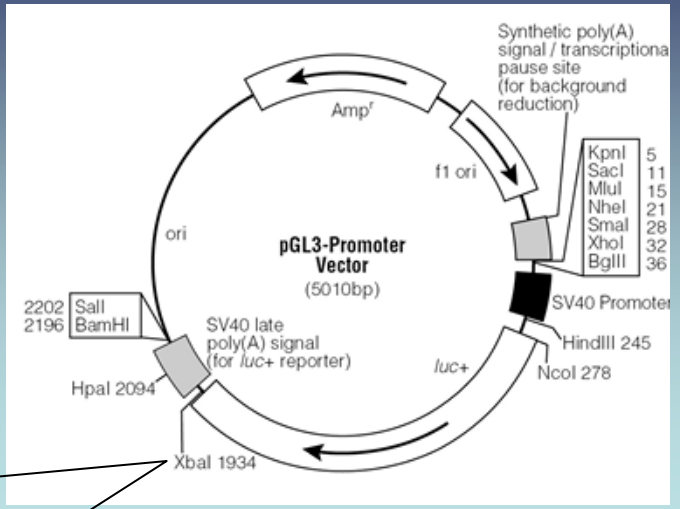
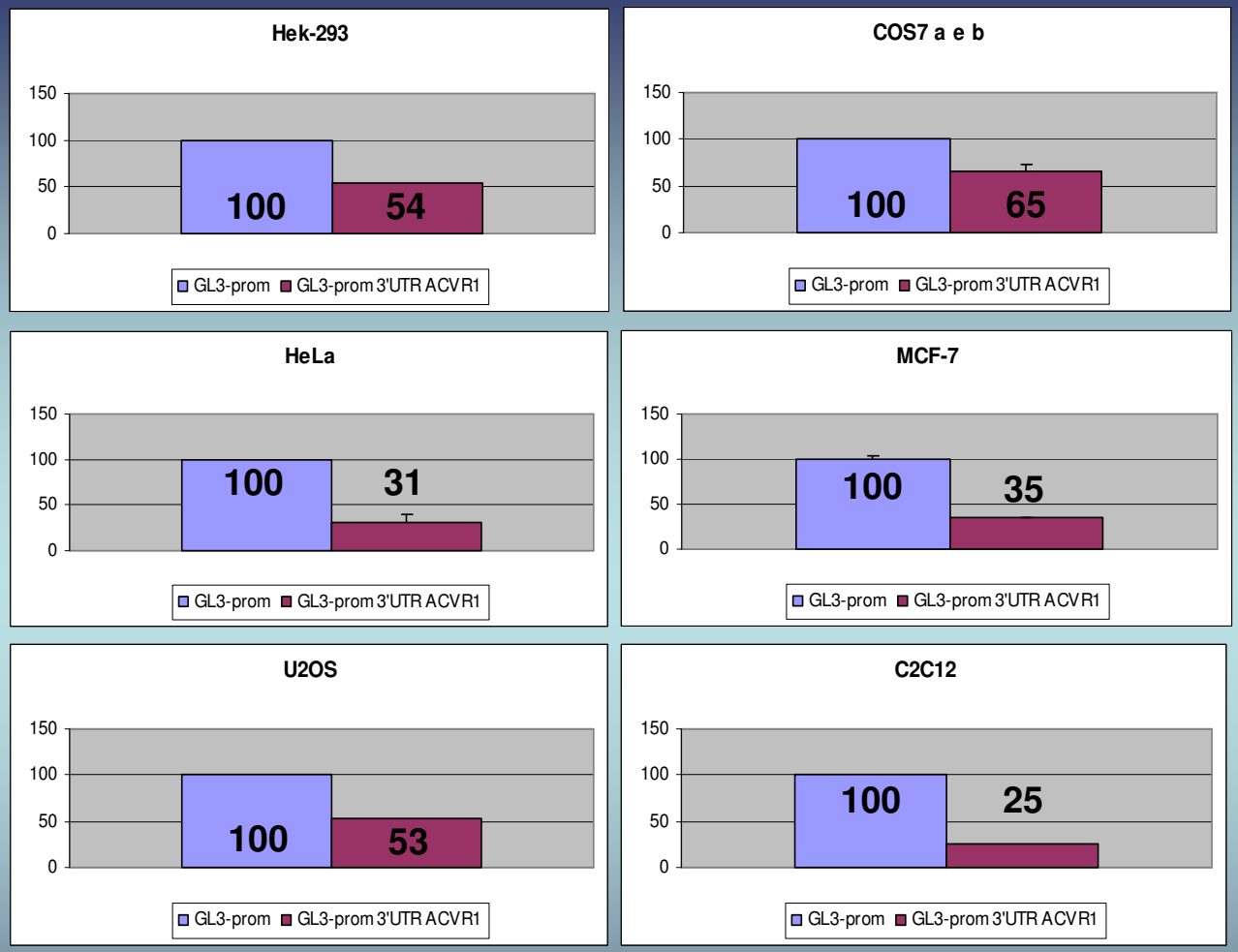


❖**Subcloning of *ACVR1* 3'*UTR* region downstream the coding sequence of the firefly Luciferase reporter gene.**

❖ **Evaluation of the Luciferase activity compared to that of the pGL3-prom empty vector in different cell lines.**

pGL3-promoter vector empty *versus* pGL3-prom *ACVR1* 3'UTR



- The presence of the 3'*UTR* sequence of *ACVR1* causes a decrease in the activity of the reporter gene (Luciferase) in all the tested cell lines.
- 3'*UTR* sequence of *ACVR1* can be target of different mechanisms of post-transcriptional regulation.

The expanding universe of RNA

It is now clear that different levels of regulation in gene expression do exist.

3% coding RNA

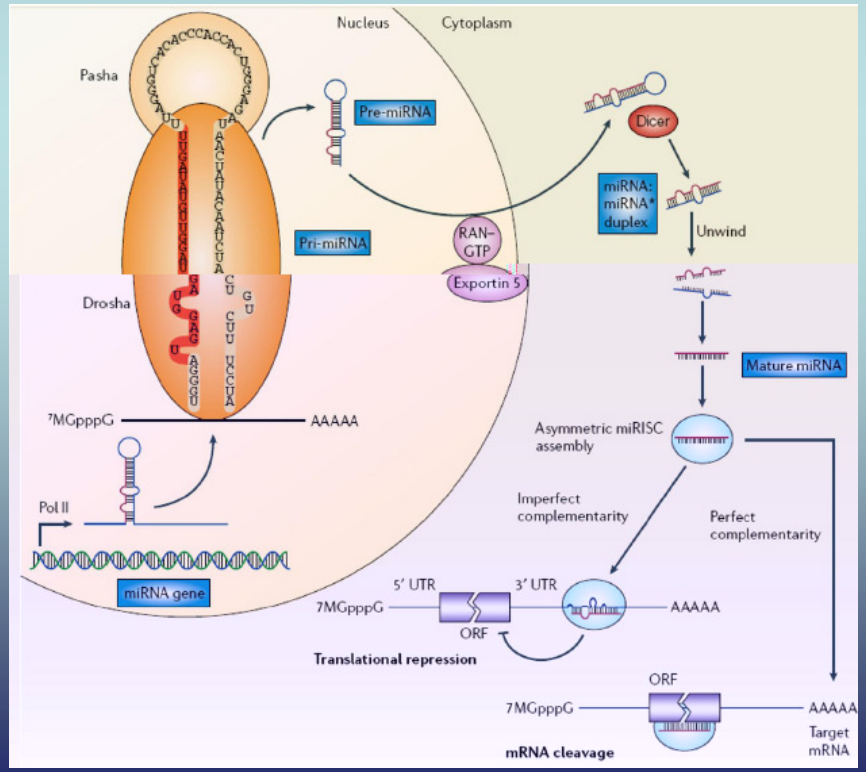
97% non-coding RNA
- housekeeping RNA (rRNA, tRNA, snRNA,…)
- regulatory RNA (scRNA, siRNA, gRNA, microRNA…)

Recently, a growing body of the literature has focused on the role of microRNAs (miRNAs) as crucial players of post-transcriptional regulation of gene expression, both in physiological conditions (development, differentiation, growth, etc.) and in disease (cancer, inflammation, etc.).

microRNA

MicroRNAs are small 20-22 nt non-coding RNA that play important gene-regulatory roles in animals and plants by pairing to the 3'*UTR* mRNAs of protein-coding genes . Around 30% of human genes is expected to be regulated by the action of microRNA.

They have a pleiotropic effect, a single microRNA can modulate several genes.
A single gene can be targeted by several microRNAs involved in the fine tuning of its expression.



microRNAs can bind the 3'*UTR* sequences of protein coding genes at specific sites

Gene silencing
✓mRNA degradation
✓Translational repression

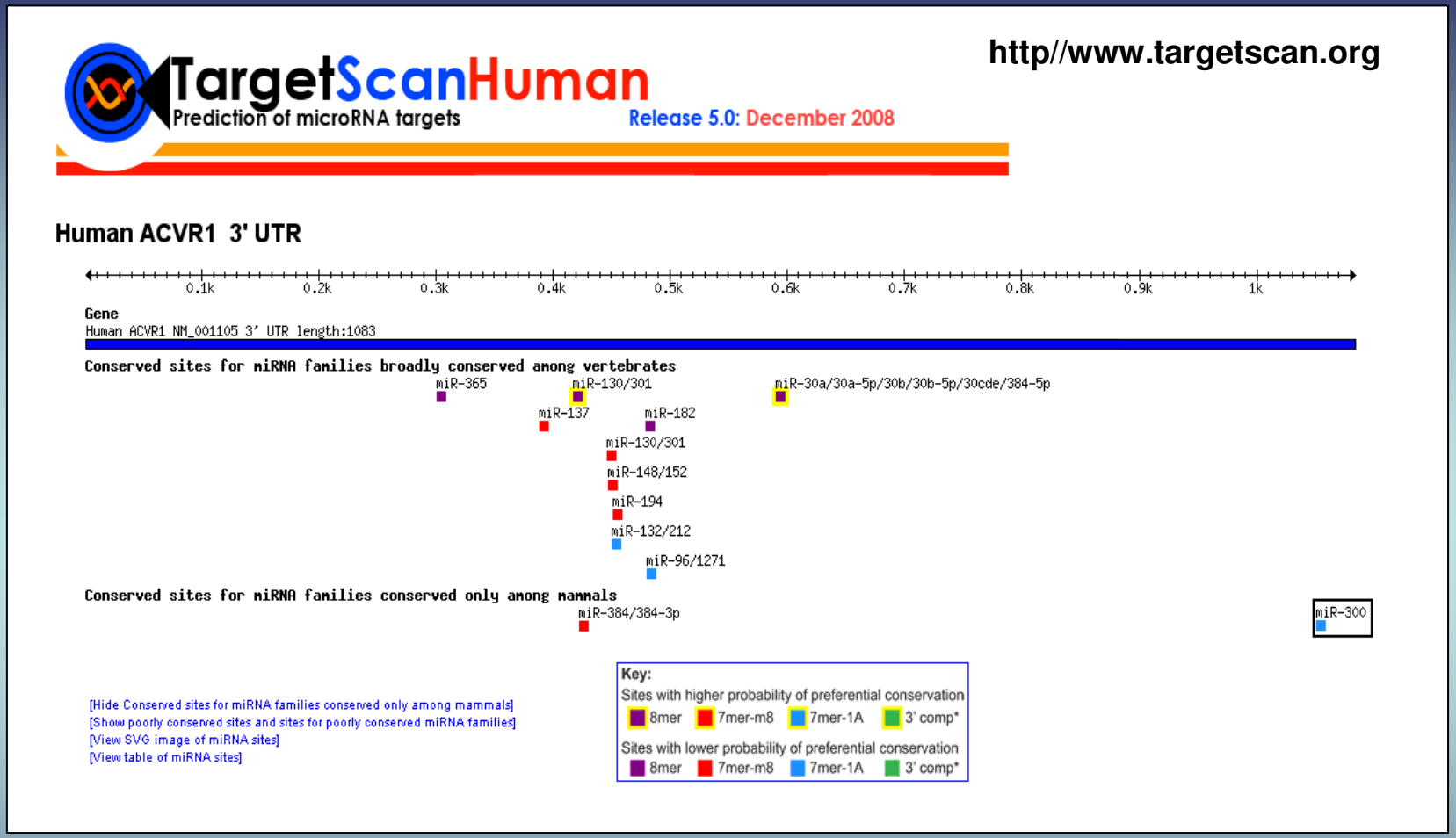
Positive regulation
mRNA stability

Role of miRNA in post-transcriptional regulation of *ACVR1*

Different web-based tools are now available to scan sequences for the presence of putative microRNA binding sites.

TargetScan:	**http//www.targetscan.org**
PicTar:	**http://pictar.mdc-berlin.de/**
miRbase:	**http://microrna.sanger.ac.uk**

ACVR1 and microRNA



In silico **analysis revealed that several conserved putative binding sites for miRNA are predicted in the 3'*UTR*** **of the gene and need to be experimentally verified.**

TargetScan References:

1) Benjamin P Lewis et al., ***Cell***, 120:15-20 (2005).

2) Andrew Grimson, etal., ***Molecular Cell***, 27:91-105 (2007).

3) Robin C Friedman et al., ***Genome Research***.

How to choose microRNA to be tested?

- microRNA with binding sites highly conserved among species.
- Binding sites recognized by different programs.
- Candidate microRNA.

Binding sites recognized by 3 programs

Micro RNA	PicTar	TargetScan	
hsa-miR-301a: 405 432	P	T	-14.46/-19.81
hsa-miR-130a: 406 435	P	T	-16.35/-18.22
hsa-miR-148a: 406 434	P	T	-11.18/-12.05
hsa-miR-182: 458	P	T	-12.34
hsa-miR-152: 436	P	T	-12.21
hsa-miR-384: 412	P	T	-11.47

Binding sites recognized by at least 2 programs

Micro RNA	PicTar	TargetScan	
hsa-miR-130b: 403 433	P		
hsa-miR-301a: 405 432	P	T	-14.46/-19.81
hsa-miR-130a: 406 435	P	T	-16.35/-18.22
hsa-miR-30c: 576 605	P		-15.92/-12.22
hsa-miR-365: 185 290		T	-10.25/-14.17
hsa-miR-137: 372		T	-13.49
hsa-miR-148a: 406 434	P	T	-11.18/-12.05
hsa-miR-30d: 576	P		-16.4
hsa-miR-30e: 576	P		-14.28
hsa-miR-182: 458	P	T	-12.34
hsa-miR-152: 436	P	T	-12.21
hsa-miR-384: 412	P	T	-11.47
hsa-miR-148b: 435	P		-11.8
hsa-miR-381: 798		T	-13.59

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CTGGGACCTA ATGCTGGCCT GACTGGTTGT CAGAATGGAA TCCATCTGTC  100
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miR-365
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TGTTGCAAAG GTAGGGACTG GAGGAACACA GAGAAATCCT AAAAGAGATC  300
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                                              miR-137
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                  miR-130a/b miR-384       miR130/301/148/152
CTGTGCTTCT CTTCTTTATT GCACTAGGAA TTCTTTGCAT TCCTTACTTG  450
                                 miR-182
CACTGTTACT CTTAATTTTA AAGACCCAAC TTGCCAAAAT GTTGGCTGCG  500
                               miR-384
TACTCCACTG GTCTGTCTTT GGATAATAGG AATTCAATTT GGCAAAACAA  550
                                                miR-30c/d/e
AATGTAATGT CAGACTTTGC TGCATTTTAC ACATGTGCTG ATGTTTACAA  600
              miR-384
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GCTTATTTTT ATGTGGTCTT ATGATTTTAT TACAGAAATG TTTTTAACAC  750
TATACTCTAA AATGGACATT TTCTTTTATT ATCAGTTAAA ATCACATTTT  800
           miR-381/300
AAGTGCTTCA CATTTGTATG TGTGTAGACT GTAACTTTTT TTCAGTTCAT  850
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                               miR-381/300
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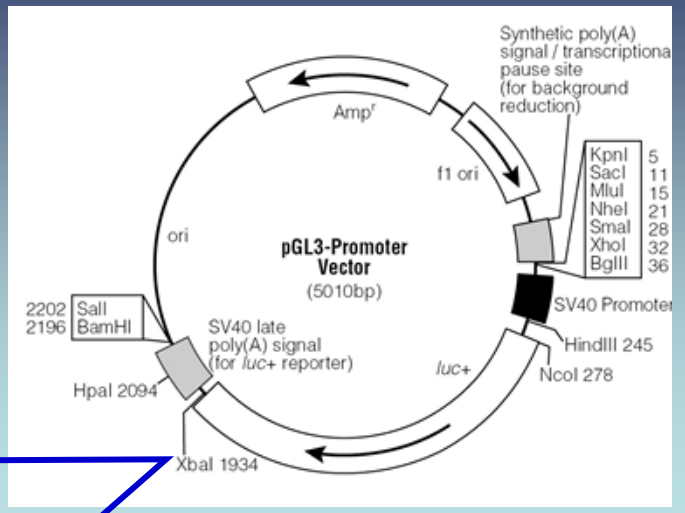
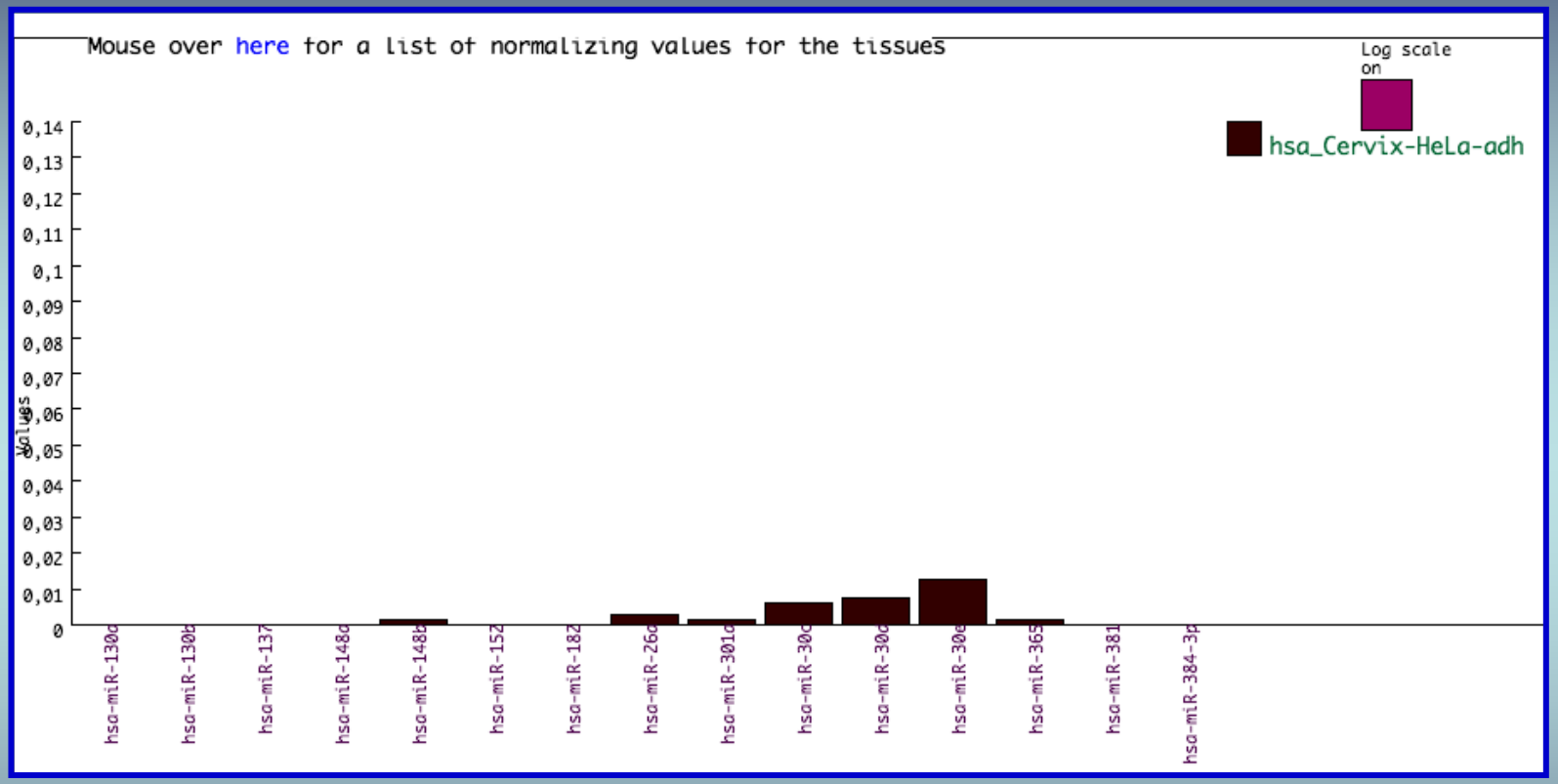


❖**Cotransfection of pGL3-Prom- *ACVR1* 3'UTR with precursor of selected microRNA (pre-MIR, Ambion) in HeLa cells.**

❖**Evaluation of the luciferase activity.**

Expression of the selected microRNA in HeLa cells according to the microRNA Data Base (www.microrna.org)



HeLa cells show little, if any, expression of the selected microRNAs.

3'*UTR* - *ACVR1* and microRNA

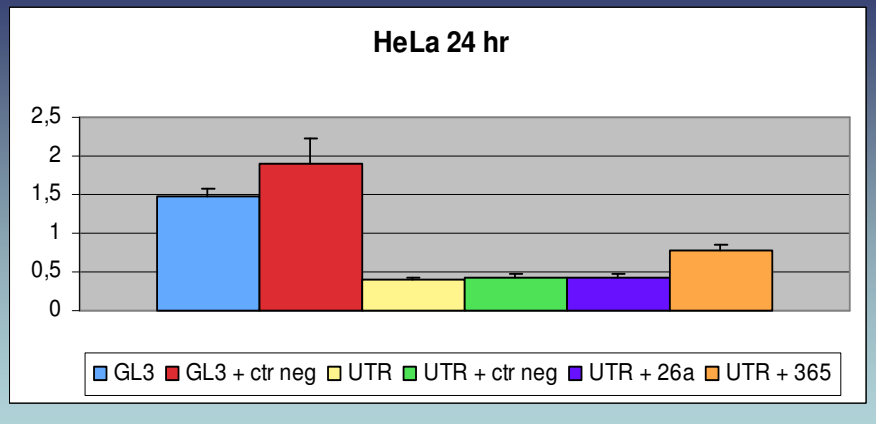
Transfection protocol (24 wells plate)

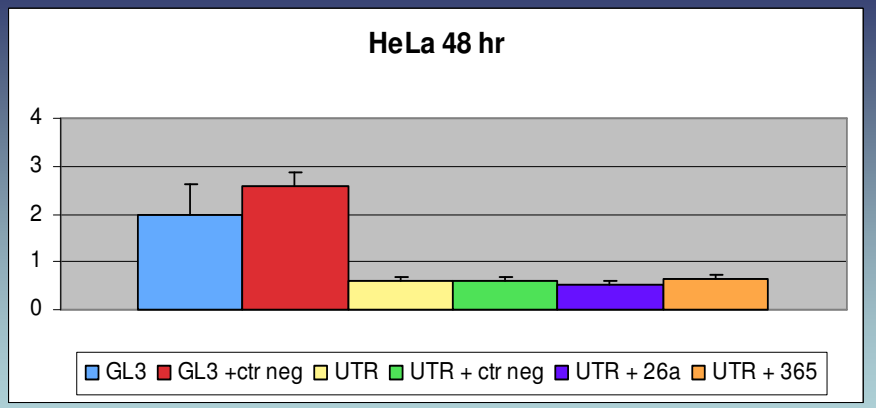
Cells are plated and transfected at the same time

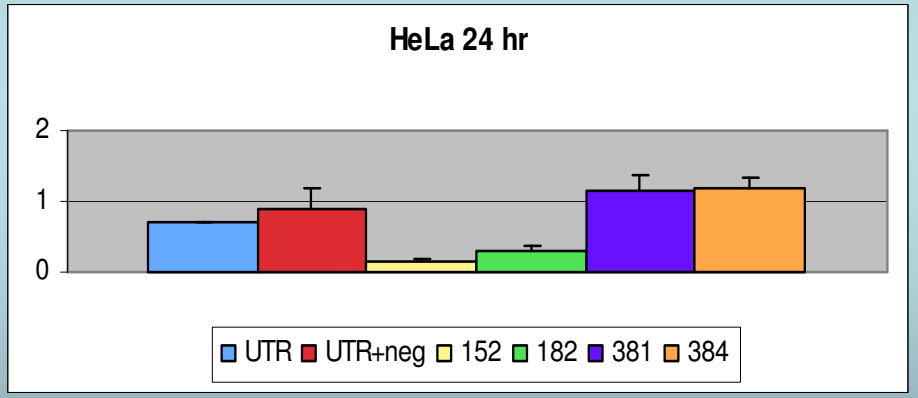
- 90 000 cells/well HeLa
- 400 ng GL3 or UTR
- 5 ng Renilla

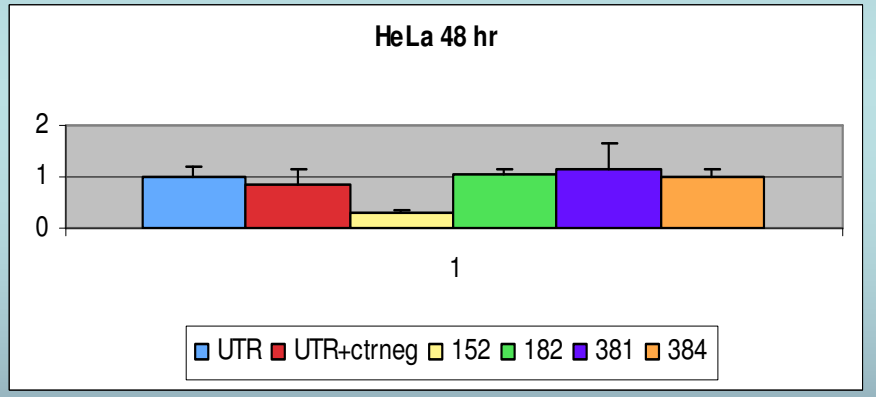
-15 pmoles miR precursor
- siPORT *Neofx Transfection agent*
- STOP at 24 and 48 hr
- Luciferase/renilla activity detection with Luminometer

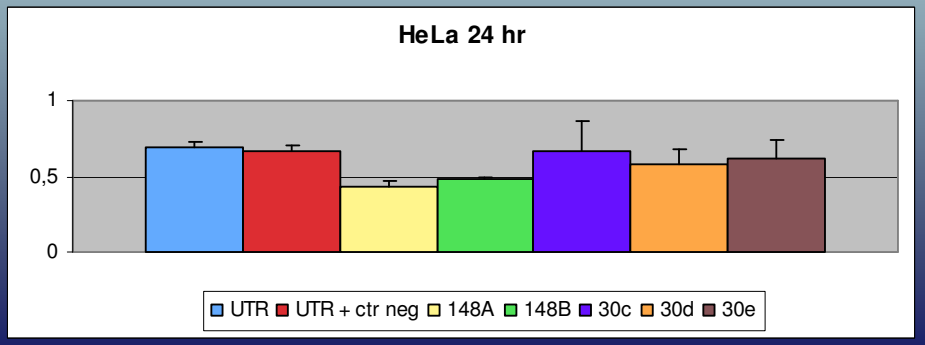
3'*UTR* - *ACVR1* and microRNA



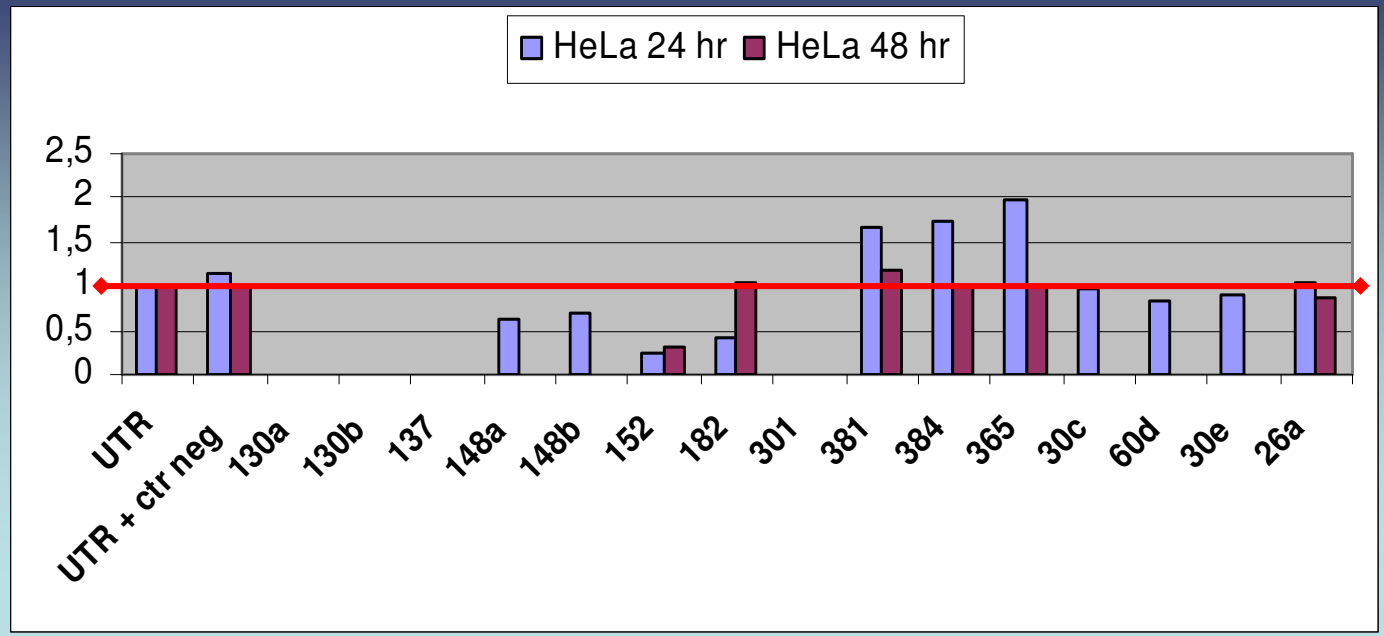








Effect of selected microRNA co-transfection on Luciferase activity



Reporter gene activity in HeLa

miR-152 stable decrease
miR-182 transient decrease

miR-381 transient increase
miR-384 transient increase
miR-365 transient increase

miR-148a mild decrease
miR-148b mild decrease

miR-30c/30d/30e no effect
miR-26a no effect

- We have found that the 3'*UTR* of *ACVR1* can be target of post-transcriptional regulation (see reporter gene activity of empty pGL3-prom vector *vs* pGL3-prom + 3'*UTR* of *ACVR1*).

- We have found that microRNA can have both a positive and negative effect on *ACVR1* expression as assessed by gene reporter activity.

Moreover…

The regulation of mRNA stability and translation are essential in the control of gene expression and regulation of these two processes allows a cell to rapidly respond to changes in intracellular and extracellular stimuli.

A number of sequence elements control the stability/availability of a mRNA either by stimulating or inhibiting degradation. Most of these elements are located in the 3'*UTR* end of the genes.

Among these modulating elements, in mammalian cells the sequence elements rich in adenosine and uridine, called **AU-rich elements (AREs)**, were identified by their ability to target host mRNAs towards rapid degradation.

ACVR1 3'*UTR* and ARE sequences

The 3'*UTR* sequence of the *ACVR1* gene is classified as containing a Class I Cluster 5 ARE

```
CATTTTCATA GTGTCAAGAA GGAAGATTTG ACGTTGTTGT CATTGTCCAG   50
CTGGGACCTA ATGCTGGCCT GACTGGTTGT CAGAATGGAA TCCATCTGTC  100
TCCCTCCCCA AATGGCTGCT TTGACAAGGC AGACGTCGTA CCCAGCCATG  150
TGTTGGGGAG ACATCAAAAC CACCCTAACC TCGCTCGATG ACTGTGAACT  200
GGGCATTTCA CGAACTGTTC ACACTGCAGA GACTAATGTT GGACAGACAC  250
TGTTGCAAAG GTAGGGACTG GAGGAACACA GAGAAATCCT AAAAGAGATC  300
TGGGCATTAA GTCAGTGGCT TTGCATAGCT TTCACAAGTC TCCTAGACAC  350
TCCCCACGGG AAACTCAAGG AGGTGGTGAA TTTTTAATCA GCAATATTGC  400
CTGTGCTTCT CTTCTTTATT GCACTAGGAA TTCTTTGCAT TCCTTACTTG  450
CACTGTTACT CTTAATTTTA AAGACCCAAC TTGCCAAAAT GTTGGCTGCG  500
TACTCCACTG GTCTGTCTTT GGATAATAGG AATTCAATTT GGCAAAACAA  550
AATGTAATGT CAGACTTTGC TGCATTTTAC ACATGTGCTG ATGTTTACAA  600
TGATGCCGAA CATTAGGAAT TGTTTATACA CAACTTTGCA AATTATTTAT  650
TACTTGTGCA CTTAGTAGTT TTTACAAAAC TGCTTTGTGC ATATGTTAAA  700
GCTTATTTTT ATGTGGTCTT ATGATTTTAT TACAGAAATG TTTTTAACAC  750
TATACTCTAA AATGGACATT TTCTTTTATT ATCAGTTAAA ATCACATTTT  800
AAGTGCTTCA CATTTGTATG TGTGTAGACT GTAACTTTTT TTCAGTTCAT  850
ATGCAGAACG TATTTAGCCA TTACCCACGT GACACCACCG AATATATTAC  900
TGATTTAGAA GCAAAGATTT CAGTAGAATT TTAGTCCTGA ACGCTACGGG  950
GAAAATGCAT TTTCTTCAGA ATTATCCATT ACGTGCATTT AAACTCTGCC 1000
AGAAAAAAAT AACTATTTTG TTTTAATCTA CTTTTTGTAT TTAGTAGTTA 1050
TTTGTATAAA TTAAATAAAC TGTTTTCAAG TCAAAaaaaa aaaaaaaaaa 1100
```

Class I several dispersed copies of the AUUUA motif.
Class II at least 2 overlapping UUAUUUA(U/A)(U/A) nonamers (citokines…).
Class III much less defined, they are U-rich regions but contain no AUUUA pentamer.

Modular structure of *ACVR1* 3'*UTR* sequence

```
CATTTTCATA GTGTCAAGAA GGAAGATTTG ACGTTGTTGT CATTGTCCAG   50
CTGGGACCTA ATGCTGGCCT GACTGGTTGT CAGAATGGAA TCCATCTGTC  100
TCCCTCCCCA AATGGCTGCT TTGACAAGGC AGACGTCGTA CCCAGCCATG  150
TGTTGGGGAG ACATCAAAAC CACCCTAACC TCGCTCGATG ACTGTGAACT  200
miR-365
GGGCATTTCA CGAACTGTTC ACACTGCAGA GACTAATGTT GGACAGACAC  250
TGTTGCAAAG GTAGGGACTG GAGGAACACA GAGAAATCCT AAAAGAGATC  300
 miR-365
TGGGCATTAA GTCAGTGGCT TTGCATAGCT TTCACAAGTC TCCTAGACAC  350
                                          miR-137
TCCCCACGGG AAACTCAAGG AGGTGGTGAA TTTTTAATCA GCAATATTGC  400
                  miR-130a/b miR-384      miR130/301/148/152
CTGTGCTTCT CTTCTTTATT GCACTAGGAA TTCTTTGCAT TCCTTACTTG  450
                                  miR-182
CACTGTTACT CTTAATTTTA AAGACCCAAC TTGCCAAAAT GTTGGCTGCG  500
                                miR-384
TACTCCACTG GTCTGTCTTT GGATAATAGG AATTCAATTT GGCAAAACAA  550
                                                  miR-30c/d/e
AATGTAATGT CAGACTTTGC TGCATTTTAC ACATGTGCTG ATGTTTACAA  600
               miR-384
TGATGCCGAA CATTAGGAAT TGTTTATACA CAACTTTGCA AATTATTTAT  650
TACTTGTGCA CTTAGTAGTT TTTACAAAAC TGCTTTGTGC ATATGTTAAA  700
GCTTATTTTT ATGTGGTCTT ATGATTTTAT TACAGAAATG TTTTTAACAC  750
TATACTCTAA AATGGACATT TTCTTTTATT ATCAGTTAAA ATCACATTTT  800
           miR-381/300
AAGTGCTTCA CATTTGTATG TGTGTAGACT GTAACTTTTT TTCAGTTCAT  850
ATGCAGAACG TATTTAGCCA TTACCCACGT GACACCACCG AATATATTAC  900
TGATTTAGAA GCAAAGATTT CAGTAGAATT TTAGTCCTGA ACGCTACGGG  950
GAAAATGCAT TTTCTTCAGA ATTATCCATT ACGTGCATTT AAACTCTGCC 1000
                                  miR-381/300
AGAAAAAAAT AACTATTTTG TTTTAATCTA CTTTTTGTAT TTAGTAGTTA 1050
TTTGTATAAA TTAAATAAAC TGTTTTCAAG TCAAAaaaaa aaaaaaaaaa 1100
```

microRNA and ARE interacting protein can compete for the binding to the *ACVR1* 3'UTR sequence.

ARE sequences have a destabilizing effect of ACVR1 mRNA. This can be counteracted in some conditions by the binding of specific miRNA.

Istituto G. Gaslini
Genova

Serena Cappato
Dr Marzia Mura
Dr Anwar Baban
Prof. Roberto Ravazzolo
S.C. Genetica Molecolare e Citogenetica

Dr Maja Di Rocco
U.O. Pediatria II

Center for Advanced Biotechnology (CBA)
Genova
Dr Sara Tavella
Laboratory of Regenerative Medicine

Acknowledgements

We thank the patients and their families and in particular we are grateful to the "**FOP Italia Association**" for their fundamental help and their enthusiastic support to our work.

We acknowledge the contribution of Dr M. Lerone, Dr M.T. Divizia, Dr . Alpigiani, Dr M.C. Schiaffino, Prof G. Ferraccioli, Dr. Paola Ferrari who referred patients.