DIAGNOSI MOLECOLARE E RICERCA SULLA F.O.P. IN ITALIA

Verbania, 16-17 Aprile, 2010

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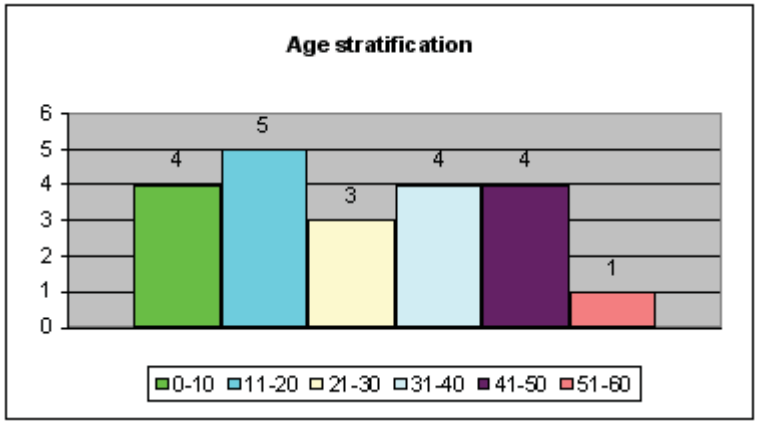
Il Laboratorio esegue la diagnosi molecolare mediante ricerca di mutazioni nel gene ACVR1 su individui con diagnosi clinica di F.O.P. o con sospetta diagnosi

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

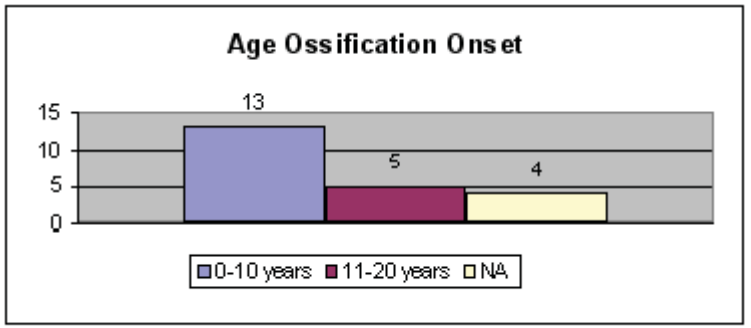
	Pt	Age	Sex	Age Ossification onset	Great toe malf.	Mutation	Other
1	FOP1	33 y	M	na	y	c.617G>A, nd	
2	FOP2	5 y	M	15 months	y	c.617G>A, *de novo*	Hydrocephalus secondary to a posterior bulbar expansive lesion of unknown origin (surgical correction)
3	FOP3	10 y	F	4 y	y	c.617G>A, *de novo*	
4	FOP4	5 y	F	20 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
21	FOP27	13 y	M	8 y	y	c.774G>T, R258S	Pt from Morocco
22	FOP29	15 m	M	> 1 y	y	c.617G>A, R206H	Pt from UK
23	FOP30	12 y	F	-	y	c.617G>A, R206H	
24	FOP31	49 y	F	7 y	y	c.617G>A, nd	Dysmorfic thumbs
25	FOP34	5 y	F	?	y	c.774G>C, *de novo*, R258S	Ossification head and neck

Pt, patient code; nd, mutation origin not determined; na, not available; 36 patients have been screened, 25 with confimation of the clinical diagnosis, 11 with exclusion of FOP.

Age stratification	
0-10 years	4
11-20 years	5
21-30 years	3
31-40 years	4
41-50 years	4
51-60 years	1
	21



Age Ossification Onset	
0-10 years	13
11-20 years	5
Not available (NA)	4
	22



Ricerca sui meccanismi della F.O.P.

Si esegue grazie ai contributi economici di

- F.O.P. Italia
- Ministero dell'Università
- Ministero della Salute

Regolazione del gene ACVR1

La conoscenza dei meccanismi di regolazione permette di comprendere come è controllata la quantità di espressione del gene

Nella F.O.P. la mutazione del gene è associata a un eccesso di funzione dell'intera via di segnalazione delle BMP

Un modo per limitare la funzione del gene e della via funzionale ad esso collegata potrebbe basarsi su un intervento nel meccanismo che regola la sua espressione

Analogamente alla maggior parte dei geni umani, anche l'espressione di ACVR1 viene regolata a livello di regioni del gene che si trovano alle sue estremità

- Promotore/Promotori

- 5'-UTR

- 3'-UTR

Study of the regulation of ACVR1 expression

- Characterization of the structure and composition of the *ACVR1* transcripts.
- Definition and characterization of sequences playing a role in the regulation of *ACVR1* expression.

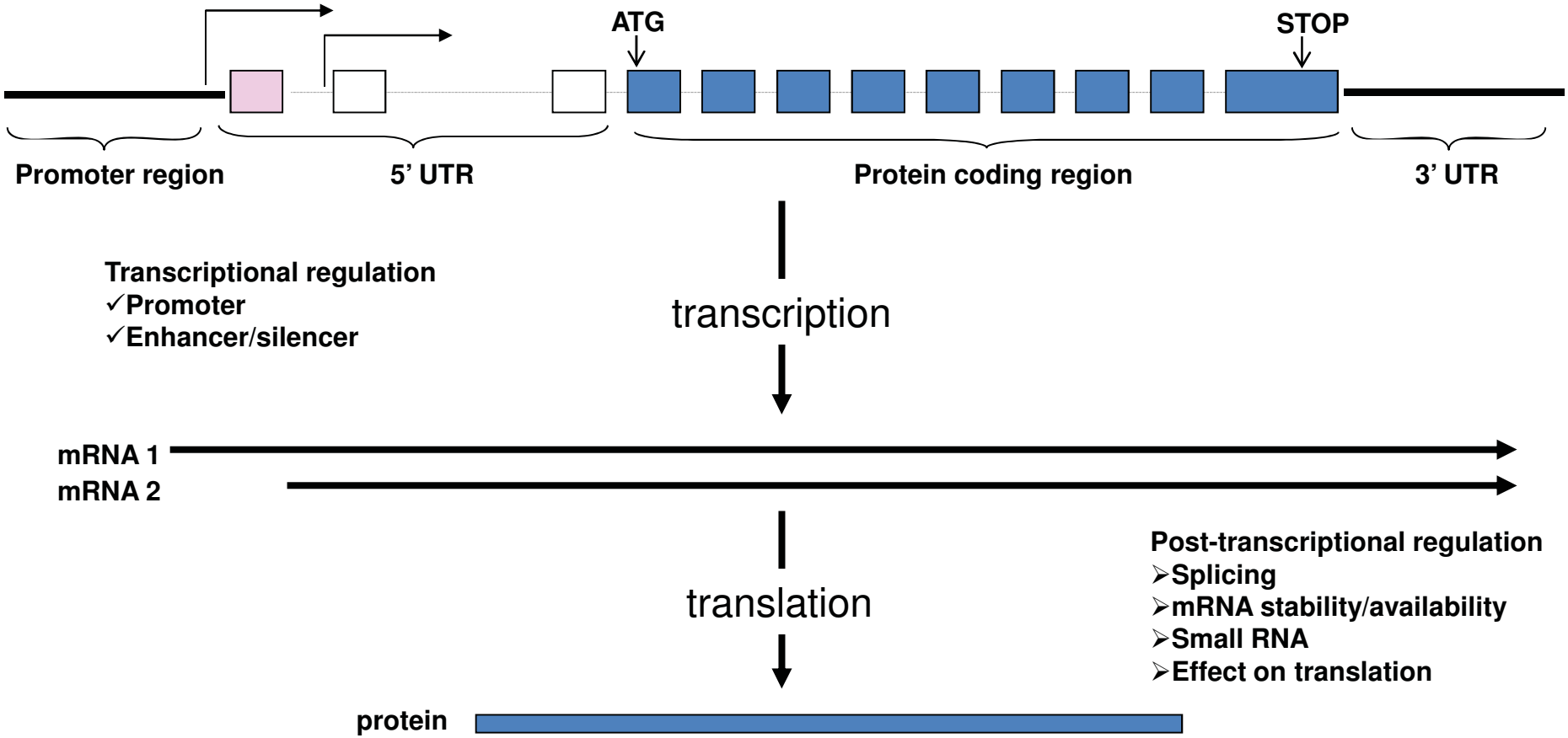
  - ✓**5'*UTR* sequences**
    Promoter region
  - ✓**3'*UTR* region**

Generation of a cellular model suitable for the high throughput screening of small molecules with potential pharmacological effect on the BMP/ACVR1 mediated pathway.

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.



Study of the regulation of ACVR1 expression

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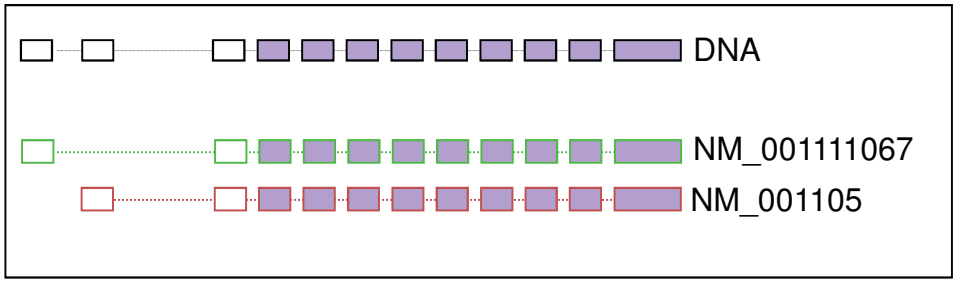
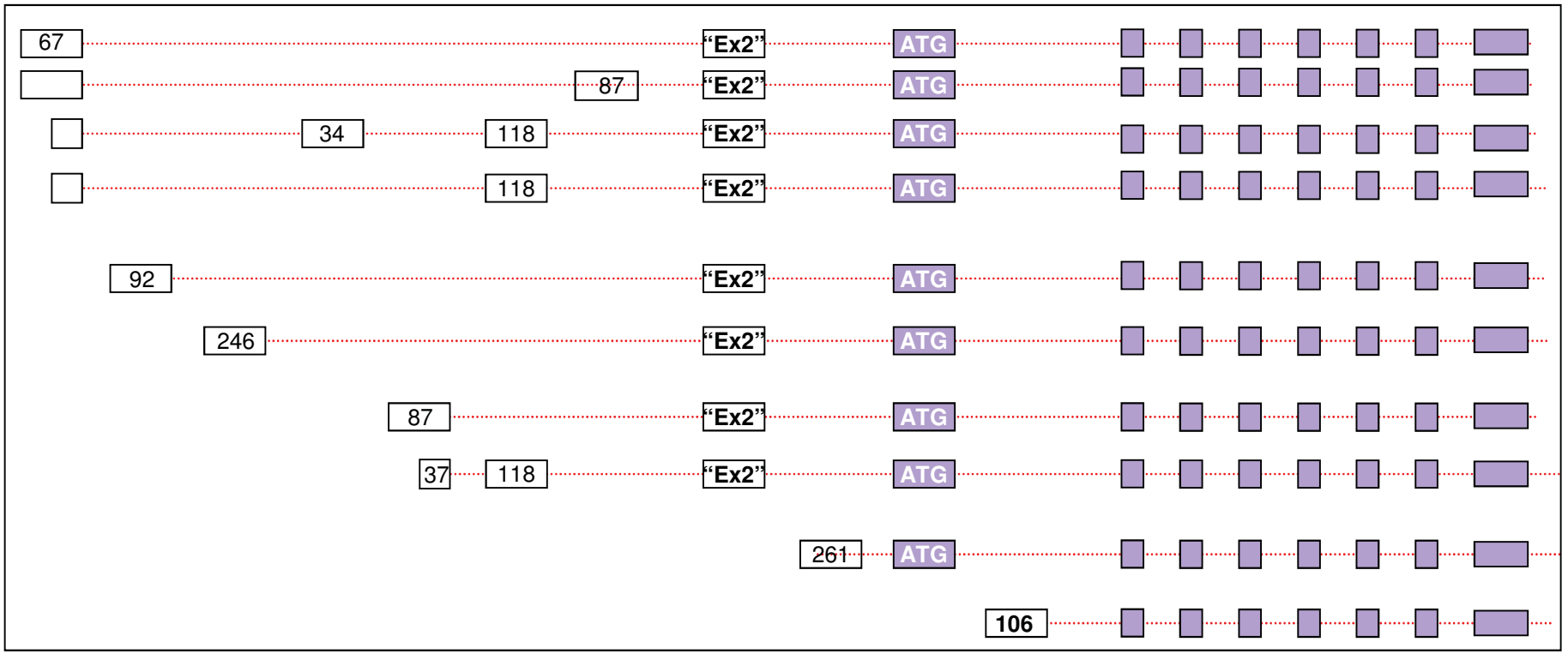
Generation of a cellular model suitable for the high through-put screening of small molecules with potential pharmacological effect on the BMP/ACVR1 mediated pathway.

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

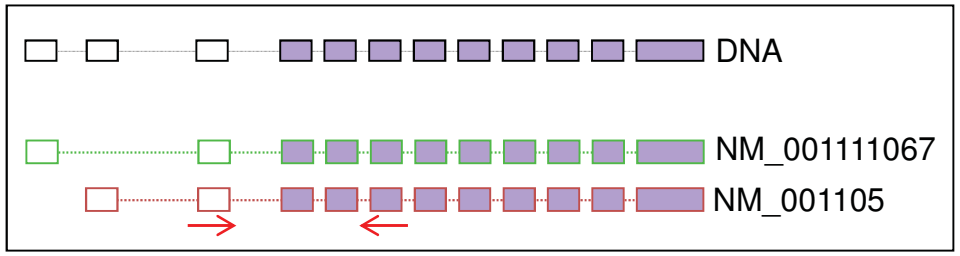
The past.....



Looking at the ESTs in the *ACVR1* genomic region....The present...

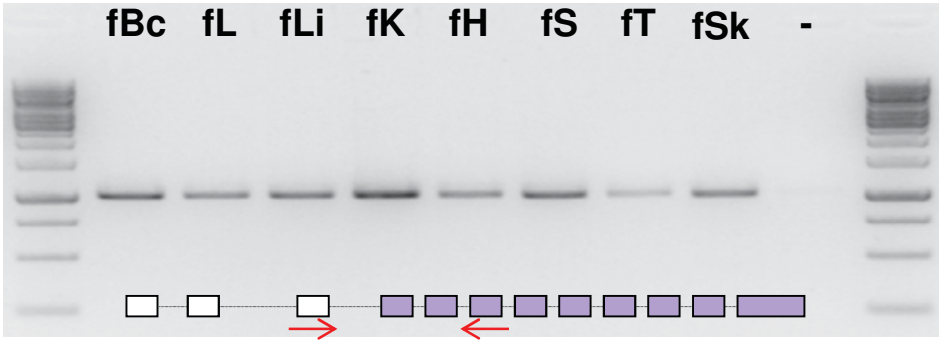


ACVR1 expression profile

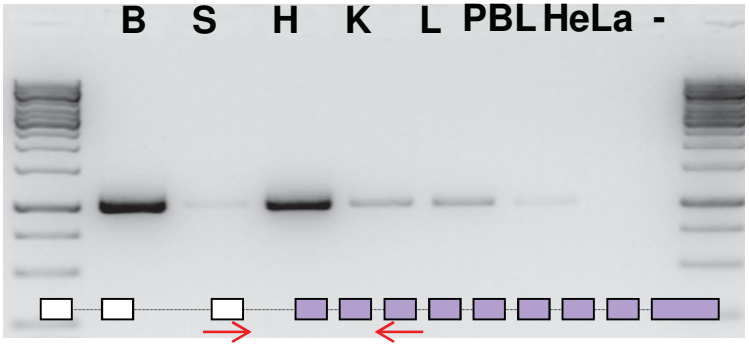


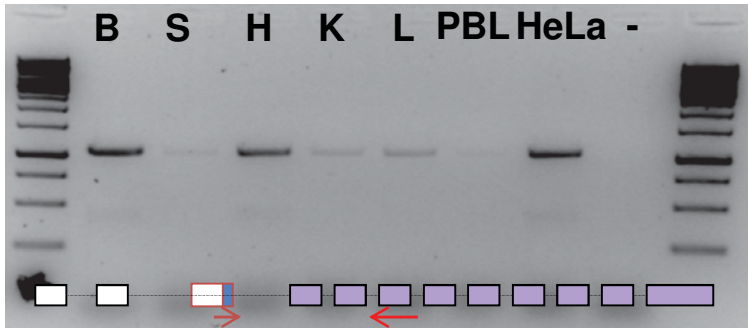
cDNA from commonly used cell lines

cDNA from fetal tissues



cDNA from adult tissues

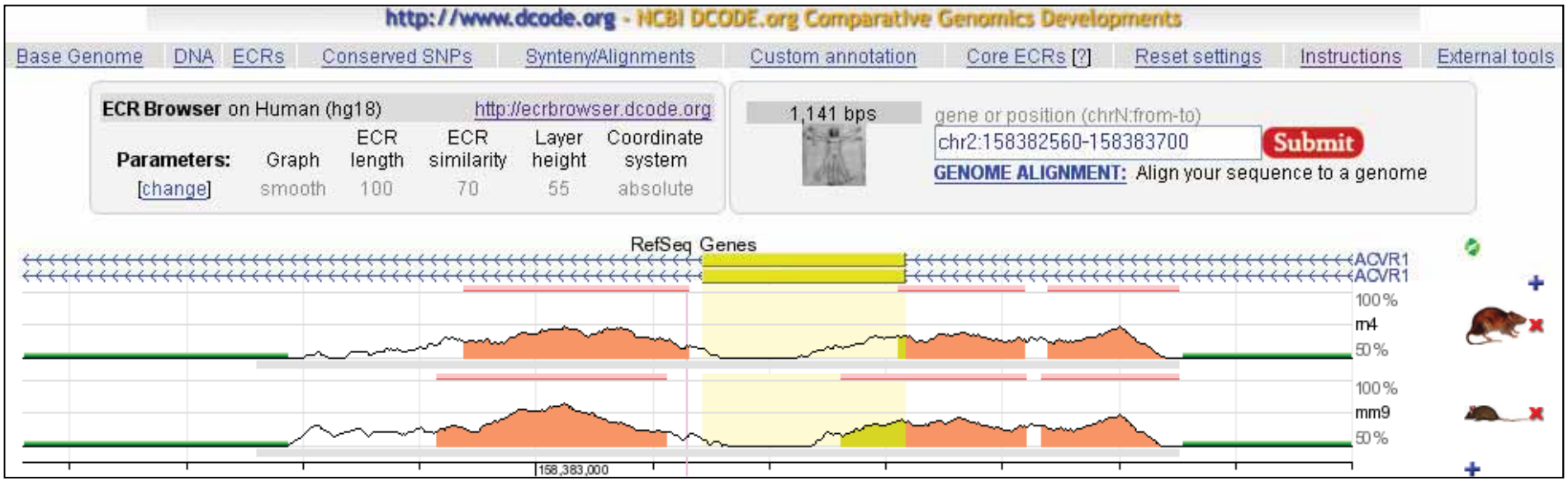




We have identified a new transcript isoform showing an insertion of *116 bp* between the untranslated exon 2 and first protein coding exon.

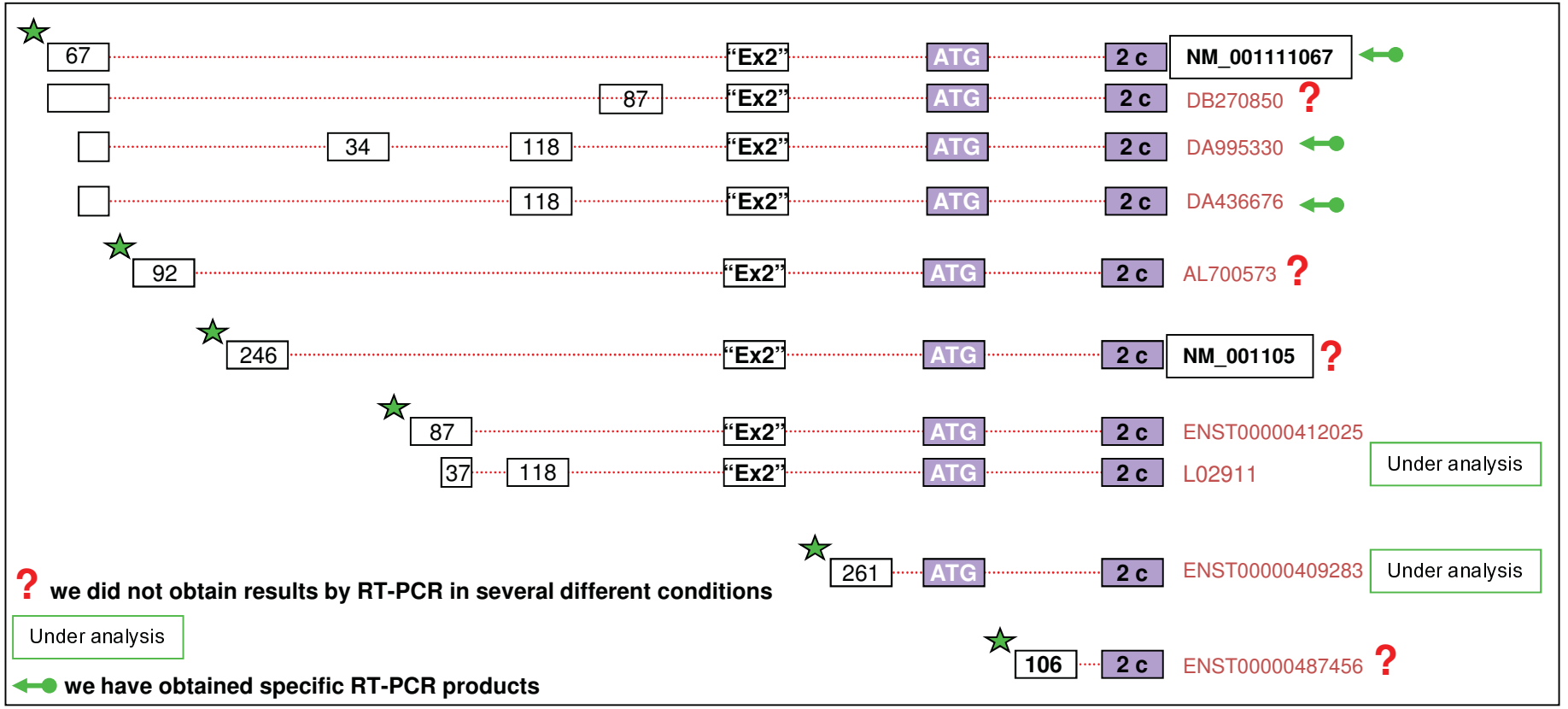
This is due to an alternative splicing leading to the incorporation of 116 bp of the 5'end of intron 3 in the mRNA.

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.



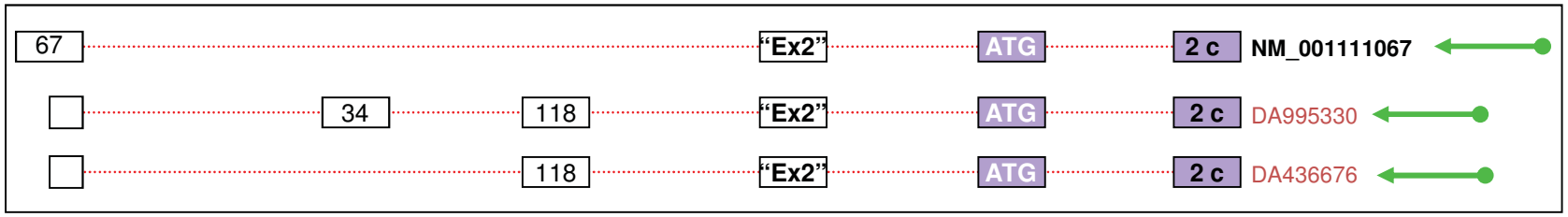
This sequence can have a role at post-transcriptional level and we are currently investigating in this direction.

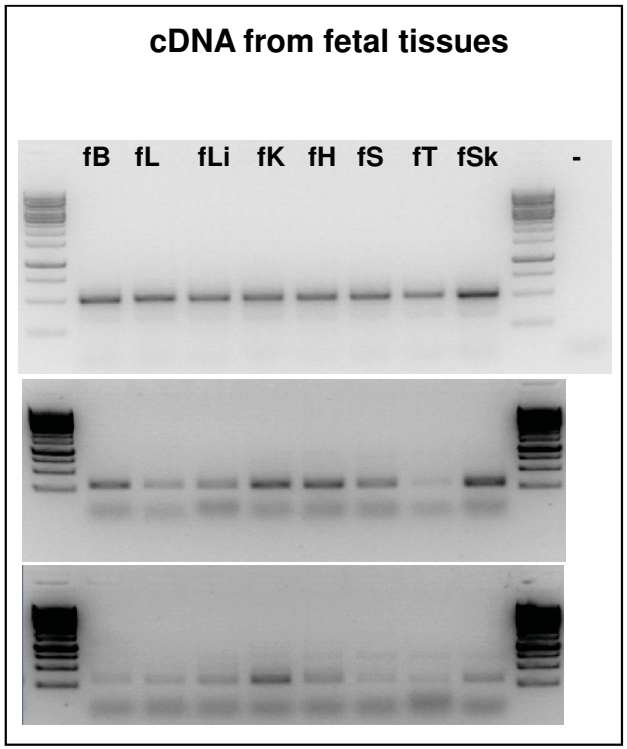
About *ACVR1* new putative transcripts.....



This indicates the putative presence of different transcription start sites (★) and of different transcripts showing the alternative combination of several 5'UTR exons (white rectangles) with common protein coding sequences (violet rectangles).

Expression profile of putative new isoforms

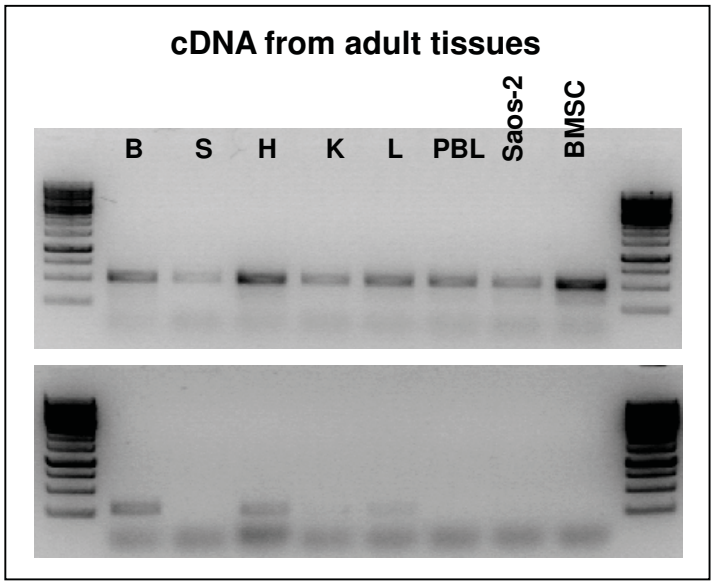




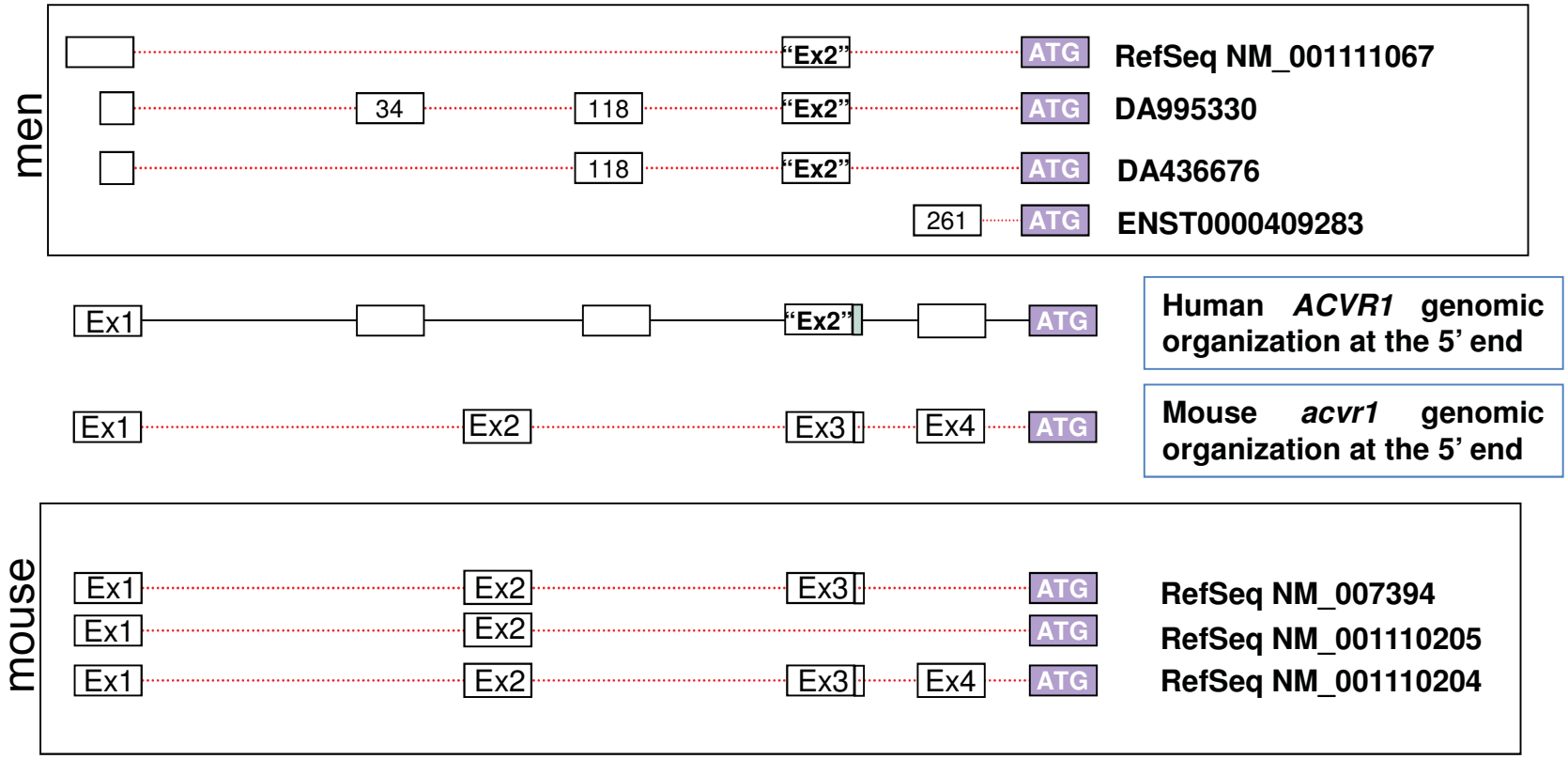
NM_001111067

DA436676

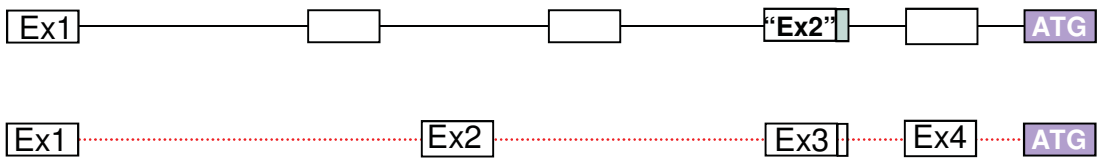
DA995330



Of Mice and Men.....and transcripts

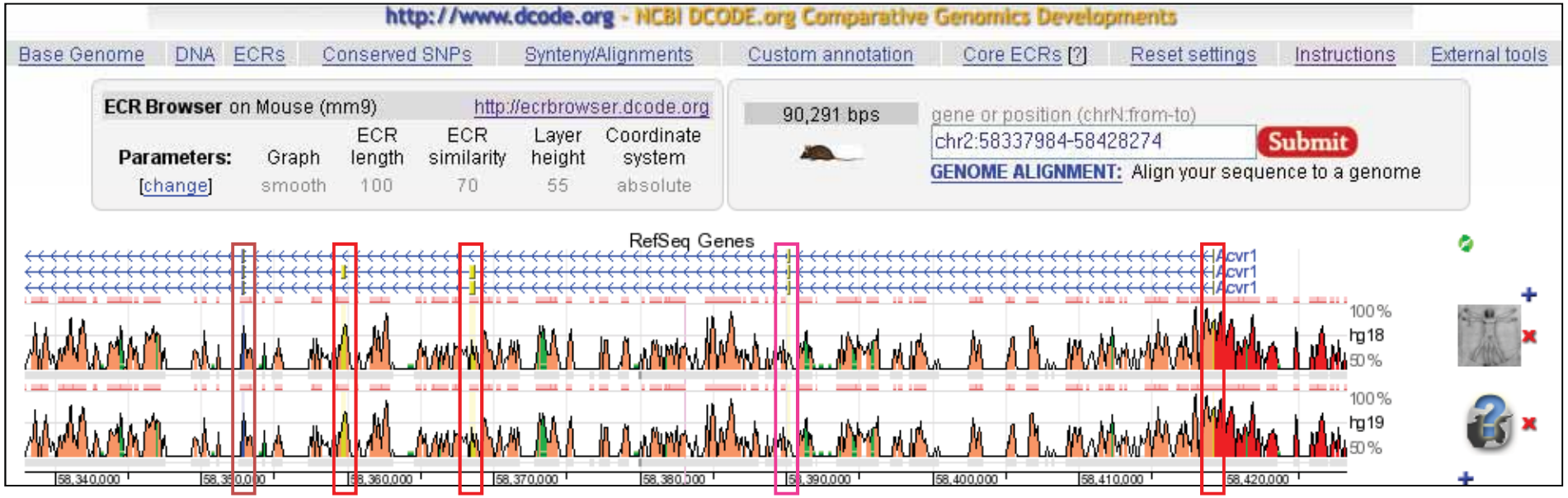


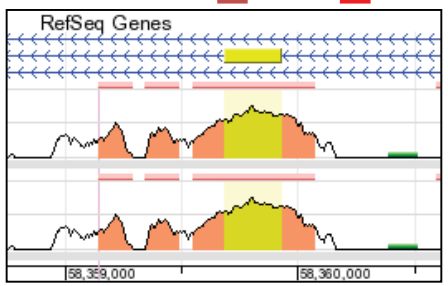
Of Mice and Men.....and conservation



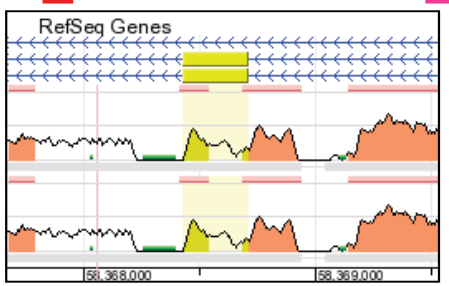
Human *ACVR1* genomic organization at the 5' end

Mouse *acvr1* genomic organization at the 5' end

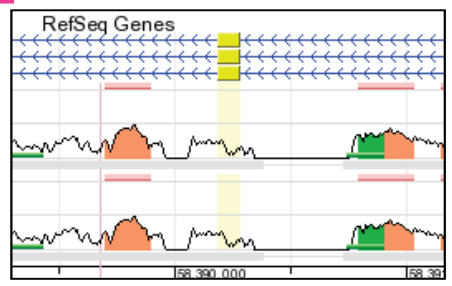




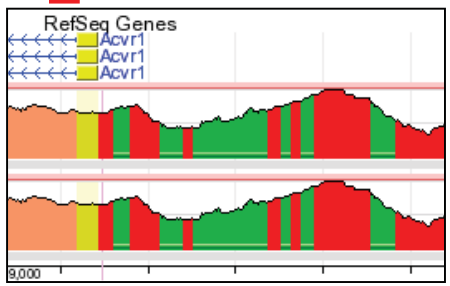
Mouse *acvr1* exon 4



Mouse *acvr1* exon 3



Mouse *acvr1* exon 2



Mouse *acvr1* exon 1

Qual è il ruolo funzionale di queste diverse regioni alternative all'estremo 5'-UTR?

C'è una regolazione differenziale per le diverse forme di mRNA?

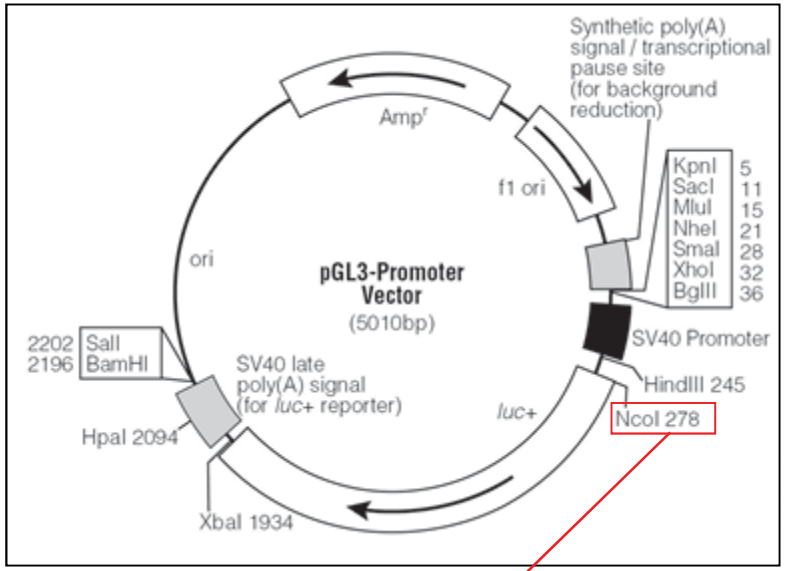
C'è un effetto sulla stabilità del mRNA o sulla capacità di tradurre il mRNA in proteina?

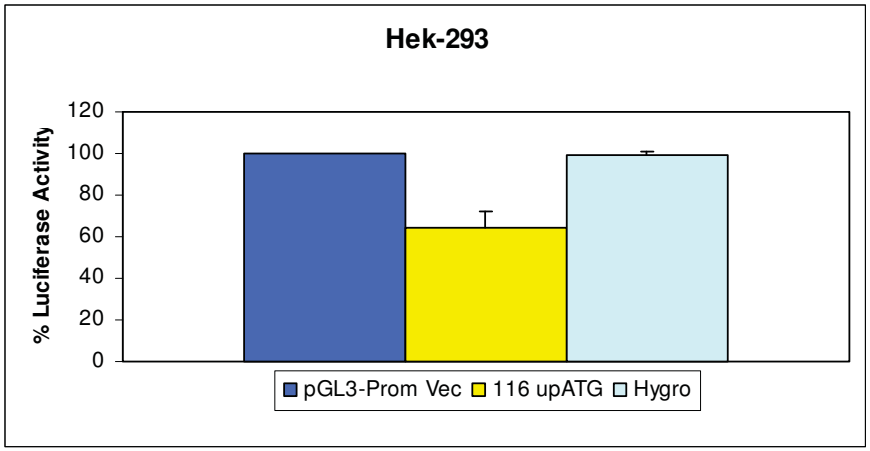
Functional analysis of 5'UTR sequences

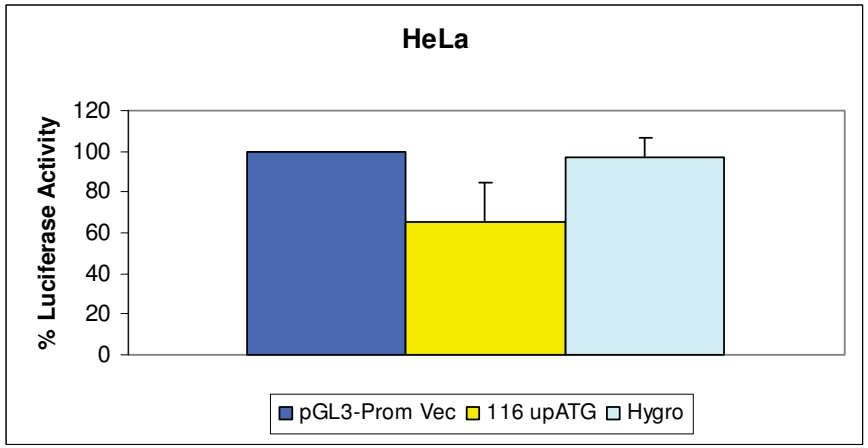
(the example of the 116 bp insertion)

Protocol

- Amplification by RT-PCR of the fragment to be tested with *NcoI* tails.
- Subcloning into the *NcoI* site of the pGL3-Promoter vector, this will cause the inclusion of the fragment under analysis in the reporter gene mRNA as 5'UTR sequence.
- Transfection in different cell lines.
- Evaluation of Luciferase activity and Real-time PCR on reporter mRNA.







Commento

Il tratto di sequenza che può essere inserito nel mRNA di ACVR1 o può non esserlo esercita un effetto di inibizione dell’espressione

Study of the regulation of ACVR1 expression

- 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**

Generation of a cellular model suitable for the high through-put screening of small molecules with potential pharmacological effect on the BMP/ACVR1 mediated pathway.

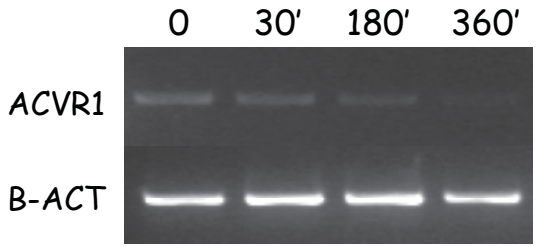
ACVR1: TRANSCRIPT STABILITY ANALYSIS

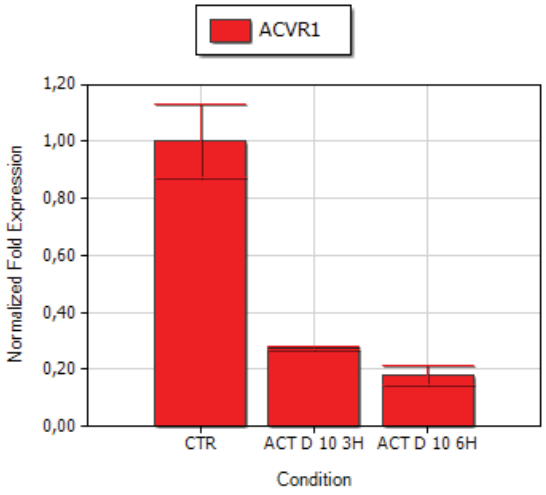
C2C12 cells (that normally express ACVR1), processed in the presence and absence of actinomycin D and doxorubicin (transcription inhibitors)

→ TIME COURSE with selected concentrations

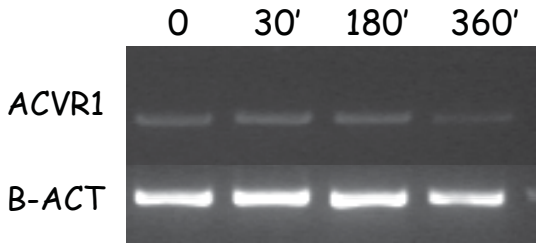
→ mRNA QUANTIFICATION

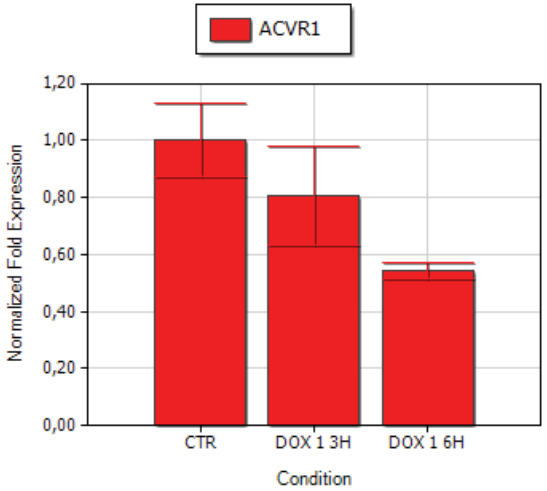
Actinomicina 10 µg/ml





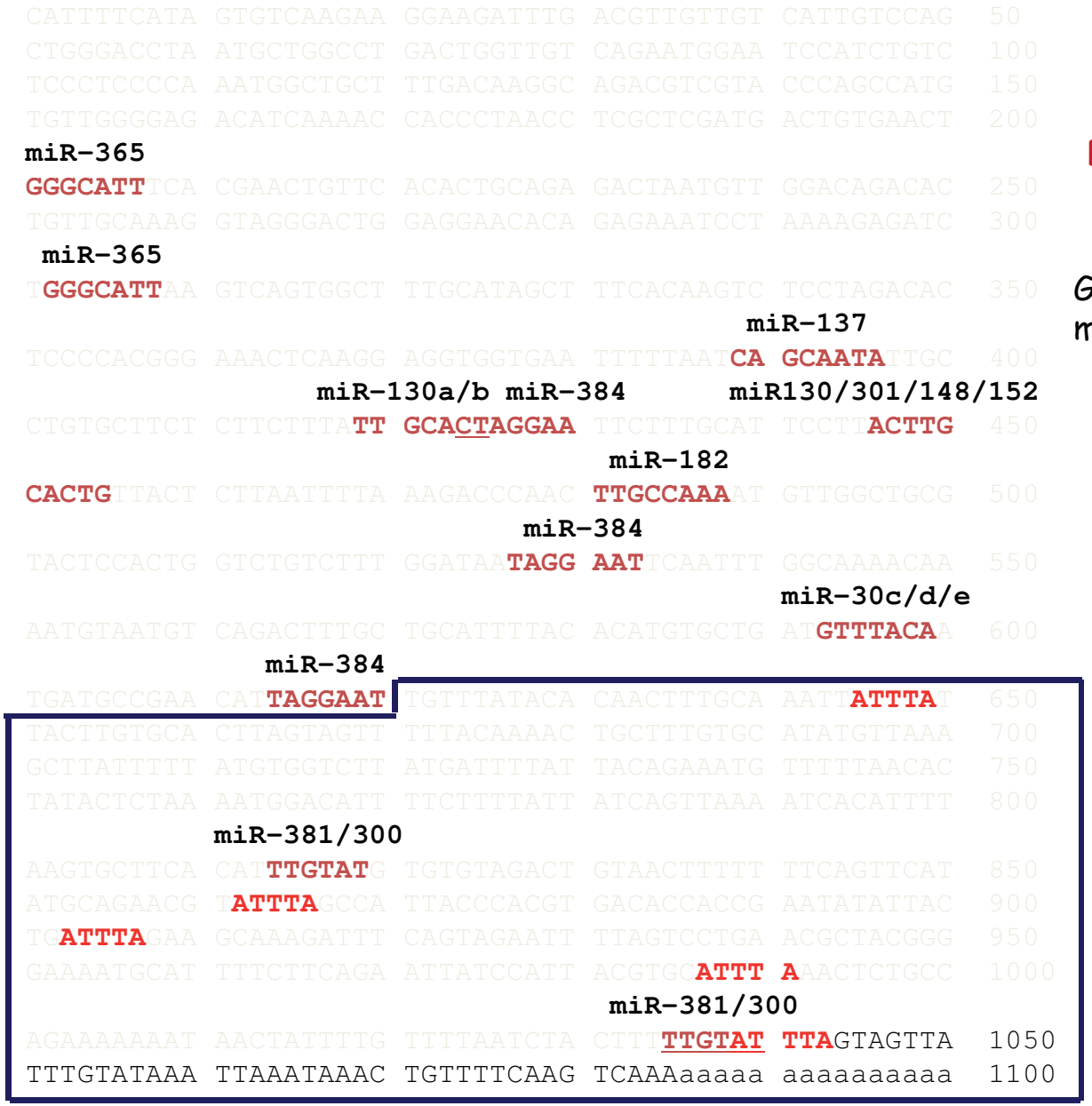
Doxorubicina 1 µM





ACVR1 TRANSCRIPT IS UNSTABLE

ACVR1 3'UTR



REGION CONTAINING miRNA

Gene silencing
mRNA degradation

Positive regulation
mRNA stability

REGION CONTAINING ARE

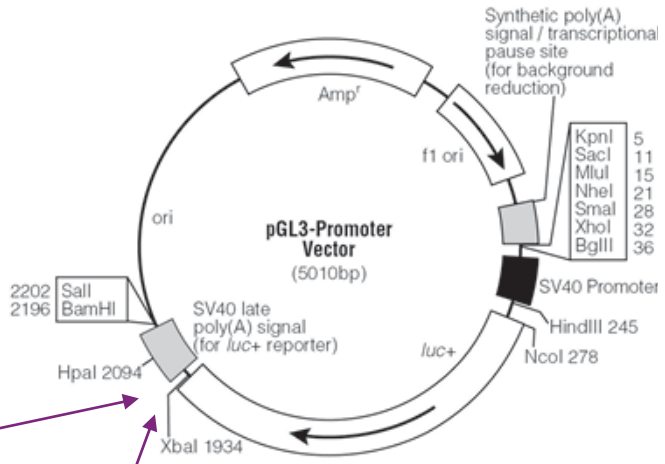
Group I class 5

Possible destabilizing effect

microRNAs and proteins that bind ARE sequences could inetrvene in the post-transcriptional regulation of the gene.

- Regions containing miRNA and ARE sequences are subcloned in pGL-3 promoter vector and transfected in HeLa cells:
  - pGL3 + miRNA region
  - pGL3 + sequences ARE region

- Luciferase activity assessment



miRNA region

```
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
GGGCATT
 miR-365
 GGGCATT
                                        miR-137
                                        CA GCAATA
                  miR-130a/b miR-384    miR130/301/148/152
                     TT GCACTAGGAA      ACTTG
                                  miR-182
CACTG                             TTGCCAAA
                               miR-384
                               TAGG AAT
                                        miR-30c/d/e
                                        GTTTACA
            miR-384
            TAGGAAT
```

ARE region

```
                                                ATTTA
TACTTGTGCA CTTAGTAGTT TTTACAAAAC TGCTTTGTGC ATATGTTAAA  700
GCTTATTTTT ATGTGGTCTT ATGATTTTAT TACAGAAATG TTTTTAACAC  750
TATACTCTAA AATGGACATT TTCTTTTATT ATCAGTTAAA ATCACATTTT  800
          miR-381/300
          TTGTAT
AAGTGCTTCA CATTTGTGTA TGTGTAGACT GTAACTTTTT TTCAGTTCAT  850
ATGCAGAACG TATTTAGCCA TTACCCACGT GACACCACCG AATATATTAC  900
TGATTTAGAA GCAAAGATTT CAGTAGAATT TTAGTCGTGA ACGCTACGGG  950
GAAAATGCAT TTTCTTCAGA ATTATCCATT ACGTGTATTT A AACTCTGCC 1000
                                  miR-381/300
AGAAAAAAAT AACTATTTTG TTTTAATCTA CTTTTGTGTAT TTAGTAGTTA 1050
TTTGTATAAA TTAAATAAAC TGTTTTCAAG TCAAAaaaaa aaaaaaaaaa 1100
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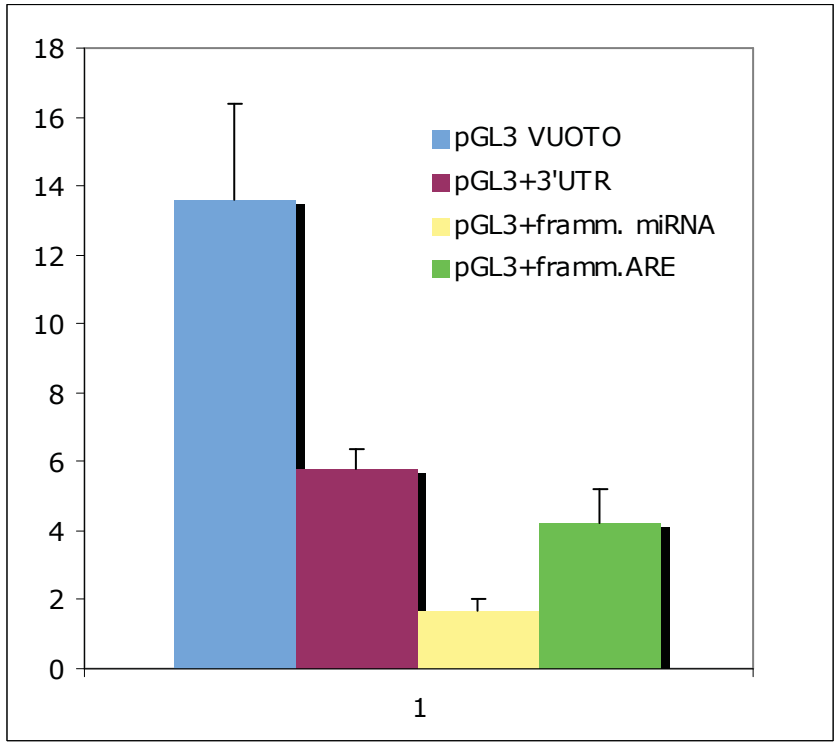


The presence of the 3'UTR sequences of ACVR1 causes a decreased activity of the reporter gene; transfection of the fragment containing miRNA further reduces luciferase activity.
The 3'UTR region of the ACVR1 gene may be involved in post-transcriptional regulation of ACVR1.

Role of miRNA in the regulation of ACVR1

Selection based on bioinformatics analysis considering conservation among species and recognition by at least two different programs.

What about miRNA selected…

miR-365: Regulates cell proliferation, resulting down-regulated in arrest of proliferation, senescence and quiescence. From the clinical point of view it is overexpressed in cases of breast cancer.

miR-148b: is involved in the mechanisms of bone generation, is essential for regulating the process of osteogenesis: has been shown that it up-regulates the osteogenic differentiation of mesenchymal stem cells.

miR-384: as observed in different cell lines it has a protective role against the induction of apoptosis.

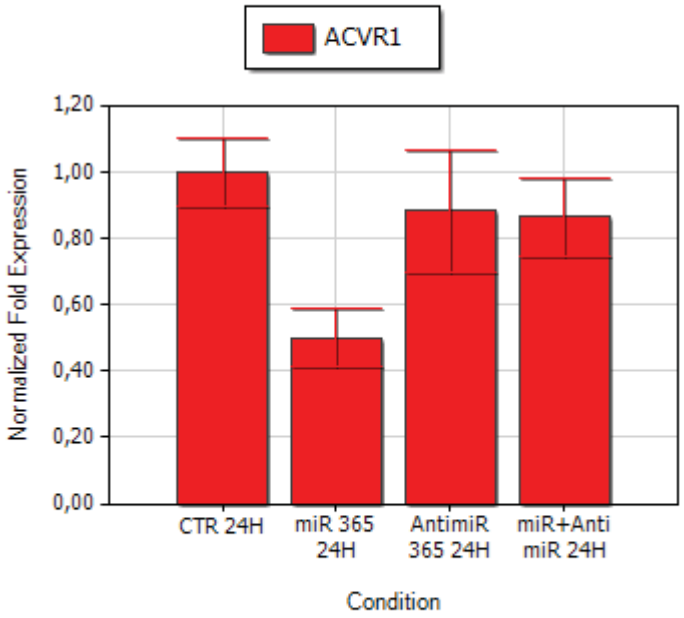
miR-381: is a candidate for the control of dendritic development and spine, has been shown that its expression is essential for dendritic outgrowth of hippocampal neurons.

TRANSFECTION through nucleofection : - control negative Cy3
- Pre-miR,
- Anti-miR
- co-transfection Pre-miR+Anti-miR

miR 365 assay

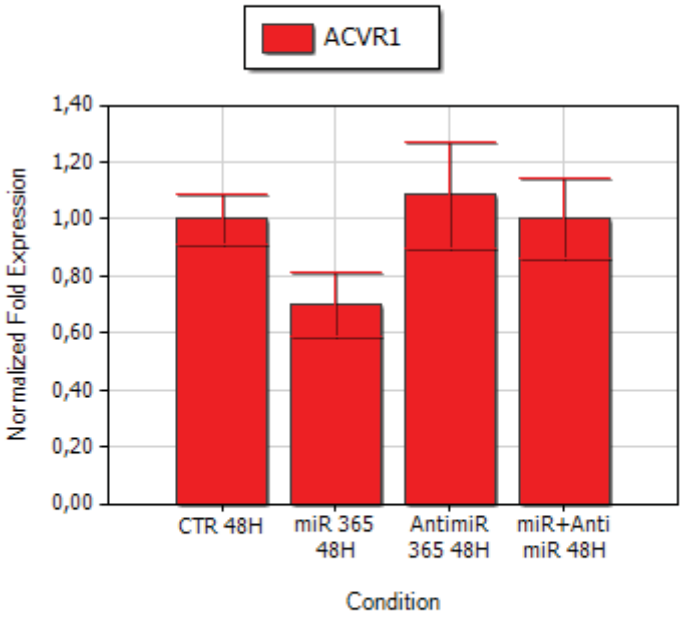
HeLa

24H



Gene Expression : RIEPILOGO_2009-10-26_1208.opd

48H

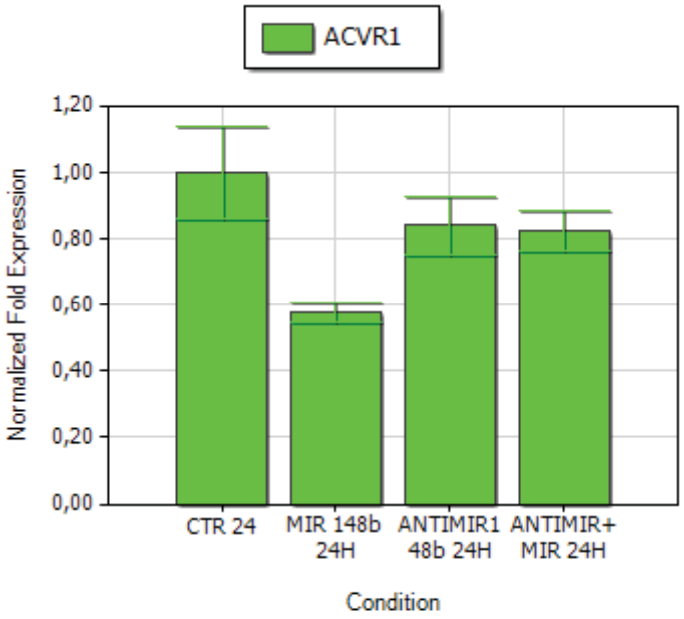


Gene Expression : RIEPILOGO_2009-10-26_1208.opd

miR 148b assay

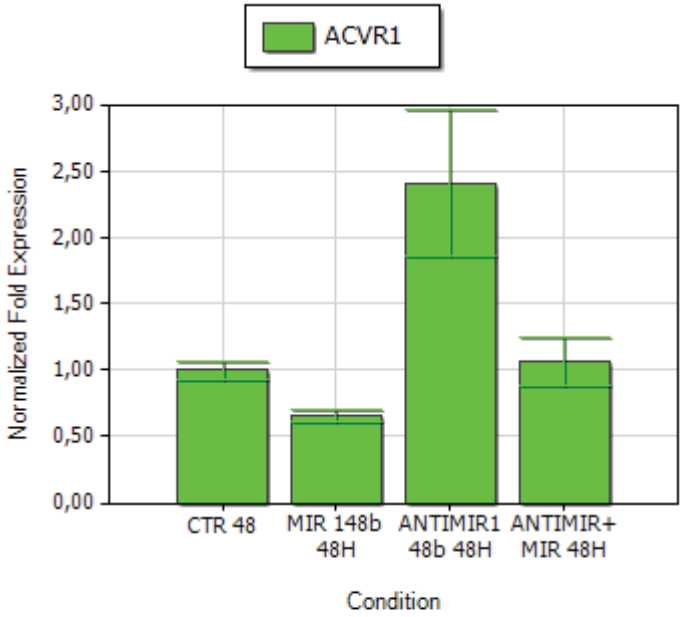
HeLa

24H



Gene Expression : ANALISIData 2009-11-30 1124.opd

48H

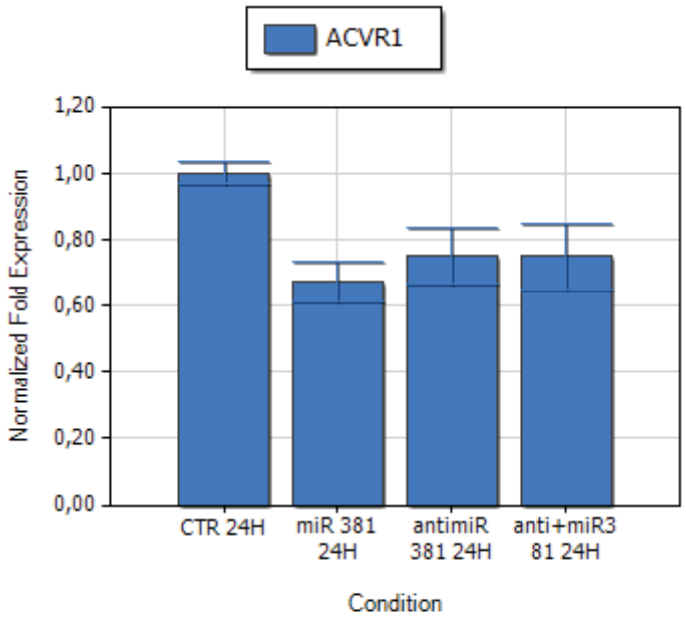


Gene Expression : ANALISIData 2009-11-30 1124.opd

miR 381 assay

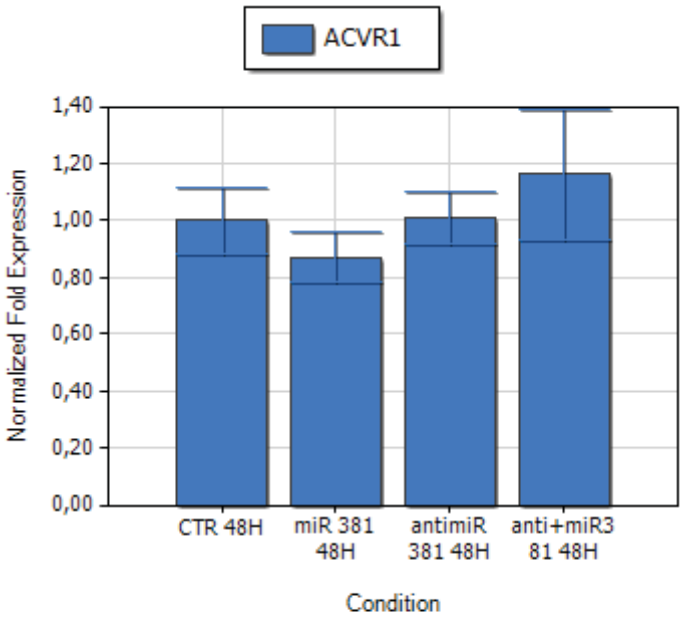
HeLa

24H



Gene Expression : ANALISIData 2010-01-15 1458.opd

48H

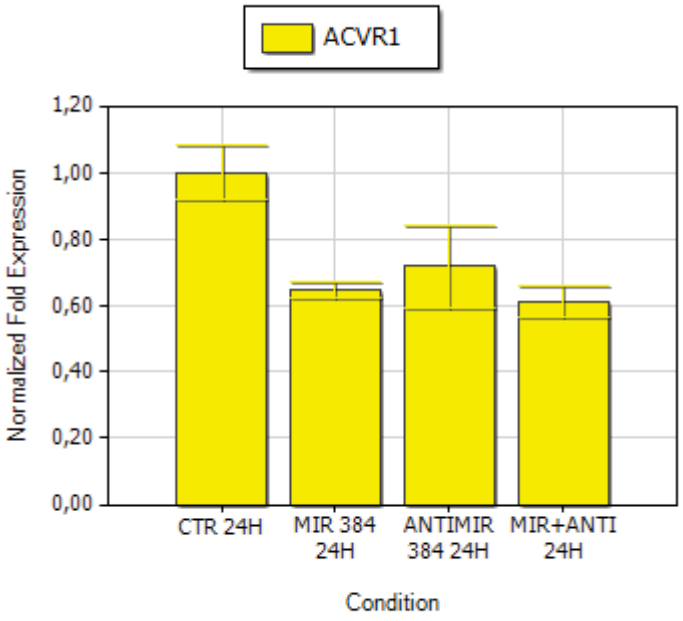


Gene Expression : ANALISIData 2010-01-15 1458.opd

miR 384 assay

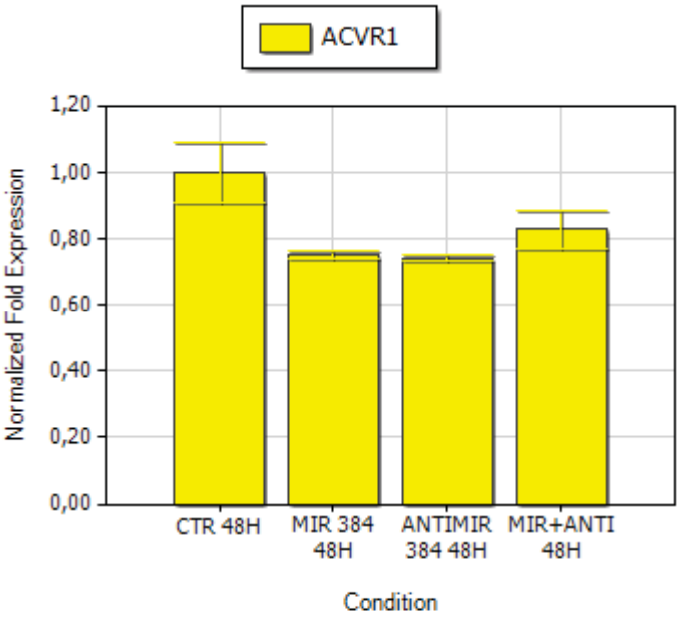
HeLa

24H



Gene Expression : ANALISIData 2010-02-19 1044.opd

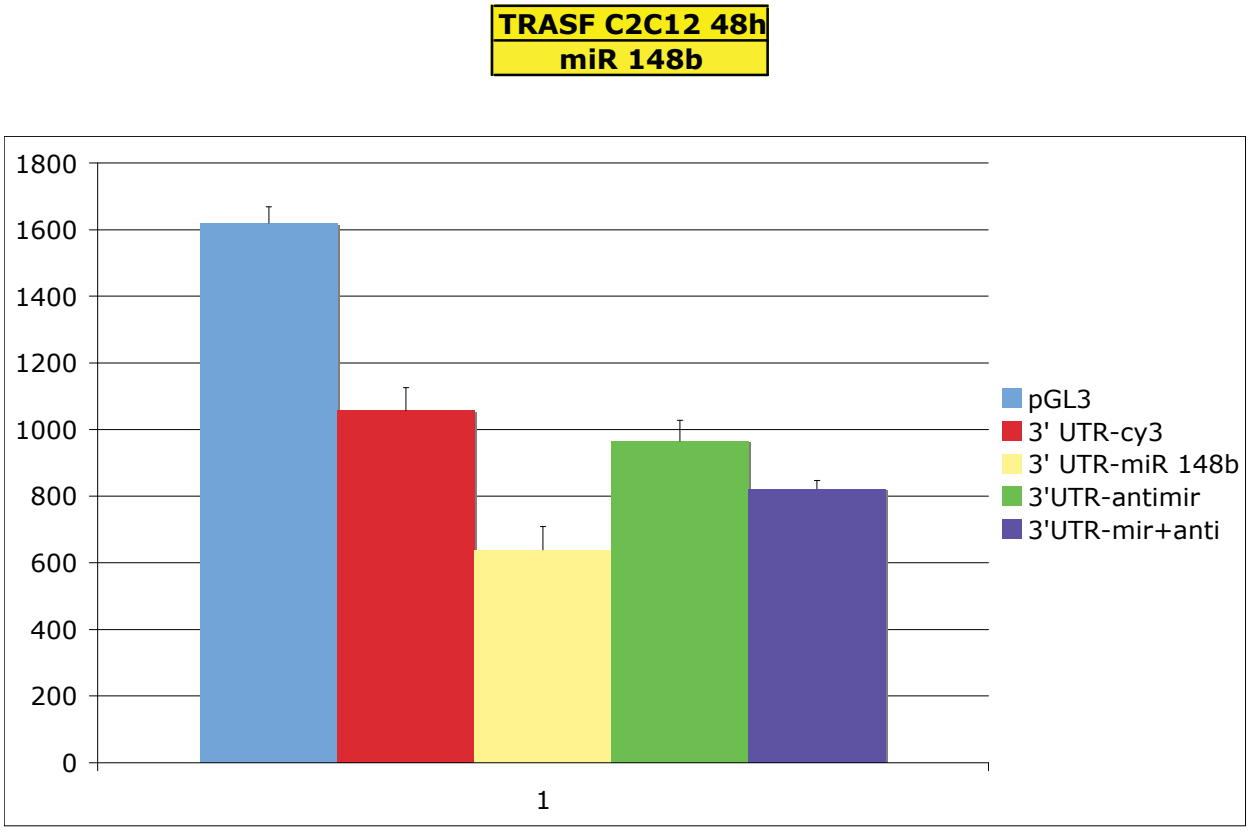
48H



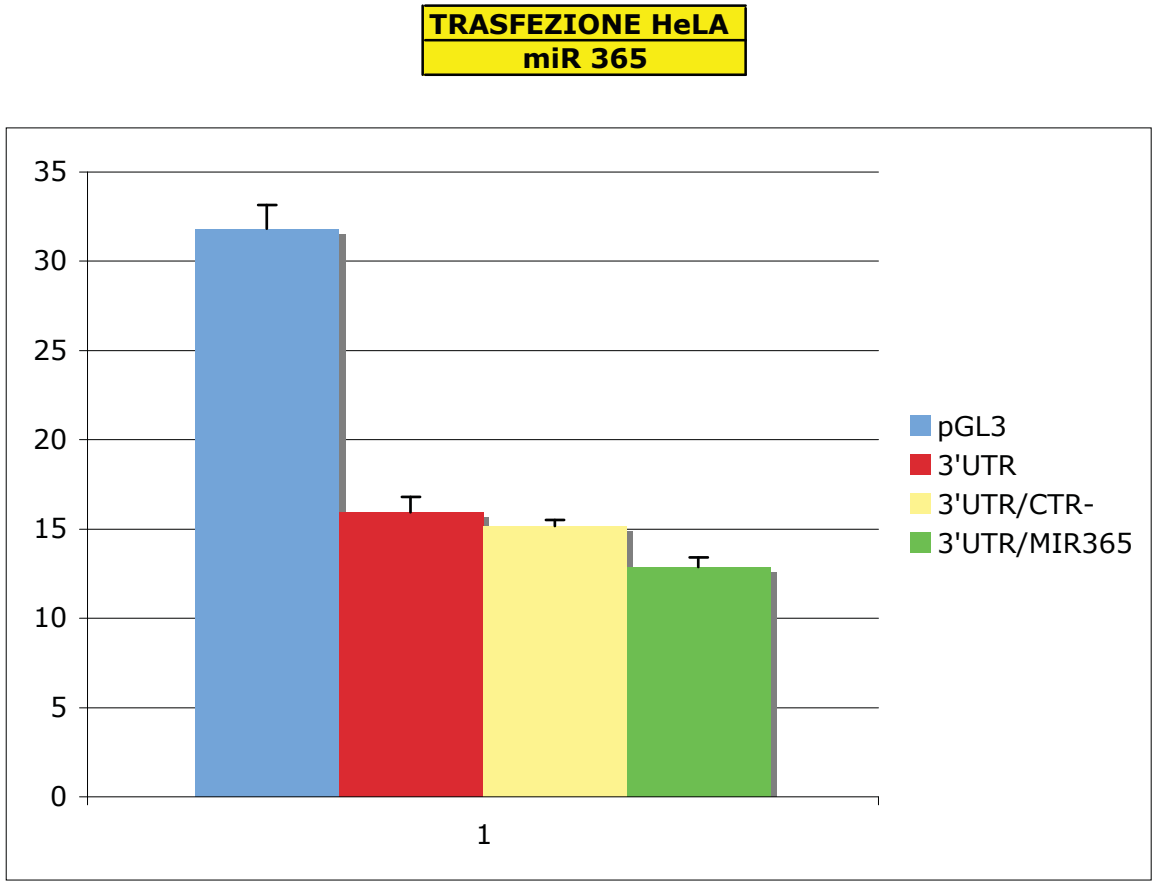
Gene Expression : ANALISIData 2010-02-19 1044.opd

- Co-transfection of ACVR1 3'UTR and selected miRNA

- Luciferase activity assessment



The miR-148b presence reduces further the reporter gene activity induced by the 3'UTR of ACVR1. His antagonist anti-miR restores the effect induced by the 3'UTR only.



The miR-365 presence further reduces the decrease of the reporter gene induced by the 3'UTR of *ACVR1*.

Commento

Nella regione 3’-UTR si esercita un regolazione dell’espressione in termini di inibizione dell’espressione.

Alcuni microRNA che riconoscono sequenze nel 3’-UTR sembrano agire come inibitori.

Un obiettivo ambizioso

Trovare dei composti chimici che esercitno un effetto di inibizione sulla funzione della via delle BMP

Bisogna mettere a punto un metodo di analisi

US agencies collaborate to speed up innovative drug development

In the United States, the Food and Drug Administration (FDA) and the National Institutes of Health (NIH) have embarked upon an initiative that will accelerate the journey of bringing innovative medicinal products to market. The new collaboration twines translational research with regulatory science - both key to transforming *"biomedical discoveries into products that benefit people"*. A Joint NIH-FDA Leadership Council will oversee the initiative to *"help ensure that regulatory considerations form an integral component of biomedical research planning and that the latest science is integrated into the regulatory review process"*. A Request for Applications that will make $USD 6.75 million available over three years will be issued. A press release states that the *"research supported through this initiative should add to the scientific knowledge base by providing new methods, models or technologies that will inform the scientific and regulatory community about better approaches to evaluating safety and efficacy in medical product development"*. Orphan drugs represent some 20% of all innovative products.

orphaNews Europe, 7 Aprile 2010

Due importanti Agenzie negli Stati Uniti, NIH e FDA, collaborano per accelerare lo sviluppo di farmaci innovativi

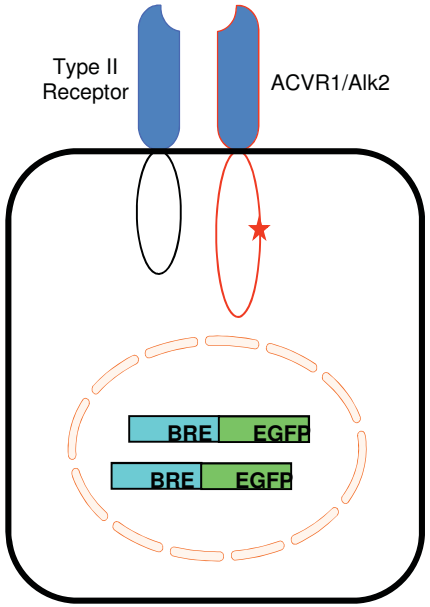
Study of the regulation of ACVR1 expression

- Characterization of the structure and composition of the *ACVR1* transcripts.
- Definition and characterization of sequences playing a role in the regulation of *ACVR1* expression.

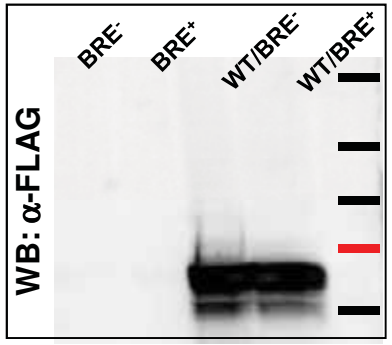
  - ✓**5'*UTR* sequences**
    Promoter region
  - ✓**3'*UTR* region**

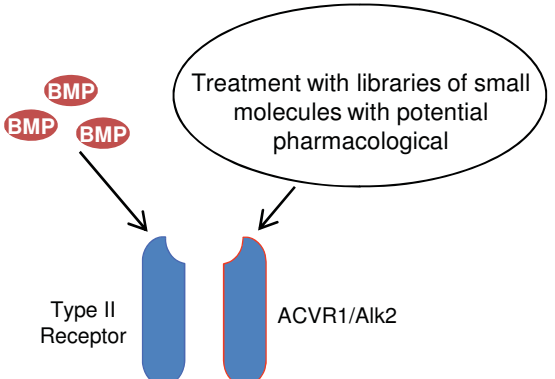
Generation of a cellular model suitable for the high through-put screening of small molecules with potential pharmacological effect on the BMP/ACVR1 mediated pathway.

A) Generation of stable cell lines expressing both ACVR1/ALK2 receptor (wild-type and mutated form) and the reporter gene (EGFP) controlled by a BMP responsive element in C2C12 cells.

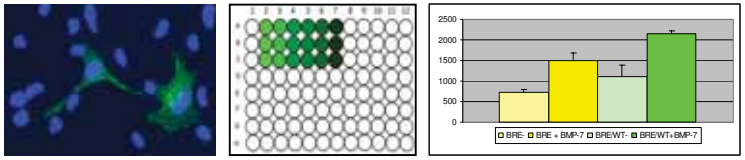


Check for the ACVR1 construct expression by WB analysis



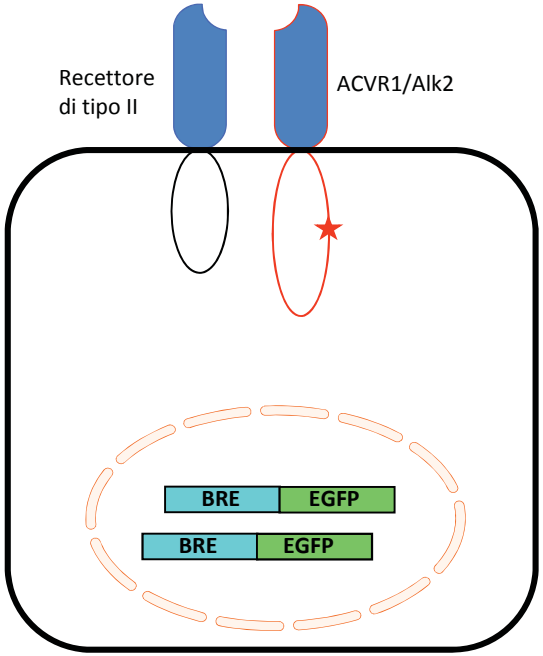


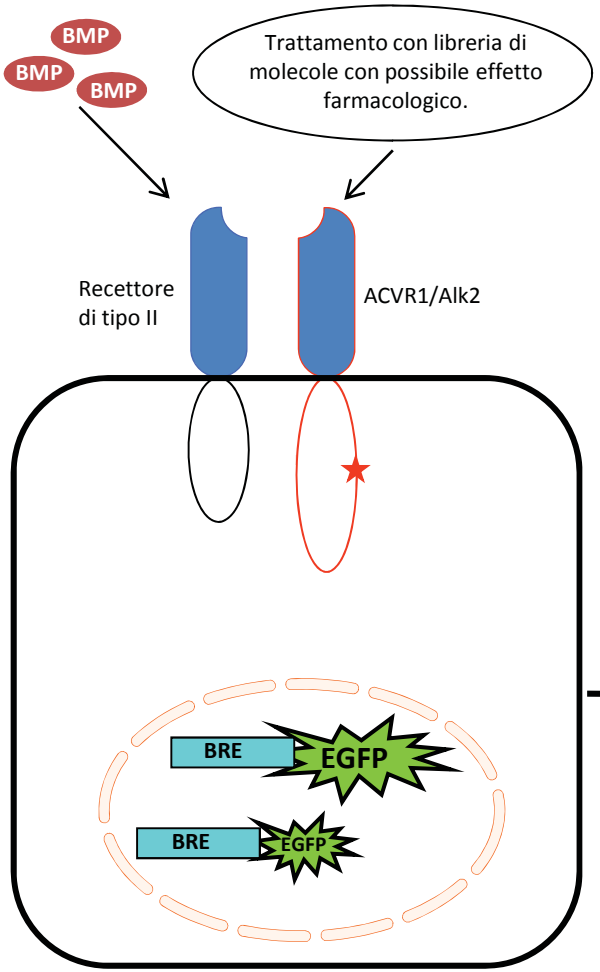
The effect of the tested molecules on the BMP-ACVR1 pathway is evaluated with a plate- reader instrument through the detection and measurement of the fluorescent signal emitted by the reporter gene, directly on living cells.



A) Generazione sistema cellulare
Introduzione del recettore ACVR1/ALK2 mutato (stella rossa) e del gene reporter (EGFP) controllato da un elemento responsivo all'attivazione delle BMP (BRE-EGFP) in cellule C2C12.

B) Trattamento delle cellule così ottenute con una libreria di molecole farmacologiche in presenza e assenza di stimolazione con BMP e valutazione del loro effetto in termini di variazione nella emissione di fluorescenza.





L'effetto delle molecole in analisi viene valutato mediante rilevazione e misurazione (lettore piastre) del segnale fluorescente emesso dal gene reporter