The Role of the 3’UTR Region in the Regulation of the ACVR1/Alk-2 Gene Expression

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Abstract

Background: The ACVR1/Alk-2 gene, encoding a BMP type I receptor, is mutated in Fibrodysplasia Ossificans Progressiva, a severe form of heterotopic ossification. Regulation of ACVR1/Alk-2 expression, still poorly understood, is likely to be controlled by transcriptional and post-transcriptional mechanisms. In our work, we focused on the functional role of the 3’UTR region of the gene and on microRNAs as possible modulators of the ACVR1/Alk-2 expression.

Results: The ACVR1/Alk-2 3’UTR region consists of a 1.1 kb sequence harboring several putative, well-conserved binding sites for miRNAs in its proximal half, and AU-rich elements in the distal one, as assessed by bioinformatic analysis. The functional role of this region was tested in presence of transcription inhibitors and in transfection experiments in different cell lines, with a ACVR1/Alk-2/3’UTR reporter construct. By this transfection-based approach, we have also verified that three microRNAs, among those predicted to target ACVR1/Alk-2 gene by in silico analysis, can bind its 3’UTR sequence thereby modulating its expression.

Conclusion: In this work we demonstrated that the ACVR1/Alk-2 transcript is unstable in presence of inhibitors of transcription. Functional analysis of the 3’UTR region by Luciferase reporter assays showed that it plays an inhibitory role on ACVR1/Alk-2 gene expression. Moreover, we found that specific miRNAs are involved in modulating ACVR1/Alk-2 gene expression as suggested by binding sites prediction in its 3’UTR sequence. In particular, we found that mir148b and mir365 were able to down-regulate ACVR1/Alk-2 expression, whereas mir26a showed a positive effect on its mRNA. Our data contribute to elucidate some of the mechanisms intervening in the modulation of ACVR1/Alk-2 expression. Considering that no specific and effective treatment of FOP is available, clarifying the basic mechanisms of the ACVR1/Alk-2 gene biology may provide means to develop innovative therapeutics approaches.

Introduction

A large class of small noncoding RNAs, known as microRNAs (miRs), functions as regulators of a broad range of cellular processes by modulating gene expression. The active molecule consists of short single-stranded RNA of about 22 nucleotides in length processed from longer (80–85 nt) precursor. The miRBASE database (release 17, April 2011) includes 16,772 entries, 1,424 of which are human miRs. Computer prediction of binding sites for miRs, among those predicted to target ACVR1/Alk-2 gene expression, still poorly understood, is likely to be controlled by transcriptional and post-transcriptional mechanisms. In our work, we focused on the functional role of the 3’UTR region of the gene and on microRNAs as possible modulators of the ACVR1/Alk-2 expression.

Results: The ACVR1/Alk-2 3’UTR region consists of a 1.1 kb sequence harboring several putative, well-conserved binding sites for miRNAs in its proximal half, and AU-rich elements in the distal one, as assessed by bioinformatic analysis. The functional role of this region was tested in presence of transcription inhibitors and in transfection experiments in different cell lines, with a ACVR1/Alk-2/3’UTR reporter construct. By this transfection-based approach, we have also verified that three microRNAs, among those predicted to target ACVR1/Alk-2 gene by in silico analysis, can bind its 3’UTR sequence thereby modulating its expression.

Conclusion: In this work we demonstrated that the ACVR1/Alk-2 transcript is unstable in presence of inhibitors of transcription. Functional analysis of the 3’UTR region by Luciferase reporter assays showed that it plays an inhibitory role on ACVR1/Alk-2 gene expression. Moreover, we found that specific miRNAs are involved in modulating ACVR1/Alk-2 gene expression as suggested by binding sites prediction in its 3’UTR sequence. In particular, we found that mir148b and mir365 were able to down-regulate ACVR1/Alk-2 expression, whereas mir26a showed a positive effect on its mRNA. Our data contribute to elucidate some of the mechanisms intervening in the modulation of ACVR1/Alk-2 expression. Considering that no specific and effective treatment of FOP is available, clarifying the basic mechanisms of the ACVR1/Alk-2 gene biology may provide means to develop innovative therapeutics approaches.
support the idea that the effect of mutation in the ACVR1/Alk-2 gene is activation of the SMAD-dependent signaling pathway.

The identification and characterization of the mechanisms intervening in the regulation of ACVR1/Alk-2 gene expression are complementary to studies aimed at identifying and characterizing the alteration of molecular and cellular mechanisms underlying heterotopic ossification process typical of FOP. Skeletal manifestations in this disease are the final result of a variety of biological processes that involve inflammatory/immune mechanisms and stimulation of multipotent progenitors to undertake an endochondral osteogenic process. This makes difficult to find a single cellular model that recapitulates the multistep and multicellular pathogenic mechanism, but requires to verify that, at least as an initial approach, some of the regulatory mechanisms are common to different cell types or there are significant differences.

Mechanisms of ACVR1/Alk-2 expression regulation have not been studied in deep up to now, therefore we have started to analyze the 3′UTR region to verify the possibility of post-transcriptional regulation by using cell lines of different origin and describe here the regulatory role of some miRs acting at the level of this region.

Results

ACVR1/Alk-2 gene is widely expressed in different tissues and cell lines and, like other receptors of important and critical signaling pathways, is likely to be targeted by different regulatory mechanisms of its gene expression.

The 3′UTR region of the ACVR1/Alk-2 gene consists of a 1.1 kb sequence highly conserved among species, in particular the percentage of identity between human and mouse is around 85% (Fig. 1A and Fig. S1). Extensive bioinformatic analysis of this region suggested the presence of sequences or conserved structural motifs with a potential functional role in post-transcriptional regulation of ACVR1/Alk-2 expression. The ACVR1/Alk-2 3′UTR is defined by two putative functional modules characterized by a proximal region containing several binding sites for miR and a more distal sequence harboring AU-rich Elements (ARE) represented by AUUUA pentamers. In particular for miRs, we applied different miR target prediction tools (see Materials and Methods section) to search for ACVR1/Alk-2-targeting miRs by which several miRs were predicted to target the ACVR1/Alk-2 3′UTR (not shown).

To carry out experimental work we applied different criteria to choose candidate miRs: a) recognition of binding site by at least two different dedicated programs utilizing different selection methods; b) involvement in bone related processes. This allowed us to selected three miRs: hsa-miR-148b (mir148b), hsa-miR-365 (mir365) and hsa-miR-26a (mir26a) (Fig. S1 and Fig. 2). Binding sites for mir148b and mir365 were recognized by all the dedicated programs applied; mir26a, although predicted by the miRanda software only, was considered as an interesting candidate because of previous work indicating its involvement in the post-transcriptional regulation of the BMP signaling pathway during in vitro osteogenesis by targeting SMAD1 [7].

Considering the possibility of post-transcriptional regulation at the level of mRNA stability as predicted by in silico analysis, we have quantified the transcript by both semi-quantitative and quantitative RT-PCR (qRT-PCR) in C2C12 cells treated for different times with known transcription inhibitor Actinomycin D (ActD). As shown in Figure 1B and C, the ACVR1/Alk-2 mRNA was significantly decreased after 3 and 6 hours in presence of the ActD transcription inhibitor. In particular, we found that expression of ACVR1/Alk-2 was reduced by about 75 and 80% after 3 and 6 hours of treatment with ActD (Fig. 1C and Fig. S2, panel A, black diamonds). Conversely, the mRNA of the ACVR1/Alk-2 gene appeared to be stable upon treatment of cells with the known translation inhibitor Cycloheximide (Chx), rather showing a slight but reproducible expression increase (Fig.S2, panel A, black squares). This phenomenon has been already described for genes known to have an unstable mRNA, and is considered due to the Chx effect on the synthesis of proteins involved in mRNA metabolism and stability [8,9]. When cells were treated with both ActD and Chx in combination, we could still observe a time-dependent reduction in the ACVR1/Alk-2 mRNA, although the slope of the decrease was attenuated (Fig. S2, panel A, grey triangles). Moreover, incubation of cells with Chx did not affect Luciferase activity by the expression construct carrying the 3′UTR sequence of the gene (Fig. S2, panel B).

To investigate the possible role of the 3′UTR region on post-transcriptional regulation of ACVR1/Alk-2 expression, the 3′UTR sequence of ACVR1/Alk-2 was subcloned into the pGL3-Promoter expression vector downstream of the Luciferase coding sequence (Fig. 3A). As indicated by our in silico analysis, the miR recognition sequences are mainly clustered in the proximal region of the ACVR1/Alk-2 3′UTR, therefore we also tested separately the functional contribution of the proximal half and of the distal region by generating two different Luciferase constructs (Fig. 3A, 3′UTR M and A). After transfection in different cell lines, the reporter gene activity was measured. As shown in Figure 3B, we found that the presence of the ACVR1/Alk-2 3′UTR sequence induced a decrease in Luciferase in all the tested cell lines, indicating that the region could affect the expression of the reporter gene at post-transcriptional level. Moreover, we found that both deletion fragments contribute to reduce the gene reporter expression, however, the strongest effect was observed with the whole 3′UTR sequence (Fig. 3B).

In order to verify if miRs play a role in the regulation of ACVR1/Alk-2 expression by targeting its 3′UTR, as predicted by bioinformatic means, the three selected miRs, mir148b, mir365 and mir26a commercially available as double stranded pre-miR (Table S2), were co-transfected with the pGL3-ACVR1/Alk-2-3′UTR construct in C2C12 and U2OS cell lines. The above miRs are expressed in these cell lines as well as in the HeLa cell line, although at different level (see Fig. S3).

At 24 and 48 hours after transfection, the Luciferase activity was measured and compared to that obtained by transfecting cells with scrambled non-targeting pre-miR as negative control and the respective specific anti-miRs. As shown in Figure 4, transfection of mir148b (Fig. 4A) and mir365 (Fig. 4B) reduced the reporter gene activity, in both cell lines, compared to that detected after co-transfection of the pGL3-ACVR1/Alk-2-3′UTR construct with the scrambled miR used as negative control. The observed downregulation of the reporter gene activity was specific because it was not observed with the control non-targeting miR and was reversed by the specific anti-miRs.

Conversely, we found that mir26a, co-transfected with pGL3-ACVR1/Alk-2-3′UTR construct, induced a significant increase in Luciferase activity compared to the control, suggesting a positive role on ACVR1/Alk-2 expression (Fig. 4C).

The effect of miRs on Luciferase activity may be linked to an action on the stability of reporter gene mRNA mediated by the presence of the 3′UTR of ACVR1/Alk-2 or, alternatively, to an effect on its translation. Therefore, we decided to evaluate the effect at the RNA level by studying the effect of the selected miR on the endogenous ACVR1/Alk-2 mRNA by RT-qPCR.

As shown in Figure 5, we found that mir148b (Fig. 5A) and mir365 (Fig. 5B) induced a downregulation of ACVR1/Alk-2 mRNA.
mRNA expression in U2OS and HeLa cells, both at 24 and 48 hours after transfection, with little if any effect in C2C12 cells. Conversely, and in accordance with reporter gene experiments, mir26a transfection caused an increase of ACVR1/Alk-2 mRNA expression in the three tested cell lines (Fig. 5C).

In order to further confirm the specificity of the effect of the selected miR on ACVR1/Alk-2 expression, site directed mutagen-
esis was used to modify the miR target sites in the 3′UTR sequence by changing 4 nucleotides of the miR seed (Fig. 2). Each of the selected miRs was then transfected in combination with both mutated and wild-type pGL3-ACVR1/Alk-2-3′UTR constructs in U2OS cells, and the effect on Luciferase activity evaluated after 24 hours (Fig. 6). The three miRs under analysis exerted their own effect through the binding at the putative site recognized by predictive bioinformatic analysis. For mir148b two putative binding sites have been identified and subjected to mutagenesis. As shown in Fig. 6A, seed mutation of the first site prevented the action of mir148, that was on the contrary apparently unaffected by mutation of the second consensus site (see Fig. 2 for position and seed sequence).

Also for mir365 two different potential binding sites were predicted in the ACVR1/Alk-2 3′UTR (Fig. 6B). Mutagenesis of the single sites, in particular of the seed of the first recognition sequence, partially abolished the action of mir365. As observed for mir148b, when both mir365 seeds were mutated we observed not only a rescue in Luciferase activity but also an increase in Luciferase activity, again suggesting that the two sites may cooperate and are necessary for mediating the action of the two miRs in targeting the 3′UTR of ACVR1/Alk-2 gene.

Finally, seed mutation of the mir26a binding site prevented the positive effect on ACVR1/Alk-2 expression mediated by the interaction of mir26a itself with its recognition sequence (Fig. 6C). As the pairing region of mir26a includes the first of the AUUUA modules (Au-rich element) mapping in the distal half...

Figure 2. Predicted miR recognition sequences in the 3′UTR of the ACVR1/Alk-2 gene. For each of the selected miRs, duplexes between miRNA sequences (middle) and wild-type (bottom) 3′UTR or harboring the corresponding mutated seed (top) are shown. Numbers on the left side indicate the matching starting position in the ACVR1/Alk-2 3′UTR, G:U wobble ($) and Watson-Crick pairing (|) are also indicated. miRVS scores rank the efficiency of the predicted binding sites, good scores are ≤-0.1 [33].
doi:10.1371/journal.pone.0050958.g002
of the 3'UTR, and it is reported that ARE sequences intervene and sometimes interfere with miRs in the post-transcriptional regulation of gene expression, we have altered the corresponding ATTTA sequence by site directed mutagenesis and generated constructs carrying either the single ACCCA mutated element (ATTTA mut) or a double mutant carrying also the mir26a seed mutation (ATTTA mut/mir26a mut). After transfection with both the negative Cy3-labeled control and mir26a, Luciferase activity of the different constructs was evaluated. As shown in Figure 6C, change of the AUUUA motif both by itself or in combination with mutation of seed of mir26a, resulted in a partial loss of the positive effect observed in presence of mir26a, with no significant difference in the activity of the double mutant compared to that of the mir26a seed.

**Discussion**

Regulation of gene expression is a crucial process in cell biology taking place by multiple control mechanisms at different levels. Recently, a growing body of the literature has focused on the role of miRs, as crucial players of post-transcriptional regulation of gene expression, both in physiological conditions and in disease [10]. miRs are mainly involved in negative regulation of gene expression through two basic modes of action, one based on mRNA decay and the other on translational repression (followed by mRNA decay). A complicating factor is that these processes are closely linked in the cell [1].

Mechanisms and factors intervening in the regulation of ACVR1/Alk-2 gene expression are still poorly investigated, although the encoded protein, a type I receptor for BMPs, is involved in fundamental biological processes during embryo development, osteogenic differentiation of progenitor mesenchymal cells [11–15], and various pathological conditions including cancer [16,17]. Since mutations in the gene, proved to induce gain of function, are associated with the severely disabling FOP for which no specific treatment is available, understanding mechanisms of regulation should increase the identification of possible targets for new therapeutic strategies.

The pathogenic mechanisms responsible for FOP are still largely unclear. Moreover, the histological features of the early FOP lesions and the observation that stimuli such as trauma or infections may initiate or exacerbate the acute phases of the disease [18], suggests that inflammation and the immune response, together with the presence of the mutated receptor, are a central issue in FOP.

In this work we focused our attention on the role of the 3'UTR sequence in the modulation of the ACVR1/Alk-2 gene expression at post-transcriptional level. We found that the introduction of the 3'UTR region of ACVR1/Alk-2 in a Luciferase reporter gene assay was able to induce a decrease of its expression, supporting the idea
that the region could be involved in the regulation of gene expression.

Data present in GenBank indicate that ACVR1/Alk-2 transcript is expressed in many different cell types raising the question whether many different cell types might possess different mechanisms to modulate its expression according to the biological processes in which they are involved.

Our bioinformatic analysis on the 1.1 kb 3’UTR region suggested the presence of sequences or conserved structural motifs with a potential functional role in post-transcriptional regulation of ACVR1/Alk-2 expression. We found that the 3’UTR region may be divided in two putative functional modules characterized by a proximal half containing most of the predicted binding sites for miRs and a more distal sequence harboring several AUUUA pentamers.

Our finding that ACVR1/Alk-2 mRNA is unstable in presence of transcription inhibitors supported the bioinformatic prediction and our hypothesis that it could be subjected to post-transcriptional regulation mechanisms, and, at this level, one of the possible mechanisms involves the action of miRs. Moreover, a recently published article reported that ACVR1/Alk-2 is repressed by binding of mir30c to its 3’UTR, during adipocytes differentiation [19].

Selection of miRs among those suggested by in silico analysis was based on prediction by different specialized programs for mir148b and mir365 (see Materials and Methods section) at two different and conserved putative binding sites. For mir26a, binding was
predicted only by one program, the miRanda software, however, as already mentioned we considered it an interesting candidate [7].

For mir148b and mir365 our findings were in accordance with the effect of most miRs, since they were able to downregulate ACVR1/Alk-2 expression, whereas mir26a exerted an unexpected positive effect. All three miRs acted by binding the predicted consensus sequences, as demonstrated by the abolition of their effects after mutagenesis of the respective binding elements.

mir148b belongs to the mir148 family of miRs comprising also mir148a and mir152 (see [20]). They largely share the consensus binding site sequence, in particular seeds of mir148a and 148b differ by two nucleotides in position 7. We decided to focus on mir148b since it was reported to exert an effect on osteogenic
differentiation of mesenchimal stem cells [21]; tumorigenic processes [22,23] and the innate immune response [20].

Studies on mir365 indicate that it can be involved in regulation of genes involved in the control of cell cycle and senescence [24]; in apoptosis of endothelial cells [25]; in brown fat differentiation by inhibiting myogenesis [26]; in triggering chondrocytes proliferation and maturation [27]; in targeting the expression of IL-6 gene [28].

![Figure 6. Binding site specific mutagenesis prevents the modulation of ACVR1/Alk-2 expression operated by the selected miRs.](image)

Mutagenesis of miRs binding sites was introduced in the two seed sequences predicted for mir148b (A) and mir365 (B), indicated as Seed1 and Seed 2, and in the mir26a seed sequence (C). Site specific mutagenesis was also performed to abolish the first AUUUA element contained in the mir26a pairing region (ATTTA mut), alone or in combination with the mutation of the mir26a seed sequence (double mutant mir26 mut/ATTTA mut). Each pair of bars represents Luciferase activity in the presence of negative control miR (-), considered as 100%, or the specific miR (+). Student’s t-test was applied to evaluate differences between the effect of specific mir compared to that of the negative control for each construct, \( p<0.05** \), \( p<0.01** \), \( p<0.001*** \). doi:10.1371/journal.pone.0050958.g006
Regarding mir 26a, it was reported to affect the osteogenic differentiation of human adipose tissue-derived stem cells induced by BMP treatment. Its expression increases progressively during the differentiation process and, in terminal differentiated cells, contributes to silencing of the BMP pathway by targeting the SMAD1 protein [7]. It is considered a tumor suppressor gene as its expression is downregulated in different forms of cancer and this correlates with poor prognosis [29–31]. In the immune system, mir26a appears to intervene in the IFNβ pathway in primary macrophages [32].

mir26a showed an upregulating effect on ACVR1/Alk-2 expression, contrary to the most typical effect of miRs, mainly inducing negative regulation of gene expression. A similar “paradoxical” upregulating effect of miR was described in condition of response to particular stimuli, such as culture in serum deprivation, for the TNFα gene, which is highly regulated at post-transcriptional level, by mechanisms correlated with the presence of particular sequence elements rich in A and U nucleotides (AU-rich Elements, ARE) in the 3'UTR region [33,34].

The ACVR1/Alk-2 3'UTR sequence has a structure highly reminiscent of that of the TNFα gene and harbors five AUUUA motifs, the first of which is located at the mir26a pairing region, immediately upstream of the seed (see Fig. S1). We propose that the apparent positive regulation of ACVR1/Alk-2 induced by mir26a could be due to interaction of these two functional elements (mir26a binding site and ARE). Results of mutational analyses indicated that mutagenesis of the AUUUA motif induced a decrease of the effect of mir26a on Luciferase activity both by itself and in combination with the mutation of the mir26a seed, probably as a result of the perturbation of the pairing region for mir26a.

As the role of inflammation and of the immune system is relevant for FOP pathogenesis, it is very interesting to note that the structural and functional features of the 3'UTR sequence of ACVR1/Alk-2, are reminiscent of what is observed for genes encoding cytokines or molecules involved in inflammation pathways, thus suggesting for ACVR1/Alk-2 expression similar mechanisms of regulation. Indeed, many cytokine miRNAs are expressed as short lived transcripts subjected to highly regulated post-transcriptional controls, involving the presence of miR binding sites and ARE motifs that can affect mRNA stability [35].

Our work highlights the relevance of miRs in gene expression regulation, and in particular, we provide the first evidence that mir148b, mir365 and mir26a can target ACVR1/Alk-2 expression. miRs are considered valuable cellular targets for the development of specific and innovative therapeutic interventions and this could be of particular interest for FOP, as no specific treatment is presently available to cure the disease.

Materials and Methods

Bioinformatic Analysis

Extensive bioinformatic analysis of ACVR1/Alk-2 3’UTR sequence was performed using different programs available on line. The conservation profile was obtained with the tools for comparative genomics of the VISTA genome Browser (http://pipeline.lbl.gov/cgi-bin/gateway).

Four different programs were applied to identify putative sites for miRs: TargetScan5.1 (http://www.targetscan.org/), PicTar (http://pictar.mdc-berlin.de/), miRBase (http://www.mirbase.org/), and miRanda (http://www.microrna.org). For each miR we have indicated the miRanda predicted binding sites (Fig. 2) [36]. We also used a program for AU-rich elements-containing sequence detection available at http://brp.kfshtc.edu.sa/ARED/.

PCR Amplification of ACVR/Alk-2 3’UTR Sequence

The 3’UTR region of the ACVR1/Alk-2 gene (UCSC Feb 2009, chr2:158,592,958-158,594,042) was obtained by PCR from genomic DNA of a control individual with the oligonucleotides specified in Table S1. The products corresponding to the entire 3’UTR sequence or to the derived fragments (M and A, see Fig. 3A) were inserted in the XbaI restriction site of the pGL3 Promoter Vector (Promega), downstream of the Luciferase reporter cDNA. Constructs were checked by sequencing.

Cell Culture, Treatment and Transfection

Different established cell lines were used for experiments: HeLa, U2OS, and C2G12 cells. These cell lines were already available in our laboratory and were originally purchased from ATCC (ATCC-LGC Standards Partnership). Cells were cultured in DMEM medium with 10% FBS, penicillin-streptomycin (50 U/ml) and 2 mM glutamine. HeLa cells were cultured in MEM medium with 10% FBS, penicillin-streptomycin (50 U/ml), 2 mM glutamine, 1 mM sodium pyruvate and 1 mM non essential aminoacids. Cells were grown at 37°C with 5% CO2.

To check the stability of the ACVR1/Alk-2 mRNA, C2G12 cells, were treated for different times (0–3–6 hours) in presence or absence of transcription inhibitors Actinomycin D 10 μg/ml (AcD, Sigma) and Doxorubicin 1 μM (Dox, Sigma) (not shown), and the mRNA expression was quantified by quantitative RT-qPCR. C2G12 cells were also treated with the protein synthesis inhibitor Cycloheximide at 10 μg/ml (Chx, Enzo Life Sciences) both as single treatment or in combination with AcD.

To carry out the functional study, reporter constructs carrying the entire 3’UTR of ACVR1/Alk-2 and the two derived fragments, were transfected in the indicated cell lines with the Lipofectamine 2000 transfecting agent according to the protocol provided by the manufacturer (Invitrogen, Life Technology). In order to normalize the transfection efficiency, the reporter constructs under analysis were always transfected together with the pRL-SV40 Renilla expression vector. Each experiment was performed in triplicate.

Subsequently, to investigate the possible role of miR on post-transcriptional regulation of ACVR1/Alk-2 expression, we selected three miRs by bioinformatic analysis, filtered on the basis of the conservation of their binding sites among species and recognized by at least two of the softwares used.

Selected miRs were transfected in the different cell lines as commercially available double stranded pre-miR molecules (pre-MIR, Ambion, Life Technology), cells were also transfected with a negative control (AM17120 Cy3 labeled pre-miR negative control n. 1, Ambion, Life Technology,) or with the corresponding anti-miR molecule (Ambion, Life Technology) (Table S2). After 24 and 48 hours post-transfection, cells were harvested and processed for assessing the Luciferase activity or for total RNA extraction to be used in RT-qPCR experiments.

The Dual Luciferase Reporter Assay System from Promega was used to measure Luciferase activity according to the provided protocol. For Luciferase assay a minimum of two independent transfection experiments were performed, with each point in triplicate.

RNA Extraction and cDNA Synthesis

Total RNA was extracted from cells using the RNeasy Mini Kit from Qiagen. RNA quantity was measured with Nanodrop Spectrophotometer (Thermoscientific), and first strand cDNA

PLOS ONE | www.plosone.org 9 December 2012 | Volume 7 | Issue 12 | e50958
was synthesized with the Advantage RT-for-PCR Kit (Becton Dickinson) according to the manufacturer’s instructions.

Quantitative Reverse Transcription PCR (RT-qPCR)

The effect of individual miRs on endogenous ACVR1/Alk-2 transcript was evaluated through RT-qPCR using specific TaqMan Gene Expression Assay (Applied Biosystems) (Table S2). Samples were measured in triplicate and the results were normalized using TaqMan Gene Expression Assays for two reference genes: β-Actin and GAPDH. qPCR was run on the iQ5 instrument from Bio-Rad, and Data analysis was performed using the provided Bio-Rad iQ5 software for Gene Expression Study.

Mutagenesis

Quickchange Lightning site-directed mutagenesis kit from Stratagene was used to mutagenize four bases in the seed of each of the the miR binding sites in ACVR1/Alk-2 3′UTR constructs (as indicated in Fig. 2), following the provided protocol. Oligonucleotides used in mutagenesis are indicated in Table S1.

miRNA Endogenous Expression

The RNA fraction containing miRNA was isolated from the different cell lines HeLa, C2C12, and U2OS, by using mirVana miRNA Detection kit (Ambion), cDNA was synthesized with TaqMan MicroRNA Reverse Transcription Kit and the expression profile of mir140b, mir365 and mir26a was evaluated by RT-qPCR (Table S2 for probe specification), using specific TaqMan MicroRNA Assays (Applied Biosystems). Each assay was performed in triplicate.

Statistical Analysis

All the experiments were performed in triplicate and repeated at least twice independently (two - up to five times). Where indicated Unpaired Student’s t-Test (two tails) was applied to verify statistical significance of the observed variations.

Supporting Information

Figure S1 Comparison between Human and mouse ACVR1/Alk-2 3′UTR sequences (Clustal alignment). * indicates the full conservation of the corresponding nucleotide position. Pairing region of selected miRs and the ARE sequences-containing module are highlighted (light grey and dark grey respectively).

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