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MiRNA let-7g regulates skeletal myoblast motility via Pinch-2

S. Boudoukha [a,b,c], T. Rivera Vargas [a,b], I. Dang [d], J. Kropp [a,b], S. Cuvellier [e], A. Gautreau [d], A. Polesskaya [a,b,*]

[a] CNRS FRE 3377, CEA Saclay, F-91191 Gif-sur-Yvette, France
[b] Univ Paris-Sud, FRE 3377, F-91191 Gif-sur-Yvette, France
[c] Department of Biochemistry and Biophysics, University of California, San Francisco, CA, USA
[d] Centre de Recherche de Gif, Laboratoire d'Enzymologie et Biochimie Structurales, CNRS UPR3082, Avenue de la Terrasse, 91198 Gif-sur-Yvette Cedex, France
[e] Inserm U1016, Institut Cochin, INSERM U1016, CNRS 8104, Université Paris Descartes, F-75014 Paris, France

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ABSTRACT

Post-transcriptional regulation of gene expression by RNA-binding proteins and by small non-coding RNAs plays an important role in cell biology. Our previous results show that in murine skeletal myoblasts, the expression of Pinch-2, a focal adhesion remodeling factor that regulates cell motility, is repressed by an RNA-binding protein IMP-2/Igf2bp2. We now show that the expression of Pinch-2 is also regulated by the miRNA let-7g. Let-7g and IMP-2 repress Pinch-2 expression independently of each other. A knock-down of let-7g leads to an increase in Pinch-2 expression, and to a decrease of cell motility, which can be reversed by a simultaneous knock-down of Pinch-2. We conclude that let-7g controls the motility of mouse myoblasts in cell culture by post-transcriptionally regulating the expression of Pinch-2.

1. Introduction

Post-transcriptional regulation of gene expression depends on multiple and complex mechanisms that fine-tune the levels of protein synthesis from specific mRNA transcripts. The main regulatory factors of post-transcriptional regulation are various RNA-binding proteins, as well as small non-coding RNAs, such as microRNAs (miRNAs). Some of the best-characterized examples of such regulation by RNA-binding proteins include the studies of the IMP/Igf2bp1-3 (Insulin-Like Growth Factor 2 mRNA-binding proteins 1, 2, and 3) family and its mRNA targets [1], reviewed in [2]. Thus, IMP-1/ZBP-1 (zipcode-binding protein 1) binds to a group of mRNAs coding for key regulatory factors for cellular motility and adhesion (β-actin, the Arp2/3 complex, alpha-actinin, E-cadherin) [3]. The functions of IMP-1 in human cancer cells include stabilization of focal adhesions and maintenance of the cellular capacity for directional motility [4]. Other studies have also shown a link between cellular motility, cellular adhesion, and the expression of members of IMP family [5].

Focal adhesions (FAs) are large protein complexes that directly regulate cell anchorage and cell motility, and also have an important impact on cell proliferation, stress response and signal transduction (reviewed in [6]). These structures are highly dynamic and can rapidly disassemble and reassemble following an interaction with numerous FA regulatory proteins. Pinch-2 protein can interact with the FAs and induce their remodeling in human and mouse cells, and an overexpression of Pinch-2 leads to dramatic changes in cell spreading and cell motility [7,8]. Thus, the expression levels of Pinch-2 have to be regulated by the cell in order to ensure a normal functioning. The migration capacity of myoblasts is particularly important in skeletal muscle development and maintenance, where the cells frequently have to undergo directed, long-range migration in order to form new muscle, or to regenerate the damaged muscle tissue (reviewed in [9]). We have earlier shown that the expression of Pinch-2 is very efficiently regulated at the post-transcriptional level by an RNA-binding protein IMP-2/Igf2bp2 in mouse and human muscle cells [10]. In the current study, we are describing another post-transcriptional regulatory mechanism that controls Pinch-2 expression in mouse myoblasts.

We now show that the expression of Pinch-2 depends on Argonaute 2 (Ago-2), a key regulatory protein in RNA interference (RNAi), indicating that Pinch-2 mRNA can be a target of RNAi in

* Corresponding author at: CNRS FRE 3377, CEA Saclay, F-91191 Gif-sur-Yvette, France.
E-mail address: anna.polesskaya@cea.fr (A. Polesskaya).

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mouse myoblasts. Indeed, we find that in addition to IMP-2, the expression of Pinch-2 is repressed by the microRNA (miRNA) let-7g-5p (further referred to as let-7g). Let-7g belongs to one of the most widely and abundantly expressed, conserved, and functionally important miRNA families known to date (reviewed in [11,12]). In human, this family consists of ten highly similar miRNAs (let-7a to i, miR-98 and miR-202) that share the same seed sequence, and in most cases were shown to target the same mRNAs and to have redundant functions (reviewed in [11]).

We show that the 3′UTR of Pinch-2 mRNA has four functionally important sites for let-7g. A knock-down (KD) of let-7g or of IMP-2 significantly increased Pinch-2 mRNA and protein levels, and a simultaneous KD of let-7g and IMP-2 led to an additional increase of Pinch-2, suggesting that the repression by IMP-2 and let-7g occurs by two independent mechanisms. Finally, we show that let-7g is important for the motility of mouse myoblasts, and that this function of let-7g depends on Pinch-2 expression levels.

2. Results

2.1. Expression of Pinch-2 in mouse myoblasts is suppressed by Ago-2 protein

To characterize the mechanisms of Pinch-2 post-transcriptional regulation in mouse myoblasts C2C12, we have decreased the expression of key RNAi regulatory protein Ago-2 by three different siRNAs and analyzed the expression of Pinch-2 mRNA and protein. In mammalian cells, Ago-2 is known to be recruited to the target mRNAs by specific short non-coding RNAs (microRNAs, or miRNAs). Subsequently, miRNA-recruited Ago-2 containing complexes can induce translational repression and/or degradation of the target mRNAs (reviewed in [13]).

The results of RT-qPCR and Western blot assays in C2C12 myoblasts depleted of Ago-2 show an up-regulation of endogenous Pinch-2 mRNA and protein in Ago-2 depleted cells (Fig. 1A and B). We concluded that Pinch-2 can be a target of miRNA regulation in mouse myoblasts.

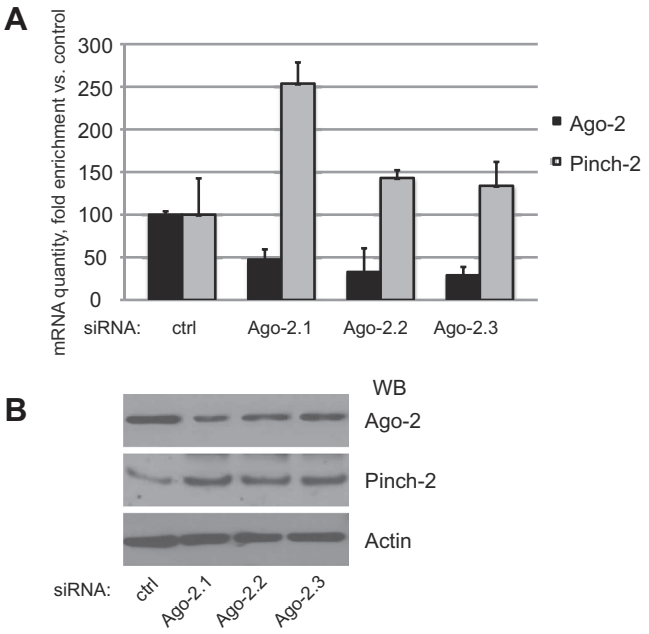

Fig. 1. Expression of Pinch-2 in mouse myoblasts is suppressed by Ago-2 protein. (A) Pinch-2 mRNA levels were quantified by RT-qPCR in C2C12 myoblasts transfected with the indicated siRNAs and collected 48 h post-transfection. Control: Cyclophilin A. (B) Western blot showing the protein levels of Ago-2, Pinch-2 and β-actin in cells described in A. Ago-2. 1–3 stand for three distinct siRNAs against Ago-2. We have subsequently studied the expression of endogenous let-7g in C2C12 myoblasts (Fig. 2D), and have demonstrated that inhibition of let-7g expression by antisense LNA (Locked Nucleic Acid) inhibitors was very efficient (Fig. 2E). A decrease of let-7g dramatically increased the levels of endogenous Pinch-2 mRNA and protein (Fig. 2F and G). As a positive control for the efficiency of let-7 inhibition, we have monitored two well-known let-7 targets, IGF2BP1 and HMGA2 (14). In a complementary gain-of-function assay, transfection of mimic let-7g molecules led to a slight, but highly reproducible inhibition of Pinch-2 expression (Fig. 2H). The slight effect of ectopic let-7g on endogenous Pinch-2 is hardly surprising, given the abundance and variety of endogenous let-7 miRNAs.

2.2. Pinch-2 expression is directly regulated by the miRNA let-7

We have analyzed the 3′UTR of PINCH-2 using the software available at http://genie.weizmann.ac.il/pubs/mir07/mir07_prediction.html, and identified a number of potential miRNA-binding sites. Among these predictions, we were interested in four potential let-7 binding sites at positions 28, 67, 189, and 205 (Fig. 2A). Let-7 miRNA family is widely expressed, conserved and very well studied, and the best characterized let-7 targets are known to possess multiple let-7 sites in their 3′-UTRs [14]. In order to study the effect of let-7 on the expression of Pinch-2, we have cloned the 3′UTR of Pinch-2 into the luciferase reporter construct psiCheck2. The expression of the Renilla luciferase in this construct is regulated by the 3′UTR of interest, whereas the Firefly luciferase serves as an internal control of the transfection efficiency. Co-transfection of the psiCheck2-Pinch-2 construct with let-7g or control miRNAs showed that the 3′UTR of Pinch-2 is strongly and specifically regulated by let-7g miRNA (Fig. 2B). In addition to an irrelevant negative control miRNA, we have included in our experiments miR-193b, a miRNA that is expressed in myoblasts, but is not predicted to target Pinch-2. Site-directed mutagenesis of let-7 sites in the 3′UTR of Pinch-2 showed that all four sites are necessary for this repression, because their individual inactivation did not significantly release the let-7 dependent repression (Fig. 2C).

We have studied the expression of endogenous let-7g in C2C12 myoblasts, which was found to be relatively stable in proliferating or in differentiating cells (Fig. 2D). Subsequently, we have demonstrated that inhibition of let-7g expression by antisense LNA (Locked Nucleic Acid) inhibitors was very efficient (Fig. 2E). A decrease of let-7g dramatically increased the levels of endogenous Pinch-2 mRNA and protein (Fig. 2F and G). As a positive control for the efficiency of let-7 inhibition, we have monitored two well-known let-7 targets, IGF2BP1 and HMGA2 [14]. In a complementary gain-of-function assay, transfection of let-7g mimic led to a slight, but highly reproducible inhibition of Pinch-2 expression (Fig. 2H). The slight effect of ectopic let-7g on endogenous Pinch-2 is hardly surprising, given the abundance and variety of endogenous let-7 miRNAs.

2.3. Let-7 and IMP-2 act independently to suppress the expression of Pinch-2

In our previous work, we have shown that Pinch-2 expression is down-regulated in C2C12 cells by the RNA-binding protein IMP-2, which interacts with the 3′UTR of Pinch-2 mRNA [10]. We have studied the regulation of endogenous Pinch-2 mRNA and protein by let-7g and IMP-2 by co-transfecting let-7g antisense inhibitor and/or two different siRNAs directed against IMP-2. In agreement with previous results, individual inhibition of let-7g or IMP-2 partially released the repression of Pinch-2 in C2C12 cells. A simultaneous inhibition of IMP-2 and let-7g led to a dramatic de-repression of Pinch-2 mRNA and protein (Fig. 3A and B). The de-repression obtained by a KD of let-7g and IMP-2 together was more important than the sum of the effects obtained by individual KDs of let-7g or of IMP-2. These results indicate that let-7g and IMP-2 repression factors can act independently on Pinch-2 3′UTR,

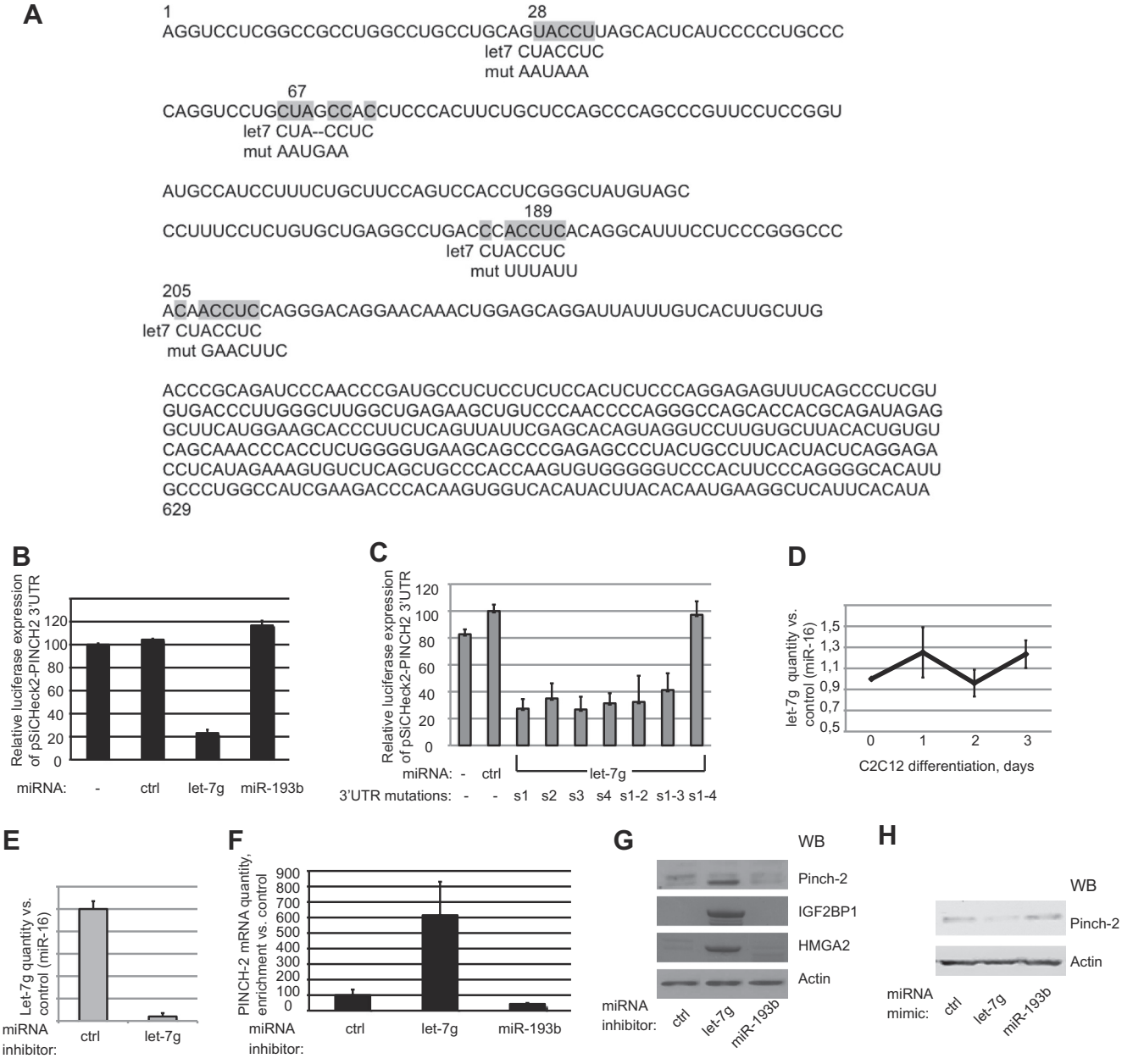

Fig. 2. Pinch-2 expression is directly regulated by the miRNA let-7. (A) A schema of the 3′UTR of mouse Pinch-2 with the potential target sites of let-7 miRNA shown in grey, numbers (28, 67, 189, 205) correspond to the positions of the first nucleotide of each target site, starting from the beginning of the 3′UTR. Let7: alignment with the seed sequence of let-7 family. Mut: mutations to potential let-7 target sites. (B) The 3′UTR of Pinch-2 was cloned into the pSiCheck.2 reporter vector after the reporter gene (Renilla luciferase) and the relative levels of Renilla versus Firefly luciferase (the latter serving as a control of the transfection efficiency) were measured in C2C12 cells co-transfected with the indicated miRNA precursors, 24 h post-transfection. (C) The potential let-7 target sites in the 3′UTR of pSiCheck.2-Pinch-2 were mutated individually or together, as indicated. Shown are the relative levels of Renilla versus Firefly luciferase measured in C2C12 cells co-transfected with control of let-7g miRNA precursors, 24 h post-transfection. (D) The expression of endogenous let-7g in C2C12 myoblasts in proliferation and differentiation was evaluated by RT-qPCR in three independent experiments (biological triplicate). (E) The efficiency of let-7g inhibition by LNA antisense inhibitor was evaluated by RT-qPCR. (F, G) The levels of endogenous Pinch-2 mRNA (F) and protein (G) were studied in C2C12 cells transfected with the indicated miRNA inhibitors and collected 48 h later. The levels of known let-7 targets IGF2BP1 and HMGA2 are monitored in (G) to evaluate the efficiency and specificity of let-7 inhibitor. (H) The levels of endogenous Pinch-2 protein in C2C12 cells transfected with the indicated miRNA mimics and collected 48 h later.

and that their simultaneous absence can have a synergistic positive effect on the expression of Pinch-2, probably by facilitating a recruitment of supplementary RNA-binding factors.

Consistent with these observations, inhibition of let-7g or of another let-7 family member, miR-98, in C2C12 myoblasts led to a remodeling of focal adhesions (FAs), similar to what can be observed in IMP-2 knock-down cells. FAs, here stained with vinculin or phospho-tyrosine antibody, appear as small individual structures in control cells, and have a strong tendency to cluster and/or fuse together in cells with inhibited let-7g/miR-98 or IMP-2 (Fig. 3C and D). This effect is consistent with what was previously observed in Pinch-2 overexpressing cells [10].

2.4. Let-7 inhibits the motility of C2C12 myoblasts in a PINCH-2 dependent manner

We and others have previously shown that up-regulation of Pinch-2 decreases the cellular motility [7,10]. Therefore, it was interesting to find out whether the levels of let-7 impacted upon the motility of C2C12 myoblasts. We have performed loss- and

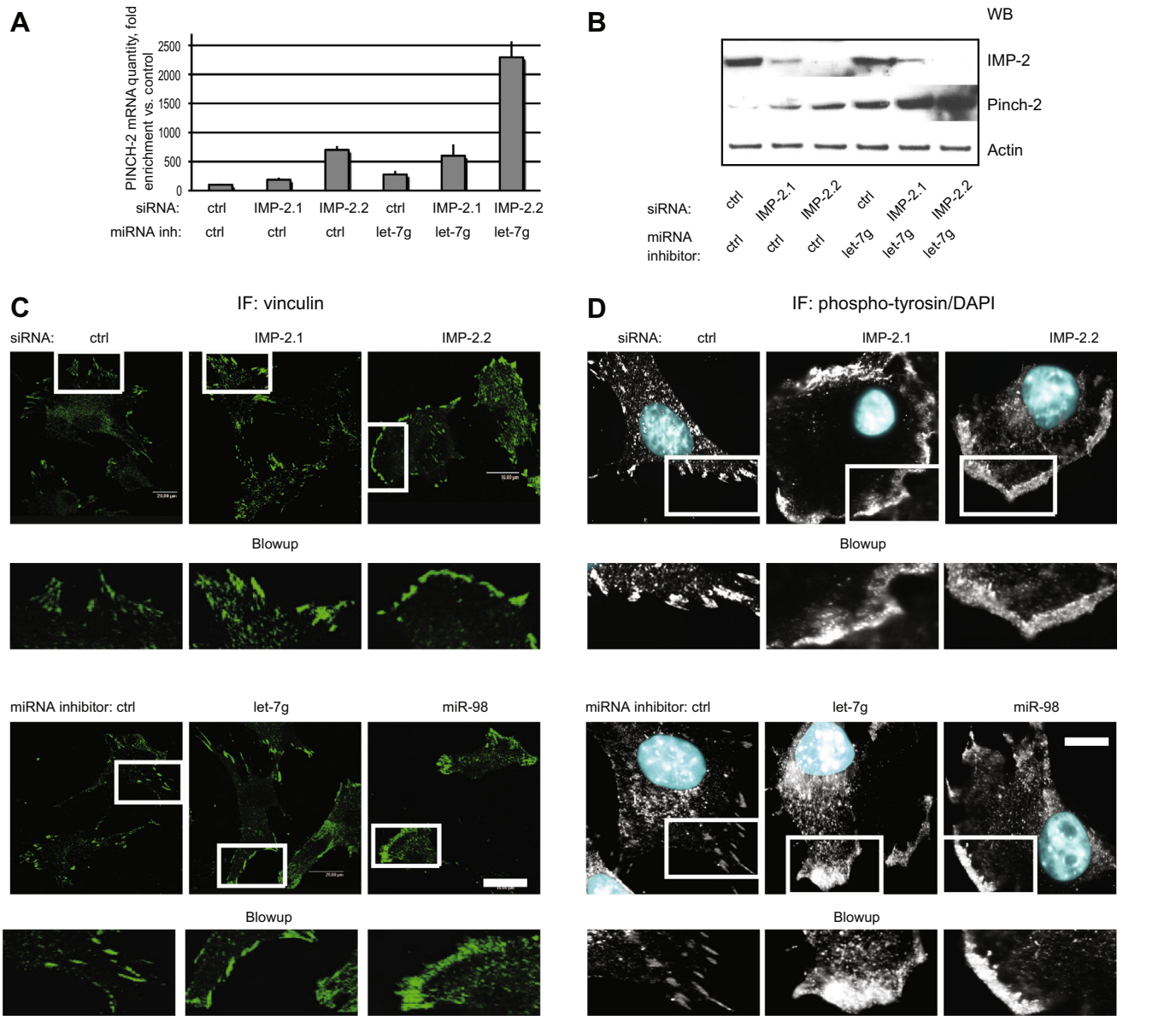

Fig. 3. Let-7 and IMP-2 act independently to suppress the expression of Pinch-2. (A, B) The levels of endogenous Pinch-2 mRNA (A) and protein (B) were studied in C2C12 cells transfected with the indicated miRNA inhibitors and siRNAs, and collected 48 h later. IMP-2.1 and .2 stand for two distinct siRNAs against IMP-2. (C) Confocal microscopy images of focal adhesions (FAs) (stained with anti-vinculin antibody) of C2C12 cells transfected with the indicated miRNA inhibitors and siRNAs, and collected 48 h later. Bar, 16 μm. (D) Epifluorescent images of FAs (stained with anti-phosphotyrosine antibody) of C2C12 cells treated as in (C). Bar, 11 μm.

gain-of-function experiments with antisense let-7g inhibitors and let-7g mimic, respectively, and have shown that inhibition of let-7g decreases the motility of C2C12 myoblasts in Pinch-2 dependent manner, as evaluated by the wound test (Fig. 4A). In this assay, the cells are transfected with the relevant miRNA inhibitors or mimics, and 48 h later a wound is made in the cellular monolayer. The relative speed of the closing of the wound corresponds to the changes in the cell motility. The decrease of let-7g levels led to a slowing-down of the closing of the wound, an effect that could be rescued by a simultaneous KD of Pinch-2 (Fig. 4A). The efficiency of Pinch-2 KD was analyzed by Western blot (Fig. 4B). Conversely, the transfection of ectopic let-7g miRNA led to an increase in the closing of the wound, as compared to the control (Fig. 4C). Thus, we concluded that let-7g can positively regulate the motility of C2C12 myoblasts. As let-7g expression levels do not change the proliferation rate of myoblasts [15], variations in cell numbers were not expected to contribute to the differences in the rate of the wound closure in the current study. We confirmed this by performing the let-7g loss-of-function wound test experiments with and without mitomycin C treatment to inhibit proliferation (Fig. 4A and data not shown), and did not observe any impact of proliferation on the relative efficiency of the wound closure between let-7g KD and control cells in these experiments.

To evaluate the relative importance of Pinch-2 up-regulation in the overall decrease of cellular motility by let-7g KD, we have performed a quantitative cell migration assay using time lapse microscopy followed by a reconstruction of cell trajectory (Fig. 4D). Cell speed is defined as the average of all instantaneous speed for all cells. Directionality (d/D) is represented by the ratio of the shortest linear distance from the start to the end point (d) divided by the total track distance migrated by an individual cell (D). The results have clearly demonstrated that an inhibition of let-7g decreased the speed and directionality of C2C12 myoblasts by 33% and 20%, respectively (Fig. 4E). This effect was fully reversed when let-7g

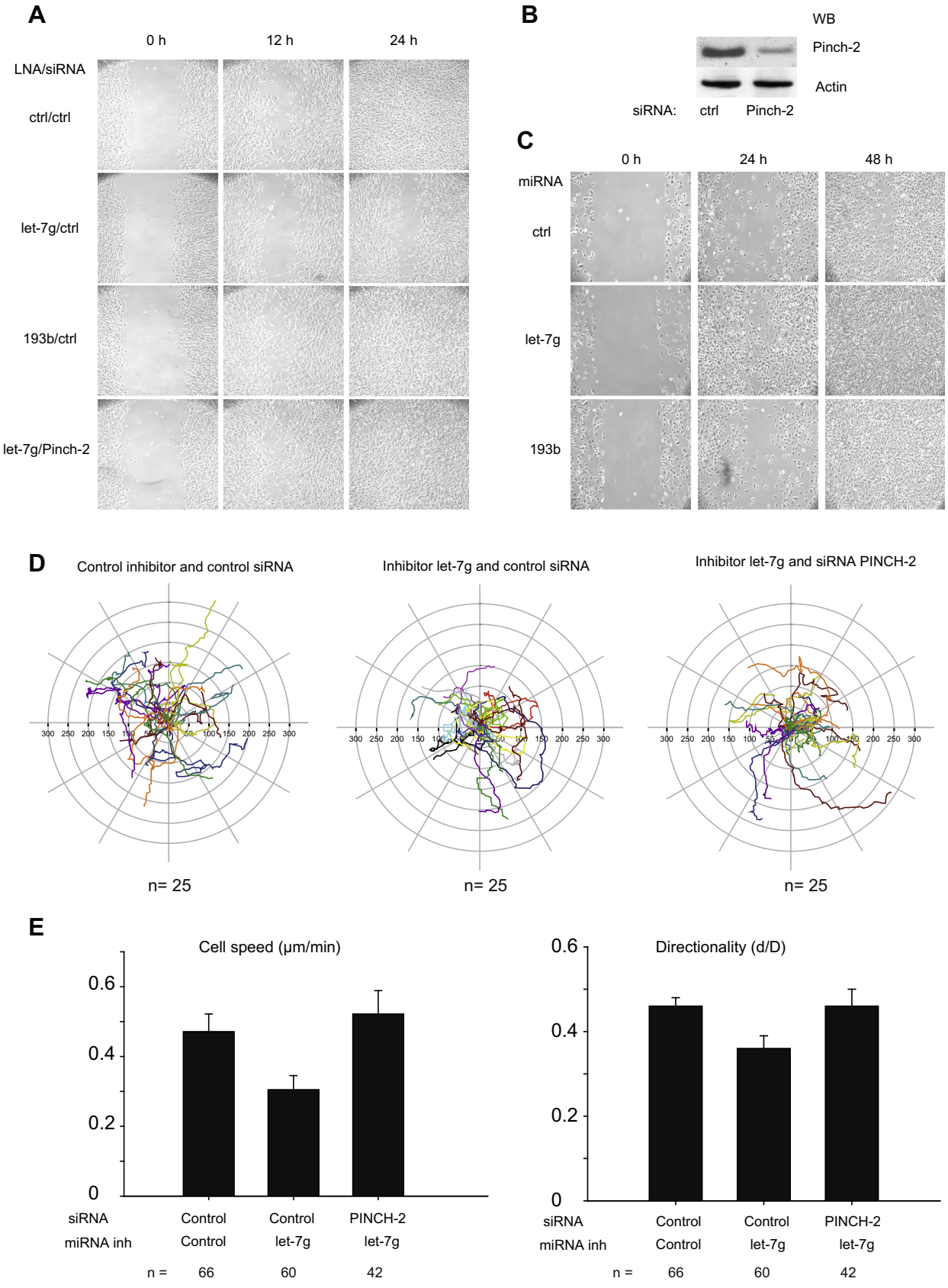

Fig. 4. Let-7 inhibits the motility of C2C12 myoblasts in a PINCH-2 dependent manner. (A, C) Phase-contrast images of C2C12 cells transfected with the indicated miRNA inhibitors (A) or precursors (C). The cells were treated (A) or not (C) with mitomycin C to inhibit proliferation, wound test was induced 48 h post-transfection, and the images were taken 0, 12 and 24 h (A) or 0, 24, and 48 h (B) later. (B) The efficiency of Pinch-2 siRNA used in (A) was analyzed by Western blot. (D) C2C12 cells were transfected with the indicated siRNAs and miRNA inhibitors and the cell mobility was quantified as described in Section 4. A radar plot representation of cell mobility was generated using SigmaPlot (10.0). (E) A quantification of cell speed and directionality in C2C12 cell culture transfected with the indicated siRNAs and miRNA inhibitors (miRNA inh). Cell numbers are indicated for each experimental point (*n*).

and Pinch-2 were inhibited simultaneously (Fig. 4E). Therefore, let-7g controls the motility of C2C12 myoblasts by regulating the expression of the focal adhesion protein Pinch-2.

3. Discussion

The focal adhesion (FA) protein Pinch-2 protein is capable to remodel the composition of the FA complexes, thus modifying the spreading and motility of cells [7,8,10,16]. In many cell and tissue types, such as skeletal muscle, the capacity for long-range directional motility is essential for the initial formation of the tissue during embryonic development, as well as for its regeneration in adult life [17]. Thus, it was very interesting to elucidate the molecular mechanisms that control the expression of Pinch-2 on the post-transriptional level in skeletal muscle myoblasts.

We have shown that there are four potential let-7 binding sites in the 3′UTR of Pinch-2, and together, these sites are necessary for a repression of Pinch-2 protein expression by let-7g. A mutation of each of these sites, or even of three sites out of four, does not release let-7g dependent repression. Interestingly, let-7g appears to repress Pinch-2 mRNA and protein independently of Pinch-2 post-transcriptional repressor IMP-2. To obtain a maximal expression level of Pinch-2, it is necessary to inactivate both let-7g and IMP-2. Therefore, the mRNA of Pinch-2 has multiple target sites for at least two post-transcriptional repressor factors in its 3′UTR. This tight, multiple-level control of Pinch-2 expression is consistent with the dramatic phenotypes induced by an overexpression of this protein, and with its functional importance in human and mouse cells.

Let-7 is one of the best-studied miRNA families, with numerous well-characterized mRNA targets and functions in development, differentiation and cancer (reviewed in [11,12]). Let-7 can have between one and six binding sites, mostly within the 3′UTR, but also within the coding region of its mRNA targets [18], reviewed in [19]. Whereas many studies identify individual and specific mRNA targets and functions for let-7 miRNAs, it was also suggested that they can simultaneously regulate the expression of thousands of genes, when overexpressed or inhibited in HeLa cells [20].

The mechanisms employed by let-7 miRNAs to specifically regulate given mRNA targets in a cell- and/or tissue-dependent manner can potentially include 3′UTR shortening via alternative polyadenylation [21,22], masking or exposing of specific target sites by RNA-binding proteins [23], subcellular compartmentalization of miRNA and/or its target mRNA [24], and others. However, these molecular mechanisms of let-7 target specificity have not been addressed experimentally so far. In this respect, Pinch-2 emerges as a very interesting novel target of let-7g, because it is efficiently down-regulated by this miRNA in normal skeletal myoblasts, leading to an increase in cellular motility. However, a recent study suggests that let-7a, b, and g can down-regulate the motility of breast cancer cells by acting on the actin cytoskeleton pathway [25]. This cell-type dependent action of let-7 raises an extremely intriguing possibility that these miRNAs can act in opposite ways while regulating the motility of normal versus cancer cells.

4. Materials and methods

Detailed experimental protocols are available on request.

4.1. Cell culture, transfections, RNAi assays, miRNA mimics and inhibitors

Cell culture media and additives were purchased from Invitrogen. C2C12 mouse myoblasts were purchased from ATCC and cultured as recommended.

All the transfections were performed using LipoRNAiMAX (Life Technologies), according to manufacturer's instructions. SiRNA duplexes were transfected at 20 nM. Efficiency of RNAi was evaluated by qRT-PCR and/or Western blot.

SiRNA target sequences:

IMP-2.1: TCGGGTAAAGTGGAATTGCAT
IMP-2.2: GGCATCAGTTTGAGGACTATT
PINCH-2: ACCCTTGGGCTTGGCTGAGAA
AGO-2.1: CACTATGAATTGGACATCAAA
AGO-2.2: CACGTTCATCGTGGTGCAGAA
AGO-2.3: AAGGGTAAAGTTTACCAAAGA
Control, Irrelevant siRNA (Qiagen): AACTGCGGTGGGCT AGACCAT

MiRNA inhibitors (LNA™ oligos) were purchased from Exiqon and transfected at the final concentration of 75 nM. The sequences were: miR-193b; GCGGGACTTTGAGGGCCAGT, let-7g; ACTGTACAAACTACTACCTC, miR-98; ACAATACAACTTACTACCTC, control; GTGTAACACGTCTATACGCCCA. MiRNA precursors (let-7g PM11758, miR-193b PM12383, Control#2 AM17111) were purchased from Ambion and transfected at the final concentration of 50 nM.

4.2. Wound healing and quantitative cell migration assay

For the wound healing test, cells were seeded at a density of 5×10^4 in 200 μl of 2% FBS DMEM in 6 well plates and transfected with 20 nM siRNA using LipoRNAiMAX (Life Technologies). After 48 h, the monolayers were wounded by scratching with a plastic pipette tip. Images of wound healing were taken every 24 h and analyzed using an inverted microscope. For experiments with mitomycin C treated cells, the amount of cells per well was doubled, and the cells were treated with 20 μg/ml mitomycin C for 2 h prior to scratching. Images of wound healing were taken every 12 h in these experiments.

For cell migration assays, C2C12 cells were transfected as described above with indicated LNA antisense oligonucleotides and siRNAs, and incubated for 48 h. 5×10^3 of the cells were subsequently seeded on ibidi μ-slide 8 well (Biovalley) coated with 10 μg/ml of fibronectin (Invitrogen) and spread for 2 h before imaging. Time lapse microscopy was done during 24 h with image acquisition every 10 min using phase contrast illumination and a Plan-Apochromat 20×/0.80 air objective. Random migration was measured using the plug-in MTrackJ, from Erik Meijering on ImageJ software. Cell speed is defined as the average of all instantaneous speed for all cells. Directionality (d/D) is represented by the ratio of the shortest linear distance from the start to the end point (d) divided by the total track distance migrated by an individual cell (D). Based on the coordinates obtained with MTrackJ, radar plot representation of cell mobility was generated using SigmaPlot (10.0).

4.3. RT-qPCR

RT-qPCR primer sequences: PINCH-2: fw CGGATTCTGTGGTGAATTTGTCA; rev CTGGCAGATGAATTTGCCCAA; Ago-2: fw AAGTC GGACAGGAGCAGAAA; rev GAAACTTGCACTTCGCATCA; Cyclophilin A: fw GTCAACCCCACCGTGTTCTT; rev CTGCTGTCTTTGGGACCTTGT.

4.4. Luciferase assays

The 3′UTR of Pinch-2 was cloned into the MCS of pSiCheck.2 reporter vector (Promega), the vector was transfected into C2C12 myoblasts using Lipofectamine 2000. 24 h later, the relative values

of Renilla versus Firefly luciferase were measured in transiently transfected C2C12 cells using the DualGlow kit (Promega) and a Mitras LB 940 luminometer (Berthold Technologies).

4.5. Site-directed mutagenesis

Directed mutagenesis of potential let-7 target sites in the 3′UTR of Pinch-2 was performed using the QuikChange protocol from Stratagene and the following primers:

site 1 fw AGGTCCTCGGCCGCCTGGCCTGCCTGCAAATAATAGCA CTCATCCCCCT, rev AGGGGGATGAGTGCTATTATTTGCAGGCAGGCC AGGCGGCCGAGGACCT, site 2 fw GCACTCATCCCCCTGCCCCAGGTC CTGAATGAATCCCACTTCTGCTCCAGCCC, rev GGGCTGGAGCAGAAGT GGGATTCATTCAGGACCTGGGGCAGGGGGATGAGTGC, site 3 fw CT TTCCTCTGTGCTGAGGCCTGACCCTTTATTCAGGCATTTCCTCCCGGG, rev CCCGGGAGGAAATGCCTGAATAAAGGGTCAGGCCTCAGCACAGA GGAAAG, site 4 fw CATTTCCTCCCGGGCCCACAGAACTTCGGGACA GGAACAAACTGGAG, rev CTCCAGTTTGTTCCTGTCCCGAAGTTCTGT GGGCCCGGGAGGAAATG.

4.6. Antibodies for Western blot and Immunofluorescence

IMP-2, rabbit antibodies kindly provided by Prof. F.C. Nielsen; Pinch-2, rabbit polyclonal, kind gift of Pr. Reinhard Fässler; phospho-tyrosin, clone 4G10, mouse monoclonal, kind gift of Dr. Eric Rubinstein; vinculin, clone V9131; Ago-2, clone 11A9; β-actin, clone AC-15 (Sigma); HMGA2, AF3184 (R&D Systems); IGF2BP1, sc-21026 (Santa Cruz).

Immunofluorescence: C2C12 mouse myoblasts were seeded on collagen-coated glass cover slides. Cells were fixed with 4% paraformaldehyde for 15 min at room temperature and permeabilized with 0.2% Triton X-100 in PBS for 20 min. After blocking with a solution containing 1% BSA and 5% serum in PBS for 1 h at room temperature, the cells were incubated with the primary antibody 1 h at room temperature, washed 3 × 5 min in PBS, and visualized with Alexa Fluor 488 or Alexa 568 conjugated anti-mouse or anti-rabbit (Invitrogen). Nuclei were stained for 2 min with DAPI (Sigma) diluted 1 μg/ml in PBS. Images were acquired using a fluorescent microscope Axiovert 2000 (Zeiss), Hamamatsu camera, and SimplePCI software. Confocal image acquisitions were carried out on a Zeiss LSM510 Meta confocal microscope with a Plan Neofluar 100×, NA = 1.3 oil immersion objective. Pinhole apertures were set to one Airy unit for each wavelength.

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