MicroRNA *let-7* establishes expression of β_2-adrenergic receptors and dynamically down-regulates agonist-promoted down-regulation

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Although β_2-adrenergic receptors (β_2AR) are expressed on most cell types, mechanisms that establish expression levels and regulate expression by chronic agonist remain unclear. The 3′ UTR of *ADRB2* has a conserved 8-nucleotide seed region that we hypothesized is targeted by the *let-7* family of miRNAs leading to translational repression. In luciferase assays with transfected cells, luc-β_2WT3′UTR had decreased expression when cotransfected with *let-7f*, but a mutated luc-$\beta_2$3′UTR lacking the seed was unaffected by *let-7f*; a mutated *let-7f* also had no effect on luc-β_2WT3′UTR expression. *ADRB2* mRNA was in greater abundance in immunoprecipitates of Ago2, a core component of the miRNA-induced silencing complex, when cells were transfected with *let-7f*, but not with a mutated *let-7f*, indicating a direct interaction with the silencing mechanism. H292 cells transfected with *let-7f* caused ∼60% decrease in native β_2AR expression, but transfection with *let-7f*–specific locked nucleic acid anti-miRNA increased β_2AR expression by ∼twofold. We considered that an increase in *let-7f* leading to greater repression of translation contributes to agonist-promoted down-regulation. Paradoxically, in cells and in lungs from mice treated in vivo, an ∼50% decrease in *let-7f* occurs during long-term agonist exposure, indicating a counterregulatory event. Consistent with this notion, *let-7f* locked nucleic acid transfection caused depressed agonist-promoted down-regulation. Thus, *let-7f* miRNA regulates baseline β_2AR expression and decreases in *let-7f* evoked by agonist attenuate down-regulation. This positive feedback loop has not previously been described for a G protein-coupled receptor and its miRNA. Methods to decrease *let-7f* expression in targeted cells may increase therapeutic responses to β-agonist by increasing β_2AR expression or minimizing tachyphylaxis.

desensitization | airways | noncoding RNA | gene regulation

G protein-coupled receptors (GPCRs) regulate a large repertoire of physiological functions and their pathways are the most commonly targeted by current therapeutic agents (1). Most GPCRs undergo agonist-promoted desensitization, defined as a loss of signaling during continuous activation by agonist (2). Such regulation limits potential deleterious effects from overstimulation and is considered critical to the integration of the multiple signals received by the cell. Early events in agonist-promoted desensitization are phosphorylation of the receptor by G protein-coupled receptor kinases, which promote the binding of β-arrestins that interdict between receptor and G protein, acting to partially uncouple receptor signaling to effectors (2). For some GPCRs, these events also lead to internalization of the receptor to the intracellular space, where routing back to the cell surface or to degradation pathways take place. With more prolonged exposure to an agonist, a loss of the net complement of cellular receptors is frequently observed, which is termed down-regulation. In physiologic settings where stimulation is persistent, down-regulation is readily quantified, such as with elevated catecholamines in heart failure and the down-regulation of cardiac β-adrenergic receptors (βAR) (3, 4). The down-regulation process can also limit the therapeutic effectiveness of chronic agonist administration, termed tachyphylaxis, as has been demonstrated at the cellular and physiologic levels for β-agonists in the treatment of asthma (5, 6). The molecular basis for the various components of agonist-promoted GPCR down-regulation, and in particular for that of the prototypic β_2AR, is not well-defined. Indeed, the mechanisms in play that establish baseline expression of β_2AR in a given cell, an obvious determinant of the response to endogenous or exogenous agonist, are also not known. Differences (or changes) in transcription (7), mRNA stability (8, 9), and receptor processing (10, 11) have been described as potential mechanisms that establish baseline β_2AR expression or agonist-promoted down-regulation. At the level of transcriptional control, multiple transcription factors appear to interact with the promoter and 5′-upstream regions of the intronless *ADRB2* gene (12).

Recently, a different level of regulation of many genes has been delineated, which involves RNA–RNA interactions rather than the canonical protein-dependent mechanisms. Indeed, nearly half of all mammalian genes are now predicted to be regulated by small noncoding RNAs (13, 14). Within the small noncoding RNAs, the miRNAs represent one such group with regulatory potential. MicroRNAs are small, single-stranded molecules approximately 21 nucleotides in length. They have the capacity to modulate protein expression levels in response to changing environmental stimuli by controlling protein synthesis in a highly specific spatiotemporal pattern (15, 16). This type of miRNA:mRNA interaction, usually occurring at the 3′ UTR, leads to translational repression, likely through steric hindrance of RNA polymerase actions (17, 18). It has been proposed that this mode of action provides cells the capacity to regulate protein expression levels in a controlled manner yet maintain a full complement of mRNA for a given gene (19). Here we define a unique mechanism by a member of the *let-7* family of miRNAs that sets the baseline level of expression of β_2AR, and is regulated by agonist leading to a counteraction to down-regulation.

Results

***ADRB2* Is a Target for *let-7f*.** To determine if β_2AR mRNA is potentially targeted by miRNAs, we used bioinformatic analyses (TargetScan, PicTar, MicroCosm, and the microRNA.org re-

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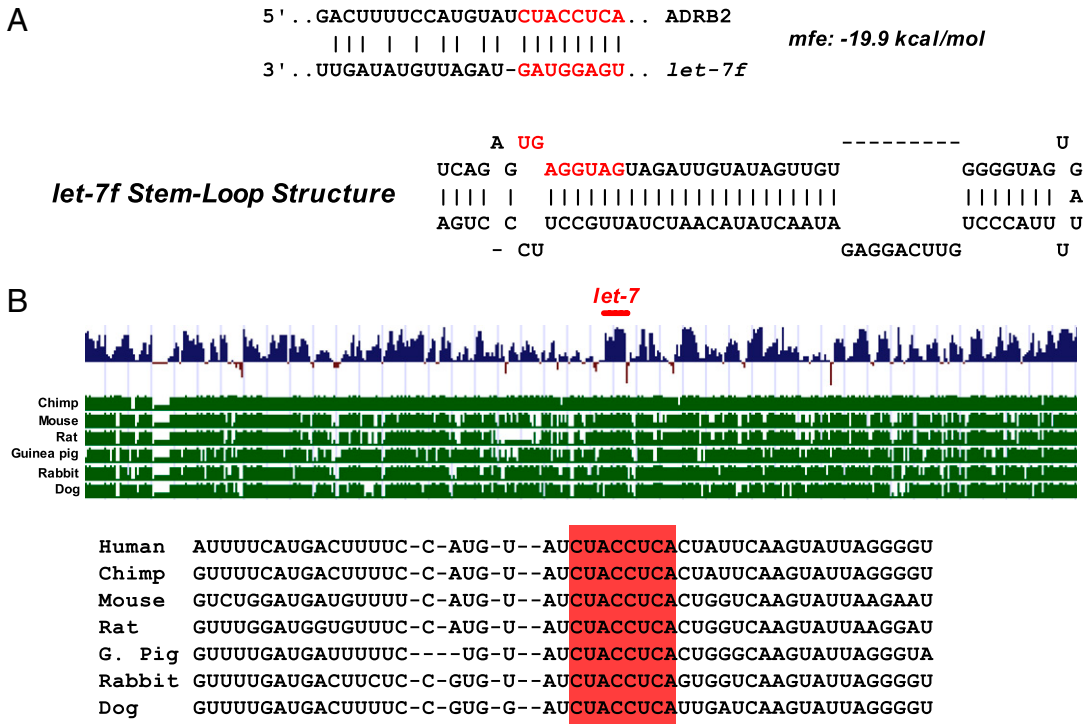

Fig. 1. Sequence analysis of *ADRB2* 3′ UTR. (*A*) Comparison of the predicted *let-7* stem-loop structure with a predicted 8-nucleotide binding site within the 3′ UTR of *ADRB2*. The mfe for the binding was calculated to be −19.9 kcal/mol. (*B*) Conservation plot of the 8-nulceotide predicted *let-7* binding region within *ADRB2* 3′ UTR among various species generated from the University of California Santa Cruz Genome Browser (http://genome.ucsc.edu).

source) deriving structural and minimum free energy (mfe) binding predictions. Fig. 1*A* shows the predicted *let-7f* stem loop structure and a mfe of −19.9 kcal/mol associated with the interaction between *ADRB2* mRNA and *let-7f*. This β_2AR sequence was found to be fully conserved across multiple species, with the *let-7f* binding site located at virtually the same location within the 3′ UTR (Fig. 1*B*). To test these prediction models that the *let-7* family of miRNAs can functionally interact with the 3′ UTR of *ADRB2*, HEK293 cells were used for transfecting β_2AR-3′UTR–based constructs. HEK293 were transfected with a construct consisting of firefly luciferase followed by the 3′ UTR of *ADRB2* (pGL3-β_2WT3′UTR) and a mutated 3′ UTR (mfe = −15.8 kcal/mol) lacking the predicted *let-7f* binding domain (pGL3-β_2Mut3′ UTR), as well as WT or a mutated *let-7f* (mfe = −12.6 kcal/mol). The characteristics of these mutated constructs are shown in Fig. 2*A*. As shown in Fig. 2*B*, luciferase activity decreased with cotransfection of WT *let-7f* in a dose-dependent fashion, consistent with *let-7f*–mediated translational repression. In contrast, *let-7f* had no effect on firefly luciferase expression when the mutated 3′ UTR construct lacking the predicted *let-7f* binding site was transfected (Fig. 2*C*). In addition, the mutated *let-7f* that lacked the seed region for the *ADRB2* 3′UTR interaction (*let-7f*-mut) had no effect when coexpressed with the pGL3-β_2WT3′UTR construct (Fig. 2*C*). These studies with WT and mutated *ADRB2* 3′ UTR, and WT and mutated *let-7f*, indicate that the observed effects of *let-7f* on *ADRB2* 3′ UTR-based repression are not a result of off-target or nonspecific interactions that might occur because of miRNA transfection. Taken together, these studies point toward an interaction between *let-7f* and a specific region of the *ADRB2* 3′ UTR that acts to repress expression.

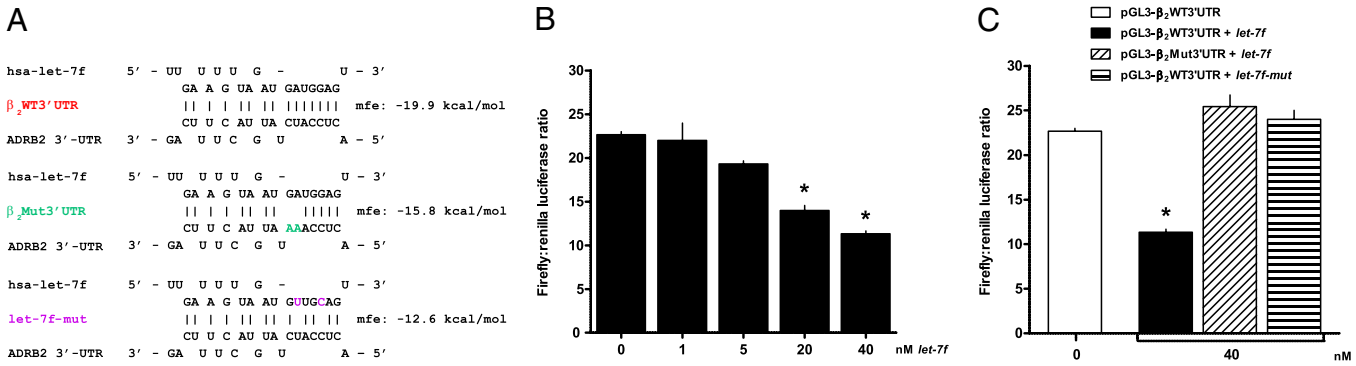

Fig. 2. *let-7f* alters expression of *ADRB2* 3′ UTR reporter genes. (*A*) Characteristics of the mutated *ADRB2* 3′UTR and *let-7f* constructs with mfe predictions. (*B*) Cotransfection of *let-7f* decreased pGL3-β_2WT3′UTR in a dose-dependent fashion in HEK293 cells. *P = <0.05 vs. mock transfection, n = 4 experiments. (*C*) Mutation of the seed region of *ADRB2* 3′UTR, or of *let-7*, ablates effects on reporter expression. *P < 0.05 vs. mock transfection, n = 4 experiments.

***Let-7f* Promoted Interaction of *ADRB2* mRNA and the RNA Silencing Complex Component Ago2.** Most miRNAs are transcribed as pri-miRNA. These molecules have a characteristic stem-loop structure and are the target of cleavage by the microprocessor complex consisting of Drosha and DGCR8 (20). The resulting 60 to 70 nucleotide pre-miRNA is then exported to the cytoplasm by the Ran-GTP–dependent nuclear receptor Exportin-5, where it is further processed by the Rnase III enzyme Dicer into ~21 nucleotide miRNA:miRNA* duplexes. Finally, one strand of this duplex (termed guide miRNA) is assembled into a ribonucleoprotein known as miRNA-induced silencing complex (miRISC); the other strand (termed "passenger strand") is degraded. The core component of miRISC is Ago2, a member of the Argonaute endonuclease family, which binds the remaining guide strand to silence target mRNAs (21). If β_2AR is regulated by the proposed miRNA pathway, then overexpression of *let-7f* should lead to an increase in Ago2-bound *ADRB2* mRNA. To test this, H292 (a human airway epithelial cell line that natively express β_2AR) extracts were immunoprecipitated with an Ago2 antibody, and the immunoprecipitates probed quantitatively for *ADRB2* mRNA by real-time RT-PCR. The specificity of the antibody and the immunoprecipitation method is shown in Fig. 3*A*, using Western blotting of the immunoprecipitates where the precipitating antibody was either the Ago2 antibody or IgG. Control (mock-transfected), *let-7f*-, and *let-7f*-mut–transfected cells were studied. Immunoprecipitated Ago2 protein was observed only with Ago2 antibody and not with IgG, and Ago2 was expressed at comparable levels under the conditions of all three transfections. When these complexes were then subjected to *ADRB2*-specific real-time RT-PCR, marked differences in *ADRB2* mRNA content were observed (Fig. 3*B*). In these cells, a modest enrichment of *ADRB2* mRNA was observed in Ago2 vs. IgG immunoprecipitates in the nontransfected cells. With cotransfection of *let-7f*, a significant increase in *ADRB2* mRNA was detected in Ago2 immunoprecipitates. In contrast, extracts from cells transfected with the *let-7f*-mut construct showed no enrichment of *ADRB2* mRNA from Ago2 immunoprecipitates over that of the nontransfected controls.

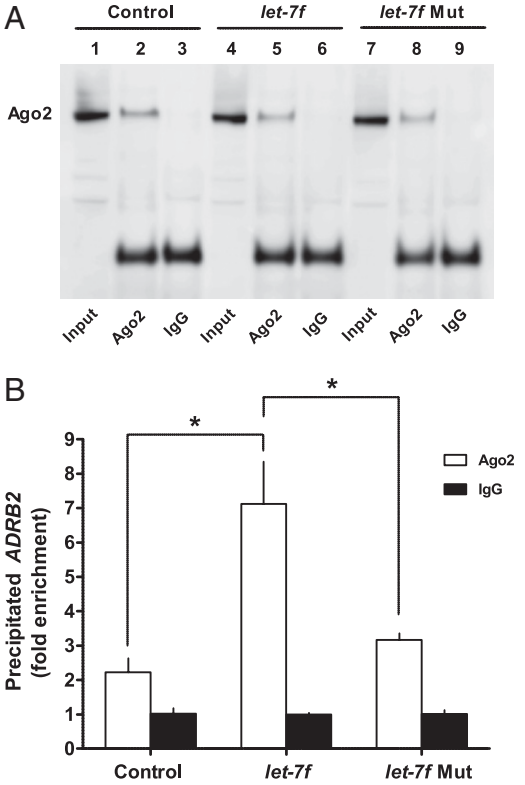

Fig. 3. *let-7* increases Ago2-associated *ADRB2* mRNA. (*A*) Western blots from lysates of H292 cells transfected with either *let-7f* of *let-7f*-mut. Extracts were immunoprecipitated with either Ago2 or IgG and blotted with Ago2 antibody. The upper band represents the 97-kDa endogenous input (lanes 1, 4, 7) and immunoprecipitated (lanes 2, 5, 8) Ago2 protein. Lower bands are 55-kDa IgG heavy chains. (*B*) RNA from immunoprecipitated materials was converted to cDNA by reverse transcriptase and quantified by real-time PCR using *ADRB2* specific primers. **P* < 0.05, *n* = 3 experiments.

***Let-7f* Regulates Endogenous β_2AR Expression and Limits Agonist-Promoted Down-Regulation.** The above studies indicate a specific interaction between *let-7f* miRNA and the 3′ UTR of *ADRB2*, which results in repression of a reporter gene. In addition, the studies indicate that there is a direct correlation between the amount of transfected *let-7f* and the extent of expression. The enrichment of *ADRB2* mRNA from Ago2 immunoprecipitates from cells transfected with *let-7f* further validates a miRNA-based effect of *let-7* for β_2AR expression. We next assessed whether *let-7* regulates baseline expression of β_2AR protein natively expressed on H292 cells by increasing *let-7f* expression and functional *let-7f* knock-downs. β_2AR protein expression on cell membranes was determined by quantitative ^{125}I-cyanopindolol (^{125}I-CYP) radioligand binding. Transfection of *let-7f* resulted in an ~60% decrease in β_2AR expression (Fig. 4*A*). No change in expression was found when *let-7f*-mut was transfected. Endogenous *let-7f* activity was inhibited by transfection of a *let-7f*–specific locked nucleic acid (LNA) anti-miRNA. As shown in Fig. 4*A*, *let-7f* LNA increased native β_2AR expression by ~twofold over control cells. These studies confirm that native β_2AR protein expression is specifically modulated by *let*-7, with a range of influence amounting to fourfold (~50 to ~200 fmol/mg).

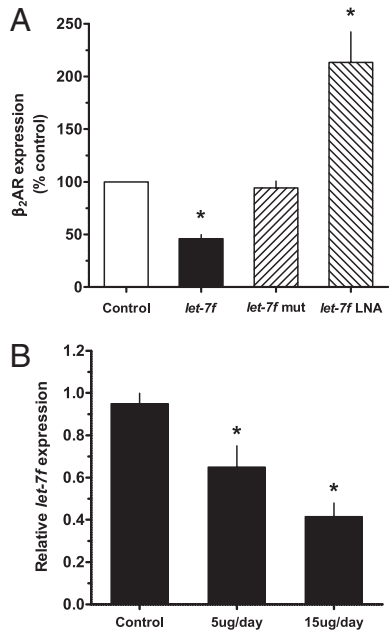

Fig. 4. Native β_2AR expression is modulated by increasing and decreasing *let-7*. (*A*) H292 cells were transfected with *let-7f*, *let-7f*-mut, *let-7f* LNA, or scrambled (scr) LNA. β_2AR protein expression was quantitated by ^{125}I-CYP radioligand binding. Control cells are either mock-transfected or, for the LNA experiments, cells transfected with scr LNA. **P* < 0.01 vs. control, *n* = 4. (*B*) Long-term in vivo agonist treatment decreases *let-7f* miRNA expression in mouse lung. Mice were treated by intraperitoneal doses of 5 or 15 μg fenoterol daily for 7 d and *let-7* miRNA determined by real-time PCR. **P* < 0.05 vs. control, *n* = 3 for each condition.

We hypothesized that *let-7f* might be up-regulated with agonist exposure, and thus the increased repression of translation would be a component of the agonist-promoted down-regulation process. However, we found a 33 ± 7% decrease in *let-7f* expression in H292 cells after 12 h of exposure to 10 μM of the β_2AR agonist fenoterol ($P < 0.05$, $n = 7$). Using pharmacologically relevant doses of fenoterol delivered for 7 d to mice, we noted a dose-dependent decrease of up to 50% in lung *let-7f* expression compared with lungs from sham-treated mice (Fig. 4*B*). Given that these conditions readily evoke a loss of β_2AR expression (Fig. 5) (22, 23), we then considered that agonist-promoted decreases in *let-7f* are a mechanism to limit the extent of agonist-promoted down-regulation. Thus, the net effect on β_2AR expression from prolonged agonist exposure includes a counter-regulatory loss of miRNA-mediated translational repression. To further examine this effect, H292 cells were transfected with *let-7f* LNA or a scrambled LNA control in the absence or presence of 12-h agonist exposure. We expected that the effect of lowering *let-7f* by agonist exposure and also decreasing the effect of the remaining *let-7f* by LNA would lead to decreased down-regulation, as translational repression would be minimized by the two mechanisms. As shown in Fig. 5, this was indeed the case, with agonist-promoted down-regulation amounting to ∼91% in control cells and agonist-promoted down-regulation in the face of *let-7* LNA transfection being ∼74%.

Discussion

GPCRs serve as critical elements in the maintenance of homeostasis at the cell, organ, and organism levels under normal physiologic states and during pathophysiologic conditions. These receptors are targets for pharmacologic therapy with receptor agonists and antagonists for a wide range of diseases, and have also been implicated in aberrant responses contributing to disease. A key aspect of signaling by most GPCRs is the capacity to adapt to a changing environment. This plasticity is a necessary attribute because the influences that impact a given state are highly dynamic, thus requiring receptors to adjust signaling along timeframes ranging from seconds to days. Of particular interest have been the mechanisms by which a cell regulates the level of receptor expression and how expression is affected by chronic agonist exposure. In regards to the former, "basal" receptor expression is considered a major determinant of the response to endogenously generated or exogenously administered agonist (24). With prolonged agonist exposure, many GPCRs display a decrease in expression, which is termed down-regulation, and is one component of tachyphylaxis to administered agonists; down-regulation from elevated levels of endogenous agonist is also apparent (3, 4). For the prototypic β_2AR, these adaptive responses, such as rapid phosphorylation by G protein-coupled receptor kinases, and many of the mechanisms that are apparent over longer time courses have been documented (2, 25–27). The mechanisms responsible for β_2AR down-regulation have been shown to occur at the level of transcription (7), mRNA stability (8, 9), and protein degradation (10, 11). Here we show an additional component, translational repression by *let-7f*, which has a two-pronged effect on receptor expression (Fig. 6). Basal levels of β_2AR expression appear to be effected by this mechanism, and indeed *let-7*–dependent regulation resulted in protein expression levels that span at least a fourfold range. Of note, measured β_2AR expression varies between individuals by about this same extent in many tissues, such as heart (28), skeletal muscle (29), peripheral lung (30), lymphocytes (30), and airway epithelial cells (5), supporting the notion that this variation in expression is within the physiologic range. We do not claim, however, that this *let-7* mechanism is the only means by which basal levels of β_2AR are set, but rather it is one mechanism that results in changes within the known range in relevant tissues. Interestingly, *let-7f* binding sites are also predicted in the 3′ UTRs of the β_1AR and β_3AR subtypes (704–710 and 521–527 nucleotides from the stop codons, respectively) but are not found in genes for the three α_1AR subtypes or the three α_2AR subtypes. This finding suggests that the *let-7* mechanism defined in the present report may be a βAR-specific mechanism within the adrenergic receptor family.

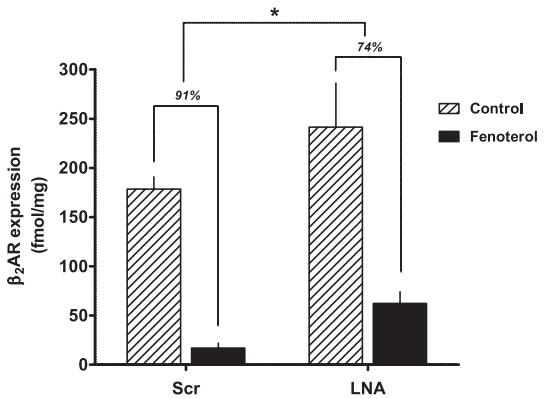

Fig. 5. Effects of *let-7f* miRNA on agonist-promoted β_2AR down-regulation. H292 cells were transfected with scr *let-7f* LNA (representing the control condition) or *let-7f* LNA to depress *let-7f* effects. Cells were treated with carrier (0.1 mM ascorbic acid, control) or carrier with 10 μM fenoterol for 12 h, washed, and ^{125}I-CYP binding performed to quantitate β_2AR expression. *Down-regulation less than scr control, $P < 0.05$, $n = 3$.

An unexpected finding was the effect of prolonged agonist exposure on *let-7f* expression. Given the efficient control of β_2AR expression exhibited by *let-7f*, we assumed that it participated in agonist-mediated down-regulation by enhanced expression and thus increased gene silencing. However, we found that *let-7f* expression was decreased by prolonged agonist exposure. Given that the net effect of such exposure is decreased β_2AR expression, the decrease in *let-7f*, which causes an increase in β_2AR expression, appears to be a counterregulatory event that attenuates down-regulation (Fig. 6). Several reports indicate that GPCR activation can alter miRNA expression, which has been linked to altered expression of other proteins (31). Furthermore, forskolin treatment of cardiomyocytes has been shown to increase *miR-1* miRNA expression (32). However, we are unaware of a feedback loop as reported here for the β_2AR being previously shown between any GPCR and miRNAs that regulate its expression. In the present report, activated β_2AR leading to decreased *let-7* would be considered a positive feedback loop, in that decreased *let-7* diminishes *ADRB2* translational repression and thus shifts the equilibrium toward an increase in β_2AR expression. To our knowledge, no positive feedback loops regulating human β_2AR expression by any mechanism have been reported. The previously characterized regulatory events that accompany agonist activation result in a loss of expression or function. It is not entirely unexpected, however, that "buffering" mechanisms would also be present, essentially providing additional control nodes to moderate negative feedback. Finally, we note that only two other groups of miRNAs, represented by *miR-15* and *miR-30*, have predicted binding sites within the 3′ UTR of the β_2AR (Fig. S1). The functional effects of these miRNAs will require additional study.

In summary, we have shown here that translational repression imposed by the binding of *let-7f* to the 3′ UTR of the β_2AR regulates baseline expression. Such regulation can be demonstrated in transfected cells and natively expressing cells, and is highly specific because mutations of the 3′ UTR or *let-7f* abolish the effect. The range of control over β_2AR expression is within the physiological range. In addition, agonist activation of the β_2AR decreases *let-7f* expression, leading to an attenuation of agonist-promoted down-regulation. Taken together, these stud-

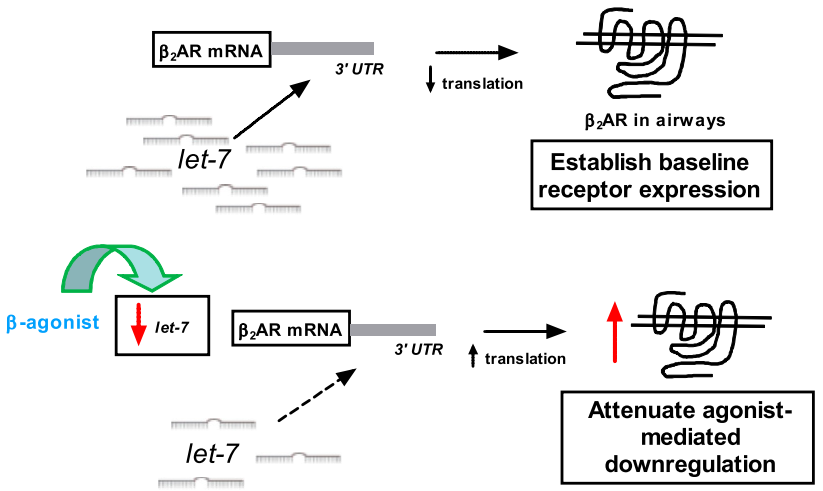

Fig. 6. Dual role of *let-7f* in regulation of β_2AR protein expression. Under static conditions, *let-7f* actively represses translation and establishes basal levels of β_2AR expression. During agonist activation, *let-7f* levels decrease, resulting in depressed *ADRB2* gene silencing and thus an attenuation of agonist-promoted down-regulation.

ies suggest that genetic or pharmacologic means of modulating *let-7* expression in target tissues, such as heart or lung, may improve therapeutic outcome by increasing baseline β_2AR expression or attenuating tachyphylaxis.

Methods

Constructs and Luciferase. To generate the ~600-bp β_2 3′ UTR, the following PCR primers were used to amplify this fragment from human genomic DNA: forward primer: 5′-AGCAGTTTTTCTACTTTTTA AAGAC-3′ and reverse primer: 5′-AGGCAACAGCACTCCAGTCAAG-3′. This PCR product was subsequently cloned into the firefly luciferase pGL3-Control vector downstream of the luciferase ORF at the XbaI site to generate pGL3-β_2WT3′UTR. Orientation and sequence accuracy were confirmed by sequencing. To generate the $\beta_2$mut3′UTR, site-directed mutagenesis (Strategene) was used to mutate the first two nucleotides (CU to AA) within the *let-7* seed region of β_2WT3′UTR. The *let-7f*-mut miRNA containing mutations within its seed region was obtained from Invitrogen. For luciferase assays, HEK293 cells were seeded in 96-well plates. Transfections used lipofectamine 2000 (Invitrogen) with pGL3-β_2WT3′UTR, pGL3-$\beta_2$mut3′UTR, *let-7f*, or *let-7f*-mut, and renilla luciferase (pRL-SV40), which was used as a transfection control. Luciferase activity was measured using the dual-luciferase reporter assay (Promega). Forty-eight hours after transfection, cells were lysed and luciferase activities were measured on a Victor^3 Multilabel Counter (Perkin-Elmer). Data are reported as the firefly:renilla luciferase activity ratios.

Immunoprecipitation. Immunoprecipitation studies were performed on H292 cells (ATCC) transfected with *let-7f* or a scrambled control, using a modification of previously described methods (33). Briefly, 1 mg of whole-cell lysate was precleared with protein A/G agarose and then incubated with 1:50 Ago2 antibody (Cell Signaling) or control IgG (Santa Cruz) for 1 h at 4 °C. After addition of 50 μL of protein G agarose, the lysates were incubated for 12 h at 4 °C. Agarose beads were washed by serial centrifugation with lysis buffer, high salt, and then low-salt buffer. RNA was extracted with TRIzol (Invitrogen), precipitated with isopropanol in the presence of glycogen to aid in pellet recovery, and treated with DNase I (Applied Biosystems). Complementary DNA was generated using SuperScript III reverse transcriptase and quantitated by real-time PCR as described elsewhere (34). The 18s rRNA was used for normalization. Western blots were performed on the immunoprecipitated material, as previously described (35, 36), with the following modifications: 25 μL of gel-loading buffer was directly loaded on the agarose pellet and the protein was denatured for 3 min at 100 °C. Membranes were blotted with antihuman Ago2 (Wako) at a titer of 1:200. The ECL Advanced Western Blotting kit (GE Healthcare) was used for detection and images were acquired using the Fuji LAS-3000 camera (Fujifilm Medical Systems) and quantitated using the associated software.

Radioligand Binding and Down-Regulation. β_2AR expression levels were determined by quantitative ^{125}I-CYP radioligand binding, as previously described (37). Briefly, H292 cells were transfected with either *let-7f* (40 nM final concentration) (Invitrogen) or *let-7f* LNA (25 nM final concentration) (Exiqon) and detached after 48 h by scraping in 5 mM Tris, 2 mM EDTA, pH 7.40 and centrifuged at 30,000 × *g* for 10 min at 4 °C. Lysates were then homogenized in 500 μL of buffer containing 75 mM Tris, 12 mM $MgCl_2$, and 2 mM EDTA, pH 7.40, which was also used as the incubation buffer for radioligand binding. ^{125}I-CYP binding was carried out for 2 h at 25 °C, and bound radioligand separated from free by vacuum filtration over glass-fiber filters (Whatman), which were counted in a γ counter. Nonspecific binding was determined by coincubations with 10 μM propranolol, and all tubes contained 100 μM GTP. For receptor down-regulation studies, H292 cells were transfected with either *let-7f* LNA or an LNA scrambled control; after 48 h, cells were treated with the β_2AR-specific full-agonist fenoterol for an additional 12 h followed by washing three times with cold PBS and ^{125}I-CYP binding, as described above. Results are expressed as femtomole per milligram or as a percentage of the expression of cells under a control condition.

Mouse Studies. The mouse studies were approved by the Institutional Animal Care and Use Committee at the University of Maryland School of Medicine. Twelve- to 18-wk-old FVB mice (Taconics) were administered intraperitoneal injections of fenoterol at 5 μg/d or 15 μg/d for 7 consecutive days; controls were administered saline. Following drug treatment, lungs were harvested and RNA extracted using TRIzol reagent. For detecting *let-7f* levels, real-time PCR as described above was used to determine expression levels with U6 snRNA used as an internal control.

MicroRNA Target Prediction and Sequence Alignment. Multiple species alignment of the β_2AR 3′ UTR was performed using the University of California Santa Cruz Genome Browser (http://genome.ucsc.edu) (38). To identify potential miRNA binding sites within the 3′ UTR of β_2AR, the following bioinformatic databases were used: TargetScan (http://www.targetscan.org) (39), the microRNA.org resource (http://www.microrna.org) (40), MicroCosm (http://www.ebi.ac.uk/enright-srv/microcosm) (41), and PicTar (http://pictar.mdc-berlin.de) (42). MicroRNA-mRNA free energies were determined using RNAhybrid (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid) (43).

Data Analysis and Statistics. All statistical calculations were performed using Prism (GraphPad). Comparisons were by two-way paired or unpaired *t* tests, with $P < 0.05$ considered significant. Comparisons among multiple conditions were performed by ANOVA followed by post hoc *t* tests. Data are expressed as mean ± SEM of *n* experiments.

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