miR-142-3p Is Involved in CD25+ CD4 T Cell Proliferation by Targeting the Expression of Glycoprotein A Repetitions Predominant

Qihui Zhou, Sonja Haupt, Iryna Prots, Katja Thümmler, Elisabeth Kremmer, Peter E. Lipsky, Hendrik Schulze-Koops and Alla Skapenko

*J Immunol* published online 6 May 2013
http://www.jimmunol.org/content/early/2013/05/05/jimmunol.1202993

Supplementary Material

http://www.jimmunol.org/content/suppl/2013/05/06/jimmunol.1202993

Subscription

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
miR-142-3p Is Involved in CD25+ CD4 T Cell Proliferation by Targeting the Expression of Glycoprotein A Repetitions Predominant

Qihui Zhou,* Sonja Haupt,* Iryna Prots,*-1 Katja Thümmler,*-2 Elisabeth Kremmer,+ Peter E. Lipsky,‡ Hendrik Schulze-Koops,* and Alla Skapenko*

Because of the numerous targets of microRNAs (miRNAs), functional dissection of specific miRNA/mRNA interactions is important to understand the complex miRNA regulatory mechanisms. Glycoprotein A repetitions predominant (GARP) is specifically expressed on regulatory CD25+ CD4 T cells upon their activation. GARP has a long 3′ untranslated region containing five highly conserved regions suggesting miRNA regulation of its expression. Although GARP is physiologically expressed on a cell subset characterized by stringent control of proliferation, amplification of the GARP gene has been found in many tumors characterized by uncontrolled proliferation. In this study, we investigated in detail miRNA regulation of GARP expression, in particular by miR-142-3p, and dissected the functional outcome of miR-142-3p/GARP mRNA interaction. We demonstrate that miR-142-3p binds directly to the 3′ untranslated region of GARP and represses GARP protein expression by Argonaute 2–associated degradation of GARP mRNA. Functionally, miR-142-3p-mediated regulation of GARP is involved in the expansion of CD25+ CD4 T cells in response to stimulation. The data indicate that miR-142-3p regulates GARP expression on CD25+ CD4 T cells and, as a result, their expansion in response to activation. Our data provide novel insight into the molecular mechanisms controlling regulatory T cell expansion. They may also have implications for understanding tumor cell biology. The Journal of Immunology, 2013, 190: 000–000.

The importance of posttranscriptional silencing pathways, in particular those involving microRNAs (miRNAs), in many biological processes has been recently highlighted. miRNAs are endogenous ~23 nt RNAs that pair to the 3′ untranslated region (UTR) of protein-coding genes to direct their posttranslational repression (1). The expression of miRNAs is often subject to regulation in response to cellular stimulation to allow for precise gene expression in different cell types and under different conditions (2, 3). The finding that inactivation of miRNA biogenesis by the deletion of the RNase III endonuclease Dicer leads to prenatal lethality in mice demonstrates the crucial importance of miRNAs in mammal development (4). The role of miRNAs during development has been attributed to differentiation of stem cells and the formation of different tissues (5). Additionally, differentiation of the immune system as well as effector functions of immune cells are centrally regulated by miRNAs (6). Lineage-specific deletion of Dicer leads to compromised T cell development (7, 8) and a developmental block at the pro– to pre–B cell transition in B cell maturation (9). Heretofore, miR-17-92, -181a, and -150 have been identified as specific miRNAs directly regulating T and B cell lineage development (9–11), whereas miRNA-155 and miRNA-182 have been identified as specific miRNAs necessary for clonal expansion of activated effector CD4 T cells and for eliciting effector functions of T and B cells (12–14). The central involvement of miRNAs in regulation of the optimal evolution of an immune response became apparent by analysis of the effect of a Dicer knockout on regulatory T cells. Conditional deletion of Dicer in CD4 T cells or further in regulatory T cells diminished the numbers of regulatory T cells and resulted in severe autoimmunity in mice (15, 16). Dissection of the network of individual miRNAs and their gene targets leading to this phenotype is the subject of current investigation. Leucine-rich repeat containing 32, also known as glycoprotein A repetitions predominant (GARP), is a member of the leucine-rich repeat family that exhibits evolutionary similarity to TLRs (17). GARP is specifically expressed by regulatory CD25+ CD4 T cells in response to activation (18). The 2-kb-long 3′UTR of GARP contains five highly conserved regions (Supplemental Fig. 1). Conservation in the 3′UTR is indicative of the presence of evo-
lutionarily selected, important regulatory sequences such as miRNA recognition sites (19). Considering this, the possibility of miRNA involvement in the regulation of GARP expression is likely. GARP has been proposed to serve as a receptor for latent TGF-β, although the relevance of this receptor/ligand interaction is not clear (20, 21). Interestingly, numerous studies have reported an amplification of the GARP gene in tumors, in particular in those with an invasive, metastasizing or treatment-resistant potential, suggesting a role of this gene product in regulating aggressive tumor biology once deregulated (22–29). Clarification of GARP miRNA regulation might therefore have an implication in determining the role of GARP and its regulatory miRNAs in immune responses. Moreover, it might be useful for understanding the pathogenesis of aggressive behavior of certain tumors.

In this study, we report that GARP expression is miRNA regulated. We identified miR-142-3p as the miRNA regulating GARP expression in CD4 CD25+ T cells. The miR-142-3p-mediated regulation of GARP expression correlated with the expansion of CD4 CD25+ T cells in response to activation. Our report provides new insight into the function of miR-142-3p during regulatory T cell effector function and delineates a possible role of miR-142-3p in the control of regulatory T cell homeostasis.

Materials and Methods

Antibodies

Monoclonal FITC-labeled anti-CD3/PE-labeled anti-CD4 dual Tag Ab (UCHT-1/Q4120, Sigma-Aldrich, St. Louis, MO), FITC-labeled anti-CD4 (Q4120, Sigma-Aldrich), and PE-labeled anti-CD25 Ab (M-A251; BD Pharmingen, San Jose, CA) were used for control of purification of CD25+ and CD25− CD4 T cells. For the surface GARP staining, monoclonal rat anti-human GARP Ab (clone 7H2) was used. PE-labeled anti-rat IgG (Sigma-Aldrich) were used for detection. For ribonucleaseprotein (RNP) immunoprecipitation (IP), anti-human Aga1 and anti-human Aga2 (provided by Dr. G. Meister, Regensburg, Germany) were used. Rat IgG (Sigma-Aldrich) was used as control for IP.

Cell lines

Human embryonic kidney (HEK)293 cells and the T cell leukemia line (Jurkat) were from the American Type Culture Collection. HEK293 cells were cultivated twice a week with DMEM supplemented with 10% FCS, penicillin G (50 U/ml), and 50 µg/ml streptomycin (all from Life Technologies Invitrogen). Jurkat cells were maintained in RPMI 1640 supplemented with 10% FCS, penicillin G (50 U/ml), and 50 µg/ml streptomycin (all from Life Technologies Invitrogen).

Generation of Abs against GARP

Rats were immunized with 5 × 10^7 Rat-2 cells overexpressing human GARP. Fusion was performed using standard procedures. Supernatants were tested by FACS on transfected HeLa cells expressing GARP Clone 7H2 (IgG2a) was used in this study.

T cell isolation and culture

CD25+ and CD25− CD4 T cells were purified from human PBMCs by a human CD4+CD25+ regulatory T cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Where indicated, T cells were stimulated with anti-CD3/28-coated beads (Dynabeads human T-activator CD3/CD28, Life Technologies Invitrogen) and 100 resonance units/ml recombinant human IL-2 (Chiron, Emeryville, CA). All donors provided written informed consent. The study was approved by the Ethics Committee of the University of Munich.

RNA extraction and reverse transcription

Total RNA was extracted using the RNeasy Plus Mini kit (Qiagen, Hilden, Germany) following the manufacturer’s instructions. One microgram was reverse transcribed into cDNA by avian myeloblastosis virus reverse transcriptase (Promega, Mannheim, Germany) in 50 mM Tris-HCl (pH 8.3), 50 mM KCl, 10 mM MgCl2, 0.5 mM spermidine, and 10 mM DTT for 1 h at 42°C.

Real-time PCR analysis

GARP mRNA, miR-142-3p, and miR-424 were detected using TaqMan assays (h00194136_m1, 000464, and 000604, respectively; Applied Biosystems, Carlsbad, CA). RNU6B was used as an endogenous miRNA control (001093; Applied Biosystems).

miRNA mimics and antagonons

miRNA mimics (negative control CN-00100, miR-142-3p C-300610, miR-181a C-300552, miR-181b C-300554, miR-181c C-300556, miR-381 C-300690, miR-424 C-300717, miR-497 C-300765, miR-551a C-300868, miR-661 C-300981, miR-662 C-300982) and miRNA hairpin inhibitors, antagonirs (negative control IN-001005, inhibitor miR-142-3p IH-300610, and inhibitor miR-424 IH-300717) were purchased from (Dharmacon/Thermo Scientific, Lafayette, CO).

Plasmid constructs

Forward (5’-CACCATGAGACCCAGATCCTGC-3’) and reverse (5’-TTAGGCTTTATAGTGTTGGTTAAAC-3’) primers were used for GARP coding sequence amplification. Kozak sequence was introduced into the forward primer for direct cloning into the pcDNA3.1 vector (Life Technologies Invitrogen). Forward (5’-CCTCGAGAGACCCAGATCCTGC-3’) and reverse (5’-CCGCGGACATTCAGGTAGG-3’) primers were used for GARP 3’UTR amplification. Restriction sites of Xhol and SacII shown underlined were introduced into the forward and reverse primers, respectively, for consecutive cloning steps. The PCR products for the GARP coding sequence and the 3’UTR were cloned directly into either a pcDNA3.1 or pcR2.1 TOPO vector (Life Technologies Invitrogen), respectively. Cloned sequences were confirmed by sequencing. The GARP 3’UTR fragment was released from the pcR2.1-GARP-3’UTR vector using Xhol and SacII restriction endonucleases (New England Biolabs, Ipswich, MA) and inserted into the pcDNA3.1-GARP coding sequence vector between Xhol and SacII sites.

Overlapping fragments of the GARP 3’UTR were amplified from pcR2.1-GARP-3’UTR and cloned into pmirGlo (Promega) downstream of the luciferase sequence or into pEGFP-C1+ which was modified to contain a stop codon at the 3’ end of the open reading frame. Primer pairs for fragment amplification were designed to contain a Pmel restriction site in the forward primer and a Sull restriction site in the reverse primer sites.

Perfect matching sequences of miRNAs (canonical miRNA recognition elements [MREs]), predicted MREs of miRNAs within the GARP 3’UTR (GARP MREs), or mutated GARP MREs and their complementary strand were synthesized as single-stranded 20-nt oligonucleotides by Eurofins MWG Operon (Ipswich, MA) and inserted into the pmirGlo vectors downstream of the luciferase gene. For subsequent cloning, overhangs generating a Pmel restriction site (sense oligonucleotide, 5’-AAAC, antisense oligonucleotide, 5’-TGGT) and an Nhel restriction site (sense oligonucleotide, 5’-G, antisense oligonucleotide, 5’-CTAGC) were introduced at the 5’ and 3’ end of the oligonucleotides, respectively. Additionally, a NotI restriction site was introduced into the oligonucleotides for cloning.

To silence GARP expression in GARP-expressing HeLa cells, in silico–generated GARP-targeting small interfering RNA (siRNA) or scrambled siRNA (Life Technologies Invitrogen) were cloned into the pcDNA6.2-GW/EmGFP miRNA expression vector (Life Technologies Invitrogen).

Luciferase assay

HEK293 cells (50,000) were transfected with 50 ng pmirGlo constructs and 25 nM miRNA mimics or 250 nM antagonirs, using lipofectamine (Life Technologies Invitrogen). CD25+ CD4 T cells were transfected with 1 µg DNA and 250 nM antagonirs using an Amaxa human T cell Nucleofector kit (Lonza, Cologne, Germany). Twenty-four hours after transfection, luciferase activity was measured using a Dual-Glo luciferase assay (Promega).

T cell proliferation and suppression assays

CD25+ or CD25− CD4 T cells were labeled with 10 µM CFSE (Life Technologies Invitrogen). Where indicated, T cells were transfected with 250 nM antagonirs, 25 nM miRNA mimics, or 2–4 µg vector of interest and cotransfected with 1 µg pdRed2-N1 (Miltenyi Biotec). When proliferation of vector-transfected T cells was evaluated, CFSE dilution was followed in Red2-expressing T cells.

RNP IP

RNP IP was performed based on the protocol by Beitzinger and Meister (30).
FIGURE 1. miR-142-3p and miR-424 recognize their MREs within the GARP 3'UTR. (A) Schematic presentation of the GARP 3'UTR. In silico predicted miRNA binding sites in the 3'UTR of GARP mRNA are shown as dashes. Positions are indicated by the nucleotide numbers indicated above. Numbers in parentheses indicate the running number of MREs when more than one was predicted. Eleven miRNAs were predicted by miRbase target (purple; http://www.mirbase.org), five by Diana Lab (blue; http://diana.cslab.ece.ntua.gr/micro), five by Pictar (red; http://pictar.mdc-berlin.de), and one by Target Scan (green; http://targetscan.org). The full-length GARP 3'UTR was subdivided into 10 overlapping fragments. (B) Fragments 1–10 were cloned into the pEGFP vector downstream of the GFP gene and transfected into freshly isolated CD25+ CD4 T cells. Mean fluorescence intensity of GFP was analyzed 24 h after transfection by flow cytometry. Results were normalized to the value of cells transfected with a control vector without an insert. Statistical analysis was performed by a Student t test. (C) MREs for miR-142-3p, miR-181a, miR-181b, miR-181c, and miR-424 predicted in the distal part of the GARP 3'UTR were cloned into the pmirGlo luciferase reporter vector downstream of the firefly luciferase gene and (Figure legend continues)
Cell cycle analysis
Cells were synchronized in the G1/S boundary by double thymidine block (2 mM thymidine; Sigma-Aldrich), released for up to 48 h, and analyzed after propidium iodide staining by FACS using the CycleTest Plus DNA reagent kit (BD Pharmingen).

Western blot
HeLa cell lysates were separated in a 7% polyacrylamide gel and transferred onto nitrocellulose. The blots were probed with specific Abs for GARP, actin (Sigma-Aldrich), p53 (DO-1; Calbiochem, Bad Soden, Germany), p21/Cip1, or p27/Kip1 (New England Biolabs) and developed with ECL.

Immunofluorescence
HeLa cells were seeded onto microscope slides, stained with DAPI, FITC-labeled anti-CD3 (both Sigma-Aldrich), or the GARP antiserum followed by biotin-conjugated goat anti-rabbit IgG and cyanine 5–labeled streptavidin (Dianova, Hamburg, Germany). Images were captured on the Leica TCS SP5 confocal laser scanning microscope (Leica Microsystems, Heidelberg, Germany).

Results
The distal part of the GARP 3′UTR contains miRNA binding sequences capable of posttranscriptional regulation of gene expression
Based on the distribution of predicted MREs within the GARP 3′UTR, 10 fragments of the GARP 3′UTR were cloned separately into the pEGFP reporter vector downstream of the GFP coding sequence (Fig. 1A). The influence of the inserted sequences on GFP expression in CD25+ CD4 T cells was evaluated by flow cytometry as shown in Fig. 1B. Of the 10 constructs, only the vector containing the distal part of the GARP 3′UTR (fragment 10) showed significantly lower GFP expression indicating the presence of sequences capable of posttranscriptional regulation of protein expression.

To dissect whether and which miRNAs can recognize fragment 10 of the GARP 3′UTR to foster posttranscriptional regulation, MREs of nine miRNAs predicted by in silico analysis to be localized within this fragment (miR-142-3p, miR-181a, miR-181b, miR-181c, miR-381, miR-424, miR-497, miR-551, and miR-661) were cloned separately into the pmirGlo luciferase reporter vector downstream of the luciferase gene. For control, reporter vectors containing complementary sequences of miRNAs (canonical MREs) were used. Luciferase activity was measured after cotransfection of miRNA mimics and the respective vectors into HEK293 cells. Cotransfection of miRNA mimics and the respective vectors resulted in a significant reduction of luciferase activity, whereas mimics of miR-181a, miR-181b, or miR-181c did not have an effect on reporter gene expression (Fig. 1D). When MREs for miR-142-3p and miR-424 were mutated as shown in Fig. 1E, the inhibitory effect of the mimics was abrogated, confirming the specificity of miRNA/mRNA binding (Fig. 1F). Importantly, only a single putative recognition site for miR-142-3p was predicted within the full-length GARP 3′UTR.

miR-142-3p but not miR-424 recognizes the GARP 3′UTR in CD25+ CD4 T cells
miR-142-3p has previously been reported to be expressed in CD25+ CD4 T cells (31). Analysis of the expression of miR-142-3p and of miR-424 in the current system confirmed the expression of miR-142-3p in CD25+ CD4 T cells and CD25− CD4 T cells and revealed low expression levels of miR-424 in CD4 T cells (Fig. 2A). We then analyzed the effect of both endogenously expressed miRNAs on the luciferase activity of the vectors. Transfection of CD25+ CD4 T cells with a vector containing just the predicted GARP miR-142-3p MRE without any cotransfection resulted in diminished luciferase activity as compared with the control vector (Fig. 2B). Transfection with the vector containing the miR-424 MRE yielded luciferase activity similar to that of the control vector (Fig. 2B). These findings indicate that miR-142-3p is expressed endogenously to a sufficient extent to downmodulate expression of the reporter gene, whereas endogenous expression of miR-424 is not sufficient to regulate the reporter gene construct. Indeed, inhibition of the biological activity of miR-142-3p by cotransfection with an antagonim to miR-142-3p restored the luciferase activity of the miR-142-3p MRE vector, whereas inhibition of miR-424 biological activity had no effect on the activity of the miR-424 MRE reporter gene construct (Fig. 2B).

In additional experiments, transfection of the distal part of the GARP 3′UTR alone into CD25+ CD4 T cells led to diminished activity of the reporter gene as compared with transfection of a control reporter gene construct without the GARP 3′UTR (Fig. 2C). Cotransfection of the GARP 3′UTR reporter vector with an antagonim to miR-142-3p or mutation of the putative miR-142-3p MRE in the GARP 3′UTR restored the luciferase activity of the vector (Fig. 2C). In contrast, neither inhibition of miR-424 biological activity nor mutation of its binding site could restore luciferase activity (Fig. 2C). Thus, the data identify miR-142-3p as the miRNA involved in posttranscriptional regulation of GARP expression in CD25+ CD4 T cells.

miR-142-3p initiates GARP translational repression via Argonaute 2
The seed region of miR-142-3p (GUAGUGU) perfectly matches the predicted miR-142-3p MRE in the GARP 3′UTR (Fig. 1E), indicating that the inhibition of GARP expression via miR-142-3p may occur through Argonaute (Ago)2–mediated mRNA cleavage (32). Thus, we performed RNP IP using Abs against Ago1 and Ago2. Both GARP mRNA and miR-142-3p were immunoprecipitated together with Ago2 from Jurkat cells transfected with a vector containing the GARP coding sequence followed by the cotransfected with 25 nM miRNA or scrambled miRNA (scr) into HEK293 cells. Luciferase activity was analyzed 24 h after transfection. Vectors containing canonical sequences of MREs (can) and the pmirGlo vector without an insert (ctrl) were used as positive and negative controls, respectively. (D) The pmirGlo luciferase reporter vector containing the distal 400-bp fragment of the GARP 3′UTR downstream of the firefly luciferase gene was cotransfected with 25 nM miR-142-3p, miR-181a, miR-181b, miR-181c, or miR-424 miRNA or with scrambled miRNA into HEK293 cells. Luciferase activity was analyzed 24 h after transfection. (E) Mutations in the region complementary to the seed sequence (shown in bold) of miR-142-3p and/or miR-424 were generated by site-directed mutagenesis as indicated. (F) pmirGlo luciferase reporter vectors containing the distal 400-bp fragment of the GARP 3′UTR with mutated binding sites as shown in (E) were cotransfected with miRNA mimics into HEK293 cells. Luciferase activity was analyzed 24 h afterward. Means ± SD of at least three experiments are shown. Statistical analysis was performed by ANOVA. *p < 0.05, **p < 0.01, ***p < 0.001.
GARP 3′UTR. GARP mRNA was detected only in the Ago2 immunoprecipitates, whereas miR-142-3p was present in both Ago1- and Ago2-containing RNP complexes, as expected (Fig. 3A). To dissect the simultaneous occurrence of miR-142-3p and GARP mRNA in the Ago2-containing RNP complex, biological activity of miR-142-3p was inhibited by an antagonim. Cotransfection with an antagonim to miR-142-3p prevented GARP mRNA loading into the Ago2-associated RNP complex, whereas miR-142-3p loading into both Ago1 and Ago2 RNPs was prevented (Fig. 3B), confirming that miR-142-3p mediates loading of GARP mRNA into the Ago2-associated RISC. Thus, posttranscriptional regulation of GARP expression involves miR-142-3p–mediated recruitment of GARP mRNA into the Ago2-associated RISC.

Endogenous expression of miR-142-3p regulates GARP expression in CD25+ CD4 T cells

We next investigated expression of GARP and miR-142-3p in CD25+ CD4 T cells in response to activation. GARP mRNA and protein expression were upregulated within 24 h in CD25+ CD4 T cells in response to activation and disappeared 3 d later (Fig. 4A), consistent with the loading of GARP mRNA into the Ago2-associated RISC responsible for mRNA cleavage (Fig. 3). miR-142-3p was also markedly upregulated in response to T cell activation; however, upregulation was first noted at day 2 after stimulation and diminished the following day (Fig. 4A). The upregulation of miR-142-3p in response to CD25+ CD4 T cell activation occurred after the upregulation of GARP mRNA and immediately preceded its downmodulation.

To evaluate whether this rise of miR-142-3p causes downmodulation of GARP expression, we assessed the effect of an antagonim to miR-142-3p on GARP expression in cells transfected just before the miR-142-3p expression peak at day 1 after stimulation. The antagonim delayed the downmodulation of GARP expression (Fig. 4B). The effect of miR-142-3p blockage was obvious on both surface protein and mRNA expression. An ∼4-fold reduction in the miRNA expression underpinned the downmodulation. Alternatively, when cells were treated with a mimic of miR-142-3p before activation, the upregulation of GARP expression was markedly diminished as observed again on protein as well as on mRNA level (Fig. 4C). It was supported by an ∼4-fold increase in the miRNA level in response to transfection with the miR-142-3p mimic. Taken together, these observations demonstrate that upregulation of miR-142-3p in response to activation correlates with GARP expression on CD25+ CD4 T cells and confirm miR-142-3p as the miRNA controlling endogenous GARP expression in CD25+ CD4 T cells in response to activation.

miR-142-3p is involved in modulation of CD25+ CD4 T cell proliferation

To provide insight into the biological function of miR-142-3p in CD25+ CD4 T cells, we again interfered with the biological activity of the miRNA by using an antagonim or mimic of miR-142-3p and, in addition to GARP expression, followed the proliferative capacity of the cells in response to activation (Fig. 5). Antagonim treatment of CD25+ CD4 T cells resulted in an immediate reduction of miR-142-3p expression level and a subsequent upregulation of miR-142-3p and GARP expression levels.
ulation of GARP mRNA and protein expression throughout the follow-up period (Fig. 5A). The proliferation of CD25+ CD4 T cells treated with an antagonim to miR-142-3p upon stimulation was modestly but significantly increased (Fig. 5A). In contrast, treatment of the cells with a mimic of miR-142-3p led to a reduction of proliferation accompanied by an increase in miR-142-3p level and a diminution of GARP mRNA and protein expression (Fig. 5B).

miR-142-3p–mediated modulation of GARP expression correlates with T cell proliferation

CD25+ CD4 T cells do not express GARP physiologically but do express miR-142-3p to a level comparable to that of CD25+ CD4 T cells. To verify the role of miR-142-3p/GARP interaction in T cell proliferation, we therefore analyzed proliferation of primary CD25+ CD4 T cells in response to enforced GARP expression (Fig. 6). For this, we generated vectors containing the GARP coding sequence and downstream sequences of the wild-type or mutated GARP miR-142-3p MRE. Transfection of CD25+ CD4 T cells with a vector encoding the naked GARP coding sequence led to ectopic expression of GARP on the surface of CD25+ CD4 T cells (Fig. 6A). The presence of GARP miR-142-3p downstream of the GARP coding sequence markedly reduced this ectopic GARP expression. Mutation of the MRE fully restored GARP expression. When the proliferative capacity of the transfected cells was analyzed, it became apparent that insertion of the
GARP miR-142-3p MRE considerably diminished the proliferation of CD25⁺ CD4 T cells (Fig. 6B, 6C). The presence of the mutated variant of GARP miR-142-3p MRE, however, did not affect T cell proliferation. These data correspond well with the observation that the interference with miR-142-3p biologic activity influences cell proliferation and indicate that the miR-142-3p–regulated GARP expression might in fact modulate proliferation of CD25⁺ CD4 T cells.

**GARP modulates cell proliferation**

To explore the link between GARP and the proliferative capacity of cells in greater detail, we overexpressed GARP in different cell lines physiologically not expressing GARP, such as HeLa or Jurkat cells. Ectopic GARP expression did not influence the proliferation behavior of the cell lines (Fig. 7A). Remarkably, however, when the proliferating capacity of individual clones was examined, profound differences in cell growth of the clones expressing different amounts of GARP were observed (Fig. 7B, 7C). Clones expressing lower amounts of GARP on the surface proliferated slower than did those expressing higher amounts, as shown for three representative clones in Fig. 7B and 7C. The changes in cell growth of clones were reflective of a slowing of their cell cycle progression and of accumulation of cell cycle–inhibiting proteins p53, p21/Cip1, and p27/Kip (Fig. 7C). Next, we examined the impact of diminishing GARP expression in a high-expressing clone by transfection with a vector expressing GARP-targeting siRNA (Fig. 7D). Expression of GARP siRNA reduced the level of GARP and also suppressed the proliferative capacity as compared with clones transfected with scrambled siRNA. In contrast, it did not influence the proliferative capacity of GARP-nonexpressing control cell lines. These observations confirm that diminishing of GARP expression attenuates cell proliferation.

**Discussion**

In this study, we dissected miRNA regulation of GARP expression in CD4 CD25⁺ T cells. We identified miR-142-3p as the miRNA facilitating GARP mRNA incorporation into the Ago2-associated RISC and initiating its subsequent degradation. miR-142-3p, therefore, regulates the short time window during which GARP is present on the surface of regulatory T cells after their activation. Because GARP appears to be involved in the proliferation of CD4 CD25⁺ T cells, this mechanism might represent a means to control regulatory T cell homeostasis, as neutralization of miR-142-3p resulted in increased proliferation.

miRNAs represent a mechanism for posttranscriptional regulation of the expression of thousands of miRNAs enabling cus-
expressed or upregulated, is currently accepted as an evolutionary counterpart. Interestingly, miR-142-3p is abundant already in nonactivated CD25+ CD4 T cells. miRNA/target interactions, in mRNA degradation as the cause for the sequential presence of both mRNAs (19). Indeed, CD25+ CD4 T cells transfected with an antagonist to miR-142-3p directly after activation express much higher levels of GARP.

The 3’UTR of GARP is remarkably long (~2000 bp) and contains five highly conserved sequences. Depending on whether dampening of protein output was beneficial, inconsequential, or harmful, the miRNA recognition sites were conserved, neutral, or selectively avoided during evolution. Usually not only the miRNA-targeting sites but also sequences in their vicinity are highly conserved throughout evolution (19). The miR-142-3p MRE is located in the distal 187-bp-long conserved segment that shares 76.2% homology with the mouse GARP 3’UTR. The miR-142-3p MRE itself differs in only two nucleotides between human and mouse sequences. Experimental interference with biological activity of miR-142-3p in mouse T cells yields similar effects on GARP expression as in human T cells (Supplemental Fig. 4). Collectively, these considerations indicate an important biological function for this miRNA in the posttranscriptional regulation of GARP expression. Besides miR-142-3p, a number of miRNAs were predicted by in silico analysis of this region to be capable of mRNA recognition. However, only miR-142-3p was regulatory in CD4 T cells. The conserved region surrounding MREs is thought to be necessary for engagement of RNA-binding proteins that play a key role to ensure successful miRNA regulation by either masking the miRNA recognition sites at distinct developmental stages and preventing miRNA-mediated repression, or by changing the RNA structure and thereby inducing the binding of miRNA and subsequent miRNA-mediated repression, or by stabilizing miRNA/mRNA complexes (35–38). It seems that there is no control of the activation-induced miR-142-3p-mediated GARP repression via an RNA-binding protein. In nonactivated CD25+ CD4 T cells, however, interaction of miR-142-3p with GARP mRNA might conceivably be influenced by this kind of regulation mechanism, as both miR-142-3p and GARP mRNA are present simultaneously.

The list of miR-142-3p putative targets consists of hundreds of genes. Because of so many targets, it is difficult to assign the function of a specific interaction of miR-142-3p with GARP mRNA with absolute certainty and to rule out indirect effects. Indeed, 18 additional predicted target genes for miR-142-3p could be identified to be part of cell cycle processes codified by gene ontology analysis. An approach to discount such concerns is to disrupt the putative miRNA/mRNA interaction either by using antisense reagents, or by mutation of the binding site, or by perturbation of the binding site by homologous recombination (19). Mutation of the miR-142-3p MRE either individually or in the context of GARP 3’UTR identified miR-142-3p as the miRNA posttranscriptionally controlling GARP expression on CD25+ CD4 T cells. By using an antagonist to miR-142-3 we augmented endogenous GARP expression in CD25+ CD4 T cells, which was accompanied by an increased proliferative capacity of the cells. Both findings strongly argue for the observed proliferation phenomenon as a direct consequence of the interaction of miR-142-3p with GARP mRNA, and they decrease the hypothetical concern of indirect effects. This is further supported by the finding that mimics of miR-142-3p diminished GARP expression associated with decreasing cell proliferation. Finally, miR142-3p was able to modulate proliferation of primary CD25+ CD4 T cells ectopically.
expressing GARP when the specific MRE was present downstream of GARP but failed to do so when the MRE was mutated. These data strongly argue for a direct role of miR142-3p/GARP interaction in regulating CD4 T cell proliferation.

Given the association between GARP expression and CD25+ CD4 T cell proliferation, a tight regulation of GARP expression and therefore the mechanisms regulating its expression might be a prerequisite of functional regulatory T cell homeostasis. It is conceivable that nonactivated CD25+ CD4 T cells, which are not strongly proliferative and expand only homeostatically to keep the population stable, express low levels of GARP. Following specific stimulation, Ag-specific regulatory T cells expand. Thus, they upregulate GARP on their surface and initiate expansion. Through the miR-142-3p-mediated GARP mRNA degradation pathway, however, they downregulate GARP expression and ensure controlled expansion. Originally GARP has been reported to serve as a receptor for the nonactive form of TGF-β (20, 21). The biological function of a receptor exposing TGF-β in its nonactive form on the surface of regulatory T cells is, however, not completely understood, as no conversion into the active form occurs (21). Nevertheless, downmodulation of GARP expression by siRNA resulted in attenuation of the suppressive capacity of regulatory T cells (20), highlighting the necessity of GARP for regulatory T cell function. Our data provide an alternative explanation for GARP function in regulatory T cells, as the mechanism controlling their expansion although the precise molecular mechanism is also still elusive.
Slowing the cell cycle in combination with expression of cell cycle inhibitors p53, p21/Cip1, and p27/Kip could contribute to the influence of GARP on cell proliferation. The exact connection between GARP and these molecules requires additional investigations. The finding that the GARP gene is an oncogene often amplified in various solid tumors (24, 28, 29) supports our hypothesis of an important role of the GARP/miR-142-3p interplay in controlling cellular proliferation and function. Importantly, however, the GARP effect on proliferation is modulating rather than absolute, although this effect may be critical in tumor cells, as deregulation of GARP expression contributes to their aggressiveness. The tight regulation of GARP expression through miR-142-3p might, therefore, create a short time window during which GARP is abundant on the surface of regulatory T cells, permitting the initiation and, alternatively, subsequent dampening of their proliferation to prevent their unopposed outgrowth. Although intriguing, our data are suggestive and further in vivo studies will verify the role of miR-142-3p/GARP interactions on T cell homeostasis in a living organism.

Acknowledgments

We thank C. Schnabel for expert technical assistance. We thank Dr. G. Meister and Dr. M. Beitzinger for providing anti-Ago Abs and helpful technical advice. We are grateful to Dr. V. Heissmeyer for fruitful discussions and critical comments.

Disclosures

The authors have no financial conflicts of interest.

References

13. Z. H. Feng. 2009. miR-142-3p restricts cAMP production in CD4+CD25+ TREG cells by targeting AC9 mRNA.

miRNA142-3p–MEDIATED REGULATION OF PROLIFERATION
SUPPLEMENTARY FIGURE 1

Human Feb. 2009 chr11:76368569-76381044

Alignment 1
Mouse Jul. 2007
chr7 (-)
105639038-105650342
Criteria: 70%, 100 bp
Regions: 12

Resolution: 4
Window size: 100 bp

Repeats:
- LINE
- LTR
- SINE
- RNA
- DNA
- Other

contig
gene
exon
UTR
CNS
mRNA

100%
50%

76366000 76366900 76367800 76368700 76369600 76370500 76371400 76372300 76373200 76374100 76375000 76376000 76377000 76378000 76379000 76380000 76381000
Suppl. Figure 1. Schematic presentation of conserved non-coding sequences (CNS) in the GARP gene locus. Fragment of chromosome 11 including the GARP gene is displayed. Exons are depicted in purple. The 3’UTR is shown in light blue. CNS regions were selected according to a conservation identity between mouse and human sequences by VISTA Genome Browser.

Suppl. Figure 2. Algorithm-based miRNAs that do not recognize their MREs in the GARP 3’UTR. MREs for miR-381, miR-497, miR-551a, and miR-661 predicted in the distal part of GARP 3’UTR were cloned into the pmirGlo luciferase reporter vector downstream of the firefly luciferase gene and cotransfected with 25nM respective miRNA or scrambled miRNA (scr) into HEK293 cells. Luciferase activity was analyzed 24h after transfection. Vectors containing canonical sequences of MREs (canon) and the pmirGlo vector without an insert were used as positive and negative controls, respectively. Mean ± SD of at least two experiments is shown.

Suppl. Figure 3. Kinetic of GARP expression within the first 24h after stimulation. CD25+ CD4 T cells were isolated and stimulated with anti-CD3 for a 24h. GARP mRNA expression was examined by real-time PCR. Relative expression normalized to cyclophilin is demonstrated. One representative of two independent experiments is shown.

Suppl. Figure 4. Interference with miR-142-3p biological activity in mouse T cells. CD25+ CD4 T cells were purified from spleen and lymph nodes of Black 6 mice, transfected either with an antagonim or with mimics of miR-142-3p and stimulated for four 24h with anti-CD3/CD28 beads. GARP expression was assessed by flow cytometry. Numbers indicate mean fluorescence intensity of labeled cell. One representative experiment out of three independent experiments is shown.