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The human Rgr oncogene is overexpressed in T cell malignancies and induces transformation by acting as a GEF for Ras and Ral

Kwame Osei-Sarfo, Ph.D.[1], **Laura Martello, Ph.D.**[2], **Sherif Ibrahim, M.D., Ph.D., F.C.A.P.**[3], and **Angel Pellicer, M.D., Ph.D.**[1]

[1] Department of Pathology, New York University Langone Medical Center, New York, NY, USA

[2] Division of Gastroenterology & Hepatology, SUNY Downstate Medical Center, New York, NY, USA

[3] Department of Hematopathology, New York University Langone Medical Center, New York, NY, USA

Abstract

The Ras superfamily of GTPases is involved in the modification of many cellular processes including cellular motility, proliferation and differentiation. Our laboratory has previously identified the RalGDS related (Rgr) oncogene in a DMBA-induced rabbit squamous cell carcinoma and its human orthologue, hRgr. In the present study, we analyzed the expression levels of the human hRgr transcript in a panel of human hematopoietic malignancies and found that a truncated form (diseased-truncated; Dtr-hrgr) was significantly overexpressed in many T-cell derived neoplasms. Although the Rgr proto-oncogene belongs to the RalGDS family of guanine nucleotide exchange factors (GEFs), we show that upon the introduction of hRgr into fibroblast cell lines it is able to elicit the activation of both Ral and Ras GTPases. Moreover, *in vitro* guanine nucleotide exchange assays confirm that hRgr promotes Ral and Ras activation through GDP dissociation, which is a critical characteristic of GEF proteins. hRgr has guanine nucleotide exchange activity for both small GTPases and this activity was reduced when a point mutation within the catalytic domain (CDC25) of the protein, (cd) Dtr-hRgr, was utilized. These observations prompted the analysis of the biological effects of hRgr and (cd) hRgr expression in cultured cells. Here, we show that hRgr increases proliferation in low serum, increases invasion, reduces anchorage dependence, and promotes the progression into S phase of the cell cycle; properties that are abolished or severely reduced in the presence of the catalytic dead mutant. We conclude that the ability of hRgr to activate both Ral and Ras is responsible for its transformation-inducing phenotype and it could be an important contributor in the development of some T cell malignancies.

Correspondence: Angel Pellicer, M.D., Ph.D., New York University Langone Medical Center, Department of Pathology, 550 First Avenue, MSB 594, New York, NY, USA, 212-263-5342 (phone), 212-263-8211 (fax), Angel.Pellicer@nyumc.org.

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Keywords

Rgr; GEFs; Ras GTPases; Ral GTPases; oncogene; T-cell lymphoma

Introduction

Small GTPase proteins are molecular switches that transduce extra- and intracellular signals by cycling between inactive GDP-bound and active GTP-bound states. The cycling between inactive and active forms is regulated by guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs). GEFs activate these GTPases by inducing the dissociation of GDP and the binding of GTP, which is present at higher concentration in cells. GAPs inactivate GTPases by hydrolyzing the γ-phosphate group, resulting in a GDP-bound molecule (Takai *et al.*, 2001). The superfamily of GTPases can be subdivided into five distinct classes (Ras, Rho, Rab, Sar1/Arf and Ran) based on sequence homology (Lundquist, 2006; Takai *et al.*, 2001). Ras genes, the more extensively studied members, typically are mutated and/or activated in approximately 30% of human cancers and are frequently mutated in several animal tumor models (Bos, 1989). Mutations involving one of the three Ras isoforms have been described in several malignancies including, but not limited to, squamous head and neck tumors, pancreatic adnocarcinoma, colorectal malignancies, lung adenocarcinomas, acute leukemias and myelodysplastic syndromes (Corominas *et al.*, 1989; Rodenhuis, 1992).

Shortly upon the identification of Ras GTPases, Ral GTPases (Ral A and Ral B) were isolated and found to be downstream members of the Ras signaling pathway. (Chardin and Tavitian, 1986). More specifically, Ral GTPases were found to be activated by Ras GTPases through the activation of a GEF for these GTPases, namely Ral guanine nucleotide dissociation stimulator (RalGDS) (Albright *et al.*, 1993). Previous studies, through inhibiting the expression of Ral GTPases, have shown that the activation of Ral GTPases cooperates with Ras-induced cellular transformation (Urano *et al.* 1996; Lim *et al.* 2005). For many years, it was assumed that Ral GTPases merely facilitated the cascade of Ras GTPase signaling events. Recent studies have determined that the effectors of Ral GTPases have been implicated in vesicle transport (Camonis and White, 2005; Rosse *et al.*, 2006), endocytosis (Jullien-Flores *et al.*, 2000), gene transcription (Frankel *et al.*, 2005), development of filopodia (Ohta *et al.*, 1999) and cellular proliferation (Smith *et al.*, 2006). In addition to these effectors, recent research has determined that improper activation of Ral GTPases has been implicated in oncogenesis through its role in metastasis (Lim *et al.*, 2005; Lim *et al.*, 2006; Oxford and Theodorescu, 2003), resistance to anoikis (Zahir *et al.*, 2003), activation of transcription factors (Frankel *et al.*, 2005) and inappropriate cell survival signals (Chien *et al.*, 2006; Smith *et al.*, 2006).

Like Ras GTPases, Ral GTPases have their own cadre of activating proteins or GEFs. Based on the elucidation of the first RalGEF, RalGDS (Albright *et al.*, 1993; Hofer *et al.*, 1994), several other GEFs for Ral GTPases have been uncovered including Rlf (Wolthuis RM., 1996), Rgl 1–3 (Shao and Andres, 2000; Sood *et al.*, 2000; Takaya *et al.*, 2007), RalGPS 1–2 (Ceriani *et al.*, 2007; Tazmini *et al.*, 2009) and AND-34 (Gotoh *et al.*, 2000). The majority

of these GEFs are activated by Ras GTPases through a Ras binding domain (RBDs); whereas, RalGPS 1–2 and AND-34 do not have detectable RBDs. Since several RalGEFs do not have RBDs, studies have confirmed that Ral activation can be achieved through Ras-independent mechanisms (Linnemann *et al.*, 2002). Several groups have observed Ras-independent activation of Ral GTPases through signals propagated by the Ca^{2+} ionophore ionomycin (Hofer *et al.*, 1998), β-arrestins and the formyl-Met-Leu-Phe receptor (Bhattacharya *et al.*, 2002), nitric oxide (Mittar *et al.*, 2004) and JAK/STAT 3 activation (Senga *et al.*, 2001)

The RalGDS related (Rgr) oncogene was isolated in our laboratory from a DMBA-induced squamous cell carcinoma and was found to induce tumorigenesis in the nude mouse assay (D'Adamo *et al.*, 1997). Protein sequencing analysis determined that Rgr had 40% sequence identity to RalGDS and up to 72% identity within the CDC25 domain (D'Adamo *et al.*, 1997). The mechanism of enhanced guanine nucleotide dissociation caused by Rgr was due to overexpression through a loss of translational regulation by the elimination of 5′ inhibitory sequences (Hernandez-Munoz *et al.*, 2003). The human orthologue of Rgr (hRgr) was also identified in our laboratory by screening a human testis cDNA library and was found to have 13 exons, of which the first three exons contained a 5′ untranslated region (UTR) (Leonardi *et al.*, 2002). Further analysis of the hRgr sequence found a significant nucleotide similarity to an EST from Jurkat cells, which is a T-cell acute lymphoblastic leukemia (T-ALL) derived cell line (Leonardi *et al.*, 2002). Northern blotting and reverse transcription-PCR (RT-PCR) of Jurkat cells demonstrated a severely truncated transcript of hRgr (Leonardi *et al.*, 2002). In addition, the truncated transcript initiated protein translation by using a start codon within an intronic region that allows the open-reading frame to be maintained through exon 9–13. Interestingly, Rgr does not contain a Ras binding domain (RBD) or a Ras exchange motif (REM) typically seen in RalGEFs (D'Adamo *et al.*, 1997; Leonardi *et al.*, 2002). However, the lack of the RBD and REM motifs has been observed in other RalGEFs including RalGPS1 (Tazmini *et al.*, 2009), RalGPS2 (Ceriani *et al.*, 2007) and AND-34 (Gotoh *et al.*, 2000).

In this study, we investigated the importance of the hRgr-truncated form in human leukemias and in the biological and biochemical aspects of cellular transformation. In addition, we show that the abnormal transcript of hRgr is overexpressed in a wide range of human hematological malignancies. Our results reveal that the expression of the truncated protein produced by the abnormal transcript of hRgr can induce cellular transformation through the activation of Ras and Ral GTPases.

Results

The abnormal hRgr transcript is overexpressed in T-cell malignancies

In order to determine the expression patterns of the abnormal hRgr transcript in hematological malignancies, a Quantitative Real Time (QRT)-PCR screen of T-cell and B-cell neoplasms and normal tissues was conducted. To detect the presence of the abnormal transcript, we utilized primers from the intronic region that becomes exonic at the 5′ end of Exon 9 and from a portion of the normal Exon 9 sequence (Figure 1a). As shown in Table 1, the abnormal hRgr transcript was only observed in T-cell neoplasms including T-cell acute

lymphoblastic leukemia (T-ALL), peripheral T-cell lymphomas (PTCL), mycosis fungoides, anaplastic large T-cell lymphoma (T-ALCL), adult T-cell lymphoma leukemia (T-ATLL) and angioimmunoblastic T-cell lymphoma (T-AILD). Figure 1b, which quantifies the expression of the abnormal transcript from selected samples, shows that the abnormal transcript is highly overexpressed in T-cell neoplasms. In addition, the amplicon produced by the QRT-PCR of a T-ALL sample was sequenced and was found to be located on chromosome 22 at the expected location of hRgr (data not shown).

For a comparative analysis, we sought to observe the putative expression of the normal hRgr transcript in the samples previously mentioned. Thus, QRT-PCR analysis was used to characterize the expression pattern of the normal transcript by using primers directed to Exon 5 (Figure 1a), which is not present in the abnormal hRgr transcript. Here, we find that the normal transcript has a low expression pattern in both T-cell and B- cell neoplasms as well as in normal tissues (Figure 1c).

These data confirm that both a truncation and the exonic expression of a portion of an intronic sequence lead to the overexpression of the abnormal hRgr transcript only in neoplasms derived from the T-cell lineage. Additionally, these data suggest that a genomic alteration, possible due to a rearrangement on chromosome 22, may initiate the abnormal expression of hRgr culminating in the development or maintenance of T-cell malignancies.

Rgr activates Ras and Ral GTPases in vivo

Since previous studies from our laboratory have demonstrated that the ability of rabbit Rgr to transform cells is dependent on Ras (Hernandez-Munoz et al., 2000), we wanted to confirm this ability for hRgr. To determine this property, we used effectors for activated Ras (Ras binding domain of Raf-1) and Ral A (Ral binding domain of Ral binding protein 1, RalBP 1) to measure the amount of active or GTP-bound Ras and Ral in cells stably expressing Rgr (R-Rgr and Dtr-hRgr). In addition, we used a catalytic-dead mutant of Dtr-hRgr [(cd) Dtr-hRgr], which has a point mutation within the catalytic or CDC25 domain. We found that the expression of Dtr-hRgr in NIH3T3 cells increases the activation of Ras GTPases, compared to nontransfected NIH3T3 cells (Figures 2a and c). Consistent with these results, NIH3T3 cells that stably express Dtr-hRgr and R-Rgr increased the activation of Ral A compared to nontransfected NIH3T3 cells and NIH3T3 cells expressing an “empty” pCGN vector (Figures 2b and c). Although slight, Ras and Ral GTPase activation was reduced in cells expressing (cd) Dtr-hRgr. Interestingly, cells that expressed R-Rgr had a higher amount of Ral A activation, compared to those expressing constitutively active N-Ras (N-Ras V12), which served as a positive control (Figure 2b). This observation is appropriate because of the dual nature of Rgr-induced activation of Ras and Ral GTPases. Rgr could activate Ral A by Ras-dependent events (as a GEF for Ras) and also directly activate Ral A (as a GEF for Ral). The activation of Ras and Ral A GTPases by hRgr could account for the induction or maintenance of T-cell malignancies.

Rgr enhances GDP dissociation from N-Ras, Ral A and Ral B

To gain a better perspective concerning the mechanism of Ras and Ral GTPases activation by Rgr, the mant-GDP exchange assay was utilized. This assay measures the ability of a

GEF to dissociate GDP from a GTPase by observing the amount of mant-GDP released over time. For this analysis, we used the full-length truncated hRgr (Tr-hRgr), which is a derivative of hRgr that lacks the 5′-UTR of the full-length hRgr (Figure 1a). Tr-hRgr was used to determine if only the complete coding sequence of hRgr could induce a higher amount of GDP dissociation on Ras and Ral GTPases. It is important to note that Tr-hRgr is a purely synthetic form that has not been naturally observed. Here 6x-Histidine-tagged-Rgr fusion proteins (Tr-hRgr, Dtr-hRgr and R-Rgr) have the ability to dissociate GDP from Ral A (Figure 3a and Supplementary Figure 1), Ral B (Figure 3b and Supplementary Figure 1) and N-Ras (Figure 3c and Supplemental Figure 2). Between the hRgr constructs (Tr-hRgr and Dtr-hRgr), we did not observe any significant differences in mant-GDP dissociation. Also, we found that the catalytic dead mutants of Dtr-hRgr, (cd) Dtr-hRgr, and Tr-hRgr, (cd) Tr-hRgr, had comparable dissociation properties to the negative control experiment samples (the GTPase alone and the GTPase incubated with GST, a nonGEF molecule; Figure 3).

In this study, we found that the more characterized GEFs for Ral A and Ral B (RalGDS, Rlf and RalGEF 2) and N-Ras (Sos and RasGRP 1) had a higher rate of mant-GDP dissociation within the first 200 seconds compared to the Rgr constructs (Supplementary Figures 1 and 2). Even though the Rgr constructs had less dissociation activity than the more characterized GEFs for N-Ras and Ral GTPases, these results confirm that Rgr has a role in the activating properties for Ras and Ral GTPases through the dissociation of GDP.

hRgr alters biological and biochemical processes and initiates cellular transformation

To determine the importance of Rgr-mediated cellular transformation, we characterized the alteration of biological activities of Rgr-stably expressing NIH3T3 cell lines. Previous results confirmed that rabbit Rgr (R-Rgr) could induce cellular transformation (D'Adamo *et al.*, 1997; Hernandez-Munoz *et al.*, 2003; Hernandez-Munoz *et al.*, 2000). Since we have demonstrated that the abnormal hRgr transcript can modulate the activation of Ras and Ral GTPases, it was necessary to confirm that hRgr-induced activation of these GTPases could contribute to cellular transformation. NIH3T3 cells expressing Dtr-hRgr and R-Rgr promoted cellular proliferation in low serum (1%) conditions (Figure 4a). These results were significant by Day 9 as demonstrated by the Kruskal-Wallis Rank Sum test and ANOVA when compared to nontransfected NIH-3T3 cells. Supporting the role of hRgr-induced transformation, cells expressing (cd) Dtr-hRgr had reduced proliferation when compared to those expressing Dtr-hRgr. In addition to increased cellular proliferation, Dtr-hRgr expression in NIH3T3 cells promoted increased colony formation in soft agar, compared to nontransfected NIH3T3 cells (Figure 4b).

As Figure 5 shows, both Dtr-hRgr and R-Rgr can also alter invasive properties *in vivo*. Stably-expressing Rgr cells were subjected to the modified Boyden chamber assay, which measures the ability of transformed cells to invade and migrate through an extracellular matrix. Consistent with the biological results observed above, we found that Dtr-hRgr expressing cells could enhance the invasive properties of transformed cells. We found that there was close to three times the amount of cellular invasion in cells expressing Dtr-hRgr, relative to nontransfected NIH3T3 cells. In addition, cells expressing (cd) Dtr-hRgr had invasive properties that were comparable to nontransfected NIH3T3 cells (Figure 5). Taken

together, the biological assays described herein confirm that the expression of the abnormal hRgr protein can alter biological properties, which were consistent with the results observed in the *in vitro* and *in vivo* Ras and Ral GTPases activation analyses.

To determine a biochemical connection to the biological processes altered by Rgr, cell cycle progression analysis was conducted by flow cytometry. Analysis of cell cycle progression showed that Dtr-hRgr and R-Rgr caused a moderate, but significant, increase in S phase events (Figure 6). Consistent with this observation, we found that there was a reduction of G_0/G_1 events in cells expressing Dtr-hRgr or R-Rgr. These results suggest that Rgr can drive cell cycle events, which could be a contributing factor in the induction of a malignant phenotype.

The abnormal hRgr transcript contributes to the malignant phenotype of T-cell malignancies

In this study as well as in previous studies (Leonardi et al., 2002), it has been established that the abnormal hRgr transcript is overexpressed in T-ALL derived cell lines and neoplasms. To fully understand the influence of transcript expression in the maintenance of a malignant phenotype in these cell lines, RNA interfering (RNAi) oligonucleotides directed against hRgr were synthesized to inhibit the expression of the abnormal hRgr transcript. RNAi oligonucleotides directed against two regions of hRgr were introduced into T-ALL 1 and Jurkat cells, which were cultured in low serum conditions, and cell cycle events were measured. Here, we found that Jurkat cells (Figure 7a) and T-ALL 1 cells (Figure 7b) transfected with hRgr RNAi oligonucleotides (hRgr Oligo 1) had a significant reduction in S phase and an increase in G_0/G_1 phase in comparison to nontransfected cells or those transfected with the non-targeting oligonucleotides. As an experimental control, we confirmed using QRT-PCR analysis that hRgr Oligo 1 decreased hRgr expression by at least 65% (Supplementary Figure 3). These data indicate that proper translation of the abnormal hRgr transcript stimulates cellular proliferation by promoting the entrance of these cells into the S phase. The results from these experiments are consistent with the postulated role in hRgr-induced transformation for T-cell neoplasms.

Discussion

Previous studies in our laboratory have isolated Rgr as a GEF with significant homology to RalGDS with the ability to dissociate guanine nucleotides from Ral GTPases (D'Adamo *et al.*, 1997; Hernandez-Munoz *et al.*, 2000). In addition to its dissociation activity, it was determined that Rgr could induce cellular transformation through the loss of translational regulation by the elimination of 5′ inhibitory sequences (Hernandez-Munoz *et al.*, 2003). The human version of Rgr (hRgr) was identified in our laboratory through the screening of a human testis cDNA library (Leonardi *et al.*, 2002). Through additional examination, a severely truncated transcript was observed in an mRNA sequence derived from an EST of the Jurkat cell line, which is a T-ALL derived cell line. Also, this truncated transcript initiated translation using a portion of the 5′ intronic sequence preceding Exon 9 that becomes exonic in the abnormal form (Leonardi *et al.*, 2002). The goal of this study was to

further characterize the biological and biochemical roles that hRgr have in cellular transformation.

Abnormal hRgr is an oncogene expressed in T-cell malignancies

In this study we performed an extensive screening of various hematological malignances and normal tissues to determine the expression patterns of hRgr's transcripts, both normal and abnormal. Consistent to previous results (Leonardi *et al.*, 2002), we detected the abnormal hRgr transcript in a wide range of T-cell neoplasms and its levels of expression were significantly elevated. In the same hematological neoplasms and normal tissue samples, we established that the normal transcript was very lowly expressed. This is consistent with the low levels of expression observed for the wild type Rgr transcript in most rabbit tissues (D'Adamo et al 1997). RNAi knockdown demonstrated that blocking translation of Dtr-hRgr could lead to reduced cell cycle progression. These data suggest that hRgr may have a role in maintaining the malignant phenotype of T-ALL derived cell lines.

These observations advance the hypothesis that a genomic modification or rearrangement may cause the overexpression of the abnormal transcript, which contains an intronic sequence that becomes exonic. The long arm of chromosome 22 is highly recognized as a participant of the Philadelphia translocation, which involves reciprocal translocation of a portion of chromosome 22 with chromosome 9 (Rowley, 1973). More specifically, this chromosomal translocation, t(9;22)(q34;q11), causes the juxtaposition of *BCR* and *ABL* genes forming an oncogenic fusion protein (Muller *et al.*, 1991). It was further determined that genomic translocations or rearrangements on chromosome 22 were identified in 90–95% of chronic myelogenous leukemias (CMLs), 20% of adult acute lymphoblastic leukemias (ALLs), 5% of pediatric ALLs and 2% of acute myeloid leukemias (AMLs) (Kurzrock *et al.*, 2003). Due to the proximity of hRgr to the region involving the translocation of *BCR* on chromosome 22, it is highly probable that hRgr may participate in the Philadelphia translocation. It has been demonstrated that the *BCR* gene translocation can extend over a 200 kb region (Chissoe *et al.*, 1995), which is well within the reach of hRgr on chromosome 22. At this point we are not in a position to suggest that hRgr plays a role in this disease, but this would be an intriguing possibility to explore in the future. Another possible mechanism for the generation of the abnormal hRgr transcript is through genomic rearrangements caused by microdeletions. Studies have confirmed the involvement of microdeletions of chromosome 22 in adult ALL (Paulsson *et al.*, 2008) and mesotheliomas (Sekido *et al.*, 1995).

hRgr activates Ras and Ral GTPases

Here, the abnormal version of hRgr (Dtr-hRgr) can activate Ras and Ral GTPases *in vivo* and *in vitro*. Previous studies have demonstrated that Rgr can activate Erk 1/2, JNK 1/2 and p38 through activating the Ras GTPase signaling pathway (Hernandez-Munoz *et al.*, 2000). These data suggest that hRgr would activate these kinases *in vivo*, since they are downstream participants of the Ras GTPase signaling pathway. Additionally, we found that the introduction of a point mutation within the catalytic domain of hRgr can severely impair its guanine nucleotide dissociation activity *in vitro*. This result is consistent with other

studies in which a similar point mutation was introduced into the CDC25 domain of Sos (Hall *et al.*, 2001).

Although both human and rabbit Rgr constructs were able to activate Ras and Ral GTPases *in vitro*, the amount of GDP dissociation was considerably less than GDP dissociation initiated by the better characterized Ras and Ral GEFs (i.e. Sos, RasGRP, RalGDS, RalGEF 2, etc). This weaker dissociation activity could suggest that Rgr may require a cooperating molecule in order to stimulate more robust dissociation activity. For example, Sos facilities the GDP dissociation from Ras and can couple this activity to Rac GTPases through its Dbl and pleckstrin homology (PH) domains (Sondermann *et al.*, 2004). This phenomenon suggests the possibility that hRgr may interact with other proteins resulting in the activation of these GTPases. Nevertheless as evident by the *in vivo* and *in vitro* activation of Ras and Ral GTPases, hRgr displays its oncogenic mechanism through its dissociation activity. The dual activation capabilities reported here for hRgr provide the molecular bases for its transforming activity, which allows a single molecule to activate both Ras and Ral.

hRgr can modulate biological and biochemical activities important for cellular transformation

As confirmed through biological assays, Dtr-hRgr can modify several biological functions including cellular proliferation, migration and invasion, and anchorage independence. Interestingly of all of the biological assays performed in this study, Dtr-hRgr had a stronger effect on the migratory and invasive properties of murine fibroblasts. Several studies have implicated Ral GTPases in the mobilization of Sec 5 and Exo 84 of the exocyst, driving cellular migration and facilitating cellular transformation (Oxford *et al.*, 2005; Rosse *et al.*, 2006). In addition, a mutation within the CDC25 domain of hRgr reduced the malignant phenotype in these fibroblasts, confirming that hRgr initiates transformation by guanine nucleotide dissociation.

These results are consistent with studies that have demonstrated that both Ral A and Ral B GTPases can cooperate with other oncogenes resulting in cellular transformation (Rosse *et al.*, 2006; Tchevkina *et al.*, 2005). Although hRgr can activate both Ras and Ral A GTPases *in vitro*, perhaps it may have a stronger dissociation activity on Ral *in vivo*. Although Ral A and Ral B have 85% similarity to each other, the interactions of these isoforms with specific RalGEFs may vary. For instance, specific RalGEFs can modify downstream activities of Ral A and Ral B in cytokinesis (Ceriani *et al.*, 2007). Ral A can be activated by RalGDS and RalGPS 2 through Ras-dependent and Ras-independent mechanisms (Cascone *et al.*, *2008*). Similar GEF specificity has been observed for the activation of Ral B by RGL and RalGPS 1 in a Ras-dependent and Ras-independent manner, respectively (Cascone *et al.*, 2008). Perhaps, in line with these studies, hRgr may have a specific role *in vivo* with a particular GTPase.

Cooperation of different GEFs could enhance transformation

Blocking the translation of Dtr-hRgr using small interfering RNA reduced, but did not completely abolish, cell cycle progression in T-ALL derived cell lines. This observation suggests that hRgr is not the only oncogene driving transformation in these cell lines.

Recently, interesting studies characterizing Ras GTPase activation in lymphocytes demonstrated a cooperation among Ras GEFs, Sos and RasGRP 1. Structural analysis of Sos confirms that Sos has two GTP-bound Ras binding pockets (Margarit *et al.*, 2003). Loading one of these pockets with an active form of Ras enhances GEF activity of Sos, suggesting a positive feedback mechanism for the activation of Ras by Sos (Boykevisch *et al.*, 2006). In lymphocytes, Ras can be activated through Sos- or RasGRP-mediated guanine nucleotide dissociation mechanisms. Small interfering RNA studies confirmed that RasGRP 1, a RasGEF that is specifically expressed in T lymphocytes, and Sos could act in concert to activate Ras GTPases in T lymphocytes (Roose *et al.*, 2007). Since the catalyst of Sos-induced Ras activation is its own product (i.e. GTP-bound Ras), it is necessary for T lymphocytes to have an additional molecule capable of creating a GTP-bound Ras molecule (Das *et al.*, 2009; Roose *et al.*, 2007). In this study, we found that abnormal hRgr is overexpressed in malignant T-lymphocytes and has the ability to dissociate GDP, which suggests that hRgr could provide activated Ras molecules (albeit in a lower amount compared to RasGRP 1) to initiate Sos' positive feedback loop.

In this report, we have shown that the abnormal hRgr is an oncogene that augments cellular transformation through the activation of Ras and Ral GTPases. Since the abnormal hRgr transcript was observed in several subsets of T-cell neoplasms, this study suggests that hRgr should be investigated as a diagnostic tool for determining subsets of T-cell malignancies, as a potential prognostic marker and/or as a target for the development of therapeutic compounds against these malignancies.

Materials and Methods

Further details and additional methods are provided as Supplementary Information.

Cell Culture and Patient materials

Detailed descriptions of cell culture and patient samples are provided in the Supplementary Experimental Procedures.

Plasmid construction and generation of catalytic dead (cd) hRgr mutants

Description is provided in Supplementary Experimental Procedures.

Transfections

NIH3T3 cells were transfected by the calcium phosphate precipitation method, as previously described (Wigler *et al.*, 1978). Descriptions are provided in Supplementary Experimental Procedures.

RNA preparation and quantitative real-time (QRT-PCR)

QRT-PCR was performed using iCYCLER iQ5 with a SYBR green master mix (Bio-Rad, Hercules, CA, USA). Gene-specific oligonucleotides were used for abnormal hRgr, normal hRgr and human Glyceraldehyde 3-phosphate dehydrogenase (GAPDH); see details in Supplementary Experimental Procedures.

Cell cycle analysis

At least 10 000 events of ethanol-fixed, RNase A-treated and propidium iodide-stained cells were acquired using a BD LSR II flow cytometer (BD Biosciences, San Jose, CA, USA). Resolution of specific cell cycle stages was determined by ModFit LT software (Verity Software House, Topsham, ME, USA). See Supplementary Experimental Procedures for more details.

Biological assays to determine cellular invasion, cellular proliferation and cellular anchorage independence

Cellular invasion, cellular proliferation and cellular anchorage independence were measured by the modified Boyden chamber assay, MTT assay for cellular proliferation and soft agar assay for anchorage independence, respectively. See Supplementary Experimental Procedures for more details.

Western blot analysis

Western blots were performed as previously described (Garcia-Espana *et al.*, 2005). Membranes were blocked for 1 h with Odyssey Blocking Buffer (LI-COR Biosciences, Lincoln, NE, USA), filters were incubated with the appropriate antibodies for Cullin 1 (Invitrogen, Carlsbad, CA, USA) or hybridoma clone (12CA5) HA epitope (gift from Dr. D. Levy, NYU Langone Medical Center, NY, NY, USA). Then, the filters were incubated with IRDye 800 anti-goat IgG (LI-COR Biosciences) and scanned on the Odyssey imager (LI-COR Biosciences). When necessary, the filters were subjected to densitometry by analysis with Odyssey Application software, version 3.0 (LI-COR).

Ras and Ral A pull-down assays

Ras and Ral A activation detection assays were performed using Active Ras and Ral Pull-Down and Detection Kits (Thermo Fisher Scientific, Rockford, IL, USA). See Supplementary Experimental Procedures for more details.

Fusion protein expression and purification

For the detailed description for the purification of 6X His fusion proteins of Tr-hRgr, Dtr-hRgr, (cd) Dtr-hRgr, R-Rgr, Sos, RasGRP 1, RalGEF 2, Rlf, RalGDS, N-Ras, Ral A or Ral B expressed in ArticExpress *E. coli* (Stratagene, Santa Clara, CA, USA), see the Supplementary Experimental Procedures

Guanine nucleotide dissociation assays

Guanine nucleotide dissociation assays were conducted on N-Ras, Ral A or Ral B loaded with mant-GDP. See Supplementary Experimental Procedures for more details.

Statistical analysis

Analysis of variation (ANOVA) was used to analyze differences between a control group and experimental groups, with *representing $P<0.05$; **$P<0.01$; and ***$P<0.001$. For the MTT assay for cellular proliferation ANOVA was used to treat each well as a testing unit and the Kruskal-Wallis test was used to treat each plate as the testing unit.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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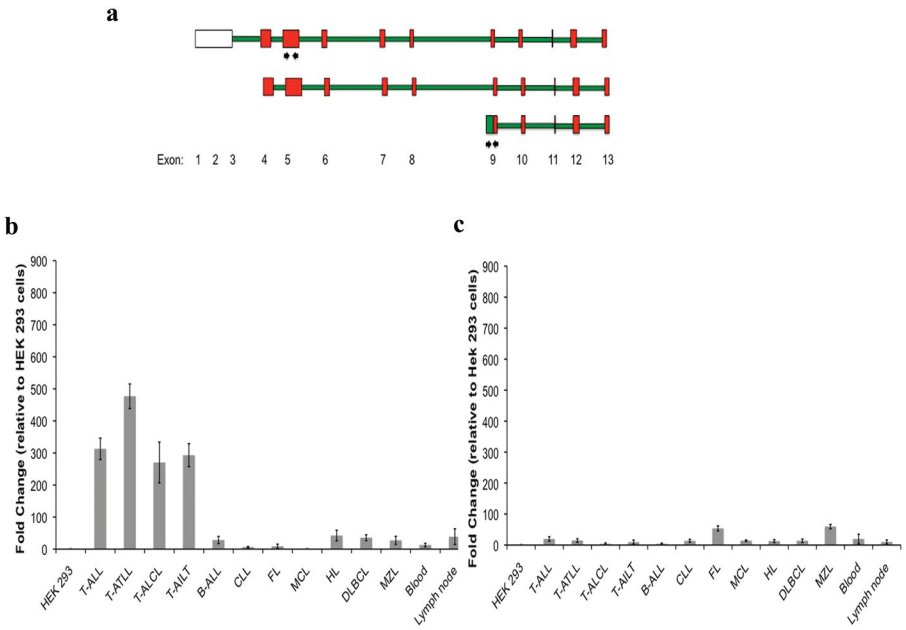

Figure 1.

Expression of the abnormal and normal hRgr transcripts. The abnormal hRgr transcript is overexpressed only in T-cell neoplasms, while the normal hRgr transcript has low levels of expression in T- and B-cell neoplasms and in normal tissues. (**a**) The genomic organization of the normal (Fl-hRgr; top), full-length truncated (Tr-hRgr; middle) and abnormal (Dtr-hRgr; bottom) hRgr transcripts. The untranslated region (UTR) is represented by the white box; exons by red vertical boxes; introns by green horizontal boxes. The green vertical box designates the coding sequence that is present in the abnormal transcript, which is intronic in the normal transcript. The black arrows represent primer sets designed against specific regions of hRgr. (**b**, **c**) QRT-PCR analysis of selected T-and B-cell neoplasms and normal tissues to determine the expression of the abnormal (**b**) and normal (**c**) hRgr transcripts, respectively. Total RNA was harvested from cyropreserved patient samples, reverse transcribed and amplified using specific hRgr primers for the abnormal and normal transcripts. Data in this QRT-PCR analysis were normalized by using primers specific for human GAPDH. Results represent at least 4 independent experiments.

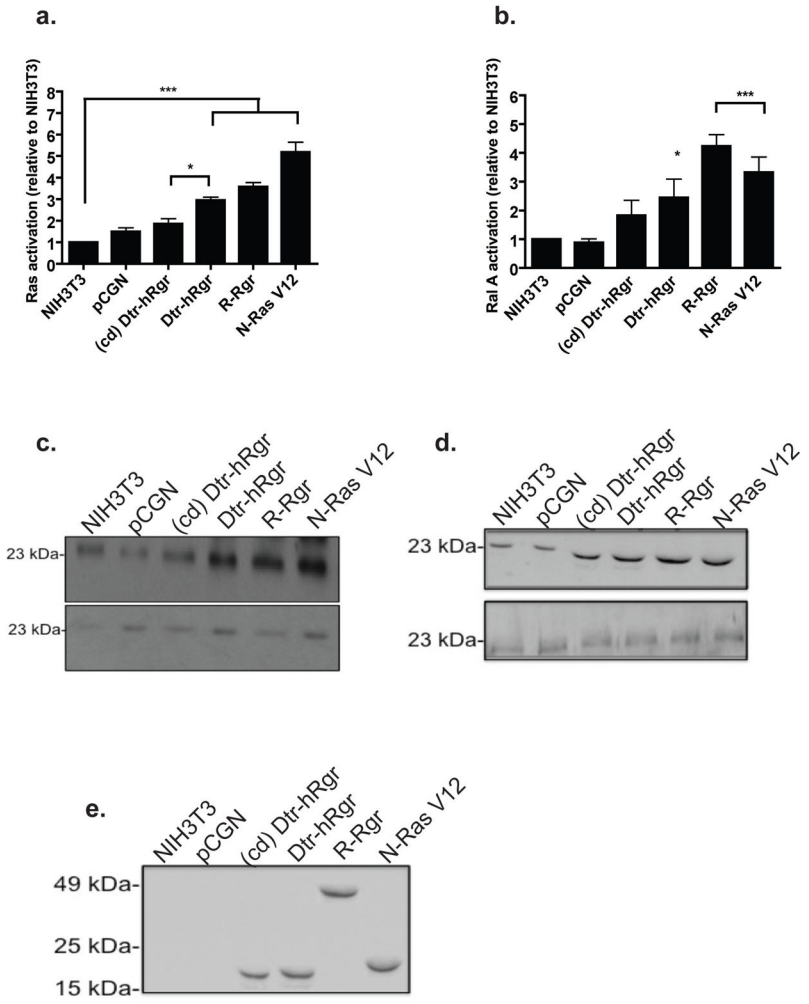

Figure 2.

hRgr increases the levels of GTP-bound forms of Ras and Ral A *in vivo*. (**a**, **b**) Cumulative expression of activated Ras (**a**) and Ral A (**b**) GTPases relative to nontransfected NIH3T3 cells in three independent experiments; **P*<0.05 and ****P*<0.001. (**c**, **d**) A representative experiment for Ras (**c**) and Ral A (**d**) activation, in which protein lysates (1 mg) extracted from nontransfected NIH3T3 cells and stably expressing Rgr and N-RasV12 cells were used to recover the GTP-bound forms of Ras (with GST-Raf 1-RBD fusion protein) and Ral A (with GST-Ral BP1-Ral binding domain fusion protein); upper panels. Total Ras and Ral A present in 20 μg of the lysates used for the activation assays; lower panels. (**e**) SDS-PAGE analysis to determine the expression of (cd) Dtr-hRgr, Dtr-hRgr, R-Rgr and N-Ras V12 with anti-HA antibody.

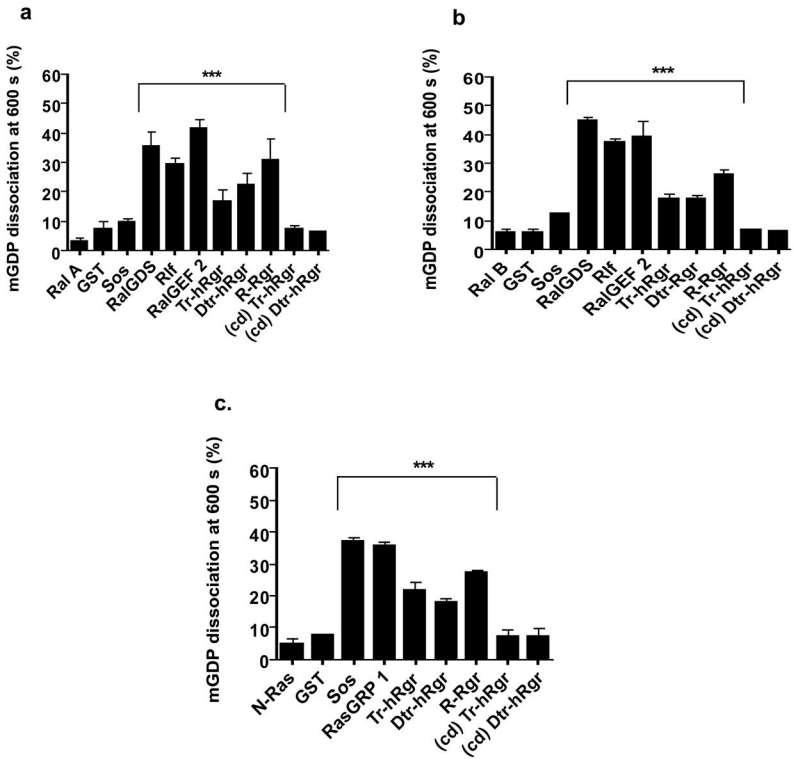

Figure 3.

hRgr has GDP dissociation activity on Ral A, Ral B and N-Ras. *In vitro* fluorescence-based guanine nucleotide exchange assays were performed to determine the amount of GDP dissociation from Ral A, Ral B and N-Ras induced by Rgr and more characterized GEFs for Ras and Ral GTPase. Purified 6XHIS recombinant fusion GTPase proteins, preloaded with mant-GDP were incubated with GTP and purified (GST or a GEF) and GDP dissociation was measured as the decrease in fluorescence for 600 s. Tr-hRgr, Dtr-hRgr and R-Rgr had the ability to dissociate mant-GDP from Ral A (**a**), Ral B (**b**) and N-Ras (**c**). However, this dissociation was typically less than the more characterized GEFs for Ral A and B (RalGDS, Rlf and RalGEF 2) and N-Ras (Sos and RasGRP 1). Additionally, the addition of a point mutation within the catalytic region of hRgr, (cd) Tr-hRgr and (cd) Dtr-hRgr, severely perturbed this dissociation activity. Data shown are representative of at least three experiments, except for (cd) Tr-hRgr and (cd) Dtr-hRgr (n=2); ***$P<0.001$.

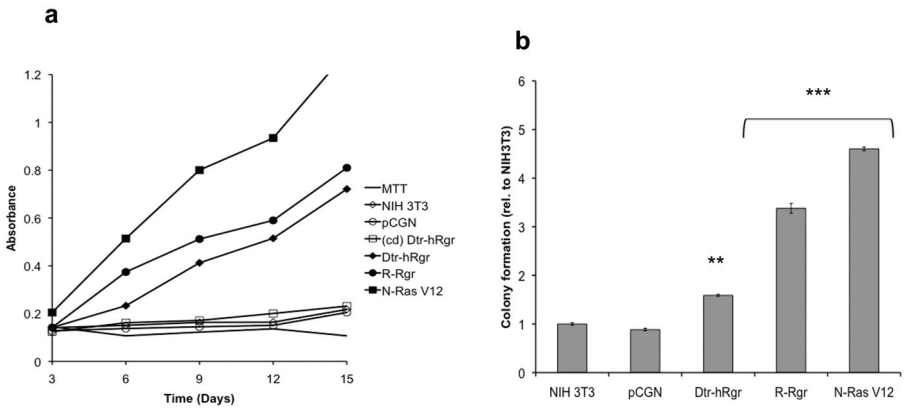

Figure 4.

hRgr expression increases proliferative growth and anchorage independence in NIH3T3 cells. (**a**) Cells stably expressing Dtr-hRgr and R-Rgr increase cellular proliferation over 15 days when compared to those expressing (cd) Dtr-hRgr, an empty vector and nontransfected NIH3T3 cells. Cell proliferation was determined by the MTT assay, in which 500 cells were cultured in low serum (1%) conditions. Every three days for 15 days, cellular proliferation was measured by the absorbance due to the conversion of yellow MTT to purple formazin. Data are represented by 6 independent experiments performed in triplicate. Statistical relevance was determined the Kruskal-Wallis test (using each plate as a testing unit) and ANOVA (using each well as a testing unit). (**b**) Fibroblasts that express Dtr-hRgr and R-Rgr reduce anchorage dependence, relative to nontransfected NIH3T3 cells. The soft agar assay for anchorage independence was conducted by plating 1 000 cells (expressing empty pCGN, Dtr-hRgr, R-Rgr and N-Ras V12) resuspended in 0.25% onto 0.5% solidified agar. The formation of colonies was scored four weeks post plating. These data are representative of three independent experiments, performed in triplicate; $^{**}P<0.01$ and $^{***}P<0.001$.

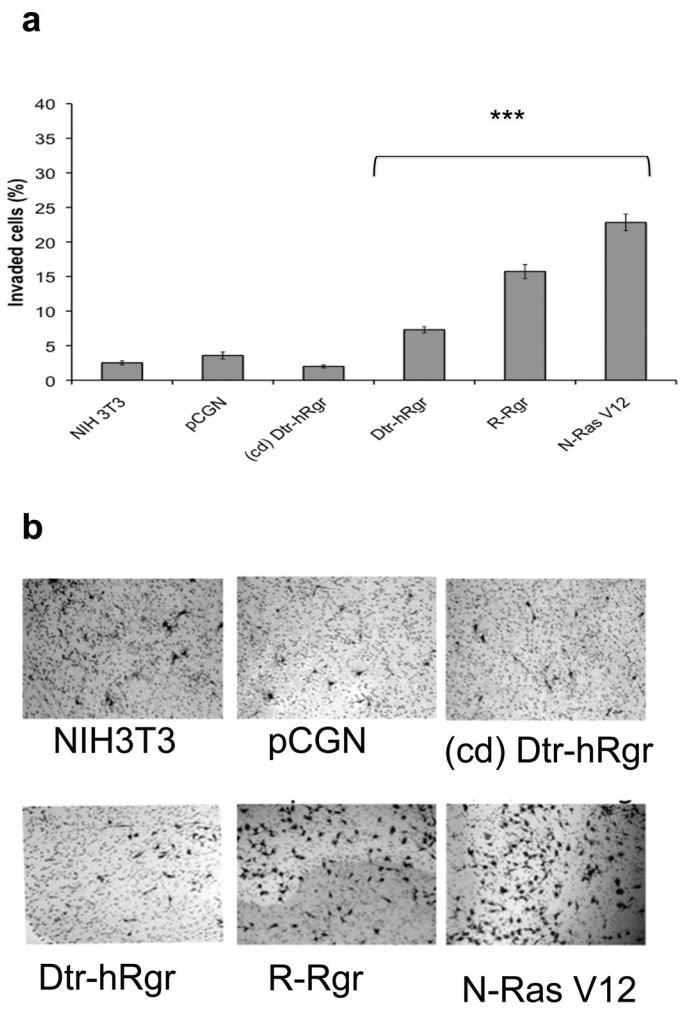

Figure 5.

NIH3T3 cells that express Dtr-hRgr have increased invasion properties and this phenotype can be perturbed in cells expressing (cd) Dtr-hRgr. (**a**) Invasion assay using nontransfected NIH3T3 cells or those stably expressing an empty vector, (cd) Dtr-hRgr, Dtr-hRgr, R-Rgr and N-Ras V12 were carried out in 0.125 μg/μl Matrigel-coated Boyden chamber stimulated with conditioned medium, containing 10% bovine serum. Data are representative of at least 4 independent experiments; ***P<0.001. (**b**) Pictographs of an individual representative experiment, in which data were acquired from three randomly selected fields

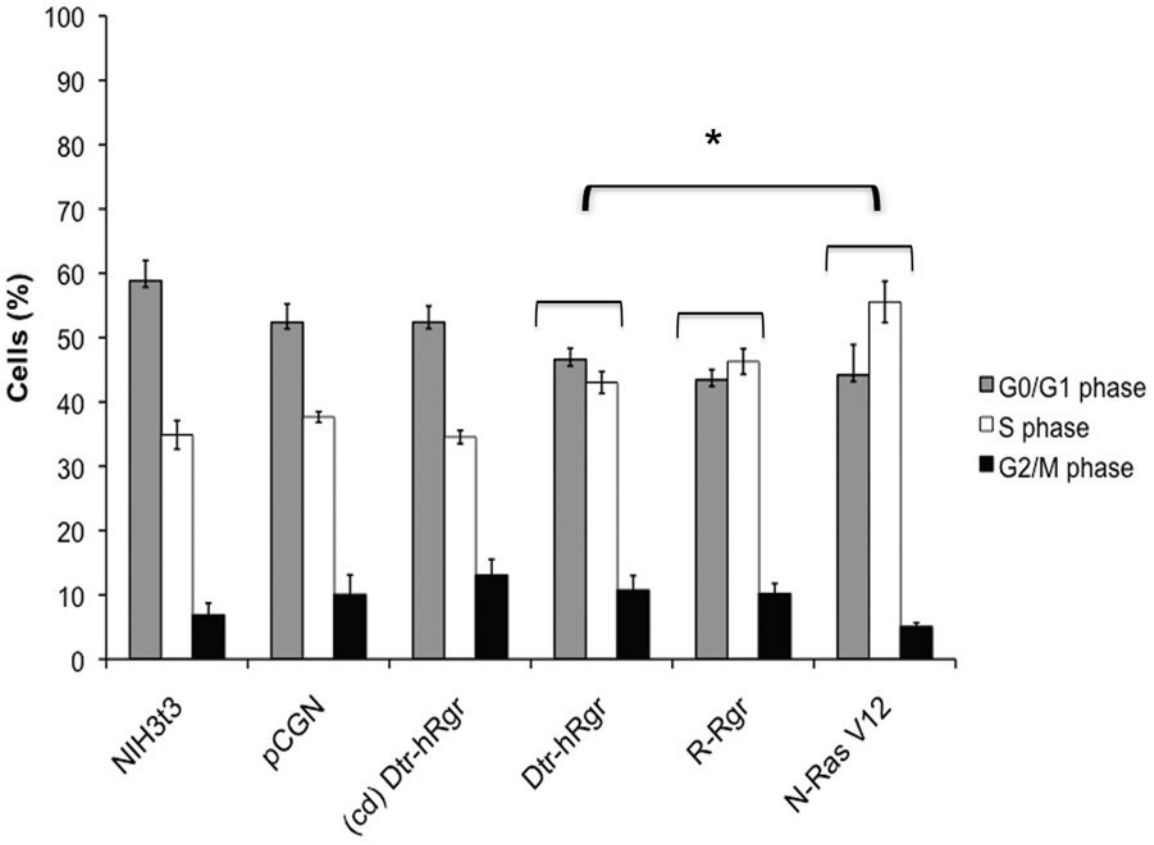

Figure 6.

hRgr expression in NIH3T3 increases the proportion of cells in S phase and reduce those in G_0/G_1 phase. Stably (cd) Dtr-hRgr, R-Rgr and N-Ras V12 expressing NIH3T3 cells (10 000) were subjected to propidium iodide staining to determine hRgr's role in cell cycling events. Data are representative of at least three independent experiments; *P<0.05.

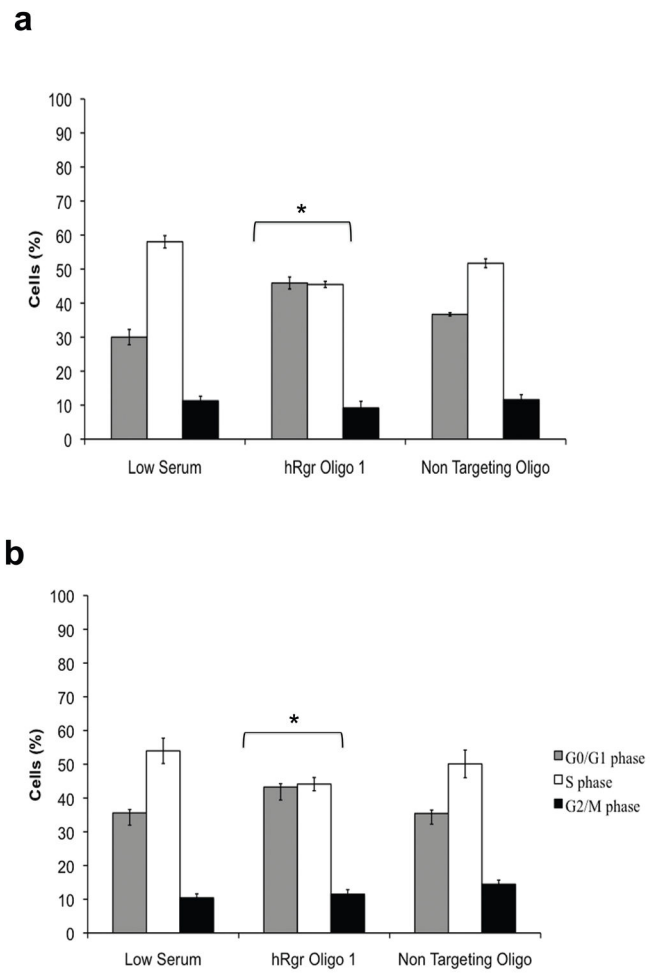

Figure 7.

Inhibiting the abnormal hRgr transcript reduces the proliferation of T-ALL derived cell lines. (**a**, **b**) Abnormal hRgr transcript silencing with hRgr specific siRNA (hRgr oligo 1) or negative control siRNA (nontargeting oligo) oligonucleotides in Jurkat (**a**) and T-ALL 1 (**b**) cell lines cultured in low serum conditions, respectively. The effects of hRgr silencing were assessed by propidium iodide (PI) staining; the reduction of hRgr expression caused an increase in cells in G_0/G_1 phases and a reduction in those in S phase. Data are representative of four independent experiments; **P*<0.05.

Table 1

The presence of the abnormal hRgr transcript in patient samples

T-cell neoplasms *	Expression of abnormal hRgr	Number of samples
T-ALL	11	11
PTCL	2	2
MF	1	1
T-ALCL	2	2
T-ATLL	1	1
T-AILT	2	2
TOTAL	19	19
B-cell neoplasms **	**Expression of abnormal hRgr**	**Number of samples**
B-ALL	0	2
CLL	0	2
MCL	0	3
FL	0	3
HL	0	2
DLBCL	0	2
MZL	0	2
TOTAL	0	16
Normal Tissue	**Expression of abnormal hRgr**	**Number of samples**
Thymus	0	1
Spleen	0	2
Tonsil	0	1
Liver	0	1
Adenoid	0	1
Blood	0	1
Lymph node	0	2
TOTAL	0	9

* Abbreviations used for T-cell neoplasms: T-cell acute lymphoblastic leukemia (T-ALL), Peripheral T-cell lymphoma (PTCL), Mycosis fungoides (MF), Anaplastic large T-cell lymphoma (ALCL), Adult T-cell leukemia/lymphoma (T-ATLL) and Angioimmunoblastic T-cell lymphoma (T-AILT).

** Abbreviations used for B-cell neoplasms: B-cell acute lymphoblastic leukemia (B-ALL), Chronic lymphocytic leukemia (CLL), Mantle cell lymphoma (MCL), Follicular lymphoma (FL), Hodgkin's lymphoma (HL), Diffuse large B-cell lymphoma (DLBCL) and Marginal zone lymphoma (MZL).