An RNA dependent RNA polymerase formed by hTERT and the RNase MRP RNA

Yoshiko Maida¹, Mami Yasukawa¹, Miho Furuuchi¹, Timo Lassmann², Richard Possemato³, Naoko Okamoto¹, Vivi Kasim¹, Yoshihide Hayashizaki², William C. Hahn³,⁴,†, and Kenkichi Masutomi¹,⁵,†

¹Cancer Stem Cell Project, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045 JAPAN
²RIKEN Omics Science Center, RIKEN Yokohama Institute, 1-7-22 Suehiro-cho, Tsurumi-ku, Yokohama 230-0045 JAPAN
³Department of Medical Oncology, Dana-Farber Cancer Institute and Departments of Medicine, Brigham and Women’s Hospital and Harvard Medical School, 44 Binney Street, Boston, MA 02115 USA
⁴Broad Institute of Harvard and MIT, 7 Cambridge Center, Cambridge, MA 02142 USA
⁵PREST, Japan Science and Technology Agency, 4-1-8 Honcho Kawaguchi, Saitama 332-0012 JAPAN

Abstract
Constitutive expression of telomerase in human cells prevents the onset of senescence and crisis by maintaining telomere homeostasis. However, accumulating evidence suggests that the human telomerase catalytic subunit (hTERT) contributes to cell physiology independent of its ability to elongate telomeres. Here we show that hTERT interacts with the RNA component of mitochondrial RNA processing endoribonuclease (RMRP), a gene that is mutated in the inherited pleiotropic syndrome Cartilage-Hair Hypoplasia. hTERT and RMRP form a distinct ribonucleoprotein complex that exhibits RNA dependent RNA polymerase (RdRP) activity and produces double-stranded RNAs that can be processed into small interfering RNA in a Dicer-dependent manner. These observations identify a mammalian RdRP composed of hTERT in complex with RMRP.
Keywords

Telomerase reverse transcriptase; RNA dependent RNA polymerase; RMRP; siRNA

Telomerase is a ribonucleoprotein complex that elongates telomeres. Although several proteins interact with telomerase 1–4, the minimal components of active telomerase include the catalytic telomerase reverse transcriptase (TERT) and a non-coding RNA (TERC) that encodes the template to synthesize telomeric DNA 5. Telomere homeostasis mediated by telomerase maintains genomic stability and regulates cell lifespan 6. Mutations in hTERT, hTERC or dyskerin, a telomerase-associated nucleolar protein involved in rRNA maturation 7, are found in dyskeratosis congenita, a syndrome characterized by ectodermal dysplasia and bone marrow failure, and mutations of hTERT have been reported in aplastic anemia and idiopathic pulmonary fibrosis 8. Moreover, alterations in the regulation of telomeres and telomerase contribute to malignant transformation by affecting genomic integrity and cell immortalization 6.

However, accumulating evidence suggests that hTERT exhibits activities beyond telomere maintenance 9–13 and forms several intracellular complexes 2–4. In particular, overexpression of TERT induces increased tumor susceptibility 9,10 and disrupts stem cell function independent of telomere maintenance 12 while suppression of hTERT expression alters global chromatin structure 11. Indeed, some of these telomere-independent functions of TERT do not require the expression of hTERC 12.

Identification of a second RNA that interacts with hTERT

To identify hTERT partners, we stably overexpressed a tandem affinity peptide (TAP)-tagged hTERT protein in HeLa-S cells, isolated hTERT immune complexes, and identified a heterogeneous mixture of 38 RNA sequences associated with hTERT (Supplementary Fig. 2; Supplementary Table 1). We found that 5% of the sequences corresponded to hTERC and the RNA component of mitochondrial RNA processing endoribonuclease (RMRP). RMRP is a 267 nt non-coding RNA that is a small nucleolar (sno) RNA, like hTERC, and is also found in mitochondria 8,14. Mutations of RMRP are found in the pleiotropic inherited syndrome, Cartilage-Hair Hypoplasia (CHH) 15.

From a single immune complex, we confirmed that either overexpressed or endogenous hTERT interacts with RMRP and hTERC by isolating TAP-hTERT (Fig. 1a) or endogenous hTERT (Fig. 1b) complexes in both HeLa and 293T cells under conditions where we failed to recover the ribozyme RNase P. We also found that the abundance of hTERT-RMRP and hTERT-hTERC complexes was similar even though hTERC was expressed at five-fold higher levels than RMRP in these cells (Fig. 1c; Supplementary Fig. 3).

To characterize the interaction of hTERT and RMRP, we used TERT truncation mutants and found that the aminoterminal end of hTERT (1–531) was necessary for interactions with RMRP (Supplementary Fig. 4). This region overlaps with two regions required for the binding of hTERC 8,16. These observations demonstrate that hTERT and RMRP form a novel ribonucleoprotein complex distinct from the hTERT-hTERC enzyme.

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The hTERT-RMRP complex exhibits RdRP activity

To test whether RMRP substitutes for hTERC to reconstitute telomerase activity, we combined recombinant hTERT with hTERC or RMRP RNAs transcribed in vitro. Although we detected telomerase activity with hTERT and hTERC (Supplementary Fig. 5), we failed to detect telomerase activity when hTERT and RMRP were co-incubated.

TERT has also been shown to act as a terminal transferase17, and hTERT shares sequence similarity to both viral reverse transcriptases and RNA dependent RNA polymerases (RdRPs)18. RdRPs participate in the endogenous RNAi pathway and in the regulation of posttranscriptional gene silencing19–23. To examine whether the hTERT-RMRP complex exhibits RdRP and/or terminal transferase (TT) activity, we established an RNA synthesis activity assay with recombinant hTERT protein (Supplementary Fig. 6) and RNA molecules transcribed in vitro. We predicted three modes that the hTERT-RMRP complex might use to elongate RNA: [i] as an RdRP that uses a de novo synthesized RNA primer to elongate a complementary strand (Fig. 2a left panel); [ii] as an RdRP that uses a 3’ fold-back (back-priming) configuration of template RNA as a primer (middle panel); or [iii] as a TT (right panel). Viral RdRPs24,25 have been shown to use the first two modes to prime RdRP activity, and cellular RdRP in fission yeast26 and fungi23 use similar priming mechanisms to produce double-stranded (ds) RNAs that serve as precursors for RNAi.

We found that recombinant hTERT and RMRP produced 2 different products depending on the salt concentration (Fig. 2b; Supplementary Fig. 7). Specifically, we found ~267 nt (corresponding to sense RMRP) and ~534 nt sized products (hereafter referred to as sense + antisense RMRP products) under high salt conditions and RMRP-sized products under low salt conditions. To discriminate among these modes, we treated the products of the RdRP assay with RNase T1 (Fig. 2c), under conditions that favor the digestion of single-stranded RNA. RNase T1 treatment eliminated the ~267 nt RMRP-sized RNA products produced under low salt concentrations (data not shown), indicating that 32P-UTP was incorporated by TT activity.

In contrast, under high salt conditions, we found two RNAs (~267 nt and ~534 nt) that collapsed into a single ~267 nt band after treatment with RNase T1 (Fig. 2c). To eliminate the possibility that the sense + antisense product represented partially denatured RNAs, we treated the products of the RdRP assay with bacterial RNase III to digest dsRNA and found that only the input ~267 nt RNA remained (Fig. 2d). Furthermore, when we left out adenine or guanine ribonucleotides, we failed to detect the sense + antisense product (Fig. 2e). These observations confirm that the ~534 nt sense + antisense products are formed by RdRP activity and represent a ds hairpin structure created by an RNA molecule composed of sense and antisense strands of RMRP.

To confirm that the interaction of hTERT and RMRP was required for RdRP activity, we performed an RdRP activity assay using combinations of recombinant mutant hTERT proteins and RMRP. We failed to detect RdRP reaction products when hTERT and hTERC were co-incubated (Supplementary Fig. 8). Moreover, when we used the hTERT-HT1 mutant that does not bind RMRP (Supplementary Fig. 4), we failed to observe labeled RNA.
products (Supplementary Fig. 8) under conditions where we detected two different RNA products in reactions containing wild-type hTERT and RMRP. We previously described a catalytically inactive hTERT mutant (DN hTERT) that fails to elongate telomeres11,27. We confirmed that the recombinant DN hTERT mutant retained the ability to bind RMRP (Fig. 2f) but that the DN hTERT-RMRP complex lacked detectable RdRP activity (Fig. 2f). Thus hTERT serves as the catalytic subunit for both the telomerase reverse transcriptase and RdRP activities.

The hTERT-RMRP RdRP produces ds RNA

These observations suggest that the hTERT-RMRP RdRP synthesizes ds RNA in a template-dependent manner. To confirm the synthesis of the RMRP complementary strand, we used the sense strand of RMRP as a probe in Northern blotting. We detected the antisense strand of RMRP in reactions containing recombinant WT hTERT protein and RMRP but not in reactions containing DN hTERT and RMRP (Fig. 2g). Furthermore, we detected the sense + antisense product in the RdRP assay using the antisense strand of RMRP as a probe (Supplementary Fig. 9). These observations indicate that the hTERT-RMRP RdRP produces ds RNAs in template-dependent manner in vitro.

To determine whether the hTERT-RMRP RdRP uses a back priming mechanism, we examined the priming process using hTERT and RMRP as a model system and found that elongation products appeared in time-dependent manner (Fig. 2h; Supplementary Fig. 10). To assess whether the RMRP RNA forms a 3’ fold-back configuration, we generated 3’ RMRP truncation mutants and failed to find any reaction products (Fig. 2i). Thus, unlike what has been described for other cellular RdRPs, the hTERT-RMRP RdRP exhibits a restricted preference for RNA molecules that can be used as a template. Indeed, when we incubated purified recombinant hTERT together with total cellular RNA and 32P-UTP, we identified a limited number of labeled RNAs (Fig. 2j). Although the secondary structure adopted by RMRP to create the 3’ fold-back is not known, these observations suggest that RMRP can itself serve as a primer for the polymerization process using a 3’ fold-back structure.

To ascertain whether this RdRP activity also occurs in vivo, we used the sense strand of RMRP as a probe and found ~534 nt RNAs that contain antisense RMRP in RNA derived from 293T, HeLa and MCF7 cells (Fig. 3a; Supplementary Fig. 11, Supplementary Fig. 12). Moreover, we detected both sense + antisense and sense products using RMRP antisense strand probe (Fig. 3b). These observations confirmed that the ~534 nt products contain both sense and antisense RMRP sequences. To determine whether hTERT was necessary for the appearance of antisense RMRP in cells, we examined the levels of the complementary RMRP strand in cells: (i) that do not express hTERT and hTERC (VA-13)28; (ii) that transiently express low levels of hTERT (BJ)27,29,30; and (iii) that constitutively express hTERT (293T and HeLa). We also introduced a control vector or a vector that encodes hTERT in VA-13 and BJ cells. We detected the complementary RMRP strand using both a quantitative RNase protection assay with a sense strand probe that detects antisense RMRP (Fig. 3c; Supplementary Fig. 13) and Northern blotting with both sense and antisense strand-specific RMRP probes (Fig. 3d and Supplementary Fig. 11a). The levels of antisense RMRP
correlated with the expression of hTERT (Fig. 3c,d). These observations confirmed that the hTERT-RMRP RdRP produces ds RMRP in vivo.

**Effects of the hTERT-RMRP complex on RMRP expression**

To assess the consequences of overexpressing the hTERT-RMRP complex on RMRP levels, we introduced RMRP into cells that lack hTERT expression (VA-13), that transiently express hTERT in a cell-cycle dependent manner (BJ fibroblasts) and that constitutively express hTERT (VA-13 and BJ fibroblasts expressing ectopic hTERT, HeLa and MCF7 cells). Upon expressing RMRP in cells lacking hTERT (VA-13), we found that RMRP levels were increased (Fig. 4a; Supplementary Fig. 14). In contrast, in cells that express hTERT, we found that the steady state levels of RMRP were decreased when RMRP was overexpressed regardless of the promoter that was used to express RMRP (Fig. 4a; Supplementary Fig. 14). We also found that forced expression of hTERT in VA-13 or BJ cells suppressed RMRP expression (Fig. 4b; Supplementary Fig. 15). Consistent with these findings, suppression of hTERT in HeLa cells led to increased RMRP expression (Fig. 4c).

Since the 3’ end of RMRP was essential for hTERT-RMRP activity (Fig. 2i), we examined the effects of expressing RMRP truncation mutants lacking 3’ ends and found that only truncation mutants lacking intact 3’ ends were readily overexpressed (Fig. 4d). These observations demonstrate that RMRP expression levels are dependent on the hTERT-RMRP RdRP and suggest that RMRP levels are controlled by an RdRP-dependent, negative feedback control mechanism.

**Identification of siRNAs derived from RMRP**

In other organisms, RdRPs synthesize ds RNAs that are processed into active siRNAs. Since manipulating hTERT and RMRP levels affected RMRP expression, we hypothesized that the hTERT-RMRP complex produces RMRP-specific siRNA to regulate RMRP levels. To test this possibility, we used sense and antisense probes corresponding to RMRP (nucleotides 21–40) in Northern blotting and found ds 22 nt RNAs (Fig. 4e; Supplementary Fig. 11b). Since siRNAs contain 5’ monophosphates and 3’ hydroxyl groups, we characterized the chemical nature of the small RNA ends. We found that calf intestinal phosphatase (CIP) slowed the migration of these short RNAs and subsequent incubation with polynucleotide kinase (PNK) and ATP restored the mobility of the short RNAs, indicating that either the 5’ or 3’ end of this small RNA is monophosphorylated (Fig. 4f and data not shown). Moreover, incubation with PNK in the absence of ATP did not alter the migration (Fig. 4f), and oxidation and β-elimination treatment increased the migration of these small RNAs (Fig. 4g), indicating that the 3’ ends bear vicinal 2’, 3’ dihydroxyls. Together, these observations confirm that these small RNAs contain 5’ monophosphate and 3’ hydroxyl groups and therefore share the size and chemical composition of known siRNAs.

To demonstrate that ds RNAs produced by the hTERT-RMRP RdRP are processed into siRNA, we suppressed the expression of Dicer with two distinct Dicer-specific shRNAs. Suppression of Dicer to levels that partially inhibited the processing of miR-16 (Fig. 5a; Supplementary Fig. 16) led to diminished levels of the siRNAs derived from RMRP (Fig. 7).
When we suppressed Dicer expression in HeLa, 293T or MCF7 cells, we found that endogenous RMRP levels increased up to 3.7 fold (Fig. 5b). Suppressing Dicer expression in VA-13 cells that lack hTERT did not affect the levels of single-stranded RMRP (Fig. 5b) but did increase levels of the elongated sense + antisense RMRP products in cells that constitutively express hTERT (Supplementary Fig. 17). Moreover, we found that only the sense strands of these endogenous RMRP-specific siRNAs were associated with human Ago2 (Fig. 5c). These observations indicate that the endogenous RMRP-specific siRNAs are processed by the RNA-induced silencing complex, similar to other small RNAs that are processed into siRNA.

To confirm that these small RNAs act as siRNA, we identified small RNAs from total RNA that hybridized to probes spanning RMRP, synthesized siRNA corresponding to the identified sequences and tested the consequences of introducing this siRNA in HeLa, 293T and MCF7 cells. We found that the synthesized siRNA suppressed endogenous RMRP levels (Supplementary Fig. 18). These observations provide evidence that similar to other cellular RdRPs, the TERT-RMRP RdRP synthesizes ds RNAs that serve as a precursor for siRNA.

Discussion

Here we demonstrate that hTERT and RMRP form distinct ribonucleoprotein complex that exhibits the ability to produce ds RNAs (Supplementary Fig. 1). Like RdRPs found in other organisms, the hTERT-RMRP complex produces ds RNAs that serve as substrates for the generation of siRNA. However, unlike other cellular RdRPs, the hTERT-RMRP RdRP exhibits a strong preference for RNA templates that can form 3’ fold-back structures. Since other cellular RdRPs have been identified using assays that require primer independent RdRP activity, the substrate specificity of the hTERT-RMRP RdRP may, in part, account for the difficulty in identifying mammalian enzymes that exhibit RdRP activity.

Although the cellular RdRPs described to date do not exhibit a primer requirement, several viral RdRPs use both primer-dependent and primer-independent mechanisms, and fungal and yeast RdRPs are also able to employ a back-priming mechanism. Since TERT is a closed right-handed polymerase evolutionarily related to both reverse transcriptases and viral RdRPs, these observations are consistent with prior observations that indicate that right-handed RdRPs exhibit primer-dependent RdRP polymerase activity.

Using RMRP as a template, the hTERT-RMRP RdRP produces ds RNAs that are processed by Dicer into 22 nt ds RNAs that contain 5’ monophosphate and 3’ hydroxyl groups and are loaded into Ago2, confirming that these short RNAs represent endogenous siRNAs. Recent work has shown that in oocytes and embryonic stem cells, endogenous siRNA can also be formed by the transcription of complementary sense and antisense strands. Thus, in mammals at least two mechanisms lead to the production of ds RNAs that are processed into siRNA. Further work will be necessary to determine whether there are tissue-dependent differences in the use of these two mechanisms and if other mammalian RdRPs exist.
We found that the hTERT-RMRP RdRP regulates RMRP levels through a negative feedback control mechanism. The identities and functions of the RNAs other than RMRP that serve as templates for the hTERT-RMRP RdRP remain to be identified (Fig. 2j). However, since endogenously encoded siRNAs suppress L1 retrotransposition in human cells42, these observations suggest that the hTERT-RMRP complex may regulate the expression of other genes by generating siRNAs.

Since mutations in RMRP are found in CHH15, these findings suggest that perturbation of the hTERT-RMRP complex is involved in the pathogenesis of this disorder. The involvement of hTERT in two syndromes characterized by stem cell failure (CHH and dyskeratosis congenita)7,8,43 suggests that ribonucleoprotein complexes containing hTERT play a critical role in stem cell biology. Indeed, overexpression of mTERT in mice lacking mTERC leads to defects in normal hair follicle stem cell function12 at least in part by altering gene expression programs related to stem cell function44. In mammals, TERT may regulate both telomere biology and gene expression through these two ribonucleoprotein complexes.

**METHODS SUMMARY**

RNAs that bind hTERT were identified from HeLa-S cells expressing a TAP-epitope tagged version of hTERT. RNAs that bound to hTERT after two rounds of purification were analyzed using an Experion capillary electrophoresis device (Bio-Rad Laboratories, Inc. CA, USA) to visualize RNA species. For RNA cloning and the sequencing, the same samples were separated using a 7 M Urea/15% polyacrylamide gel, and RNAs recovered from gel were cloned using the small RNA cloning Kit (TaKaRa). Purified GST-hTERT was isolated from E. coli and incubated with either hTERC or RMRP transcribed in vitro to assess the ability of such complexes to exhibit telomerase or RdRP activity. RNAi was used to suppress hTERT and to show that the hTERT-RMRP complex also produces ds RNA in cells. Northern blotting with sense and anti-sense probes specific for RMRP (nt 21–40) identified 22 nt ds, small RNAs that contained a 5’ monophosphate and a 3’ hydroxyl group, which were loaded into human Ago2. To determine the function of these RMRP-derived small RNAs, a chemically synthesized siRNA corresponding to these small RNAs (siRNA: 5’-gctactacactgaggactc-3’; Dharmacon) was transfected into HeLa, 293T and MCF7 cells.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. hTERT and RMRP interact

**a**, Detection of *RMRP* and *hTERC*. RNA species associated with TAP-hTERT complexes from a single immunoprecipitation were isolated and subjected to RT-PCR. RT (−) indicates the absence of reverse transcriptase. Right panel shows the levels of TAP-hTERT.

**b**, hTERT interacts endogenous *RMRP*. hTERT complexes from 293T and HeLa cells were isolated with an anti-hTERT antibody and associated RNAs were subjected to RT-PCR.

**c**, RNAs purified from hTERT complexes isolated from HeLa-S cells expressing TAP-hTERT or a control vector or 293T cells were subjected to Northern blotting.
Figure 2. hTERT and RMRP exhibit RdRP activity

a, Predicted RNA products produced by RdRP or TT activity. b, RNA products produced by the RdRP activity derived from recombinant hTERT and RMRP. c,d Treatment of RNA products with RNase T1 (c) or bacterial RNase III (d). e, RdRP assay performed in the presence of ribonucleotides (middle) or in the absence of adenine (left lane) or guanine (right lane) ribonucleotides. A and G are present within the first 5 nt of the predicted complementary strand of RMRP. f, DN-hTERT binds RMRP but lacks RdRP activity. TERT immune complexes were isolated from 293T cells expressing FLAG-tagged hTERT or FLAG-tagged DN hTERT. RdRP activity is shown in lower panel. g, Northern blotting to detect complementary sequence of RMRP. h, Time course of RdRP activity. i, RNA products produced by recombinant hTERT and truncation mutants of RMRP transcribed in vitro. Faint signals at the 200 nt, 120 nt and 60 nt are hTERT TT products. j, RNA products produced by the RdRP activity derived from recombinant hTERT or DN hTERT and total RNA. A limited pool of RNAs serve as templates for RdRP activity.

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Figure 3. Identification of ds RNA synthesized by the hTERT-RMRP RdRP

**a.** Northern blotting to detect complementary sequence of RMRP in cell lines. (+) indicates samples treated with RNase. **b.** Northern blotting to detect the RMRP sense strand. **c,** hTERT expression correlates with the levels of antisense RMRP detected by RNase protection assay. Vector denotes cells infected with a control vector. **d,** hTERT expression correlates with the levels of the sense (S) + antisense (AS) RMRP products detected by Northern blotting. The bottom panel shows U2 RNA levels.
Figure 4. Effects of ds RNA produced by the hTERT-RMRP RdRP

a, Semi-quantitative RT-PCR for total RMRP and retrovirally delivered RMRP (ectopic) in cell lines expressing control or RMRP expression vectors. Promoters used to express RMRP are indicated. The relative intensity of RMRP is noted below each panel. See Supplementary Fig. 14.

b, RT-PCR for total RMRP. See Supplementary Fig. 15.

c, Effects of suppressing hTERT on RMRP levels. A control shRNA (sh-GFP) or 2 different hTERT-specific shRNAs were stably introduced into HeLa cells.

d, Effects of RMRP mutants on RMRP levels. RT-PCR was used to detect RMRP levels in (c, d).

e, Detection of small RNA species in human cells. Northern blotting to detect small RNAs (22 nt in length) using antisense (left panel) and sense (right panel) probes derived from nt 21–40 of RMRP.

f, g, Analysis of the termini of the small RNA species identified in (e). Total RNA was incubated with the indicated enzyme (f), or oxidation-β-elimination reactions (g) were performed. Northern blotting was performed with antisense probe. CIP = calf intestinal phosphatase. PNK = polynucleotide kinase. ATP-indicates samples lacking ATP.
Figure 5. Production of RMRP-derived endogenous siRNAs depends on Dicer

**a.** Effect of suppressing Dicer on RMRP-derived small RNAs. Northern blotting was performed to detect [1] small RNAs using the antisense strand of RMRP as a probe in the indicated cells expressing control shRNA (sh-GFP) or Dicer-specific shRNAs (sh-Dicer #1 and sh-Dicer #2), [2] pre-miR-16 and mature miR-16 using a miR-16 specific probe, and [3] U6 RNA. See Supplementary Fig. 16. **b.** RT-PCR for total RMRP from cell lines expressing control shRNA or Dicer-specific shRNAs. The relative intensity of RMRP is noted at the bottom of the panel. **c.** RMRP-derived small RNAs are associated with Ago2. Human Ago2
immune complexes were isolated using anti-hAgo2-specific antisera or pre-immune sera, and small RNAs were detected by Northern blotting. Blotting of oligonucleotides (RMRP 20–41 and RMRP AS 41-20) is also shown.