The emerging role of RNA polymerase I transcription machinery in human malignancy: a clinical perspective

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Abstract: Ribosome biogenesis – the complex and highly coordinated cellular process leading to the production of ribosomes – is strictly dependent on the activity of RNA polymerase I (Pol I) transcriptional machinery. Pol I activity is continually increased in proliferating cells to sustain the increased demand for ribosome production and protein synthesis, which are necessary for appropriate cell growth and division. The integrity of the process of ribosome biogenesis represents an important sensor of cellular stress: when this process is altered, a tumor suppressor response is triggered, which leads to proliferative arrest. The present review focuses on the possible implications of Pol I targeting in the treatment of human malignancies.

Keywords: RNA polymerase I inhibitors, ribosome biogenesis, cell cycle control, cancer, Pol I

Introduction

The genome of eukaryotic cells is transcribed by means of three different enzymes, which carry out the synthesis of different types of RNA: RNA polymerase I (Pol I) synthesizes the precursor rRNA 45S, which, when mature, forms the major RNA part of the ribosome; RNA polymerase II (Pol II) synthesizes the precursors of mRNAs and most small nucleolar RNA, small nuclear RNA, and microRNAs;3 and RNA polymerase III (Pol III), which synthesizes transfer RNAs, rRNA 5S, and other small RNAs.4

Of the three polymerases, RNA Pol I is considered the most actively engaged (or, the “busiest”), since it accounts for at least 60% of cell transcriptional activity.5 RNA Pol I activity is further increased in proliferating cells and/or neoplastic cells to sustain increased production of the ribosomes required for the protein synthesis necessary for unbridled cell growth.

The present review focuses on both the role of RNA Pol I in human malignancies and the possible implications of its targeting in cancer treatment. To introduce the topic, a brief summary of both the structure of the Pol I machinery and the functional importance of its molecular components is provided.
factors at the rDNA promoter. In mammalian cells, the action of two of these basal factors, upstream binding factor (UBF) and selectivity factor 1 (SL1), synergistically regulates Pol I recruitment to the rDNA gene promoter and transcriptional initiation. UBF binds both to a specific region upstream of the rDNA core promoter, termed the “upstream core element,” and to a guanine-cytosine-rich element in the core promoter, thus allowing the subsequent binding of SL1, which confers promoter specificity, and the engagement of Pol I. After initiation, the transcript may be elongated by Pol I. The elongation phase of rDNA gene transcription appears to be less finely regulated than initiation; however, there is evidence indicating that rRNA synthesis might also be regulated during this step. The transcription ends at specific sequence signals – termed “terminator elements” – with the aid of specific termination factors, such as both DNA-bound transcription terminator factor I (TTF-I) and Pol I transcript release factor (PTRF). TTF-I also affects transcription initiation by Pol I and binds several sites upstream of the Pol I promoter.

Figure 1 summarizes the organization of the ribosomal gene unit and shows the major basal factors binding to the promoter region that are required for the transcription of ribosomal genes transcription. Mammalian cells contain hundreds of tandemly repeated rDNA genes. The transcription of ribosomal genes by Pol I produces 45S RNA precursor molecules, which are then specifically site-modified and processed to generate the mature 18S, 5.8S, and 28S rRNAs. Mature rRNAs are then assembled with 5S rRNAs (which are transcribed in the nucleoplasm by RNA Pol III) and with ribosomal proteins to form the large 60S and the small 40S subunits of mature ribosomes. The large 60S subunit contains one each of the 28S, 5.8S, and 5S RNAs, together with 47 ribosomal proteins, whereas the small 40S subunit contains one 18S RNA and 32 ribosomal proteins. Since the rate of ribosome production in the cell is strictly dependent on RNA Pol I activity, rRNA transcription is considered the limiting step in ribosome biogenesis.

The role of ribosomal biogenesis in malignancy
Long before a series of studies conducted from the late 1950s through the early 1960s made it possible to establish that the nucleolus is the site of ribosome production, investigation into the relationship between ribosome biogenesis and cancer had been begun unknowingly. In fact, in the late nineteenth century, “nucleolar hypertrophy” – which we now know is the morphological counterpart of an upregulated ribosome biogenesis – had started to attract the attention of tumor pathologists as a peculiar morphological aspect of cancer cells. Since then, the relationship between nucleolar function and cancer has been studied in great depth and, more recently, studies have been devoted mainly to clarifying the cause of the upregulated ribosome production in cancer cells, and to ascertaining whether an upregulated ribosome biogenesis of normal cells might be responsible for their neoplastic transformation. The major results obtained by these two research topics will be reported separately.

Why ribosome biogenesis is upregulated in cancer cells
In proliferating cells, there is a higher biosynthetic demand for increasing the production of cell constituents, which should ensure that daughter cells have the necessary complement for survival and normal functioning. This result is accomplished by the increased synthesis of proteins which, in turn, is induced by an upregulation of the rate of
ribozyme production. The generally high ribosome biogenesis in cancer cells is a consequence of the fact that ribosome production is mainly controlled by the same products of proto-oncogene and tumor suppressor genes that also control cell proliferation. In fact, in cancer cells, the changes in either tumor suppressors or proto-oncogenes, which are responsible for uncontrolled cell proliferation, also upregulate ribosome biogenesis.

Regarding the relationship between oncogenes and ribosome biogenesis, it is worth noting that cell proliferation is stimulated by mitogens and growth factors that activate the c-myc proto-oncogene product (MYC), which is the major factor responsible for variations in the ribosome biogenesis rate. In fact, all the steps of ribosome biogenesis are controlled by MYC: the product of this oncogene increases Pol I activity by recruiting SL1 to promoters, stimulates ribosomal protein synthesis by enhancing Pol II transcription, and stimulates Pol III transcription by activating transcription factor for polymerase III B (TFIIB).

Mitogens and growth factors also trigger the extracellular signal-regulated kinase (mitogen-activated protein kinases/extracellular signal-regulated kinases) pathway. This leads to the activation of both Pol I transcription, through the phosphorylation of UBF, and Pol III transcription, through the phosphorylation of TFIIIB. Also, mammalian target of rapamycin (mTOR) is stimulated by mitogens and growth factors. The activated mTOR induces Pol I transcription by activating UBF and transcription initiation factor 1A, and Pol III transcription by facilitating the association of TFIIB and transcription factor for polymerase III C with 5S rRNA genes.

In this context, two other factors, nuclear ErbB2 and the proto-oncogene PELP1, have been described to stimulate rRNA transcription even though their mechanism of action on the Pol I transcription complex has not yet been clearly clarified.

Regarding the relationship between tumor suppressors and ribosome biogenesis, it should be remembered that the tumor suppressors which modulate cell cycle progression also control ribosome biogenesis. Retinoblastoma protein (pRB) negatively controls the passage through the G1/S phase restriction point by binding to E2F1, a transcription factor whose target gene products are necessary for entry and progression through the S phase. pRB inhibits both rRNA synthesis by binding to UBF and Pol III transcription by binding to TFIIB. During the G1 phase, a phosphorylation of pRB occurs which functionally inactivates the tumor suppressor, thus allowing the cell to progress to the S phase. At the same time, phosphorylation of pRB, in addition to facilitating cell cycle progression, also induces a progressive increase in the rRNA transcription from the G1 phase to G2 phase by removing its inhibitory effects on Pol I and Pol III function. p53, the other major tumor suppressor which negatively controls cell cycle progression, directly hinders ribosome biogenesis. p53 binds to the selectivity factor SL1, which is necessary for Pol I recruitment to the rRNA gene promoter, and to TFIIB, thus inhibiting Pol I and Pol III transcription, respectively. This inhibitory effect is reduced in cycling cells as a consequence of a downregulation of p53 expression. p14ARF contributes to the p53-mediated control of ribosome biogenesis. The Arf gene is induced by a series of stress signals such as hyperproliferative signals emanating from oncogenic Ras and overexpressed MYC, and p14Arf helps the stabilization of p53 by binding to Hdm2, which is the factor responsible for p53 degradation. This tumor suppressor, in addition to activating the p53 pathway, reduces the ribosome biogenesis rate both by hindering UBF recruitment on the Pol I transcription complex and by downregulating the activity of “nucleophosmin,” a multifunctional protein involved in rRNA processing. Lastly, another important tumor suppressor involved in the control of ribosome biogenesis is phosphatase and tensin homolog deleted in chromosome 10 (PTEN), which represses Pol I transcription by disrupting the SL1 complex.

Since the neoplastic transformation is characterized by either the uncontrolled activity of oncogenes or the inactivation of tumor suppressors, all the data just reported indicate that some very frequent changes in proto-oncogenes and tumor suppressor genes in a variety of human cancers, which are responsible for the loss of the normal control mechanisms of cell proliferation and cell cycle progression, are also responsible for an enhanced ribosome biogenesis. In fact, MYC overexpression and the aberrant activation of the mitogen-activated protein kinases/extracellular signal-regulated kinases pathway, which are very frequently observed in human cancers, both result in increased rRNA synthesis; pRB inactivation due to genetic changes strongly reduces its braking power on rRNA transcription, as well as TP53 mutations, resulting in p53 inactivation, which characterizes about 50% of all human tumors. Also, the Arf gene may be mutated or silenced in cancers, thus it may enhance ribosome biogenesis both directly and through action on p53 stabilization. Lastly, the repressive action on Pol I transcription by PTEN may be lost in human cancers in which the tumor suppressor is deleted or mutated.

We may conclude that both the nucleolar hypertrophy and the upregulated ribosome biogenesis that frequently
characterize cancer cells are the consequences of the changes in proto-oncogene and tumor suppressor protein expression that control cell proliferation: the highly variable severity of these changes explains the highly variable nucleolar size and function in cancer.\textsuperscript{56–48}

From the teleological point of view, the upregulation of ribosome biogenesis in cancer cells appears to be an advantage for cancer growth. In fact, the acquired upregulated ribosome biogenesis may allow the complement of constituents necessary to always achieve the appropriate division in dividing cells, independently of the loss of cell cycle progression checkpoints.

At the same time, the enhanced ribosome biogenesis accelerates the cell cycle progression and, consequently, the cell proliferation rate.\textsuperscript{49}

### Upregulated ribosome biogenesis and neoplastic transformations

Can an upregulation of ribosome biogenesis be responsible for a neoplastic transformation? Many experimental data are consistent with an affirmative answer to this question. The depletion of TTF-1-interacting-protein-5 – a component of the nucleolar remodeling complex which keeps a portion of ribosomal genes in a silent heterochromatin organization – not only increases rRNA transcription but also induces a transformed phenotype in NIH3T3 cells.\textsuperscript{50}

The loss of MTG16a, a ribosomal gene repressor, increases ribosome biogenesis and induces morphological and molecular changes which are typical of breast cancer initiation in breast epithelial cells.\textsuperscript{51} Further, the importance of an enhanced ribosome biogenesis in tumor development was demonstrated by the fact that the lymphomagenesis occurring in E\textsubscript{\textmu}–MYC\textsuperscript{+}/+ transgenic mice – in which MYC is overexpressed in the B-cell compartment – was reduced by their intercrossing with RPL24\textsuperscript{+/-}, as well as with RPL38\textsuperscript{+/-} mice.\textsuperscript{52,53} In these mice, the level of ribosome biogenesis and protein synthesis was lowered remarkably, thus restoring the increased protein synthesis in E\textsubscript{\textmu}–MYC\textsuperscript{+} transgenic mice to normal levels and suppressing the oncogenic potential of MYC.

With regard to human pathology, a series of epidemiological studies has shown that many diseases with different etiologies, associated with an increased risk of developing cancer, are also characterized by the focal and systemic release of factors which stimulate ribosome biogenesis.

There is evidence that chronic inflammation,\textsuperscript{54,55} obesity,\textsuperscript{56,57} and type 2 diabetes\textsuperscript{58} represent important risk factors for the onset of malignancies. Interestingly, all these conditions exhibit an increased plasma level of the inflammatory cytokine, interleukin 6 (IL-6),\textsuperscript{59,60} which has been proved to stimulate rRNA transcription (Brighenti et al, unpublished data, 2013). Further, in obese and early stage type 2 diabetes patients, a higher plasma level of insulin is also present,\textsuperscript{50} enhancing per se ribosome biogenesis.\textsuperscript{61} What is the cause that lets an enhanced ribosome biogenesis increase the risk of cancer onset? The answer lies in the mechanism by which ribosome biogenesis controls the function of p53. A large body of data has shown that a perturbation in any of the several steps of ribosome biogenesis causes p53 stabilization and activation,\textsuperscript{62–65} whereas an enhancement of rRNA transcription is responsible for a downregulation of p53 expression and activity.\textsuperscript{61} p53 is regulated by murine double minute 2 (MDM2; HDM2 [ie, human double minute 2] in humans), which adversely controls p53 activity in two ways: by binding to the protein, thus interfering with its transactivation activity, and by facilitating p53 proteasomal degradation, acting as an E3 ubiquitin ligase.\textsuperscript{66–68} After the inhibition of rRNA transcription, the ribosomal proteins, no longer used for ribosome building, bind to and inactivate MDM2, thus allowing p53 stabilization.\textsuperscript{68,69} Conversely, when rRNA transcription is stimulated, the ribosomal protein that binds and inactivates MDM2 is reduced, thus resulting in a greater degradation of p53.\textsuperscript{65} This was clearly demonstrated in human cell lines exposed to either IL-6 or insulin, in which both the amount of p53 and the p53-mediated response to cytotoxic stresses decreased.\textsuperscript{61} Therefore, cells with upregulated ribosome biogenesis would have greater difficulty in activating their tumor suppressor mechanisms, thus be more subject to neoplastic transformation. This may be the root of the increased risk of developing cancer in those human pathological conditions in which high plasma levels of IL-6 and insulin in responsive tissues induce an upregulation of ribosome biogenesis with the consequent downregulation of p53-related tumor suppressor potential.\textsuperscript{70}

### Targeting Pol I for the treatment of human cancer

Currently, chemotherapy is a widely chosen option for treating cancer, either before or after the surgical removal of tumor masses. Chemotherapeutic agents may be broken down into several families according to their mechanism of action: alkylating drugs, anthracyclines, antimetabolites, DNA damaging agents, spindle poisons, topoisomerase inhibitors, oncoprotein-targeting agents, and antihormone drugs. With the exception of the last two classes, the other chemotherapeutic agents exert their toxic action on cancer
cells by damaging DNA, hindering DNA synthesis, and disrupting the mitotic process. Therefore, the targets of these drugs are mainly proliferating cells, which are abundant in cancer tissues. Unfortunately, this toxic action is also exerted on the proliferating cells of normal tissues, thus establishing strict pharmacological limits to the use of these anticancer drugs. This has led to the need for continuous efforts to find new therapeutic strategies that more specifically address cancer cells.

Considering that an ongoing, appropriate ribosome production is necessary both for cell cycle progression and for the generation of viable cells, a new therapeutic approach to killing cancer cells based on the specific inhibition of Pol I activity has been recently proposed.\textsuperscript{71–75}

At first glance, a similar approach based on the inhibition of ribosome biogenesis would appear to be even less specific for cancer cell elimination than the above-reported classic chemotherapeutic procedures. In fact, unlike the DNA synthesis, the synthesis of rRNA occurs in both proliferating and resting cells. What is more, the specific inhibition of the Pol I activity might sound less efficient than a large series of chemotherapeutics which, in addition to the inhibition of DNA synthesis, also hinder the rRNA production at either the transcription or processing level.\textsuperscript{71} However, a series of data dealing with the relationship between ribosome biogenesis and cell proliferation has indicated that inhibition of the Pol I activity may result in specific damage to neoplastic cells, at least in some instances. Indeed, there is evidence that the inhibition of rDNA transcription may have specific lethal effects on cancer cells in the following two conditions: (1) when cancer cells have lost checkpoint mechanisms which control the cell cycle progression and (2) when cancer cells are characterized by an upregulated ribosome biogenesis. In fact, in both cases, the inhibition of rRNA synthesis has been demonstrated to have deleterious effects only on cancer cells, while sparing normal tissues.\textsuperscript{71–76}

Regarding the first condition, it has been shown that a transient, selective inhibition of the Pol I-dependent transcription induced by actinomycin D exposure caused cell cycle arrest in pRB and p53 proficient cells but not in pRB- and p53-deficient cells.\textsuperscript{76} The cell cycle arrest observed in pRB- and p53-proficient cells, due to the activation of the p53-pRB checkpoint pathway, was reversible: after recovery of ribosome biogenesis, cell cycle progression started up again, and cells could achieve a ribosomal complement sufficient for giving rise to normal daughter cells (Figure 2A). This was not the case for pRB- and p53-deficient cells in which rRNA inhibition did not alter the cell cycle progression and cells underwent a progressive ribosome depletion that led to apoptotic death (Figure 2B).\textsuperscript{76} Therefore, these data indicate

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\caption{Different effects of RNA polymerase I (Pol I) inhibition according to the function of the p53/pRb pathway. (A) Pol I inhibition induces G1/S cell cycle arrest through activation of the p53/pRb pathway. (B) In cells lacking the functional p53/pRb pathway, the inhibition of Pol I does not induce cell cycle arrest; rather, without a sufficient ribosomal complement, the cell divides and dies.}
\end{figure}
that the selective inhibition of Pol I-dependent transcription may be very useful in killing cancer cells lacking the two tumor suppressors, without having significant effects on the proliferation of noncancerous cells in which the two tumor suppressors are functioning normally.

The other pathological cancer condition in which the inhibition of rDNA transcription may selectively destroy cancer, but not normal cells, is that of tumors characterized by highly upregulated ribosomal biogenesis. This has been clearly demonstrated in a study conducted using the CX-5461 inhibitor of RNA synthesis in a transgenic murine model of spontaneous lymphoma (Eμ-MYC), in which MYC is overexpressed in lymphocytes of the B lineage. CX-5461 (Cylene Pharmaceuticals Inc, San Diego, CA, USA), is a powerful small molecule that selectively inhibits Pol I-driven transcription. It was demonstrated that CX-5461 disrupts the binding of the SL1 transcription factor to the rDNA promoter, thus preventing the initiation of rRNA synthesis. The drug inhibits Pol I transcription without having almost any effect on DNA replication, protein translation, and general cellular transcription.

MYC overexpression was responsible for the high elevation of ribosome biogenesis in malignant B cells from Eμ-MYC transgenic mice. Administration of CX-5461 to mice caused p53-dependent apoptotic death in neoplastic but not normal B cells. This different sensitivity to CX-5461 exposure may be explained by considering the mechanism involved in the MDM2 inactivation by the ribosomal protein binding, with the consequent p53 stabilization, after the inhibition of rDNA transcription (Figure 3). Since the synthesis of rRNA requires the stoichiometric production of ribosomal proteins, in cells with low rRNA synthesis activity, and therefore with low ribosomal protein production, even a marked reduction of rDNA transcription should not make ribosomal proteins available in an amount sufficient to inactivate MDM2 (Figure 3A). In contrast, in the case of cells with highly upregulated ribosome biogenesis, a slight reduction in rRNA molecules would make ribosomal proteins free to an extent sufficient for inactivating MDM2 (Figure 3B).

**Conclusion**

Up to now, research efforts in the chemotherapeutic treatment of cancer have mainly addressed the development of drugs that hinder the neoplastic cell division by either inhibiting DNA synthesis or damaging the mitotic process, or by blocking abnormally upregulated pathways which stimulate cell proliferation. The results achieved over the past few years indicate that an appropriate ribosome biogenesis is necessary for adequate cell growth, which, in turn, is necessary for both normal cell cycle progression and cell division.
Since the selective disruption of ribosome biogenesis by hindering rRNA synthesis has been proven to arrest cell cycle progression and/or cause apoptotic cell death, the targeting factors of the rDNA transcription complex may represent a promising new strategy for the chemotherapeutic treatment of cancers. Indeed, available data suggest that the specific inhibition of rRNA transcription could be used for the selective killing of cancer cells characterized by either a highly enhanced ribosome biogenesis or a deranged function of the p53 and pRB3 tumor suppressors.

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Disclosure

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