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Characterization of the angiogenic activity of zebrafish ribonucleases

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Summary

Ribonucleases (RNases) have recently been identified from zebrafish and shown to possess angiogenic and bactericidal activities. Zebrafish RNases (ZF-RNases) have three intramolecular disulfide bonds, a characteristic structural feature of angiogenin (ANG), different from the typical four disulfide bonds of the other members of the RNase A superfamily. They also have a higher degree of sequence homology to ANG than to RNase A. It has therefore been proposed that all RNases evolved from these ANG-like progenitors. Here we characterize in detail the function of ZF-RNases in various steps in the process of angiogenesis. We report that ZF-RNase-1, -2, and -3 bind to the cell surface specifically and are able to compete with human ANG (hANG). Similar to hANG, all three ZF-RNases are able to induce phosphorylation of Erk1/2 MAP kinase. They also undergo nuclear translocation, accumulate in the nucleolus, and stimulate ribosomal RNA (rRNA) transcription. However, ZF-RNase-3 is defective in cleaving rRNA precursor (pre-rRNA) even though it has been reported to have an open active site and has higher enzymatic activity toward more classic RNase substrates such as yeast tRNA and synthetic oligonucleotides. Together with the findings that ZF-RNase-3 is less angiogenic than ZF-RNase-1, -2, and hANG, these results suggest that ZF-RNase-1 is the ortholog of hANG and that the ribonucleolytic activity of ZF-RNases toward the pre-rRNA substrate is functionally important for their angiogenic activity.

Keywords

ribonuclease; angiogenin; angiogenesis; zebrafish; amyotrophic lateral sclerosis

Introduction

The vertebrate RNase superfamily has over 100 members from fish, amphibians, reptiles, birds and mammals [1]. Several members of this superfamily are endowed with special activities, in addition to catalysis, including angiogenic [2], antifertility [3], anti-pathogen [4], cytotoxic [5], and immunosuppressive [6] activities. The ability to degrade RNA is essential for most of these RNases to perform their special activities even though the natural substrates for most of the family members are yet unknown. The exceptions are human RNases 3 [7] and 7 [8], for which the microbicidal activity remain when the RNase catalytic activity is suppressed.

One of the most interesting special activities of the RNase superfamily is the angiogenic activity, which is represented by hANG [9]. While mammalian ANG form a distinct subfamily of RNases with several active members [10], angiogenic RNases have also been identified in birds [11] and fish [12-15]. Two zebrafish RNases, ZF-RNase-1 and -2 have been shown to be angiogenic in an early study, whereas no angiogenic activity was observed for ZF-RNase-3 [14]. However, all of them have been recently reported to have microbicidal activity [12], similar to some isoforms of mammalian ANG [16] and the chicken leukocyte RNase A-2 [11].

Some interesting features of ANG have been documented [2], mainly through studies with hANG. A key feature is that ANG has several orders of magnitude lower ribonucleolytic activity than that of RNase A but this enzymatic activity is essential for ANG to induce angiogenesis [17]. Another key step in the process of ANG-mediated angiogenesis is the specific interaction with endothelial cells, which triggers a wide range of cellular responses, including migration [18], proliferation [19], and tubular structure formation [20]. ANG also undergoes nuclear translocation where it accumulates in the nucleolus, binds to the ribosomal DNA (rDNA) promoter, and stimulates rRNA transcription [21]. Nuclear translocation of ANG in endothelial cells is independent of microtubules and lysosomes [22], but is strictly dependent on cell density [23]. Nuclear translocation of ANG in endothelial cells decreases as cell density increases, and it ceases when cells are confluent [23]. This tight regulation of nuclear translocation of ANG in endothelial cells ensures that the nuclear function of ANG is limited only to proliferating endothelial cells [24]. However, this cell density-dependent regulation of nuclear translocation of ANG is lost in cancer cells. ANG has been found to undergo constitutive nuclear translocation in a variety of human cancer cells [25]. One reason for constitutive nuclear translocation of ANG in cancer cells has been proposed to be the constant demand for rRNA in order to sustain their continuing growth [25].

Recently, ANG has been demonstrated to be the first “loss-of-function” mutated gene in amyotrophic lateral sclerosis (ALS) [26]. Since the original discovery of ANG as an ALS candidate gene [27], a total of 14 missense mutations in the coding region of ANG have been identified in 35 of the 3170 ALS patients of the Irish, Scottish, Swedish, North American, and Italian populations [26-30]. Ten of the 14 mutant ANG proteins have been prepared, characterized, and shown to be not angiogenic [26,31]. ANG is the only loss-of-function gene so far identified in ALS patients and is actually the second most frequently mutated gene in ALS. Mouse ANG is strongly expressed in the central nervous system during development [32]. Human ANG is strongly expressed in both endothelial cells and motor neurons of normal human fetal and adult spinal cords [26]. Wild type ANG has been shown to stimulate neurite outgrowth and pathfinding of motor neurons in culture and to protect hypoxia-induced motor neuron death, whereas the mutant ANG proteins not only lack these activities but also induce motor neuron degeneration [33]. Therefore, a role of ANG in motor neuron physiology and a therapeutic activity of ANG toward ALS can be envisioned. To reveal the role of ANG in motor neuron physiology, one approach would be to create and characterize ANG knockout mice. However, although humans have only a single ANG gene, mice have six [34]. It is not possible to knockout all of them simultaneously because they are spread out over ~8 million bp.

The zebrafish offers an excellent alternative model to study the role of ANG in motor neuron development and disease mechanisms. The development of the transparent embryos ex utero is fast, and several thousand phenotypic mutations are available for study. Furthermore, the embryos are easy to manipulate, and target genes can be easily knocked down by morpholino antisense compounds. Zebrafish has been used as an animal model for studying angiogenesis [35], ALS [36], and spinal muscular atrophy [37].

Four paralogs of RNases have been identified from zebrafish [12,14]. Significant polymorphism exists in three of the four paralogs [13]. These paralogs have been named RNases ZF-1a-c, -2a-d, -3a-e, and -4 [13]. ZF-RNase-1 and -2 have been shown to have angiogenic activity in the endothelial cell tube formation assay, whereas ZF-RNase-3 was not angiogenic under the same conditions [14]. Crystal structures of ZF-RNase-1a and -3e revealed that the enzyme active site of ZF-RNase-1 is blocked by the C-terminal segment [13] in a way resembling that of hANG [38], whereas that of ZF-RNase-3 is open as found in the nonangiogenic RNase A [13]. These findings have set the foundation for further characterization of zebrafish RNases so that they can be selectively targeted for studies of disease mechanism such as tumor angiogenesis and neurodegeneration. In the present study, we investigated the activities of ZF-RNase-1, -2, and -3 in various steps of the angiogenesis process, including cell surface binding, MAP kinase activation, nuclear translocation, rRNA transcription and processing.

Results

ZF-RNase-3 has low angiogenic activity

ZF-RNase-1 and -2 have been previously shown to induce the formation of tubular structures of cultured endothelial cells but ZF-RNase-3 failed to do so [14]. Only one dose (200 ng/ml) was used in this early experiment. Therefore, we determined the dose-dependent angiogenic activities of ZF-RNases. Figure 1 shows that ZF-RNase-1 induced tube formation (indicated by arrows) of cultured human umbilical vein endothelial (HUVE) cells at a concentration as low as 50 ng/ml. For ZF-RNase-2, the angiogenic activity started to be detected at 100 ng/ml. No detectable activity was observed for ZF-RNase-3 at a concentration up to 200 ng/ml, consistent with the previous report [14]. However, tubular structures started to form at 500 ng/ml and extensive network formed when the concentration of ZF-RNase-3 reached 1 μ g/ml. Recombinant WT hANG in the same serial dilution was used as positive control. H13A hANG, an inactive variant in which the catalytic His-13 has been replaced with Ala [39], was used as negative control (data not shown). These results indicate that ZF-RNase-3 is not completely devoid of angiogenic activity but rather has a reduced potential.

ZF-RNases bind to HUVE and HeLa cells specifically

ANG-stimulated angiogenesis is a multistep process including binding to the cell surface, activation of cellular signaling kinases such as Erk 1/2 and AKT, nuclear translocation, stimulation of rRNA transcription and processing of rRNA precursor [40]. We therefore studied the effect of ZF-RNases on these individual steps in the angiogenesis process. We have previously shown that, in addition to sparsely cultured endothelial cells [24], tumor cells are also target cells for ANG [25,41]. Tumor cells are more practical than endothelial cells for studying cellular interactions of ANG because they respond to ANG in a cell density-independent manner [25], whereas the activity of ANG diminishes in endothelial cells when cell density increases [19]. Therefore, the ability of ZF-RNases to bind to specific sites on target cells was first examined in HUVE cells and then in HeLa cells in more detail.

All three isoforms of ZF-RNases were found to bind to the surface of HUVE cells cultured in sparse density. The binding assays were carried out at 4 °C to minimize internalization and nuclear translocation. Competition experiments with unlabeled hANG showed that binding of ZF-RNases to HUVE cells is inhibited by hANG. Figure 2A shows the percent of inhibition with a 200-fold molar excess of hANG was able to compete for the binding of 125 I-labeled hANG, ZF-RNase-1, 2, and 3 to HUVE cells by 81 ± 10 , 77 ± 9 , 67 ± 8 , and $69 \pm 10\%$, respectively (Fig. 2A). Unlabeled RNase A, at the same concentration, did not compete for binding of 125 I-labeled hANG, ZF-RNase-1, 2, and 3 to HUVE cells (less than 5% in all cases).

These results indicate that ZF-RNases compete with hANG for the same binding sites in HUVE cells.

Figure 2B shows that ZF-RNase-1, -2, and -3 bind to HeLa cells in a way very similar to that of hANG. In these experiments, total binding was obtained in the absence of unlabeled proteins. Nonspecific binding was obtained in the presence of a 200-fold molar excess of unlabelled proteins. Specific binding was then calculated by subtracting the values of nonspecific binding from those of total binding. It is noticeable that binding of all three ZF-RNases and hANG to HeLa cells are saturable. The specific bindings of ZF-RNase-1 and hANG to HeLa cells were about 70% of the total binding, a typical value of hANG binding to its target cells [42]. However, the specific bindings of ZF-RNase-2 and -3 were around 50% of the total binding.

Scatchard analyses of the specific binding data revealed that the K_d for ZF-RNase-1, -2, and -3 are 0.38 ± 0.06 , 0.40 ± 0.07 , and 0.58 ± 0.07 μM , with a total of 3.73 ± 0.74 , 1.23 ± 0.27 , and 0.77 ± 0.26 millions specific binding sites per cell, respectively (Fig. 2B, insets). Under the same condition, hANG has a K_d of 0.22 ± 0.05 μM with the total binding site of 4.3 ± 0.71 millions per cell. Thus, ZF-RNase-1 has the strongest and highest binding to the cell surface, and ZF-RNase 3 has the lowest binding.

Next, we examined whether ZF-RNases also compete with hANG for the same binding sites in HeLa cells. For this purpose, cells were incubated with ^{125}I -labeled ZF-RNase or hANG at a fixed concentration of 60 nM in the presence of increasing unlabeled hANG up to a concentration that is 200-fold molar excess of the labeled ligands. As shown in Figure 2C, unlabeled hANG competed with ^{125}I -labeled ZF-RNases for binding to HeLa cells to various degrees. In the presence of 20- to 200-fold molar excess (1.2 to 12 μM) of unlabeled hANG, the amount of remained binding of ^{125}I -labeled ZF-RNase-1 was indistinguishable from that of ^{125}I -labeled hANG. Interestingly, at the concentration lower than 1.2 μM (20-fold molar excess), the amount of remained ^{125}I -ZF-RNase-1 on cell surface was actually somewhat lower than that of ^{125}I -hANG. At lower concentration of unlabeled hANG (0.6 μM , 10-fold molar excess), the amount of remained ^{125}I -ZF-RNase-2 was the same as that of ^{125}I -ZF-RNase-1, whereas that of ^{125}I -ZF-RNase-3 was significantly higher. At higher concentration of unlabeled hANG, a significant higher amount of ^{125}I -labelled ZF-RNase-2 and -3 remained bound on the cell surface than that of ^{125}I -ZF-RNase-1. For example, in the presence of 12 μM hANG (200-fold molar excess), the amount of remained binding of ^{125}I -ZF-RNase-1, -2, and -3, was 17, 45, and 56%, respectively, of the total binding in the absence of competitors. Thus, among the three zebrafish RNases, ZF-RNase-1 most closely resembles that of hANG and ZF-RNase-3 is the most different one in their binding to the cell surface. Most importantly, these results demonstrated that ZF-RNases and hANG share at least some of the common binding sites on the surface of human cells.

ZF-RNases induce Erk1/2 phosphorylation in HUVE cells

Binding of hANG to endothelial cells has been shown to induce second messenger responses including diacylglycerol and prostacyclin, and to activate cellular signaling kinases such as Erk1/2 MAP kinase [43] and AKT [44]. We therefore examined whether Erk can be activated by ZF-RNases. HUVE cells were examined for their response in Erk1/2 phosphorylation upon stimulation of ZF-RNases. Figure 3 shows that all three ZF-RNases are able to activate Erk1/2 in HUVE cells. Phosphorylation of Erk1/2 occurred as early as 1 min upon stimulation of ZF-RNases and remained for at least 30 min, similar to the observations previously reported with hANG [43].

ZF-RNases undergo nuclear translocation in HUVE and HeLa cells

Next, we examined the ability of ZF-RNases to undergo nuclear translocation, a step known to be essential for the biological activity of hANG [45]. First, indirect immunofluorescence was used to determine cellular localization of ZF-RNases in endothelial cells. Sparsely cultured HUVE cells were incubated with 1 µg/ml hANG and ZF-RNases for 1 h. Cellular localization of hANG was detected by the anti-hANG monoclonal antibody 26-2F and visualized with an Alexa 488-labeled goat anti-mouse IgG. A similar approach was applied to ZF-RNases with an anti-ZF-RNases polyclonal antibody and an Alexa 488-labeled goat anti-rabbit IgG. DAPI staining was performed to visualize the nuclei. The merge of the green (Alexa 488) and blue (DAPI) staining indicated that all three ZF-RNases are localized in the nucleus with punctate nucleolus staining, in a way very similar to that of hANG (Fig. 4A). The polyclonal antibody used in this study was raised with ZF-RNase-3 as the immunogen, but was found to recognize all three ZF-RNases in immunodiffusion and Western Blotting (data not shown). No nuclear staining was visible in untreated cells (negative control) or when the primary antibody was omitted or replaced with a non-immune IgG (data not shown). The subnuclear localization of ZF-RNases is somewhat different from that of hANG and among the 3 ZF paralogs. The significance of the difference in subnuclear compartments is currently unknown but nucleolar accumulation is obvious in all cases.

¹²⁵I-labeled ZF-RNases were used to confirm the findings of indirect immunofluorescence. For these experiments, HeLa cells were used instead of HUVE cells to obtain adequate radiolabeled proteins from the nuclear fractions because it is known that nuclear translocation of ANG in endothelial cells decreases as the cell density increases so it was not practical to enhance the signal strength by increasing the cell density of endothelial cells. Confluent HeLa cells were incubated with ¹²⁵I-labeled ZF-RNases in serum-free DMEM at 37 °C for 1 h. Cells were then lysed and the nuclear fraction was isolated and analyzed by SDS-PAGE and autoradiography. As shown in Figure 4B, a strong band with a MW of 14 kDa was detected from the nuclear fractions of HeLa cells incubated with ¹²⁵I-labeled hANG (lane 2), ZF-RNase-1 (lane 4), -2 (lane 6), and -3 (lane 8). It is noticeable that a band with MW of 28 kDa was also detected from the nuclear fractions, which was not present or was under the detection limit in the preparation of iodinated hANG and ZF-RNases (lanes 1, 3, 5, and 7). A similar enrichment of the dimeric form of hANG in the nucleus has been previously reported in human umbilical artery endothelial cells [23]. Some lower MW bands of ZF-RNase-2 (lane 6) and -3 (lane 8) were also detected in the nuclear fractions. The significance of the presence of these minor forms of ZF-RNases in the nucleus was not yet clear. But these results clearly demonstrated that nuclear translocation of ZF-RNases occurs in both HUVE and HeLa cells.

ZF-RNases stimulate rRNA transcription

hANG has been shown to bind to the promoter region of rDNA and stimulate rRNA transcription [21,46]. ANG-stimulated rRNA transcription in endothelial cells has been demonstrated to be essential for angiogenesis induced by a variety of angiogenic factors and has been proposed as a cross-road in the process of angiogenesis [24]. Moreover, ANG-mediated rRNA transcription has also been shown to play a role in proliferation of cancer cells [25,41]. Therefore, we measured the activity of ZF-RNases in stimulating rRNA transcription in HeLa cells. Subconfluent HeLa cells were incubated with 1 µg/ml of ZF-RNases for 1 h and the total RNA was extracted, and analyzed by Northern blotting with a probe specific to the initiation site of 47S rRNA precursor. Cells incubated in the absence of exogenous proteins and in the presence of 1 µg/ml hANG were used as negative and positive controls, respectively. The membrane was stripped, reblotted with a probe specific for β-actin, and the results were used as the loading control. Figure 5 shows that all 3 ZF-RNases were able to stimulate an increase in the steady-state level of the 47S rRNA precursor (Fig. 5A, left panel). Densitometry data shows that ZF-RNase-1, -2, and -3 all have comparable activity as that of hANG (Fig. 5A, right

panel). Quantitative RT-PCR was also used to assess rRNA transcription stimulated by ZF-RNases. Figure 5B shows that the cellular level of the 47S/45S rRNA precursor increased 7.21 ± 0.12 , 5.97 ± 0.11 , 6.07 ± 0.09 , and 5.85 ± 0.12 fold in the presence of hANG, ZF-RNase-1, 2, and 3, respectively. The primers used for qRT-PCR recognize both 47S and 45S rRNA, which may explain the more significant difference seen in qRT-PCR (Fig.5B) than in Northern blotting (Fig.5A). Together, these results demonstrate that all 3 ZF-RNases are able to stimulate rRNA transcription in HeLa cells.

ZF-RNase-3 is defective in mediating rRNA processing

rRNA is transcribed as a 47S precursor that is processed into 18S, 5.8S, and 28S mature rRNA [47]. rRNA processing is a multi-step process in which the initial cleavage occurs at the 5'-external transcription spacer (A_0 site) [48]. Cleavage at A_0 is a prerequisite for all the subsequent processing and maturation events. It has been shown that the sequence of the A_0 site, as well as that of the downstream 200 nt are well conserved from *Xenopus* to humans [49-51]. An endoribonuclease has been implicated in A_0 cleavage, although its identity has not yet been determined [52]. Our preliminary studies suggest that ANG is one of the candidate endoribonuclease involved in the cleavage at the A_0 site in the process of rRNA maturation (W. Yu and G.-f. Hu, unpublished). In order to know whether zebrafish RNases play a role in rRNA processing, we carried out an *in vitro* enzymatic assay using a specific RNA substrate containing the sequence of A_0 site and the flanking regions. First, a 43 nt substrate was used to compare the product profile of hANG and ZF-RNases. Figure 6A shows that a major product corresponding to a cleavage at the putative A_0 site (cucuuc) was generated by both hANG and ZF-RNase-1 (indicated by arrows). In contrast, bovine pancreatic RNase A degraded this substrate into much smaller fragments, whereas ZF-RNase-3, under the same conditions, did not cleave the substrate. Interestingly, the products of ZF-RNase-2, consisted of two major bands (indicated by arrow heads), were different from that of ZF-RNase-1 and hANG. The reasons for the different substrate specificities of ZF-RNase-1 and -2 are unknown at present, but these results may suggest that the ZF-RNase-1 and -2 may have different biological functions. ZF-RNase-1 is clearly an ortholog of hANG. The activity of ZF-RNases in cleaving rRNA precursor was further examined with a 17 nt substrate that also containing the A_0 site but with shorter flanking regions at both 5'- and 3'- ends. The results are shown in Figure 6B, which confirms that ZF-RNase-1 and -2 were able to cleave the pre-rRNA substrate but ZF-RNase-3 failed to do so. It is to note that the enzymatic activity of ZF-RNase-1 is lower toward the 43 nt substrate (Fig. 6A) and is higher toward the 17 nt substrate (Fig. 6B) than that of ZF-RNase-2. The product pattern of ZF-RNase-1 is similar to that of hANG with both substrates. These results indicate that the ribonucleolytic activity and specificities of the three ZF-RNases are different toward the pre-rRNA substrate. ZF-RNase-1 shares similar enzymatic properties with hANG in the cleavage of pre-rRNA, whereas ZF-RNase-3 has the lowest activity under these conditions. It has been known that the released RNA fragment from A_0 cleavage of pre-rRNA precursor is rapidly degraded and is therefore not readily detectable by Northern blotting (51).

Discussion

ANG is the fifth member of the pancreatic RNase superfamily [2]. It was originally isolated from the conditioned medium of HT29 human colon adenocarcinoma cells based on its angiogenic activity [9]. ANG has been shown to play a role in tumor angiogenesis. Its expression is upregulated in many types of cancers [53]. Extensive works on the structure and function relationship [38,54,55], mutagenesis [39,56], cell biology [19,42], and experimental tumor therapy [57-59] have been carried out and the role of ANG in tumor angiogenesis is now very well established. More recently, a novel function of ANG in motor neuron function has been discovered. Loss-of-function mutations in the coding region of ANG gene were identified

in ALS patients [26-31] and ANG has been shown to play a role in neurogenesis [32,33], which raised considerable interest in understanding the role of ANG in motor neuron physiology and in therapy of motor neuron diseases [60]. *ANG* gene knockout in a mouse model might be complicated because of the existence of 6 isoforms and 4 pseudogenes [34]. Timely, zebrafish RNases were recently identified and shown to be more closely related to ANG than to RNase A both structurally and functionally [12-14]. In light of the powerful genetic tools available in zebrafish model [35-37], it can be envisioned that they will be a convenient model for elucidating the role of ANG in angiogenesis and neurogenesis. We therefore set out to determine which zebrafish RNase most closely resembles ANG functionally. We dissected the role of ZF-RNase-1, -2, and -3 in each of the individual steps in the process of ANG-induced angiogenesis including cell surface binding, signal transduction, nuclear translocation, rRNA transcription, as well as pre-rRNA processing. Our results indicate that ZF-RNase-1 is the ortholog of hANG and that ZF-RNase-3 is the most different one among the three paralogs. It is therefore likely that knockout ZF-RNase-1 will suffice for investigating the function of hANG.

All three ZF-RNases are able to bind to the cell surface in a specific, saturable and competeable manner. The K_d and the total binding sites of ZF-RNases are not significantly different from that of hANG, suggesting that they all have the same cell surface receptor. We have also demonstrated that ZF-RNases activate Erk in HUVE cells as did hANG, indicating that these binding are productive. Moreover, all three ZF-RNases were found to undergo nuclear translocation where they accumulate in the nucleolus. These findings are functionally significant as it has been shown that ANG undergo nuclear translocation in endothelial [22, 23,45] and cancer [25,41] cells and that this process is essential for its biological activity. Nuclear translocation of ANG occurs through receptor-mediated endocytosis [45] and is independent of microtubule system and lysosomal processing [22]. ANG seems to enter the nuclear pore by the classic nuclear pore input route [61]. It can be hypothesized that ZF-RNases utilize the same machinery as that of ANG in the nuclear translocation process.

Upon arriving at the nucleus, ANG accumulates in the nucleolus [45] where ribosome biogenesis takes place. Nuclear ANG has been shown to bind to the promoter region of rDNA [46] and to stimulate rRNA transcription [21,24]. Cell growth requires the production of new ribosomes. Ribosome biogenesis is a process involving rRNA transcription, processing of the pre-rRNA precursor and assembly of the mature rRNA with ribosomal proteins [62-64]. Therefore, rRNA transcription is an important aspect of growth control. It is also important for maintaining a normal cell function as proteins are required for essentially all cellular activities. Our results demonstrated that all three ZF-RNases are able to stimulate rRNA transcription to a similar degree as hANG (Fig. 5).

ANG has a unique ribonucleolytic activity that is several orders of magnitude lower than that of RNase A but is important for its biological activity [17]. Extensive studies on site-directed mutagenesis have shown that ANG variants with reduced enzymatic activity also have reduced angiogenic activity. Structural work indicated that one of the reasons for ANG to have a reduced ribonucleolytic activity is that the side chain of Gln 117 occupies part of the enzymatic active site so that substrate binding is compromised [38,65]. Recent structural work has shown that a similar blockage of the enzyme active site occurs in ZF-RNase-1 but not in ZF-RNase-3 [13], which offered an excellent explanation of the relatively higher ribonucleolytic activity of ZFRNase-3 toward yeast tRNA and synthetic oligonucleotides [13,14]. These differences in the structures of ZF-RNase-1 and -3 also seem to explain the lack of angiogenic activity of ZF-RNase-3 [14]. Here, we show that ZF-RNase-3 is actually much less active toward a pre-rRNA substrate. Since rRNA is transcribed as a 47S precursor that is processed by a series of cleavage events to generate the mature 18S, 5.8S, and 28S rRNA, these results may suggest that ZF-RNase-3 is defective in mediating pre-rRNA processing. However, ZF-RNase-3 has a robust

ribonucleolytic activity toward yeast tRNA or synthetic dinucleotides [13,14]. Therefore, a digestive function of ZF-RNase-3 can not be excluded at present. Of note, the product pattern of ZF-RNase-1 and hANG is identical when pre-rRNA was used as substrate. Thus, our results provide an alternative explanation and a further characterization of the lower angiogenic activity of ZF-RNase-3, and suggest that specificity and activity toward rRNA substrate is important for angiogenesis.

We have therefore demonstrated that ZF-RNase-1 most closely resembles hANG in mediating the key individual steps of the angiogenesis process and that the most likely reason for the diminished angiogenic activity of ZF-RNase-3 is its defect in mediating rRNA processing.

Experimental Procedures

Preparation of ANG and ZF-RNases

Recombinant ZF-RNases, wild type (WT) human ANG (hANG) and the H13A hANG variant were prepared and characterized as described previously [14,66].

Cell cultures

Human umbilical vein endothelial (HUVE) cells were cultured in EBM-2 basal endothelial cell culture medium containing the EGM-2 Bullet kit (Cambrex). HeLa cells were cultured in DMEM + 10% FBS.

Protein iodination

ZF-RNases and hANG (100 µg) were labeled with 1 mCi of carrier-free Na¹²⁵I and Iodobeads according to the manufacturer's instructions. Labeled proteins were desalted on PD10 columns equilibrated in PBS. The specific activity of labeled proteins was approximately 1.5 µCi/µg of protein.

Endothelial cell tube formation angiogenesis assay

HUVE cells were seeded in Matrigel-coated 48-well plates (150 µl/well) at a density of 4×10^4 per well in 0.15 ml of EBM-2 basal medium. ZF-RNases, WT and H13A hANG were added to the cells at different concentrations and incubated at 37 °C for 4 h. Cells were fixed with phosphate-buffered glutaraldehyde (0.2%) and paraformaldehyde (1%), and photographed.

Cell surface binding assays

HUVE cells were seeded in 6-well plates at a density of $1 \times 10^4/\text{cm}^2$ and cultured in human endothelial serum-free medium (HEM, Invitrogen) + 5% FBS + 5 ng/ml basic fibroblast growth factor (bFGF) for 24 h. Cells were washed with HEM + 1 mg/ml BSA three times at 4 °C and incubated with 50 ng/ml of ¹²⁵I-labeled ZF-RNases and hANG in the absence and presence of 10 µg/ml unlabeled hANG.

HeLa cells were seeded in 24-well plates at a density of 1×10^5 per well. After 24 h, 200 µl of binding buffer (25 mM Hepes, pH 7.5, 1 mg/ml BSA in DMEM), containing increasing concentrations of the labeled proteins with or without 200-fold molar excess of unlabelled protein, were added to the cells. After 1 h incubation of at 4 °C, cells were washed three times with PBS containing 0.1% BSA. Bound materials were released by treating the cells with 0.7 ml of cold 0.6 M NaCl in PBS for 2 min on ice. Released radioactivity was measured with a gamma counter. Total binding was determined in the absence of unlabeled proteins. Non-specific binding was determined in the presence of 200-fold molar excess of unlabelled proteins at each concentration. Specific binding was calculated by subtracting the non-specific binding

from the total binding. K_d and total binding sites were calculated from the Scatchard equation of the specific binding data. Each value was the mean of triplicates. For competition experiments with hANG, cells were incubated at 4 °C in 200 μ l of binding buffer containing a constant 60 nM of 125 I-labeled protein and increasing concentrations of unlabeled hANG.

Western blotting analysis of Erk phosphorylation

HUVE cells were seeded at a density of 5×10^4 cells per well of 6-well plate in HEM supplemented with 5% FBS and 5 ng/ml bFGF at 37 °C under 5% of humidified CO₂ for 24 h, washed with serum-free HEM three times and serum-starved in HEM for another 24 h. The cells were then washed again three times with prewarmed HEM and incubated with 1 μ g/ml ZFRNases at 37 °C for 1, 5, 10 and 30 min. Cells were washed with PBS and lysed in 60 μ l per well of the lysis buffer (20 mM Tris-HCl, pH 7.5, 5 mM EDTA, 5 mM EGTA, 50 mM NaF, 1 mM NH₄VO₄, 30 mM Na₄P₂O₇, 50 mM NaCl, 1% Triton X-100, 1x complete protease inhibitor cocktail). Protein concentrations were determined chromometrically with a microplate method. Samples of equal amounts of protein (50 μ g) were subject to SDS-PAGE and Western blotting analyses for phosphorylation of Erk1/2 with an anti-phosphor-Erk antibody. A parallel gel was run for detection of total Erk1/2 with an anti-Erk antibody.

Immunofluorescence

HUVE cells were seeded on coverslips placed in 6-well plates at a density of 5×10^4 per well, and cultured in full medium overnight. The cells were washed with serum-free HEM and incubated with 1 μ g/ml ZF-RNases or hANG at 37 °C for 1 h. The cells were then washed with PBS and fixed in -20 °C methanol for 10 min, blocked with 30 mg/ml BSA and incubated with 10 μ g/ml anti-ZF-RNase polyclonal antibody or anti-hANG monoclonal antibody (26 -2F) at 4 °C overnight. Anti-ZF-RNase polyclonal antibody was prepared using ZF-RNase-3 as the immunogen. This antibody recognizes all three isoforms of ZF-RNases but not hANG and RNase A as determined by Western blotting. It does not stain untreated HUVE and HeLa cells in immunofluorescence experiments. After extensive washing with PBS, the bound primary antibodies were visualized by Alexa 488-labeled goat F(ab')₂ anti-rabbit and anti-mouse IgG, respectively.

Nuclear translocation of 125 I-labelled ZF-RNases

Confluent HeLa cells (2.5×10^5 cells/well in 6-well plates) were incubated with labeled proteins (1 μ g/ml) for 1 h at 37 °C in serum-free DMEM. At the end of incubation, cells were washed three times with PBS at 4 °C for 5 min and once with 50 mM Gly, pH 3.0, for 2 min on ice. The cells were then detached by scraping and lysed for 30 min on ice with 0.5% Triton in PBS containing 1x protease inhibitor cocktail. The cell lysates were centrifuged at $1000 \times g$ for 5 min and the nuclear fractions were washed twice with PBS, and analyzed by SDS-PAGE and autoradiography.

Northern blot analyses

Subconfluent HeLa cells were incubated with ZF-RNases or hANG (1 μ g/ml) at 37 °C for 1 h. Total RNA was extracted with Trizol reagent and separated on agarose-formaldehyde gels, and transferred to a nylon membrane. The probes for 47S rRNA and β -actin have the sequences of 5'-ggtcgccagaggacagcgtgtcag-3' and 5'-ggagccgtgtgtcagcagcagcgcgG-3' that hybridize with nucleotides 2–25 of 47S rRNA and nucleotide 57–83 of β -actin mRNA, respectively. The probes were freshly labeled with γ -³²P-ATP by T4 polynucleotide kinase. The densitometry scans of the gel were analyzed with software Scion Image for Windows (version beta 4.0.2).

Quantitative RT-PCR (qRT-PCR) analysis of 47S rRNA

cDNA was synthesized using Quantitect Reverse Transcription kit from 1 µg of DNase-treated total RNA. Real-time qRT-PCR on cDNAs was carried out on Light CyclerO 480 SYBR Green I Master with the Light Cycler 480 Detection System (Roche), Cycling conditions were: 95 °C, 5 min; (95 °C, 10 sec; 60 °C, 10 sec) x 40; 72 °C, 15 sec. The primers used for the PCR were designed with PrimerDesigner 2.0 software and have the following sequences: forward, 5'-CTCGCCAAATCGACCTCGTA-3'; reverse, 5'-CACGAGCCGAGTGATCCAC-3', which are complementary to nucleotides 6603–6622 and 6635–6653 of the 47S rRNA (GenBank accession number U13369), respectively. The primers were first confirmed for their ability to amplify the correct replicon by RT-PCR. qRT-PCR were performed in triplicate and the results were analyzed using the comparative Ct method normalized against the housekeeping gene GAPDH and HPRT [67]. The range of expression levels was determined by calculating the standard deviation of the DCt [68].

Cleavage of rRNA precursor

Two substrates, both containing the A₀ cleavage site of rRNA precursor, with the sequences of 5'-uggccggccggccuccgcuccggggggcucucgaucgaugu-3' and 5'-gggggggcucucgaucgaugu-3', respectively, were used. These substrates were synthesized by IDT, purified by HPLC, and end-labeled with T4 polynucleotide kinase and (γ-³²P)-ATP. The radio-labeled substrate, 1 pmol, was mixed with unlabeled substrate, 4 pmol, and incubated with 1 pmol of ZF-RNase-1, -2, -3, RNase A or hANG in a final volume of 15 µl reaction buffer containing 50 mM Tris-HCl, 50 mM NaCl, 0.5 mM MgCl₂, pH 7.4, at 37 °C. Therefore, the final concentrations of enzyme and substrate were 0.06 and 0.3 µM, respectively. After incubation, an aliquot of 5 µl samples were removed and mixed with RNA sequencing loading buffer (95% formamide, 18 mM EDTA, 0.025% SDS, 0.025% xylene cyanol, 0.025% bromophenol blue). The samples were analyzed in 20% acrylamide/7 M urea sequencing gel in 1 × TBE buffer. After electrophoresis, the gel was wrapped by plastic films and put at -80 °C for 30 min. The frozen gel was then autoradiographed.

Abbreviations

ALS, amyotrophic lateral sclerosis
 ANG, angiogenin
 bFGF, basic fibroblast growth factor
 hANG, human angiogenin
 HEM, human endothelial serum-free medium
 HUVE, human umbilical vein endothelial
 RNase, ribonuclease
 rDNA, ribosomal DNA
 rRNA, ribosomal RNA
 WT, wild type
 ZF-RNase, zebrafish ribonuclease

References

1. Beintema, JJ.; Breukelman, HJ.; Carsana, A.; Furia, A. Evolution of Vertebrate Ribonucleases: Ribonuclease A Superfamily.. In: D'Alessio, G.; Riordan, JF., editors. Ribonucleases: Structures and Functions. Academic Press; San Diego: 1997. p. 245-269.
2. Riordan, JF. Structure and Function of Angiogenin.. In: D'Alessio, G.; Riordan, JF., editors. Ribonucleases: Structures and Functions. Academic Press; New York: 1997. p. 446-466.
3. Matousek J. Aspermatogenic effect of the bull seminal ribonuclease (BS RNase) in the presence of anti-BS RNase antibodies in mice. Anim Genet 1994;25(Suppl 1):45–50. [PubMed: 7943983]

4. Harder J, Schroder JM. RNase 7, a novel innate immune defense antimicrobial protein of healthy human skin. *J Biol Chem* 2002;277:46779–46784. [PubMed: 12244054]
5. Youle RJ, D'Alessio G. Antitumor RNases. In: D'Alessio G, Riordan JF, editors. *Ribonucleases Structures and Function*. Academic Press; San Diego: 1997. p. 491-514.
6. Tamburrini M, Scala G, Verde C, Ruocco MR, Parente A, Venuta S, D'Alessio G, Tamburrini M, Scala G, Verde C, Ruocco MR, Parente A, Venuta S, D'Alessio G. Immunosuppressive activity of bovine seminal RNase on T-cell proliferation. *Eur J Biochem* 1990;190:145–148. [PubMed: 2364942]
7. Rosenberg HF. Recombinant human eosinophil cationic protein. Ribonuclease activity is not essential for cytotoxicity. *J Biol Chem* 1995;270:7876–7881. [PubMed: 7713881]
8. Huang YC, Lin YM, Chang TW, Wu SJ, Lee YS, Chang MD, Chen C, Wu SH, Liao YD. The flexible and clustered lysine residues of human ribonuclease 7 are critical for membrane permeability and antimicrobial activity. *J Biol Chem* 2007;282:4626–4633. [PubMed: 17150966]
9. Fett JW, Strydom DJ, Lobb RR, Alederman EM, Bethune JL, Riordan JF, Vallee B. Isolation and characterization of angiogenin, an angiogenic protein from human carcinoma cells. *Biochemistry* 1985;24:5480–5486. [PubMed: 4074709]
10. Strydom DJ. The angiogenins. *Cell Mol Life Sci* 1998;54:811–824. [PubMed: 9760990]
11. Nitto T, Dyer KD, Czapiga M, Rosenberg HF. Evolution and function of leukocyte RNase A ribonucleases of the avian species, *Gallus gallus*. *J Biol Chem* 2006;281:25622–25634. [PubMed: 16803891]
12. Cho S, Zhang J. Zebrafish ribonucleases are bactericidal: implications for the origin of the vertebrate RNase A superfamily. *Mol Biol Evol* 2007;24:1259–1268. [PubMed: 17347156]
13. Kazakou K, Holloway DE, Prior SH, Subramanian V, Acharya KR. Ribonuclease A homologues of the zebrafish: polymorphism, crystal structures of two representatives and their evolutionary implications. *J Mol Biol* 2008;380:206–222. [PubMed: 18508078]
14. Pizzo E, Buonanno P, Di Maro A, Ponticelli S, De Falco S, Quarto N, Cubellis MV, D'Alessio G. Ribonucleases and angiogenins from fish. *J Biol Chem* 2006;281:27454–27460. [PubMed: 16861230]
15. Pizzo E, Varcamonti M, Di Maro A, Zanfardino A, Giancola C, D'Alessio G. Ribonucleases with angiogenic and bactericidal activities from the Atlantic salmon. *FEBS J* 2008;275:1283–1295. [PubMed: 18279393]
16. Hooper LV, Stappenbeck TS, Hong CV, Gordon JI. Angiogenins: a new class of microbicidal proteins involved in innate immunity. *Nat Immunol* 2003;4:269–273. [PubMed: 12548285]
17. Shapiro R, Riordan JF, Vallee BL. Characteristic ribonucleolytic activity of human angiogenin. *Biochemistry* 1986;25:3527–3532. [PubMed: 2424496]
18. Hu G, Riordan JF, Vallee BL. Angiogenin promotes invasiveness of cultured endothelial cells by stimulation of cell-associated proteolytic activities. *Proc Natl Acad Sci U S A* 1994;91:12096–12100. [PubMed: 7991590]
19. Hu GF, Riordan JF, Vallee BL. A putative Angiogenin receptor in Angiogenin-responsive human endothelial cells. *Proc Natl Acad Sci U S A* 1997;94:2204–2209. [PubMed: 9122172]
20. Jimi S, Ito K, Kohno K, Ono M, Kuwano M, Itagaki Y, Ishikawa H. Modulation by bovine angiogenin of tubular morphogenesis and expression of plasminogen activator in bovine endothelial cells. *Biochem Biophys Res Commun* 1995;211:476–483. [PubMed: 7540839]
21. Xu ZP, Tsuji T, Riordan JF, Hu GF. The nuclear function of angiogenin in endothelial cells is related to rRNA production. *Biochem Biophys Res Commun* 2002;294:287–292. [PubMed: 12051708]
22. Li R, Riordan JF, Hu G. Nuclear translocation of human angiogenin in cultured human umbilical artery endothelial cells is microtubule and lysosome independent. *Biochem Biophys Res Commun* 1997;238:305–312. [PubMed: 9299500]
23. Hu G, Xu C, Riordan JF. Human angiogenin is rapidly translocated to the nucleus of human umbilical vein endothelial cells and binds to DNA. *J Cell Biochem* 2000;76:452–462. [PubMed: 10649442]
24. Kishimoto K, Liu S, Tsuji T, Olson KA, Hu GF. Endogenous angiogenin in endothelial cells is a general requirement for cell proliferation and angiogenesis. *Oncogene* 2005;24:445–456. [PubMed: 15558023]

25. Tsuji T, Sun Y, Kishimoto K, Olson KA, Liu S, Hirukawa S, Hu GF. Angiogenin is translocated to the nucleus of HeLa cells and is involved in ribosomal RNA transcription and cell proliferation. *Cancer Res* 2005;65:1352–1360. [PubMed: 15735021]
26. Wu D, Yu W, Kishikawa H, Folkerth RD, Iafrate AJ, Shen Y, Xin W, Sims K, Hu GF. Angiogenin loss-of-function mutations in amyotrophic lateral sclerosis. *Ann Neurol* 2007;62:609–617. [PubMed: 17886298]
27. Greenway MJ, Alexander MD, Ennis S, Traynor BJ, Corr B, Frost E, Green A, Hardiman O. A novel candidate region for ALS on chromosome 14q11.2. *Neurology* 2004;63:1936–1938. [PubMed: 15557516]
28. Conforti FL, Sprovieri T, Mazzei R, Ungaro C, La Bella V, Tessitore A, Patitucci A, Magariello A, Gabriele AL, Tedeschi G, Simone IL, Majorana G, Valentino P, Condino F, Bono F, Monsurro MR, Muglia M, Quattrone A. A novel Angiogenin gene mutation in a sporadic patient with amyotrophic lateral sclerosis from southern Italy. *Neuromuscul Disord* 2007;18:68–70. [PubMed: 17703939]
29. Gellera C, Colombrita C, Ticozzi N, Castellotti B, Bragato C, Ratti A, Taroni F, Silani V. Identification of new ANG gene mutations in a large cohort of Italian patients with amyotrophic lateral sclerosis. *Neurogenetics* 2008;9:33–40. [PubMed: 18087731]
30. Greenway MJ, Andersen PM, Russ C, Ennis S, Cashman S, Donaghy C, Patterson V, Swingle R, Kieran D, Prehn J, Morrison KE, Green A, Acharya KR, Brown RH Jr, Hardiman O. ANG mutations segregate with familial and ‘sporadic’ amyotrophic lateral sclerosis. *Nat Genet* 2006;38:411–413. [PubMed: 16501576]
31. Crabtree B, Thiagarajan N, Prior SH, Wilson P, Iyer S, Ferns T, Shapiro R, Brew K, Subramanian V, Acharya KR. Characterization of human angiogenin variants implicated in amyotrophic lateral sclerosis. *Biochemistry* 2007;46:11810–11818. [PubMed: 17900154]
32. Subramanian V, Feng Y. A new role for angiogenin in neurite growth and pathfinding: implications for amyotrophic lateral sclerosis. *Hum Mol Genet* 2007;16:1445–1453. [PubMed: 17468498]
33. Subramanian V, Crabtree B, Acharya KR. Human angiogenin is a neuroprotective factor and amyotrophic lateral sclerosis associated angiogenin variants affect neurite extension/pathfinding and survival of motor neurons. *Hum Mol Genet* 2008;17:130–149. [PubMed: 17916583]
34. Cho S, Zhang J. Ancient expansion of the ribonuclease A superfamily revealed by genomic analysis of placental and marsupial mammals. *Gene* 2006;373:116–125. [PubMed: 16530354]
35. Childs S, Chen JN, Garrity DM, Fishman MC. Patterning of angiogenesis in the zebrafish embryo. *Development* 2002;129:973–982. [PubMed: 11861480]
36. Lemmens R, Van Hoecke A, Hersmus N, Geelen V, D'Hollander I, Thijs V, Van Den Bosch L, Carmeliet P, Robberecht W. Overexpression of mutant superoxide dismutase 1 causes a motor axonopathy in the zebrafish. *Hum Mol Genet* 2007;16:2359–2365. [PubMed: 17636250]
37. Winkler C, Eggert C, Gradl D, Meister G, Giegerich M, Wedlich D, Lagerbauer B, Fischer U. Reduced U snRNP assembly causes motor axon degeneration in an animal model for spinal muscular atrophy. *Genes Dev* 2005;19:2320–2330. [PubMed: 16204184]
38. Acharya KR, Shapiro R, Allen SC, Riordan JF, Vallee BL. Crystal structure of human angiogenin reveals the structural basis for its functional divergence from ribonuclease. *Proc Natl Acad Sci U S A* 1994;91:2915–2919. [PubMed: 8159679]
39. Shapiro R, Vallee BL. Site-directed mutagenesis of histidine-13 and histidine-114 of human angiogenin. Alanine derivatives inhibit angiogenin-induced angiogenesis. *Biochemistry* 1989;28:7401–7408. [PubMed: 2479414]
40. Adams SA, Subramanian V. The angiogenins: an emerging family of ribonuclease related proteins with diverse cellular functions. *Angiogenesis* 1999;3:189–199. [PubMed: 14517418]
41. Yoshioka N, Wang L, Kishimoto K, Tsuji T, Hu GF. A therapeutic target for prostate cancer based on angiogenin-stimulated angiogenesis and cancer cell proliferation. *Proc Natl Acad Sci U S A* 2006;103:14519–14524. [PubMed: 16971483]
42. Badet J, Soncin F, Guitton JD, Lamare O, Cartwright T, Barritault D. Specific binding of angiogenin to calf pulmonary artery endothelial cells. *Proc Natl Acad Sci U S A* 1989;86:8427–8431. [PubMed: 2813401]
43. Liu S, Yu D, Xu ZP, Riordan JF, Hu GF. Angiogenin activates Erk1/2 in human umbilical vein endothelial cells. *Biochem Biophys Res Commun* 2001;287:305–310. [PubMed: 11549292]

44. Kim HM, Kang DK, Kim HY, Kang SS, Chang SI. Angiogenin-induced protein kinase B/Akt activation is necessary for angiogenesis but is independent of nuclear translocation of angiogenin in HUVE cells. *Biochem Biophys Res Commun* 2007;352:509–513. [PubMed: 17125737]
45. Moroianu J, Riordan JF. Nuclear translocation of angiogenin in proliferating endothelial cells is essential to its angiogenic activity. *Proc Natl Acad Sci U S A* 1994;91:1677–1681. [PubMed: 8127865]
46. Xu ZP, Tsuji T, Riordan JF, Hu GF. Identification and characterization of an angiogenin-binding DNA sequence that stimulates luciferase reporter gene expression. *Biochemistry* 2003;42:121–128. [PubMed: 12515546]
47. Eichler DC, Craig N. Processing of eukaryotic ribosomal RNA. *Prog Nucleic Acid Res Mol Biol* 1994;49:197–239. [PubMed: 7863007]
48. Maden BE. The numerous modified nucleotides in eukaryotic ribosomal RNA. *Prog Nucleic Acid Res Mol Biol* 1990;39:241–303. [PubMed: 2247610]
49. Kass S, Craig N, Sollner-Webb B. Primary processing of mammalian rRNA involves two adjacent cleavages and is not species specific. *Mol Cell Biol* 1987;7:2891–2898. [PubMed: 3670298]
50. Miller KG, Sollner-Webb B. Transcription of mouse rRNA genes by RNA polymerase I: in vitro and in vivo initiation and processing sites. *Cell* 1981;27:165–174. [PubMed: 7326749]
51. Mougey EB, Pape LK, Sollner-Webb B. A U3 small nuclear ribonucleoprotein-requiring processing event in the 5' external transcribed spacer of *Xenopus* precursor rRNA. *Mol Cell Biol* 1993;13:5990–5998. [PubMed: 8413202]
52. Craig N, Kass S, Sollner-Webb B. Nucleotide sequence determining the first cleavage site in the processing of mouse precursor rRNA. *Proc Natl Acad Sci U S A* 1987;84:629–633. [PubMed: 3027694]
53. Tello-Montoliu A, Patel JV, Lip GY. Angiogenin: a review of the pathophysiology and potential clinical applications. *J Thromb Haemost* 2006;4:1864–1874. [PubMed: 16961595]
54. Acharya KR, Shapiro R, Riordan JF, Vallee BL. Crystal structure of bovine angiogenin at 1.5-Å resolution. *Proc Natl Acad Sci U S A* 1995;92:2949–2953. [PubMed: 7708754]
55. Chavali GB, Papageorgiou AC, Olson KA, Fett JW, Hu G, Shapiro R, Acharya KR. The crystal structure of human angiogenin in complex with an antitumor neutralizing antibody. *Structure* 2003;11:875–885. [PubMed: 12842050]
56. Shapiro R, Vallee BL. Identification of functional arginines in human angiogenin by site-directed mutagenesis. *Biochemistry* 1992;31:12477–12485. [PubMed: 1281426]
57. Kao RY, Jenkins JL, Olson KA, Key ME, Fett JW, Shapiro R. A small-molecule inhibitor of the ribonucleolytic activity of human angiogenin that possesses antitumor activity. *Proc Natl Acad Sci U S A* 2002;99:10066–10071. [PubMed: 12118120]
58. Olson KA, Byers HR, Key ME, Fett JW. Prevention of human prostate tumor metastasis in athymic mice by antisense targeting of human angiogenin. *Clin Cancer Res* 2001;7:3598–3605. [PubMed: 11705882]
59. Olson KA, Fett JW, French TC, Key ME, Vallee BL. Angiogenin antagonists prevent tumor growth in vivo. *Proc Natl Acad Sci U S A* 1995;92:442–446. [PubMed: 7831307]
60. Kishikawa H, Wu D, Hu GF. Targeting angiogenin in therapy of amyotrophic lateral sclerosis. *Expert Opin Ther Targets* 2008;12:1229–1242. [PubMed: 18781822]
61. Moroianu J, Riordan JF. Identification of the nucleolar targeting signal of human angiogenin. *Biochem Biophys Res Commun* 1994;203:1765–1772. [PubMed: 7945327]
62. Comai L. The nucleolus: a paradigm for cell proliferation and aging. *Braz J Med Biol Res* 1999;32:1473–1478. [PubMed: 10585627]
63. Melese T, Xue Z. The nucleolus: an organelle formed by the act of building a ribosome. *Curr Opin Cell Biol* 1995;7:319–324. [PubMed: 7662360]
64. Stoykova AS, Dabeva MD, Dimova RN, Hadjiolov AA. Ribosome biogenesis and nucleolar ultrastructure in neuronal and oligodendroglial rat brain cells. *J Neurochem* 1985;45:1667–1676. [PubMed: 3850925]
65. Russo N, Shapiro R, Acharya KR, Riordan JF, Vallee BL. Role of glutamine-117 in the ribonucleolytic activity of human angiogenin. *Proc Natl Acad Sci U S A* 1994;91:2920–2924. [PubMed: 8159680]

66. Shapiro R, Harper JW, Fox EA, Jansen HW, Hein F, Uhlmann E. Expression of Met-(−1) angiogenin in *Escherichia coli*: conversion to the authentic less than Glu-1 protein. *Anal Biochem* 1988;175:450–461. [PubMed: 3071185]
67. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* 2002;3:RESEARCH0034. [PubMed: 12184808]
68. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 2001;29:e45. [PubMed: 11328886]

Fig. 1.

Angiogenic activity of zebrafish RNases. HUVE cells were seeded in Matrigel-coated 48-well plates (150 μ l/well) at a density of 4×10^4 /well. Zebrafish RNases and hANG were added at the final concentration indicated and incubated for 4h. Tubular structures were indicated by arrows. Bar, 250 μ m.

Fig. 2.

Binding of zebrafish RNases to HUVE and HeLa cells. (A) HUVE cells. ¹²⁵I-labeled proteins (60 nM) were incubated with HUVE cells for 1 h at 4 °C in the absence or presence of unlabeled hANG. Bound proteins were detached with 0.6 M NaCl and the amount of detached proteins was determined by gamma counting. Data shown are percentage of inhibition by 12 μM (200-fold molar excess) of unlabeled hANG. (B) HeLa cells. ¹²⁵I-labeled proteins were incubated with HeLa cells for 1 h at 4 °C in the absence (Δ, total binding) or presence (□, nonspecific binding) of a 200-fold molar excess of the unlabelled proteins. Specific bindings (▲) were obtained by subtracting the non-specific binding from the total binding. Values were normalized to 1 × 10⁶ cells. Insets, Scatchard analyses of the specific binding data. (C) Competition between hANG and ZF-RNases in binding to HeLa cells. Cells were incubated for 1 h at 4 °C with 60 nM of the ¹²⁵I-labeled ZF-RNase-1 (○), ZF-RNase-2 (■), ZF-RNase-3 (□), and hANG (●) in the presence of increasing concentrations of unlabeled hANG. Data shown are percentage of inhibition at the given concentration of unlabeled hANG.

Fig. 3.

Zebrafish RNases induce Erk1/2 phosphorylation in HUVE cells. HUVE cells were cultured at a density of 5×10^3 cells per cm^2 in full medium for 24 h, starved in serum-free HEM for another 24 h, and stimulated with 1 $\mu\text{g}/\text{ml}$ ZF-RNases for 1, 5, 10, and 30 min. Cell lysates were analyzed for Erk1/2 phosphorylation by Western blotting with an anti-phosphorylated Erk1/2 antibody. A parallel gel was run in each experiment and analyzed for total Erk1/2 with an anti-Erk1/2 antibody.

Fig. 4.

Nuclear localization of zebrafish RNases. (A) nuclear translocation of ZF-RNases in HUVE cells. Cells were incubated with 1 μ g/ml of hANG or ZF-RNases at 37 °C for 1h. hANG was visualized with 26–2F and Alexa 488-labeled anti-mouse IgG. ZF-RNases were visualized with anti-ZF-RNases polyclonal antibody and Alexa 488-labeled anti-rabbit IgG. Insets, higher magnification images of nuclear ZF-RNases. (B) nuclear translocation of 125 I-labeled RNases in HeLa cells. HeLa cells were cultured in six-well plates (2×10^5 cells/well) and incubated for 1 h at 37 °C with 1 μ g/ml of the 125 I-labeled hANG and ZF-RNases. Nuclear fractions were isolated and analyzed by SDS-PAGE and autoradiography. Lanes 1, 3, 5, and 7; purity of the 125 I-labeled hANG, ZF-RNase-1, -2, and -3, respectively. Lanes 2, 4, 6, and 8; nuclear fractions isolated from cells treated with 125 I-labeled hANG, ZF-RNase-1, -2, and -3, respectively.

Fig. 5.

Zebrafish RNases stimulate rRNA transcription. HeLa cells were incubated at 37 °C for 1h in the absence or presence of 1 µg/ml of ZF-RNases or hANG. Total cellular RNA was isolated by Trizol. (A) Northern blot analyses. Left panel, total RNA was extracted and analyzed with probes specific for 47S rRNA and for actin mRNA. Right panel, relative density of 47S rRNA to actin mRNA. * indicates $p < 0.01$. (B) Quantitative RT-PCR analyses. Both 47S and 45S rRNA are amplified with the primer set used in these experiments. Data shown are means plus SD of the triplicates.

Fig. 6.

Cleavage of pre-ribosomal RNA by zebrafish RNases. RNA substrates with the sequence corresponding to the A₀ cleavage site (cucuuc) of the 47S pre-rRNA and the flanking regions were chemically synthesized and end-labeled with ³²P. The radio-labeled RNA (1 pmole) was mixed with 4 pmole of unlabeled substrate, and was incubated with 1 pmole of enzyme in 15 µl of 50 mM Tris, pH 8.0, containing 50 mM NaCl and 0.5 mM MgCl₂ at 37 °C. (A) cleavage of a 43 nt substrate (5'-uggccggccggccuccgcucccggggggcucuucgaucgaugu-3') by hANG, RNase A, and ZF-RNase-1, -2, and -3, at 37 °C for 15 min. (B) cleavage of a 19 nt substrate (5'-gggggggcucuucgaucg-3') by hANG, ZF-RNase-1, -2, and -3 for 1 and 5 min. The reactions were terminated by adding an equal volume of 20% perchloric acid. RNA was extracted, separated on a 20% urea-polyacrylamide gel, and visualized by autoradiography. No proteins were added to the controls.