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Argonaute proteins in mammals, revealing individual functions

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Liesbeth Kerkhof

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Supervised by Joost Kluiver

Abstract
Argonaute (Ago) proteins are part of the RNA-induces silencing (RISC) complex and play a key role in RNA interference. They can bind small RNA, which leads the Ago proteins to their (partial) complementary mRNA. Ago proteins silence the gene function of the target mRNA by either RNA degradation (also known as 'slicing') or translation inhibition. Argonaute proteins are found not only in mammals, but also in plants, worms, fughi, archaea and certain bacteria and are highly conserved between species.

In humans, there are four Argonaute proteins, Ago1-4. They are ubiquitously expressed in various cell types and seem to have overlapping functions. Only Ago-2 stands out for its unique slicer activity and is also located on a different chromosome than Ago1,3,4. The different chromosome location might be the reason only Ago2 contains the slicer activity. But why are there four different types of Argonaute proteins, when they seem to have similar functions? In *Drosophila* and *C. elegans,* the individual Ago proteins have specialized functions. Since the high conservation between species of the Ago proteins, human Argonautes might also have individual functions. However, the functions of the four humans Argonautes and the reason why there are four Ago proteins, remain unclear. Since Ago proteins are associated with several diseases like cancer, fragile X syndrome and autoimmune diseases, it is important to understand the functions of the individual Ago proteins.

The aim of this paper is to learn why there are four different human Argonaute proteins and reveal their individual functions. The results show that Ago2 is not capable of operating on its own and all human Ago proteins can load the same pool of miRNAs. This rises the theory that the four Ago proteins serve as a backup mechanism in case one of the Ago proteins is deficient. The combination of different Ago proteins could be evolutionary beneficial and leads to higher survival in case an Argonaute protein is not functioning.

However, the results also show that Ago3,4 expression levels are much lower in various tissues than Ago1,2 expression levels. This creates the theory that four Ago proteins are not evolutionary beneficial and Ago3,4 might disappear in the future, due to redundancy.

In conclusion, it is too soon to draw solid conclusions about why there are four human Ago proteins. Both the backup theory and the redundancy theory could occur. More research is necessary to determine their individual functions and reveal why all four are required.

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1.0 Introduction

Argonaute (Ago) proteins are essential compounds of the RNA-induced silencing complex (RISC) and play a key role in different RNA gene silencing pathways also known as RNA interference (RNAi). Argonaute proteins can bind to different types of small RNA, including small interfering RNA (siRNA), microRNA (miRNA) and Piwi- interacting RNA (piRNA)[1]. The small RNAs lead the Argonaute proteins to their (partial) complementary sequence, silencing it by either RNA degradation, also known as 'slicing', or by inhibiting translation.

Argonaute proteins are found not only in mammals, but also in plants, worms, fungi, archaea and certain bacteria and are highly conserved between species. The name Argonaute originates from the Ago1 mutant of the plant Arabidopsis thaliana described by Bohmert et al[2]. Because of the mutation, leaves develop abnormally resulting in a squid-like leave morphology resembling the Argonauta argo squid.

In mammals, the Argonaute family can be subdivided into the Ago subfamily(Ago 1-4) and the Piwi subfamily (PIWIL1/HIWI, PIWIL2/HILI, PIWIL3, and PIWIL4/HIWI2). Ago1-4 subfamily is ubiquitously expressed in various cell types like thymus, heart, kidney, testes[3]. The Piwi subfamily appears to be only expressed in hematopoietic stem cells[4] and germ cell lines[3].

Human Argonaute proteins have a molecular weight of approximately 100kDa[5] and seem to have similar functions in RNAi. Only Ago2 stands out for its unique slicer activity. Ago1, Ago3 and Ago4 genes are clustered together and located on chromosome 1p34.3, while the Ago2 gene is located on chromosome 8q24.3[6]. Since the four Ago proteins have overlapping functions in RNAi (the process of RNAi is discussed in 'RNA silencing pathways'), it seems remarkable that the proteins are located on different chromosomes. Does this clustering give Ago1,3,4 the same functions, or do they have specific functions which explains why there are four different types of human Ago proteins? It has not yet been determined whether this clustering of Ago 1,3,4 results in similar functions. However, since Ago2 is the only Ago protein that contains slicer activity, it might be possible that the slicer activity is the consequence of the different chromosomal location.

Argonaute proteins seem to be associated with diseases like systemic autoimmune diseases, cancer and Fragile X syndrome. In systemic autoimmune diseases, an autoimmune response is initiated to components of the RNAi pathway , in which Argonaute proteins play key roles[7]. Ago protein levels are elevated in epithelial skin cancer[8], colon cancer[9], breast cancer[10] and serous ovarian carcinoma[11]. In Fragile X syndrome, there is no expression of the Fragile x mental retardation protein (FMRP). Ago1 was described to be essential for normal functioning of FMRP, which is required for normal neuronal development[12]. Because Argonaute proteins are associated with several diseases, it is important to identify the specific Ago functions in human gene silencing.

As mentioned earlier, Ago proteins have been demonstrated in many species and are highly conserved. Studies in *Caenorhabditis elegans, Drosophila Melanogaster* and *Arabidopsis thaliana* revealed that different members of Ago proteins have specialized functions in RNA mediated gene silencing. RISC-silencing therefore not only dependents on the complementarity between the smallRNA and its target mRNA, but also the type of Ago which serves its own specialized function. In *D. melanogaster* and *C. elegans* for example, perfectly matched siRNA is actively loaded into the Ago2 RISC complex, where bulged miRNAs are loaded into the Ago1 RISC complex[13]. It seems like multiple Ago family members have evolved into specific functions, to ensure RNA mediated gene silencing is effective. Because Ago proteins are highly conservated between species, human Argonautes might also have individual functions. However, the individual functions of the human Argonautes and the reason why there are four Ago proteins, remain unclear.

The aim of this paper is to better understand the functions of the Ago proteins in

mammals and learn why there are four different human Argonaute proteins. The overall concept of Argonaute proteins functioning in RNAi will be discussed. Also in terms of where these proteins are located and which functional domains they contain. After that, a summary of the Argonaute protein functions is given and a conclusion is given about why there are four different human Argonaute proteins.

2.0 Argonaute characteristics

2.1 Argonaute Distribution

Ago1-4 are not localized randomly throughout the cell. Localization studies showed that they seem to concentrate in specific cytoplasmic foci, also known as cytoplasmic processing bodies (P-bodies). P-bodies are aggregates of specific mRNAs and several proteins, like Ago. They store silenced mRNAs which can leave the P-bodies again to yet undergo translation. They also store mRNAs that are predetermined of deadenylation and degradation. P-bodies are also called scaffolding centers of miRNA function. Some proteins are core components of P-bodies, others are involved in recognizing mRNAs. Ago proteins possibly target mRNAs to these P-bodies to silence translation and promote degradation[14]. However, analyses in mammalian cells showed that Ago2 is also localized in the cytoplasm as well as in so called stress granules(SGs). These are granules that are induced under conditions of cellular stress. It is not clear whether Ago1,3,4 are also localized in the cytoplasm and it has not yet been determined which location is essential for optimal Ago protein functioning[6].

However, Liu et al., 2005 found that without P-body component proteins, the RISC activity is inhibited. They state that in consequence, Ago mutants cannot bind miRNA and therefore cannot localize to the P-bodies. They suggest that Ago proteins have to be incorporated in mRNA protein complexes (mRNPs) via miRNA-mRNA interactions to be able to localize the P-bodies and initiate target mRNA degradation and silencing, however more research on this subject is necessary[15].

Leung A. et al., 2006 revealed that siRNA and miRNA gene silencing remained intact in cells without microscopically visible P-bodies and stress granules[16]. In contrast to what Liu et al suggests, this indicates that RNAi processes can occur in the diffuse cytoplasm. When cellular stress is initiated, SGs are formed. mRNAs that are required for stress response and repressed by Ago proteins under normal conditions can yet be translated. They also found that Ago proteins migrate from the cytoplasm to the SGs during the stress response instead of migrating from the P-bodies, which was initially thought.

Yongjun et al., 2010 investigated the role of Ago proteins in gene silencing at the progestoron receptor promoter[17]. For altering gene promoter activity, Ago proteins must also be located in the nucleus. They found that Ago proteins are mostly found in the cytoplasm, but are also found in the nucleus.

These contradicting results indicate that more research is necessary to further reveal the distribution of Argonaute proteins in the cell and their associations with P-bodies and SGs.

2.2 Argonaute functional domains

In the Ago gene, there are two common domains; the N-terminal PAZ (Piwi–Argonaute–Zwille) domain and the C-terminal PIWI (P-element induced wimpy testis) domain. The PIWI domain is approximately 400 amino acids in length and contains the MID and PIWI subdomains. These domains are found in all Argonaute proteins. Figure 1A shows an overview of the different domains.

The PAZ domain is only found in the Argonaute family and in Dicer, both involved in RNAi. It is approximately 130 amino acids in length and is thought to be a protein-protein interaction domain. After being processed by Dicer and Drosha (see the ‘RNA silencing

pathways' paragraph) the miRNA or siRNA is left with a two 3' end overhang. The PAZ domain contains aromatic residues (Tyr309, Tyr314, His269 and Tyr277) which can bind to this 3' overhang by making hydrogen bonds with the oxygens of the phosphate between the two bases in the overhang[18]. Because of this binding the PAZ domain can distinguish them from degraded RNAs that are derived from other pathways[19]. The PAZ domain also displays an OB-fold(oligonucleotide/oligosaccharide binding fold). The OB-fold functions as a single stranded nucleic acid binder. Overall, the PAZ domain can provide a guide strand mediated target recognition.

Structural studies showed that the PIWI domain folds the same way as RNase H[20]. RNase H enzymes cleave RNA by using a DNA template. In Ago proteins the PIWI interacts with the 5' end of the guide strand smallRNA. RNA cleavage requires an catalytic center containing an Asp-Asp-His motif and the binding of a divalent cation for activation[21]. Some Ago proteins contain this cleavage ability which makes them able to cleave the target RNA bound the small RNA which is also referred to as slicer activity. While all Ago proteins contain this PIWI cleavage domain, only Ago2 is known for its slicing capabilities. This remarkable finding will be discussed later in this paper (see the 'Ago2 cleavage activity' paragraph).

The MID domain is the third domain that lies between the PAZ and the PIWI domain, therefore referred to as MID. This domain contains a high basic pocket, which binds the characteristic 5' phosphates of miRNA or siRNA and so anchors them onto the Ago protein[22]. An overview of the Argonaute protein domains and the way Ago folds around smallRNA and mRNA is showed in figure 1.

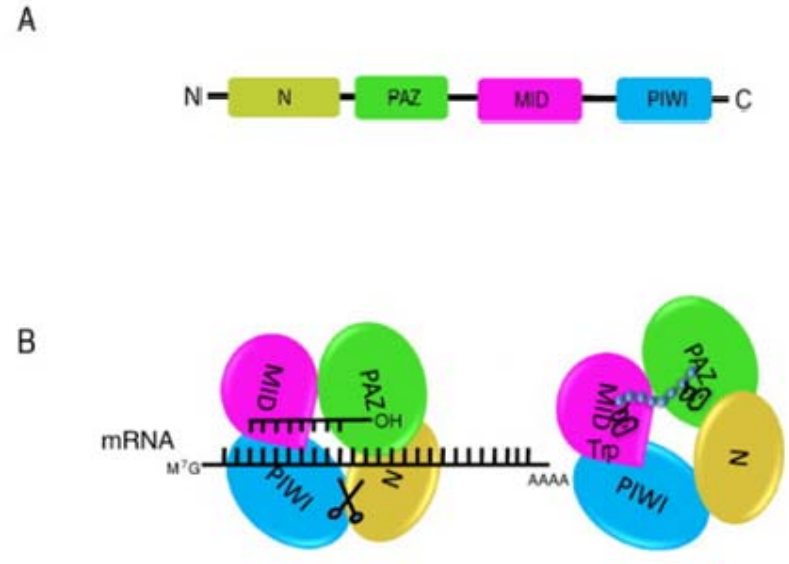


Figure 1. (adapted from Jing Yang, Y. Adam Yuan, 2009).
A. Domain structure of an Argonaute protein. The PAZ domain (green), which is important for guide strand mediated target recognition, the PIWI domain containing MID (pink) for anchoring and PIWI (blue) for cleavage. **B.** Model of the Argonaute protein with smallRNA onto mRNA.

3.0 RNA silencing pathways

Gene silencing requires miRNA of siRNA, also referred to as small RNA. Small RNAs are double stranded and are 19-25 nucleotides in length. As mentioned before they contain 5' phosphate groups and 3' 2-nt overhangs.

SiRNAs are produced from long double stranded RNA. Dicer, an endoribonuclease, cleaves the double stranded RNA(dsRNA) into smaller dsRNA of approximately 20-25 nucleotides called siRNA. The double stranded siRNA is unwound into two single stranded RNA strands (ssRNA) by RHA (RNA helicase A). The so called passenger stand will then be discarded, while the guide strand will be incorporated into the RNA induced silencing complex (RISC). RHA promotes RISC complex assembly by promoting the association of siRNA with Ago2[24]. The siRNA will guide the RISC complex to the perfectly complementary target mRNAs where Ago2 cleaves the target mRNA, which then will be degraded and will lead to loss of gene function.

The RISC complex is a large multi-protein complex. Gregory RI et al 2005, showed that the human RISC complex contains Ago2, Dicer and TRBP (human immunodeficiency virus (HIV) Transactivating Response RNA Binding Protein) by immune-purification and size-exclusion chromatography[25]. The RISC complex is also known as the RISC loading complex for its ability to cleave pre-miRNA and load the guide miRNA into Ago2[25].

MiRNA molecules are approximately 22 nucleotides in length and characterized by their hairpin structure. Unlike siRNAs, miRNAs are encoded in the genome and are transcribed by RNA polymerase II. The transcript is also known as primary miRNA (pri-miRNA) and is approximately 70 nucleotides in length. The pri-miRNA is then processed by the microprocessor complex which contains Drosha (another ribonuclease) and the double standed RNA binding protein DiGeorge syndrome region 8 gene (DGCR8)[24]. The pri-miRNA hairpin is recognized by the DGCR8 and then associates with Drosha. Drosha cleaves the RNA approximately eleven nucleotides from the hairpin base into precursor miRNA (pre-miRNA). The pre-miRNA is then transported to the cytoplasm by the export receptor Exportin-5 where the hairpin structure is recognized by Dicer[26]. Dicer interacts with the 3' end of the hairpin and cleaves the loop attached to the 3' and 5' arms into a 21-25 nucleotides long dsRNA intermediates containing the characteristic 5' phosphate groups and the 2-nt 3' overhang as mentioned before[16]. The dsRNA is separated into ssRNA where the guide strand (mature miRNA) is incorporated into the RISC complex. The miRNA recognizes target sequences which are most found in the 3' untranslated region of the mRNA, where the imperfect base pairing miRNA;mRNA leads to translation repression or leads to removal of the poly A tail of the mRNA which causes destabilization and degradation[27].
Figure 3 below shows an overview of the siRNA and miRNA pathway.

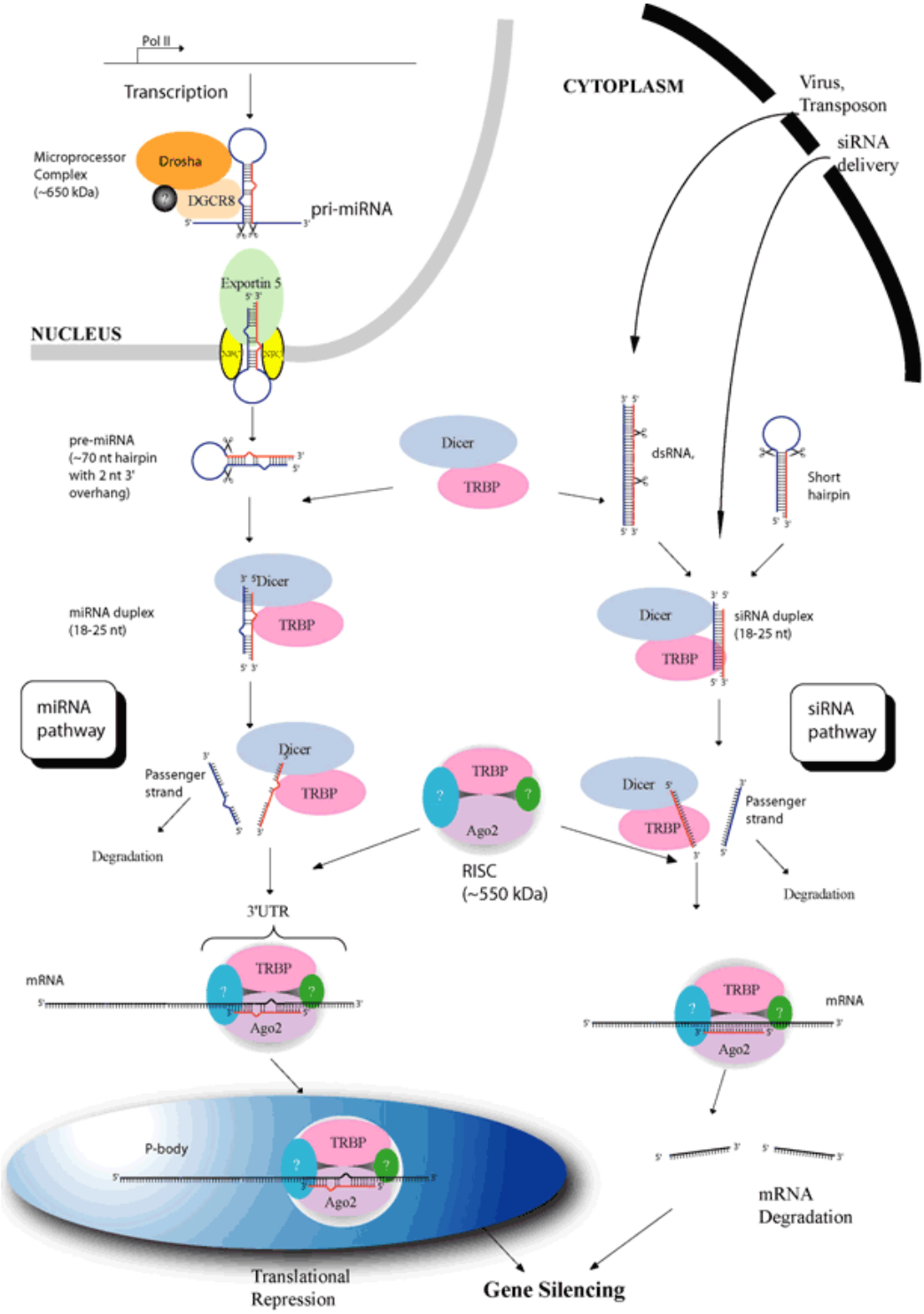


Figure 3 (adapted from Man Lung Yeung et al., 2005).
An overview of the miRNA and siRNA pathways in gene silencing. (Left) Double stranded Pri-miRNA is transcribed by RNA polymerase II and then processed by Drosha and DGCR8 into pre-miRNA. Pre-miRNA is transported to the cytoplasm by exportin-5 and there cleaved by Dicer. It is then separated into single strand RNA and incorporated into the RISC complex. Imperfect base pairing of the mRNA and the miRNA leads to translation repression. (Right) double stranded SiRNAs are cleaved by dicer and separated into single stranded siRNA. The guide strand is incorporated into the RISC complex and binds to the perfect complementary target mRNA. Ago2 then cleaves the target mRNA which will be degraded and will lead to loss of gene function.

4.0 Argonaute functions

So far, it seems that human Ago1-4 have overlapping functions in RNA mediated gene silencing. But if their functions are basically the same, not four different Ago proteins would be necessary, due to redundancy. It is possible that many individual Ago functions are still undetected. Revealing these functions might explain why all four Ago proteins are required. It is also possible that individual Ago proteins serve tissue specific functions. This could also explain why there are four Ago proteins necessary.

RNA trimming is also an interesting feature to identify Ago specific functions. RNA trimming is a type of post-transcriptional RNA processing where the 3' and 5' ends of the miRNA can be cleaved. It is a similar process like RNA splicing, although splicing cleaves in the middle of the sequence. Since the 3' and 5' end of miRNAs are important in interacting with the PAZ and PIWI domain respectively, RNA trimming might be an interesting feature. It is known that miRNAs associated with Argonautes have a trimmed 3' end in Drosophila[33], but whether this also occurs in mammalian miRNAs was not determined yet.

Studies revealed that the exact functions of the Ago proteins remain unclear. Below, an overview of important results of several recent studies about Argonaute functions is described.

4.1 Tissue specific Argonaute expression

To examine the expression levels of Ago1-4, Valdmanis P et al., 2012 used quantitative RT-PCR of all four human Ago in several body tissues like the brain, kidneys, placenta, skeleton, and testes[23]. Figure 2 below shows the results.

If individual Ago proteins have tissue specific functions, the expression levels of individual Ago proteins would be higher in certain tissues. Figure 2 shows that on average, Ago1 and Ago2 expression was higher than Ago3 and Ago4 expression in the skeletal muscle, brain, placenta, kidney and testes. Overall expression of Ago3 and Ago4 were low in all tissues. For Ago3,4 it seems unlikely, that they have tissue specific functions, since figure 2 shows low Ago3,4 expression in all tissues. Their explanation of the lower expression of Ago3 and Ago4 is that possibly Ago3 and Ago4 contain more so called rare codons. They found that some rare codons like those for valine (GTA) were more frequent in Ago3 and Ago4. Particularly in the N-terminal region. These rare codons would be responsible for RNA instability and could lead to RNA degradation. They also determined that Ago3 and Ago4 proteins levels were declined. This would also be due to the level of rare codons. They were not able to find a solid explanation for the low expression levels of Ago3,4, but they state that certain sequence features in the first half of the mRNA must be responsible.

Figure 2 also shows that Ago2 is the only high Ago expressed in the placenta. This might indicate that Ago2 has more specific functions in the placenta. Apart from that, figure 2 does not suggest that Ago proteins have tissue specific functions. Repeating this experiment might give more clearance about Ago tissue specific expression.

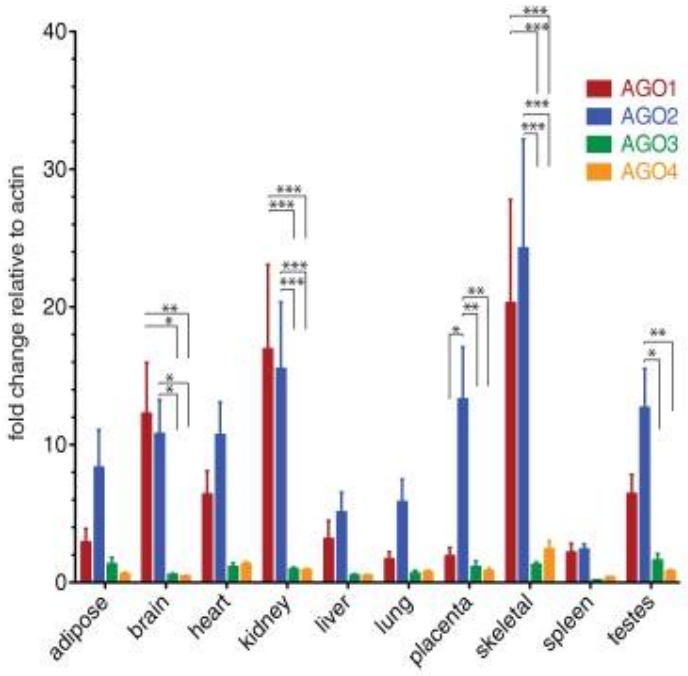


Figure 2. Adapted from Veldmanis et al., 2012
Expression levels of human Argonaute proteins in various tissues. Ago3,4 expression levels are much lower than Ago1,2 expression levels. Ago2 is the only Ago that with high expression in the placenta. This might indicate that is serves specific functions in the placenta. Apart from that, Ago proteins do not seem to have tissue specific functions, according to figure 2.

4.2 Ago2 cleavage activity

We do know that Ago2 is the only Argonaute protein that exhibits mRNA cleavage activity. As mentioned before, Ago2 is located on a different chromosome than Ago1,3,4. The same pattern is found in mice. Mice also have Ago1,3,4 genes on mouse chromosome 4qD2.2 and Ago2 on mouse chromosome 15qD3[23].

Structural studies identified the PIWI domain as the domain responsible for the mRNA cleavage because it resembles RNase H. The Asp-Asp-His motif was thought to be required for cleavage in this domain. Studies of the catalytic center showed that the histidine residue is absent in Ago1 and in Ago4 one of the aspartates is lost. Ago3 on the other hand does have all three residues but is still does not show slicing activity[15]. Since all Ago proteins contain the PIWI domain and Ago3 also contain the Asp-Asp-His motif but has no cleavage capabilities, it is clear that an unknown feature gives Ago2 its cleavage capabilities.

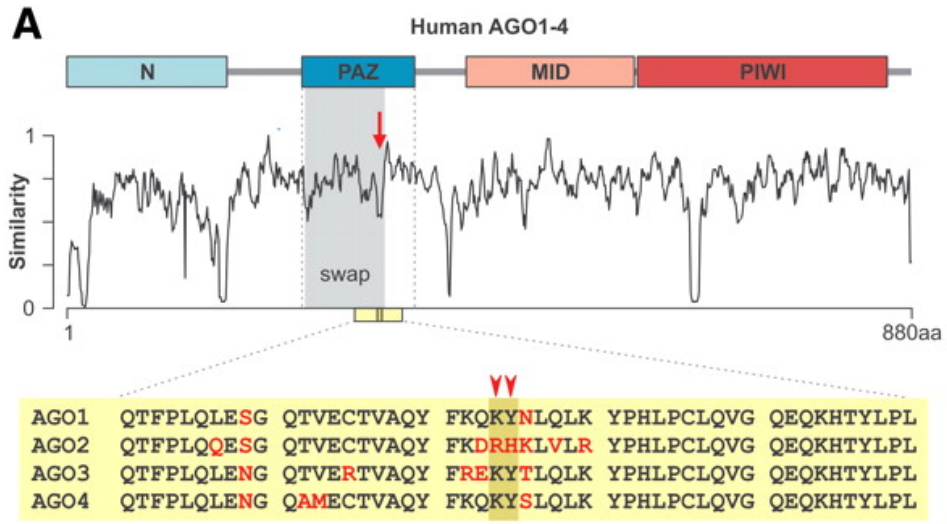
Liu J. et al., 2004 studied whether the PIWI domain of Ago2 provided the cleavage activity to RISC[15]. Therefore they changed two aspartates in the Asp-Asp-His motif into alanine. They predicted that this mutation would inactivate the RISC cleavage. They found that Ago2 did indeed not show RISC cleavage assembly in vitro or in vivo, but it still had the ability to bind siRNA[15]. This would indicate that the mRNA cleavage capabilities of Ago2 would lie in the Asp-Asp-His motif residues. They cannot explain why Ago1,3,4 do not have the cleavage capabilities, but hypothesized that Ago1,3,4 likely fail to coordinate the metal ion or that the structure of the active site is distorted. They also stated that RNase H, which resembles the cleavage activity of Ago2 is proposed to have two metal ions, this might suggest that the non-cleavage Ago proteins might be incapable binding the second metal ion[15]. Others hypothesized that post-translational modifications like phosphorylation or methylation might be associated with the cleavage capabilities of Ago proteins[6].

It is clear that the Asp-Asp-His residues along with the metal ion and possible another

unknown factor play an important role in creating an active cleavage domain. All these factors must be located very precisely, so they can coordinate a site where cleavage can occur.

4.3 3'end Ago2 associated miRNA trimming

Juvvuna PK et al., 2012 investigated whether mammalian miRNAs are also trimmed at the 3' end by measuring the average length of the miRNA during nervous system development[28]. miRNA can be 19 to 25 nucleotides long. They found that the average length of several miRNAs was reduced during nervous system development due to the 3' end miRNA trimming. Dicer was found to process the miRNA into 22-mers where trimming resulted into 21-mers. They linked this finding to an increase in Ago2. Only Ago2 loaded miRNAs were efficiently trimmed and not Ago1,3,4 loaded miRNAs. They explained that only Ago2 is efficient because it contains a special structural motif located in the PAZ domain, containing a RH sequence where the other Agos contain a KY motif. To check if this motif really was responsible for the trimming, they made reciprocal swap mutants of Ago1 with the Ago2 PAZ domain motif(Ago1-RH-PAZ) and Ago2 with the Ago1 PAZ domain (Ago2-KY-PAZ) motif. Remarkably, they found that trimming was more effective in Ago1-RH-PAZ compared to the wild type-Ago1. This is remarkable since trimming of Ago1-loaded miRNA is not efficient. They also found that in Ago2-KY-PAZ trimming was suppressed. This indicates that the RH motif is responsible for the trimming capabilities of Ago2. Figure 4 below shows an overview of the PAZ sequence of all Ago proteins(A). B shows that overall Ago2 is more capable of trimming than Ago1, but when Ago2 contains the Ago1 motif its trimming capabilities decline. Equally, Ago1 is more capable of trimming when it contains the Ago2 motif.



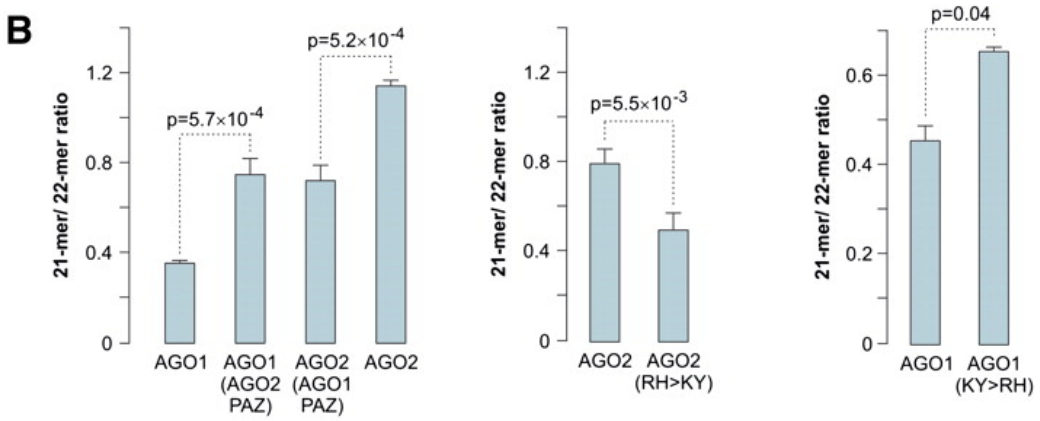


Figure 4 (adapted from Juvvuna PK et al., 2012).
A. PAZ domain sequences of Ago1-4, red arrows indicate the specific motif (RH/KY). **B.** The WT-Ago and mutant-Ago trimming activity.

They explain that the change in PAZ motif results in a loss in hydrogen bonds between the KY motif and the miRNA 3' end. This weakened interaction makes the Ago2 miRNA 3' end more accessible to trimming exonucleases. This study indicates that miRNA is shortened due to 3'end trimming only in Ago2 associated miRNA and not in Ago1,3,4, due to the specific PAZ domain motif.

4.4 Ago2 required for post-implantation development

Morita et al., 2007 used a homozygote Ago2 knockout mice model and showed that the loss of Ago2 leads to embryonic death in mice[29]. The embryos were misshapen and the cell size was irregular. The homozygote mutants showed slightly larger cells which might indicate cell cycle arrest. Remarkably, this was not the case in the Ago1,3,4 knockout of Liu et al., 2004[15]. Morita et al. stated that Ago2 is required for post-implantation development via RNAi. It seems like the Ago2 has a significant function in mouse development. Since Ago1,3,4 do not show this phenotype, it is clear that this cannot be compensated by the remaining Ago proteins.

4.5 Ago1 and Ago2 bind siRNA more efficient than Ago3 and Ago4

Because more is known about Ago2 functioning than the other human Ago members, Hong Su et al., 2009 also used a mouse Ago knockout model to study whether Ago1,3,4 were also necessary in miRNA mediated gene silencing[13]. They found that all the Ago members are required in RNA mediated gene silencing and bind bulged miRNA indistinguishably[13]. So, for miRNA they are all equally used. In the perfect complementary siRNA on the other hand, Ago1 and Ago2 seem to be more efficient in binding than Ago3 and Ago4. This indicates that although Ago1-4 show overlapping functions in RNA gene silencing, individual Agos do seem to have preferences in binding miRNA or siRNA, so a sorting mechanism does seem to be present in human Ago as well. Below, Figure 5 shows an overview of the Ago family member preferences of binding smallRNA.

Figure 5. (adapted from Hong Su et al., 2009).
All human Ago family members bind the bulged miRNA whereas only Ago1 and Ago2 seem to be effective in binding the not bulged siRNA.

Ago1-4 also seemed to be essential in mouse embryonic stem cell survival[13]. The gene silencing was found defective in Ago1-4 -/- embryonic stem cells along with induced hAgo2 indicating that Ago2 alone is not sufficient enough.

4.6 Ago1 and Ago2 might function as RNA chaperones

Wang et al., 2009 studied the assembly of the RISC complex by using a minimal assay system composed of smallRNA and recombinant human AGO proteins[30]. They demonstrated that Ago1 and Ago2 possess a RISC loading activity. As mentioned before, double stranded siRNA is separated by RHA, Wang et al. showed that unlike siRNA, double stranded miRNA is separated by Ago1 and Ago2. They stated that Ago1 and Ago2 act as chaperones instead of RNA helicases. They hypothesized that the Ago-guide strand interaction is weak and other RISC interacting proteins stabilize the Ago-guide strand interaction[30]. RNA chaperones also facilitate RNA strand annealing. They showed that Ago1 had a low affinity for ssRNAs compared to Ago2 which might indicate that Ago1 can facilitate miRNA strand dissociation before the loading onto Ago2. Another possibility is that Ago1 facilitates guide strand – target mRNA association followed by Ago2 recruitment. In conclusion, they state that Ago1 and Ago2 proteins might function as RNA chaperones because of the miRNA strand separation and the guide strand annealing to mRNA targets[30].

4.7 Ago2 in gene silencing and activation of progesterone promoter

As mentioned before, Ago proteins can also be found in the nucleus and so might be able to also have an effect on gene silencing or activation of promoters. Yongjun et al., 2010 examined whether Ago proteins might have a role in silencing and activating the progesterone receptor promoter[17]. By using antigen RNAs (agRNAs) they were able to target the promoter and in this way distinguish them from other small RNAs. They found that in gene silencing, primary Ago2 but also Ago1 was recruited to a noncoding transcript overlapping the promoter. They state that Ago2 must have a primary role in silencing the promoter, but they do not rule out Ago1 involvement.

In RNA-mediated activation Ago2 seems to have a primary role as well[17]. RNA mediated activation is a process of activating gene expression by small RNAs. It is also known as ‘small RNA induced gene activation’. Ago1 was not found to be recruited in this process.

Ago3 and Ago4 seem to have no essential role in gene silencing or activation of this promoter and are not recruited to the noncoding transcript. The role of Ago1 in the progesterone promoter gene silencing remains controversial, since Ago1 is recruited, but its function remains unclear.

4.7 Similar pool of miRNA is associated with Ago1-4 in human skin

The functional significance of the human Ago proteins is poorly understood. Knockout models are an useful method to investigate the functions of individual Argonautes. Wang D et al., 2012 used several knockout models[31]. First they found that the overall expression levels of several miRNAs in the skin was reduced with 10% in the Ago1 knockout model, 30% in the Ago2 knockout model and 80% in the Ago1&2 knockout model. In the Ago1 knockout model, the expression of Ago2 and Ago3 was up-regulated. This indicates that the remaining Ago proteins can, to a certain level, compensate the loss of one Ago protein in the skin. However, when both Ago1 and Ago2 are missing, miRNA expression is significantly impaired(80% reduction) despite the increased Ago3 level. Still, in the Ago1&2 knockout, all the miRNAs were loaded similarly onto Ago3. This makes Ago3 as much capable of miRNA loading as Ago1 and Ago2. It could therefore be that since RNAi has major influences on biological processes, four different Ago proteins are necessary in terms of a backup mechanism.

Next, they found that individual Ago proteins load the same pool of miRNAs into the RISC complex. Ago2 was found to be associated with the most miRNA, followed by Ago1, Ago3 and Ago4. They state that this is due to the Ago expression levels, because Ago2 is also expressed the most, followed by Ago1, Ago3 and Ago4. They conclude that the abundance of a single Ago protein is a causal factor for the miRNA distribution, indicating that the Ago protein that is expressed the most will load the most miRNAs. This occurs without preferences in miRNA. All Argonaute proteins equally load miRNAs into the RISC complex in the skin and there are thus far no signs of miRNA sorting preferences.

Finally, they found that despite the fact that Ago2 inactivation and not Ago1,3,4 inactivation results in embryonic lethality15, Ago2(with unique slicer activity) knockout did not seem give problems in skin development. Neither did Ago1. However, Ago1&2 knockout did give developmental defects of the skin. These findings indicate that both Ago1 and Ago2 are not essential in miRNA associated skin development.

5.0 Conclusion

Human Ago proteins are key players in RNA mediated gene silencing and thereby influencing many biological processes including cell division, death and differentiation, immunity and metabolic control, development regulation[32]. Therefore, Ago proteins are as well associated with diseases like cancer, fragile X syndrome and autoimmune diseases. Because of the effect Ago proteins might have in these processes, it is imaginable that modulating Ago proteins can have great future therapy implications in such related diseases. Revealing the possible interactions with other proteins or factors in specific tissues might clarify these pathophysiologic Ago associated processes.

Ago proteins exist throughout many species and are highly conserved. In some species different members of Ago proteins have specialized functions. It seems like multiple Ago family members have evolved into specific functions to ensure RNA mediated gene silencing is effective. In human Argonautes only Ago2 stands out for its unique activities such as cleavage and trimming. There are two possibilities why Ago2 has these abilities and Ago1,3,4 have not; Ago2 gained these capabilities or Ago1,3,4 lost them over time. This could both be due to mutations, which could explain the differences between Ago2 and Ago1,3,4 in the PAZ and PIWI domain, necessary for cleavage and trimming.

Since Ago3 is equally capable of loading miRNA and all Argonaute proteins can randomly load the same pool of miRNAs, it is possible that four different Ago proteins are necessary in terms of a backup mechanism. RNAi has major influences on biological processes and it is of evolutionary importance that this is accomplished correctly. Because this seemed evolutionary beneficial, it resulted in four Argonaute proteins that have overlapping functions and can therefore function as a compensatory backup mechanism in case one of the proteins is deficient.

But it is also possible that the present four human Argonaute proteins are redundant enough and are now a product of a transient evolutionary phase in which it strives to an Argonaute family with more individual functionality, like some species do. The way Argonaute proteins are expressed might support this theory. Ago2, with the most unique abilities is expressed the most and is therefore associated with the most miRNA. Ago1 also seems to have some unique activities along with Ago2 and therefore has got the second highest expression levels. Ago3,4 are expressed less than Ago1,2 and do not seem to have unique activities. They might be redundant and disappear in the future.

Although Ago2 is the only Ago with slicing activity, Hong Su et al,. 2007 indicated that Ago2 is not capable of operating on its own in mouse embryonic stem cell survival. It is clear that the combination of different Ago proteins is evolutionary beneficial and will lead to higher survival in case an Argonaute protein is not functioning. But three extra Argonaute proteins seems a little exaggerated. Maybe research should focus more on Ago1,3,4, since a lot is known about Ago2. Knockout studies are highly useful in revealing individual Ago functions. For now we cannot draw solid conclusions about these theories, because too little is known about Ago1,3,4 and more research is necessary.

6.0 References

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