Investigating the regulation of miRNA biogenesis and Argonaute2 by RNA binding proteins

Patrick Peter Connerty

Supervisor: Gyorgy Hutvagner

Thesis Submitted for the Degree of Doctor of Philosophy (Science)
University of Technology, Sydney
2016
Declaration

I declare that the work presented within this thesis is, unless stated otherwise, entirely my own; that all references cited herein have been consulted by myself; and that this work has not been previously accepted for a higher degree.

Patrick Peter Connerty

I certify that Patrick Peter Connerty has carried out research under my supervision and has fulfilled the conditions of the relevant ordinance and regulation for the completion of a PhD degree.

Gyorgy Hutvagner

25/6/2016
Acknowledgements:

Firstly, I would like to thank my supervisor Gyorgy Hutvagner for teaching me so much and offering me guidance during the course of both my Honours and PhD. This has all been made possible by your patience and mentorship. Secondly, I would like to thank Sarah Bajan for constantly helping me in and out of the lab and showing me how to do practically everything for my PhD. Also, my co supervisor Nham Tran for teaching me the basics to make me the scientist I am today.

Next, I would like to thank Mum and Dad for the never ending support and putting up with me when I decided to stay at university for 7 years straight. My sister, Dominique, for proof reading and being there for me, and my wonderful partner Harui for being so understanding when I had to miss weekend dates to work in the lab!

Finally, thanks to all the wonderful friends I have made over the course of my PhD, both in and out of uni. Sam and Rob (KGB), Peter and Elliot for accompanying me on those hour long coffee breaks while we waited for incubations. Kallo, Michael, Ng and Xuan (The Squad) for kicking back with me every Friday down at the ho market. You guys have made the last four years so memorable.
Table of Contents

Abstract: ........................................................................................................................ 15

1. Introduction: ........................................................................................................... 16
   1.1 microRNAs – Key regulators of gene expression: ............................................... 16
   1.2 Biogenesis of miRNAs: ........................................................................................ 17
       1.2.1 Expression of primary miRNAs: .................................................................... 17
       1.2.2 Processing of pre-miRNas: .......................................................................... 18
       1.2.3 Transport of pre-miRNAs to the cytoplasm: .................................................. 18
       1.2.4 Pre-miRNA processing: ................................................................................. 18
       1.2.5 Ago2 and the RNA Induced Silencing Complex (RISC): ............................... 19
   1.3 Mechanisms of miRNA-mediated post-transcriptional regulation: ..................... 23
       1.3.1 miRNA-Mediated regulation of translation initiation: .................................. 25
       1.3.2 miRNA-Mediated regulation of translation elongation: ............................... 27
   1.4 RNA binding proteins in the miRNA pathway: ...................................................... 32
   1.5 Aims and Hypothesis: .......................................................................................... 55
       1.5.1 The miRNA biogenesis factors, p72/DDX17 and KHSRP regulate the protein level of Ago2 in human cells: .................................................................................. 55
       1.5.2 Transient inhibition of Dicer elevates the RNA and protein level of Ago2 in human cells ............................................................................................................ 55
       1.5.3 Dicer regulates pri-miRNA biogenesis ........................................................... 56
       1.5.4 Increasing miRNA and miRNA target abundance affects miRNA biogenesis 56
2. Materials and Methods: ............................................................................................. 57
   2.1. General Materials and Methods: ......................................................................... 57
       2.1.1 Buffers: .......................................................................................................... 57
       2.1.2 Reagents: ...................................................................................................... 58
       2.1.3 Oligonucleotides, RNA mimics, PCR primers, siRNAs: ................................. 60
       2.1.4 Plasmids: ....................................................................................................... 63
       2.1.5 Antibodies: .................................................................................................... 64
       2.1.6 Cell Culture maintenance: ............................................................................. 65
       2.1.7 Transfection of human cells with siRNA and miRNA mimics: ......................... 67
       2.1.8 Transfection of human cells with plasmid DNA: ............................................ 68
       2.1.9 Geldanamycin treatment: .............................................................................. 68
       2.1.10 Protein extraction: ....................................................................................... 69
       2.1.11 Western Blot Analysis: ................................................................................ 69
       2.1.12 Luciferase Assay: ........................................................................................ 71
3. The miRNA biogenesis factors, p72/DDX17 and KHSRP regulate the protein level of Ago2 in human cells:

3.1 Introduction: ................................................................. 78
3.2 Results: ......................................................................... 81
  3.2.1 The RNA-binding proteins p72 and KHSRP stabilise the protein level of human Ago2: ................................................................. 81
  3.2.2 P72 and KHSRP regulate Ago2 post-transcriptionally: .................... 87
  3.2.3 P72 and KHSRP regulate Ago2 protein level through influencing the level of cellular miRNAs: ................................................................. 89
  3.2.4 KHSRP and p72 regulate the level of unloaded Ago2: ......................... 92
3.3 Discussion: ................................................................. 95

4. Transient inhibition of Dicer elevates the RNA and protein level of Ago2 in human cells:

4.1 Introduction: ................................................................. 97
4.2 Results: ......................................................................... 100
  4.2.1 Impairing the expression of human Dicer with siRNA elevates the mRNA and protein level of human Ago2: ................................................................. 100
  4.2.2 Human Dicer regulates Ago2 post-transcriptionally: ....................... 103
  4.2.3 Inhibition of Dicer elevates the levels of unloaded Ago2: ..................... 104
4.3 Discussion: ................................................................. 108

5. Dicer regulates pri-miRNA biogenesis:

5.1 Introduction: ................................................................. 112
5.2 Results: ......................................................................... 113
  5.2.1 Short hairpin RNA (shRNA) induced knockdown of Dicer in T-REX 293 cells increases the level of pri-let-7a: ................................................................. 113
  5.2.2 siRNA induced knockdown of Dicer affects pri-miRNA biogenesis: ......... 116
  5.2.3 Inducible deletion of Dicer increases the level of pri-miR-155 in mouse embryonic fibroblasts: ................................................................. 122
5.3 Discussion: ................................................................. 125

6. Increasing miRNA and miRNA target abundance affects miRNA biogenesis: ...... 129
List of Figures and Tables:

1. Introduction:

<table>
<thead>
<tr>
<th>Figure No.</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>miRNA biogenesis pathway</td>
<td>22</td>
</tr>
<tr>
<td>1.2</td>
<td>Mechanisms of post-transcriptional gene regulation mediated by miRNAs</td>
<td>24</td>
</tr>
</tbody>
</table>
2. Materials and Methods:

<table>
<thead>
<tr>
<th>Table No.</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Primer Sequences for miRNA qPCR.</td>
<td>60</td>
</tr>
<tr>
<td>2.2</td>
<td>Primer Sequences for gene qPCR.</td>
<td>61</td>
</tr>
<tr>
<td>2.3</td>
<td>siRNAs</td>
<td>61</td>
</tr>
<tr>
<td>2.4</td>
<td>miRNA mimics</td>
<td>62</td>
</tr>
<tr>
<td>2.5</td>
<td>Plasmids</td>
<td>63</td>
</tr>
<tr>
<td>2.6</td>
<td>Antibodies</td>
<td>64</td>
</tr>
<tr>
<td>2.7</td>
<td>Recipe for 40% 5:1 bis-acrylamide Gels</td>
<td>71</td>
</tr>
<tr>
<td>2.8</td>
<td>Recipe for master mix used in miRNA cDNA synthesis.</td>
<td>74</td>
</tr>
</tbody>
</table>
2.9 Recipe for master mix used in mRNA cDNA synthesis

2.10 Recipe for qPCR 10 μL reactions.
3. The miRNA biogenesis factors, p72/DDX17 and KHSRP regulate the protein level of Ago2 in human cells:

Figure No.  

3.1  p72 and KHSRP stabilise human Ago2 protein.  83

3.2  p72 stabilises human Ago2 protein.  84

3.3  KHSRP stabilises human Ago2 protein.  85

3.4  p72 and KHSRP stabilise human Ago2 protein in U2Os and HEK293 cells.  86

3.5  p72 and KHSRP regulated Ago2 via post-transcriptional mechanisms.  88

3.6  miRNA abundance regulates Ago2 protein level.  90

3.7  miRNA abundance regulates Ago2 protein level (2).  91

3.8  p72 and KHSRP regulate the level of unloaded Ago2.  94
4. Transient inhibition of Dicer elevates the RNA and protein level of Ago2 in human cells.

<table>
<thead>
<tr>
<th>Figure No.</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1</td>
<td>Dicer knockdown stabilises human Ago2 protein.</td>
<td>101</td>
</tr>
<tr>
<td>4.2</td>
<td>Dicer stabilises human Ago2 protein in a range of human cells.</td>
<td>102</td>
</tr>
<tr>
<td>4.3</td>
<td>Dicer regulates Ago2 via a post-transcriptional mechanism.</td>
<td>106</td>
</tr>
<tr>
<td>4.4</td>
<td>Dicer increases the level of low molecular weight (LMW)-RISC.</td>
<td>107</td>
</tr>
</tbody>
</table>
5. Dicer regulates pri-miRNA biogenesis:

<table>
<thead>
<tr>
<th>Figure No.</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.1</td>
<td>shRNA induced Dicer knockdown increases pri-let-7 levels.</td>
<td>115</td>
</tr>
<tr>
<td>5.2</td>
<td>Dicer knockdown affects pri-miRNA production in HEK293 cells.</td>
<td>117</td>
</tr>
<tr>
<td>5.3</td>
<td>Dicer knockdown affects pri-miRNA production in HeLa cells.</td>
<td>119</td>
</tr>
<tr>
<td>5.4</td>
<td>Dicer knockdown affects pri-miRNA production in U2OS cells.</td>
<td>121</td>
</tr>
<tr>
<td>5.5</td>
<td>Cre-Lox induced Dicer knockdown increases pri-mir-155 levels.</td>
<td>124</td>
</tr>
</tbody>
</table>
6. Increasing miRNA and miRNA target abundance affects miRNA biogenesis:

<table>
<thead>
<tr>
<th>Figure No.</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.1</td>
<td>Increased miRNA level recovers miR-21 in cells with impaired p72.</td>
<td>133</td>
</tr>
<tr>
<td>6.2</td>
<td>Increased miRNA level recovers <em>let-7</em> in cells with impaired KHSRP.</td>
<td>136</td>
</tr>
<tr>
<td>6.3</td>
<td>Increased miRNA level recovers <em>let-7</em> and miR-21 in Dicer knockdown cells.</td>
<td>139</td>
</tr>
<tr>
<td>6.4</td>
<td>Increased miRNA target results in increased miRNA stability.</td>
<td>143</td>
</tr>
<tr>
<td>6.5</td>
<td>Increased perfect miRNA target results in increased miRNA stability.</td>
<td>144</td>
</tr>
</tbody>
</table>
7. Discussion:

<table>
<thead>
<tr>
<th>Figure No.</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.1</td>
<td>Knockdown of Dicer influences pri-miRNA production.</td>
<td>159</td>
</tr>
<tr>
<td>7.2</td>
<td>Addition of miR-17 duplex to the cell results in an increase in pri-miRNA and mature miRNA levels.</td>
<td>163</td>
</tr>
<tr>
<td>7.3</td>
<td>Addition of miR-17 duplex to the cell results in an increase in pri-miRNA and mature miRNA levels.</td>
<td>164</td>
</tr>
</tbody>
</table>
Abstract:

microRNAs (miRNAs) are small non-coding RNAs which post-transcriptionally regulate gene expression. As miRNAs control many important biological processes it is important that their own production is highly controlled too. A range of auxiliary proteins involved in regulating miRNA biogenesis have been documented extensively, highlighting the complexity of the miRNA pathway. This study identifies novel roles of RNA binding proteins which are both canonical and auxiliary to the miRNA biogenesis pathway.

Here, we demonstrate that inhibition of p72 and KHSRP decreases Ago2 protein stability through disturbing miRNA biogenesis and therefore miRNA abundance. Furthermore, we have demonstrated that Ago2 is subject to multiple types of regulation as transient knockdown of Dicer stabilises Ago2 protein despite a decrease in miRNA abundance via an unknown mechanism.

Additionally, we have established that miRNA biogenesis is subject to a possible negative feedback mechanisms in which impairment of Dicer function both promotes and inhibits pri-miRNA production in a pri-miRNA and cell specific manner.

Finally, this study provides evidence to suggest that both mature miRNA levels and miRNA target abundance can stabilise miRNA biogenesis and promote pri-miRNA production in the absence of key and auxiliary proteins involved in miRNA biogenesis.
1. Introduction:

1.1 microRNAs – Key regulators of gene expression:

microRNAs (miRNAs) are short (~22 nucleotide) ribonucleic acid (RNA) molecules that regulate gene expression post-transcriptionally. miRNAs bind to complementary mRNA sequences. This binding represses the translation of the bound mRNA and leads to the inhibition of protein synthesis (Ha & Kim 2014). miRNA regulated gene expression is required for many aspects of the cell cycle, organism development and the immune response. An imbalance in miRNA levels can contribute to the development of diseases, including certain types of cancers (Iorio & Croce 2012). In fact numerous cancers exhibit a dependency on the expression of a single oncogenic miRNA, also known as an ‘oncomiR’. miRNAs also play a role in skeletal muscle development and proliferation (Williams et al. 2009), development of the heart (Zhao et al. 2007), and the suppression of neuron development (Le et al. 2009). miRNAs also play a vital role in the immune response, including the development and differentiation of T cells (Jeker & Bluestone 2013), proliferation of monocytes and neutrophils (Fontana et al. 2007), and the release of virus combating interferons (Sedger 2013). miRNAs are required for the healthy development of an organism, with alterations in miRNA biogenesis leading to the development of diseases and eventually becoming fatal.
Therefore, miRNAs may serve as reliable biomarkers and the regulation of their expression can potentially be used therapeutically to treat a range of diseases and infections (Li & Rana 2014).

1.2 Biogenesis of miRNAs:

The biogenesis of miRNAs is a multi-step process outlined below (Figure 1.1):

1.2.1. Expression of primary miRNAs:

The canonical miRNA biogenesis pathway begins with the polymerase II mediated transcription of primary-miRNAs (pri-miRNAs) from miRNA genes (Lee et al. 2004). The majority of miRNAs are encoded in introns of genes, however, there are also miRNAs which originate from the exons of protein coding genes (Rodriguez et al. 2004). This suggests that some mRNA can produce both miRNAs and proteins (Cai et al. 2004). The pri-miRNA is transcribed as containing a ~80 nucleotide stem loop containing a mature miRNA sequence and a two-nucleotide overhang at its 3’ end (Lee et al. 2002). While most miRNAs are transcribed independently of each other, some are transcribed together in large groups known as miRNA clusters (Altuvia et al. 2005).
1.2.2. Processing of pre-miRNAs:

Once transcribed, the stem loop of the pri-miRNA is cleaved into the precursor miRNA (pre-miRNA) by the Microprocessor complex. In its most basic form the Microprocessor complex contains a single nuclear RNase III Drosha and a DGCR8 (Di George syndrome critical region protein 8) dimer (Han et al. 2004; Nguyen et al. 2015). Drosha cleaves the pri-miRNA into a ~70 nucleotide pre-miRNA stem loop (Nguyen et al. 2015; Gregory et al. 2004). However, another pathway which is independent of Drosha exists which generates mirtrons. These miRNA are found within short introns and the pre-miRNA is produced by splicing machinery, rather than the Microprocessor complex (Ruby et al. 2007).

1.2.3 Transport of pre-miRNAs to the cytoplasm:

After the Microprocessor cleaves the pri-miRNA into a pre-miRNA, the pre-miRNA is exported from the nucleus to the cytoplasm via the Exportin 5 pathway (Yi et al. 2003). Exportin 5 specifically recognises the end structure of pre-miRNAs (Lund et al. 2004) and protects the pre-miRNA from degradation (Wang et al. 2011).

1.2.4 Pre-miRNA processing:

Once in the cytoplasm a pair of proteins, the RNase III Dicer and the trans-activator RNA (tar)-binding protein (TRBP), process the pre-miRNA.
Dicer is a large ~200 kDa RNAse III like protein which recognises the double-stranded portion of the pre-miRNA with affinity to the 3’ overhang at the stem loops base (Bartel 2004). Dicer dsRNA recognition is directly mediated by the PAZ domain (Lau et al. 2012). The helicase forms a clamp adjacent to the RNase III active site and allows recognition of pre-miRNA loops (Lau et al. 2012). Dicer measures from the monophosphorylated 5´ end of the precursors and positions the molecule for cleavage approximately 21-24 nucleotides away (Park et al. 2011). This produces the ~22 nucleotide miRNA/miRNA* duplex (Kim 2005). Each RNase domain cleaves one strand of the precursor and releases the hairpin loop (MacRae et al. 2007).

TRBP binds with Dicer and promotes pre-miRNA cleavage in an RNA-structure-specific manner (Fukunaga et al. 2012). TRBP can also direct Dicer to cut the pre-miRNA at different positions. This process produces variations in mature miRNA sequences, known as isomiRs, from a single pre-miRNA. This allows a single pre-miRNA to generate a variety of mature miRNAs (Fukunaga et al. 2012; Morin et al. 2008). TRBP stabilises the interaction between Dicer and Argonaute 2 (Wang et al. 2009), promoting the transfer of the miRNA duplex to the RNA induced silencing complex (RISC).

1.2.5 Ago2 and the RNA Induced Silencing Complex (RISC):

miRNAs need to be associated with an Argonaute protein to conduct gene regulation. The miRNA loaded into an Argonaute protein forms the minimal RNA induced silencing
complex (RISC) (Miyoshi et al. 2005; Liu et al. 2004; Rivas et al. 2005; Meister et al. 2004; Khvorova et al. 2003; Schwarz et al. 2003). The RISC then binds to target mRNAs and represses gene expression through direct cleavage, translational repression or RNA degradation.

There are four Argonautes encoded in mammals (Ago 1-4), however, only Ago2 has endonuclease activity and thus exclusively carries out RNAi dependent gene silencing (Liu et al. 2004). Ago2 is a ~95 kDa bi-lobed protein which is formed from four globular domains; an amino-terminal N, a PAZ domain, a middle (MID) domain and a PIWI domain (Song et al. 2004; Elkayam et al. 2012; Schirle & MacRae 2012).

Early biochemical data demonstrated that miRNA duplexes cannot be loaded into an Ago in vitro suggesting that there is a mechanism which governs the loading of a mature miRNA into an Ago protein and the separating of the miRNA duplex (Rivas et al. 2005). It was later determined that loading of the miRNA duplex into the RISC is dependent on heat shock proteins HSC70 and HSP90 (Iwasaki et al. 2010; Johnston et al. 2010). In an ATP dependent manner, HSP90 mediates a conformational change in the Ago so that it can uptake the mature miRNA strand of the duplex (Johnston et al. 2010; Iwasaki et al. 2010). This conformational change stretches the Ago protein like a ‘rubber band’ upon loading, which then springs to a relaxed state. During this process, the Ago N terminal removes one of the strands from the duplex in an ATP independent manner (Kwak & Tomari 2012). The selection of the strand is determined by the thermodynamic properties of the miRNA duplex. The strand with the less stable 5’ end will more efficiently be
incorporated into RISC, while the other strand is more prone to degradation (Khvorova et al. 2003; Schwarz et al. 2003). Ago2, loaded with a miRNA, binds with its complimentary mRNA and either cleaves it or recruits other proteins such as GW182 to repress translation (Iwakawa & Tomari 2015).
Figure 1.1: Biogenesis of miRNA. Pri-miRNA is transcribed from the miRNA gene by polymerase II. Pri-miRNA is processed into pre-miRNA by the Microprocessor complex containing Drosha and DGCR8, and then it is exported from the nucleus via Exportin 5 into the cytoplasm. Once in the cytoplasm Dicer cleaves the pre-miRNA forming miRNA/miRNA* duplex. One strand of the duplex is subsequently incorporated into an Ago to form the RISC. The RISC loaded with the miRNA is now ready to repress target mRNA.
1.3 Mechanisms of miRNA-mediated post-transcriptional regulation:

RISC carries out small RNA-directed gene silencing in the miRNA pathway (Pratt & MacRae 2009). miRNAs are loaded into and direct the RISC to a target mRNA 3´ UTR, the most frequent area for miRNA targeting. This down regulates gene expression by three post-transcriptional mechanisms; sequence-specific mRNA cleavage, translational repression and mRNA degradation (Iwakawa & Tomari 2015; Yekta et al. 2004). The mechanism utilised depends on the levels of complementarity between the target mRNA and the miRNA, and the endonuclease activity of the Ago protein. RISC cleaves the target mRNA if the miRNA is incorporated into a cleavage competent Ago and has an almost perfect complementarity to its target mRNA. Although common in plants, direct cleavage of target mRNAs is a rare event in humans and has been documented only in specific cases, such as miR-196 targeting HOXB8 (Yekta et al. 2004).

The minimum level of complementarity needed to achieve translational repression is limited to the miRNA ‘seed region’, nucleotides 2-7 of the 5´ end of the miRNAs (Lewis et al. 2005). If the miRNAs do not have sufficient base pairing to cleave the target, or they bind to an Ago protein that has no endonuclease activity, they will repress translation without sequence specific cleavage (Iwakawa & Tomari 2015). This is achieved through repression of translational initiation, elongation or mRNA decay (Figure 1.2).
Figure 1.2: Mechanisms of post-transcriptional gene regulation mediated by microRNAs. If the miRNA has perfect complementarity to the target mRNA then the target mRNA will be cleaved by a cleavage competent Ago protein. However, if complementarity is restricted to only the seed region and the Ago is not able to cleave, then an inhibition of translation will occur.
1.3.1 miRNA-Mediated regulation of translation initiation:

Translation initiation begins with the initiation factor eIF3 binding to the 40S ribosomal subunit and the eIF4F complex which comprises of scaffolding proteins eIF4A and eIF4G and the cap binding eIF4E (Reviewed by Jackson, Hellen, and Pestova 2010). The poly-(A) binding protein (PABP) then associates with eIF4G and binds to the poly-(A) tail of the mRNA. This results in the circularisation of the mRNA and brings the 5’ and 3’ ends of the mRNA into close proximity. The 40S ribosomal subunit and other initiation factors can then scan the mRNA for the AUG start codon and recruit the ribosomal subunit 60S to form an elongation-competent 80S (Reviewed by Jackson, Hellen, and Pestova 2010).

miRNAs can regulate translation initiation by interrupting any of the processes detailed above, however, the most studied mechanism of translational inhibition is the GW182, also known as TNRC6C, dependent pathway (Gu & Kay 2010). GW182 interacts with Ago2 and is an essential component of the mechanism that allows miRNA to inhibit translation (Ding & Han 2007; Eulalio et al. 2008). GW182 competitively binds with PABP and blocks it from binding to the poly-(A)-tail (Zekri et al. 2013). This inhibits translation initiation by preventing the mRNA from changing to a closed loop conformation (Gu & Kay 2010).

miRNA can use alternate mechanisms to inhibit translational initiation. Ago2 interferes with the function or binding of eIF4E, the initiation factor which directly binds to the 5’ cap (Humphreys et al. 2005). Ago2 has a m7G cap binding-like motif which shares homology
with the 5´cap-interacting motif of eIF4E and is thought to effectively compete for binding to the cap, therefore, interfering with ribosomal subunit recruitment (Kiriakidou et al. 2007). Furthermore, repression through inhibiting ribosomal 80S assembly via RISC interaction with the protein eIF6 has also been documented (Chendrimada et al. 2007). These studies suggest that many steps of translational initiation are inhibited by miRNAs.
1.3.2 miRNA-Mediated regulation of translation elongation:

Studies conducted in both worms and mammalian cells have reported that mRNAs which have been repressed by miRNAs remain associated with translating polysomes while the production of proteins are still inhibited (Seggerson et al. 2002; Gu et al. 2009; Nottrott et al. 2006; Maroney et al. 2006). This indicates that miRNA-directed inhibition of target gene expression occurs after the initiation steps of translation (Gu et al. 2009; Nottrott et al. 2006; Maroney et al. 2006). The RISC has also been found to associate with translating polysomes in both mammalian and plant systems, further suggesting that the elongation stage of translation is a target of miRNA-mediated repression (Lanet et al. 2009; Vasudevan et al. 2007; Kim et al. 2004; Nottrott et al. 2006).

The post-initiation inhibition of translation by miRNAs could also result from a high rate of ribosome drop-off during translational elongation, resulting in incomplete protein products (Petersen et al. 2006; Orang et al. 2014). Furthermore, a knockdown of RACK1 (a component of the eukaryotic 40S subunit) was found to inhibit miRNA mediated translational repression. RACK1 depletion impairs the association of miRNA complex with the translating ribosomes (Jannot et al. 2011). This data shows that RACK1 can contribute to the recruitment of RISC to the site of translation and supports a post-initiation mode of miRNA-mediated gene repression.
1.3.4 miRNA-Mediated mRNA deadenylation and decay - Separate mechanisms or partners in action?

Besides regulating transcriptional initiation and elongation, miRNAs can also inhibit gene expression through sequence independent deadenylation and degradation of target mRNAs. In fact, while early reports documented miRNAs as inhibiting gene expression through translational repression (Olsen & Ambros 1999), more recent studies have shown that mRNA degradation via deadenylation accounts for roughly 80% of overall repression (Guo et al. 2010).

In the case of mammals, miRNAs can promote mRNA destabilisation through GW182, recruiting the deadenylase complex CCR4-NOT to the target mRNA (Fabian et al. 2012; Braun et al. 2011; Huntzinger et al. 2013; Kuzuoğlu-Öztürk et al. 2012). Studies have shown that a knockdown of CCR4-NOT significantly decreases miRNA-mediated deadenylation and mRNA decay, indicating the complexes role in mRNA decay (Behm-Ansmant et al. 2006; Fabian et al. 2012).

miRNAs can also promote mRNA decay through increasing the accessibility of the poly-(A)-tail to deadenylases. Recent studies have revealed that GW182 tethering to the mRNA dissociates PABP and promotes mRNA decay (Moretti et al. 2012). After deadenylation, target mRNAs undergo 5´ to 3´ mRNA decay. In this, target mRNAs are deadenylated, decapped and degraded by the cytoplasmic 5´-to-3´ exonuclease XRN1 (Eulalio et al. 2009; Behm-Ansmant et al. 2006; Bail et al. 2010). Furthermore, Ago2 co-
immunoprecipitates with the decapping enzyme complex subunit DCP2, suggesting that
the RISC can also initiate decay by recruiting the decapping enzyme. To support this,
studies have reported that miRNA targets levels increase when decapping activators are
depleted (Behm-Ansmant et al. 2006; Eulalio et al. 2007; Braun et al. 2011).

Currently, whether miRNAs repress translation at the initiation or elongation phase or
work through inducing mRNA decay is debated. In Drosophila studies, Djuranovic et al
demonstrated that luciferase reporters containing miRNA binding sites consistently
underwent translational repression before mRNA decay. Furthermore, they observed that
deadenylation, the main step of mRNA degradation, is not required for the translational
repression of reporters, suggesting that translational repression is indeed the method of
miRNA mediated mRNA repression (Djuranovic et al. 2012). Likewise, a separate study
by Bazzini et al revealed that, in zebra fish, translational repression is the main initiator of
miRNA-mediated mRNA regulation. During zebra fish embryo development miR-430
targets and rapidly depletes maternal mRNAs four days after fertilisation. Bazzini et al
analysed the translational status and stability of all miR-430 targets during fish embryo
development using ribosome profiling analysis followed by high throughput RNA
sequencing. Their data revealed that during the induction of miR-430, the target mRNAs
first experienced translational repression followed by mRNA decay. Furthermore, slowing
deadenylation of target mRNAs did not influence translational repression (Bazzini et al.
2012). Together, these studies argue that miRNA-mediated mRNA repression occurs
through translational repression and the degradation of the targeted mRNAs is the
consequence of stalled translation.
Additional studies, however, have shown conflicting evidence. Eichhorn et al, proposed that previous studies demonstrating miRNA translational repression had the caveat of utilising reporter genes rather than endogenous transcripts (Béthune et al. 2012; Djuranovic et al. 2012). They argue that the repression attributed to translational repression of the reporters exceeds the levels of typical endogenous mRNAs. Similarly, the magnitude of repression observed for reporters vastly exceeds endogenous mRNA repression levels and thus, the translational repression observed was not reflective of endogenous miRNA action (Eichhorn et al. 2014). Furthermore, they argue that the fish embryo model presents a system with an unusual mRNA metabolism where mRNAs with short poly-(A)-tails are stable and is therefore not applicable to human cells except for at the earliest time points of miRNA mediated repression (Eichhorn et al. 2014).

Eichhorn et al put forward that although when miRNA-mediated mRNA decay was first reported, it was thought to be a secondary result of translational repression, their data suggests that this is not the case and that mRNA decay is actually just a slower process. After analysing the stability of mRNAs in a range of human cells transfected with miRNAs, using ribosome profiling analysis followed by deep sequencing of mRNAs, they found that the vast majority of mRNAs targeted by miRNAs were initially regulated by translational inhibition, after which, degradation soon took over to be the sole mRNA regulating mode of action. They argue that, at least in the case of mammalian cells, detectable mRNA decay occurs after a lag because mRNA decay takes longer than inhibiting translation initiation. However, the lag in decay does not last long, and time point experiments show
that decay soon dominates as the main form of miRNA-mediated repression (Eichhorn et al. 2014).

Though conflicting evidence exists at the moment, the mode of miRNA mediated action (whether translational repression or mRNA degradation) could be defined by various factors. For example, certain animal models and stages of cellular growth have been shown to provide stability to mRNAs with short poly-(A)-tails and thus favour translational repression over mRNA degradation (Subtelny et al. 2014). This suggests that the mode of action chosen could be defined by the current cellular environment and change as the cell develops. Furthermore, the sequence of the miRNA or even the target mRNA could perhaps define which method is utilised to inhibit gene expression. Considering that miRNAs need perfect complementarity to achieve Ago2 mediated cleavage, translational repression and mRNA degradation could have co-evolved as a fall back system to account for nucleotide mutations in mRNA sequence and allow for a level of gene inhibition in the absence of perfect complementarity. However, considering that miRNAs are important gene regulators and their dysregulation has been documented in a number of disease states; it would be evolutionarily advantageous and unsurprising if miRNAs had multiple mechanisms of action and mediated mRNA expression not through a single method, but through both translation repression and mRNA decay.
1.4 RNA binding proteins in the miRNA pathway:

As miRNAs control practically every cellular process, it is important that their own production is highly regulated. As a result multiple mechanisms exist by which the cell can inhibit or promote miRNA biogenesis (Ha & Kim 2014). One of these mechanisms is protein modification of the canonical proteins of the miRNA biogenesis pathway. For example, post translational modification of Ago proteins through hydroxylation can stabilise Ago proteins and thus promote miRNA action within the cell. Furthermore, Phosphorylation at distinct sites of Ago is important for correct protein localization and efficient small RNA binding (Meister 2013).

Alternatively, miRNA biogenesis can be controlled by direct regulation of the processing intermediates of the miRNA pathway. For example, RNA editing involves the conversion of an adenosine to inosine in the stem region of a pri-miRNA. This chemical editing results in a pre-miRNA that cannot be efficiently processed by Dicer (Kawahara et al. 2007). Furthermore, RNA methylation of pri-miRNAs can also regulate miRNA processing. Studies have shown that the methylation of the 5’ monophosphate of pre-miR-145 inhibits Dicer-mediated processing (Xhemalce et al. 2012).

However, the key mechanisms in the regulation of miRNA biogenesis is the vast number of auxiliary RNA binding proteins in the miRNA pathway which both promote and inhibit miRNA processing at various stages in the pathway using a variety of methods. In the review below we have collected and described most of the RNA-binding protein mediated regulations, as this is the main theme of this thesis.
STATEMENT OF CONTRIBUTION OF OTHERS


I attest that Research Higher Degree candidate Patrick Connerty was the joint primary contributor to the development of this publication. This extensive contribution included: synthesising and developing the intellectual scope of the literature review and writing the manuscript.

Aliriza Ahadi

Gyorgy Hutvagner
RNA binding proteins in the miRNA pathway

Patrick Connerty 1,†, Alireza Ahadi 2,† and Gyorgy Hutvagner 2,*

Abstract: microRNAs (miRNAs) are short ~22nt ribonucleic acids which post-transcriptionally regulate gene expression. miRNAs are key regulators of all cellular processes and the correct expression of miRNAs in an organism is crucial for proper development and cellular function. As a result, the miRNA biogenesis pathway must be highly regulated itself. In this review we outline the basic steps of miRNA biogenesis and miRNA mediated gene regulation focusing on the role of RNA binding proteins (RBPs). We also describe multiple mechanisms that regulate the canonical miRNA pathway which depend on a wide range of RBPs. Moreover, we hypothesise that the interaction between miRNA regulation and RBPs is potentially more widespread based on the analysis of available high-throughput datasets.

Keywords: keyword 1; miRNA 2; RNA binding protein 3. Drosha, Dicer, Argonaute, CLIP

1. Introduction

MicroRNAs (miRNAs) are an abundant class of small regulatory RNAs about 19-22 nucleotides (nt) in length [1]. They have been predicted to regulate the expression of more than 60% of mammalian genes and play fundamental roles in most biological processes including multiple diseases [2, 3]. The canonical miRNA pathway starts with the transcription of miRNA genes by RNA polymerase II, which results in the production of the primary miRNA (pri-miRNA). The ~80 base pair long stem loops of the pri-miRNAs are released from the primary transcript generating the precursor miRNA (pre-miRNA). The pre-miRNA itself undergoes multiple processing steps before the mature miRNA is finally generated [4]. Incorporated into one member of the Argonaute (Ago) protein family in the RNA induced silencing complex (RISC), a mature miRNA binds typically to the 3′ untranslated region (UTR) of the targeted messenger RNA (mRNA) [1] and inhibits its translation via various mechanisms [5], The key determinant of target recognition is a short sequence homology between the miRNA seed sequence (the 2nd-8th nucleotides of the miRNA) and the targeted mRNA [6]. The maturation and function of miRNAs are highly dependent on the coordinated action of several RNA-binding proteins (RBP) [7]. Some of these proteins present unique protein domains that are characteristic of proteins involved in small RNA processing and small RNA mediated gene regulatory events [8].

The processing and action of miRNAs are extensively regulated by auxiliary factors to ensure cell/tissue specific functions or adequate response to environmental and cellular stimuli. One of the largest groups of proteins that influence the miRNA pathways are RNA-binding proteins (RBP). The application of advanced biochemical methods such as the use of a variety of cross-linking immunoprecipitations (CLIPs) with the key proteins of the miRNA pathway, accelerated the identification of RBPs that are
In this review, we describe the characteristics and functions of RBPs that are necessary for the production of miRNAs and miRNA mediated gene expression as well as RBPs that regulate these processes in mammalian cells. We have also carried out a basic bioinformatics exercise to compute the potential scale of miRNA and RBP interactions using available CLIP data. Based on this we suggest a more widespread interaction between RBPs and miRNA complexes in the targeting step of miRNA mediated gene regulation.

2.1. Core RNA binding proteins of miRNA processing and miRNA mediated gene regulation

2.1.1. pri-miRNA processing by the Microprocessor

The mammalian pri-miRNA contains a double stranded hairpin stem, a terminal loop and two single stranded flanking regions [11]. Recent studies identified additional sequence and structural elements that are necessary for efficient miRNA production. These include the length of the hairpin stem structure, the UGU motif in the apical loop, a GHG motif in the stem, and a UG and CNNC motif in the basal region of the pri-miRNA [12–14]. The first step in miRNA biogenesis is the recognition of these motifs and the cleavage of the miRNA hairpins from the primary transcript. This is carried out by the coordinated action of the Microprocessor complex. The Microprocessor is a heterotrimeric complex, which is made up of two proteins: an RNA III enzyme Drosha, and two RNA-binding proteins DGCR8 (DiGeorge Critical Region 8) [12, 15–18].

Drosha (also known as RNASEN) is a Class II RNase III enzyme that characteristically contains two tandem RNase III domains and a dsRNA-binding domain. Drosha is an Mg2+ dependent endonuclease that has roles in miRNA biogenesis, ribosomal RNA processing, and viral defence [19].

DGCR8, is the other component of the Microprocessor complex [15–17]. It contains a nuclear localization signal, two dsRBDs (double stranded RNA-Binding domains), an RNA-binding heme domain (Rhed), and a C-terminal tail (CTT) [11, 20–22]. DGCR8 is dimerized through the Rhed domain and binds to Drosha with its C-terminal region [20, 23].

Recent biochemical data using purified recombinant Drosha and DGCR8 revealed that DGCR8 recognizes the apical UGU motif and binds to the pri-miRNA stem with its dsRBD domains. It also stabilises Drosha with its CTT. In a cleavage competent Microprocessor, Drosha is positioned at the basal UG motif that is at the junction of the single stranded flanking regions and the stem of the hairpin [12]. Drosha cleaves the 5’ and 3’ arms of the pri-miRNA, 11 base pairs away from the single-stranded flank/double-stranded junction [18]. It was recently demonstrated that a CNNC motif, downstream from the stem-ssRNA junction, is also required for efficient mammalian pri-miRNA processing by the binding of the SRp20 splicing factor through its RNA recognition motif (RRM) [13] (Figure 1a).
Figure 1. RNA binding proteins of the canonical miRNA biogenesis pathway: (a) Sequence motives and proteins that required for efficient pri-miRNA processing by the Microprocessor complex. (b) Proteins required for pre-miRNA processing (c) Argonaute and GW182 dependent inhibition of translation initiation. (d) miRNA mediated inhibition of translation initiation that is not required GW182.

Both Drosha and DGCR8 have miRNA independent functions. Drosha recognizes and cleaves hairpins in mRNAs including the mRNA of DGCR8 that results in an auto regulatory circuitry of the Microprocessor [24, 25]. DGCR8 also has RNA-binding functions that are independent from the Microprocessor. For example, a HITS-CLIP screening of RNA targets of DGCR8 in human cells revealed that DGCR8 binds to and mediates cleavage of small nucleolar RNAs (snoRNAs) [10].

2.1.2. pre-miRNA processing

After Microprocessor processing the produced pre-miRNAs are exported from the nucleus to the cytoplasm via the Exportin 5 pathway [26, 27]. In the cytoplasm, pre-miRNAs are further cleaved by Dicer, another RNAse III enzyme, with the help of RNA-binding co-factors, Protein Kinase, Interferon-Inducible Double Stranded RNA Dependent Activator (PACT) and HIV-1 TAR RNA-binding protein (TRBP) [28–31].
Dicer is a RNase III enzyme which contains an ATPase/RNA helicase, a PAZ domain (Piwi, Argonaute and Zwille domain, a domain only present in proteins involved in small RNA mediated gene regulation) [8], two catalytic RNase III domains, a domain of unknown function (DUF283) and a C-terminal dsRBD [32]. Dicer was first recognised for cleaving dsRNA into small interfering RNA (siRNA) [33, 34] but was later discovered to also process miRNAs [28, 29].

The PAZ domain of DICER binds to the 3' end of the small RNA substrates and also recognizes the 5’ terminal phosphate of an authentic miRNA precursor [35–38]. The RNA helicase of Dicer recognises the hairpin loop structures of pre-miRNAs and is able to differentiate between pre-miRNA and other double stranded RNAs [39–42]. Dicer uses the region between its PAZ and RNase III domains as a ‘molecular ruler’ to produce small RNAs with consistent size [43]. Both RNase III domains of Dicer cleaves the pre-miRNA with each RNase domain cleaving one strand of the small RNA duplex [44] (Figure 1b).

Although Dicer is capable of cleaving pre-miRNA and dsRNA alone, its activity is modulated by protein interactors. Two particular proteins well documented in this role are the related PACT and TRBP [30, 31]. Both of these Dicer binding proteins have three dsRBDs, one of which facilitates protein-protein interaction by binding to the helicase domain of Dicer [45–47]. Both proteins stabilize Dicer [48], affect the fidelity of miRNA processing [47, 49, and 50] and influence the subsequent strand selection of the miRNA [30, 47, and 51].

2.1.3. Argonautes and the RNA Induced Silencing Complex (RISC)

Dicer cleavage produces a short (21-23 nt long) double stranded RNA with a characteristic 2 nt overhang at the 3’ end [52, 53]. One of the strands of the miRNA duplex is incorporated into an Argonaute (Ago) protein, forming the minimal effector RNA induced silencing complex (RISC) [54–59].

Argonautes are bi-lobed proteins which are formed from four globular domains of an N-terminal, a PAZ domain (similar to the PAZ domain found in Dicer), a middle (MID) domain and a PIWI domain [60–62]. The N-terminal domain facilitates small RNA loading and the unwinding of the RNA duplex generated by Dicer [63]. The PAZ domain recognizes and anchors to the 3’ end of the miRNA [64]. The MID domain binds the 5’ terminal monophosphate moiety and the 5’ terminal nucleotide of the miRNA guide strand. The PIWI domain accommodates the miRNA-target RNA duplex and it folds similarly to RNase H [60].

Ago2 (one of the four mammalian Argonautes) is the only Ago that possesses endonuclease activity and can initiate the cleavage of the passenger strand of an extensively base-paired small RNA duplex or the targeted mRNA if it is perfectly complementary to the Ago2 bound small RNA. [60, 65–68]. An increasing amount of evidence also suggests that cleavage competent Argonautes also play a role in the processing of a pri-miRNA in C.elegans and in the Dicer dependent and independent maturation of pre-miRNAs in mammals and worms [69–72].

2.1.4. RNA-binding proteins involved in miRNA mediated gene regulation

Since the majority of mammalian miRNAs only share limited complementary to their targets and miRNAs also associate with Argonautes that lack endonuclease activity, target mRNA cleavage is a rare event in mammals. Therefore miRNAs in mammals mainly inhibit the translation of miRNAs and degrade targeted RNA in a non-sequence specific manner. These processes also involve a range of RNA-binding proteins.
The key proteins of a known mechanism of miRNA mediated translational repression and RNA decay are the members of the TNRC6C/GW182 protein family [73]. They contain an N-terminal, Gly and Trp (GW/WG) repeats in its Argonaute Hook Domain and a RNA recognition motif (RRM) [74]. The TNRC6C protein binds to the PIWI domain of Ago through their GW/WG repeats and localises Ago to the processing bodies (P-bodies) [75]. It also binds to the poly (A)-binding protein (PABP) [76] inhibiting its interaction with the cap binding protein eIF4G that is necessary for translation initiation [77,78]. Ago bound TNRC6Cs also recruit the deadenylase complex CCR4: NOT (Carbon catabolite repression 4- negative on TATA-less) which in turn recruits the DEAD box helicase DDX6 and facilitates the degradation of the miRNA target [76, 79–82] (Figure 1c).

Recent studies in flies and mammalian systems showed that miRNAs could repress translation in a GW182 independent way by inhibiting the formation of the eIF4F complex which recruits the translationally competent mRNA to the ribosomes [83–85]. RISCs facilitate the release of ATP-dependent RNA helicase proteins (eIF4A) from the eIF4F complex and therefore inhibit translation of the miRNA targeted mRNA [84, 85] (Figure 1d).

2.2. RNA-binding proteins that regulate miRNA biosynthesis

miRNA processing is regulated at each step of the maturation pathway by multiple RBPs to ensure tissue specific expression or proper response to environmental and cellular stimuli. [4,7,86,87]. RBPs can facilitate or inhibit miRNA processing either by recognizing and binding to RNA sequences or structures, or altering the function of the machinery involved in a specific step of processing (Figure 2).
Figure 2. RNA binding proteins that regulate (a) pri-miRNAs and (b) pre-miRNAs biogenesis by recognizing sequences or structures on the hairpin RNA. RBPs labelled with green are promoting miRNA processing and PBPs coloured with orange are inhibitors of the miRNA pathway. (c) RBPs that regulate the stability and turnover of mature miRNAs.

2.2.1. The regulation of pri-miRNA processing

Altering the structure of pri-miRNAs to promote the binding or action of the Microprocessor is a common form of regulating pri-miRNA processing. For example, p72 (DDX17) is a DEAD-box helicase subunit of the Microprocessor [17,88,89] which facilitates pri-miRNA processing by binding to the 3' flanking segments of the stem of the pri-miRNA hairpin [88,90,91]. While p72 is not required for the efficient production of all miRNAs, the production of a large subset of miRNAs are inhibited by the loss of p72 [88]. Also, data generated by p72 CLIP experiments showed that 160 pri-miRNAs bind to p72 and most of these associations were mapped to the stem of the pri-miRNAs [90]. p68 (DDX5), is also a DEAD-box helicase subunit of the Microprocessor complex [17,89] that is required for the efficient processing of a specific subset of pre-miRNAs [92]. p68 recognizes the internal bulges of the pri-miRNA stem and unwinds them in an ATP-dependent manner which promotes the binding of the Microprocessor complex [93].

RBPs can also regulate the processing of pri-miRNAs by recognizing and binding to the terminal loop of the pri-miRNAs [94]. For example, the heterogenous ribonucleoprotein A1 (hnRNPA1) is required for the efficient processing of pri-miR-18a, which is part of miR-17-92 cluster [95]. hnRNPA1 promotes pri-miR-18a processing by binding to its stem loop and generating a relaxed loop structure that is more
accessible for the Microprocessor and enhances Drosha cleavage [95]. Conserved miRNA loops have been proposed to have the potential to bind other RBPs mainly from the hnRNP protein family that may also influence pri-miRNA processing [96].

The loop can also provide a platform for proteins that inhibit pri-miRNA processing. For instance, Lin28 negatively regulates let-7a biogenesis by binding to the pri-miRNA loop sequence which inhibits the effective association of the Microprocessor [97].

In addition to recognizing structures on pri-miRNAs, RBPs can influence pri-miRNA processing via binding to specific sequences. Interestingly, hnRNPA1, besides promoting miR-18 processing, can also have inhibitory role in pri-miRNA processing. It binds to the GGG sequence of the pri-let-7a loop and displaces another RBP, KHSRP, a KH-type splicing protein, which is necessary for efficient pri-let-7a and pre-let-7a processing [94,98]. Another heterologous nuclear RNA-binding protein, hnRNPA2B1 binds to m6A sites (a site of methylation of the N6 nitrogen in adenosine, the most common internal modification of eukaryotic messenger RNA) located in the flanking regions of a subset of pri-miRNAs and facilitates the recruitments of the Microprocessor complex [99]. pri-miR-7 processing is also facilitated by direct binding of an RBP. In this case Serine/Arginine-Rich Splicing Factor 1 (SF2/ASF) recognises sequence motives on the miRNA stem region [100]. Fused in sarcoma (FUS) binds to the GU-rich elements of pri-miRNAs and promotes pri-miRNA processing of a distinct subset of miRNAs [101] (Figure 2a).

RBPs could also regulate pri-miRNA biogenesis by affecting the function of the Microprocessor without apparent binding to pri-miRNAs. Early proteomics study showed that members of the FET protein family (which is composed of FUS, EWS and TATA Box Binding Protein Associated Factor, (TAF15) proteins) co-immunoprecipitate with the Microprocessor [17] and subsequently all FET proteins have documented roles in the processing of miRNAs [102]. FUS controls the biogenesis of a subset of miRNAs by directly interacting with the Microprocessor and recruiting it to the transcription sites of miRNAs [101,103]. Ewings sarcoma protein (EWS) affects miRNA biogenesis by inhibiting the expression of Drosha possibly by binding to its promoter region [104].

2.2.2. The regulation of pre-miRNA processing

Similar to pri-miRNA processing, the turnover and further maturation of pre-miRNAs are also extensively regulated by auxiliary RBPs. The majority of these RBPs bind to the pre-miRNA and either affect the stability of the RNA or modulate Dicer binding and/or function.

KHSRP regulates the processing of a subset of miRNAs by binding to G-rich regions of the terminal loops of their precursors [98]. This binding promotes Dicer association to the pre-miRNA and subsequently increases the cleavage rate of the precursors. TAR DNA-binding protein-43 (TDP-43) regulates the processing of two miRNAs, pre-miR-143 and 547, by binding to UG rich motifs in their respective terminal loops [105]. The cold-stress induced protein RBM3 regulates pre-miRNA production through binding pre-let-7 and pre-miR-16 directly and promoting their association with Dicer [106].

miRNA precursors can also be subjected to modifications and RNA nuclease activities that decrease their stability and result in impaired miRNA production. The most extensively studied mechanism that regulates miRNA turnover is the inhibition of miRNA processing mediated by Lin28. Lin28 binds to GGAG sequences in the terminal loop of the pre-miRNA of let-7, miR-107, miR-143 and miR-200c and recruits two Terminal Uridylyl Transferases (TUTases) that polyuridynylate the 3’ end of the pre-miRNA. This leads to the degradation of the precursor [107–109]. Currently, MCPIP1 (monocyte chemoattractant protein-MCP-induced Protein 1) is the only example of an endo-RNAse that regulates the stability of select pre-miRNAs by endonucleolytic cleavage [110]. MCPIP1 cleaves the terminal loops of the
precursors which inhibit the binding of Dicer and accelerate the turnover of the pre-miRNAs [110]. Dicer recognizes and cleaves pre-miRNAs that are monophosphorylated at their 5’ ends [38,111,112]. The RNA-methyltransferase BCDIN3D interferes with the processing of pre-miR-145 by methylating its 5’ nucleotide that prevents Dicer binding and processing [113] (Figure 2b).

RBP s could also regulate miRNA biogenesis by affecting the expression or stability of the canonical proteins of the miRNA pathway. For example, the RBP AU-binding factor 1 (AUF1) regulates the general miRNA biosynthesis by inhibiting Dicer expression. AUF1 binds to the coding regions and the 3’ UTR of the Dicer mRNA and accelerates its turnover and decreases its expression [114].

2.2.3. Regulation of the turnover of mature miRNAs

Increasing number of studies show that the turnover rate of mature miRNAs are not uniform suggesting the existence of regulatory mechanisms that regulate the stability of individual miRNAs [115–117]. The exact mechanism of this phenomenon has not been revealed yet but RBPs that degrade or modify mature miRNAs have been identified in a wide range of organisms [118].

In mammals 5’ to 3’ (XRN1) and 3’ to 5’ exonucleases (RRP41 and PNPase) have been demonstrated to degrade a subset of miRNAs [119,120]. QKI, a member of the signalling transduction and activation of RNA (STAR) family represents a unique regulatory mechanism in which QKI and its isoforms directly bind to miR-20 and stabilize it[121] (Figure 2c).

2.2.4. RNA-binding proteins that influence the recognition and regulation of miRNA targets.

In mammals miRNAs mainly bind to the 3’ UTR of the target mRNAs [122]. The 3’ UTR is also a hotspot for RNA-binding proteins that regulate maturation, stability, transfer, localization and translation of mRNAs [123,124]. Therefore it is inevitable that the miRNA loaded RISCs interact with other RBPs when numerous RBPs may bind to the same 3’ UTR. These interactions could result in the inhibition of miRNA action either by competing for the same binding motif or restructuring the RNA so that it becomes inaccessible for RISC complexes. On the other hand, RBPs could also change the structure of the 3’ UTRs to favour miRNA binding that facilitates miRNA mediated gene regulation. A particular RBP could be either an inhibitor or an enhancer of miRNA targeting depending on the context of its association with the mRNAs (Figure 3).
Figure 3. RNA binding proteins can promote and inhibit miRNA action. Restructuring the target RNA by RBPs could result in miRNA targeting (a) or inhibition of RISC binding to the targeted RNA (c). (b) RBPs assisting miRNA targeting via direct binding to RISC. (d) RBPs and miRNA complexes can compete to target the same mRNA via overlapping target sites.
HuR could regulate miRNA action through diverse mechanisms. For instance, HuR was shown to compete with a few miRNAs over potential binding sites and could inhibit miRNA targeting on select mRNAs (Figure 3d). It relieves the translational repression of TOP2A, CAT-1, COX-2 and ERBB2 by masking the miRNA target sites for miR-548c-3p, miR-122, miR-16 and miR-331 respectively [125–127]. HuR could also inhibit miRNA mediated gene regulation by preventing the RISC dependent dissociation of eIF4A from the translation initiation complex [85] (Figure 1d). On the other hand, HuR could also facilitate miRNA mediated gene regulation. It promotes let-7a binding by increasing binding site accessibility to the c-MYC 3' UTR [128] (Figure 3a).

Polyprimidine tract binding protein (PTB) or hnRNP, an RBP with well described function in splicing and alternative splicing, I also have dual functions in miRNA mediated gene regulation by promoting or inhibiting miRNA targeting through an RNA dependent interaction with the RISC [129].

DND1 also inhibits the targeting potential of multiple miRNAs. Binding to the U rich regions of target sites of miR-221 and miR-372, DND1 protects certain mRNAs from miRNA mediated repression [130]. Interestingly, DND1 function in regulating miRNA mediated gene expression is conserved since it regulates the accessibility of miRNAs in zebrafish to regulate early development [131].

In cancer cells, coding region determinant binding protein (CRD-BP) competes with miR-340 on the 3' UTR of MITF and with miR-183 on the βTrCP1 transcript preventing their downregulation [132,133].

In addition, RNA Binding Motif Protein 38 (RBM38) inhibits miRNA targeting by binding to U-rich region in the proximity of miRNA target sites [127].

2.2.4.1. Transcriptome wide identification of RBPs that modulate miRNA mediated gene regulation

Cross-linking immunoprecipitation (CLIP) using Ago specific antibodies is being used to experimentally identify the Ago bound transcriptome that includes transcripts which are targeted by miRNAs [9,134]. This method has also been used to identify the footprint of different RBPs on whole transcriptomes [10,135–140]. Combining these approaches revealed that the interaction between miRNA targeting and RBP binding are much more widespread than was originally thought.

Ago2 CLIP combined with PTB knock down experiments showed that PTB could inhibit or facilitate miRNA mediated gene regulation either to bind to miRNA target sites or restructure the 3’ UTR of the co-targeted mRNAs [141]. PAR-CLIP analysis of Pumilo (PUM) revealed that PUM sites co-localize 50 nt to the proximal region of specific miRNA sites and enhance miRNA action in human B cells [142]. CLIP based analysis also revealed the interactions of two RBPs, Moloney Leukemia Virus 10 (MOV10) and Fragile X Mental Retardation Protein (FMRP) in regulating miRNA mediated gene regulation. MOV10 was mapped to bind GC-rich sequences to facilitate miRNA targeting while FMRP could counteract this cooperativity by binding to or near MOV10 sites [143].

2.3. Comparison of Ago2 and RBPs binding in HeLa cells using published CLIP data

To investigate to what extent the binding site of AGO2 and other RBPs could overlap in a genomic scale, we carried out a preliminary bioinformatics analysis in which we compared the results of CLIP experiments carried out with a range of RBPs including Eukaryotic initiation factor 4AIII (eIF4AIII) [136], hnRNPC[144], PTB[141], T-cell intracellular antigen 1(TIA1)[145], TIA1-like 1 (TIAL1)[145], U2 small nucleolar RNA auxiliary factor 65 (U2AF65) [144] and Upframeshift 1 UPF1[140] in HeLa cells and
compared these data generated with Ago2 CLIP data\[141\]. Our preliminary results show that a considerable number of Ago2 CLIP-ed sites overlap with CLIP-ed sites derived from at least one RBP (Table 1).

**Table 1. Identification of overlapping RBP and Ago2 binding sites**

<table>
<thead>
<tr>
<th>RNABP</th>
<th>Number of binding sites</th>
<th>Number of overlaps with Ago2 binding sites</th>
<th>Percentage of overlap in Ago2</th>
</tr>
</thead>
<tbody>
<tr>
<td>eIF4AIII</td>
<td>364659</td>
<td>35872</td>
<td>21%</td>
</tr>
<tr>
<td>hnRNPC</td>
<td>438360</td>
<td>6422</td>
<td>4%</td>
</tr>
<tr>
<td>PTB</td>
<td>308980</td>
<td>40618</td>
<td>24%</td>
</tr>
<tr>
<td>TIA1</td>
<td>21884</td>
<td>5029</td>
<td>3%</td>
</tr>
<tr>
<td>TIAL1</td>
<td>51751</td>
<td>9029</td>
<td>5%</td>
</tr>
<tr>
<td>U2AF65</td>
<td>1122142</td>
<td>40904</td>
<td>24%</td>
</tr>
<tr>
<td>UPF1</td>
<td>141390</td>
<td>14748</td>
<td>9%</td>
</tr>
</tbody>
</table>

The analysis of PAR-CLIP, HITS-CLIP and iCLIP datasets obtained from the StarBase version 2\[146,147\] shows that ~50% of Ago2 interaction sites in HeLa cells co-localize with the binding site of at least one RBP (Table 1). Interestingly, we also found that the sequenced read numbers are significantly (P<0.05) higher if they are covering sites that are recognized by Ago2 and other RBPs. This suggests that these sites are hotspots for RNA binding; therefore, inherently could be subjected to interaction such as competition and/or cooperation between different RBPs.

3. Concluding remarks

RBPs are key proteins not only involved in generating miRNAs and carrying out their function but they regulate miRNA processing and action at each step of the miRNA pathway. High-throughput identifications of RBP binding sites on mRNAs suggest that RBPs interaction with miRNA mediated gene regulation is potentially more widespread than the number of the verified interactions would suggest. Most of the RBPs that have documented roles in the regulation of the miRNA pathway act in a very specific environment. Therefore, it is very likely that most of these predicted potential interactions are the manifestation of responses to cellular or environmental stimuli or specific to developmental stage or cell type.

4. Materials and Methods

Bioinformatics:

CLIP-seq data (iCLIP, PAR-CLIP and HITS-CLIP) generated with AGO2 and multiple RBPs including eIF4AIII, hnRNPC, PTB, TIA1, TIAL1, U2AF65 and UPF1 in HeLa cells were downloaded from StarBase v2.0. The RBPs and Ago2 bound RNA fragments were mapped to human genome version hg19. RNA fragments that were bind to AGO2 as well as to any of the analysed RBPs with at least one nucleotide
overlap were counted and the corresponding sequencing read numbers were quantified. Significance calculation was carried out using one paired t-test in SPSS statistics tool.

Acknowledgments: We would like to thank Sarah Bajan for reading and commenting on the manuscript. This work was supported by the Australian Research Council (ARC) Future Fellowship. G.H. is an ARC Future Fellow.

Conflicts of Interest: The authors declare no conflict of interest.

References
14. Fang, W.; Bartel, D. P. The Menu of Features that Define Primary MicroRNAs and Enable De Novo Design of MicroRNA Article The Menu of Features that Define Primary MicroRNAs and Enable De Novo Design of


47. Wilson, R. C.; Tambe, A.; Kidwell, M. A.; Noland, C. L.; Schneider, C. P.; Doudna, J. A. Dicer-TRBP Complex


62. Schirle, N. T.; MacRae, I. J. The crystal structure of human Argonaute2. Science 2012, 336, 1037–40.


81. Rouya, C.; Siddiqui, N.; Morita, M.; Duchaine, T. F.; Fabian, M. R.; Sonenberg, N. Human DDX6 effects


© 2015 by the authors; licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons by Attribution (CC-BY) license (http://creativecommons.org/licenses/by/4.0/).
1.5 Aims and Hypothesis:

This study aims to characterise novel roles of RNA binding proteins in the miRNA biogenesis pathway. Each aims and hypothesis correlates to its respective results chapter which is presented in this thesis.

1.5.1: The miRNA biogenesis factors, p72/DDX17 and KHSRP regulate the protein level of Ago2 in human cells:

**Hypothesis:** We hypothesise that a knock down of the RNA binding proteins KHSRP and P72 regulate the stability of Ago2, the main effector protein of miRNA pathway via reducing the miRNA abundance

**Aim:** To determine if the RNA binding proteins KHSRP and P72 regulate the stability of Ago2 at a transcriptional, post-transcriptional or post-translational level. Furthermore, we aim to determine if miRNA abundance is related to the stability of Ago2. We will achieve this by utilising the methods of siRNA transfection, western blot and qPCR.

1.5.2: Transient inhibition of Dicer elevates the RNA and protein level of Ago2 in human cells

**Hypothesis:** We hypothesise that the transient inhibition the key miRNA biogenesis protein Dicer regulates the stability of Ago2.

**Aim:** To determine if inhibition of Dicer regulates Ago2 stability at a transcriptional, post-transcriptional or post-translational level. Also, we aim to determine whether the pool of Ago2 regulated by the inhibition of Dicer is loaded and involved in RNAi or unloaded. We will achieve this by utilising methods of siRNA transfection, western blot and qPCR.
1.5.3: Dicer regulates pri-miRNA biogenesis

**Hypothesis:** We hypothesis that the pre-miRNA processing protein Dicer, regulates pri-miRNA biogenesis.

**Aim:** To investigate the effect of Dicer knockdown on pri-miRNA levels in a range of cells. We aim to achieve this by utilising a variety of knockdown methods such as shRNA, siRNA and cre-lox genetic knock down. Pri-miRNA levels will be measured and quantified using qPCR.

1.5.4: Increasing miRNA and miRNA target abundance affects miRNA biogenesis

**Hypothesis:** We hypothesis that increasing miRNA and miRNA target abundance affects miRNA biogenesis.

**Aim:** To investigate the relationship between miRNA levels and miRNA target abundance on the miRNA biogenesis pathway. We aim to transfect increasing amounts of miRNA-mimic into cells and investigate the effect this has on the miRNA biogenesis. Furthermore, we aim to transfect in increasing amounts of miRNA target in the form of luciferase plasmids containing miRNA target sites, and in investigate the effect this has on the miRNA biogenesis. We aim to detect changes in miRNA biogenesis by measuring expression of miRNA transcripts.
2. Materials and Methods:

2.1. General Materials and Methods:

2.1.1 Buffers:

- Tris-Glycine-SDS Running Buffer (14% w/v Glycine, 3% w/v Tris pH 8.6, 1% SDS w/v)
- Tris-Glycine-Transfer Buffer (14% w/v Glycine, 3% w/v Tris, 20% methanol)
- TBS-Tween (Tris-Buffered-Saline or TBS-T) (100mM Tris pH 7.5, 150mM NaCl, 0.1% Tween-20 v/v)
- NP-40 Lysis Buffer (50mM Tris pH 7.5, 150mM NaCl, 1% NP-40, 1 Protease Tablet)
- Antibody Stripping Buffer (15% w/v Glycine, 1% SDS w/v, 1% Tween-20 v/v, pH to 2.2)
- 2 X SDS Protein Loading Buffer (125mM Tris 6.8 pH, 4% SDS w/v, 20% Glycine w/v, 200mM DTT, Bromophenol Blue)
- Phosphate Buffered Saline (PBS) (NaCl 8% w/v, KCL 2% w/v, 14% Na₂HPO₄ w/v, 2.4% KH₂PO₄ w/v)
- Ponceau Stain (0.1% (w/v) Ponceau S in 5% acetic acid.)
- BSA antibody storage (5% w/v BSA, 1X TBS, 0.1% Sodium Azide)
- Polysome Extraction Buffer (0.5% (v/v) NP-40130 mM KCl, 10 mM MgCl2, 10 μg/ml cycloheximide, 0.2 mg/ml heparin, 2.5 mM DTT, 0.4 units/μl RNasin)
2.1.2 Reagents:

- DMEM (Dulbecco’s modified Eagle medium) (Invitrogen)
- Opti-MEM (Invitrogen)
- FCS (Fetal Calf Serum) (Invitrogen)
- TrypLE Express (Invitrogen)
- Recovery Cell Culture Freezing Medium (Invitrogen)
- Pen-Strep (Penicillin Streptomycin) (Invitrogen)
- Passive Lysis Buffer (Promega).
- MG132 (Sigma-Aldrich)
- Dual-Luciferase® Reporter (DLR™) Assay (Promega)
- Cycloheximide (Sigma-Aldrich)
- Geldanamycin (Integrated Sciences)
- Trizol (Thermofisher)
- BCA Assay (Bicinchoninic acid) (Thermo-Scientific)
- TEMED (Tetramethylethylenediamine) (Bio-rad)
- Taqman® gene expression master mix (Applied Biosystems)
- Multiscribe Reverse Transcriptase(Applied Biosystems)
- RNase Inhibitor(Applied Biosystems)
- Lipofectamine RNAiMax (Invitrogen)
- Lipofectamine 2000 (Invitrogen)
- RT-Buffer Mix -10X (Applied Biosystems)
- 10X RT Random Primers (Applied Bioystems)
- DMSO (Sigma-Aldrich)
- Proteinase K (VWR)
- Blasticidin (Sigma-Aldrich)
- Doxycyclin (Sigma-Aldrich)
- Zeocin (Sigma-Aldrich)
- 4-hydroxytamoxifen (Sigma-Aldrich)
2.1.3 Oligonucleotides, RNA mimics, PCR primers, siRNAs:

<table>
<thead>
<tr>
<th>Name</th>
<th>Catalogue Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>U75</td>
<td>001219</td>
</tr>
<tr>
<td>hsa-let-7a</td>
<td>002283</td>
</tr>
<tr>
<td>hsa-let-7e</td>
<td>002406</td>
</tr>
<tr>
<td>hsa-miR-21</td>
<td>000397</td>
</tr>
<tr>
<td>hsa-miR-17</td>
<td>002421</td>
</tr>
<tr>
<td>hsa-miR-19</td>
<td>000395</td>
</tr>
<tr>
<td>mmu-miR-155</td>
<td>002571</td>
</tr>
</tbody>
</table>

Table 2.1 Primer Sequences for miRNA qPCR. All primers were purchased from Applied Biosystems Taqman.
<table>
<thead>
<tr>
<th>Name</th>
<th>Assay ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>EIF2C2(Ago2)2</td>
<td>Hs01085579_m1</td>
</tr>
<tr>
<td>KHSRP</td>
<td>Hs01100874_g1</td>
</tr>
<tr>
<td>Dicer 1</td>
<td>Hs00229023_m1</td>
</tr>
<tr>
<td>pri-hsa-miR-21</td>
<td>Hs03302625_pri</td>
</tr>
<tr>
<td>pri-hsa-let-7d</td>
<td>Hs03295173_pri</td>
</tr>
<tr>
<td>pri-hsa-let-7e</td>
<td>Hs03302562_pri</td>
</tr>
<tr>
<td>pri-hsa-miR-17</td>
<td>Hs03295901_pri</td>
</tr>
<tr>
<td>DDX17 (p72)</td>
<td>Hs00428758_m1</td>
</tr>
<tr>
<td>pri-mmu-miR-155</td>
<td>Mm03306395_pri</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Hs04420697_g1</td>
</tr>
<tr>
<td>18S</td>
<td>Hs03003631_g1</td>
</tr>
</tbody>
</table>

Table 2.2 Primer Sequences for gene qPCR. All primers were purchased from Applied Biosystems Taqman.

<table>
<thead>
<tr>
<th>Target</th>
<th>siRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>KHSRP</td>
<td>ON-TARGET SmartPool Anti-KHSRP(Dharmacon)</td>
</tr>
<tr>
<td>Dicer 1</td>
<td>ON-TARGET SmartPool Anti-Dicer (Dharmacon)</td>
</tr>
<tr>
<td>DDX17 (p72)</td>
<td>ON-TARGET SmartPool Anti-DDX17(Dharmacon)</td>
</tr>
<tr>
<td></td>
<td>siGenome Smartpool Anti-DDX172 (Dharmacon)</td>
</tr>
<tr>
<td>p68</td>
<td>CUCUAAUGUGGAGUGCGAC</td>
</tr>
</tbody>
</table>

Table 2.3 siRNAs.
<table>
<thead>
<tr>
<th>miRNA</th>
<th>miRNA mimic</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-17</td>
<td>miRVana miRNA mimic miR-17-3p (Thermofisher)</td>
</tr>
</tbody>
</table>

Table 2.4 miRNA mimics.
2.1.4 Plasmids:

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>psi-CHECK2 let-7 x8</td>
<td>Iwasaki et al, 2009</td>
</tr>
<tr>
<td>psiCHECK2 let-7 x0</td>
<td>Iwasaki et al, 2009</td>
</tr>
<tr>
<td>pCMV5 FLAG::Ago2 plasmid</td>
<td>Johnson et al, 2010</td>
</tr>
<tr>
<td>psiCHECK2 let-7 x3</td>
<td>Johnston et al, 2010</td>
</tr>
<tr>
<td>psiCHECK2 Mutated let-7 x3</td>
<td>Johnston et al, 2010</td>
</tr>
</tbody>
</table>

Table 2.5 Plasmids.
2.1.5 Antibodies:

<table>
<thead>
<tr>
<th>Primary Antibody Target</th>
<th>Antibody Used (Company)</th>
<th>Dilution in BSA solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ago2</td>
<td>Rat Monoclonal Anti-Ago2 Clone 11A9 (Sigma Aldrich)</td>
<td>1:1000</td>
</tr>
<tr>
<td>Tubulin</td>
<td>Mouse Monoclonal-Anti-α-Tubulin Clone DM1A. (Sigma Aldrich)</td>
<td>1:5000</td>
</tr>
<tr>
<td>KHSRP</td>
<td>Rabbit Monoclonal Anti-KSRP (Cell Signalling Technology)</td>
<td>1:1000</td>
</tr>
<tr>
<td>FLAG</td>
<td>Monoclonal ANTI-FLAG® M2 mouse (Sigma-Aldrich)</td>
<td>1:1000</td>
</tr>
<tr>
<td>Dicer</td>
<td>Dicer Antibody Rabbit (H-212) (Santa Cruz Biotechnology)</td>
<td>1:1000</td>
</tr>
<tr>
<td>Actin</td>
<td>C2 sc-8432 Mouse Monoclonal IgG(Santa Cruz Biotechnology)</td>
<td>1:5000</td>
</tr>
<tr>
<td>RPS6</td>
<td>Anti-RPS6 antibody Rabbit (Abcam)</td>
<td>1:1000</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Secondary Antibody Target</th>
<th>Antibody Used (Company)</th>
<th>Dilution in 5% Milk TBS-T</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat IgG</td>
<td>Donkey Polyclonal Secondary Antibody to Rat IgG (Abcam)</td>
<td>1:5000</td>
</tr>
<tr>
<td>Mouse IgG</td>
<td>Donkey Polyclonal Secondary Antibody to Mouse IgG (Abcam)</td>
<td>1:5000</td>
</tr>
<tr>
<td>Rabbit IgG</td>
<td>Anti-Rabbit IgG, HRP-linked Antibody (Cell Signalling Technology)</td>
<td>1:5000</td>
</tr>
</tbody>
</table>

Table 2.6 Antibodies.
2.1.6 Cell Culture maintenance:

HeLa, HEK293 and U2OS cells were grown at 37°C, 5% CO2 in DMEM supplemented with 2mM L-glutamine and 10% fetal calf serum and 1% pen-strep. Cells were grown in T75 flasks at 37°C and passaged once every 7 days. Cells were washed with 5mL PBS and split with 1.5mL of TRIple Express (Invitrogen). Cells were diluted 1:10 (1mL of cell suspended in PBS to 10mL of supplemented DMEM). Cell lines were discarded after 20 passages.

2.1.6.1 Propagation and induction of T-REX293 cells:

T-REX cells (Invitrogen) are transgenic cells that express the Tet-repressor protein in which a plasmid pTER-Dcr_sh2 (targeting Dicer) was transfected. In the presence of tetracycline/ doxycycline the plasmid encodes a short-hairpin RNA (sh-RNA) which targets the gene of interest and represses it (Schmitter et al, 2006). After T-REX Knockdown cells had grown to 100% confluence they were split. T-REX Dicer Knockdown cells were grown in the presence of 50 μg/mL zeocycin and 10 μg/mL blasticidin. Clones were then grown in 6 well plates in the presence of 1 μg/mL doxycycline to express Dicer specific shRNA. Control cells were exempt from the tetracycline/doxycycline treatment.
2.1.6.2 Propagation and induction of Mouse Embryonic Fibroblasts Dicer Knockdown cells:

Dicer flox/flox Mouse Embryonic Fibroblasts (MEF) are a transgenic cell line that utilise Cre-Lox recombination to delete specific sites of DNA. Two transgenic mice, one expressing Cre-protien, the other with lox flanked target gene are bred to produce a Cre-expressing mouse with a lox flanked gene of interest. The Cre protein is a site-specific DNA recombinase that catalyses the recombination of DNA between specific Lox sites. The Lox sites contain specific binding sites for Cre and facilitate recombination. A Cre recombinase protein binds to the lox site forming a dimer. This dimer then binds to the other lox site dimer and forms a tetramer. The DNA is cut at both loxP sites by the Cre protein. The strands are then re-joined with DNA ligase and the target DNA is spliced out (Reviewed by Sauer & Henderson, 1988). Cells were grown in DMEM supplemented with 2mM L-glutamine and 10% foetal calf serum and 1% pen-strep. After MEF cells had grown to confluence they were split and grown in 500nM OHT-4-hydroxytamoxifen to induce cre-recombinase activity. Control cells were exempt from the tamoxifen treatment.

After 24, 48, 72, 96 and 120 hours cell were harvested for RNA and Protein Samples.

Special thanks to Gantier Labs for supplying us with the Dicer flox/flox MEF cell line.
2.1.7 Transfection of human cells with siRNA and miRNA mimics:

Hela, HEK293 and U2OS cells were the cell lines transfected. The transfections were conducted in six well plates. Solutions of 500μL OptiMEM plus 2μL of 20μM siRNA (final concentration 20nM) or miRNA mimic and 500μL OptiMEM plus 3μL RNAimax were prepared for each well of a six well plate. Solutions were incubated at room temperature for 5 minutes then mixed together and incubated at room temperature for 15 minutes.

Cells were treated with TRypLE Express (Invitrogen), split, suspended in 10ml PBS centrifuged for 5 minutes at 14,000 x g. Cells were then counted using a haemocytometer and diluted in antibiotic free DMEM to a final count of 1x10^6 cells per half mL of solution. To each well of a six well plate 1mL of siRNA-RNAimax-OptiMEM solution was added as well as 500μL of cell suspension. 3 hours after transfection 1mL of supplemented DMEM was added to each well. 24 hours after the initial transfection, cells were washed in PBS and a secondary dose of 2μL of specific 20μM siRNA and 1mL OptiMEM was administered to each well. 3 hours after transfection 1mL of supplemented DMEM was added to each well. Cells were then harvested 24 hours after secondary transfection.
2.1.8 Transfection of human cells with plasmid DNA:

HeLa cells were co-transfected with 20nM siRNA as outlined above and increasing amount of FLAG::Ago2 expressing plasmid using Lipofectamine 2000 (Invitrogen). Each well of a 6 well plate was treated with 1mL OptiMEM, a specified amount FLAG::Ago2 plasmid and 3μL of Lipofectamine 2000. After 48 hours post initial transfection cells were harvested followed by the purification of RNA and proteins.

In the case of luciferase expressing psiCHECK2 plasmids, HeLa cells were transfected with KHSRP siRNA as outlined above. 24 hours after initial transfection cells were re-transfected with KHSRP siRNA as well as luciferase plasmids at 160 ng plasmid per well using Lipofectamine 2000 (Invitrogen) following the manufacturer’s instructions. 24 hours later cells were harvested for RNA and protein purification or lysed for luciferase assay.

2.1.9 Geldanamycin treatment:

HeLa cells were transfected with siRNA as described above. 48 hours after transfection, Geldanamycin was diluted in DMSO and added to cells for 18 hours at a final concentration of 10 μM. Cells were then harvested for RNA and protein purification.
2.1.10 Protein extraction:

Cells were washed in 1 X PBS and each well of a six well plate was lysed in 200μL of NP-40 Lysis Buffer. Lysate was collected and then centrifuged for 5 minutes at 10,800 rpm at 4°C. Supernatant was then collected. Protein concentration was determined by BCA Assay Kit (Thermo-Scientific) as per manufacturer’s instructions. 30μL of 1M DTT (final concentration 100mM) and 75μL of 2 X SDS Bromophenol dye were then added to samples. The samples were then heated for 10 minutes and stored at -20°C.

2.1.11 Western Blot Analysis:

Equal amounts of protein were loaded into a 10% SDS-polyacrylamide tris-glycine gel. Tris-glycine gels (Table 2.7) were run with tris-glycine-SDS (TGS) running buffer. Protein was then transferred from the gel to PVDF membranes (Millipore) using a wet transfer apparatus (Invitrogen) in Tris-glycine-methanol transfer buffer. Membranes were blocked with 5% fat-free milk powder (w/v) in TBST (Tris-Buffered Saline and 0.05% (v/v) Tween-20) for 1 hour at room temperature. Following this, membranes were washed extensively with TBST, and then incubated with primary antibody (Table 2.6) at 4°C overnight. After removing the membrane from the primary antibody, the membrane was then washed thoroughly in TBST, and then incubated with their corresponding horseradish peroxidase (HRP)-conjugated secondary antibody (Table 6.0) diluted in 5% skim milk powder (w/v)
in TBS-T for 120 minutes at room temperature. Membranes were washed in TBST and developed using either Supersignal West Pico Chemiluminescence (ECL) substrate (Thermo Scientific) or Amersham ECL Prime Western Blotting Detection Reagent (GE Lifesciences). Images were visualized using LAS-3000 Imaging System (Fuji) and an Amersham Imager 600 (Amersham). Western blots were quantified using ImageJ software.
**Polyacrylamide gels for Western Blotting**

<table>
<thead>
<tr>
<th>Stock mL</th>
<th>10% Gel (8mL total)</th>
<th>Stacking Gel (3.5mL total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H(_2)O</td>
<td>3.88mL</td>
<td>2.56mL</td>
</tr>
<tr>
<td>1.5M Tris 8.8</td>
<td>2.0mL</td>
<td>-</td>
</tr>
<tr>
<td>1M Tris 6.8</td>
<td>-</td>
<td>0.5mL</td>
</tr>
<tr>
<td>40% Acrylamide</td>
<td>2.0mL</td>
<td>0.4mL</td>
</tr>
<tr>
<td>20% SDS</td>
<td>40μL</td>
<td>20 μL</td>
</tr>
<tr>
<td>10% APS</td>
<td>40μL</td>
<td>20 μL</td>
</tr>
<tr>
<td>TEMED</td>
<td>8 μL</td>
<td>4 μL</td>
</tr>
</tbody>
</table>

Table 2.7 Recipe for 40% 5:1 bis-acrylamide Gels.

2.1.12 Luciferase Assay:

24 hours after psiCHECK2 plasmid transfection HeLa cells were lysed in 100μL of passive lysis buffer (Promega) per well of a 24 well plate. Samples were run in triplicates. Luciferase activity was quantified using a dual luciferase reporter assay system (Promega). Luminescence was measured on a Microlumat Plus LB96V microplate Luminometer.
2.1.13 Sucrose Cushion:

HeLa cells were treated with 50 μg/ml cycloheximide for 5 minutes. Cells were then washed in PBS, trypsinised and centrifuged (4°C, 2 mins, 2000 rpm) to form a cell pellet. The pellet was lysed in ice-cold polysome extraction buffer and centrifuged (4°C, 10 mins, 14000 rpm). The supernatant was then layered onto a 0.5 M sucrose cushion and samples were spun at 107,000 g for 45 mins at 4°C in an Optima Max Ultracentrifuge (Beckman Coulter), the pellet was re-suspended in 2x SDS loading dye.

2.1.14 RNA isolation:

Cells were washed in 1 X PBS and then each well was lysed with 500μL TRIzole (Invitrogen). RNA was isolated using DirectZol RNA miniprep kit (Zymo Research) as per manufacturer’s instructions. RNA concentrations were determined using the Nano-drop ND-1000 spectrophotometer. Samples were frozen in liquid nitrogen and stored at -80°C.
2.1.15 qPCR:

RNA samples were diluted to 100ng/μL and a master mix was prepared for each sample depending on if miRNA or mRNAs were being assayed (Table 2.8 and Table 2.9). Samples were then run in the Eppendorf-Vapoprotect at 25°C for 10 minutes, 37°C for 120 minutes and 85°C for 5 minutes. Samples were diluted 1:4 with RNase free water then stored at -20°C. cDNA samples were then prepared into master mixes for qPCR (Table 2.10).
<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount per sample (μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X RT Buffer</td>
<td>1.5</td>
</tr>
<tr>
<td>dNTP (100mM)</td>
<td>0.15</td>
</tr>
<tr>
<td>RNase Inhibitor (50 units/μL)</td>
<td>0.2</td>
</tr>
<tr>
<td>Primer</td>
<td>0.25 (of each 5x primer)</td>
</tr>
<tr>
<td>Reverse Transcriptase (50 units/μL)</td>
<td>1.0</td>
</tr>
<tr>
<td>RNA</td>
<td>2.0 (total 200ng)</td>
</tr>
<tr>
<td>RNase Free Water</td>
<td>8.95</td>
</tr>
<tr>
<td>Total Volume</td>
<td>15μL</td>
</tr>
</tbody>
</table>

Table 2.8 Recipe for master mix used in miRNA cDNA synthesis.
<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount per sample (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X RT Buffer</td>
<td>2.0</td>
</tr>
<tr>
<td>dNTP (100Mm)</td>
<td>0.8</td>
</tr>
<tr>
<td>RNase Inhibitor (50 units/ µL)</td>
<td>1.0</td>
</tr>
<tr>
<td>10X RT Random Primers</td>
<td>2.0</td>
</tr>
<tr>
<td>Reverse Transcriptase (50 units/ µL)</td>
<td>1.0</td>
</tr>
<tr>
<td>RNA</td>
<td>2.0 (total 200 ng)</td>
</tr>
<tr>
<td>RNase Free Water</td>
<td>10.2</td>
</tr>
<tr>
<td>Total Volume</td>
<td>20 µL</td>
</tr>
</tbody>
</table>

Table 2.9 Recipe for master mix used in mRNA cDNA synthesis

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount per sample (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Taqman® Master Mix 2X</td>
<td>5.0</td>
</tr>
<tr>
<td>Probe (20x)</td>
<td>0.5</td>
</tr>
<tr>
<td>cDNA</td>
<td>2.0</td>
</tr>
<tr>
<td>RNase Free Water</td>
<td>2.5</td>
</tr>
<tr>
<td>Total Volume</td>
<td>10 µL</td>
</tr>
</tbody>
</table>

Table 2.10 Recipe for qPCR 10 µL reactions.
cDNA samples were run in PCR-7500 Real Time PCR System-ABI for 40 cycles at 50°C - 20 minutes for holding, 95°C - 10 minutes for holding, 95°C - 15 seconds and 60°C - 1 minute cycling stages. Each sample was run in triplicate. Samples were then quantified in graph form as per the method outlined by Haimes *Demonstration of a ΔΔCq Calculation Method to Compute Relative Gene Expression from qPCR Data, 2010* (Haimes et al. 2010). Graphs were created in Igor Pro.

2.1.16 Statistical analysis:

Student t-test, two-tailed, homoscedastic, was used to determine statistical significance. A P-value of <0.05 was considered statistically significant. All statistical analysis was calculated in Microsoft excel.
3. The miRNA biogenesis factors, p72/DDX17 and KHSRP regulate the protein level of Ago2 in human cells:

**STATEMENT OF CONTRIBUTION OF OTHERS**

Patrick Connerty, Sarah Bajan, Judit Remenyi, Frances V. Fuller-Pace, Gyorgy Hutvagner. The miRNA biogenesis factors, p72/DDX17 and KHSRP regulate the protein level of Ago2 in human cells. BBA - Gene Regulatory Mechanisms, BBAGRM-16-7R1 (Submitted)

I attest that Research Higher Degree candidate Patrick Connerty was the primary contributor to the development of this publication. This extensive contribution included: synthesising and developing the data for and writing the manuscript.

Production Note:
Signature removed prior to publication.

Production Note:
Signature removed prior to publication.

Sarah Bajan

Fuller-Pace Frances

Production Note:
Signature removed prior to publication.

Production Note:
Signature removed prior to publication.

Judit Remenyi

Gyorgy Hutvagner
3.1 Introduction:

miRNAs are key post-transcriptional regulators of gene expression. miRNAs undergo several processing steps before they are loaded onto the RNA induced silencing complex (RISC) and are engaged in gene repression. In general, primary miRNAs (pri-miRNA) are processed in the nucleus by the Microprocessor complex into a precursor miRNA (pre-miRNA) (Denli et al. 2004). The precursor is then transported to the cytoplasm (Yi et al. 2003), where Dicer cleaves it into a double stranded miRNA complex (Hutvágner et al. 2001). One strand of the miRNA is then loaded onto an Argonaute protein which uses the bound strand as a guide for the sequence-specific targeting of mRNAs (Hammond et al. 2001; Martinez et al. 2002; Schwarz et al. 2003; Khvorova et al. 2003).

miRNAs control highly regulated biological pathways such as cell differentiation, proliferation and the immune response; therefore, it is not surprising that miRNA-mediated gene regulation itself is also tightly regulated. Transcription of miRNAs, miRNA processing and miRNA maturation are all influenced by auxiliary factors that modulate general miRNA biosynthesis and function or regulate the biogenesis of a subset of miRNAs (Ha & Kim 2014).

p72 (DDX17) and KH-type splicing regulatory protein (KHSRP), are two RNA-binding proteins that are necessary for the production of subsets of miRNAs (Suzuki et al. 2009;
Dardenne et al. 2014; Trabucchi et al. 2009). p72 is a DEAD-box helicase subunit of the Drosha complex which is required for altering pri-miRNA structure to facilitate Drosha processing (Suzuki & Miyazono 2013). KHSRP associates with pre-miRNA loops and promotes the recruitment of Dicer to pre-miRNAs (Trabucchi et al. 2009). KHSRP interacts with single-strand AU-rich-element-containing mRNAs and plays a vital role in mRNA decay, and binds to RNA-degrading enzymes and thus can promote rapid RNA degradation (Gherzi et al. 2010). Additionally, KHSRP auto-regulates let-7 production and in turn regulates Dicer expression through a negative feedback regulatory mechanism, as Dicer mRNA is a target of let-7 (Tokumaru et al. 2008; Repetto et al. 2012; Ha & Kim 2014).

An increasing amount of evidence shows that miRNA-mediated gene regulation is regulated through post-translational modification of Ago2, the main effector protein of the miRNA pathway in mammals (Johnston & Hutvagner 2011). Inhibition of HSP90 (Heat Shock Protein 90), a chaperone required for the loading of miRNAs into Argonaute proteins, leads to the degradation of unloaded Ago2 protein. This mechanism is mediated by the proteasome and therefore likely involves the ubiquitination of Ago2 (Iwasaki et al. 2010; Johnston & Hutvagner 2011). Recent studies have shown that decreasing miRNA levels induces the reduction of Ago2 protein levels post-transcriptionally (Martinez & Gregory 2013; Smibert et al. 2013). In these studies the roles of both the proteasome (Smibert et al. 2013) and the lysosome were demonstrated (Martinez & Gregory 2013).
Here we report that inhibiting auxiliary factors to the miRNA pathway, p72 and KHSRP, which promote the processing of abundant miRNAs at distinct steps of miRNA maturation, also leads to reduced levels of Ago2 protein in human cells. We also demonstrate that the loss of Ago2 is regulated post-transcriptionally and the lowered Ago2 expression is a direct consequence of the decreased miRNA levels in the cell. We also have evidence that this mechanism affects the pool of Ago2 that is not loaded with miRNAs.
3.2 Results:

3.2.1 The RNA-binding proteins p72 and KHSRP stabilise the protein level of human Ago2:

For an unrelated study, we tested the consequences of down-regulating three RNA-binding proteins; p68, p72 and KHSRP, in HeLa cells using gene specific siRNAs. Surprisingly, we observed that the loss of both p72 and KHSRP reduce Ago2 protein level (Figure 3.1). We decided to further study this unexpected finding.

First, we used gene-specific siRNAs to knockdown p72 and KHSRP expression by carrying out transfection of siRNAs. qPCR analysis of the mRNA level of p72 and KHSRP (Figure 3.2 A and Figure 3.3 A) and Western blotting of KHSRP (Figure 3.3 D) confirmed that the knockdown was successful and resulted in more than an 80% reduction of gene expression in both cases.

Both RNA-binding proteins have a well-described role in the regulation of processing of miRNAs. p72 is required for the production of subsets of miRNAs, which includes miR-21, by facilitating pri-miRNA processing (Suzuki & Miyazono 2013). KHSRP is also involved in the regulation of the processing of certain miRNAs including let-7a (Trabucchi et al. 2010; Repetto et al. 2012). Therefore, to test if the downregulation of p72 and KHSRP impairs miRNA processing, we used qPCR to quantify miR-21 and let-7a miRNA levels in the p72 and KHSRP targeted samples respectively. We observed a strong
reduction in the steady-state level of both miRNAs (Figure 3.2 B and Figure 3.3 B) in the samples where p72 and KHSRP were knocked down. As a negative control, we assayed the level of miR-19 which does not require the activity of p72 or KHSRP for production. miR-19 level was undisturbed in both the p72 and the KHSRP knocked down cells (Figure 3.2 C and Figure 3.3 C). These data suggest that we were able to successfully impede the function of p72 and KHSRP.

After the initial characterization of the p72 and KHSRP knockdowns we tested the effects of their impaired functionality on endogenous Ago2. In both cases we confirmed our initial observation that the reduction of p72 or KHSRP decreases the protein level of Ago2 (Figure 3.2 D and Figure 3.3 D).

Next, we tested if this phenomenon is specific in HeLa cells or if p72 and KHSRP knockdowns affect Ago2 level in other immortalized human cells. Knocking down the expression of p72 and KHSRP in HEK 293 and U2OS cells also resulted in a marked decrease in the protein level of Ago2 (Figure 3.4 A and B).
Figure 3.1 p72 and KHSRP stabilise human Ago2 protein. Hela cells were transfected with non-targeting siRNA (c), siRNA targeting p68 (p68), p72 (p72) and KHSRP (KHSRP) in triplicate experiments. Drosha, Dicer and Ago2 protein were visualised by Western blot. p72 and KHSRP knockdown reduce Ago2 protein levels, while p68 knockdown does not. GAPDH was used as a loading control.
Figure 3.2: p72 stabilises human Ago2 protein. HeLa cells were treated with non-targeting (Control) siRNA and siRNA targeting p72 (p72) in triplicate experiments. (A) The p72 knockdown was confirmed by measuring the relative p72 mRNA/18S RNA levels using Taqman qPCR (* = p<0.05). (B) Knocking down p72 resulted in a decrease of the level of mature miR-21 (*** = p<0.001). (C) miR-19 is not affected by p72 knockdown. The relative miR-21/U75 and miR-19/U75 RNA levels were quantified using Taqman small RNA qPCR. (D) The reduction of p72 destabilizes the human Ago2 protein. Human Ago2 was visualized by Western blotting and the relative Ago2/Tubulin protein level was calculated. The numbers above the panels indicate relative Ago2/Tubulin protein levels compared to the first control lane. (E) Knocking down p72 does not decrease the level of human Ago2 mRNA. RNA levels were quantified using Taqman qPCR and the relative Ago2 mRNA/18S RNA levels were compared.
Figure 3.3: KHSRP stabilises human Ago2 protein. HeLa cells were treated with non-targeting (Control) siRNA and siRNA targeting KHSRP (KHSRP) in triplicate experiments. (A) The KHSRP knockdown was confirmed by measuring the relative KHSRP mRNA/18S RNA levels using Taqman qPCR and Western blotting of KHSRP protein (** = p<0.01) (B) Knocking down KHSRP resulted in a decrease of the level of mature let-7a. (C) miR-19 is not affected by KHSRP knockdown. The relative let-7a/U75 RNA and miR-19/U75 levels were quantified using Taqman small RNA qPCR. (*** = p<0.005) (D) The inhibition of KHSRP destabilizes the human Ago2 protein. Human Ago2 was visualized by Western blotting and the relative Ago2/tubulin protein level was calculated. The numbers above the panels indicate the relative Ago2/tubulin protein levels compared with the first control. (E) Knocking down KHSRP does not decrease the level of human Ago2 mRNA. RNA levels were quantified using Taqman qPCR and the relative Ago2 mRNA/18S RNA levels were compared.
**Figure 3.4:** p72 and KHSRP stabilise Ago2 protein in a range of human cells. (A) U2OS cells and (B) HEK293 cells were treated with siRNA targeting p72 and KHSRP (*= unspecific band). p72 knockdown was confirmed by measuring the relative p72 mRNA/18S RNA levels using Taqman qPCR (** *= p<0.01). KHSRP knockdown was confirmed via western blot. Tubulin was used as a loading control. The numbers above the panels indicate the relative Ago2/tubulin protein levels compared with the first control.
3.2.2 P72 and KHSRP regulate Ago2 post-transcriptionally:

The first step in investigating the mechanism of p72 and KHSRP influence on the protein level of Ago2 was to determine if the two proteins alter the transcription of Ago2 or they regulate its expression post-transcriptionally. We used qRT-PCR to quantify the mRNA levels of Ago2 from p72 and KHSRP knocked down cells that displayed reduced Ago2 protein expression. We could not measure a significant difference in the steady state level of Ago2 mRNA between the control and p72/KHSRP targeted samples suggesting that these two proteins control Ago2 expression post-transcriptionally (Figure 3.2 E and Figure 3.3 E).

To further confirm that Ago2 expression is post-transcriptionally regulated by p72 and KHSRP we transfected an increasing amount of FLAG tagged Ago2 (Johnston & Hutvagner 2011) expressing plasmid into HeLa cells in which p72 and KHSRP were targeted with siRNAs. We used qPCR and Western blotting to quantify p72 mRNA (Figure 3.5 A) and KHSRP protein levels (Figure 3.5 B) respectively to confirm successful knockdowns. Western blot analysis revealed that when both KHSRP and p72 expression was knocked down, FLAG::Ago2 levels were also decreased despite being independently transcribed from endogenous Ago2 (Figure 3.5 A and B). This data confirms that p72 and KHSRP affect the expression of Ago2 at a post-transcriptional level. We have also noted that the regulatory effect of p72 and KHSRP could be saturated since HeLa cells transfected with the highest concentration of the FLAG::Ago2 plasmid showed no change in Flag tagged Ago2 expression between the p72/KHSRP knocked down and control cells (Figure 3.5 A and B).
Figure 3.5: p72 and KHSRP regulate Ago2 via a post-transcriptional mechanism. p72 (A) and KHSRP (B) was knocked down in HeLa cells using siRNAs followed by the transfection of increasing amount of FLAG tagged human Ago2 (FLAG::Ago2). The efficiency of p72 knockdown was verified with qPCR by calculating the relative level of the p72 between the knockdown and control cells. 18S RNA was used to normalize expression (A). The efficiency of KHSRP knockdown was verified with Western blotting. The relative KHSRP expression levels are indicated at the bottom of the panel. Tubulin was used as a loading control (B). The recombinant FLAG tagged Ago2 was detected by Western blotting using a FLAG antibody. The FLAG::AGO2 protein levels were quantified and their relative expression in the knocked down cells compared to the control cells were calculated. The relative FLAG::Ago2 expressions are indicated on the top of the panels. Tubulin was used as a loading control. (-): cells transfected with non-targeting siRNA.
3.2.3 P72 and KHSRP regulate Ago2 protein level through influencing the level of cellular miRNAs:

An increasing amount of evidence suggests that, in addition to auxiliary factors such as p72/KHSRP, the miRNA pathway is also modulated through post-translational modification of the effector protein Ago2 (Johnston & Hutvagner 2011). In addition, unloaded Argonaute is degraded by the proteasome (Smibert et al. 2013) and a decrease of miRNA production via the inhibition of key miRNA processing proteins such as Drosha, DGCR8 and Dicer, results in a drop of the level of Ago2 protein (Smibert et al. 2013; Martinez & Gregory 2013). Since knocking down both p72 and KHSRP resulted in a decrease of miRNA abundance it is a possibility that the reduced expression of Ago2 is a response to the decline in miRNA levels.

To test this hypothesis we transfected an increasing amount of mature miRNA into HeLa cells followed by knocking down p72 and KHSRP. In this experiment we used a mature miR-17 mimic because production of this miRNA is not regulated by either of these two RNA-binding proteins. Also, by providing the miRNA in its mature form we circumvent all processing steps that may be impaired by the downregulation of p72 and KHSRP prior to miR-17 incorporation into Ago2. Our results clearly show that increasing the level of available miRNA restores the level of the Ago2 protein in both experiments (Figure 3.6 A and B and Figure 3. 7 A and B).
**Figure 3.6:** miRNA abundance regulates Ago2 protein level. p72 (A) and KHSRP (B) were knocked down in HeLa cells using siRNAs followed by the transfection of increasing amount of a miR-17 mimic. (A) The efficiency of p72 knockdown was verified with qPCR by calculating the relative level of the p72 between the knockdown and control cells. 18S RNA was used to normalize expression. (B) The efficiency of KHSRP knockdown was verified with Western blotting. Tubulin was used as a loading control. miR-17 expression was measured using Taqman qPCR. U75 was used to normalize expression. The relative miR-17/U75 levels compared to the cells that were not transfected with miR-17 mimic were plotted. Endogenous Ago2 level was quantified with Western blotting using Tubulin as a loading control. The relative Ago2 levels between the knocked down cells and cells transfected with non-targeting siRNAs (-) are indicated above the panels.
Figure 3.7: miRNA abundance regulates Ago2 protein level. p72 (A) and KHSRP (B) were knocked down in HeLa cells using siRNAs followed by the transfection of miR-17 mimic. The efficiency of p72 knockdown was verified with qPCR by calculating the relative level of the p72 between the knockdown and control cells (Right panel). 18S RNA was used to normalize expression. The efficiency of KHSRP knockdown was verified with Western blotting. Tubulin was used as a loading control. miR-17 expression was measured using Taqman qPCR. U75 was used to normalize expression. The relative miR-17/U75 levels compared to the cells that were not transfected with miR-17 mimic were plotted (lower panel). Endogenous Ago2 level was quantified with Western blotting using Tubulin as a loading control. The relative Ago2 levels between the knocked down cells and cells transfected with non-targeting siRNAs (-) are indicated above the panels.
3.2.4 KHSRP and p72 regulate the level of unloaded Ago2:

Next, we wanted to determine which pool(s) of Ago2 are regulated by KHSRP and/or p72. Ago2 is a part of both low (LMW-RISC) and high molecular weight (HMW-RISC) Ago2 containing protein complexes. LMW-RISC consists of unloaded Ago2 and Ago2 loaded with a small RNA (minimal RISC) which performs RNAi (Rivas et al. 2005). HMW-RISC contains miRNA loaded Ago2 that is possibly associated with the translational machinery (La Rocca et al. 2015).

We and others have previously shown that the HSP90 machinery has a crucial role in the loading of Argonaute with small duplex RNAs and its activity is required for the stabilization of unloaded Ago1 and 2 (Izumi et al. 2013; Johnston et al. 2010). In order to test if KHSRP and p72 regulate Ago2 that is not associated with miRNAs we knocked down these proteins in HeLa cells followed by a treatment with Geldanamycin (GD), a potent inhibitor of HSP90 activity. As expected, inhibition of HSP90 in the control cells resulted in a significant 70% drop in Ago2 protein level (Figure 3.8). However, GD induced a more subtle decrease of Ago2 level in the p72 and KHSRP knocked down cells (50% and 30% respectively, Figure 3.8). This suggests that in cells with impaired p72/KHSRP function there are less available unloaded Ago2. When we compared the expression level of Ago2 between the control and the KHSRP/p72 knocked down cells after GD treatment we observed no difference in the steady state level of Ago2 (Figure 3.8). This strongly suggests that KHSRP and p72 influence the level of unloaded Ago2
and it is very unlikely that they have an additional effect on the amount of Ago2 that binds to small RNAs and are engaged in gene regulation.
Figure 3.8: p72 and KHSRP regulate the level of unloaded Ago2. p72 and KHSRP were knocked down in HeLa cells using siRNAs followed by mock (M) and Geldanamycin (GD) treatments (10μM for 18 hours). The efficiency of p72 knockdown was verified with qPCR by calculating the relative level of the p72 between the knockdown and control cells. 18S RNA was used as a loading control (**p<0.001). The efficiency of KHSRP knockdown and Ago2 level were verified with Western blotting. Tubulin was used as a loading control. The top set of values above the panel show the relative Ago2 level between the knockdown cells and the cells that were transfected with non-targeting siRNA (c). The second set of values above the panel show the relative Ago2 levels comparing the mock treated and GD treated cells.
3.3 Discussion:

In this study we have shown that impairing the function of KHSRP and p72, two RNA-binding proteins with auxiliary functions in diverse steps of miRNA maturation, results in a significant decrease of Ago2 protein in several transformed human cell lines. We have demonstrated that p72 and KHSRP regulate Ago2 post-transcriptionally. Our data support the notion that the reduction of Ago2 protein level is very likely a consequence of the decrease of abundant miRNAs in the cells due to insufficient miRNA processing. We have presented supporting evidence that the p72/KHSRP mediated regulatory mechanism targets unloaded Ago2 and they unlikely influence the quantity of small RNA associated Ago2 complexes.

Our data support the recent findings that investigated the effects of miRNA abundance on the stability of mammalian and fly Ago2 (Smibert et al. 2013; Martinez & Gregory 2013). Those studies demonstrated that interfering with essential factors of miRNA biogenesis, by inhibiting the function of DGCR8/Dicer in mouse MEF and ESCs and Drosha in fly S2 cells, results in a drop in the level of Ago2 protein by the lysosome and proteosome respectively (Martinez & Gregory 2013; Smibert et al. 2013). Here we further demonstrate that a similar mechanism exists in the highly transformed HeLa cell line. Our finding that the miRNA level regulates the level of the unloaded Ago2 in HeLa cells supports the hypothesis that the proteasome is a key factor regulating Ago2 that are not bound to small RNA (Johnston et al. 2010; Smibert et al. 2013). However, we cannot
exclude the possibility that Ago2 level is regulated differently in embryonic stem cells (Martinez & Gregory 2013).

One of our key results here indicates that auxiliary proteins, not essential to the processing of all miRNAs, can also influence miRNA levels to a degree that the key effector protein is destabilized. Increasing numbers of auxiliary proteins have been identified in the miRNA pathway that either regulate the processing of all miRNAs or influence the maturation of a subset of miRNAs (Ha & Kim 2014). Most of these proteins have well described roles in diverse signalling pathways and are regulated by various cellular and environmental stimuli (Winter et al. 2009). Therefore, the regulation of auxiliary proteins of the miRNA pathway could alter the functionality of the whole miRNA pathway by modulating the level of unloaded Ago2 that would, in time, affect Ago2 complexes that are engaged in gene regulation. This could be applied to proteins that specifically regulate the processing of a subset of miRNAs since characteristically, only a few miRNAs contribute dominantly to the total miRNA level in mammalian cells (Landgraf et al. 2007).
4. Transient inhibition of Dicer elevates the RNA and protein level of Ago2 in human cells:

4.1 Introduction:

In humans, one of the most abundant small regulatory RNAs are microRNAs (miRNAs). They play a key role in post-transcriptional regulation of gene expression by targeting complementary sequences within mRNAs and repressing their translation (Ha & Kim 2014).

miRNA biogenesis begins with RNA polymerase II transcribing the miRNA transcript, known as the primary miRNA from a miRNA gene (Lee et al. 2004). The pri-miRNA is then processed by the Drosha/DGCR8 Microprocessor into a pre-miRNA (Denli et al. 2004). The pre-miRNA is then transported out of the nucleus to the cytoplasm where the RNase III enzyme Dicer cleaves it into a miRNA duplex (Yi et al. 2003; Hutvágner et al. 2001). The duplex is then loaded into Ago2 protein by the chaperone protein HSP90 (Iwasaki et al. 2010; Johnston et al. 2010). One strand of the duplex is degraded and the other remains loaded in the Ago2 as a guide strand. Ago loaded with a miRNA form the minimal components of the RISC (Hammond et al. 2001). The RISC can then inhibit gene expression by binding to target mRNAs and either degrading the mRNA or inhibiting translation (Martinez et al. 2002; Schwarz et al. 2003; Khvorova et al. 2003).
Affecting the abundance of key miRNA biogenesis proteins influences the levels of primary, precursor and mature miRNAs. Knockdown of Drosha or DGCR8 results in an accumulation of pri-miRNA but a decrease of the downstream precursor and mature miRNAs (Han et al., 2004). Likewise, knocking down of Dicer results in an accumulation of pre-miRNAs but a decrease in mature miRNAs (Grishok et al. 2001; Hutvágner et al. 2001).

In the case of Ago2, over expression of Ago2 results in a significant increase in mature miRNA levels, while Ago2 knockout MEFs and hematopoietic cells have dramatically reduced mature miRNAs (Diederichs & D. A. Haber 2007; O’Carroll et al. 2007). Furthermore, an accumulation of pre-miRNAs have been observed in Ago2 knockouts suggesting a role for Ago2 in Dicer mediated pre-miRNA processing (Diederichs & D. A. Haber 2007).

However, recent research has demonstrated that genetic knock out experiments in murine and fly embryonic cells showed that eliminating the function of the key proteins of pri- and pre-miRNA processing, DGCR8 and Dicer, severely reduce Ago2 levels via directly reducing miRNA abundance (Smibert et al. 2013; Martinez & Gregory 2013). Interestingly, in immortalised human cells the genetic knock out of Dicer resulted in no significant change in Ago2 levels despite a reduction in mature miRNAs (Bogerd et al. 2014).
Here we show, that the transient inhibition of Dicer using siRNAs leads to increased levels of both Ago2 mRNA and protein in a range of human cells. We have evidence that the elevation of Ago2 is regulated post-transcriptionally. Furthermore, we have demonstrated that this mechanism affects the pool of Ago2 that is not loaded with miRNAs.
4.2 Results:

4.2.1 Impairing the expression of human Dicer with siRNA elevates the mRNA and protein level of human Ago2:

Genetic knock out and knockdown experiments in murine and fly embryonic cells have shown that eliminating the function of the key proteins in pri- and pre-miRNA processing severely reduce Ago2 levels in mice and fruit flies (Martinez & Gregory 2013; Smibert et al. 2013). However, recent data has demonstrated that knocking out Dicer in HEK 293 cells does not alter the protein level of human Ago2. We decided to investigate the effect of Dicer on the stability of Ago2 in HeLa cells. HeLa cells were treated with sequential transfection of Dicer targeting siRNA and 72 hours later the protein level of Dicer and Ago2 were quantified (Figure 4.1 A). We also measured the mRNA level of Dicer and Ago2 as well as the expression level of two abundant miRNAs, let-7a and miR-21 (Figure 4.1 B and C). The marked decrease of the level of both miRNAs suggests that the function of Dicer was successfully impaired. To our surprise, the reduced Dicer activity reproducibly resulted in a significant increase in both the mRNA and the protein level of Ago2 (Figure 4.1 A and B).

Next, we tested if this phenomenon is specific in HeLa cells or present in other immortalized human cells. Impairing Dicer in HEK 293 and U2OS cells also resulted in a marked increase in the mRNA and protein level of Ago2 (Figure 4.2 A and B).
Figure 4.1: Dicer knockdown stabilises human Ago2 protein. HeLa cells were treated with non-targeting (Control) siRNA and siRNA targeting Dicer (Dicer). (A) Dicer knockdown increases Ago2 protein. Dicer and Ago2 were visualized by Western blotting and the relative Dicer/Tubulin and Ago2/Tubulin protein level was calculated. The numbers above the panels indicate relative protein levels compared to the control lane. (B) Knocking down Dicer resulted in a decrease of Dicer mRNA (left panel) and an increase in Ago2 mRNA (right panel). The relative mRNA/18S RNA levels were quantified using Taqman qPCR. (** = p<0.001). (C) The reduction of Dicer results in a decrease of the level of mature miR-21 (left panel) and mature let-7a (right panel). The relative miR-21/U75 RNA and let-7a/U75 levels were quantified using Taqman small RNA qPCR. (** = p<0.05)
Figure 4.2: Dicer stabilises human Ago2 protein in a range of human cells. (A) U2OS and (B) HEK293 cells were transfected with either non-targeting (control) or Dicer siRNA (Dicer). Dicer and Ago2 were visualized by Western blotting and the relative Dicer/Tubulin and Ago2/Tubulin protein level was calculated. The numbers above the panels indicate relative protein levels compared to the control lane. Dicer knockdown was further confirmed in U2OS (C left panel) and HEK293 (D left panel) by measuring the relative Dicer mRNA/18S RNA levels using Taqman qPCR (** = p<0.001). Dicer knockdown increases Ago2 mRNA in both U2OS (C right panel) and HEK293 (D right panel). The relative mRNA/18S RNA levels were quantified using Taqman qPCR (** = p<0.005).
4.2.2 Human Dicer regulates Ago2 post-transcriptionally:

Since the inhibition of Dicer resulted in the accumulation of both the mRNA and the protein levels of Ago2, we tested whether Dicer regulates Ago2 at the transcriptional or post-transcriptional level. First, we transfected HeLa cells with both Dicer targeting siRNAs and an increasing amount of FLAG-tagged Ago2 expressing plasmid (FLAG::Ago2). Western blot analysis revealed that when Dicer was knocked down, despite being independently transcribed from endogenous Ago2, FLAG::Ago2 levels were increased (Figure 4.3 A). This is an indication that Dicer may regulate the level of Ago2 post-transcriptionally.

One obvious post-transcriptional regulation that could result in the upregulation of Ago2 is the impaired miRNA pathway as a consequence of the Dicer knockdown, as Ago2 has been documented to be regulated by miRNAs (J. Zhang et al. 2014). miRNAs mainly target mRNAs at the 3’ UTR sequences and since the transfected FLAG::Ago2 plasmid mRNA lacks the 3’ UTR of endogenous Ago2, it is very unlikely that the observed upregulation of Ago2 is miRNA dependent. However, we cannot exclude the possibility that miRNAs target Ago2 by recognizing sequences in the coding region (Brümmer & Hausser 2014).
4.2.3 Inhibition of Dicer elevates the levels of unloaded Ago2:

There are two pools of Ago2 which exist in the cell. These are Ago2, which is not loaded or loaded with only a small RNA, and Ago2 associated with other RISC machinery and engaged in translational repression (La Rocca et al. 2015). We wanted to determine which pool of Ago2 was being increased by Dicer knockdown.

First we tested if knocking down Dicer influences the level of the pool of Ago2 that are not bound to small RNAs. We knocked down the expression of Dicer in HeLa cells followed by a treatment with the HSP90 inhibitor Geldanamycin (GD). GD is a potent inhibitor of the HSP90 chaperone complex, which is vital for loading Ago2 with double stranded miRNAs (Iwasaki et al. 2010). It also stabilizes unloaded Ago2 which is subjected to proteasomal degradation in the absence of HSP90 activity (Johnston et al. 2010). Western blot analysis revealed that inhibition of HSP90 in Hela cells transfected with control siRNA resulted in an expected significant 70% drop in Ago2 protein level (Figure 4.3 B). Interestingly, Ago2 accumulated in the GD treated Dicer knocked down cells; however this increase of Ago2 level was less than the accumulation of Ago2 if Dicer was knocked down without GD treatment (4.2 fold compared to 11.3 fold in Figure 4.3 B). This data suggests that impairing Dicer function stabilizes the unloaded Ago2 protein.
Ago2 containing complexes can be distinguished by their molecular weights. The unloaded and small RNA loaded Ago2 represent a low molecular weight fraction (LMW-RISC) in the cell while the RISC engaged in translational repression is a part of a heavy molecular weight protein complex (HMW-RISC) (La Rocca et al. 2015). These markedly different complexes can be separated by fractionation experiments (La Rocca et al. 2015). To further confirm that the accumulation of Ago2 in the Dicer impaired cells affect the unloaded fraction of Ago2 complexes, we fractionated HeLa cells transfected with control siRNA and siRNA targeting Dicer using a sucrose cushion. This method of fractionation was developed to enrich the ribosome bound proteome and we previously have shown that it also separates LMW and HMW-RISC complexes (Halbeisen et al. 2009; Jannot et al. 2011). Downregulation of Dicer again resulted in a marked increase of Ago2 protein and mRNA level (Figure 4.4 A and B). Our fractionation data clearly shows that the increased level of Ago2 only accounted for the accumulation of Ago2 in the non-ribosomal, LMW fraction, supporting the hypothesis that inhibition of Dicer increases unloaded or small RNA loaded Ago2 (Figure 4.4 C).
Figure 4.3: Dicer regulates Ago2 via a post-transcriptional mechanism. (A) Dicer was knocked down in HeLa cells using siRNA followed by the transfection of increasing amount of FLAG tagged human Ago2 (FLAG::Ago2). The efficiency of Dicer knockdown was verified with Western blot. Tubulin was used as a loading control. The recombinant FLAG tagged Ago2 was detected by Western blotting using a FLAG antibody. The FLAG::AGO2 protein levels were quantified and their relative expression in the knocked down cells compared to the control cells were calculated. The relative FLAG::Ago2 expressions are indicated on the top of the panels. Tubulin was used as a loading control. (-): cells transfected with non-targeting siRNA. (B) Dicer regulates the level of unloadd Ago2. Dicer was knocked down in HeLa cells using siRNAs followed by mock (M) and Geldanamycin (GD) treatments (10μM for 18 hours). The efficiency of Dicer knockdown and Ago2 level were verified with Western blotting. Tubulin was used as a loading control. The top set of values below the panel show the relative Ago2 level between the knockdown cells and the cells that were transfected with non-targeting siRNA.
Figure 4.4: Dicer increases the level of low molecular weight (LMW) -RISC. HeLa cells were treated with non-targeting (Control) siRNA and siRNA targeting Dicer (Dicer) then fractionated with ultracentrifugation through a sucrose gradient. (A) Dicer and Ago2 were visualized by Western blotting and the relative Dicer/Tubulin and Ago2/Tubulin protein level was calculated. The efficiency of Dicer knockdown and Ago2 level were verified with Western blotting. Tubulin was used as a loading control. (Panel two) Dicer mRNA knockdown was verified by qPCR by calculating the relative level of the p72 between the knockdown and control cells. 18S RNA was used to normalize expression. (B) The reduction of Dicer results in a decrease of the level of mature miR-21. The relative miR-21/U75 RNA levels were quantified using Taqman small RNA qPCR (***=p<0.001). (C) Dicer knockdown increases Ago2 mRNA. The relative Ago2/GAPDH RNA levels were quantified using Taqman small RNA qPCR (*=p<0.05). (D) Dicer increases the level of LMW-RISC. Dicer knockdown cells have increased Ago2 protein levels in the light (L) fraction of the cell compared to controls. RPS6 was used as a control to verify successful fractionation.
4.3 Discussion:

In this study we have shown that impairing the function of Dicer, the protein that processes pre-miRNAs, results in a significant increase in the level of both mRNA and protein levels of Ago2, the key protein of the RISC, in several immortalized human laboratory cell lines. We have provided evidence that Dicer regulates Ago2 level post-transcriptionally and that the elevated Ago2 level is likely an increase in the pool of unloaded Ago2. Our data demonstrates that Ago2 responds differently to the transient siRNA mediated inhibition of human Dicer, compared to the genetic knockout of DGCR8 and Dicer in murine, fly and human cells (Martinez & Gregory 2013; Smibert et al. 2013; Bogerd et al. 2014; Kim et al. 2016). Studies in murine and fly embryonic cells demonstrated a marked decrease in Ago2 stability upon DGCR8 and Dicer deletion (Martinez & Gregory 2013; Smibert et al. 2013). The depletion of Ago2 was found to be a direct result of decreased miRNA abundance (Martinez & Gregory 2013; Smibert et al. 2013). However, MEF, HEK293 and HCT116 Dicer (-/-) cells in which Dicer was depleted with targeted genetic knock out techniques showed no measurable changes in Ago2 protein levels compared to the sister cell lines (Bogerd et al. 2014; Kim et al. 2016; Frohn et al. 2012). The stabilization of Ago2 in Dicer (-/-) HEK293 cells has been explained by its binding to a pool of small RNAs with unknown origin and function that replaced miRNAs, therefore able to stabilize free Agos (Bogerd et al. 2014).

In this study we provided evidence that the initial inhibition of Dicer by siRNA, elevates both the mRNA and protein level of Ago2, despite a significant reduction in miRNA
abundance. The simplest explanation of these conflicting data is that inhibition of Dicer affects cellular pathways that are involved in depleting unloaded Argonautes. So far, two such mechanisms were described. Martinez et al, showed that unloaded Ago2 is degraded by the proteasome in MEF cells while in fly S2 cells, the lysosome was indicated to be responsible for the depletion of Ago2 that are not bound to small RNAs (Martinez & Gregory 2013; Smibert et al. 2013). Both pathways are regulated by miRNAs therefore; it is possible that the impairment of Dicer resulted in the downregulation of these mechanisms and in turn unloaded Ago2s are accumulated (Kurashige et al. 2012; Frankel & Lund 2012). Since long term inhibition of these cellular pathways are lethal, we hypothesise that the selected Dicer (−/−) clones from HEK293 and HCT116 overcame the limited proteasome and lysosome activities and in the long term, Ago2 became loaded with small RNAs that do not require Dicer for their processing. An increasing number of studies have shown that Ago2 is involved in miRNA processing and could process subsets of miRNAs independently of Drosha and Dicer such as mirtrons, tRFs and the newly identified intronic miRNAs dubbed Agotrons (Hansen et al. 2016; Ruby et al. 2007; Kim et al. 2016). In fact, Hansen et al found that the Agotrons were stabilised by increased Ago2 expression and enriched in Dicer knockdown HEK293 cells (Hansen et al. 2016).

We also observed a marked increase in the mRNA level of Ago2 after inhibiting Dicer. We have excluded the possibility of transcriptional activation of Ago2, however, it is still possible that Ago2 mRNA is stabilised post-transcriptionally that in turn results in an elevated Ago2 protein level. Ago2 mRNA has been shown to be regulated by miRNA-mediated repression, therefore, the relief from miRNA-mediated repression followed by
the downregulation of Dicer would be a logical explanation for the detected increase of Ago2 mRNA level. However, the miRNAs known to regulate Ago2 expression target its 3′ UTR and are not expressed abundantly in the cell lines used in this study (Guo et al. 2015; J. Zhang et al. 2014). Transfection of recombinant Ago2, without its 3´ UTR, also showed increased Ago2 expression in Dicer knocked down cells. This makes miRNA-mediated regulation that targets the 3´ UTR of Ago2 an unlikely mechanism for explaining the elevated Ago2 mRNA level. miRNA have been shown to be able to inhibit gene expression by recognizing complementary sequences on the coding sequences of mRNAs (Reczko et al. 2012; Xu et al. 2014). Therefore, we cannot formally rule out the possibility that the elevated Ago2 mRNA and protein level is a consequence of a so far uncharacterized miRNA dependent regulation that targets Ago2 in the CDS. A similar mechanisms has been reported in the regulation of Dicer which is targeted by let-7 at multiple sites on the coding region of the Dicer mRNA (Tokumaru et al. 2008).

Recent studies have demonstrated that induction of cellular stress can re-localise Ago2 and decrease miRNA-mediated RNAi. Detzer et al found that transfection of phosphorothioate modified oligonucleotides induces cell stress and directs Ago2 to stress granules (Detzer et al. 2011). Interestingly, they also noted that translocation of Ago2 to the stress granules resulted in a significant decrease in RNAi (Detzer et al. 2011). Furthermore, they observed that Ago2 shows increased stability under cell stress and that the half-life of Ago2 increased from ~4 hours to over 48 hours (Detzer et al. 2011). This data indicates cellular stress can stabilize Ago2 protein. It is possible that the sudden and massive drop in Dicer expression triggers a stress response in the cell
and localises Ago2 to the stress granules. This could explain the increased Ago2 stability as well as a dissociation of Ago2 from the translational machinery to LMW-RISC observed in this study. This could also be why this phenomenon is not observed in Dicer knock out cells.
5. Dicer regulates pri-miRNA biogenesis:

5.1 Introduction:

As miRNAs are important gene regulators, it is important that miRNA biogenesis is highly regulated and well understood. The main proteins of canonical miRNA biogenesis are Drosha/DGCR8, Dicer and Argonaute. Depleting or inhibiting the function of these proteins has significant effects on miRNA biogenesis. Studies have shown that knockdown of Drosha or DGCR8 results in an accumulation of pri-miRNA and a decrease in the downstream pre-miRNA and mature miRNA levels (Han et al. 2004; Schmitter et al. 2006). Likewise, a knockdown of Dicer causes an accumulation of pre-miRNA and a reduction in mature miRNA (Grishok et al. 2001; Hutvágner et al. 2001). Furthermore, Ago2 knockout MEFs and hematopoietic cells demonstrate greatly reduced stability of mature miRNAs (Diederichs & D. a. Haber 2007; O’Carroll et al. 2007; Schmitter et al. 2006).

In this study we investigated the role of Dicer, the main protein involved in the processing of pre-miRNAs into mature miRNAs, in pri-miRNA biogenesis. Using a variety of systems to deplete Dicer, we observed that impairing Dicer function affects the pri-miRNA levels of multiple miRNA species. We also found that the effect of Dicer on individual pri-miRNAs is cell specific and occurs in both human and mouse cells. This data suggests a feedback mechanism that senses the level of Dicer and effects the production or stability of pri-miRNA.
5.2 Results:

5.2.1 Short hairpin RNA (shRNA) induced knockdown of Dicer in T-REX 293 cells increases the level of pri-let-7a:

While it has been established that knockdown of Dicer results in an accumulation of pre-miRNAs and a reduction of mature miRNAs, recent studies have documented that mature miRNAs can enhance the processing of pri-miRNAs (Zisoulis et al. 2012). We aimed to investigate the effects of inhibition of Dicer on pri-miRNA processing.

The first step to investigating this was to knockdown Dicer. To achieve this, we utilised an inducible gene inhibitory system that is based on short hairpin RNAs (shRNA) that are processed by the cells into siRNAs and target endogenous genes. T-REX 293 cells are a transgenic human embryonic kidney 293 (HEK293) cell line which expresses an inducible shRNA targeting Dicer (Schmitter et al. 2006). The T-REX cell line was chosen for its efficient shRNA system which can maintain a longer inhibition of gene expression. The cells contain an integrated and inducible shRNA gene targeting Dicer. Transcription of this shRNA gene is inhibited by doxycycline repressors binding to the shRNA gene promoter. Cells were grown in the presence of Zeocin for 120 hours to competitively bind to the Zeocin repressors and relieve the shRNA gene promoter. This then induces shRNA expression. Following this treatment, RNA was purified from control and induced cells.
To confirm that Dicer expression had been successfully impaired, we used qPCR to measure Dicer mRNA levels in Dicer-targeting-shRNA expressing T-REX 293 cells. qPCR analysis revealed that Dicer mRNA levels had decreased 65% compared to non-induced controls (Figure 5.1 A). However, to ensure that the observed reduction in Dicer had a functional effect on miRNA biogenesis we also measured the levels of three mature miRNAs (let-7a, miR-21 and miR-19a) using Taqman small RNA qPCR. These miRNAs were chosen as they are all abundantly expressed in T-REX 293 cells. Our results showed that mature miRNA levels for all three miRNAs tested were reduced >50% in Dicer-targeting-shRNA induced cells compared to the non-induced controls (Figure 5.1 B). This confirmed that the reduction of Dicer expression had a functional impact on miRNA biogenesis.

Next, we investigated the effect of Dicer knockdown on pri-miRNA biogenesis. Using qPCR we measured the expression of pri-let-7a and pri-miR-21 in induced cells compared to controls. While there was no significant change in pri-miR-21 expression in Dicer impaired cells, pri-let-7a levels were significantly increased by almost 120% (Figure 5.1 C). This data suggests that Dicer knockdown has an impact on the biogenesis or turnover of at least a subset of pri-miRNAs.
**Figure 5.1:** shRNA induced Dicer knockdown (KD) increases pri-let-7 levels. HEK293-TREX cells containing an inducible gene encoding a Dicer targeting shRNA were treated with 50μg/mL Zeocin and 10μg/mL Blastcoid to induce shRNA expression. (A) The Dicer knockdown was confirmed by measuring the relative Dicer mRNA/18S RNA levels using Taqman qPCR. (B) Knocking down Dicer resulted in a decrease of the level of mature let-7a, miR-21 and miR-19a. The relative miRNA/U75 RNA levels were quantified using Taqman small RNA qPCR. (C) The reduction of Dicer results in an increase in pri-let-7a levels compared to control cells. (D) pri-miR-21 levels do not change upon induction of Dicer KD. The relative pri-miRNA levels were quantified using Taqman qPCR and GAPDH as a control.

* = P value < 0.05  
** = P value < 0.005  
*** = P value < 0.001
5.2.2 siRNA induced knockdown of Dicer affects pri-miRNA biogenesis:

Next, we wanted to determine if the regulation of pri-miRNAs by Dicer was a general phenomenon or was specific to the T-REX 293 cells. To achieve this, we measured pri-miRNA levels in Dicer siRNA treated wild type HEK293, Hela, and U2OS cells generated in Chapter 4 (Chapter 4.2).

**5.2.2.1 siRNA induced knockdown of Dicer in wild type HEK293 cells:**

The first cell line we tested was wild type HEK293 in order to determine if Dicer mediated regulation of pri-miRNA expression is specific to the T-REX system. Wild type HEK293 cells were transfected with Dicer targeting siRNA encapsulated in RNAimax transfection reagent (Chapter 4). siRNAs are synthetic small RNA molecules which utilise the RNAi pathway to target mRNAs in a sequence specific manner. 24 hours post transfection, cells were washed and treated with a second equal dose of Dicer targeting siRNA to ensure Dicer gene expression was efficiently impaired. qPCR analysis of Dicer mRNA confirmed that Dicer had been successfully decreased in siRNA treated wild type HEK293 cells (Figure 4.2 B). qPCR was then used to measure the expression levels of pri-miR-21, pri-miR-17 and pri-let-7a. Dicer knockdown HEK293 cells showed a stark increase in both pri-miR-17 and pri-let-7a and no change in pri-miR-21 levels compared to controls (Figure 5.2 A, B and C). This data indicates that Dicer regulates the same pri-miRNAs in both wild type and T-REX 293 cells.
Figure 5.2: Dicer knockdown affects pri-miRNA production in HEK293 cells. HEK293 cells were treated with non-targeting (Control) siRNA and siRNA targeting Dicer. (Chapter 4, Figure 4.2) The Dicer knockdown was confirmed by measuring the relative Dicer mRNA/18S RNA levels using Taqman qPCR (A) pri-miR-17 levels were increased upon Dicer depletion (** = P value < 0.005). (B) Knocking down Dicer results in a significant increase in pri-let-7a levels (*** = P value < 0.001). The relative pri-miRNA/GAPDH RNA levels in each experiment were quantified using Taqman qPCR.
Next we investigated the effect of siRNA induced Dicer knockdown in HeLa cells. HeLa cells were sequentially transfected with Dicer targeting siRNA as described in 5.2.2.1 and then RNA was harvested. qPCR analysis of Dicer mRNA confirmed that Dicer had been successfully depleted in siRNA treated HeLa cells (Figure 4.1). pri-miRNA levels were then quantified using qPCR. pri-miR-17 and pri-let-7a levels were significantly decreased, 85% and 80% respectively, upon depleting Dicer (Figure 5.3 A and B). However, pri-miR-21 levels were increased 4 fold (Figure 5.3 C). These results were starkly different from the results obtained from the Dicer impaired HEK293 cells, indicating that Dicer dependent pri-miRNA regulation is cell specific.
Figure 5.3: Dicer knockdown affects pri-miRNA production in HeLa cells. HeLa cells were treated with non-targeting (Control) siRNA and siRNA targeting Dicer (Chapter 4, Figure 4.1). (A) Knocking down Dicer results in a decrease of the level of pri-miR-17 (* - P value < 0.05) and (B) a decrease in pri-let-7a levels (** = P-value < 0.005). The relative pri-miRNA/GAPDH RNA levels were quantified using Taqman qPCR. (C) Dicer knockdown in HeLa cells results in a significant increase in pri-miR-21 levels (*** = P-value < 0.001). pri-miR-21/GAPDH RNA levels were quantified using Taqman qPCR.
5.2.2.3 siRNA induced knockdown of Dicer in U2OS cells:

Next we investigate the effect of Dicer knockdown in pri-miRNAs in U2OS cells. U2OS cells were sequentially transfected with Dicer targeting siRNA (Figure 4.2 A). qPCR analysis of siRNA treated U2OS cells reported no change in pri-miR-17 levels, a 2 fold increase in pri-let-7a levels and a 50% decrease in pri-miR-21 levels upon Dicer knockdown (Figure 5.4 A, B and C). Interestingly, these changes in pri-miRNA expression were different from HEK293 and HeLa Dicer impaired cells. Together this data suggests that Dicer regulates pri-miRNA expression in a cell specific manner.
Figure 5.4: Dicer knockdown affects pri-miRNA in U2OS cells. U2OS cells were treated with non-targeting (Control) siRNA and siRNA targeting Dicer (Dicer) (Chapter 4, Figure 4.2). The Dicer knockdown was confirmed by measuring the relative Dicer mRNA.18S RNA levels using Taqman qPCR. (A) Knocking down Dicer has no effect on the level of pri-miR-17. (B) Knocking down Dicer results in a significant increase in pri-let-7a levels (* = P value < 0.05). (C) pri-miR-21 levels were reduced following Dicer knockdown (** = P value < 0.005). The relative pri-miRNA/GAPDH RNA levels in each experiment were quantified using Taqman qPCR.
5.2.3 Inducible deletion of Dicer increases the level of pri-miR-155 in mouse embryonic fibroblasts:

Numerous studies have demonstrated that miRNA biogenesis can differ between species (Reviewed by Kim et al, 2009). We wanted to determine if Dicer dependent regulation of pri-miRNAs was specific to humans or present in other mammals. To achieve this we knocked out Dicer in Mouse Embryonic Fibroblast (MEF) cells using the Cre-Lox recombinase system. The Cre-Lox system uses the bacterial Cre-Recombinase protein to splice out a target gene which has been flanked by two Lox sites (Sauer 1998). The Cre-protein is fused with the mutated ligand-binding domain for the human estrogen receptor (ERt). Upon treatment with the estrogen receptor antagonist tamoxifen, the Cre-ERt construct is able to penetrate the nucleus and induce targeted splicing. Cre-ERt binds to the tamoxifen with greater affinity than endogenous estrogens. This allows Cre-ERt to remain cytoplasmic in cells which are untreated. Cre-recombinase expression was induced in Dicer flox/flox MEF cells (Gantier et al. 2011) by treating cells with tamoxifen. This treatment was then continued for 7 days. Time points were taken at 3, 6 and 7 days after initial treatment. To verify that Dicer was targeted efficiently, we measured if its corresponding mRNA levels were reduced. qPCR confirmed that Dicer mRNA levels were significantly decreased in all time point samples. Dicer levels were decreased by 50% at day 3 and 80% by day 6. However, at day 7 Dicer levels had recovered to 50% (Figure 5.5 A). These results are expected as the Cre-Lox system is not 100% efficient and a small amount of cells will not undergo the Cre-Lox recombination. Over time they will outgrow the unhealthy cells which have had Dicer spliced out and a recovery of Dicer will
be observed. After Dicer knockdown was confirmed, we then quantified the expression of miR-155 in all time points. Initially at day 3 miR-155 expression was reduced by a modest 20%, however, by day 6 it was reduced by 90%. Interestingly, although Dicer mRNA levels had recovered in our day 7 sample, miR-155 expression was reduced 99% compared to control MEF cells (Figure 5.5 B).

After confirming that the Cre-Lox system had achieved a functional knock out of Dicer in MEFs we then used qPCR to measure the expression of pri-miR-155 at day 3, 6 and 7 time points. While there was no significant change in pri-miR-155 expression detected at day 3, by day 6 there was a marked increase in pri-miR-155 levels in Dicer depleted cells when compared to controls (Figure 5.5 C). Likewise, in samples collected 7 days post induction, pri-miR-155 levels had almost doubled in Dicer depleted cells (Figure 5.5 C). This data indicates that Dicer dependent pri-miRNA regulation is present in multiple species.
Figure 5.5: Dicer flox/flox mouse embryonic fibroblasts (MEF) targeting the Dicer gene were treated to induce recombinase dependent Dicer KO. Cells were grown over 7 days with time points collected at days 3, 6 and 7. (A) The Dicer knockdown was confirmed by measuring the relative Dicer mRNA/18S RNA levels using Taqman qPCR. Cells harvested on Day 6 showed the lowest levels of Dicer mRNA compared to controls, followed by days 3 and 7. (B) Knocking down Dicer results in a decrease of the level of mature mouse miR-155. The relative miR-155/U75 RNA levels were quantified using Taqman small RNA qPCR. (C) The reduction of Dicer results in an increase in pri-miR-155 levels compared to control cells. The relative pri-mi155 levels were quantified using Taqman qPCR and GAPDH as a control.

* = P value < 0.05
** = P value < 0.005
5.3 Discussion:

In this study we have demonstrated that impairing Dicer function, the main effector protein of pre-miRNA processing, has a significant impact on the expression of select pri-miRNAs in a range of mammalian cell types. We also present data that suggests that the nature of regulation of each family of pri-miRNA, whether up or down regulated, is dependent on cell type.

While we did not determine the mechanism of action by which Dicer regulates pri-miRNA processing, our data supports recent studies that have shown that mature miRNAs can regulate miRNA biogenesis by directly binding to pri-miRNA sequences. Studies by Tang et al, demonstrated that in mouse cells, miR-709 is able to negatively regulate the biogenesis of miR-15a/16-1 (Tang et al. 2012). miR-709, a miRNA predominately found in the nucleus, competitively binds to the pri-miR-15a/16-1 transcript and represses Microprocessor processing. As a result the increase of miR-709 causes an accumulation of pri-miR-15a/16-1 and a decrease of mature miR-15a/16-1 (Tang et al. 2012). This work demonstrates that mature miRNAs can directly inhibit the processing of pri-miRNAs. Our data could be explained by this model of mature/pri-miRNA regulation. Upon depletion of Dicer and an impairment of mature miRNA production, we observed a decrease in pri-miR-17 and pri-let-7a expression in Hela cells (Figure 5.3 A and B) as well as a decrease of pri-miR-21 in U2OS (Figure 5.4 C). It is possible that inhibition of Dicer decreases the expression of unidentified pri-let-7a and pri-miR-21 inhibitory mature miRNAs and therefore increases the efficiency of pri-miRNA processing.
Furthermore, it has been demonstrated that *let-7* is able to regulate its own processing through an auto regulatory mature/pri-miRNA loop. CLIP-seq analysis of sequences bound to one of the *C.elegans* Argonaute protein, ALG-1, revealed a *let-7* interaction site in the *let-7* primary transcript (Zisoulis et al. 2012). Surprisingly, disruption of ALG-1 binding to pri-*let-7a* results in reduced pri-miRNA processing, implying a new role for mature miRNAs in promoting pri-miRNA biogenesis (Zisoulis et al. 2012). Moreover, Zisoulis et al demonstrated that human Ago2 associated with a range of pri-*let-7* isoforms, indicating that miRNA/pri-miRNA regulation exists in other species (Zisoulis et al. 2012). Our data could suggest that a similar regulatory system exists for other miRNAs. Inhibiting Dicer resulted in an accumulation of pri-*let-7a* in all systems tested except for siRNA induced Dicer knockdown in HeLa cells (Figure 5.1 B, Figure 5.4 B, Figure 5.5 B). Likewise, pri-miR-21 and pri-miR-17 levels were increased in Dicer depleted HeLa and HEK293 cells respectively. It is possible that these pri-miRNAs are subject to a similar mature/pri-miRNA regulatory loop described by Zisoulis et al and as a consequence a loss of mature miRNA results in pri-miRNA accumulation. Similarly, from analysing our Cre-Lox MEF Dicer knockout data it is evident that as mature miR-155 expression decreases there is an accumulation of pri-miR-155 (Figure 5.5 B and C). This trend continued even when Dicer mRNA levels began to recover, strongly suggesting that pri-miR-155 accumulation is coupled to mature miR-155 (Figure 5.5 A, B and C). This phenomenon could be similar to the accumulation of pre-miRNA which has been reported in Dicer knockdown cells (Grishok et al. 2001; Hutvágner et al. 2001), however, that does not explain why pri-miRNA accumulation is not a general mechanism and currently, we
do not know if mouse miRNAs would be subject to this type of general regulation. Further studies into possible miRNA binding sites of each pri-miRNA would need to be investigated to verify any possible mature miRNA/pri-miRNA interactions.

As pri-miRNAs are the earliest initial upstream products of miRNA biogenesis and are transcribed directly from miRNA genes, we cannot rule out the possibility that Dicer influences pri-miRNA production at a transcriptional level. While it is well known that miRNAs regulate gene expression post-transcriptionally, recent studies have demonstrated that mature miRNAs can regulate genes at the transcriptional level. For example, Zhang et al demonstrated that miRNAs can increase transcription via interacting with promoter TATA-box motifs (Y. Zhang et al. 2014). They found that a certain subset of miRNAs, including miR-145, miR-16 and let-7i, were associated with the RNA Pol II. Furthermore, it was demonstrated that over expression of these miRNAs enhances the IL-2 promoter activity and elevates IL-2 mRNA and protein production in HEK293 cells via targeting TATA-box motifs (Y. Zhang et al. 2014). This data indicates that mature miRNAs can interact with core transcriptional machinery and promote transcription. It is possible that in the case of Dicer depletion, the resulting reduction of endogenous miRNAs inhibits pri-miRNA transcription by dysregulating gene promoters. This would explain our observation of a decrease in pri-miR-17 and pri-let-7a in HeLa cells (Figure 5.3 A and B) and a decrease of pri-miR-21 in U2OS (Figure 5.4 C).

Although miRNAs can promote gene expression at a transcriptional level, recent studies have also shown that miRNAs can also inhibit transcription. miR-423-5p has been found
to decrease RNA Pol II occupancy and increased histone H3 lysine 9 dimethylation (H3K9me2) of the progesterone receptor gene promoter, suggesting a form of miRNA induced chromatin-level silencing (Younger & Corey 2011). Likewise, separate studies reported that miR-10 suppresses hoxd4 expression via methylating the hoxd4 gene promoter. When the nucleus of MCF-7 cells were transfected with miR-10, hoxd4 expression was significantly reduced, however, this inhibition was relieved upon treatment with DNA methyltransferase inhibitors (Tan et al. 2009). Impaired function of Dicer would reduce miRNA dependent transcriptional inhibition and result in increased pri-miRNA transcription, thus explaining the increase in pri-miRNA levels we measured in this study.

In this study we demonstrated that inhibition of Dicer affects pri-miRNA levels in both a miRNA and cell specific manner, however, one limitation of our approach is that only a limited number of miRNAs were analysed. It would be worth noting that in future research next generation sequencing or a micro-array could be utilised to analyse large families of miRNAs and reveal any patters in miRNA regulation following Dicer inhibition.

In conclusion, our data demonstrates that Dicer, a protein traditionally thought to regulate pre-miRNA processing, regulates the expression level of pri-miRNAs. Our data indicates a novel role for Dicer in the miRNA biogenesis pathway and further exhibits the complexity in the regulation of miRNA biogenesis.
6. Increasing miRNA and miRNA target abundance affects miRNA biogenesis:

6.1 Introduction:

miRNAs are important gene regulators that control the majority of cellular process. miRNA biogenesis is a strictly regulated pathway in which disruption of upstream processing events could lead to a decrease in mature miRNA level. Knockdown of Drosha, Dicer or Ago2, the key proteins of miRNA biogenesis, results in a decrease in mature miRNA levels (Han et al. 2004; Schmitter et al. 2006; Grishok et al. 2001; Hutvágner et al. 2001; Diederichs & Haber 2007; O’Carroll et al. 2007). Furthermore, as outlined earlier in Chapter 3, even the knockdown of auxiliary proteins to the miRNA biogenesis pathway such as the RNA-binding proteins p72 and KHSRP also decreases the levels of at least a subset of miRNAs.

Changes in miRNA abundance can have a serious impact on the cell and the miRNA biogenesis pathway. Altered miRNA expression has been linked to the development of various diseases such as cancer (Ha & Kim 2014). Furthermore, recent studies have shown that Ago2 stability is post-transcriptionally coupled to mature miRNA levels, with a decrease in miRNA expression leading to a similar change in Ago2 protein level (Smibert et al. 2013; Martinez & Gregory 2013). Like many biological systems, miRNA biogenesis is subject to a number of positive and negative feedback loops. For example, studies have shown that mature let-7 can promote its own production by binding to let-7 pri-miRNA
(Zisoulis et al. 2012). Furthermore, let-7 expression negatively regulates Dicer, the main effector protein of pre-miRNA processing, in a regulatory feedback loop (Tokumaru et al. 2008). Likewise, Ago2 mRNA is regulated negatively by miRNAs (Zhang et al. 2014; Leonov et al. 2015).

In this study we investigated the role miRNA abundance has on regulating miRNA biogenesis. We observed that over expressing a miRNA stabilises miR-21 and let-7 levels in cells where proteins required for miRNA biogenesis, such as p72, KHSRP and Dicer, have been depleted. We also found that increasing mature miRNA levels elevates pri-miRNA expression in p72 and KHSRP depleted cells. Furthermore, we show that increasing miRNA target abundance increases miRNA stability. This data suggests a feedback mechanism that senses the levels of miRNA or miRNA target within a cell and adjusts either miRNA biogenesis, turnover or stability accordingly.
6.2 Results:

6.2.1 Transfection of miRNA duplex recovers miR-21 in cells with impaired p72 function:

Our studies, previously outlined in Chapter 3, tested the effect of miRNA abundance on Ago2 protein stability. As part of that study we investigated whether transfecting mature miRNA into a cell could recover the protein level of Ago2 in p72 and KHSRP depleted cells. p72 is a subunit of the Drosha complex which is required for altering pri-miRNA structure to facilitate Drosha processing of miR-21 and other miRNAs and accordingly, knockdown of p72 results in a decrease in miRNA level (Suzuki & Miyazono 2013). HeLa cells were transfected with a miR-17 mimic and subsequently treated with p72 targeting siRNA. The miR-17 miRNA mimic duplex is not subject to Drosha or Dicer processing and as a result it can circumvent the miRNA biogenesis pathway. To confirm that p72 had been successfully targeted we used qPCR to measure p72 mRNA levels in siRNA treated cells. qPCR analysis revealed that p72 mRNA had decreased 80% and over 70% in miRNA mimic transfected and untransfected cells respectively compared to cells transfected with a non-targeting siRNA (Figure 6.1 A).

Next, to confirm the transfection of miR-17 mimic was successful, we measured the mature miR-17 levels in both miRNA mimic transfected and untransfected cells. Using qPCR analysis we measured no significant change in endogenous miR-17 level in the control or p72 knockdown cells, confirming that miR-17 is not affected by p72 knockdown (Figure 6.1 B). However, in miR-17 mimic transfected cells we measured a ~1000-fold
increase in the level of mature miR-17 compared to endogenous miR-17 levels (Figure 6.1 B).

Next, we measured the level of miR-21 in the p72 depleted cells with and without added miR-17 mimic. As documented previously, p72 knockdown decreased mature miR-21 levels significantly (Figure 6.1 C). However, p72 knockdown cells transfected with miR-17 showed no significant change in miR-21 levels compared to non-targeting siRNA treated control cells transfected with miR-17. This was unexpected as even though p72, the miRNA biogenesis auxiliary factor responsible for miR-21 production, was depleted, miR-21 levels were increased. To test whether the increase of mature miR-21 was due to an increase in the primary transcript expression we used qPCR to measure the relative expression levels of pri-miR-21 in p72 knockdown cells and p72 knockdown cells transfected with miR-17. A modest accumulation of pri-miR-21 was detected in p72 knockdown cells that were not transfected with miR-17. This was expected because the Microprocessor activity was impaired by the inhibition of p72 resulting in an accumulation of pri-miRNA due to reduced pri-miRNA processing. However, with the addition of miR-17, pri-miR-21 levels increased 4 fold compared to controls treated with non-targeting siRNA and transfected with miR-17 (Figure 6.1 D). This data suggests that directly increasing mature miRNA abundance with miRNA mimic can influence miRNA processing in a system in which miRNA biogenesis is inhibited.
Figure 6.1: Increased miRNA levels recovers miR-21 in cells with impaired p72. HeLa cells were transfected with miR-17 miRNA mimic then subsequently treated with p72 targeting siRNA. (A) The p72 knockdown was confirmed by measuring the relative p72 mRNA/18S RNA levels using Taqman qPCR. (B) miRNA transfection was confirmed by measuring relative mature miR-17 levels using Taqman small RNA qPCR. U75 was used as an endogenous control. (C) The transfection of miR-17 results in a recovery in miR-21 levels in p72 impaired cells (D) Pri-miR-21 expression increases upon miR-17 transfection. The relative pri-miRNA levels were quantified using Taqman qPCR and GAPDH as a control.

* = P value < 0.05
*** = P value < 0.001
6.2.2 Transfection of miRNA duplex recovers the members of the let-7 miRNA family in cells with impaired KHSRP function:

Next, to determine if this observed phenomenon was specific to p72 and miR-21 or general to other miRNAs, we decided to repeat the experiment in HeLa cells depleted of KHSRP. KHSRP is a protein involved in the regulation of RNA splicing and is required for the efficient processing of pre-let-7 to mature let-7 (Trabucchi et al. 2009). HeLa cells were transfected with a miR-17 mimic and subsequently treated with KHSRP targeting siRNA. Western blot analysis confirmed that KHSRP expression had been successfully lowered in siRNA treated cells (Figure 6.2 A).

To confirm that the transfection of miR-17 mimic was successful we quantified the mature miR-17 levels in both miRNA mimic treated and untreated cells. Using qPCR analysis we detected no significant change in endogenous miR-17 in control or KHSRP knockdown cells, confirming that miR-17 is not regulated by KHSRP (Figure 6.2 B). However, in miR-17 mimic treated cells miR-17 levels showed a ~1000 fold increase (Figure 6.2 B).

As KHSRP is required for the production of the let-7 family of miRNAs (Trabucchi et al. 2009), we used qPCR to measure both let-7e and let-7a expression in our KHSRP knockdown cells. As reported by Trabucchi et al, we also observed a significant decrease in both mature let-7e and let-7a levels when KHSRP was impaired (Figure 6.2 C). Similar to our previous findings described above we also observed an increase in the level of both let-7 isoforms in KHSRP knockdown cells which were transfected with the miR-17
mimic (Figure 6.2 C). Furthermore, when we measured pri-miRNA levels, the transfection of miR-17 resulted in elevated expression of both pri-let-7e and pri-let-7a compared to scramble siRNA treated controls transfected with miR-17 (Figure 6.2 D). This data confirms that increasing the level of mature miRNA can increase the expression of other miRNAs.
Figure 6.2: Increased miRNA levels recovers let-7a in cells with impaired KHSRP. HeLa cells were transfected with miR-17 miRNA mimic then subsequently treated with KHSRP targeting siRNA. (A) The KHSRP knockdown was confirmed by Western Blot. (B) miRNA transfection was confirmed by measuring relative mature miR-17 levels using Taqman small RNA qPCR. U75 was used as an endogenous control. (C) The transfection of miR-17 results in a recovery in let-7a and let-7e family levels in KHSRP impaired cells (D) pri-let-7a and pri-let-7e expression increases upon miR-17 transfection. The relative pri-miRNA levels were quantified using Taqman qPCR and GAPDH as a control.

* = P value < 0.05
*** = P value < 0.001
6.2.3 Transfection of miRNA duplex recovers let-7a and miR-21 in cells with impaired Dicer function:

p72 and KHSRP are required for the production of only a subset of miRNAs (Suzuki et al. 2009; Trabucchi et al. 2009). We next tested if increasing miRNA abundance, through the use of miRNA mimics which circumvent the biogenesis pathway, can recover miRNA production when the core machinery of the miRNA biogenesis pathway is impaired. Dicer is responsible for the processing of pre-miRNA to mature miRNA for over 95% of miRNAs (Kim et al. 2016), therefore, we decided to investigate the effect of miRNA levels in Dicer knockdown cells. HeLa cells were transfected with miR-17 mimic and subsequently treated with Dicer targeting siRNA. Western Blot and qPCR analysis confirmed that Dicer expression had been successfully decreased in siRNA treated cells (Figure 6.3 A). Likewise, using qPCR analysis we determined that miR-17 transfection was successful. We measured a 50% reduction in endogenous miR-17 in Dicer knockdown cells (Figure 6.3 B). However, in miR-17 mimic treated cells miR-17 expression was elevated ~1000 fold (Figure 6.3 B).

Next, we tested if miR-17 transfection could recover the level of miR-21 and let-7a in Dicer depleted cells. qPCR analysis showed that levels of both miR-21 and let-7a were significantly reduced in Dicer knockdown cells (Figure 6.3 C and D). However, the addition of miR-17 resulted in an increase in both of these miRNAs even though Dicer, a key protein of the miRNA biogenesis pathway, was severely inhibited (Figure 6.3 C and
D). This data confirms that increasing miRNA abundance can influence miRNA biogenesis in the absence of key miRNA biogenesis proteins.
**Figure 6.3:** Increased miRNA levels recovers *let-7a* and miR-21 in Dicer knockdown cells. HeLa cells were transfected with miR-17 miRNA mimic treated and siRNA targeting Dicer. (A) The Dicer knockdown was confirmed by measuring the relative Dicer mRNA/18S RNA levels using Taqman qPCR (B) miRNA transfection was confirmed by measuring relative mature miR-17 levels. miR-17 transfection recovers (C) miR-21 and (D) *let-7a* in Dicer depleted cells. All miRNAs were quantified using Taqman small RNA qPCR. U75 was used as an endogenous control.

* = P value < 0.05  
** = P value < 0.005  
*** = P value < 0.001
6.2.4 Increasing abundance of miRNA target results in increased miRNA stability:

Studies have shown that an increase in miRNA target abundance can increase miRNA levels (Kuchen et al. 2010). We investigated if increasing the amount of miRNA targets could recover miRNA levels in the absence of miRNA biogenesis processing machinery. To achieve this we devised an experiment where KHSRP depleted HeLa cells were transfected with either a control (psiCheck2) or let-7 regulated (psiCHECK2::x8) plasmids (Iwasaki et al. 2009). The psiCHECK2::x8 Dual Luciferase reporter construct contains eight let-7 binding sites arranged in tandem in the 3’ UTR of the Renilla luciferase, and is therefore subject to translational repression dependent regulation by endogenous let-7. The control luciferase gene lacks miRNA binding sites, and as a result should be exempt from let-7 targeting.

Firstly, we confirmed that the knockdown of KHSRP was successful. Western blot analysis revealed that KHSRP protein was starkly reduced in siRNA treated cells (Figure 6.4 A). Next, using Dual Luciferase assay we measured the Renilla/Firefly expression of both control and psiCHECK2::x8 plasmids. In non-targeting siRNA treated cells the expression level of let-7 targeted reporter was ~60% of the reporter that is not targeted by let-7. However, in the KHSRP knocked down cells the difference in repression between the let-7 targeted reporter and control plasmid luciferase was significantly less. let-7 targeted reporter expression was elevated by ~20% compared to the reporter that is not targeted by let-7 (Figure 6.4 A). This data reinforced the published finding that inhibiting
KHSRP causes a reduction of mature *let-7* and this results in an impaired *let-7* mediated translational repression (Trabucchi et al. 2009).

Next we investigated if the addition of miRNA targets had any effect on miRNA levels. qPCR analysis revealed that *let-7* levels were decreased ~50% in KHSRP knockdown cells with no additional miRNA targets. However, we observed a stark increase in the level of *let-7a* in KHSRP knockdown cells expressing the *let-7* regulated reporter (Figure 6.4 B). Likewise, miR-21, which also required KHSRP for its maturation (Trabucchi et al. 2009), displayed identical behaviour with increased levels in cells expressing the *let-7* regulated reporter (Figure 6.4 C). This data demonstrates that miRNAs levels are increased by miRNA target abundance independent of the actual binding affinity between miRNA and its target.

To test if this phenomenon could be detected when we add a target that is cleaved by *let-7* as a siRNA, a similar experiment was conducted using an alternative set of reporter constructs. HeLa cells in which KHSRP had been knocked down were transfected with control mutant plasmid (psiCHECK2 Mutated *let-7* x3) or a 3x plasmid (psiCHECK2 *let-7* x3) (Johnston et al. 2010). The 3x plasmid contains three binding sites in the 3´ UTR of the Renilla luciferase transcript with perfect complementarity to *let-7*. In the control construct, the *let-7* binding sites have been mutated so that they are no longer recognised by endogenous miRNAs, and the expression of Renilla luciferase is not susceptible to small RNA-mediated regulatory pathways.
Western blot analysis revealed that KHSRP protein was starkly reduced in siRNA treated cells (Figure 6.5 A). Next, using Dual Luciferase assay we measured the Renilla/Firefly expression of both control mutant and let-7 regulated reporters (Figure 6.5 A). In non-targeting siRNA treated cells the let-7 regulated reporter expression was ~70% of the reporter with mutated let-7 binding sites (Figure 6.5 A). Interestingly, we still measured a marked decrease in the let-7 regulated reporter expression when KHSRP was depleted (Figure 6.5 A). However, it is possible that the ~50% decrease of let-7a level in the KHSRP knocked down cells is not enough to detect changes in the enzymatic activity of the RISC that performs RNAi (Figure 6.5 B).

Next we tested if the addition of miRNA targets with perfect complementarity had any effect on miRNA stability. qPCR analysis revealed that let-7 levels were decreased ~60% in KHSRP knockdown cells expressing the control mutant reporter (Figure 6.5 B). However, as seen in our previous experiment, we observed an increase in the level of let-7a in KHSRP knockdown cells which contained the let-7 regulated reporter (Figure 6.5 B). Likewise, miR-21 levels were also increased in cells expressing let-7 regulated reporter (Figure 6.5 C). Together this data confirms that both targets of translational repression and RNAi can stabilise miRNAs.
Figure 6.4: Increased miRNA target results in increased miRNA stability. HeLa cells were transfected with either control plasmid containing no let-7 targeting sites, or a 8x plasmid containing eight tandem let-7 sites in the 3'UTR of the renilla gene. Cells were then transfected with siRNA targeting KHSRP. (A) KHSRP knockdown was confirmed via western blot. Actin was used as a loading control. Luciferase reporter activity was confirmed with renilla/firefly assay. Reporter expression was decreased in 8x compared to control plasmid. (B) Let-7a levels are recovered in KHSRP impaired HeLa cells transfected with plasmid containing 8x let-7 binding sites. Let-7 levels were quantified using Taqman small RNA qPCR. U75 was used as an endogenous control. (C) miR-21 levels were recovered in KHSRP impaired HeLa cells transfected with plasmid containing 8x let-7 binding sites. miR-21 levels were quantified using Taqman small RNA qPCR. U75 was used as an endogenous control.

* = P value < 0.05
** = P value < 0.005
*** = P value < 0.001
Figure 6.5: Increased levels of perfect miRNA target results in increased miRNA stability. HeLa cells were transfected with either plasmid containing 3x perfect let-7 binding sites, or a 3x mutated let-7 binding sites in the 3'UTR of the renilla gene. (A) KHSRP knockdown was confirmed via western blot. Actin was used as a loading control. Luciferase reporter expression was decreased in 3x perfect plasmid compared to mutant 3x plasmid controls. (B) let-7a levels were recovered in KHSRP impaired HeLa cells transfected with plasmid containing 3x let-7 perfect binding sites. let-7 levels were quantified using Taqman small RNA qPCR. U75 was used as an endogenous control. (C) miR-21 levels were recovered in KHSRP impaired HeLa cells transfected with plasmid containing 3x let-7 perfect binding sites compared to mutant controls. miR-21 levels were quantified using Taqman small RNA qPCR. U75 was used as an endogenous control.

** = P value < 0.005
*** = P value < 0.001
6.3 Discussion:

In this study we have demonstrated that increasing miRNA abundance can affect miRNA biogenesis through increasing the production of pri-miRNA. We also present data that strongly suggests that miRNA stability is coupled to levels of miRNA targets.

While we did not determine the mechanism of how exogenously provided miRNA duplex can regulate miRNA biogenesis, our data partially supports recent studies which have shown that mature miRNAs can influence miRNA biogenesis. Studies by Martinez et al have demonstrated that Ago2 stability is coupled to mature miRNA abundance. They showed that knocking down key proteins of the miRNA biogenesis pathway results in a decrease in mature miRNA and that this in turn results in a decrease in Ago2 protein stability (Martinez & Gregory 2013). Likewise, Smibert et al demonstrated the same mechanism in fly cells. They documented that knocking down DGCR8 and Dicer resulted in a decrease in Ago2 stability which was able to be recovered by the addition of miRNAs (Smibert et al. 2013). These data demonstrate that miRNA abundance can affect key steps of the miRNA biogenesis pathway. The mechanism of Ago2 stability being coupled to miRNA levels could explain our data; as we increase miRNA abundance in the cell, we increase Ago2 levels (Chapter 3 Figure 3.6 and 3.7). Ago2 has been found to process both pre-miRNAs and primary miRNAs (Diederichs & D. a. Haber 2007; Kim et al. 2016; Zisoulis et al. 2012; Nottrott et al. 2006; Bossé & Simard 2010). It is possible that an increase in Ago2 protein level, brought upon by increasing amount of miRNA, is able to compensate for the lack of miRNA processing machinery, such as p72, KHSRP and
Dicer, and recover the miRNA biogenesis pathway. This would explain the recovery in miRNA levels when miR-17 is introduced to the cell.

In all cases of miR-17 transfection, miRNA recovery was coupled with an increase in respective pri-miRNA levels. As pri-miRNAs are the earliest products of miRNA biogenesis and are transcribed directly from miRNA genes, we cannot rule out the possibility that miRNA abundance influences pri-miRNA production at a transcriptional level. Recent studies have demonstrated that mature miRNAs can regulate genes at the transcriptional level (Woods et al. 2007; Y. Zhang et al. 2014). Furthermore, Pol II, the polymerase responsible for the majority of miRNA gene transcription, associates with mature miRNAs (Y. Zhang et al. 2014). One explanation of our data could be that the large increase in mature miRNAs promotes miRNA transcription through increasing the activity of Pol II. Alternatively, due to the depletion of auxiliary factors and Dicer in the system, miRNA processing becomes the rate limiting step in the biogenesis pathway. It is possible that in order to compensate for this, the system increases pri-miRNA levels to return mature miRNA levels to homeostasis. Moreover, we cannot rule out the possibility that the accumulation in pri-miRNAs is due to a decrease in the efficient turnover of pri-miRNAs as the biogenesis pathway auxiliary factors are inhibited.

An alternative interpretation is that the accumulation of pri-miRNAs is due to the actions of an unknown negative feedback mechanism which senses miRNA levels and inhibits miRNA biogenesis accordingly. The large amounts of miRNA transfected into the cell could cause a feedback response which shuts down the miRNA biogenesis pathway until
levels of small RNAs returns to homeostasis. Thus we see an accumulation of pri-miRNA when miR-17 is transfected, as the cell halts pri-miRNA processing in an attempt to decrease the amount mature miRNA to return the cell to homeostasis.

Moreover, a number of viruses have been found to encode miRNAs which hijack Ago2 and suppress host cell miRNA function (Kincaid et al. 2012). It is feasible that the large amounts of miR-17 small RNA transfected into the cell triggers the production of endogenous miRNAs in an attempt to displace exogenous small RNAs which are associating with Ago. Furthermore, the large influx of small RNAs could induce a miRNA mediated immune response in response to the ‘infection’. In fact, both miR-21 and let-7 have documented roles in promoting the immune response; miR-21 reduces the inflammation response through targeting PCDC4 and let-7 inhibits NF-KB inhibitor A20 (Kumar et al. 2015; Sheedy et al. 2010; Das et al. 2014). This could explain why we see a surge in pri-miR-21 and pri-let-7 following the transfection of miR-17 and an increase in exogenous miR-21 and let-7a levels, as the cell initiates an immune response.

One key finding of this study is that miRNAs can be stabilised by miRNA target abundance whether the target is regulated by RNAi or translational repression. We see a stabilisation of both miR-21 and let-7a levels when plasmids with let-7 binding sites are introduced to a cell, even though the machinery to produce these miRNAs is depleted. One explanation for these results is that miRNAs which are actively involved in RNAi or translational repression are stabilised by the cell and thus have an extended turnover rate. In fact, fly studies by Wee et al, demonstrated that extensive complementarity between a siRNA and
its target slows the rate that Ago2 binds to and dissociates from the target mRNA. They found that the calculated $K_{on}$, the biomolecular association rate constant for RISC binding to its target, for fly Ago2-RISC binding a fully complementary target was 10 times slower than for a seed-matching target (Wee et al. 2012). It is possible that the addition of numerous let-7 binding sites of both perfect and seed complementarity results in a longer association of Ago2 with the target reporter and as a result a stabilisation of the mature miRNA occurs even in the absence of the auxiliary protein responsible for its biogenesis.

Another interesting observation of this study was that miR-21 was stabilised along with let-7 when let-7 targets were added to the cell. miR-21 had no additional targets and should theoretically not be associated with the let-7 binding sites and thus increased stability cannot be explained by binding to targets. One interpretation of this data is that groups of miRNAs or RISCs exist together in a common complex. Interestingly, recent studies have documented that cellular miRNAs exist predominately in low molecular weight RISC complexes not associated with mRNAs (La Rocca et al. 2015). Perhaps the stabilisation of one miRNA/RISC inadvertently stabilises the entire complex containing other miRNAs. This would explain why miR-21 is stabilised alongside let-7 in cells enriched with let-7 targets. Furthermore, this hypothesis could help explain the increase in miR-21 and let-7 when we transfected miR-17 duplex. It is possible that the inhibition of auxiliary factors or Dicer followed by the transfection of miR-17 stabilises already existing Ago2-miRNA complexes through loading Ago2 with the mature miR-17. The stabilisation of Ago2 protein through increased miRNA abundance could inadvertently stabilise other miRNAs which exist together in a theoretical common complex. This would
explain why we observed an increase in miR-21 and *let-7* when miR-17 is added to the cell.
7. Discussion:

7.1 The importance of understanding the regulation of miRNA biogenesis:

The regulatory processes that control gene expression are vital to cellular function. miRNAs regulate gene expression in animals, plants and unicellular eukaryotes and control nearly all cellular processes (Ha & Kim 2014).

In humans, processes such as cell differentiation, migration, proliferation and apoptosis involve many miRNAs. miRNAs are required for the healthy development of an organism, and the alterations in miRNA expression can lead to the development of diseases and eventually be fatal (Reviewed by Lin & Gregory 2015). Therefore, understanding the mechanisms that drive miRNA production may help to provide insight into how they become dysregulated and what effects this has on the cell.

Several factors have been identified that can influence miRNA biogenesis, such as cellular stress and stages of cellular development (Romero-Cordoba et al. 2014; Finnegan & Pasquinelli 2013; Ha & Kim 2014). More directly, a large number of additional RNA binding proteins have been identified to have auxiliary roles in the miRNA biogenesis pathway (Reviewed by Connerty et al. 2015). This study identifies novel roles of RNA
binding proteins in the regulation of both Ago2 and general miRNA biogenesis, highlighting the complexity of the regulation of the miRNA pathway.

7.1.1 Auxiliary RNA binding proteins influence Ago2 protein stability:

As discussed above, many auxiliary RNA binding proteins play fundamental roles in miRNA biogenesis. Two of those proteins are the DEAD-box helicase p72 and the KH-Type Splicing protein KHSRP. We have shown that knockdown of p72 and KHSRP post-transcriptionally decrease Ago2 protein in several transformed human cell lines (Chapter 3). Our study aligns with the established model that the reduction of Ago2 protein level is very likely a consequence of the decrease of abundant miRNAs in the cells due to the lack of loadable miRNA to stabilize Ago2 (Martinez & Gregory 2013; Smibert et al. 2013).

So far there has been a number of conflicting results as to exactly how miRNA abundance regulates Ago2 stability. Two studies have demonstrated that interfering with essential factors of miRNA biogenesis, by inhibiting the function of DGCR8/Dicer in mouse MEF and ESCs and Drosha in fly S2 cells, results in a degradation of Ago2 protein by the lysosome and proteasome respectively (Martinez & Gregory 2013; Smibert et al. 2013). In this study we show that a similar mechanism exists in the highly transformed human cells, however, we have not determined which pathway unloaded Ago2 protein is degraded by.
A key point of our study is that knock down of auxiliary proteins, not essential to the processing of all miRNAs, can reduce miRNA levels to a degree that the key effector protein is destabilized. However, it should be mentioned that miR-21 and let-7, two of the miRNAs which are processed by p72 and KHSRP respectively, are two of the most abundant miRNAs in the transformed human cells used in this study (Androsavich et al. 2012; Liu et al. 2012; Roush & Slack 2008). The reduction in the two most abundant miRNAs could trigger the same cellular mechanisms which reduce Ago2 stability in the DGCR8 and Dicer depletion by Martinez et al and Smibert et al. However, one interesting point of discussion remains. We still observed a decrease in Ago2 protein levels when the cells had been flooded with p72 or KHSRP targeting siRNA. In humans, Ago2 binds to both miRNAs and siRNAs (Rivas et al. 2005), however, our data suggests that siRNAs cannot stabilize Ago2 in the same manner as miRNAs.

One of the main differences between miRNAs and siRNAs is that siRNAs exclusively bind with perfect complementarity to their target mRNA and thus induce RNA cleavage (Carthew & Sontheimer 2009). This form of regulation is rare for exogenous miRNAs in humans and it is possible that an as of yet unknown feature of translational repression stabilizes Ago2 in a way that cleavage cannot. In fact, a recent study has documented that the levels of affinity between miRNA and target can slow the dissociation rate between the RISC and the target mRNA (Wee et al. 2012). It is possible that miRNA/mRNA binding stabilizes Ago2 to a higher degree then siRNA/mRNA binding. Furthermore, another idea for consideration is that siRNAs are not as efficiently loaded into Ago2 as miRNAs, but are able to turn over quicker than miRNAs. miRNAs on the
other hand are more efficiently loaded but are slower to inhibit mRNAs via translational repression and thus associate with target mRNA longer, increasing stability. In fact, several studies have shown that miRNAs are preferentially loaded into Ago1 in flies while siRNAs are loaded into Ago2 (Czech et al. 2009; Okamura et al. 2009). Furthermore, it has been suggested that small RNA loading in human cells is analogous to fly Ago1 so it is feasible that miRNAs are loaded with preference over siRNAs, explaining why we see a stabilization of Ago2 based on miRNA abundance and not siRNAs (Yoda et al. 2010).

Together this data demonstrates that inhibiting auxiliary RNA binding proteins to the miRNA biogenesis pathway can affect miRNA abundance significantly enough to destabilize unloaded Ago2.
7.1.2 Novel roles of Dicer in the regulation of Ago2 stability and pri-miRNA production:

Dicer is the key protein of pre-miRNA processing in the canonical miRNA biogenesis pathway. Numerous studies have documented that impairment of Dicer results in either a major decrease in the protein level of Ago2 or no change at all. Studies in murine and fly embryonic cells reported a decrease in Ago2 stability upon Dicer deletion (Martinez & Gregory 2013; Smibert et al. 2013). The depletion of Ago2 was found to be a result of a decrease in miRNA levels (Martinez & Gregory 2013; Smibert et al. 2013). However, MEF, HEK293 and HCT116 Dicer (-/-) cells in which Dicer was depleted with genetic knock out showed no measurable change in Ago2 protein level compared to controls (Bogerd et al. 2014; Kim et al. 2016; Frohn et al. 2012).

In our study we have provided conflicting data to the current literature. Our results show that impairment of Dicer increases Ago2 protein stability. We demonstrated that Dicer regulates Ago2 level post-transcriptionally and that the elevated Ago2 level is likely due to an increase in the pool of LMW unloaded Ago2 (Chapter 4). Furthermore we document that Ago2 responds differently to siRNA-mediated inhibition of human Dicer, compared to the genetic knockout of DGCR8 and Dicer in murine, fly and human cells mentioned earlier (Martinez & Gregory 2013; Smibert et al. 2013; Bogerd et al. 2014; Kim et al. 2016). Studies have shown that Ago2 is depleted by both the lysosome and the proteasome degradation pathways (Martinez & Gregory 2013; Smibert et al. 2013; Johnston et al. 2010). Both pathways are regulated by miRNAs, therefore, it is possible that the
impairment of Dicer results in the downregulation of these mechanisms and in turn an
accumulation of Ago2 (Kurashige et al. 2012; Frankel & Lund 2012). Likewise, an
increasing number of studies have shown that Ago2 is involved in miRNA processing and
could process subsets of miRNAs independently of Drosha and Dicer such as mirtrons,
tRFs and the newly identified intronic miRNAs named Agotrons (Ruby et al. 2007; Kim et
al. 2016; Hansen et al. 2016). Therefore, we cannot rule out the possibility that the Ago2
becomes loaded with these Dicer independent small RNAs and protects Ago2 protein
from degradation. However, this does not explain why Martinez et al did not observe a
similar loading of Ago2 with small RNAs as both Agotrons and mirtrons are present in

One key difference between our study and previous literature is that we used siRNA to
inhibit the function of Dicer. In recent papers which observed no difference in Ago2
expression upon Dicer inhibition, the knockdown was achieved via stable genetic knock
out of Dicer through a variety of methods (Bogerd et al. 2014; Kim et al. 2016; Frohn et
al. 2012). One of the main differences in our method is that siRNA transfection results
in a sudden and massive drop in Dicer expression and an influx of small RNAs. This
large and sudden change to cell homeostasis could initiate a stress response in the cell
if an influx of small RNAs is mistaken for a viral infection (Reviewed in Reineke & Lloyd
2013). In fact, recent studies have demonstrated that induction of cellular stress can re-
localise Ago2 to stress granules and increase Ago2 protein stability (Detzer et al. 2011).
This data could explain the increased Ago2 protein stability we observed in our study.
Furthermore, another key finding of this study was that the increased stability of Ago2 protein was localized to the LMW-RISC complexes which are either unloaded or loaded only with a small RNA and not associated with translational machinery (Chapter 4) (La Rocca et al. 2015). If siRNA transfection does induce a cellular stress response and localise Ago2 to the stress granules, this would explain why we see a shift of Ago2 from HMW ribosome associated RISC to LMW RISC as the cell re-localises the RISC complexes (Detzer et al. 2011).

Moreover, since long term inhibition of the miRNA pathway is lethal we hypothesise that the selected Dicer (-/-) clones from previous studies overcame the cellular stress and thus, and in the long term, Ago2 became loaded with small RNAs that do not require Dicer for their processing. This would also explain why the increase in Ago2 is not observed in stable Dicer knock out cells (Ruby et al. 2007; Kim et al. 2016; Hansen et al. 2016).

Another key finding of this study is that impairing Dicer function has a significant impact on the expression of select pri-miRNAs in a range of mammalian cell types. Mature miRNAs can both inhibit or promote the production of miRNAs through direct binding to pri-miRNA sequences (Tang et al. 2012; Zisoulis et al. 2012). Upon depletion of Dicer and an impairment of mature miRNA production, we observed a decrease in pri-miR-17 and pri-let-7a expression in HeLa and a decrease of pri-miR-21 in U2OS (Chapter 5.
Figure 5.2 and 5.3). It is possible that inhibition of Dicer decreases the expression of unidentified pri-let-7a and pri-miR-21 inhibitory mature miRNAs and therefore increases the efficiency of pri-miRNA processing. Further studies into possible miRNA binding sites of each pri-miRNA would need to be discovered to verify any possible miRNA/pri-miRNA interactions (Figure 7.1).

Furthermore we cannot rule out the possibility that Dicer influences pri-miRNA production at the transcriptional level. miRNAs have had numerous roles documented in both the promotion and inhibition of transcription (Y. Zhang et al. 2014; Younger & Corey 2011; Tan et al. 2009). It is conceivable that in the case of Dicer depletion, the resulting reduction of endogenous miRNAs inhibits pri-miRNA transcription by inhibiting gene expression. This would explain the observed decrease in pri-miRNAs in Dicer knockdown cells. Moreover, impaired function of Dicer could reduce miRNA dependent transcriptional inhibition and result in increased pri-miRNA transcription, thus explaining the increase in pri-miRNA levels we measured in this study (Figure 7.1). However, any possible miRNA interaction with gene promoters would need to be investigated further.

An alternative explanation for this data is that the depletion of Dicer causes pre-miRNA processing to become the rate limiting step of miRNA biogenesis. As a result of this, the cell could increase pri-miRNA production through an unknown feedback mechanism in an attempt to compensate for the lack of pre-cursor processing. On the other hand, it is possible the cell senses the accumulation of pre-miRNAs caused by Dicer inhibition and
shuts down pri-miRNA processing in an attempt to prevent an overabundance of pre-miRNA molecules. Thus the increase of pri-miRNA observed is actually caused by a lack of Microprocessor activity (Figure 7.1).

In conclusion our data demonstrates that Dicer, a protein traditionally thought to regulate pre-miRNA processing, also regulates the level of pri-miRNAs and the stability of Ago2 protein. Our data indicates a novel role for Dicer in the miRNA biogenesis pathway and further exhibits the complexity in the regulation of miRNA biogenesis.
**Figure 7.1:** Knockdown of Dicer influences pri-miRNA production. We hypothesise a number of mechanisms: (1) Unidentified miRNAs which inhibit pri-miRNA processing are decreased upon Dicer knockdown. As a result pri-miRNA processing efficiency increases. (2) Unknown miRNAs inhibit transcription of pri-miRNAs. Dicer knockdown reduces the abundance of these miRNAs and as a result promotes pri-miRNA transcription. (3) The cell senses the decrease in pre-miRNA processing and through an unknown feedback mechanisms halts pri-miRNA transcription in an attempt to prevent flooding of pre-miRNA molecules.
7.1.3 Exogenously added miRNA duplex abundance increase miRNA stability:

Dysregulation of miRNA biogenesis has been documented to affect key proteins of miRNA biogenesis by a number of studies (Martinez & Gregory 2013; Smibert et al. 2013). We present novel data that further confirms that changes in miRNA abundance can affect key steps of the miRNA biogenesis pathway.

In this study we have demonstrated that increasing miRNA abundance using miRNA duplexes can affect miRNA biogenesis through increasing the production of pri-miRNA and stabilising mature miRNA levels. Likewise, in Chapter 3, we demonstrated that Ago2 stability is coupled to miRNA levels (Chapter 3 Figure 3.1 and Figure 3.2). Ago2 has documented roles in promoting the production of both precursor and primary miRNAs (Zisoulis et al. 2012; Diederichs & Haber 2007). This increase in Ago2 protein level, brought upon by an increasing amount of miRNA, could recover the miRNA biogenesis pathway despite the lack of miRNA processing machinery, such as p72, KHSRP and Dicer (Figure 7.2). This would explain the recovery in miRNA levels when miR-17 mimic is introduced to the cell.

Furthermore, when we transfected miR-17 duplex into cells which have impaired miRNA biogenesis, miRNA levels recovered and we observed an increase in respective pri-miRNA levels (Chapter 6). While we cannot rule out the possibility that the accumulation in pri-miRNAs is due to a decrease in the turnover of pri-miRNAs, as the biogenesis
pathway auxiliary factors are inhibited, there are a few other possible explanations for this phenomenon. One explanation could be that due to the depletion of auxiliary factors and Dicer, miRNA processing becomes the rate limiting step in the biogenesis pathway. It is possible that in order to compensate for this, the cells increases pri-miRNA expression to return mature miRNA levels to homeostasis. Also, as mentioned earlier, miRNAs can promote transcription (Tan et al. 2009; Younger & Corey 2011). Perhaps the large increase in mature miRNAs promotes miRNA transcription through increasing the activity of Pol II in an attempt to either saturate the remaining miRNA machinery or to transcribe miRNAs which do not rely on the canonical miRNA biogenesis machinery (Kim et al. 2016) (Figure 7.2).

An alternative interpretation is that the accumulation of pri-miRNAs is due to the actions of an unknown negative feedback mechanism which senses miRNA levels and inhibits miRNA biogenesis accordingly (7.2). The large amounts of miRNA transfected into the cell could cause a feedback response which shuts down the miRNA biogenesis pathway until levels of small RNAs return to homeostasis. Thus we see an accumulation of pri-miRNA when miR-17 is transfected, as the cell halts pri-miRNA processing in an attempt to decrease miRNA levels.

Moreover, a number of viruses have been found to encode miRNAs which hijack Ago2 and suppress host cell miRNA function (Kincaid et al. 2012). It is feasible that the large amounts of miR-17 duplex transfected into the cell triggers the production of endogenous
miRNAs in an attempt to displace the exogenous small RNAs which are associating with Ago. miR-21 and let-7, the two miRNAs which increased upon miR-17 transfection, have documented roles in promoting the immune response (Kumar et al. 2015; Sheedy et al. 2010; Das et al. 2014). The large influx of exogenous small RNAs could induce a miRNA-mediated immune response (Figure 7.3). This could explain why we see a surge in pri-miR-21 and pri-let-7 following the transfection of miR-17 and an increase in exogenous miR-21 and let-7a levels, as the cell initiates an immune response. Another possibility is that the influx of exogenous miRNAs in turns stabilise the existing miRNA complexes to circumvent the lack of available Ago2 to be loaded, and therefore we observe an increase in miRNA stability (Figure 7.3 2).
Figure 7.2: Addition of miR-17 duplex to the cell results in an increase in pri-miRNA and mature miRNA levels. (1) miR-17 increases Ago2 protein stability which in turn assists in the processing of pri-miRNA processing to account for the lack of auxiliary biogenesis proteins. (2) miR-17 transfection increases pri-miRNA transcription to compensate for the loss of auxiliary pri-miRNA processing proteins KHSRP and p72. (3) Increased miR-17 levels loads Ago2 and saturates the cell. An unknown negative feedback mechanism senses the accumulation and halts pri-miRNA processing accordingly.
Figure 7.3: Addition of miR-17 duplex to the cell results in an increase in pri-miRNA and mature miRNA levels. (1) miR-17 triggers an immune response which results in the production of pri-miR-21 and pri-let-7 to displace exogenous miR-17. (2) miR-17 stabilises Ago2 and which exists in a common complex with other RISCs and therefore indirectly stabilises other miRNAs.
7.1.4 miRNA targeting increases miRNA stability:

The final key finding of this study is that miRNAs can be stabilised by miRNA target abundance, independent of whether the target is regulated by RNAi or translational repression. In Chapter 6 we observed a stabilisation of both miR-21 and let-7a levels when plasmids with let-7 binding sites were introduced to the cells, even though the machinery to produce these miRNAs was depleted (Chapter 6 figure 6.3 and 6.4). Studies have demonstrated that extensive complementarity between a siRNA and its target slows the rate that Ago2 binds to and dissociates from the target mRNA (Wee et al. 2012). One explanation for our results is that miRNAs, which are actively involved in RNAi or translational repression, are stabilised by the cell and thus have an extended turnover rate. It is possible that the additional let-7 binding sites of both perfect and seed complementarity results in a prolonged association of Ago2 with the target reporter. This consequently stabilises the existing mature miRNA even if the cell is lacking the auxiliary protein responsible for its biogenesis.

Another significant observation of this study was that miR-21 was stabilised along with let-7 when let-7 targeted reporter plasmids were added to the cell. miR-21 had no additional targets on these constructs and therefore should not be associated with the let-7 targeted 3’ UTRs. One explanation of this phenomenon is that groups of miRNAs or RISCs associate together in a common complex. Interestingly, recent studies have documented that cellular miRNAs exist predominately in LMW-RISC complexes not
associated with mRNAs (La Rocca et al. 2015). The stabilisation of Ago2 protein through increased miRNA abundance could inadvertently stabilise other miRNAs which group together in a theoretical common complex. This would explain why we observed an increase in miR-21 and *let-7* when miR-17 is added to the cell.
7.2 Concluding remarks:

In conclusion, this study identifies novel roles of RNA binding proteins which are both canonical and auxiliary to the miRNA biogenesis pathway. We found that inhibition of p72 and KHSRP decreases Ago2 protein stability via disturbing miRNA biogenesis and therefore miRNA abundance. Furthermore, we have demonstrated that Ago2 is subject to multiple types of regulation as transient knockdown of Dicer stabilises Ago2 protein despite a decrease in miRNA abundance via a mechanism which is yet to be identified.

Additionally we have demonstrated that miRNA biogenesis is subject to possible negative feedback mechanism in which impairment of Dicer function both promotes and inhibits pri-miRNA production in a pri-miRNA and cell specific manner.

Finally, this study provides evidence to suggest that both mature miRNA levels and miRNA target abundance can stabilise miRNA biogenesis and promote pri-miRNA production in the absence of key and auxiliary proteins involved in miRNA biogenesis.
8. Bibliography:


Iwasaki, S. et al., 2010. Hsc70/Hsp90 chaperone machinery mediates ATP-dependent RISC


Martinez, N.J. & Gregory, R.I., 2013. Argonaute2 expression is post-transcriptionally coupled to


La Rocca, G. et al., 2015. In vivo, Argonaute-bound microRNAs exist predominantly in a
reservoir of low molecular weight complexes not associated with mRNA. *Proceedings of the National Academy of Sciences*, 112(3), p.201424217.


Tan, R. et al., 2012. Mouse miRNA-709 directly regulates miRNA-15a/16-1 biogenesis at the posttranscriptional level in the nucleus: evidence for a microRNA hierarchy system. *Cell*


### 9. Appendix:

#### 9.1 List of abbreviations:

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>3'</td>
<td>Three prime</td>
</tr>
<tr>
<td>5'</td>
<td>Five prime</td>
</tr>
<tr>
<td>Ago</td>
<td>Argonaute</td>
</tr>
<tr>
<td>Ago2</td>
<td>Protein argonaute-2</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium Persulfate</td>
</tr>
<tr>
<td>ALG</td>
<td>Argonaute like gene</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine tri-phosphate</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CCR4-NOT</td>
<td>Carbon catabolite repression 4- negative on TATA-less</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CDS</td>
<td>Coding Sequence</td>
</tr>
<tr>
<td>CLIP</td>
<td>Cross Linking Immunoprecipitation</td>
</tr>
<tr>
<td>CT</td>
<td>Cycle threshold</td>
</tr>
<tr>
<td>C-terminal</td>
<td>Carboxyl-terminal</td>
</tr>
<tr>
<td>DGCR8</td>
<td>Di George syndrome critical region protein 8</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified Eagle medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulphoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxy nucleoside triphosphates</td>
</tr>
<tr>
<td>dsRBD</td>
<td>Double stranded ribonucleic acid binding domain</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>eIF4E</td>
<td>Eukaryotic translation initiation factor 4E</td>
</tr>
<tr>
<td>eIF4G</td>
<td>Eukaryotic translation initiation factor 4 gamma</td>
</tr>
<tr>
<td>eIF6</td>
<td>Eukaryotic initiation factor 6</td>
</tr>
<tr>
<td>ESC</td>
<td>Embryonic Stem Cell</td>
</tr>
<tr>
<td>ERt</td>
<td>Estrogen Receptor</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal Calf Serum</td>
</tr>
<tr>
<td>GD</td>
<td>Geldanamycin</td>
</tr>
<tr>
<td>HEK293</td>
<td>Human Embryonic Kidney 293</td>
</tr>
<tr>
<td>HSP</td>
<td>Heat shock protein</td>
</tr>
<tr>
<td>KHSRP</td>
<td>KH-type splicing regulatory protein</td>
</tr>
<tr>
<td>MEF</td>
<td>Mouse Embryonic Fibroblast</td>
</tr>
<tr>
<td>miRNA</td>
<td>Micro ribonucleic acid</td>
</tr>
<tr>
<td>miRNA*</td>
<td>Micro ribonucleic acid star strand</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>NF-KappaB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>Opti-MEM</td>
<td>Opti-Minimum Essential Media</td>
</tr>
<tr>
<td>PABP</td>
<td>Poly-(A) binding protein</td>
</tr>
<tr>
<td>PAZ</td>
<td>Piwi, Argonaute and Zwille domain</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
</tr>
<tr>
<td>------------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>Pen-Strep</td>
<td>Penicillin streptomycin</td>
</tr>
<tr>
<td>PIWI</td>
<td>P-element-induced wimpy testis</td>
</tr>
<tr>
<td>POL II</td>
<td>Polymerase II</td>
</tr>
<tr>
<td>pre-miRNA</td>
<td>Precursor micro ribonucleic acid</td>
</tr>
<tr>
<td>pri-miRNA</td>
<td>Primary micro ribonucleic acid</td>
</tr>
<tr>
<td>qPCR</td>
<td>Real time polymerase chain reaction</td>
</tr>
<tr>
<td>RACK1</td>
<td>Receptor for activated kinase-C 1</td>
</tr>
<tr>
<td>RISC</td>
<td>Ribonucleic acid induced silencing complex</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNAi</td>
<td>Ribonucleic acid interference</td>
</tr>
<tr>
<td>RPM</td>
<td>Rotations Per Minute</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering ribonucleic acid</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TBS-T</td>
<td>Tris buffered saline Tween 20</td>
</tr>
<tr>
<td>TEMED</td>
<td>Tetramethylethylenediamine</td>
</tr>
<tr>
<td>tetR</td>
<td>Tetracycline Repressor</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming Growth Factor beta</td>
</tr>
<tr>
<td>TRBP</td>
<td>(tar)-binding protein</td>
</tr>
<tr>
<td>U2OS</td>
<td>Human Bone Osteosarcoma Cells</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
</tr>
<tr>
<td>V</td>
<td>Volts</td>
</tr>
</tbody>
</table>