2012

Study of MicroRNA-34a mediated post transcriptional regulation of MDM4

Pooja P. Mandke
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STUDY OF MICRORNA-34a MEDIATED POST
TRANSCRIPTIONAL REGULATION OF MDM4

A thesis submitted in partial fulfillment of the
requirements for the degree
of Master of Science

By

POOJA MANDKE
M.Sc, Pune University, 2009

2012
Wright State University
WRIGHT STATE UNIVERSITY
GRADUATE SCHOOL

September 5, 2012

I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY
SUPERVISION BY Pooja Mandke ENTITLED Study of MicroRNA-34a mediated post
transcriptional regulation of MDM4 BE ACCEPTED IN PARTIAL
FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science

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ABSTRACT

Mandke, Pooja M.S Department of Biochemistry and Molecular Biology, Wright State University, 2012. Study of microRNA-34a mediated post transcriptional regulation of MDM4

MDM4 is an important negative regulator of the tumor suppressor p53. In normal unstressed cells the activity of p53 is kept under control by MDM4 and its homologue MDM2. MDM4 is said to possess oncogenic potential based on the evidence of its overexpression in many cancers. Until recently it was believed MDM4 is constitutively transcribed; however a decrease in full length MDM4 in response to genotoxic stress was observed paving way for exploring the mechanism responsible for this. 

It was observed miR-34a a member of the miR34 family which is a direct transcriptional targets of p53 could have a potential role in regulation of MDM4 expression. The 3’untranslated region of MDM4 was also seen to contain several miR-34a binding sites.

However reporter assays with select regions of the 3’UTR revealed that the 3’UTR was unresponsive to miR-34a mediated regulation. Reassessment of the MDM4 gene revealed presence of a potential miR-34a regulatory site in the protein coding exon 11 of MDM4. This site was further considered to check for functionality in response to miR-34a modulation. A reporter with the miR-34a site from the coding region was constructed. This reporter was responsive to overexpression or inhibition of endogenous miR-34a in H1299 and MCF7 cells respectively ascertaining the functionality of this site.
A SNP leading to A>C transversion in the seed region of this miR-34a site in the exon 11 was predicted to disrupt responsiveness to miR-34a. We confirmed this by creating point mutants and performing reporter assays.

This study was designed to understand the regulation of MDM4 in absence of DNA damage conditions. Understanding the role of miR-34a in regulation of MDM4 will pave way for designing specific therapeutic strategy for reactivation of p53 via inhibition of MDM4 in cancer that overexpress MDM4 and retain wild type p53.
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I. INTRODUCTION

**P53, MDM2 and MDM4**

The gene \(TP53\), encoding transcription factor p53, is one of the most important tumor suppressors and is considered as the guardian of the genome (Efeyan 2007). As a part of its overall function it is shown to play a role in providing protection against malignant transformation and maintenance of genomic stability. The crucial role of p53 as a tumor suppressor is demonstrated by the fact that it is seen to be mutated or deleted in more than fifty percent of the human cancers. However, in cancers which possess wild type p53, p53 inactivation can result from the amplification/overexpression of its specific inhibitors MDM2 and MDM4 (also known as MDMX) (Toledo 2007).

In normal unstressed cells p53 is maintained at basal levels by the p53 binding proteins MDM2 and MDM4. MDM2 (transformed mouse 3T3 cell double minute 2) is a well-studied regulator/inhibitor of p53. MDM2 affects subcellular localization of p53 where it promotes monoubiquitination of p53 in nucleus thereby leading to its nuclear export. In the cytoplasm more ubiquitin is added to this mono ubiquitin which facilitates degradation of p53 (O'Keefe 2003). MDM2 indirectly also affects translation of p53 via binding to the ribosomal protein L26. Under conditions of stress L26 binds to the 5’ untranslated region of p53 mRNA and increases translation of p53 mRNA leading to production of more p53 protein. MDM2 however is shown to interact with L26 and target it for ubiquitin mediated proteasomal degradation causing a decrease in the L26 mediated increase in p53 translation (Ofir Rosenfeld 2008). MDM4 also affects stability and transcriptional activity of p53 protein. MDM2-dependent p53 inhibition is essential
in regulating p53 activity during embryonic development and in adult tissues (Wang 2012). MDM2 regulates the p53 turnover by binding p53 and acting as a ubiquitin E3 ligase mediating the proteasomal degradation of p53. It is seen that MDM2 abrogates the ability of p53 to induce cell cycle arrest and apoptosis by virtue of its overexpression. Its overexpression as a result of gene amplifications is seen in about 30% of human osteogenic sarcomas and soft tissue sarcomas (Chen 1998). In tumors where MDM2 is not amplified, p53 inactivation can also take place as a result of hyperactivation of MDM2 due to silencing of ARF expression (Hu 2006). Therefore, MDM2 is a key factor in tolerance of wild type p53 in nearly 50% of tumors, making it an attractive target for the development of novel anti-tumor agents (Bond 2005).

Murine Mdm4 (for transformed mouse 3T3 cell double minute 4, also Mdmx) and human ortholog MDM4 (also HDMX) are the closest analogues of Mdm2 (transformed mouse 3T3 cell double minute 2). MDM4 shares a considerable sequence homology with MDM2 and is also structurally analogous to MDM2. Like MDM2, MDM4 possesses a p53-binding domain (p53BD) encompassing approximately the first 100 amino acids, the residues required for p53 interaction are conserved in both MDM2 and MDM4 and the same residues in p53 are required for its interaction with both MDM2 and MDM4. It also has a C-terminus RING (really interesting new gene) finger region through which it interacts with MDM2 (Marine 2006, Mancini 2010).
MDM4 is also an essential inhibitor of p53 in vivo. Although structurally homologous to MDM2, MDM4 does not possess the intrinsic ubiquitin ligase activity hence does not directly participate in degradation of p53 protein. However, MDM4 stimulates the MDM2 mediated ubiquitination and degradation of p53 by binding to MDM2 through C-terminal RING domain. The ubiquitin ligase MDM2 also ubiquitinates and targets MDMX for degradation via MDM4-MDM2 RING domain interaction; and this may be an important mechanism for elimination of MDM4 during DNA damage (Tanimura 1999, Hu 2006). Recent advances in the field from biochemical and genetic studies have revealed an essential role for the MDM4 RING domain in MDM2-dependent p53 polyubiquitination and degradation. MDM2 on its own is a monoubiquitin E3 ligase for p53, but is converted to a p53 polyubiquitin E3 ligase by MDM4 through their RING-RING domain interactions. MDM4 acts as an activator as well as a substrate
of MDM2/MDM4 E3 complex (Wang 2012). Apart from its indirect role in maintaining p53 protein levels MDM4 is shown to control the transcriptional activity of p53 by binding to its N terminal domain. Genetic studies have shown that MDM4 controls p53 transcriptional activity independent of MDM2 (Francoz 2006).

Like MDM2, MDM4 is overexpressed in many cancers, especially those of breast, colon, and lung, as well as in glioma, lymphoma, and retinoblastoma (Danovi 2004), where it is thought to promote tumorigenesis by suppressing p53 function. However, conditional deletion (Grier 2006) and knockout studies (Parant 2001) have shown that MDM4 like MDM2, is required for protection against p53 lethal activity in embryonic development. This indicates a unique role of MDM4 in regulating p53 during embryonic development (Migliorini 2002). Although MDM4 can regulate p53 stability by heterodimerization with MDM2, its impact on p53 level is moderate compared to MDM2. Recent studies suggest that the major mechanism of p53 regulation by MDM4 is the formation of inactive p53-MDM4 complexes. Therefore, elimination of MDM4 is important for efficient p53 activation during stress response (Gilkes 2008).

Regulation of MDM4

As stated earlier, MDM4 is a critical negative regulator of p53 and keeps its activity in check in normal unstressed cells. However, under conditions of stress which requires the activation of p53, it is necessary to control/inhibit activity of the negative regulators of p53 viz. MDM2 and MDM4. MDM4 expression can be controlled at the level of transcription, post transcription, translation or post translation which may lead to either overexpression as seen in some cancers or inhibition of expression. The transcription control of MDM4 is an area less explored. The changes in production of transcripts of
MDM4 could not be attributed to changes in promoter activity of MDM4 (Markey 2008). However, there are reports of MDM4 expression being regulated by mitogenic signaling pathways via binding of different transcription factors to the MDM4 promoter. This mechanism may confer protection to normal proliferating cells from p53 but may contribute to hampering of p53 response during tumor development (Gilkes 2008). It was also seen that a transcript variant of MDM4 also known as HDMX could be transcribed from a p53 responsive promoter in the first intron of the HDMX gene. This variant transcript called as HDMX-L was more efficiently translated than the transcript produced from the constitutive promoter P1 of HDMX gene and produced a functional protein product (Philips 2010).

Another aspect playing a role in controlling expression of full length MDM4 could be production of alternative splice variants in response to damage conditions as reported in some cancers (Mancini 2009). The production of alternative splice variants also initiates another layer of p53 regulation. Some of the reported splice variants of MDM4 are the XALT1 which lacks the p53-binding domain and retains the COOH-terminal RING domain. The XALT2 splice variant in which only retains the p53 binding domain in the final protein. The MDM4211 variant is a highly aberrant form present only in tumor cells. The MDM4-A, MDM4-G and MDM4-S variants are also seen in certain cancers (Figure 2). The XALT2 variant which lacks the p53 binding domain may be responsible for negatively regulating the full length MDM4 variant (Chandler 2006) however the presence of XALT2 does not completely compensate for the loss of full length MDM4 variant (Markey 2008). On the other hand the XALT1 variant has only the p53 binding domain hence these two variants may function in an antagonistic manner in the cell (Chandler 2006).
Figure 2: **MDM4 and its splice variants** A) schematic of full length MDM4

B) Splice variants of MDM4


There are also reports on the control of absolute stability of MDM4 mRNA which could possibly be due to the role of microRNAs (Markey 2008). At the level of translation, the role of eukaryotic initiation factor factor 4E and 4E-BP1 could play a role in affecting the translation of MDM4 protein (Zhu 2005, Horton 2002); however no other reports are available implicating the process of translation itself. MDM4 protein itself is subject to MDM2 E3 ligase mediated proteasomal degradation as a result of phosphorylation signals (Chen 2005). Against the background of various aspects controlling MDM4 expression and activity, the area of post transcriptional control of MDM4 is interesting and one that remains less explored.
Potential Role of microRNAs in regulation of MDM4

What are microRNAs?

MicroRNAs (miRNAs) are highly conserved, endogenously expressed, non-protein-coding RNAs that play a role in regulation of gene expression. These are typically 20-25 nucleotide long and regulate gene expression in plants and animals by recognizing motifs of imperfect complementarity between the mature microRNA via a 2-7 bases region called a ‘seed region’ and 3’ untranslated region (UTR) of target transcripts. miRNA mediate regulation of gene expression by repression of translation, degradation of mRNA and mRNA decay initiated by miRNA-guided rapid deadenylation (Zhang 2007). Since the discovery of miRNAs a large number of miRNAs have been discovered and these are shown to participate in variety of homeostatic processes such as development, differentiation, cell proliferation and cell death. Since miRNAs are involved is all the important cellular processes their dysregulation has been linked to initiation and progression of cancer. Hence depending on the genes they regulate and the pathways they affect miRNAs can be termed as oncogenes or tumor suppressors (Lyammlennon 2009).

Biogenesis of microRNAs.

miRNA genes are transcribed by RNA polymerase II. The resulting transcript is capped with a specially modified nucleotide at the 5’ end and polyadenylated with multiple adenosines at the 3’end. This primary miRNA (pri-miRNA) varies from 200 nt to several kb. The Drosha ribonuclease in conjunction with DiGeorge Syndrome Critical Region Gene 8 (DGCR8), cleaves the pri-miRNA into a 60- to 70-nt precursor-miRNA (pre-
miRNA). Pre-miRNA hairpins are exported from the nucleus to the cytoplasm by Exportin-5 and Ran-GTP. In the cytoplasm, the pre-miRNA hairpin is cleaved by the enzyme Dicer with the TAR RNA-binding protein (TARP) or PKR activating protein (PACT). One miRNA strand, the guide miRNA, is taken into the RNA-induced silencing complex (RISC), of which the Argonaute proteins are a main component. In particular, Argonaute 2 (Ago2) is thought to play a central role in the miRNA biogenesis pathway. In the RISC, the guide miRNA finds and binds its complementary target mRNA. miRNAs that bind to mRNA targets with perfect matching induce mRNA degradation, whereas translational repression is induced when matching is imperfect (Lee 2004, Shomron 2009).

Figure 3: MicroRNA biogenesis

MicroRNAs in cancer

After the discovery of miRNAs several observations led researchers to believe that miRNAs could play a role in cancer. In *C. elegans* and *Drosophila* where the earliest miRNAs were discovered they were shown to be associated with functions like cell proliferation and apoptosis (Ambros 2003), suggesting that their dysregulation may pave way for proliferative diseases like cancer. Secondly, it was seen that more than half the miRNA genes were located on sites on the genome that were frequently amplified or deleted in human cancers (Calin 2004). Thirdly, compared with normal tissue, malignant tumors and tumor cell lines were found to have widespread deregulated miRNA expression (Lu 2005). However the question that remained to be answered was whether malignant transformation was a consequence or cause of altered miRNA expression.

Further studies showed miRNAs were directly involved in human cancers, including lung, breast, brain, liver, colon cancer, and leukemia (Nikitina 2012). Depending on the genes they target and the pathways they affect miRNAs may function as oncogenes or tumor suppressors. For instance microRNAs such as mir-17-92, have oncogenic potential owing to its presence in a region on chromosome 13 commonly amplified in human B-cell lymphoma. On the other hand miRNAs such as let-7 are shown to possess tumor suppressive potential as it is able to inhibit RAS expression in human cancer cell lines (Johnson 2005). Hence against this background, expression profiles of miRNAs may not only serve as important biomarkers for diagnosis of cancer but also aid in development of probable therapeutics for cancer prevention (Sassen 2008, Nohata 2012).
MiR-34 family and p53

As mentioned earlier p53 is an important tumor suppressor. P53 functions as a tumor suppressor by transcriptionally activating its target genes viz genes involved in cell cycle checkpoints, apoptosis and cellular senescence. However, against the background of the tumor suppressive function of p53 the concept of p53 mediated repression of target genes was less appreciated. A proof for this was provided by a study which showed p53 mediated downregulation of a new set of genes indicating p53 induces cell cycle arrest not only by transactivation of cell cycle inhibitors (p21) but also through repression of targets regulating proliferation at different phases of the cell cycle (Spurgers 2006). A plausible explanation was provided by the existence of microRNAs. It was possible that p53 was mediating indirect control of certain target genes at post transcriptional level via influencing expression of microRNAs (Feng 2011).

In 2007 reports showed that miR34 family members are direct transcriptional targets of p53 and might be key effectors of p53 tumor suppressor function (Bommer 2007). The miR34 family consists of 3 members: mir-34a, miR-34b and miR-34c. The miR-34a is transcribed from an independent transcript and is ubiquitously expressed with high expression in the brain tissue. The miR-34b and miR-34c share a common primary transcript and are predominantly expressed in the lung tissue. Reports have shown that miR34a and miR-34b/c are downregulated in many cancers. Apart from the confirmation of miR34a being a direct transcriptional direct of p53 its importance in p53 mediated functions was confirmed by demonstration of induction of apoptosis upon overexpression of miR34a (Hermeking 2010).
miR-34a was shown to target and repress SIRT1, a NAD-dependent deacetylase, which is known to inhibit several pro-apoptotic proteins including p53. Apoptosis induced by the re-introduction of miR-34a is seen to be p53 dependent and linked to the targeting of SIRT1 mRNA by miR-34a. Hence it can be said the regulation of SIRT1 by miR-34a is part of a positive feedback loop that leads to further activation of p53. Another feedback loop between p53 and miR-34a may involve the downregulation of the p53-inhibitor HDMX by miR-34a. Hence miR-34a is reported to be an important mediator of p53 tumor suppressive function (Hermeking 2010).

MicroRNAs as therapeutics?
Since miRNAs are linked to various aspects of cancer progression and pathogenesis, miRNA based therapeutic approaches have tremendous potential to produce cancer specific effects. Inhibition of oncogenic miRNAs or overexpression of tumor suppressive
miRNAs may help control malignant transformation. Hence that is an exciting aspect of miRNA biology.

As already stated with respect to p53, the miR-34 family has gained much attention and is seen to be down regulated in many cancers. Recent research has shown that miR-34 based therapy could be of great value. Studies have shown that impairment of tumorigenesis was seen in a xenograft model of non-small lung cancer with an intratumor or systemic administration of miR34 mimic and same was observed in case of lung tumors (Trang 2011). As it is already known attenuated expression of miR34 is observed in prostate and pancreatic cancers it could prove to be a valuable therapeutic approach for these cancers (Pramanik 2011, Liu 2011).

**Inhibition of MDM4 and MDM2 as possible therapeutic strategies**

MDM4 is overexpressed in many cancers and this acts to block the p53 tumor suppressive function. Hence, inhibition of MDM4 in cancers that retain wild type p53 may have therapeutic potential. Currently there are therapeutic strategies involving inhibition of MDM2 but response to these may be dampened in the presence of high levels of MDM4, hence this calls for MDM4 specific therapeutic strategies (Wade 2009). However, the effect of acute systemic inhibition of MDM4 on normal adult tissues had to be explored. A study in this direction showed that MDM4 was required to buffer p53 activity in adult normal tissues and their stem cells. Another important observation was that transient restoration of p53 in absence of MDM4 was not lethal and had no phenotypic consequences. As opposed to restoration of p53 in a MDM2 null background which led to p53 dependent cell death (Gembraska 2012). It was also shown that the therapeutic impact of restoring p53 in a tumor model is enhanced in the absence of MDM4, leading to a significant extension of survival over p53 restoration in the presence of MDM4. Hence, systemic inhibition of MDM4 is both a feasible and safer therapeutic
strategy for restoring p53 function in tumors that retain wild-type p53 than inhibition of Mdm2 (Garcia 2011, Gembraska 2012).

Some of the recent advances for targeted therapy for MDM4 is the development of (stapled alpha helix peptide) SAH-p53-8. The design of the SAH-p53-8 was based on the peptide sequence of the alpha helix transactivation domain of p53. This was previously designed for targeting MDM2 however studies showed 25-fold greater binding preference of SAH-p53-8 for HDMX. The mechanism of action of SAH-p53-8 involves blocking formation of the p53-HDMX interaction, and thereby restoring the p53 pathway, as seen by reduction in tumor cell viability and transcriptional upregulation of p53 targets. SAH-p53-8 was particularly effective in nutlin resistant tumors. Thus, cancers that sequester p53 via HDMX overexpression may be particularly sensitive to SAH-p53-8 or other HDMX-specific antagonists in development (Bernel 2010).

**Objective**

The objective of this study was to explore the role of miR-34a as a possible factor responsible for downregulation of MDM4 in absence of DNA damage. It was previously seen that in presence of DNA damage there was decrease in full length MDM4 transcripts. However the mechanism of this downregulation was not exactly understood. Since there was a decrease in the full length transcripts it was indicative of certain transcriptional or post transcriptional regulatory mechanisms. After, preliminary evidence of MDM4 as a potential target of miR-34a as seen through computational analysis; we sought to explore the role of miR-34a in regulation of MDM4. We hypothesized that miR-34a did regulate the expression of MDM4. Firstly qPCR and western blot analysis was done to assess response of endogenous MDM4 to miR-34a as compared to known targets of miR-34a. This was followed up by reporter assays with plasmids containing potential miR-34a target sites from MDM4 tested in background of
elevated or inhibited levels of miR-34a to confirm functionality of these miR-34a target sites in MDM4. The results obtained from this study will help in the understanding of the regulation of MDM4 by miR-34a paving way for its potential use as a therapeutic strategy for MDM4 inhibition.
II. MATERIALS AND METHODS

Cell Culture

Human non-small cell lung carcinoma cell line H1299 was cultured in Dulbecco’s modified Eagle’s medium (DMEM, Atlanta Biologicals) supplemented with 10% normal calf serum. Breast cancer cell line MCF7, human fibroblasts IMR90, and osteosarcoma cell line SAOS2 were cultured in DMEM supplemented with 10% fetal calf serum. All cell lines were obtained from the American Type Culture Collection.

Quantitative RT-PCR

MCF7 cells were transfected 48 hours after seeding (2 x 10^5) cells in 6 cm plates. Thirty nanomolar miRVANA hsa-miR-34a inhibitor (Ambion) was added to 50 µl of serum free media. Six µl lipofectamine 2000 was added to 50 µl serum free media. The two solutions were mixed in equal quantities and allowed to stand at room temperature for 20 minutes. Hundred µl of the resulting solution was added to 6 cm plates containing 2.9 ml serum free media. After 5 hours of incubation the cultures were returned to DMEM media containing 10% serum. Forty eight hours later the cultures were processed for RNA extraction.

RNA extraction

Cells were washed with phosphate buffered saline (PBS). This was followed by addition of TRIzol reagent (Invitrogen), a mixture of phenol, guanidine isothiocynate, red dye and other proprietary components that can be used to isolate RNA. The cells were scraped, collected in a centrifuge tube and passed through a syringe. This was incubated at room
temperature for 5 minutes followed by addition of 200 µl of chloroform, shaking the contents for 15 seconds and incubation at room temperature for 2-3 minutes. The contents were centrifuged at 12,000g for 15 minutes at 4°C. This resulted in separation of an aqueous and organic phase. The aqueous phase was transferred to a new tube and 5 µg of glycogen was added to it. To this was added 500 µl isopropyl alcohol, incubated at room temperature for 10 minutes and centrifuged at 12,000g for 10 minutes at 4°C. The supernatant was removed and the pellet was washed with 1ml of 75% RNase free ethanol and centrifuged at 7500g for 5 minutes at 4°C. The supernatant was again removed and the pellet was air dried for 5-10 minutes. The pellet contained RNA which was resuspended in 40 µl of RNase free water and incubated at 55-60°C for 10 minutes. Following this RNA was either stored at -80°C for future use or continued for quantification by Nanodrop ND-1000 spectrophotometer. The absorbance ratio at 260nm and 280nm was considered to determine purity of RNA, with a ratio between 1.8-2.1 was considered as optimum.

**MicroRNA extraction**

RNA extraction was carried out using the TRIzol reagent method as mentioned above. The RNA obtained by this method was further processed to enrich for microRNA using the RT²qPCR Grade miRNA Isolation Kit (SAbiosciences) according to manufacturer’s protocol.

**Reverse transcription of total RNA**

0.5 µg of RNA was reverse transcribed to cDNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). The RNA was mixed with water to bring the final volume to 9.62 µl. For one reaction the reaction mix was prepared as follows:
The contents of the reaction mix were tapped gently to mix properly and spun to eliminate bubbles. The reaction mix was added to 0.2 ml microamp tubes followed by addition of 9.62 µl RNA sample (total reaction volume 25 µl). The contents of the tube were tapped gently to mix properly and spun to eliminate bubbles. The reaction was incubated at 25°C for 10 minutes, 48°C for 30 minutes and 95°C for 5 minutes and held indefinitely at 4°C. The cDNA was diluted to 100µl prior to quantitative PCR.

**Reverse transcription of microRNA**

10 ng of the microRNA was reverse transcribed using miRNA-specific primers for hsa-miR-34a or hsa-mir213 (to normalize) (5X working stock solution) according to the TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems).
The master mix was prepared as follows:

<table>
<thead>
<tr>
<th>Components</th>
<th>Volume μl (x1)</th>
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<td>10X Reverse transcription buffer</td>
<td>1.5</td>
</tr>
<tr>
<td>100mM dNTPs</td>
<td>0.15</td>
</tr>
<tr>
<td>RNase inhibitor 20U/μl</td>
<td>0.19</td>
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<tr>
<td>multiscrIBE reverse transcriptase 50U/μl</td>
<td>1</td>
</tr>
<tr>
<td>Nuclease free water</td>
<td>4.16</td>
</tr>
<tr>
<td><strong>Total volume</strong></td>
<td><strong>7</strong></td>
</tr>
</tbody>
</table>

The final reaction was set with 7 μl of master mix and 5 μl of RNA (total quantity 10 ng). The contents were mixed gently and centrifuged. Meanwhile the primer tubes were thawed on ice, mixed by vortexing and centrifuged before addition to the reaction mixture. This 12 μl mixture was added to two different tubes followed by addition of 3 μl of 5X primers specific to miR-34a in one and miR-213 in another. These tubes were incubated at 16°C for 30 minutes, 42°C for 30 minutes, 85°C for 5 minutes and held at 4°C indefinitely. The cDNA was diluted to 50 μl prior to quantitative PCR.

**Quantitative RT-PCR**

The cDNA from both the total RNA and microRNA was used to perform quantitative RT-PCR. For this purpose 9 μl of the diluted cDNA was mixed with 11 μl of the mastermix which contains 10 μl of the 2X Taqman universal master mix (no AmpErase, Applied Biosystems) and 1 μl of the 20X of the Assay on Demand gene expression product (Applied Biosystems) for MDM4, CDK6, CCND1, GAPDH, miR-34a and miR-213. Four technical replicates of each sample were set up. The reactions were incubated at 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Target
gene expression from RNA was normalized to GAPDH and from microRNA was normalized to miR-213. SDS 2.2.2 software was used for target gene expression analysis with setting automatic Ct values, outlier removal and 95% confidence interval for RQ min/max calculations. All the reactions were performed in the ABI 7900HT sequence detection system (Applied Biosystems).

**Reporter Assays**

H1299 cells were transfected 24 hours after seeding (2.5 x 10^6) cells in 6 cm plates. The psicheck2 vector or psicheck2-ex11 reporter or psicheck2-ex11 A>C reporter, and mir-34a expression plasmid or mir-34a-mut expression plasmid, were added to serum free media (final volume of 200 µl). Six µl of lipofectamine2000 was added to 200 µl of serum free media, which was then mixed with the plasmid DNA and incubated 30 minutes at room temperature. 400 µl of the resulting DNA:lipid solutions were added to cells on 6 cm plates containing 2.5 ml serum free media. After 5 hours of incubation the cultures were replaced with DMEM media containing 10% NCS. 24 hours later the cultures were processed for the dual luciferase assay using the Promega Dual Luciferase assay system and protocol. Samples were normalized to luciferase expression in the vector-transfected cells. Paired, one-tailed t-tests were used to determine significance.

The plasmid psicheck2-AS34a contains a consensus response site for miR-34a, and was used as a positive control for response to miR-34a overexpression or inhibition.

MCF7 cells were transfected 24 hours after seeding the 24-well plates (4 x 10^4 cells/well). The reporter plasmid (0.2 µg) with or without the hsa-miR-34a inhibitor (30 nM) and the psicheck2 (vector) plasmid (0.2 µg) with or without the inhibitor (30 nM) were added to 50 µl of serum free DMEM media. Two µl of lipofectamine2000 was added to 50 µl serum free DMEM media. The two solutions were mixed in equal quantities and
allowed to stand at room temperature for 20 minutes. 100 µl of the resulting DNA:lipid solution was added to culture plates containing 2.9 ml serum free media. After 5 hours of incubation the serum free media was replaced with DMEM media containing 10% FBS. 24 hours later the cells were processed for the Dual Luciferase assay (Promega).

The Dual-Luciferase reporter (DLR) assay system provides an efficient means of performing dual reporter assays. In the DLR assay, the activities of firefly viz hluc \textit{(Photinus pyralis)} and renilla viz. Rluc \textit{(Renilla reniformis), also known as sea pansy)} luciferases are measured sequentially from a single sample. This is achieved as both the renilla (experimental reporter) and firefly luciferase (control reporter) genes are present on the same plasmid.

The cells on the plate were first cleared of any growth media and washed with phosphate buffered saline (PBS). IX passive lysis buffer (PLB) was added to plate according to the plate/well size. The culture vessel was gently rocked at room temperature and transferred to a vial or tube. For the assay the firefly luciferase reporter was measured first by adding 100 µl luciferase assay reagent II (LAR II) to 20 µl of the lysate. After quantifying the firefly luminescence, this reaction was quenched, and the renilla luciferase reaction was initiated by simultaneously adding stop & glow reagent to the same tube. The luminometer was used to quantify the luminescence generated from the two steps. The ratio of renilla luciferase to firefly luciferase was the calculated to give relative luciferase activity. Readings from six biological replicates of the same experiment were obtained and the average of these are plotted to obtain the average relative luciferase expression for each experimental condition.

**Immunoblotting**

MCF7 cells were transfected 24 hours after seeding the 6 cm plates (2 x 10⁴ cells) and
treated with 30 nM miRVANA hsa-miR-34a inhibitor (Ambion) as described above. 48 hours later the cells were processed for protein extraction.

The cells were harvested from the 6 cm plates in phosphate buffer saline (PBS) and lysed in RIPA buffer (50 mM Tris pH=8.0, 150 mM NaCl, 1% NP-40, 0.5% Deoxycholate, 0.1% SDS) (150 µl) to which was added protease inhibitor cocktail PIC (1 µl/100 µl) and NaF (to 50 µM). The contents were mixed by pipeting, incubated on ice for 30 minutes and centrifuged at 10,000 rpm for 5 minutes at 4°C. The supernatant was then taken and protein concentration measured using the Bradford Protein Assay (Biorad). The proteins were separated by SDS-PAGE. After separation the proteins were transferred onto a PVDF membrane (at 400 milliamps for 1 hour). This was followed by blocking with 5% milk in Tris-buffered saline-tween 20 (TBST) for one hour and probing with the relevant primary antibody overnight. Following 2 thirty minute washes with TBST, a horse radish peroxidase (HRP) conjugated secondary antibody was added for 1 hour and the protein band was observed following addition of Super signal West Pico chemiluminescent substrate (Thermo Scientific). The following antibodies were used: HdmX /Mdm4 A300-287A (Bethyl labs), actin AC-40 (Sigma), p53 FL393 (Santa Cruz Technology), anti-rabbit IgG HRP (Promega), anti-mouse IgG HRP (Promega).

Relative quantification of western blots was performed with ImageJ. Values shown are relative to the control condition and normalized to actin, to control for any protein loading inequalities.

**Cloning**

For the plasmid “psicheck2 Exon 11”, a 444 bp region of the coding region of exon 11 of the human MDM4 gene, surrounding the putative ORF miR-34a site, was amplified by PCR from BAC 433N15 (BACPAC Resources Center) and inserted between SgfI and NotI sites in the psicheck2 reporter plasmid (Promega) downstream of renilla luciferase.
Cloning primers were: sense 5’- AAA AAA GCG CCT TGA GGA AGG ATT GGT AT -3’ and antisense 5’- AAA AAA GCG GCC GCA GCC CCA GCC TTC TTT AGT C -3’.

PCR was set up as follows: SYBR green PCR master mix=25 µl, BAC template=1 µl, cloning primers= 2 µl each, water=20 µl, total volume=50 µl. PCR conditions :95°C=10 minutes, 40 cycles of 95°C=30 seconds, 55°C=40 seconds and 72°C=25 seconds, 72°C=5 minutes and hold at 4°C). Following PCR the digestion of insert and vector was performed.

<table>
<thead>
<tr>
<th></th>
<th>Insert digest (µl)</th>
<th>Vector digest (µl)</th>
<th>Final Concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td>AsisI</td>
<td>2</td>
<td>2</td>
<td>2 units/µl</td>
</tr>
<tr>
<td>NotI</td>
<td>1</td>
<td>1</td>
<td>1 unit/µl</td>
</tr>
<tr>
<td>NEB4 buffer</td>
<td>1</td>
<td>1</td>
<td>1X</td>
</tr>
<tr>
<td>BSA</td>
<td>1</td>
<td>1</td>
<td>1X</td>
</tr>
<tr>
<td>Shrimp alkaline phosphatase (SAP)</td>
<td>-</td>
<td>1</td>
<td>1 unit/µl</td>
</tr>
<tr>
<td>Insert (PCR product)</td>
<td>6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Psicheck2</td>
<td>-</td>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td>Total</td>
<td>10</td>
<td>10</td>
<td>-</td>
</tr>
</tbody>
</table>

The digest mixtures were incubated at 37°C for 90 minutes followed by incubation at 80°C for 20 minutes.

The next step in the protocol was to ligate the digested insert and vector. The ligation reaction mixture included: T₄ ligase=1 µl, T₄ ligase buffer=2 µl, insert and vector in the ratio 3:1, final volume made upto 10µl with water. The reaction mixture was incubated at 25°C for 10 minutes followed by overnight incubation at 16°C.
This ligation mixture was used to transform 50 µl of TOP10 cells. One vial of TOP10 cells corresponding to 50 µl volume was thawed on ice for 30 minutes. To this was added 5 µl of the ligated product (no mixing of the contents). The contents were incubated on ice for 30 minutes followed by 30 seconds incubation at 42°C. The reaction mixture was placed on ice briefly after which was added 250 µl of SOC media. This mixture was incubated horizontally in a shaker incubator at 37°C set at 225RPM for 60 minutes. Fifty µl of the reaction mixture was plated on LB+Amp plates followed by overnight incubation at 37°C. Colonies are picked randomly and inoculated in LB+Amp liquid media and again incubated overnight at 37°C. Following growth in the liquid media these are processed for plasmid extraction.

**Plasmid DNA purification.**

The plasmid DNA was extracted using the Hurricane miniprep protocol (Gerard Biotech). The bacterial culture was transferred to a 1.5ml centrifuge tube and centrifuged at 10,000g for 1 minute. The bacterial pellet was resuspended in 250 µl of Buffer A by pipetting. This was followed by addition of 250 µl of Buffer B and mixed thoroughly by inverting the tubes 10 times and lastly was added 325 µl of Buffer C and mixed gently by inverting 10 times. The mixture was centrifuged at 10,000g for 10 minutes. The supernatant was transferred to a DNA binding column and centrifuged at 10,000g for 1 minute. The liquid from the tube was discarded and to the DNA binding column was added 750 µl of 70% ethanol and centrifuged at 10,000g for 1 minute. The ethanol was discarded and the tubes were centrifuged for an additional 3 minutes to remove excess ethanol. The DNA binding column was then placed in 1.5 ml collection tubes to these was added 50 µl of preheated (65°C-70°C) sterile water and centrifuged at 10,000g for 1 minute after allowing standing at room temperature for 1 minute. Identity of all clones was verified by sequencing (Retrogen).
**Ethanol precipitation to purify DNA from the PCR product.**

The purification of the PCR product began with addition of 1/10th volume of sodium acetate (3M pH=5.2) followed by addition of 2 volumes of cold (-20°C) 100% ethanol and proper mixing of the contents. The contents were then incubated at -20°C for 20 minutes and spun at maximum speed for 10 minutes at 4°C. The supernatant was decanted followed by addition of 1ml of cold (-20°C) 70% ethanol and again spun at maximum speed for 5 minutes at 4°C. The supernatant was decanted and pipetted out. The pellet was subjected to speed vac but no heat to dry. The dry pellet was suspended in 12 µl of water.

**Site Directed Mutagenesis.**

The plasmid “psicheck2 exon 11 A>C” was generated by site-directed mutagenesis of the “psicheck2 exon 11” plasmid according to the QuikChange II Site-Directed Mutagenesis kit and protocol (Aligent Technologies). An A to C mutation was generated to correspond to the human SNP rs79824231, which lies in the seed region of the exon 11 ORF miR-34a site. Mutagenic primers were: sense 5’- CTC TCC ACG TCT GAT ATC CCT GCC AT ACCT GAA AA -3’ and antisense 5’- TTT TCA GGT ATG GCA GGG ATA TCA GAC GTG GAG AG -3’. Mutation of the target site was verified by sequencing.

**Sequence alignment**

Sequences were retrieved from GenBank and aligned using ClustalW2 v2.1 with default (slow) alignment settings. The accession and version numbers for sequences used were: *Mus musculus* EU568360.1, *Rattus norvegicus* NM_001012026.1, *Xenopus laevis* NM_001088965.1, *Pan troglodytes* XM_003308706.1, human MDM4 NM_002393, and human MDM2 NM_002392.3.
III. RESULTS

Previous work/Background

Initially it was thought that MdmX was constitutively transcribed and it is controlled at the level of translation where the expression of MdmX protein is affected probably through ubiquitin mediated proteasomal degradation catalysed by Mdm2. The same was believed to happen with respect to control of the human MDMX (HDMX or MDM4) expression. However previous work in the laboratory showed that the levels of full length MDM4 decreased in response to genotoxic stress. It was seen that the decrease in hdmx transcript levels was species specific as it was not observed in murine cell lines and independent of the p53 status, type of cell line and type of DNA damage (Figure 5).

It was also shown that apart from the established ubiquitin mediated proteasomal degradation, the decrease in full length transcripts following genotoxic stress also contributed to decrease in levels of MDM4 proteins. In order to ascertain the cause for the reduction in full length transcripts certain potential factors were explored. Firstly, change in the promoter activity as a potential cause for reduction in full length transcripts was explored. HCT116 (wt p53), HCT116 (p53-/-), MCF7 and H1299 were transfected with MDM4 promoter –luciferase gene construct or constitutive CMV-luciferase gene construct as a control. Reporter assays were performed to assess expression of the promoter construct. There was no significant change in promoter activity following DNA damage which could account for this decrease (Figure 6).
Figure 5: $hdmX$ (MDM4) is transcriptionally repressed in response to DNA damage independent of p53 status, and in non-tumor human fibroblasts. Markey M, Berberich SJ (2008) Full length MDM4 transcripts decrease following genotoxic stress. *Oncogene* 27: 6657-6666.
Figure 6: The activity of an MDM4 promoter-luciferase gene construct is unchanged by exposure to doxorubicin. The activity of the MDM4 promoter was unchanged similar to that observed with a constitutive CMV promoter-luciferase gene construct.

It was earlier seen that in response to DNA damage alternative splice variants of MDM4 were produced (Chandler 2006). Hence, production of alternative splice variants of MDM4 in response to DNA damage conditions as a possible mechanism leading to reduction of full length transcripts was studied. A loss of full-length MDM4 mRNA levels in several cell lines and concurrent induction of XALT2 mRNA was observed. Alternative splicing could not be the only mechanism leading to the loss of MDM4 transcripts based on two findings in this report. Based on the experiments where absolute quantification of XALT2 transcripts and full length transcripts indicated that the observed decrease in full length transcripts was not fully compensated by the increased levels of XALT2 variant transcripts (Figure 7B). Experiments showed that alternative splice variants could not fully explain this observed effect on full length transcripts in response to DNA damage (Figure 7).

In addition to this there was evidence of destabilization of MDM4 mRNA as seen by decreased half-life of full-length MDM4 mRNA following DNA damage. Towards understanding the basis for the MDM4 mRNA degradation, there were several reports demonstrating that miR-34a could be induced upon DNA damage and MDM4 was one of the mRNAs downregulated by miR-34a overexpression (Chang 2007). In this direction the work of the laboratory showed presence of several potential miR-34a binding sites in the 3’UTR of the MDM4 transcript. Some data from the laboratory showed a correlation between miR-34a and reduction of MDM4 transcripts. It was also seen that with the use of anti-miR- 34a to reduce miR-34a there was an increase in full-length MDM4 mRNA (Figure 8). The role of miR34a as the sole factor responsible for reduction of MDM4 levels could not be established. However, these results suggest micro-RNA expression may play an important role in the regulation of MDM4 mRNA expression and thereby impact p53 activity (Markey 2008).
Figure 7: A) Alternative transcripts of hdmX are differentially regulated in response to increasing doses of the DNA-damaging agent cisplatin B) Absolute Q–PCR analysis of full-length hdmX and XAlt2 transcripts
It was previously shown that overexpression of miR-34a led to decrease in expression of a large number of mRNAs and HDMX/MDM4 was one among them (Chang 2007). Consistent with this report the 3'UTR of MDM4 was found to possess several potential miR-34a binding sites (Markey 2008). It was also shown that when MCF7 cells were transfected with anti-34a (inhibitor for miR-34a) and non-targeting control in presence and absence of doxorubicin treatment, significant increase in MDM4 expression was observed with inhibition of miR-34a in absence of doxorubicin. However in presence of doxorubicin efficient reduction of miR-34a could not be achieved. Hence the role of miR-34a as a sole factor responsible for reduction of MDM4 could not be ascertained. However miR-34a was found to have a potential role in this phenomenon (Figure 8) (Markey 2008).

Studies have shown that miR-34a is frequently silenced or downregulated in various types of cancer as it is located on a region on the 1p chromosome which is frequently lost in certain types of cancers (He 2011). Also MDM4 is shown to be overexpressed in certain cancers (Danovi 2004). Hence the next set of experiments sought to determine the relationship between miR-34a expression and MDM4 expression, their expression was analysed in a variety of cell lines of different origins and p53 status. H1299 (non-small cell lung carcinoma, p53 null) and SAOS2 (osteosarcoma, p53 null) expressed low levels of miR-34a. MCF7 (breast carcinoma, p53 wild type), U2OS (osteosarcoma, p53 wild type) and IMR90 (primary human fibroblasts) all expressed high basal levels of miR-34a which were further induced upon doxorubicin treatment only in MCF7. Hence in absence of DNA damage conditions the expression of miR-34a was consistent with p53 mediated transcription of miR-34a (Hermeking 2011). All cell lines except SAOS2 showed downregulation of MDM4 following doxorubicin treatment (Figure 9).
Figure 8: **Effect of inhibition of miR-34a in presence and absence of doxorubicin treatment.** MCF7 cells were transfected with the indicated antimicroRNA (Neg, negative control anti-miR; mi-34a, miR-34a) prior to DNA damage with 0.5mg/ml doxorubicin (+dox).

Figure 9: **Expression of miR-34a and MDM4 in cell lines of different origin and p53 status.** qRT-PCR analysis of miR34a and MDM4 expression. Error bars show 95% confidence intervals. Double asterisks indicate paired, one-tailed t-test values, <0.01 comparing the untreated to doxorubicin treated condition for each cell line.

The previous experiment showed that there is an inverse relationship between miR-34a expression and MDM4 expression in some of the cell lines viz H1299 and MCF7. In the cell line SAOS2 it was observed that even with an induction of miR-34a after doxorubicin treatment an increase in MDM4 expression was observed. Hence an absolute relationship between expression of miR-34a and MDM4 could not be established.

However to verify the observations in H1299 and MCF7 cell line we sought to determine the effect of overexpression and inhibition of miR-34a on MDM4 expression in comparison to the effect of miR-34a on two of its known targets. Cell cycle associated genes CDK6 and CCND1 were selected as known targets of miR-34a as it has been demonstrated with previous work that ectopic overexpression of miR-34a leads to reduction in mRNA and protein levels of CDK6 and CCND1 (Sun 2008).
Effect of overexpression of miR-34a on expression of MDM4 and known miR-34a targets CDK6 and CCND1.

MCF7 cells were transfected with miR-34a overexpression plasmid and a miR-34a mut plasmid as a control (this plasmid does not produce mature miR-34a). Following 24 hours of transfection cells were subjected to GFP expression analysis to determine transfection efficiency. This was followed with RNA isolation and qRT-PCR analysis for the expression of MDM4, CDK6 and CCND1. It was seen that in comparison with the miR-34a mut control the expression of MDM4 in response to miR-34a was reduced. A reduction in expression was also observed in case of the known targets CDK6 and CCND1 (Figure 10A). Having ascertained the effect of miR-34a overexpression on mRNA levels of MDM4 this was followed up with ascertaining effect of overexpression of miR-34a on protein levels of MDM4. MCF7 cells were transfected with miR-34a overexpression plasmid and a miR-34a mut plasmid as a control. Following 48 hours of transfection cells were subjected to protein extraction and western blot analysis using antibodies for MDM4, p53 and actin. As seen with the mRNA levels of MDM4 in comparison to the miR-34a mut MDM4 protein levels were seen to decrease in response to miR-34a overexpression (Figure 10B).
Figure 10: A) **Effect of overexpression of miR-34a on mRNA levels of MDM4 and known targets of miR-34a viz. CDK6 and CCND1.** B) **Effect of overexpression on protein levels of MDM4.** Asterisks and double asterisks indicate t-test values <0.05 and <0.01, respectively, comparing the miR-34a expression plasmid to the control mutant.

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**Effect of inhibition of endogenous miR-34a on expression of MDM4 and known miR-34a targets CDK6 and CCND1.**

To verify this effect of overexpression of miR-34a on MDM4, CDK6 and CCND1 expression we sought to see if inhibition of endogenous miR-34a levels in MCF7 could alleviate the repression imposed by miR-34a on these genes. MCF7 cells were selected as they express high levels of endogenous miR-34a and MDM4. MCF7 cells were transfected with anti-miR-34a. Following 24 hours of transfection cells were subjected to RNA isolation and qRT-PCR analysis for the expression of MDM4, CDK6 and CCND1. In comparison to the untransfected control there was an increase in expression of MDM4 in response to inhibition of endogenous miR-34a. An increase in expression of CDK6 and CCND1 was also observed (Figure 11A).

MCF7 cells were transfected with anti-miR-34a. Following 48 hours of transfection cells were subjected to protein extraction and western blot analysis. Untransfected MCF7 cells served as control. In comparison to untransfected control it was seen that inhibition of endogenous miR-34a also led to increase in the protein levels of MDM4 (Figure 11B).

As mentioned before, microRNAs affect expression of their target genes by binding via their seed sequences to complimentary or near complimentary sites in the 3'UTR. Hence a 1700bp region of the 3'UTR of MDM4 expected to contain miR-34a binding sites was cloned into the psicheck2 (Promega) vector upstream of the renilla luciferase between the sgf1 and not1 restriction endonuclease sites (Figure 12).

These plasmids were used for reporter assays to check for responsiveness of the 3'UTR to overexpression of miR-34a.
A)

![Bar chart showing relative gene expression for untreated and Anti-miR-34a samples.](image)

- Untreated miR-34a inhibitor
- MDM4
- CDK6
- CCND1

B)

- Untreated
- Anti-miR-34a

- MDM4
  - 1.00
  - 1.29

- p53

- Actin
Figure 11: **Analysis of MDM4, CDK6 and CCND1 after transfection with anti-miR-34a.** A) MCF7 cells were transfected with inhibitor to miR-34a (anti-miR-34a) at 80% confluence. Untransfected MCF7 cells served as control. 24 hours post transfection cells were subjected to RNA extraction by TRIZOL followed by RNA extraction. MDM4, CDK6 and CCND1 levels were analysed using qRT-PCR and normalized to GAPDH. Error bars represent the 95% confidence intervals resulting from quadruplicate assays for both miR-34a and miR213 expression. Asterisks and double asterisks indicate t-test values <0.05 and <0.01, respectively, comparing the anti-miR34a to the untreated controls.

B) MCF7 cells were transfected with inhibitor to miR-34a (anti-miR-34a) at 80% confluence. Untransfected MCF7 cells served as control. 48 hours post transfection cells were subjected to protein extraction and western blot analysis using antibodies for MDM4, p53 and actin.
Figure 12: Schematic of the psicheck2 vector with 1700bp region of the 3’ untranslated region (UTR) cloned between the NotI and SgfI sites.

The psicheck 2 plasmid is routinely used for RNA interference studies. The plasmid has the genes for both the reporters: hRluc (Renilla luciferase experimental reporter) and hluc (Firefly luciferase control reporter). The plasmid also possesses an ampicillin resistance marker.
Overexpression of miR-34a in both MCF7 and H1299 cell lines was confirmed. Both the cell lines were transfected with miR-34a or miR-34a mut. 24 hours post transfection cells were subjected to RNA extraction followed by microRNA extraction. The expression of miR34a was quantitated by qRT-PCR. As compared to miR-34amut we could achieve high levels of expression of miR-34a in both the cell lines. The increase in H1299 which expresses low levels of endogenous miR-34a levels was 250 fold as compared to a 50 fold increase in MCF7 cells which already express high levels of endogenous miR-34a (Figure 13A and 13B).

With both the cell lines being able to express increased levels of miR-34a after transfection of miR-34a overexpression plasmid, these were transfected with psicheck2 containing the 1700 bp region of the 3’UTR of MDM4 (psicheck2-MDM4) and cotransfected with miR-34a and miR-34amut. As a control, cells were transfected with empty psicheck2 plasmid and cotransfected with miR-34a and miR-34amut. 24 hours post transfection the cells were processed for obtaining cell lysates and the reporter assay was performed. It was observed that the response of the psicheck2-MDM4 reporter was identical to the response of empty psicheck 2 to both the miR-34a and miR-34a mut. Indicating that though predicted to contain miR-34a binding sites the 3’UTR of MDM4 was unresponsive to miR-34a mediated repression (Figure 14A and 14B).
A) MCF7

B) H1299
Figure 13: qRT-PCR analysis of miR-34a after transfection with miR-34a overexpression plasmid. A) qRT-PCR of miR-34a overexpression in MCF7 cells. Mandke et al (2012) MicroRNA-34a Modulates MDM4 Expression via a Target Site in the Open Reading Frame, *PLoS One*, 7

B) H1299 cells were transfected with miR-34a overexpression plasmid at 80% confluence. H1299 cells transfected with miR-34a mut served as control. 24 hours post transfection cells were subjected to RNA extraction by TRIZOL followed by microRNA extraction. miR-34a levels were analysed using qRT-PCR and normalized to miR-213 levels. Error bars represent the 95% confidence intervals resulting from quadruplicate assays for both miR-34a and miR213 expression.

Double and triple asterisks indicate paired, one-tailed t-test values <0.01 and <0.001 respectively between control and experimental conditions.
Figure 14: **Effect of miR-34a overexpression on psicheck2-MDM4 (1700 bp 3'UTR reporter of MDM4)** in A) MCF7 cells B) H1299 cells

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Hence the 3’UTR region with potential miR-34a binding sites was not responding to miR-34a mediated downregulation.

Reassessment of the MDM4 was performed using the August 2010 release of the miRANDA software an improved version of the miRanda software. This model predicts microRNA target mRNAs based on the likelihood of downregulation by providing a mirSVR score. The model is based on information extracted from miRANDA predicted target sites. The use of this model enables prediction of several noncanonical or non-conserved microRNA target sites. The mirSVR score is interpreted as more negative the value better is the downregulation indicating a strong regulatory site.

This software did not identify any of the previous sites identified to be potential miR-34a binding sites in the 3’UTR however a strong candidate site was identified in the protein coding exon 11 of the MDM4 gene (Figure 15). As seen from the schematic the model predicts several miR-34a sites in the 3’UTR however these have a score >-0.05 indicating poor regulatory sites. However the site in the exon 11 coding region has a mirSVR score of -1.286 making this a potential strong miR-34a regulatory site (microRNA.org August 2010 release).
Figure 15: **schematic of the exon 11 coding region and 3’UTR of the MDM4 gene with probable miR-34a regulatory sites (inverted triangles).** Values indicate the mirSVR score of the respective sites.

(microRNA.org August 2010 release)

(Schematic not to scale)
Cloning of the miR-34a site in the exon 11 in the psicheck 2 vector

To determine if the new miR-34a binding site predicted in the exon 11 of MDM4 is a functional site, a reporter was constructed with this region cloned downstream of a luciferase gene in a vector called the psicheck2 vector.

BAC 433N15 was used as template to PCR amplify the predicted site. To begin with, Platinum Taq DNA polymerase was used to amplify the region expected to be approximately 120bp region in length. However, a 1% agarose gel analysis did not reveal the presence of any amplified product. Indicating this PCR had not worked. Hence another PCR was set up using RT2 SYBr green PCR master mix with BAC DNA as template and the specific primers. On a 1% agarose gel a product was obtained approximately of the size corresponding to the size of expected region. Hence RT2 SYBR green PCR master mix was used for the consequent experiments.

Since this could be the region of interest, a digestion reaction of the insert region in the PCR product and the psicheck2 vector with NotI and SgfI restriction endonucleases was set up, followed by ligation and transformation of bacterial cells. After overnight incubation at 37°C no colonies were obtained on LB+Amp plates.

We tried to address the problem by verifying the digestion of the insert, purifying the insert PCR product by ethanol precipitation and the ligation of insert to the vector. However, we were unable to get clones of bacterial cells harboring the plasmid with the 120bp region. One of the possible difficulties was the inability to probably get a pure 120bp product. This could have resulted in the problem associated with the ligation of the insert to the vector.

Having tried to address the cloning of this 120bp region we decided to use primers for a longer region of the exon 11 of MDM4 still containing the potential miR-34a binding site.
The new primers would give a product approximately 444bp long. For this purpose we again used BAC DNA as template DNA, primers for 444bp region and RT2 SYBr green PCR master mix and same PCR conditions as before, ethanol precipitation to purify the product, followed by digestion of amplified insert and vector by NotI and SgfI, ligation and transformation. On obtaining colonies on the LB+Amp plate after 24 hours incubation at 37°C; 6 single colonies were randomly selected and inoculated in LB+Amp broth and incubated overnight on a shaker incubator at 37°C. The liquid culture if turned was processed for plasmid extraction. 2 colonies grew in the overnight culture.

One of the methods to test the presence of the region of interest in the psicheck2 vector was digestion with restriction endonuclease EcoRI. The plasmid map indicates the presence of two EcoRI sites (Figure 16A). One between the two restriction sites NotI and SgfI and the other after NotI site. Hence digestion of psicheck 2 + insert upon with EcoRI should give a single product (~6500bp) corresponding to digestion of the site after NotI and inactivation of the site between NotI and SgfI due to presence of the insert. In case of absence of the insert the digestion would yield two products (732bp and 5541bp).

The plasmid extracted from these two colonies was digested with EcoRI (37°C for 60 minutes). The bands observed after separation on a 1% agarose gel did not reveal the expected pattern (Figure 16B). In case of the psicheck2+insert plasmid digestion with EcoRI gave a single product as expected but of a comparatively smaller size than expected. In case of the empty vector the digestion yielded a larger product of ~4000bp (smaller than the expected 5541 bp) and a smaller product corresponding to 732bp was not observed. Hence, due to the absence of a smaller product we could not conclusively ascertain the presence of insert in the psicheck2+insert plasmid.
Figure 16: **Elucidating presence of the insert by EcoRI mediated restriction digestion.** A) Schematic of psicheck 2 vector with 2 EcoRI and single NotI and AsisI (Sgfl) site.

B) Empty psicheck2 vector and 2 plasmids suspected to contain the insert were digested with EcoRI (incubation at 37°C for 60 mins). The products after digestion were analysed on a 1% agarose gel.
Since the pattern of digestion of the empty psicheck2 with EcoRI did not match the expected sizes, we sought to determine the quality of the psicheck2 vector by subjecting it to digestion by 6 endonucleases (BamHI, EcoRI, NotI, NcoI, SgfI and XhoI). Psicheck2 plasmid was incubated with the mentioned endonucleases for 60 minutes at 37°C. The final product was run on a 1.2% agarose gel. The expected product sizes were (BamHI= 6273bp, EcoRI=5541bp and 732bp, NcoI=3758bp, 1375bp and 463bp, NotI, SgfI, XhoI=6273bp,). We did obtain the products of expected sizes for all the restriction endonucleases except for SgfI (Figure 17). Hence from the pattern of fragments obtained from the restriction digestion it could be said that the psicheck2 vector itself was suitable for use. However since SgfI is one of the restriction sites used to clone the region of interest in the psicheck 2 and the digestion mediated by it did not match the expected fragment size the digestion with SgfI had to be addressed.

To address this problem, psicheck2 digestions with new AsisI were set up with different quantities of the enzyme itself (1µl and 2µl) and NEB3 and NEB4 buffer. The enzyme is known to have 100% activity with the use of both NEB3 and NEB4 buffers however the suitability of each had to be determined for our experimental conditions. Amongst this the combination of 2ul enzyme with NEBB4 gave the expected result of a single product indicating efficient digestion instead of a smear or range of products as seen with the other combinations. This combination was used for the further experiment (Figure 18).
Figure 17: **Restriction digestion of Psicheck 2 vector.** The psicheck2 vector was digested with 6 restriction endonucleases BamHI, EcoRI, NcoI, NotI, XhoI and SgfI (incubation at 37°C for 60 minutes). The products obtained after digestion were analysed on a 1.2% agarose gel. The sizes of the fragments obtained were compared with documented sizes.
Figure 18: **Restriction digestion of psicheck 2 with restriction endonuclease SgfI.** Psicheck2 was digested with different combinations of amount of SgfI and NEB4 buffers. The combinations considered were 1µl and 2µl of SgfI with NEB3 buffer and 1µl and 2µl of SgfI with NEB4 buffer (incubated at 37°C for 60 minutes). The products after digestion were analysed on 1% agarose gel.
We went back to the BAC 14532 and PCR amplified the 444bp region using the new primers, digestion of the amplified insert region and vector was carried out using modified conditions (2µl of SgfI and NEB4 buffer), followed by ligation and transformation of bacterial cells. Several colonies were obtained on the LB+Amp plate after overnight incubation at 37°C. Out of 6 random colonies inoculated in LB+Amp broth one could be processed further for plasmid extraction. This plasmid was further set up for EcoRI digestion to verify the presence of insert along with digestion of psicheck2 as control. Following EcoRI digestion; psicheck 2 and psicheck2+insert gave a larger product corresponding to ~6000bp. However, the smaller product (732bp) expected to see with the digestion of psicheck2 was not observed (Figure 19).

Since EcoRI digestion was inconclusive we sought to verify the presence of the insert by PCR amplification of the insert from the psicheck2+insert using the same primers used to amplify the insert sequence from the BAC. HCT116 was used as a positive control, psicheck2 (negative control) and psicheck2 + insert was the test. Both for the test and HCT116 a ~450 bp product was observed on a 1.4%agarose gel which was absent in the empty psicheck2. Hence it could be said the psicheck2+ insert could potentially have the region of interest (Figure 20A).

This plasmid was further sent for sequencing to verify the presence of the insert.

The results of the sequencing revealed the successful cloning of the ~444bp region of the exon 11 into the psicheck2 reporter (henceforth referred to as Exon11) (Figure 20B).
Figure 19: **EcoRI digestion of plasmids from one clone to ascertain presence of 444bp insert.** Empty psicheck 2 vector and psicheck2+ 444bp insert suspected to contain insert was digested with EcoRI (incubation at 37°C for 60 minutes). The products after digestion were observed on a 1.2% agarose gel.
Figure 20: **PCR to verify presence of 444bp insert in one of the engineered psicheck2 vectors.** A) PCR was set up using water (negative control), cDNA from HCT116 (positive control) and plasmid suspected to contain the 444bp insert. Primers specific for 444bp region and RT2 SYBr green PCR master mix was used to perform the PCR. B) Schematic of the psicheck2 with the 444bp region of interest between the NotI and SgfI restriction sites.
**Reporter assays**

The successful cloning of the miR-34a site from the exon 11 of MDM4 in the psicheck2 vector was followed by reporter assays to test the functionality of the miR-34a site.

**Effect of inhibition of endogenous miR-34a on exon11 reporter activity:**

Firstly we sought to determine the effect of inhibition of endogenous miR-34a on the reporter activity. If the miR-34a site found to be strong candidate site for regulation was indeed a functional site then inhibition of endogenous miR-34a by the anti-miR-34a would be able to relieve the repression imposed by presence of miR-34a. MiR-34a is expected to bind this site in the exon11 and bring about downregulation of MDM4 expression.

For this the MCF7 cell line was selected as it possesses high level of endogenous miR-34a and MDM4 expression. The expression of endogenous miR-34a was knocked down with the use of an antisense oligonucleotide (Ambion) an inhibitor of miR-34a.

To verify the inhibition of endogenous miR-34a MCF7 cells were transfected with anti-miR-34a. Untransfected cells served as a control. 24 hours after transfection MCF7 cells were subjected to microRNA extraction, reverse transcription to get cDNA and qRT-PCR analysis to check for miR-34a expression (Figure 21).

Transfection with the inhibitor was able to bring about a 80% reduction in endogenous mir-34a expression as compared to the untransfected control. This validated the use of the inhibitor for the reporter assays.
Figure 21: **qRT-PCR analysis of miR-34a after transfection with anti-miR-34a.** MCF7 cells were transfected with inhibitor to miR-34a (anti-miR-34a) at 80% confluence. Untransfected MCF7 cells served as control. 24 hours post transfection cells were subjected to RNA extraction by TRIZOL followed by microRNA extraction. miR-34a levels were analysed using qRT-PCR and normalized to miR-213 levels. Error bars represent the 95% confidence intervals resulting from quadruplicate assays for both miR-34a and miR213 expression. Double asterisks indicate t-test values <0.01, comparing the anti-miR-34a to the untreated control.
For this MCF7 cells were transfected with anti-miR-34a and co transfected with either empty psicheck2 vector or exon11 plasmid. The control cells were transfected with just the empty psicheck2 or exon11 plasmid. The plasmid psicheck2-AS34a contains a consensus response site for miR-34a, and was used as a positive control for response to miR-34a inhibition. 24 hours post transfection cell were processed for obtaining the lysates. These lysates were used for performing the Dual luciferase reporter assay (Promega).

It was observed in response to inhibition of endogenous miR-34a the activity of the exon11 reporter was found to increase in comparison with the empty vector under the same conditions and also when compared to the untransfected controls. The increase was also comparable to increase observed for the reporter with the consensus miR-34a response site (Figure 22 A, B).

**Effect of overexpression of miR-34a on exon11 reporter activity.**

Next we sought to determine the effect of overexpression miR-34a on the reporter activity. If the miR-34a site in the exon11 of MDM4 found to be strong candidate site for regulation was indeed a functional site then overexpression of miR-34a using miR-34a overexpression plasmids should be able to repress the expression of the reporter. MiR-34a is expected to bind this site in the exon11 and bring about downregulation of MDM4 expression.

For this the H1299 cell line was selected as it expresses low levels of endogenous mir-34a and MDM4. The overexpression of miR-34a was achieved by using miR-34a overexpression plasmids.
Figure 22: **Effect of inhibition of endogenous miR-34a on expression of exon11 reporter.** A) MCF7 cells were transfected with anti-miR-34a and cotransfected with either empty psicheck2 or exon11. Control cells were transfected with empty psicheck2 or exon11. 24 hours post transfection cells were processed for obtaining lysates and relative luciferase activity was measured. Error bars represent results from six independent experiments. B) Similar experiment was performed using plasmid containing a control site for miR-34a known to respond to miR-34a. Asterisks and triple asterisk indicate t-test values <0.05 and <0.001 respectively.
To verify the overexpression of miR-34a H1299 cells were transfected with miR-34a overexpression plasmids. H1299 cells transfected with miR-34amut served as the control. 24 hours after transfection H1299 cells were subjected to microRNA extraction, reverse transcription to get cDNA and qRT-PCR analysis to check for miR-34a expression (Figure 23).

Transfection with the overexpression plasmid was able to bring about a 250 fold increase in endogenous mir-34a expression as compared to the H1299 cells transfected with miR-34amut plasmid. This indicated robust induction of miR-34a expression in H1299 cells validating the use of this system for the reporter assay.

For this H1299 cells were transfected with miR-34a and cotransfected with empty psicheck2 vector or exon11. The control cells were transfected with miR-34a mut and cotransfected with psicheck2 or exon11 plasmid. The plasmid psicheck2-AS34a contains a consensus response site for miR-34a, and was used as a positive control for response to miR-34a overexpression. 24 hours post transfection cell were processed for obtaining the lysates. These lysates were used for performing the reporter assay.

It was observed in response to overexpression of miR-34a the activity of the exon11 reporter was found to decrease in comparison with the empty vector under the same conditions and also when compared to the H1299 cells transfected with miR-34amut. The decrease was also comparable to decrease observed for the reporter with the consensus miR-34a response site (Figure 24 A, B).

From both the experiments it was seen that the reporter with the new miR-34a site from the exon11 was responding to manipulation of endogenous miR-34a in the different cell lines; indicating it is indeed a functional regulatory site.
Figure 23: qRT-PCR analysis of miR-34a after transfection with miR-34a overexpression plasmid. H1299 cells were transfected with miR-34a overexpression plasmid at 80% confluence. H1299 cells transfected with miR-34amut served as control. 24 hours post transfection cells were subjected to RNA extraction by TRIZOL followed by microRNA extraction. miR-34a levels were analysed using qRT-PCR and normalized to miR-213 levels. Error bars represent the 95% confidence intervals resulting from quadruplicate assays for both miR-34a and miR213 expression. Triple asterisks indicate p-value <0.001.
A) H1299

![Graph showing relative luciferase activity for psicheck2 and Exon 11 under miR-34a-mut and miR-34a conditions.]

B) [Graph showing relative luciferase activity for psicheck2 and psi-A$\phi$34a under miR-34a-mut and miR-34a conditions.]

- miR-34a-mut
- miR-34a
Figure 24: **Effect of overexpression miR-34a on expression of exon11 reporter.** A) H1299 cells were transfected with miR-34a and cotransfected with either empty psicheck2 or exon11. Control cells were transfected with miR-34amut and cotransfected with empty psicheck2 or exon11. 24 hours post transfection cells were processed for obtaining lysates and relative luciferase activity was measured. Error bars represent results from six independent experiments. Double asterix indicate p value <0.01.

B) Similar experiment was performed using plasmid containing a control site for miR-34a known to respond to miR-34a regulation.
Site directed Mutagenesis to create point mutants corresponding to the SNP found in the miR-34a site in exon 11.

It was shown that the miR-34a seed region in the exon11 is also a site for a human single nucleotide polymorphism (SNP) (The 1000 Genome Project Consortium 2010). This SNP is an A to C transversion, the presence of which we predict would render this miR-34a site unresponsive to miR-34a mediated regulation (Figure 25A) is found at the frequency of 21.6% in the global population. Against this background all our cell lines were assessed for the presence of this SNP. However none of the cells were seen to possess this C allele instead they had the A allele.

However we set out to ascertain the effect of this SNP on miR-34a mediated regulation of MDM4. Point mutations corresponding to the A to C transversion were created by site directed mutagenesis using specific primers. 4 colonies from the colonies obtained on LB+ Amp after overnight incubation at 37°C were inoculated in LB+Amp broth. After overnight incubation at 37°C on a shaker, the broth was processed for plasmid extraction.

Digestion with BtsI was employed to test for presence of the A to C transversion in the miR-34a site. The exon 11 reporter plasmid harbors four sites for the BtsI which upon digestion is expected to produce two major products and 2 minor products indicating no loss of the BtsI sites. However successful mutation of A to C is expected to lead to loss of one of the BtsI site in the miR-34a seed region hence digestion with Bts1 is now expected to give one major product and 2 minor products (Figure 25B).

A digestion reaction of the newly obtained plasmids expected to harbor the mutation and the original exon11 plasmid with the restriction endonuclease BtsI was set up by incubation at 55°C for 60 minutes. BtsI is a very sensitive enzyme hence the BtsI was diluted in NEB4 buffer as the glycerol present in enzyme mix itself may prevent specific
cutting of the plasmid. The reaction was set using 1 µg of plasmid DNA with 1 unit of BtsI and 1:10 diluted BSA (1 µg/µl) added at 1 µl per 10 µl of total reaction volume. This managed to solve the problem as we obtained the expected products for both the exon11 (2 major products at the expected size range) and the suspected mutants (single product at ~6000bp) (Figure 25C). Hence one of them was used for the further reporter assays.

** Reporter assay to check for responsiveness of exon11 mutant reporter (exon 11 A>C) to miR-34a regulation. **

For this MCF7 cells were transfected with anti-miR-34a and co transfected with either empty psicheck2 vector or exon11 A>C plasmid. The control cells were transfected with just the empty psicheck2 or exon11 A>C plasmid. 24 hours post transfection cell were processed for obtaining the lysates. These lysates were used for performing the reporter assay (Figure 26A).

Similarly H1299 cells were transfected with miR-34a and cotransfected with empty psicheck2 vector or exon11 A>C. The control cells were transfected with miR-34a mut and cotransfected with psicheck2 or exon11 plasmid A>C. 24 hours post transfection cell were processed for obtaining the lysates. These lysates were used for performing the reporter assay (Figure 26B).

It was seen in both the cell lines the mutant reporter showed similar activity as compared to the empty vector. It was also seen that the mutant reporter showed similar activity in response to anti-miR-34a treated or untreated conditions in MCF7 cell line and miR-34a overexpression or miR-34a mut expression in H1299. Hence it was seen with the presence of A>C transversion responsiveness to miR-34a was indeed abolished.
A) 5’ cuccaCGUCUGAUACACUGCCa 3’ MDM4
5’ cuccaCGUCUGAUACCCUGCCa 3’ MDM4
3’ uguugGUCGAGUUCUGUGACGGu 5’ hsa-miR-34a

B) Digestion with BtsI- 2 major products
Pscheck2 + 444bp insert
Pscheck2 + 444bp with A>C transversion

Digestion with BtsI- 1 major product
One site lost due to A>C transversion

C)
Figure 25: **Site directed mutagenesis** A) Schematic of the miR-34a seed region in exon11 in presence and absence of the A to C tranversion.

B) Plasmid map showing restriction digestion sites for psicheck2+444bp and psicheck2+444bp with A>C transversion.

C) Digestion of the plasmids with BtsI by using extremely specific digestion conditions. The reaction was set using 1µg of plasmid DNA with 1 unit of BtsI and 1:10 diluted BSA added at 1 µl per 10 µl of total reaction volume. All the products were analysed on a 1% agarose gel.
A) 

MCF7

![Graph showing relative luciferase activity for MCF7 cells with pscheck2 and Exon 11 A>C with and without anti-miR treatment.]

B) 

H1299

![Graph showing relative luciferase activity for H1299 cells with pscheck2 and Exon 11 A>C with and without miR-34a-mut and miR-34a treatment.]
Figure 26: **Effect of miR-34a on exon 11 A>C reporter.** A) Effect of inhibition of endogenous miR-34a on expression of exon11 A>C reporter. MCF7 cells were transfected with anti-miR-34a and cotransfected with either empty psicheck2 or exon11 A>C. Control cells were transfected with empty psicheck2 or exon11 A>C. 24 hours post transfection cells were processed for obtaining lysates and relative luciferase activity was measured. Error bars represent results from six independent experiments.

B) Effect of overexpression miR-34a on expression of exon11 A>C reporter. H1299 cells were transfected with miR-34a and cotransfected with either empty psicheck2 or exon11 A>C. Control cells were transfected with miR-34amut and cotransfected with empty psicheck2 or exon11 A>C. 24 hours post transfection cells were processed for obtaining lysates and relative luciferase activity was measured. Error bars represent results from six independent experiments.

It is known that the activity of p53 is kept under check by two of its important negative regulators MDM2 and MDM4 (HDMX). An interesting negative feedback loop exists between p53 and MDM2 where p53 activates MDM2 and MDM2 in turn inhibits the activity of p53 either by inhibition of transcription of p53 or by ubiquitin mediated proteasomal degradation of p53. Hence MDM2 is known to be activated by p53 but no such report was available for MDM4. However, recently it was shown that HDMX might contain a potential p53 inducible promoter P2 in its first intron (Figure 27A). It was shown that in response to DNA damage agent leading to p53 activation production of a novel HDMX-L transcript from the P2 promoter is observed. It was also shown that this mRNA transcript is more efficiently translated than the transcript from the constitutive P1 promoter and codes for a longer form of the protein (Philips 2010).

It was also shown that 10 µM cisplatin treatments of ovarian cancer cells led to an increase in transcription from the HDMX-P2 promoter over a period of 24 hours and in comparison to transcription from the HDMX constitutive promoter (Figure 27B).
Figure 27: **Schematic of the HDMX promoter region and the effect of cisplatin on transcription from these promoters**

A) A novel promoter in intron 1 of *HDMX* contains a functional p53 binding site. A map of the 5′-end of the *HDMX* gene, showing the position of the novel exon 1β. A potential p53-binding site in intron 1 is compared with the consensus p53-binding sequence. *Inverted triangles* show the known translation start site in exon 2 and an in-frame ATG in exon 1β, initiation of translation from which would incorporate 18 additional amino acids at the N terminus of HDMX.

B) Effect of 10 μM cisplatin on transcription from HDMX-P2: OAW-42 ovarian cancer cells that express wild-type p53 were exposed to 10 μM cisplatin for the indicated times before being prepared for analysis by quantitative RT-PCR.

We sought to analyse the presence of this new transcript from the promoter P2 by quantitative RT-PCR using the forward primer 5’-TGTTTCAGCCTTCACCTGAG-3’ and reverse primer 5’-AGATCCTGCAAGCAGCTGTCA-3’ specific for the HDMX-P2 transcript. MCF7 cells were treated with 10µM cisplatin and untreated MCF7 cells were used as control. 24 hours post treatment RNA was extracted, reverse transcribed and qRT-PCR analysis was performed.

From the analysis of the amplification plot which plots fluorescence vs cycle number it could be seen that low quantity of transcript from exon 1β (HDMX-P2) was present in the untreated MCF7 cells as higher the cycle number lower is the initial amount of mRNA transcript present. However there is no increase the production of transcript from exon 1β (HDMX-P2) in the presence of 10 µM cisplatin (DNA damage agent which would lead to p53 activation). Hence we could not verify the report on role of p53 in the activation of transcription from the alternative promoter P2 in the intron of the HDMX gene (Figure 28).
Figure 28: Amplification plot for Exon 1β and GAPDH in absence and presence 10 μM cisplatin. MCF7 cells were treated with 10μM cisplatin. 24 hours after treatment RNA was extracted, reverse transcribed and qRT-PCR analysis was performed in quadruplicates.
IV. DISCUSSION

It is known that p53 is mutated or deleted in 50% of cancers contributing toward tumor development. However, in cancers retaining the wild type p53 it was realized there were certain other factors responsible for mediating tumor formation. Against this background the critical negative regulators of p53 viz. MDM2 and MDM4 gained attention. It was found that these two proteins were overexpressed in many cancers retaining wild type p53. MDM4 in fact is overexpressed in several types of cancers that retained wild-type p53 including gliomas, a number of pre-B acute lymphoblastic leukemias, tumor cell lines, and some primary tumors including breast tumors, head and neck squamous cell carcinomas, and retinoblastomas (Gilkes 2008).

In normal unstressed cells MDM4 and MDM2 keep the activity of p53 in check. However, under conditions of stress these two proteins undergo modifications that play a role in relieving the repression off p53 (Shafdan 2012). Hence the inhibition of expression of these negative regulators is considered essential for reactivation of p53.

Initially it was thought that with constitutive transcription little control was imposed on transcription of MDM4 (maybe through transcription factor binding). There were also reports of an alternative form of MDM4 being transcribed from a novel p53 responsive promoter in the first intron of MDM4 gene. However, we were unable to verify the report of production of this alternative form of MDM4 (Figure 28). Post transcriptional regulation was also not extensively looked into as a possible mechanism for regulation of MDM4 expression. With previous studies it was shown that genotoxic stress led to a
decrease in full length MDM4 transcripts (Markey 2008) Alternative splice variants of MDM4 in response to genotoxic stress were also reported (Chandler 2006) but their production could not completely compensate for the decrease in full length MDM4 that was observed (Figure 7). Having explored several factors to address this downregulation there was preliminary evidence of possible role of microRNAs in regulation of MDM4 expression. The microRNAs as small non coding RNA molecules involved in the post transcriptional regulation of gene expression have received attention in the recent years (Bartel 2004). In case of the tumor suppressor p53 the miR-34 family is considered important as the members of this family are direct transcriptional targets of p53 and aid the tumor suppressive function of p53 (Hermeking 2010).

There was evidence of inverse correlation between MDM4 levels and miR-34a levels in MCF7 cells (Figure 8). Computational analysis also showed presence of several miR-34a binding sites in the 3’UTR. However the mechanism for the down regulation had not been determined.

We wanted to analyse the potential role of miR-34a in regulation of MDM4. On analysis of cells of different origins and varying p53 status it was seen that upon DNA damage viz. treatment with doxorubicin miR-34a was induced in H1299 and MCF7 accompanied by a decrease in MDM4 expression(Figure 9). IMR90 and U2OS expressed high levels of miR-34a and though there was no induction of miR-34a there was decrease in MDM4 expression in these two cells following damage indicating role of alternative mechanisms in control of MDM4 expression. In case of SAOS2 where induction of miR-34a upon damage was accompanied by increase in MDM4 expression we speculate involvement of other factors in regulation of expression. The presence of a human single nucleotide polymorphism which is expected to render MDM4 unresponsive to miR-34a regulation was checked however this SNP was absent in the SAOS2 cell line. It could also be possible
that this cell line maybe incompetent for microRNA mediated regulation. Hence, it was seen that depending on the cell line different mechanisms may impact MDM4 expression, regulation by miR-34a being one of them (Figure 9).

The effect of miR-34a on MDM4 was verified by comparing the response of MDM4 and CDK6 and CCND1 (known targets of miR-34a) to downregulated or elevated levels of miR-34a. It was seen that both in case of overexpression of miR-34a or inhibition of miR-34a the response of MDM4 was comparable to the response of CDK6 and CCND1 (Figure 10A, 11A). This impact was also biologically significant as seen from the downregulation of protein levels of MDM4 in response to elevated levels of miR-34a or increase in protein levels in response to inhibition of endogenous miR-34a. Since microRNAs are responsible for subtle changes in gene expression we could observe only a modest impact on MDM4 protein expression (Figure 10B, 11B). Hence it could be seen that miR-34a led to modulation of MDM4 expression leading to differences in the protein expression. This indicated that mir-34a may be directly involved in regulation of MDM4.

However the 3′UTR of MDM4 which was seen to harbor potential miR-34a binding sites was unresponsive to miR-34a regulation as seen with the reporter assays performed using 1700bp region of the 3′UTR cloned in a reporter plasmid (Figure 14). Since there was an effect on MDM4 expression in presence of miR-34a and to address the possibility of indirect effect of miR-34a on MDM4 expression instead of direct binding a reassessment of MDM4 mRNA was performed using August 2010 release of MiRanda. It predicted a miR-34a regulatory site in the protein coding exon 11 of MDM4. It was possible that the older software was not able to detect this site as it is not programmed include sites in the coding region but only the ones in 3′UTR. Experimental
evidence has accumulated showing that microRNA (miRNA) binding sites within protein coding sequences (CDSs) are functional in controlling gene expression viz miR29 and miR-15 differentially expressed in postnatal aortic development downregulate elastin via 3’ UTR and coding-sequence binding sites (Ott 2011).

This has prompted researchers to develop algorithms that include both coding region sequences and 3’UTR sequences resulting in higher sensitivity of target site prediction. As seen with a larger scale study which showed that more than a thousand genes expected to be microRNA targets did not contain microRNA binding sites in the 3’UTR and these were thus not recognized by existing miRNA target prediction programs. (Reckzo 2012).

This new site found in the exon 11 of MDM4 was further cloned into a reporter vector and used to test its functionality (Figure 20). Reporter assays under similar conditions of overexpression and inhibition of miR-34a led to modulation of reporter activity verifying a direct role of miR-34a in regulating expression of MDM4 via binding of miR-34a to this new site in the exon 11 (Figure 22, 24).

This MDM4 site is of the most highly conserved type, 8mer-Al with miRSVR score -1.286 which places this site in the top 2.5% of the predicted sites (Betel 2010). The predicted region was also seen to match the seed sequences of CDK6 and CCND1 (Sun 2008). It was also seen that the structurally homologous MDM2 possesses a different codon in the same region therefore MDM2 is expected to be unresponsive to miR-34a regulation.

The mir-34a site in exon 11 is also a site for a human single nucleotide polymorphism (SNP) which causes an A to C transversion. It is suspected that the presence of this point mutation would disrupt the miR-34a seed region and thereby render it unresponsive to
miR-34a regulation. We confirmed this with reporter assays involving the use of plasmids with the point mutant (Figure 26). All our cell lines were also screened for the presence of this allele however they were found to possess the majority A allele. However the presence of the SNP would need consideration and screening in case of cell lines unresponsive to miR-34a regulation. This would also play an important role in therapeutic intervention by miR-34a as a subset of population might be unresponsive to miR-34a owing to the presence of this SNP. It was also reported that microRNA target sites in coding regions require a perfect binding along the miRNA seed region and mismatches disrupt their functionality (Reckzo 2012).

**Significance of microRNA regulatory sites in the coding region?**

There is increasing evidence of microRNA target sites in the coding region which are seen to play a role in controlling gene expression (Reckzo 2012). Hence it is worth focusing on the significance of this phenomenon observed.

There are reports of a positional bias in the location of these target sites in the coding region with these sites located at the end of the coding region and the beginning of the 3’UTR. These sites towards the very 3’ end of the transcript are more likely to be functional and evolutionarily conserved (Forman 2010).

From the point of view of evolution the sites in the 3’UTR have less selective pressure, as these sites can be created and mutated without affecting the functionality of an encoded protein. Many of these sites can be eventually be lost in the process of evolution (Lu 2008). For microRNA recognition sites in coding regions, mutations in the target site that lead to formation of a less efficient protein will be selected against and not find its
way in the population. This would lead to less likelyhood of variation in the microRNA target sites.

Another factor playing a role in localization of these sites in the coding region is the presence of polyadenylation signals. In case of presence of alternative polyadenylation signals there could be different lengths of 3’UTRs under different conditions which in some case could lead to elimination of microRNA regulatory sites (Selbach 2008). As opposed to this the sites in the coding region would be present irrespective of the presence of these alternative polyadenylation signals making it less vulnerable to change in cellular conditions (Lytle 2007). Making coding region microRNA target sites a more consistent form of regulatory mechanism.

Another level of regulation can be achieved through the splicing mechanism which could lead to inclusion or exclusion of these regulatory sites in the exon in a particular splice variant. This could potentially impose differential control on the various splice variants of a particular gene. For instance the exon 11 of MDM4 is retained in all of its known transcript variant making all the transcripts possible targets of miR-34a mediated regulation (Gu 2009).

There were also some reports indicating the influence of the length of the 3’UTR to the presence of microRNA binding sites in the coding region. In a study involving the use of DIANA-microT-CDS algorithm on comparing the length of the 3’UTR with coding region scores for microRNA binding it was see that genes with 3’UTR of less than 500nt length have a significantly higher CDS target score. This indicates that the length of the 3’UTR also creates a bias in the possible presence of these sites in the coding region (Reckzo 2012).
**Future studies**

Recent studies with MDM2 have shown that not only the primary amino acid sequence but also the length of C terminal tail present after the RING domain plays an important role in MDM2 activity (homodimerization and heterodimerization). The sequence of the C terminal tail is evolutionarily conserved. The sequence and length of the C terminal tail is also conserved in case of MDM4 protein. To see if there is possibility of translation into the 3’UTR which may alter the C terminal tail length and sequence thereby affecting its activity (Dolezelova 2012)

This work paves way for future directions of this project. The regulation of MDM4 mRNA was not due to microRNA binding sites in the 3’UTR. Based on existing reports the length of the 3’UTR needs assessment which will aid in identification of the exact role of 3’UTR of MDM4. To begin with we have started looking for the presence of 3’UTR variants by 3’Rapid amplification of cDNA ends (3’RACE). If there exist such variants we would want to identify their sequences and look for regulatory sites and signals which may aid understanding of translation of MDM4 protein.

The long term application of this study would in terms of potential clinical application to assess the efficacy of miR34a mediated downregulation of hdmx in combination with MDM2/HDM2 inhibitors directed towards reactivation of p53.

Of late, restoration of p53 function as a therapeutic approach has gained attention. Inhibition of MDM2 and MDM4 has been explored as a strategy for reactivation of p53 in tumors. Unfortunately, inhibition of MDM2 leads to activation of p53 not only in cancer cells but also normal cells leading to lethal side effects. However, it was shown that complete inhibition of MDM4 is relatively transient and a less toxic and hazardous strategy. Although it was seen that transient activity in absence of MDM4 may damage lymphoid organs and bone marrow its effect on the intestinal epithelium arguably a
critical tissue is very mild. It was seen in mouse studies that with intestinal integrity preserved all effects arising from activation of p53 in absence of MDM4 are reversible leading to increase in long term survival of mice. This was particularly seen to be effective in treatment of a lymphoma model (Garcia 2011).

There are two strategies that can be adopted in order to inhibit activity of MDM4 in cancer. First would be the use of small molecule inhibitor which would relieve the MDM4 dependent inhibition of p53 and help in restoration of p53 function. In the absence of optimal MDM4 antagonists, an alternative approach using existing MDM4 antagonists in combination with agents that sensitize cells to p53-dependent apoptosis would help in restoration of p53 (Wade 2009).

The second approach of using combination therapy is gaining attention. Although agents (e.g., Nutlin-3a) that disrupt MDM2-p53 interaction can inhibit tumor growth, they are less effective in cancer cells that express high levels of MDM4. There are reports of a benzofuroxan derivative (7-(4-methylpiperazin-1-yl)-4-nitro-1-oxido-2,1,3-benzoxadiazol-1-ium, NSC207895) that could inhibit MDM4 expression in cancer cells. Treatments of MCF-7 cells with this small-molecule MDM4 inhibitor activated p53, resulting in elevated expression of proapoptotic genes (e.g., PUMA, BAX, and PIG3). Importantly, this novel small-molecule p53 activator caused MCF-7 cells to undergo apoptosis, and acted additively with Nutlin-3a to activate p53 and decrease the viability of cancer cells (Wang 2010). In another study combined treatment with Nutlin and ABT-737 (a small molecule BH3 mimetic significantly increased apoptosis compared to either agent alone in cells with high levels of MDM4. This particular combination has also proven effective in primary isolates from AML patients (Wade 2009).
Recently it was also shown that SAH-p53-8 (small alpha helical peptide) which targets MDM4 in cells and blocks formation of the p53-MDM4 interaction, thereby restoring the p53 pathway (Bernal 2010) in combination with nutlin was a successful combination in sensitization of melanoma. Most melanomas have high levels of MDM4 and low levels of MDM2 and hence responded poorly to nutlin 3 treatments. However, these cells were sensitive to treatment with SAH-p53-8 indicating its effectiveness in induction of p53 in these cells. It was also seen that melanoma cells with high MDM4/MDM2 were sensitive to either SAH-p53-8 or nutlin-3 but even more sensitive to combination treatment (Bernal 2010). This validates the development of specific targeted therapy for MDM4 which in combination with MDM2 inhibitors can work towards effective reactivation of p53.

In terms of exploring the therapeutic potential of miR-34a several animal studies have indicated successful tumor volume reduction or induction of apoptosis with use of miR-34a. In a study involving use of mice with lung metastasized tumors a 50% reduction in tumor volume was observed upon intravenous delivery of miR-34a. In another study an increase in apoptosis was observed upon increased levels of miR-34a in a similar lung cancer model (Bader 2012). In a model of subcutaneous and orthotopic miaPACA pancreatic xenografts a systemic delivery of miR-34a was accompanied with reduction in tumor burden and increase in necrosis and apoptosis (Pramanik 2011). Similarly in xenograft models of non small cell lung cancer miR-34a administration led to increase in apoptosis and reduction in proliferation (Bader 2012). The success of miR-34a overexpression in animal models warrants its use to be tested for human studies.

One of the challenges for the transition of miR-34a based therapies from bench to bedside is the selection of an appropriate delivery system to ensure effective delivery of the therapeutic microRNA to the target tumors. The delivery technology with the best combination of efficacy, biodistribution, and safety was the NOV340 technology
SMARTICLES Marina Biotech, Bothell, WA; MirnaTherapeuticsInc., 2011), an ionizable liposome that forms a particle with a diameter of ~120 nm. The lipids and miRNA mimics are mixed under acidic conditions to facilitate efficient miRNA encapsulation and liposome formation. The pharmacology of the NOV340/miR-34a formulation was tested in an orthotopic model of hepatocellular carcinoma. It was seen that administration of this combination led to significant regression of existing tumors and prolonged their survival. The mice also appeared to be tumor free upon histologic examination (Bader 2012).

**Summary**

The results from the present study can be summarized as follows.

- A relationship between miR-34a expression and MDM4 expression was established.

- Interestingly, The 3’UTR of MDM4 was unresponsive.

- Analysis revealed the presence a functional miR-34a regulatory site in the exon 11 region of MDM4.

- However a subset of the population/cell lines may be unresponsive to miR-34a regulation due to the presence of the ‘C’ allele.

- This validates further work in the direction of exploring the potential of miR-34a as therapeutic for MDM4 inhibition in tumors overexpressing MDM4 and retaining wild type p53.

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