IDENTIFICATION AND CHARACTERIZATION OF MIR-34: A P53-REGULATED MICRORNA FAMILY

by David C Corney

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MicroRNAs (miRNAs) are a recently identified class of small non-coding RNAs that have been implicated in multiple developmental processes and disease states. While their biogenesis is relatively well understood, their transcriptional regulation is less well characterized and we therefore sought to elucidate the role of the transcription factor p53 in control of miRNAs. Through its function as a transcription factor, p53 regulates hundreds of genes involved in control over proliferation, apoptosis and senescence and mutation in the p53 pathway is identified in almost all human tumors, including epithelial ovarian cancer (EOC) which is thought to originate from the ovarian surface epithelium (OSE).

OSE cells were chosen as a model to identify p53-regulated microRNAs. Using a microarray approach, the miR-34 family of miRNAs was found to be downregulated 12-fold after Cre/*loxP*-mediated conditional *p53* inactivation in OSE in cell culture. Computational analysis identified evolutionarily conserved p53 recognition elements in the *mir-34b/c* promoter, which were deemed to be functional based upon DNA damage-induced increased miR-34b/c expression only in *p53* wild type cells. Functionally, miR-34b and miR-34c cooperated in reducing proliferation and adhesion-independent growth in soft agar when transfected into *p53*-null neoplastic OSE cells.

As a next step in characterization of miR-34 family, their potential involvement in human cancer was investigated. Expression analysis demonstrated that all three members of the miR-34 family are downregulated in human EOC and their reduced expression was correlated with *p53* mutation, promoter methylation and copy number variation. Importantly, reduced miR-34b/c expression was correlated with more advanced tumor stage. Consistent with reconstitution experiments in mouse cells, proliferation of human EOC cell line SKOV-3 was reduced after miR-34 reconstitution. Furthermore, cell invasion and motility in Matrigel chambers was also significantly reduced.

To gain a more complete understanding of the role of miR-34 family members *in vivo*, and to test the hypothesis that they function as tumor suppressors, conditional *mir-34b/c* knockout mice were generated. Characterization of these mice will elucidate the role of these miRNAs in development and in cancer initiation and progression.

BIOGRAPHICAL SKETCH

David Corney was born on April 10, 1983, in Bristol, United Kingdom. Having decided to pursue a career in the biomedical sciences from an early age, David obtained his Bachelor's degree in Applied Biological Sciences from the University of the West of England, Bristol, where in 2005 he graduated with First Class Honors. Due to the enjoyment and satisfaction obtained from performing research while an undergraduate, David decided that he wanted to continue performing research and towards this, set his sights on attaining an advanced degree. In the summer of 2005, David came to USA and started to pursue his Ph.D. degree at Cornell University, Ithaca, NY, where he joined the laboratory of Dr. Alexander Nikitin to study tumor suppressor genes in mouse models of human cancer.

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ARE	AU-rich element
BAC	Bacterial artificial chromosome
BCIP	5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt
BrdU	5-bromo-2-deoxyuridine
CLL	Chronic lymphocytic leukemia
DAPI	4',6-diamidino-2-phenylindole
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
EOC	Epithelial ovarian cancer
IRES	Internal ribosomal entry site
MEF	Mouse embryonic fibroblast
miRNA	MicroRNA
MSP	Methylation specific polymerase chain reaction
NBT	Nitro-blue tetrazolium chloride
NOS	Not otherwise specified
NSCLC	Non-small cell lung cancer
OIS	Oncogene-induced senescence
OSE	Ovarian surface epithelium
p53RE	p53-recognition element
PCR	Polymerase chain reaction
RISC	RNA-induced silencing complex
RT	Reverse transcription
RFLP	Restriction fragment length polymorphism

CHAPTER 1

INTRODUCTION*

1.1 Ovarian cancer etiology, pathogenesis and classification

It is predicted that over 21,000 women will be diagnosed with ovarian cancer and over 15,000 will die from the disease in the US during 2009 (Jemal et al., 2009). Like cancers at many other sites, 5-year survival is good if diagnosed at an early stage when the cancer is confined to the ovary. However, almost 70% of women are diagnosed at an advanced stage, at which point 5-year survival is 30%. Clearly, improved screening programs to detect epithelial ovarian cancer (EOC) at an early stage and better therapeutics to treat advanced disease are urgently required. Unfortunately, asymptomatic development in humans, combined with a scarcity of accurate animal models, has resulted in a marked lack of knowledge of the pathogenesis of ovarian cancer. However, risk factors include persistent ovulation, age and family history, the latter being largely attributed to carriers of hereditary *BRCA1* mutations, which may account for up to 10% of EOC (Risch et al., 2001).

EOC is the most prevalent type of ovarian cancer, far outnumbering the sex cord-stromal tumors and germ cell tumors. Based upon morphological criteria, EOCs are classified as serous, mucinous, endometrioid, clear cell, transitional cell, squamous cell, and mixed epithelial neoplasms, with serous being the most common subtype (Scully and Sobin, 1999). The etiology of EOC is poorly understood and although several risk factors have been

* Modified from Corney et al. (2008). Histology & Histopathology. 23 (9): 1161-1169

identified, their direct involvement remains largely unaddressed. The risk factor receiving widest attention is ovulation. Persistent ovulation was hypothesized to increase ovarian cancer incidence (Fathalla, 1971; Fathalla, 1972). Multiple studies have demonstrated that a reduction in ovulatory events by pregnancy and/or oral contraceptive decreases EOC risk (Riman et al., 2002; Risch et al., 1994; Risch et al., 1983; Titus-Ernstoff et al., 2001; Whittemore et al., 1992). The presumed origin of EOC is the ovarian surface epithelium (OSE), a single layer of cuboidal cells coating the ovary (Auersperg et al., 2001; Nikitin et al., 2004). Early in life, the OSE is generally smooth but with age becomes increasingly convoluted, resulting in an increase in number of inclusion cysts. Such cysts, which may form by pinching off of invaginations or by trapping of epithelial cells during postovulatory wound healing, may be a precursor lesion for progression to carcinoma. Advocates of the so-called incessant ovulation hypothesis argue that postovulatory wound healing by OSE proliferation may increase the frequency at which cysts and mutations arise. Yet, this hypothesis is relatively simplistic since the potentially mutagenic effects of reproductive hormones and acute inflammation are not taken into account (Bose, 2005; Bukulmez and Arici, 2000; Cramer and Welch, 1983; Fleming et al., 2006; Konishi, 2006; Mohle et al., 1985; Ness and Cottreau, 1999; Nikitin and Hamilton, 2005).

A recent study by Horiuchi et al. demonstrated that while almost 50% of tumors developed from pre-existing lesions, in the remaining patients no such pre-existing lesions had been present 12 months prior to diagnosis. Furthermore, cancers arising from pre-existing cysts were categorized as low-grade by pathological analysis, while those with no evidence of a precursor lesion were mostly of a high-grade serous carcinoma (Horiuchi et al., 2003).

This study gives significant weight to the hypothesis that high-grade serous carcinomas are not derived step-wise from low-grade tumors but instead arise *de novo* from the OSE (Shih Ie and Kurman, 2004).

1.2 Mutations in the p53 Pathway in Ovarian Cancer

Germline mutations in *BRCA1* and *BRCA2* tumor suppressor genes are the most common genetic alterations in hereditary ovarian carcinomas and can account for 10-15% of ovarian cancers (Risch et al., 2006; Risch et al., 2001), yet by far the most frequent inactivating mutation in sporadic serous EOC are in the p53 pathway. Defects in the p53 tumor suppressor pathway are present in over eighty percent of human cancers (Hahn and Weinberg, 2002; Sherr and McCormick, 2002) and have been associated with poor prognosis in ovarian carcinomas (Bali et al., 2004; Fujita et al., 1994; Hashiguchi et al., 2001; Salani et al., 2008; Tachibana et al., 2003)

Mutation of the *p53* gene at the locus 17p13.1 is the most common single genetic alteration in sporadic human serous EOC. The p53 protein contains four functional domains – a transcriptional activation domain, a tetramerization domain and two DNA binding domains. p53 protein binds a p53 recognition element (p53RE) commonly found in either promoter regions or first introns of its target genes to activate transcription (el-Deiry et al., 1992). However, in addition to transactivation properties, transcriptional repression has been described, although binding sites are less well characterized (Curtin and Spinella, 2005; D'Souza et al., 2001; Hammond and Giaccia, 2005; Hoffman et al., 2002; Imbriano et al., 2005).

Loss of wild type p53 function by inactivating or dominant-negative mutations, or gain of oncogenic mutant p53 function, severely compromises the capacity of cells for controlled proliferation. Multiple types of cellular stress, such as DNA damage as a result of UV or γ irradiation, ribosomal stress, inappropriate oncogene activation and hypoxia activate p53 by post-translational modifications, such as acetylation and phosphorylation (Toledo and Wahl, 2006; Vousden and Ryan, 2009). Depending on the cell type, severity and type of stress, p53 implements one of several responses, such as cell cycle arrest, senescence, differentiation or, if damage is severe, induction of the apoptotic cascade. p53 executes the desired response by directly binding p53REs of target genes. Over 4,000 putative target genes were identified by computational methods (Wang et al., 2001), while chromatin immunoprecipitation combined with paired-end ditag (ChIP-PET) experiments have identified over 500 genes with promoter-bound p53 protein with high confidence (Wei et al., 2006). Validated p53 targets include the Cdk inhibitor *p21*, members of the *Bcl-2* family, the death receptor *Fas* and p53 repressor *MDM2* (el-Deiry et al., 1993; Miyashita and Reed, 1995; Oda et al., 2000; Owen-Schaub et al., 1995).

While most post-translational modifications occur in residues in the C-terminal and transactivation domains, the majority of *p53* mutations are found in the DNA binding domain (Sigal and Rotter, 2000; Toledo and Wahl, 2006). Mutant p53 protein is able to act as a dominant negative by binding to and tetramerizing with wild type p53 to repress normal physiological processes of p53, possibly by inducing an inactive conformation of the DNA binding domain and reducing the ability to transactivate/repress target genes (Chene, 1998; Kern et al., 1992; Shaulian et al., 1992; Unger et al., 1993). Normally, p53

exists in a negative feedback loop with MDM2, which tightly controls both p53 and MDM2 levels in the cell. However, loss of p53 transcriptional activity as a result of dominant negative mutation may result in decreased MDM2, with the consequence of mutant p53 stabilization and increased amount of non-functional/gain-of-function mutant p53 protein (Blagosklonny, 2000).

Although *p53* mutations have been detected in all histological types of EOC, mutations in serous EOC are much more commonly observed (Table 1.1). Interestingly, *p53* mutation frequency differs between serous tumors of low and high-grade, with *p53* mutations strongly associated with high-grade serous carcinomas and rare in low-grade/borderline serous carcinomas (Kupryjanczyk et al., 1995; Kupryjanczyk et al., 1993; Skomedal et al., 1997; Zheng et al., 1995). In contrast, low-grade/borderline tumors frequently harbor mutations in *K-ras*, which are very rare in high-grade serous adenocarcinomas (Cuatrecasas et al., 1997; Diebold et al., 2003; Singer et al., 2002; Singer et al., 2003a; Singer et al., 2003b; Zheng et al., 1995). These observations have given strong support to the hypothesis that high-grade and low-grade serous carcinomas arise via discrete pathways (Shih Ie and Kurman, 2004; Singer et al., 2003a; Singer et al., 2003b; Singer et al., 2005). Lending further support to this hypothesis is the observation that *p53* is mutated in early stage high-grade carcinomas as well as adjacent dysplastic epithelium in prophylactically removed ovaries from *BRCA1* heterozygotes (Pothuir, 2001; Werness et al., 2000). This supports a model in which *p53* mutation is not only required for carcinogenesis, but is an early event in the pathogenesis of high-grade serous carcinoma.

Table 1.1 Frequency of *p53* mutations in histological subtypes of EOC.

Type of EOC (average %)		**Defective/Total cases (%)**	**Reference**
Serous	Low-grade (14%)	4/22 (18)	(O'Neill et al., 2005)
		1/12 (8)	(Singer et al., 2005)
		33/190 (17)	(Lassus et al., 2003)
		5/27 (19)	(Chan et al., 2000)
		1/13 (8)	(Salani et al., 2008)
	High-grade (68%)	30/47 (64)	(O'Neill et al., 2005)
		30/59 (51)	(Singer et al., 2005)
		167/180 (93)	(Lassus et al., 2003)
		25/46 (54)	(Chan et al., 2000)
		57/71 (80)	(Salani et al., 2008)
Clear cell (8%)		6/38 (17)	(Ho et al., 2001)
		0/4 (0)	(Otis et al., 2000)
		1/12 (8)	(Caduff et al., 1999)
Endometrioid (45%)		5/15 (33)	(Dogan et al., 2005)
		7/13 (54)	(Henriksen et al., 1994)
		13/27 (48)	(Caduff et al., 1999)
Mucinous (19%)		1/12 (8)	(Dogan et al., 2005)
		3/12 (25)	(Renninson et al., 1994)
		3/11 (27)	(Henriksen et al., 1994)
		3/21 (14)	(Caduff et al., 1999)

1.3 MicroRNAs

It had been known for many years that the *Caenorhabditis elegans* heterochronic gene *lin-4* is required for correct developmental timing and that its loss of function results in reiterations of early fates at inappropriate developmental stages (Chalfie et al., 1981). Conversely, mutation of *lin-14* results in an opposite phenotype and is completely epistatic to *lin-4* loss of function, suggesting that *lin-4* may negatively regulate *lin-14*. Despite much effort, however, no LIN-4 protein could be identified and it was not until 1993, when *lin-4* was cloned in the Ambros lab, that it was realized that the gene is non-coding (Lee et al., 1993). Rather, two transcripts of 61 nt and 22 nt in length, the later which is partially antisense complementary to the 3' untranslated region (UTR) of *lin-14* (Wightman et al., 1993), were the only detectable products – the first miRNA. However, not until the identification of a second small RNA, *let-7* (Reinhart et al., 2000), and the observation that it is widely conserved (Pasquinelli et al., 2000), did miRNAs receive attention by the wider scientific community.

Biogenesis of miRNAs starts with transcription of their primary transcript (pri-miRNA), largely by RNA polymerase II, but polymerase III for some miRNAs within Alu repeats (Borchert et al., 2006; Lee et al., 2004). The pri-miRNA, which may be several kilobases in length, is then subjected to step-wise processing by two RNase III endonucleases. Firstly, in the nucleus, Drosha together with DGCR8/Pasha processes the pri-miRNA to a ~70 nt double stranded precursor miRNA (pre-miRNA) (Landthaler et al., 2004; Lee et al., 2003). After Exportin-5 mediated export to the cytoplasm (Lund et al., 2004; Yi et al., 2003), pre-miRNA molecules are subjected to processing by

Dicer resulting in a ~22 nt RNA duplex: one stand being the mature miRNA and the other being the passenger strand (denoted in the literature as miRNA*). The miRNA duplex is unwound with the miRNA*, generally the strand with the highest stability at the 5' end, being degraded, while the mature miRNA is loaded into the RNA induced silencing complex (RISC) (Khvorova et al., 2003; Schwarz et al., 2003). Although the mechanism of miRNA unwinding is unclear, the unwinding of *let-7* can be attributed to the P68 RNA helicase (Salzman et al., 2007). In addition to Drosha-mediated processing to generate pre-miRNAs, a Drosha-independent biogenesis pathway has recently been reported (Berezikov et al., 2007; Okamura et al., 2007; Ruby et al., 2007). Mirtrons, short introns that, after splicing and de-branching, form pre-miRNA-like molecules, are a second source of pre-miRNA molecules. Once exported to the cytoplasm by Exportin-5, they are processed by Dicer to mature miRNAs and seem to function identically to canonically synthesized miRNAs. The number of identified mirtrons is far lower than that of miRNAs, however, and it would appear that Drosha-dependent miRNAs are far more common.

There are 2 possible outcomes upon binding of the miRNA-loaded RISC to a target 3' UTR (Figure 1.1). The first possibility is target cleavage, which occurs if perfect base pairing is achieved between the entire miRNA and target. The mechanism for this is target cleavage between nucleotides 10 and 11 by Ago2-containing RISC. However, while this outcome is common in plants, it is less so in animals. The second potential outcome in animals is translational repression. For this to occur perfect base pairing between miRNA/mRNA is not required and mismatches and bulges are tolerated so long as perfect base pairing is achieved at the seed region of the miRNA,

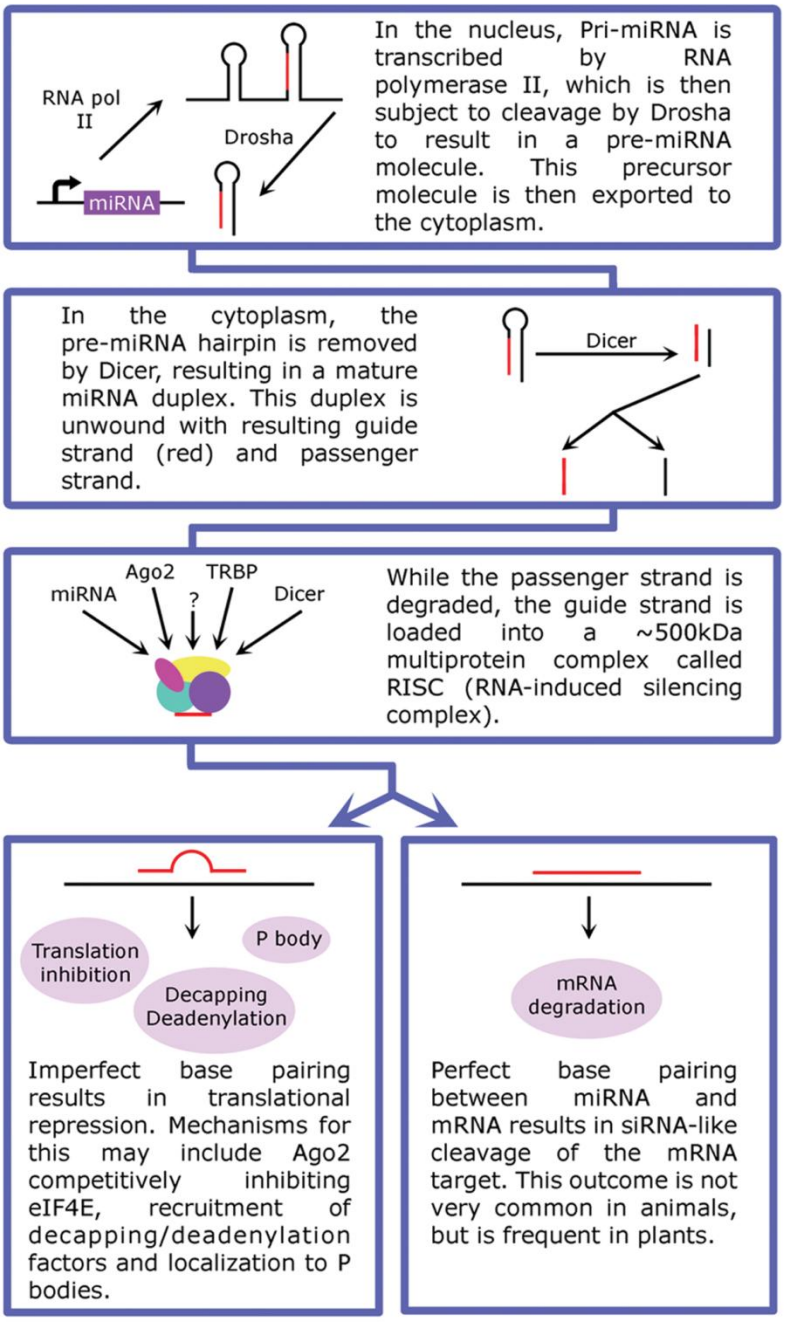

Figure 1.1 Flow diagram outlining miRNA biogenesis and function.

which encompasses nucleotides 2-8. While the outcomes of miRNA-mediated translational repression are well agreed upon, the first step in bringing this about is controversial. Experimental data currently falls into one of two groups: evidence for repression at initiation stage of translation, and evidence for repression post-initiation. Supporting the hypothesis that a post-initiation stage is repressed, reporter genes with internal ribosomal entry site (IRES)-directed expression are still repressed, which suggests that binding of the full complement of initiation factors is not required (Lytle et al., 2007; Petersen et al., 2006). Additionally, miRNA-targeted mRNAs are found to be associated with polysomes (Lytle et al., 2007; Maroney et al., 2006; Petersen et al., 2006). To the contrary, other groups have shown that during sedimentation gradient experiments, repressed mRNAs are shifted towards the top, suggesting reduced ribosome loading, and that mRNAs with IRES or a nonfunctional 5' cap are no longer repressed (Bhattacharyya et al., 2006; Humphreys et al., 2005; Pillai et al., 2007). The cause of these conflicting results has been suggested to be a result of the differing systems used by each group. For example, different transfection protocols were shown to affect the outcome of miRNA repression (Lytle et al., 2007). Recent *in vitro*, cell-free extract experiments have provided more evidence for a role at translation initiation. Using different cell extracts and protocols, several groups showed that the m^7GpppN 5' cap is required for repression, and this requirement cannot be resolved by substitution with a nonfunctional ApppN cap or IRES, while a poly [A] tail is also indispensable (Mathonnet et al., 2007; Thermann and Hentze, 2007; Wakiyama et al., 2007; Wang et al., 2006). It was shown by Mathonnet et al. that an increase in the cap binding protein eIF4e attenuates miRNA-mediated repression in a cell-free system (Mathonnet et al., 2007).

These data are consistent with the presence of a cap binding domain within RISC component Ago2 (Kiriakidou et al., 2007), suggesting that repression occurs by Ago2 competitively inhibiting eIF4e binding to the cap structure which might lead to decapping and deadenylation of the mRNA target. However, an alternative explanation of this observation was made by Eulalio et al., who showed that the domain binds GW182, not Ago2, which functions downstream in the miRNA repression pathway (Eulalio et al., 2008). In closing, it is important to bear in mind that data obtained from miRNA/mRNA reporter assays and cell-free systems is not necessarily compatible with the *in vivo* situation. It is possible that a combination of both initiation and post-initiation stages are repressed. Underlining this, Kong et al. recently showed that in addition to different transfection methods affecting mechanism of repression, the promoter used to drive mRNA expression is critical. While mRNA transcribed from *SV40* promoter are repressed at initiation stage, *TK* promoter results in post-initiation repression (Kong et al., 2008). Regarding the identity of mRNA targeted by miRNAs, computational predictions were first used based solely upon evolutionarily conserved perfect base pairing at the seed region (John et al., 2004; Krek et al., 2005; Lewis et al., 2005). In recent years, advances in computational prediction have resulted in consideration of location of target site within 3' UTR, free energy and localization near AU-rich sequence (Grimson et al., 2007; Kertesz et al., 2007). Methods to identify authentic mRNA targets of individual miRNAs by proteomic approaches are currently under development by multiple groups (Baek et al., 2008; Selbach et al., 2008).

In a highly intriguing twist, it appears that in certain contexts and circumstances, RISC can be "converted" into a translation activating complex.

Firstly, Vasudevan and Steitz demonstrated that tethering AGO2 and fragile-X-mental-retardation-related protein 1 (FXR1) to an AU-rich element (ARE) present in the 3' UTR of *TNFα* was sufficient to increase translation (Vasudevan and Steitz, 2007). In a separate paper, they went on to show the requirement of miR-369-3 in directing the AGO/FXR1 complex to the mRNA, resulting in increased translation (Vasudevan et al., 2007). Mutating the seed sequence for the miRNA binding site in the ARE prevents translation upregulation, yet mutating miR-369-3 to match the mutated binding site restores upregulation. This effect is not a peculiarity of miR-369-3, either, since the authors also demonstrate similar effects with the artificial miRNA cxcr4 system and the endogenous miRNA *let-7* on its target *HMGA2*. However, whether activation over repression is observed depends on the stage of the cell cycle – only when cells have been serum-starved and have undergone cell cycle arrest is activation observed, whereas in proliferating cells, repression is seen. While clearly an exciting observation, further work will be required to elucidate the responsible mechanisms and to establish the consequences of miRNA-mediated translation upregulation *in vivo* in a non-synchronized cell pool.

1.4 MicroRNAs in cancer

Given the large number of mRNAs predicted to be directly regulated by miRNAs, it would be expected that a large number of processes be controlled by miRNAs, and indeed this is the case. Processes under miRNA control include hematopoiesis (Chen et al., 2004; Felli et al., 2005), cardiogenesis

(Zhao et al., 2007), blastocyst implantation (Chakrabarty et al., 2007) and neuronal development (Schratt et al., 2006). Yet the majority of research has been focused on the possible roles that miRNAs may play in disease, and specifically in cancer.

The first indications of a role for miRNAs in cancer came from the laboratory of Carlo Croce. A bicistronic miRNA cluster containing *mir-15a* and *mir-16-1* at chromosome 13q14 was observed to be mutated, deleted or have reduced expression in chronic lymphocytic leukemia (CLL) (Calin et al., 2002; Calin et al., 2005). Later, germline mutations in *mir-15a/-16-1* were observed, and it was shown that these two miRNAs target anti-apoptotic *BCL-2* mRNA (Calin et al., 2005; Cimmino et al., 2005). While these reports suggested that miR-15a/-16 act as tumor suppressors, a tumor suppressor role in B cells was confirmed by deletion of *mir-15a/16-1 in vivo* resulting in CLL (Klein et al., 2010). However, conventional knockout of this cluster resulted in no developmental defect or tumor development other than the aforementioned CLL, suggesting that either the main effect of miR-15a/-16-1 is in B cells, or miR-15a/-16-1 loss is compensated by other members of the miR-15 family in non-B cells, such as by the second copy of the miR-15 and miR-16 cluster on chromosome 3 and/or the related miR-195 on chromosome 17. This hypothesis fits well with the observation that miRNAs appear to have cell type specific roles, since while miR-15a/-16 induces apoptosis by targeting *BCL-2* in megakaryoblastic cell line MEG-01, no such induction of apoptosis or change in BCL-2 protein is observed in non-hematopoietic cells (Cimmino et al., 2005; Linsley et al., 2007).

In addition to possessing growth inhibitory activities, some miRNAs, such as those in the miR-17-92 cluster, have been implicated as oncogenes.

mir-17-92 is found in the *c13orf25* locus on human chromosome 13q31, a region that is commonly amplified in multiple cancer types (Knuutila et al., 1998). Tumors arising from hematopoietic stem cells expressing both *c-myc* and a subset of miRNAs from miR-17-92 demonstrate an absence of apoptosis observed in *c-Myc*-only tumors (He et al., 2005), while overexpression of this miRNA cluster also results in greater angiogenesis in tumors (Dews et al., 2006). Subsequent experiments in mice have shown that overexpression of all members of the miR-17-92 cluster is not required for Myc-induced B-cell lymphomagenesis. Rather, overexpression of miR-19a/b recapitulates overexpression of the entire cluster (Mu et al., 2009; Olive et al., 2009). Underlying the importance of Myc in cooperating with miR-17-92, Myc directly binds canonical E-box sequences in the *mir-17-92* promoter and members of the miR-17-92 cluster target *E2F1*. This suggests an interesting signaling pathway, since Myc and E2F1 are known to induce each other's expression, while at the same time Myc represses *E2F1* translation through miRNAs (O'Donnell et al., 2005). Complicating the issue further still are reports demonstrating that E2F proteins also bind the *mir-17-92* locus, resulting in a negative feedback loop (Sylvestre et al., 2007; Woods et al., 2007).

The genomic location of miRNAs is not evenly distributed throughout the human genome. Instead, there are some chromosomes, for example chromosome 4, with a lower than expected number, and some with greater than expected miRNAs, for example chromosomes 17 and 19 (Calin et al., 2004). More importantly, however, taking a computational approach, Calin et al. demonstrate that miRNA genes are frequently found at fragile sites, regions commonly amplified or lost, common breakpoint regions and are close to

human papilloma virus integration sites (together abbreviated to CAGRs; cancer-associated genomic regions). Of 186 miRNAs studied, 98 (52.5%) were located in CAGRs and a significant number of miRNAs are located either in, or close to, homeobox clusters. Homeobox genes are transcription factors playing important roles in both normal development and carcinogenesis in a variety of cancers, including ovarian cancer (Hennessy and Mills, 2006).

1.5 MicroRNA alterations in ovarian cancer

As outlined previously, Calin et al. provided evidence that miRNA expression may be altered by changes in gene copy number by proximity to CAGRs. To gain a more detailed view of miRNA copy number alterations in cancer, Zhang et al. used array comparative genomic hybridization (aCGH) to identify miRNA loci gained/lost in ovarian cancer, breast cancer and melanoma (Zhang et al., 2006). Towards ovarian cancer, 93 primary tumors and 16 cell lines were compared to wild type DNA. A tumor-to-reference ratio less than 0.8 was considered as a copy number loss, while a value greater than 1.2 was considered as a copy gain. Of 283 miRNA loci analyzed, 105 (37.1%) were significantly altered in their copy number. Similarly, significant copy changes were observed in 206 of 283 (72.8%) miRNAs in breast cancer and 243 of 283 (85.9%) in melanomas. Quantitative RT-PCR demonstrated that expression of miRNAs was consistent with DNA copy number status for 73.1% of miRNAs, demonstrating that DNA copy number alterations may have an important effect on miRNA expression. Consistent with reports of tissue-specific expression of many miRNAs, the aCGH profile for each ovarian cancer type were not the

same, as revealed by cluster analysis. However, there are a number of copy number gains and losses shared between all three tumor types such as loss of the *mir-17-92* locus in all tumor types. In addition, the *mir-15a/16-1* locus is lost in 23.9% of ovarian and 24.7% of breast cancers. Consistent with reduced copy number, reduced miR-15a and miR-16 expression is observed in a panel of 38 high grade serous tumors (Bhattacharya et al., 2009). Interestingly, Bhattacharya et al. identify the Polycomb gene *Bmi-1* as a direct target of these two miRNAs and show that Bmi-1 expression is inversely correlated with miR-15a and miR-16 in ovarian cancer. Functionally, and similar to roles in other cancers, miR-15a or miR-16 reconstitution experiments reduces proliferation and clonal growth, the latter of which can be rescued by a miR-15a/miR-16-independent *Bmi-1* transgene.

Interestingly, Zhang et al. observed that 24.8% and 51.5% of ovarian tumors exhibited gains in copy number of the *Dicer* and *Ago2* loci. As outlined previously, Dicer and Ago2 proteins are required for efficient miRNA processing and function. Most recently it has been shown that short-hairpin RNA (shRNA)-mediated knockdown of *Dicer* and *Ago2* increases colony formation in soft agar and tumor formation *in vivo* (Kumar et al., 2007) and is a haploinsufficient tumor suppressor (Kumar et al., 2009). Consistent with this, Drosha and Dicer are associated with favorable prognosis in ovarian cancer (Merritt et al., 2008).

Given the relatively large number of ovarian cancer samples analyzed by Zhang et al., it is disappointing that no data was presented regarding the histological characteristics of each sample, since particular alterations may be correlated with particular EOC subtypes. However, this issue has been addressed, to some extent, at the transcriptional level by microarray profiling

experiments and a follow-up study by Zhang et al. (Iorio et al., 2007; Zhang et al., 2008). Iorio et al. analyzed a total of 69 primary EOC tissues, including 31 serous, 8 endometroid and 4 clear cell tumors and compared to total normal ovarian tissue. The miR-200 family, which comprises 3 members (miR-200a, -200b and -200c) was among those miRNAs significantly overexpressed, while those downregulated included miR-199a, miR-140 and miR-145. Only 2 miRNAs were downregulated in serous, endometrioid and clear cell carcinomas, miR-200a and miR-200c, while 19 were overexpressed in all three tumor types, including miR-140 and let-7d. Notably, the expression of the miR-200 family, miR-140 and let-7d is concordant with DNA copy number previously reported (Zhang et al., 2006) (Table 1.2). Functionally, the miR-200 family of miRNAs regulate ZEB1 and ZEB2 transcription factors and their reconstitution results in increased E-cadherin expression and mesenchymal-to-epithelial transition in culture (Bendoraite et al., 2010; Park et al., 2008).

In addition to miRNAs conserved between EOC histological subtypes, Iorio et al. also identified pools of miRNAs that are specifically downregulated in just one subtype, an observation that may have some implications for differential diagnosis in the future. However, while this report may be useful for diagnosis, the authors' choice of total normal ovarian tissue, rather than OSE, as normal control, limits what information can be used towards our basic understanding of miRNAome alterations in the OSE. For this reason, a follow-up study by Zhang et al. used qRT-PCR transcriptional profiling of EOC specimens and used immortalized OSE cells for comparison to identify a similar pool of dysregulated miRNAs as reported by others (Zhang et al., 2008). Importantly, and consistent with studies in other cell types, the authors identified miRNAs that were subject to epigenetic regulation and by copy

Table 1.2 MicroRNAs altered in ovarian cancer.

miRNA	**Reference**
miR-105 miR-143 miR-203 miR-373	(Iorio et al., 2007)
let-7 miR-9 miR-34 miR-140	(Iorio et al., 2007; Zhang et al., 2006)
miR-15a/-16 miR-29a/b miR-30b/d miR-181b	(Zhang et al., 2006)

number variation. DNA copy deletions at *mir-15a* and *mir-140* loci were found in over 20% of EOC specimens. Meanwhile, hemizygous deletion at the *mir-34a* locus (chromosome 1p36) were identified in 58% (7/12) of low-grade serous EOC, but not in high-grade serous tumors (Kuo et al., 2009).

1.6 Use of microRNAs as diagnostic and therapeutic tools

1.6.1 Potential diagnostic uses of microRNAs

The let-7 miRNA family is known to repress expression of the *RAS* and *HMGA2* oncogenes and its decreased expression is associated with poorer prognosis in non-small cell lung cancer (NSCLC) (Johnson et al., 2005; Mayr et al., 2007; Takamizawa et al., 2004). Shell et al. performed miRNA profiling of the NCI60 panel of human tumor cell lines (Shell et al., 2007). Members of the NCI60 panel can be grouped into 1 of 2 superclusters (SC) based upon their gene expression, with SC1 cell lines having mesenchymal and SC2 cell lines having epithelial properties. Interestingly, expression of 5 of 7 let-7 family members was reduced in SC1, whereas SC2 expressed high let-7 levels. Noticing that the mesenchymal-like SC1 cell lines share similarities with EOC, which commonly expresses mesenchymal markers (Rosano et al., 2005), the authors looked at the expression of let-7 and HMGA2 in 6 ovarian cancer cell lines. The expression of let-7 and HMGA2 was inversely correlated, as one might expect given that let-7 represses *HMGA2* expression via 7 binding sites in the *HMGA2* 3' UTR. The authors then went on to analyze HMGA2 expression by performing immunohistochemistry on a tissue microarray

containing 100 EOC samples. While no difference in HMGA2 staining intensity was observed between primary tumor sites and metastatic tumors, high HMGA2 expression significantly correlated with poor overall survival. In clinical samples, the let-7/HMGA2 relationship was conserved and importantly, let-7 and HMGA2 expression was sufficient to group patients into one of two groups. The first group, with a high HMGA2/let-7 ratio, has poor prognosis and estimated 5-year progression-free survival under 10%. In contrast, the second group, with a lower HMGA2/let-7 ratio, has a higher 5-year progression survival of about 40%.

Similarly, miR-31 has been shown to have diagnostic potential in EOC. To identify miRNAs with decreased expression in EOC, massively parallel small RNA sequencing was performed in primary OSE cultures, serous tumors and EOC cell lines (Creighton et al., 2010). miR-31 was found to be the most downregulated miRNA in tumor samples compared to primary OSE cultures and, through data mining of CGH data, found to be located at chromosome 9p21.3 which is deleted in >60% tumors. Further bioinformatic analysis revealed possible interaction with the p53 pathway. Overexpression of miR-31 in *p53*-null EOC cell lines results in apoptosis and proliferative arrest. Meanwhile, in *p53* wild type EOC cell lines, such as HEY and OVSAYO, proliferation is unchanged in miR-31-treated cells compared to negative control. Importantly, a miR-31-inactive gene expression signature was significantly correlated with poorer prognosis. Furthermore, miR-31 has recently been shown to inhibit metastasis of breast cancer in part through targeting of pro-metastatic genes RhoA, integrin-α5 and radixin (Valastyan et al., 2009a; Valastyan et al., 2009b).

In addition to utility in prognostication, miRNA profiles were shown to

classify human cancers of uncertain cellular origin with greater accuracy than a mRNA profile (Lu et al., 2005). The significance of the work performed by Lu et al. is that a miRNA expression profile was able to classify human cancers by their origin, which could be useful given that 2-4% of cases are unable to be diagnosed with any certainty by histopathological means (Pavlidis et al., 2003).

While clearly demonstrating diagnostic potential, profiling of miRNAs from tumors requires undesirable invasive procedures to obtain necessary biopsy tissue. A better alternative might be to use biomarkers present in circulating blood, and towards this, several groups have examined the presence of miRNAs in serum. Serum-derived miRNAs have been shown to be remarkably stable, resistant to RNase, extreme pH and temperature and repeated freeze-thaw cycles (Chen et al., 2008; Mitchell et al., 2008). Interestingly, resistance to RNase treatment is not due to miRNA structure, since naked miRNA spiked in to plasma samples is rapidly degraded (Mitchell et al., 2008). It remains to be understood how miRNAs are released by cells into the circulation. One possibility, however, is that the circulating miRNAs are contained within exosomes, since their existence in these small (50-90 nm) vesicles has been recently reported (Skog et al., 2008; Valadi et al., 2007).

1.6.2 Therapeutic potential of microRNAs

Given the potential of miRNAs to function either as tumor suppressors or oncogenes, a number of groups have used different approaches to increase or decrease expression of different miRNAs in the hope of inducing a therapeutic effect. Several methods to reduce, or knock down, miRNA expression have

been devised, which may be useful in the case of oncogenic miRNAs. Firstly, 2'-*O*-methyl modified oligoribonucleotides complementary to the mature miRNA transfected into mammalian cells rapidly and irreversibly bind the miRNA, preventing it from binding its target (Hutvagner et al., 2004; Meister et al., 2004). For *in vivo* knockdown "antagomirs", cholesterol–conjugated 2'-*O*-methyl-modified oligo-ribonucleotides antisense to a miRNA of interest, can be injected directly into the mouse (Krutzfeldt et al., 2005). At a dose of 240 mg per kg body weight, an antagomir directed against miR-122 results in undetectable levels of the mature miRNA for up to 23 days. Due to the relatively high doses required and the short lived response, however, Ebert et al. designed an alternative strategy based upon sequestering miRNAs by binding an artificial target expressed at high levels in the cell (Ebert et al., 2007). In this approach, a target is designed that is perfectly complementary to the miRNA with the exception of a bulge at nucleotides 9 to 12 which prevent target cleavage by Ago2. Multiple copies of the target are placed in the 3' UTR of a gene such as green fluorescent protein (GFP) and transfected into cells, generating a "miRNA sponge" transgene. The strong expression of the sponge releases miRNA-mediated repression of endogenous targets. Importantly, in principle at least, this technique could be directed to generating transgenic animals with constitutive or conditional knockdown of miRNAs.

As mentioned, some miRNAs have been described as having tumor suppressor properties and ectopic expression of these miRNAs in neoplastic tissues may have therapeutic potential. Numerous groups, including ourselves, have overexpressed miRNAs through infection of retrovirus or lentivirus expressing the miRNA of interest. This approach has the advantage of using inducible and/or cell-type specific promoters although, as with mRNA

overexpression, there are many safety concerns if this technology is to be used in humans. A second approach is delivery of mature miRNAs to a cell. These molecules are commercially available, can result in increased silencing of a miRNA target and can be delivered to cells through non-viral means (Johnson et al., 2005). Currently, however, their use has largely been restricted to transfection of cells in cell culture and while they have worked well in our hands (Corney et al., 2007; Corney et al., 2010), their usefulness *in vivo*, like siRNAs, will depend on efficient delivery to cancerous cells (reviewed by Kota et al., 2009; Whitehead et al., 2009).

1.7 Concluding remarks and project overview

Due to asymptomatic development, the initiating events of ovarian cancer remain obscure and much of our current understanding is based upon circumstantial and correlative evidences. To this end, the development of accurate mouse models of ovarian cancer is of utmost importance in expanding our knowledge of ovarian carcinogenesis. The emergence of the miRNA field has been rapid yet also widespread, given that miRNAs are present in multiple species and are predicted to regulate thousands of human mRNAs. It is plain to see, however, that we are at an early stage in our understanding of these molecules and much remains to be learnt. Yet it is equally clear that miRNAs possess a large potential to play a role in the clinic, both as diagnostic and therapeutic tools. Such potential is critically required for many cancers, but especially for ovarian cancer given late diagnosis and inadequate treatment options currently available.

Given the poor state of understanding of control of miRNAs in normal and pathological physiology, I initiated studies to gain a better understanding of the role of miRNAs in EOC. The central hypothesis of these studies is that 1) there is a direct pathway between p53 and individual and/or all miRNAs and that 2) p53-regulated miRNAs fulfill a critical role in the p53 pathway. Towards this, in Chapter 2, miRNA microarray profiling experiments were performed to identify altered miRNA expression after *p53* inactivation in primary mouse OSE cells. The most downregulated miRNAs, the miR-34 family, was shown to contain evolutionarily conserved p53 recognition elements in the *mir-34* promoters. Functionally, miR-34 reconstitution experiments reveal a role in reducing proliferation and adhesion-independent growth in neoplastic ovarian cancer cell lines, suggesting a role in tumor suppression. To gain a fuller understanding of miR-34 function and determine its clinical relevance two approaches were taken. In Chapter 3, the frequency, and mode, of miR-34 downregulation was determined in a panel of EOC clinical specimens. While decreased miR-34 expression was most correlated with *p53* mutation, promoter methylation and copy number variation were also frequently observed. Further demonstrating potential for therapeutic applications of miR-34, and of miRNAs in general, miR-34 reconstitution in a human ovarian cancer cell line resulted in decreased proliferation, motility and invasion. Finally, in Chapter 4, *mir-34b/c* conditional knockout mice were generated to allow direct testing of the hypothesis that they are tumor suppressors *in vivo*.

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CHAPTER 2

MICRORNA-34B AND -34C ARE TARGETS OF P53 AND COOPERATE IN CONTROL OF CELL PROLIFERATION AND ADHESION-INDEPENDENT GROWTH[*]

2.1 Abstract

MicroRNAs (miRNAs) are a recently discovered class of non-coding RNAs that negatively regulate gene expression. Recent evidences indicate that miRNAs may play an important role in cancer. However, the mechanism of their deregulation in neoplastic transformation has only begun to be understood. In order to elucidate the role of tumor suppressor p53 in regulation of miRNAs we have analyzed changes in miRNA microarray expression profile immediately after conditional inactivation of *p53* in primary mouse ovarian surface epithelium cells. Among the most significantly affected miRNAs were miR-34b and miR-34c, which were downregulated 12-fold according to quantitative RT-PCR analysis. Computational promoter analysis of the *mir-34b*/*c* locus identified the presence of evolutionarily conserved p53 binding sites about 3 kb upstream of the miRNA coding sequence. Consistent with evolutionary conservation, miR-34b/c were also downregulated in *p53*-null human ovarian carcinoma cells. Furthermore, as expected from p53 binding to the *mir-34b*/*c* promoter, doxorubicin treatment of wild-type but not *p53* deficient cells resulted in an increase of miR-34b/c expression. Importantly, miR-34b and miR-34c cooperate in suppressing proliferation and soft agar

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colony formation of neoplastic epithelial ovarian cells, in agreement with the partially overlapping spectrum of their predicted targets. Taken together, these results demonstrate the existence of a novel mechanism by which p53 suppresses such critical components of neoplastic growth as cell proliferation and adhesion-independent colony formation.

2.2 Introduction

MicroRNAs (miRNAs) are a recently discovered class of non-coding RNAs which control gene expression either by degradation of target mRNAs or, more commonly for animal miRNAs, by post-transcriptional repression in a mechanism similar to siRNA-mediated gene silencing. Numerous evidences point to a role for miRNAs in the etiology and pathogenesis cancer by targeting oncogenes or tumor suppressors (Esquela-Kerscher and Slack, 2006). For example, miR-15a and -16 target antiapoptotic gene *BCL-2* (Cimmino et al., 2005), while the *LATS32* tumor suppressor is targeted by miR-372 and -373 (Voorhoeve et al., 2006). Dysregulated miRNA expression may occur via a number of mechanisms, such as gene copy gain or loss (Zhang et al., 2006), germline mutation of precursor miRNA molecules (Calin et al., 2005), promoter methylation (Saito et al., 2006) or aberrant miRNA processing due to altered expression of miRNA biogenesis machinery (Thomson et al., 2006). However, the role of transcription factors in miRNA expression has received little attention. Most, if not all, miRNAs are transcribed by RNA polymerase II (Lee et al., 2004) suggesting that transcription factors involved in mRNA transcription may also regulate miRNA transcription. Supporting this hypothesis, the proto-oncogene and transcription

factor Myc has been demonstrated to bind canonical E-box sequences found upstream of the *mir-17-92* miRNA locus (O'Donnell et al., 2005).

Given the above observations, we decided to elucidate the involvement of p53 in the regulation of miRNAs. The p53 protein is a transcription factor that is frequently mutated in many types of human cancer. Cellular stress, such as DNA damage, hypoxia or inappropriate oncogene activation, activates and stabilizes p53, resulting in an anti-proliferative response, such as cell cycle arrest, apoptosis or senescence. p53 orchestrates such responses by directly activating key genes via binding two repeats of the DNA sequence 5'-PuPuPuC(A/T)(T/A)GPyPyPy-3' (el-Deiry et al., 1992). Genes known to be activated by p53 include *p21*, *Gadd45*, *Bax* and its negative regulator *Mdm2* (Levine et al., 2006).

p53 mutations are thought to be the initiating or earliest events in formation of a number of cancers, including ovarian cancer. Approximately 90% of ovarian cancers are carcinomas, which are assumed to originate from the ovarian surface epithelium (OSE), a single layer of cells coating the ovary. Due to near symptomless progression, the majority of cases are diagnosed at a late stage, at which stage prognosis is extremely poor (Nikitin and Hamilton, 2005). Based on the previously described mouse model of epithelial ovarian cancer (EOC) (Flesken-Nikitin et al., 2003), we have established a system to evaluate immediate effects of *p53* inactivation on miRNAome of the OSE within the first few passages after explantation. We report that miRNAs miR-34b and miR-34c are transcriptional targets of p53 and represent novel effectors mediating its suppression of such critical components of neoplastic growth as cell proliferation and adhesion-independent colony formation.

2.3 Materials and methods

Cell Culture. For preparation of primary cell cultures, mouse OSE cells from either *p53*$^{loxP/loxP}$ (Jonkers et al., 2001) or wild-type age-matched mice of the same FVB/N background were isolated as previously described (Flesken-Nikitin et al., 2003). All mice used for cell preparations were maintained identically, following recommendations of the Institutional Laboratory Animal Use and Care Committee. Neoplastic cell lines OSN1 and OSN 2 were generated by Cre-*loxP* mediated inactivation of *p53* and *Rb,* or *p53* individually, respectively, after 3 passages in culture. These cell lines were continually passaged upon reaching confluence using standard techniques and late passage OSN1/OSN2 cells were used in this study. Primary cultures and established mouse cell lines were maintained in DMEM/F12 (50:50 mix) supplemented with 5% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 10 ng/ml EGF, 500 ng/ml hydrocortisone, 5 µg/ml each of insulin and transferrin and 5 ng/ml sodium selenite. Human SKOV-3 cell line was obtained from ATCC and maintained according to supplier's directions.

*miRNA isolation and profiling***.** At passage three after explantation, subconfluent OSE cell cultures were treated with either Ad*Cre* or blank adenovirus in serum-free medium for two hours at 37°C/5% CO_2, cultured for a further 2 passages and processed for miRNA isolation. Total RNA was isolated using a mirVana miRNA Isolation Kit (Ambion, Austin, TX) and was highly enriched for mature miRNA species using a FlashPAGE Fractionator (Ambion, Austin, TX). 110 ng miRNA-enriched material was labeled with Cy5 using Label IT miRNA Labeling kit (Mirus Bio Corporation, Madison, WI)

according to the manufacturer's instructions and subsequently hybridized to CombiMatrix MicroRNA 4X2K Microarrays (CombiMatrix, Mukilteo, WA) containing probes against mouse miRNAs in Release 8.1 of the Sanger database.

Each slide harbored 4 microarrays, each containing 2240 probes. 400 miRNA sequences were represented by a native probe corresponding to wild type miRNA sequence (Nat) and a 2 point mutation mutant probe (Mut) to maximally disturb the binding between probe and intended target miRNA. Each probe was replicated 3 to 5 times on the array. The hybridized arrays were scanned by GenePix 4000B scanner (Molecular Devices, Sunnyvale, CA) to generate raw fluorescence intensity file. The intensities of replicate spots for the same probe sequence were averaged to represent the probe signal. Six wild-type samples and six mutant samples were analyzed. Three samples with obvious hybridization artifacts were excluded from further analysis. True signal for the intended miRNA target was obtained by subtracting Mut probe intensity from the corresponding Nat probe intensity. Some of these Nat-Mut signals were very small so data flooring was applied to raise any value below 2 to be equal to 2 and were log-transformed. To minimize slide to slide variation, biological replicate samples on each slide were averaged and the difference between sample types taken for subsequent analysis. The log-ratio values of each array were centered to have a median of 0. One-sample t test against mean of 0 was applied on the normalized *p53*-mutant/wildtype values of each gene across 3 slide hybridizations. At cutoff value of 0.1, a total of 84 genes were selected.

Quantitative RT-PCR. Stem-loop quantitative RT-PCR for mature miRNAs was performed as described (Chen et al., 2005) on an Applied Biosystems AB 7500 Real Time PCR system (Foster City, CA). All PCR reactions were run in quadruplicate and gene expression, relative to *RNU6B,* calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Bioinformatics. To identify p53 response elements in miRNA promoter regions, 5 kb 5' and 3' of each miRNA genomic sequence from Build 36 of the *Mus musculus* genome and Build 36.1 of the *Homo sapiens* genome were retrieved from UCSC Genome Browser. Retrieved sequences were analyzed by the p53MH algorithm, which searches for two copies of the p53 DNA-binding motif separated by 0-14 bp (Hoh et al., 2002).

Molecular cloning of mir-34b and mir-34c. Genomic DNA encoding *mir-34b* and/or *mir-34c* was cloned using standard molecular biology techniques. Briefly, a 347 bp, 258 bp or 795 bp fragment of mouse DNA containing *mir-34b*, *mir-34c* or both sequences, respectively, was PCR amplified with Herculase II Fusion DNA Polymerase (Stratagene, La Jolla, CA). Primers contained restriction sites for EcoRI or BamHI (Table 2.1) and PCR products were ligated into pCDH-MCS1-EF1-Puro (System Biosciences, Mountain View, CA). Infectious lentiviral particles were prepared using ViraPower Lentiviral Packaging Mix (Invitrogen, Carlsbad, CA) per the manufacturers' protocol. Viral titer was also calculated per the manufacturers' protocol and cells were transduced with approximately 1 MOI. For stable transduction, transduced cells were cultured in 4 μg/ml puromycin until non-transduced cells were all killed.

Table 2.1 miR-34b/c amplification primers for lentivirus construction.

Gene	**Forward primer***	**Reverse primer***	**Product size**
miR-34b	ATACTGAATTCGGGA GCCTGAGGCACCTCT	TGAATGGATCCGCCGG TCTCCGAGGGTTA	347 bp
miR-34c	TATCAGAATTCCAAGG CAGCGACTAGAGTC	GCAATGGATCCTCCCTA TGGCTCTGTCCTCAC	258 bp
miR-34b/c	ATACTGAATTCGGGA GCCTGAGGCACCTCT	GCAATGGATCCTCCCTA TGGCTCTGTCCTCAC	795 bp

*Underlined sequence represents EcoRI recognition site (forward primer) or BamHI recognition site (reverse primer).

Pre-miR transfection and proliferation assay. Cells seeded in either 24-well plates or 8-well chamber slides were transfected with 33 nM or 66 nM pre-miR miRNA precursor molecules (Ambion, Austin, TX) using Lipofectamine2000 (Invitrogen, Carlsbad, CA), or transduced with lentivirus encoding miR-34b and/or miR-34c and assayed for proliferation status 48 hours later. Proliferation was quantified by bromodeoxyuridine (BrdU) incorporation assay as described previously (Nikitin and Lee, 1996). For estimation of proliferative indices, three representative images were collected per well using a SPOT-RT digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI).

Soft agar assay. Soft agar assay was performed essentially as described in (Reid, 1979). Briefly, 6 cm plates were covered in a Nobel agar base layer (0.5% agar, 10% FBS and 0.2% tryptone in DMEM). A top layer containing 5 $x10^5$ cells to be assayed were suspended in DMEM containing 10% FBS, 0.2% tryptone and 0.4% Nobel agar and pipetted on top of the base layer. After 3 days, growth medium was added to prevent the gel from drying.

Statistical Analyses. For statistical testing, two-sided unpaired Student's t-tests were performed using InStat 3.05 and Prism 4.03 software (GraphPad, Inc., San Diego, CA).

2.4 Results and discussion

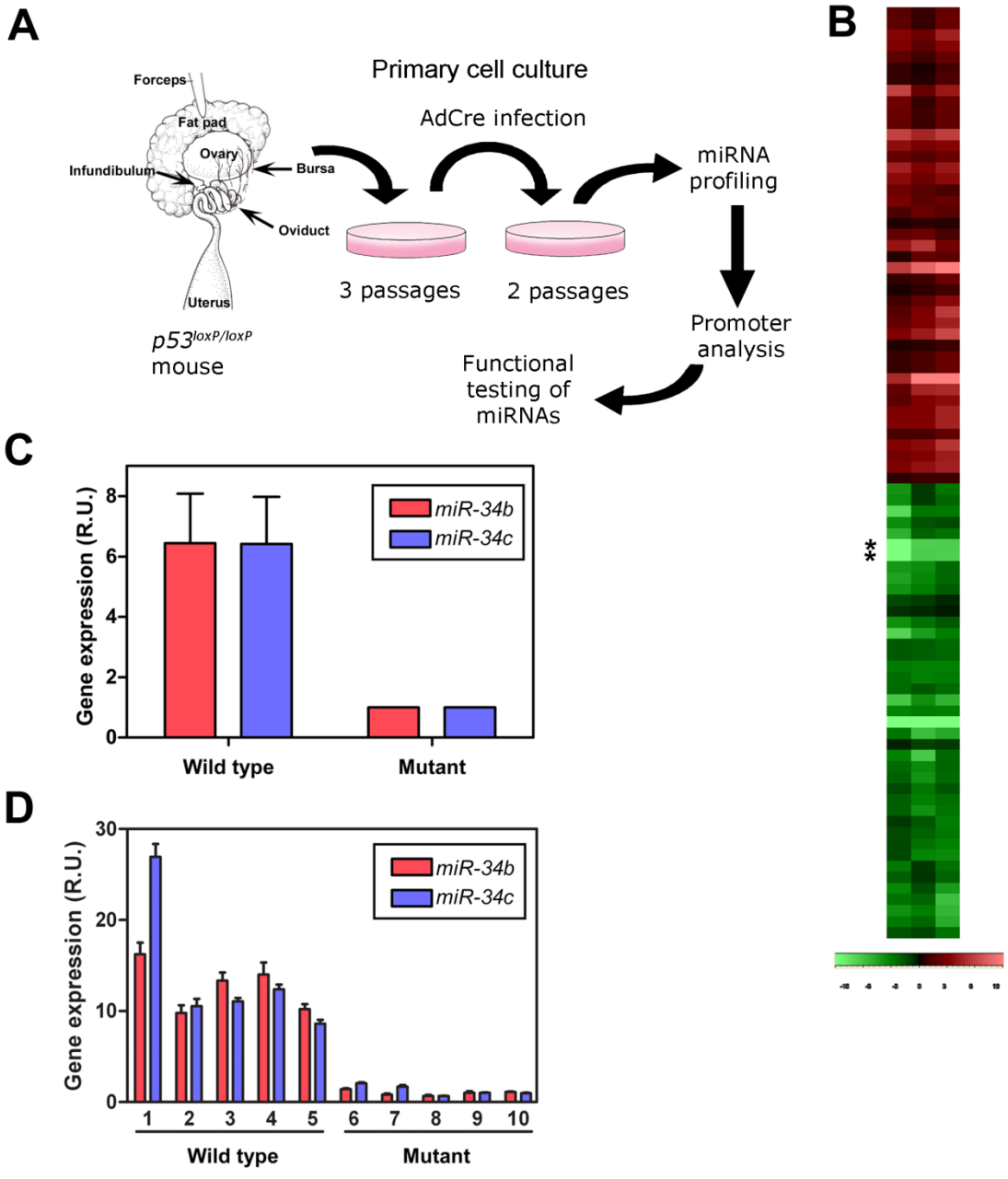
2.4.1 *p53* inactivation results in miRNAome alteration

To demonstrate that p53 either directly or indirectly regulates a subset of miRNAs, we performed miRNA microarray profiling of mouse ovarian surface epithelial cells subjected to the acute inactivation of *p53* (Figure 2.1A). A total of 84 miRNAs were significantly over or under expressed (Figure 2.1B), while the majority of miRNAs were unchanged or not expressed in either wild type or mutant, in agreement with previous data demonstrating spatiotemporal-specific expression of a high percentage of miRNAs (Liu et al., 2004). The 3 most downregulated miRNAs were the miR-34 family which consists of miR-34a, miR-34b and mir-34c. *mir-34a* is located at mouse chromosome 4qE2 while *mir-34b* and *mir-34c* are located 435bp apart on chromosome 9qA5 and appear to be coordinately expressed as a miRNA cluster. In order to confirm the microarray data (Figure 2.1C) we performed quantitative RT-PCR. Using stem loop primers, we were able to specifically amplify mature miRNA molecules and confirm approximately 12-fold down regulation of both miR-34b and miR-34c (Figure 2.1D).

2.4.2 Identification of a p53 responsive element upstream of the *mir-34b/c* locus.

In order to identify candidate p53-regulated miRNAs, we conducted an *in silico* screen for p53 responsive elements (p53REs). We focused our attention on downregulated miRNAs, since the p53 activation consensus sequence is well

Figure 2.1 miRNAome alterations after acute *p53* inactivation in primary mouse OSE cells. A, Outline of experiments. miRNA expression profile was generated after AdCre-mediated *p53* inactivation followed by computational miRNA promoter analysis for p53REs. Finally, the function of p53-dependent miRNAs was elucidated by experimental testing. B, Expression profile of top 84 miRNAs differentially expressed after acute *p53* inactivation in three independent experiments. miR-34b and miR-34c, two of the most downregulated miRNAs, are indicated by asterisks. C, Expression levels of miR-34b and miR-34c as judged by microarray analysis of 4 wild type and 5 p53 deficient (mutant) OSE cultures ($P= 0.055$ and $P= 0.046$ for miR-34b and miR-34c, respectively). D, Expression levels of miR-34b and miR-34c relative to RNU6B as judged by quantitative RT-PCR of 5 wild type and 5 mutant OSE cultures ($P<0.0001$ and $P= 0.0053$ for miR-34b and miR-34c, respectively). C, D, R.U., relative units.

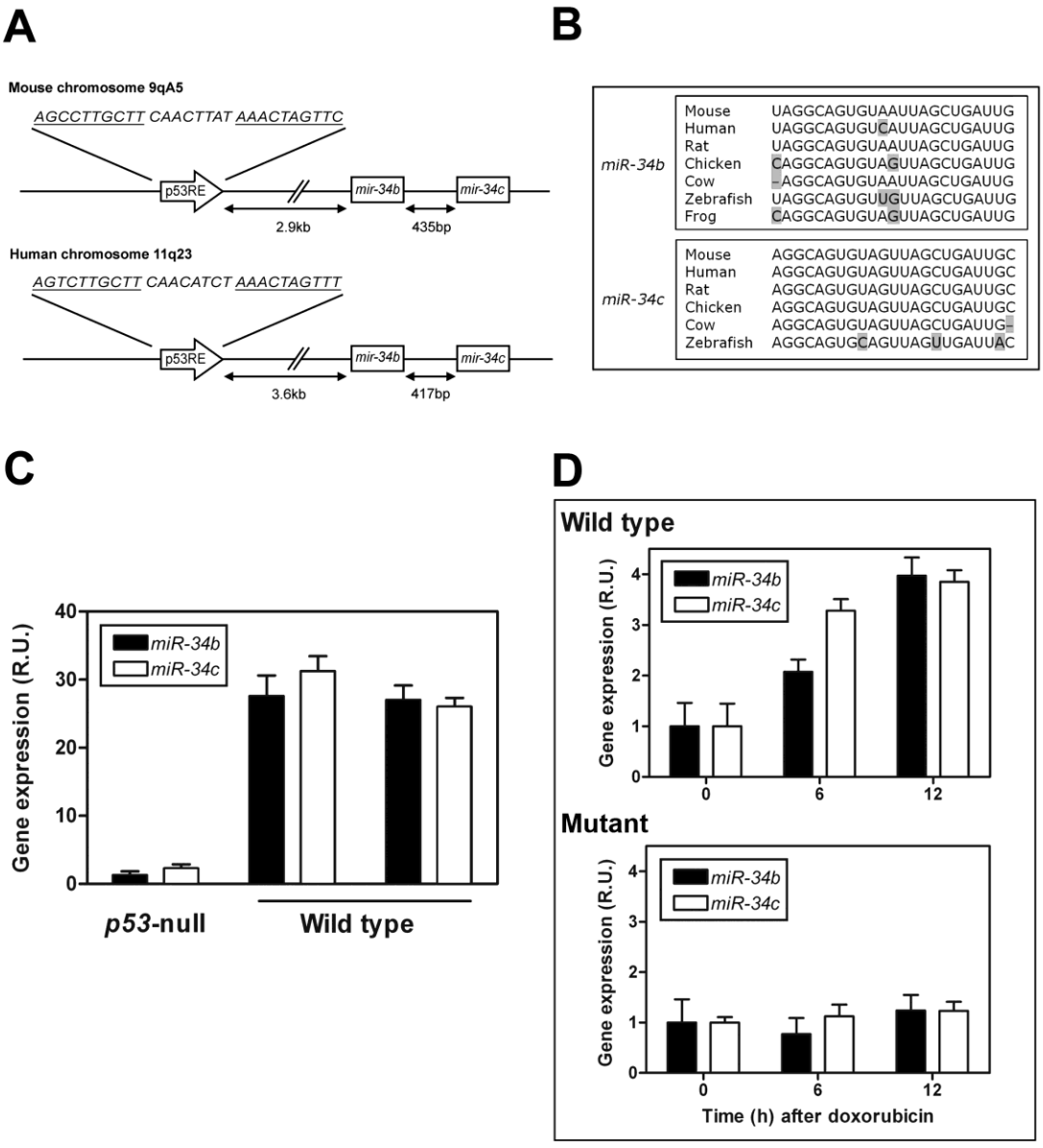

A
Forceps
Fat pad
Ovary
Infundibulum
Bursa
Oviduct
Uterus
p53loxP/loxP
mouse
Primary cell culture
AdCre infection
3 passages
2 passages
miRNA
profiling
Promoter
analysis
Functional
testing of
miRNAs
B
**
C
Gene expression (R.U.)
0
2
4
6
8
miR-34b
miR-34c
Wild type
Mutant
D
Gene expression (R.U.)
0
10
20
30
miR-34b
miR-34c
1 2 3 4 5 6 7 8 9 10
Wild type
Mutant

defined whereas in contrast the repression sequence is less so. In this respect, loss of p53-mediated transcriptional activation after *p53* inactivation will lead to a decrease in expression of the target gene (Yugawa et al., 2007). Towards this we took advantage of the p53MH algorithm (Hoh et al., 2002), which has previously identified novel p53-responsive genes (Feng et al., 2005), to search 5 kb up- and down-stream of each downregulated miRNA. Predicted p53 binding sites were identified upstream of 9 miRNAs.

Given that p53 is evolutionarily conserved, noteworthy p53 binding may also be expected to be evolutionarily conserved. Therefore we also searched the corresponding human miRNA locus for predicted binding sites. Three binding sites were conserved between human and mouse: *mir-129, mir-34b* and *mir-34c.* We decided to direct our attention towards miR-34b and miR-34c (Figure 2.2A), given that we observed a far greater reduction in expression of these two genes compared to miR-129. In addition to the conserved p53RE upstream of the miRNA locus, both miR-34b and miR-34c are remarkably well conserved between species (Figure 2.2B), suggesting that these miRNAs have critical roles in animals. Finally, in order to demonstrate that expression of miR-34b/c is lost in p53-deficient human cancer cells, we performed quantitative RT-PCR on RNA isolated from briefly cultured wild type human OSE cells and the *p53*-null cell line SKOV-3, which was derived from adenocarcinoma of the ovary. In good agreement with miR-34b/c expression in our mouse model, both miRNAs were dramatically reduced in the *p53*-null cells (Figure 2.2C).

We next decided to establish if p53 activation induces expression of miR-34b/c. Doxorubicin leads to DNA strand breaks and a physiological increase in p53 protein, partly through its stabilization by posttranslational

Figure 2.2 miR-34b and miR-34c are direct targets of p53. A, Structure of the mouse and human *mir-34b/c* locus. Computational analysis identified a p53RE approximately 3 kb upstream of the miRNA coding sequence, which is conserved between mouse and human. B, In addition to conservation of a p53RE, miR-34b and miR-34c mature sequences are remarkably well conserved among species. C, miR-34b and miR-34c expression relative to RNU6B in *p53*-null SKOV-3 human OSE cell line is considerably lower than expression in wild type human OSE. D, Doxorubicin treatment (0.5 µg/ml) of wild type OSE cultures results in a rapid increase in expression of both miR-34b and miR-34c (top, P= 0.0004). In contrast, no such induction is observed in identically treated *p53* mutant OSE cultures (bottom). C, D, R.U., relative units.


A
Mouse chromosome 9qA5
AGCCTTGCTT CAACTTAT AAACTAGTTC
p53RE
mir-34b
mir-34c
2.9kb
435bp
Human chromosome 11q23
AGTCTTGCTT CAACATCT AAACTAGTTT
p53RE
mir-34b
mir-34c
3.6kb
417bp
B
miR-34b
Mouse UAGGCAGUGUAAUUAGCUGAUUG
Human UAGGCAGUGUCAUUAGCUGAUUG
Rat UAGGCAGUGUAAUUAGCUGAUUG
Chicken CAGGCAGUGUAGUUAGCUGAUUG
Cow -AGGCAGUGUAAUUAGCUGAUUG
Zebrafish UAGGCAGUGUUGUUAGCUGAUUG
Frog CAGGCAGUGUAGUUAGCUGAUUG
miR-34c
Mouse AGGCAGUGUAGUUAGCUGAUUGC
Human AGGCAGUGUAGUUAGCUGAUUGC
Rat AGGCAGUGUAGUUAGCUGAUUGC
Chicken AGGCAGUGUAGUUAGCUGAUUGC
Cow AGGCAGUGUAGUUAGCUGAUUG-
Zebrafish AGGCAGUGCAGUUAGUUGAUUAC
C
Gene expression (R.U.)
miR-34b
miR-34c
p53-null
Wild type
D
Wild type
Gene expression (R.U.)
miR-34b
miR-34c
Mutant
Gene expression (R.U.)
miR-34b
miR-34c
Time (h) after doxorubicin

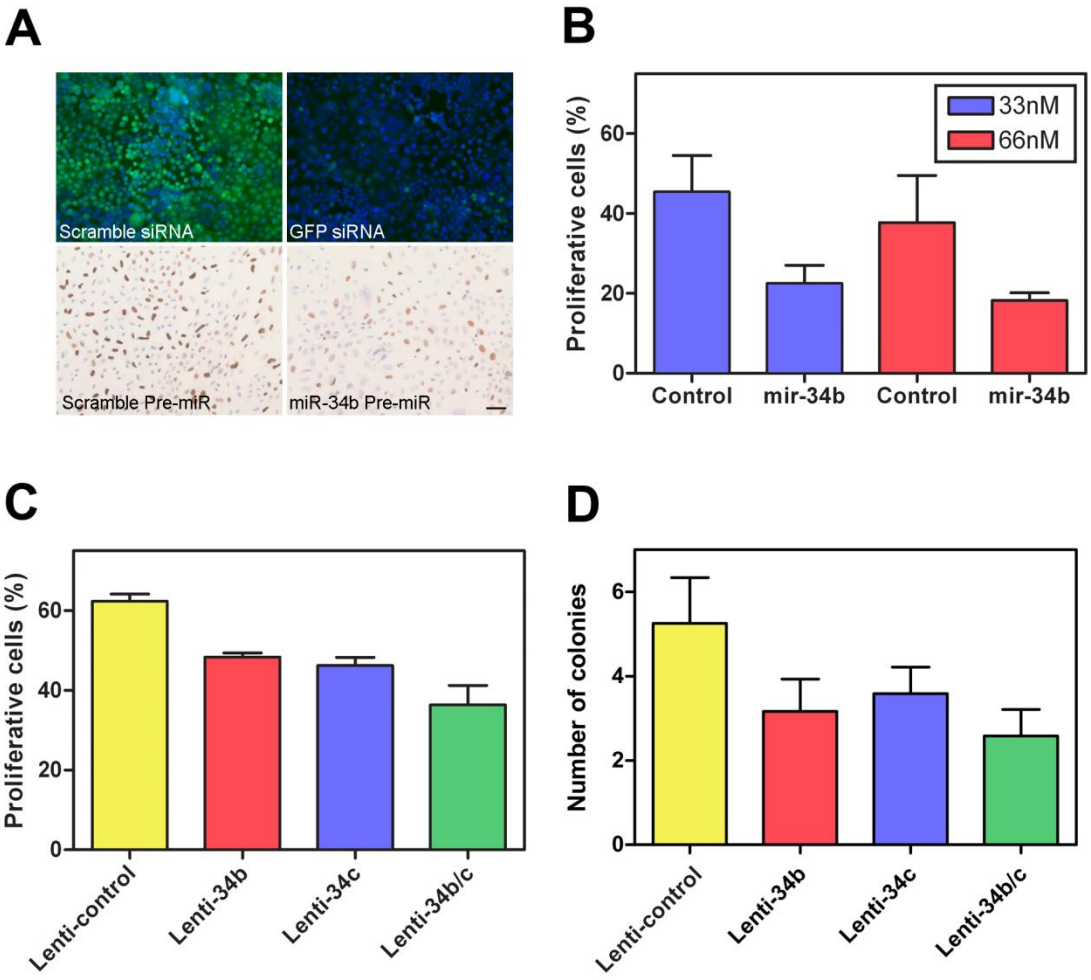
modifications via the DNA damage pathway. We therefore treated cells with 0.5 µg/ml doxorubicin. While a 4-fold increase in miR-34b/c expression was observed by 12 hours of doxorubicin exposure in wild type OSE cells, no such increase was observed in *p53* mutant OSE cells (Figure 2.2D), consistent with computational analysis predicting that these miRNAs contain a p53RE and are therefore p53-responsive.

2.4.3 miR-34b and miR-34c cooperate in reducing proliferation and adhesion-independent growth.

In order to characterize the roles of these p53-dependent miRNAs, we transfected OSN1 or OSN2 cells with synthetic miRNAs for miR-34b. 48 hours post transfection, proliferation index was determined by BrdU incorporation. A visible reduction in proliferation was observed upon miR-34b transfection compared to a non-targeting negative control molecule in OSN1 cells (Figure 2.3A), which was also somewhat dose-dependent (Figure 2.3B).

To generate cell lines with stable integrations of miR-34b and/or miR-34c, we cloned the miRNA and surrounding genomic sequence into a lentivirus vector. Quantitative RT-PCR of miR-34b and miR-34c in cells after puromycin selection demonstrated an increase in miRNA expression (Figure 2.4). Stably transduced OSN2 cells demonstrated a significant reduction in proliferation (Figure 2.3C). Interestingly, while transduction of either lenti-34b or lenti-34c individually reduced percent of proliferating cells (Mean ± SD, 48.4 ±1.8%, P= 0.0025 and 46.2 ± 3.6%, P= 0.0041, respectively, compared to 62.4 ± 3.1% for blank virus), transduction of both miRNAs reduced proliferation to an even greater extent (36.3 ± 8.4%, P= 0.0074). Furthermore,

Figure 2.3 miR-34b and miR-34c cooperate in decreasing proliferation and anchorage-independent growth. A, GFP-expressing OSN2 cells were either untreated (upper left) or transfected with 33 nM siRNA directed against GFP (upper right), demonstrating efficient knockdown. Transfection of OSN2 cells with 33 nM non-targeting synthetic miRNA molecules (lower left) or synthetic miR-34b (lower right) followed by BrdU administration. A significant decrease in cell proliferation as determined by BrdU incorporation is observed 48 hours after miR-34b transfection. B, Quantitative assessment of cell proliferation after transfection with 33 nM or 66 nM synthetic miR-34b demonstrates significantly reduced percent of BrdU incorporating cells as compared to non-targeting control molecule (33 nM: miR-34b versus control, P= 0.0042; 66 nM: miR-34b versus control, P= 0.0213). C, Quantitative assessment of OSN1 cell proliferation after transduction with either control (blank) lentivirus or lentivirus encoding for mir-34b and/or mir-34c. A significant reduction in proliferating cells was observed for each treatment compared to control lentivirus (Lenti-control versus Lenti-34b, P= 0.0025; Lenti-34c, P= 0.0041; Lenti-34b/c, P= 0.0074). D, Quantitative assessment of soft agar colony formation by OSN1 cells transduced with Lenti-34b/c demonstrates a significant decrease in colony formation in soft agar (Lenti-control versus Lenti-34b/c, P= 0.0214). *A*, GFP fluorescence with DAPI counterstain (upper), BrdU, ABC Elite method, hematoxylin counterstaining (bottom). Bar, 50 μm (*A*).


A
Scramble siRNA
GFP siRNA
Scramble Pre-miR
miR-34b Pre-miR
B
Proliferative cells (%)
33nM
66nM
0
20
40
60
Control
mir-34b
Control
mir-34b
C
Proliferative cells (%)
0
20
40
60
Lenti-control
Lenti-34b
Lenti-34c
Lenti-34b/c
D
Number of colonies
0
2
4
6
Lenti-control
Lenti-34b
Lenti-34c
Lenti-34b/c

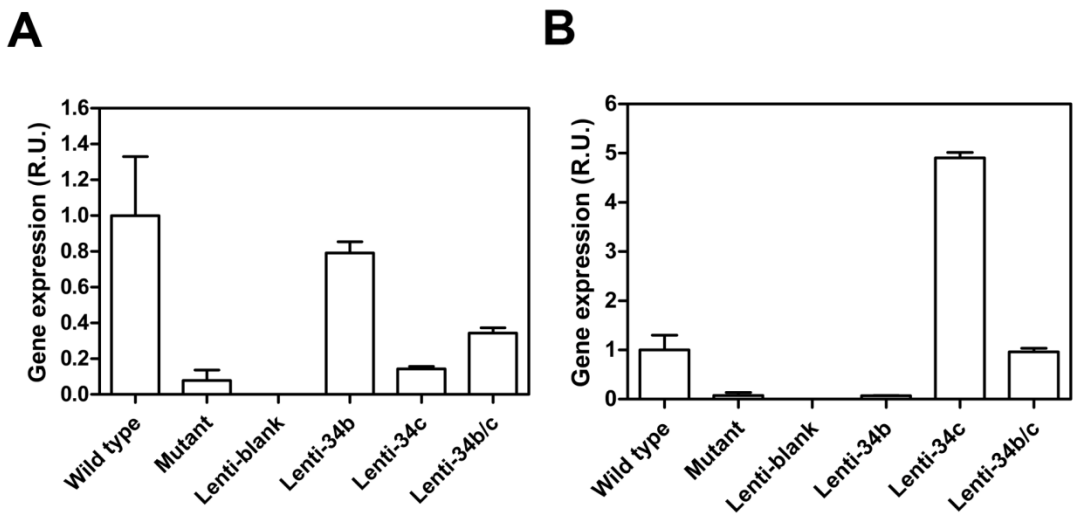


Figure 2.4 miR-34b/c expression after lentivirus transduction. OSN1 cells (*p53* mutant) were transduced with blank lentivirus or lentivirus containing genomic sequence of miR-34b and/or miR-34c. After puromycin selection, total RNA was isolated and quantitative RT-PCR performed, demonstrating increased miRNA expression. Expression of miR-34b (A) and miR-34c (B) in wild type cells and in cells after acute *p53* inactivation is shown for comparison. *A*, miR-34b expression in mutant (Mean ± SD): 0.078 ± 0.059 versus Lenti-34b: 0.792 ± 0.062, P= 0.0001, n= 4; versus Lenti-34c: 0.143 ± 0.014, P= 0.1369, n= 4; versus Lenti-34b/c: 0.343 ± 0.03, P= 0.0023, n= 4. *B*, miR-34c expression in mutant (Mean ± SD): (0.079 ±0.059) versus Lenti-34b: 0.073 ± 0.005, P= 0.8692, n= 4; Lenti-34c: 4.912 ± 0.11, P< 0.0001, n=4; Lenti-34b/c: 0.962 ± 0.077, P<0.0001, n=4. *A, B,* R.U., relative units.

a cooperative relationship was also observed when transduced OSN1 cells were cultured in soft agar (Figure 2.3D). Transduction of lenti-34b and lenti-34c reduced the number of colonies per 4x field of view from 5.3 ± 1.1 for cells transduced with blank virus to 3.2 ± 0.8 (P= 0.0534) and 3.6 ± 0.6 (P= 0.0835), respectively, whereas statistically significant reduction was observed in lenti-34b/c- transduced cells (2.9 ± 0.6 colonies, P= 0.0214). These data suggest that while

the sequences, and therefore predicted targets, of miR-34b/c are very similar, their differences have a significant consequence on biological activity and that maximal suppression of proliferation and anchorage-independent growth is achieved only when both miRNAs are expressed. In particular, Delta-like 1, Notch1, Met and Ezh2 are all predicted targets for both miR-34b and miR-34c. In contrast, Myc and Cdk6 are among predicted targets for miR-34b and E2f3, Bcl2 and Cyclin D1 for miR-34c (Griffiths-Jones et al., 2006).

2.4.4 A model for p53-dependent miRNA-mediated gene silencing.

Taken together, our data illustrate a novel mechanism for p53-mediated control of gene expression. As outlined in Figure 2.5, p53 is activated by DNA damage and directly induces expression miR-34b and miR-34c through a p53RE approximately 3 kb upstream of the coding sequence. This activation, in turn, leads to repression of target genes. Although these two miRNAs share significant sequence similarity, their predicted targets are not perfectly conserved, thereby explaining cooperative effects of miR-34b and miR-34c. It should be noted that different stimuli, such as inappropriate mitogenic signaling, hypoxia, and spindle damage, etc., may result in disparate

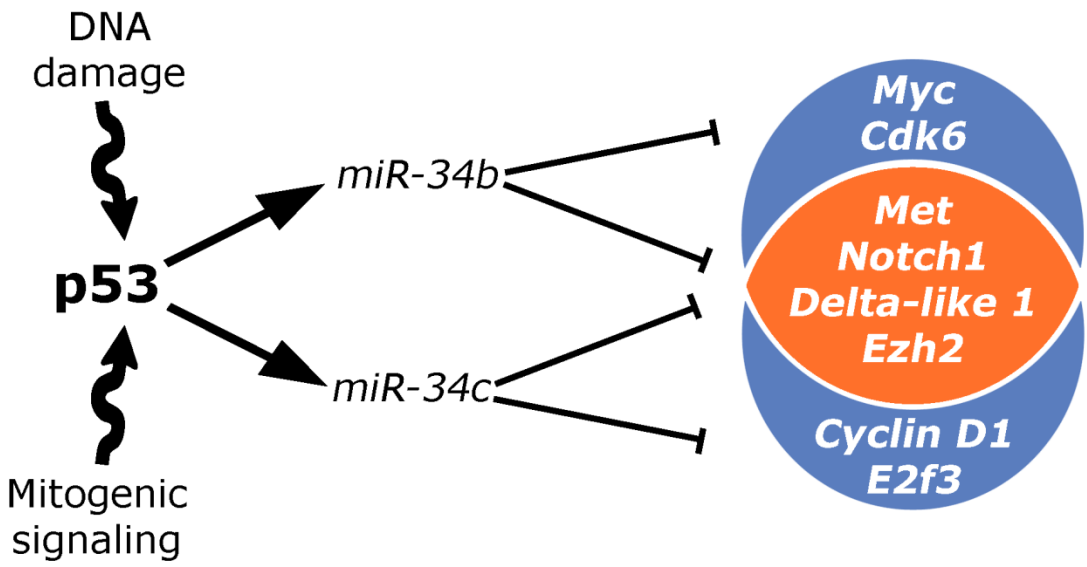


Figure 2.5 A model for p53-dependent miRNA-mediated repression of gene expression. p53 activation by DNA damage and possibly other stimuli, such as mitogenic signaling, lead to a rapid increase in expression of the p53-dependent miRNAs miR-34b and miR-34c. These miRNAs are predicted to bind a large number of target mRNAs; a selection of these targets are shown. Predicted targets of each miRNA are both independent (top and bottom) and conserved (middle).

consequences as a result of p53 binding to different subsets of its target genes (Levine et al., 2006). Whether miR-34b and miR-34c may have different extent of p53-dependant activation by other stimuli remains to be determined. The discovery of inhibitory effects of these microRNAs on such critical components of neoplastic growth as cell proliferation and adhesion-independent colony formation opens an exciting opportunity for development of novel therapeutic approaches utilizing these small molecules.

After submission of our paper, He et al. reported p53-dependent regulation of miR-34b/c in mouse embryonic fibroblasts and IMR90 fibroblasts and demonstrated reduction of cell growth and induction of senescence after ectopic expression of miR-34b/c in IMR90 fibroblasts (He et al., 2007). Taken together with our observations of miR-34b/c effects on cell proliferation and adhesion-independent growth of OSE, these results indicate that miR-34b/c play important roles in controlling carcinogenesis in various cell types, likely due to the diversity of their mRNA targets.

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CHAPTER 3

FREQUENT DOWNREGULATION OF MIR-34 FAMILY IN HUMAN OVARIAN CANCERS*

3.1 Abstract

Purpose: The miR-34 family is directly transactivated by tumor suppressor p53 which is frequently mutated in human epithelial ovarian cancer (EOC). We hypothesized that miR-34 expression would be decreased in EOC and that reconstituted miR-34 expression might reduce cell proliferation and invasion of EOC cells.

Experimental designs: miR-34 expression was determined by quantitative RT-PCR and *in situ* hybridization in a panel of 83 human EOC samples. Functional characterization of miR-34 was accomplished by reconstitution of miR-34 expression in EOC cells with synthetic pre-miR molecules followed by determining changes in proliferation, apoptosis and invasion.

Results: miR-34a expression is decreased in 100%, and miR-34b*/c in 72%, of EOC with *p53* mutation, while miR-34a is also downregulated in 93% of tumors with wild-type *p53*. Furthermore, expression of miR-34b*/c is significantly reduced in stage IV tumors compared to stage III (p= 0.0171 and p= 0.0033, respectively). Additionally, we observed promoter methylation and copy number variations at *mir-34* loci. *In situ* hybridization demonstrated that miR-34a expression is inversely correlated with MET immunohistochemical staining, consistent with translational inhibition by miR-34a. Finally, miR-34

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reconstitution experiments in *p53* mutant EOC cancer cells resulted in reduced proliferation, motility and invasion, the latter of which was dependent on MET expression.

Conclusions: Our work suggests that miR-34 family plays an important role in EOC pathogenesis and reduced expression of miR-34b*/c may be particularly important for progression to the most advanced stages. Part of miR-34 effects on motility and invasion may be explained by regulation of MET, which is frequently overexpressed in EOC.

3.2 Translational relevance

Diagnosis and treatment of EOC is particularly difficult due to poor understanding of disease pathogenesis. We show that miR-34 expression is frequently decreased in EOC due to several mechanisms, but mainly due to *p53* mutation. Importantly, reconstitution of miR-34 in human ovarian cancer cells results in decreased proliferation and invasion, at least partially by inhibition of the MET oncogene. Taken together, these data suggest that miR-34 family is important for EOC development and may be an attractive candidate for development of novel therapeutic approaches.

3.3 Introduction

Ovarian cancer is the most deadly malignancy and will lead to almost 15,000 deaths in the USA in 2009 (Jemal et al., 2009). While survival has increased slightly over the past 25 years, 5-year survival remains below 50%. A major factor for low survival is our poor understanding of the initiating events that

lead to ovarian cancer and how the disease progresses. Due to asymptomatic development and few screening options, almost 70% of women present at late stages of carcinogenesis. At an advanced stage, treatment options are severely limited, with palliative treatment most often administered in the form of debulking surgery and paclitaxel and platinum based therapeutics. However, work over the past decade using human cancer samples and mouse models have revealed new insights into the molecular basis of ovarian cancer, particularly its most common form, epithelial ovarian cancer (EOC). For example, it is well established that over 50% of high-grade serous type EOC contain *p53* mutations and alterations in the RB pathway (reviewed in Bast et al., 2009; Corney et al., 2008). Consistently, conditional inactivation of *p53* and *Rb* in the mouse ovarian surface epithelium (OSE) leads to development of poorly differentiated serous ovarian adenocarcinomas (Flesken-Nikitin et al., 2003), while K-Ras, Pten and Wnt/beta-catenin are implicated in carcinogenesis of the endometrioid EOC subtype (Dinulescu et al., 2005; Wu et al., 2007).

In recent years, the involvement of small non-coding RNAs called microRNAs (miRNAs) in cancers of many types has become unambiguous, including in ovarian cancer (Iorio et al., 2007; Zhang et al., 2008). Although the precise roles they play during carcinogenesis are still being dissected, it is clear that miRNAs can act as tumor suppressors and oncogenes by regulating processes such as proliferation and the cell cycle, apoptosis, invasion and metastasis (reviewed in Esquela-Kerscher and Slack, 2006). miRNAs have been found to be dysregulated in cancer by DNA copy number changes and epigenetic alterations, altered processing by the miRNA biogenesis machinery and through altered transcriptional activation. In particular, the transcription

factor and tumor suppressor p53 has been independently shown by several laboratories to directly transactivate genes of the miR-34 family, which is comprised of three members (Bommer et al., 2007; Chang et al., 2007; Corney et al., 2007; He et al., 2007; Raver-Shapira et al., 2007; Tarasov et al., 2007). Gene encoding miR-34a is located on human chromosome 1p36, while miR-34b and miR-34c are co-transcribed from one transcription unit on chromosome 11q23. The miR-34 family downregulates numerous important regulatory proteins and thereby presumably mediates tumor suppression (reviewed in Hermeking, 2010).

Previously, we have demonstrated that conditional inactivation of *p53* results in miR-34 downregulation in mouse OSE (Corney et al., 2007). To evaluate the potential roles of miR-34 family in human EOC, we have determined their expression level in a panel of 83 cancer tissues and found that miR-34 expression is frequently decreased in EOC, is associated with metastatic clinical stage and increased expression of receptor protein tyrosine kinase MET. Furthermore, reconstitution of miR-34 expression in EOC cells leads to reduced proliferation and invasion, as well as decreased MET levels.

3.4 Materials and methods

Clinical samples. Informed consent was obtained from patients undergoing surgery for ovarian cancer at Fox Chase Cancer Center, Philadelphia, PA and M.D. Anderson Cancer Center, Houston, TX. Sample collection was performed after approval by an Institutional Review Board and a portion of tumor tissue not required for diagnostic purposes was snap frozen in liquid nitrogen and stored at -80°C. Surgical evaluation was used to determine

clinical stage and presence of metastases, while histopathological analysis by gynecologic pathologists was performed to assess cancer type and subtype. Only tumors found to contain over 70% tumor cells were used in the study and tissue sample and clinical data was available for 83 patients (Table 3.1). Additional formalin-fixed paraffin embedded specimens were obtained from New York-Presbyterian Hospital/Weill Cornell Medical Center, New York, NY.

miR-34 nomenclature. miRNA nomenclature has recently been revised such that the miR-34b sequence has been renamed miR-34b* (i.e. the passenger strand) (Landgraf et al., 2007). Nevertheless, “star” or passenger strands of the miRNA duplex have previously been shown to be biologically important (e.g. miR-199a* and miR-10* (Migliore et al., 2008; Stark et al., 2007). Although both miR-34b strands are likely to be functional, the miR-34b strand is not predicted to bind the 3’ UTR of MET and our studies have therefore focused on the miR-34b* strand. It is worth noting that our qRT-PCR data demonstrate that both strands are present at equal quantities and are highly correlated (Figure 3.1); they therefore might be renamed miR-34b-3p and miR-34b-5p, respectively, consistent with mouse miR-34b nomenclature.

p53 mutation screening. The IARC protocol (http://www-p53.iarc.fr) was followed for *p53* mutation screening. DNA was isolated by Qiagen DNeasy mini kit (Qiagen, Valencia, CA) and exons 4-11, including splice junctions, were amplified by PCR and sequenced with both forward and reverse primers (Table 3.2). In the case sequencing data was unclear, T-vector cloning was performed, and three clones sequenced by T7 and SP6 primers.

Table 3.1 Characteristics of 83 patients with epithelial ovarian cancer.

	Characteristic	Value
Age (years)	Mean	60.8
	Range	28-87
Race (%)	White	64 (77.1)
	African American	5 (6)
	Other non-white	6 (7.2)
	Unknown	2 (2.4)
Tumor stage (%)	I	2 (2.4)
	II	4 (4.8)
	III	46 (55.4)
	IV	18 (21.7)
	TX	13 (15.7)
Histology	Serous	62 (74.7)
	Mucinous	2 (2.4)
	Endometrioid	3 (3.6)
	Clear cell	1 (1.2)
	Adenocarcinoma, NOS/undifferentiated	4 (4.8)
	Mixed	11 (13.3)

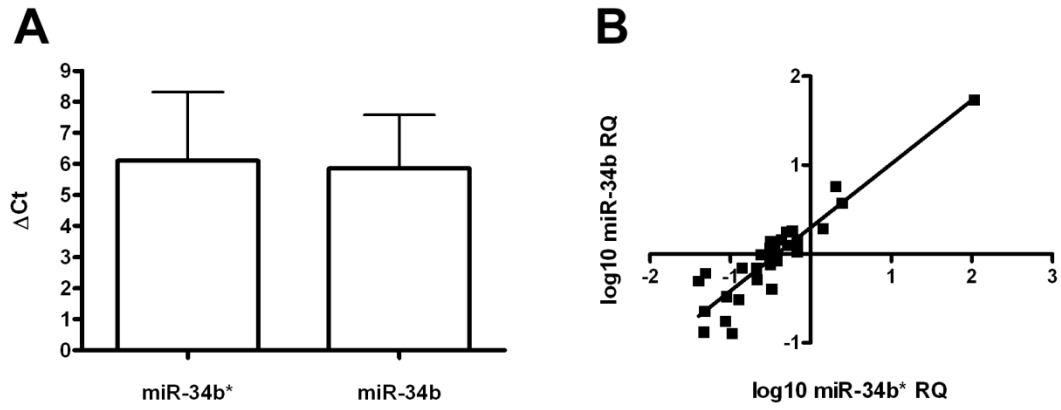


Figure 3.1 miR-34b and miR-34b* are present in equal amounts and are highly correlated. A. miR-34b* and miR-34b strands are present in equal quantities in RNA isolated from EOC specimens (n=30, p= 0.1764, 2-tailed paired t-test. B. miR-34b* and miR-34b show significant correlation in their expression (r^2= 0.8497, p<0.0001). Error bars represent standard deviation.

Table 3.2 List of primers used for *p53* mutation analysis and *mir-34a* copy number and methylation analysis.

Gene		Forward primer	Reverse primer	Probe
miR-34a		AGTGTCTTAGCTGGTTGTTGTGA	GCAGCACTTCTAGGGCAGTAT	TTGCTGATTGCTTCCTTACTATTGC
LINE1		GCTCCTGAATGACTACTGGGTACA	GTGTCTTTGTTCTCGTTGGTTTCAA	ACGAAATGAAGGCAGAAATAAA
p53	Exon 4	TGCTCTTTTCACCCATCTAC	ATACGGCCAGGCATTGAAGT	N/A
	Exon 5	TTCAACTCTGTCTCCTTCCT	CAGCCCTGTCGTCTCTCCAG	N/A
	Exon 6	GCCTCTGATTCCTCACTGAT	TTAACCCCTCCTCCCAGAGA	N/A
	Exon 7	AGGCGCACTGGCCTCATCTT	TGTGCAGGGTGGCAAGTGGC	N/A
	Exon 8/9	TTGGGAGTAGATGGAGCCT	AGTGTTAGACTGGAAACTTT	N/A
	Exon 10	CAATTGTAACTTGAACCATC	GGATGAGAATGGAATCCTAT	N/A
	Exon 11	AGACCCTCTCACTCATGTGA	TGACGCACACCTATTGCAAG	N/A
miR-34a methylated		GGTTTTGGGTAGGCGCGTTTC	TCCTCATCCCCTTCACCGCCG	N/A
miR-34a unmethylated		IIGGTTTTGGGTAGGTGTGTTTT	AATCCTCATCCCCTTCACCACCA	N/A
miR-34b/c* methylated		TTTAGTTACGCGTGTTGTGC	ACTACAACTCCCGAACGATC	N/A
miR-34b/c* unmethylated		TGGTTTAGTTATGTGTGTTGTGT	CAACTACAACTCCCAAACAATCC	N/A

Quantitative RT-PCR. Total RNA was isolated using mirVana miRNA Isolation Kit (Ambion, Austin, TX) according to the manufacturer's protocol and RNA concentration and purity determined by NanoDrop analysis. Stem-loop qRT-PCR for mature miR-34 and miR-199a* miRNAs was performed as previously described (Chen et al., 2005). For *MET* qRT-PCR, cDNA was prepared from 100 ng total RNA using SuperScript III (Invitrogen, Carlsbad, CA) and amplified with TaqMan primer/probes. All PCR reactions were performed in triplicate on an AB 7500 Real Time PCR system (Applied Biosystems Inc, Foster City, CA) and miRNA and mRNA expression normalized to *RNU6B* and *GAPDH*, respectively, using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

*MET immunohistochemistry***.** Paraffin sections of formalin-fixed tissue were stained according to modified avidin-biotin-peroxidase technique (Nikitin and Lee, 1996). The antibody used for detection of MET was CVD13 from Zymed Laboratories (dilution 1:200).

In situ hybridization. Detection of miR-34 in a panel of serous adenocarcinomas was performed by the protocol adapted from Nelson et al. (Nelson et al., 2006). In order to prevent the loss of miRNAs, we additionally applied 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) fixation as described in (Pena et al., 2009). In brief, 6 µm-thick sections of formalin-fixed paraffin-embedded material were deparaffinized, rehydrated, and fixed with EDC. After 1 hour prehybridization, a DIG-labeled LNA probe (Exiqon, Woburn, MA) was hybridized to Proteinase K-treated sections at 56°C for 16 hours. Slides were then incubated with anti-DIG-AP antibody (Roche,

Indianapolis, IN), and microRNA expression was detected by NBT/BCIP method. Methyl green was used for nucleic counter staining.

Quantitative PCR. For copy number variation analysis, DNA was isolated with a DNeasy DNA mini kit (Qiagen, Valencia, CA) and 60 ng DNA amplified with custom TaqMan real time primers and probes that were designed to amplify across the *mir-34a* locus (Table 3.2). Copy number was calculated by normalizing to *LINE1* retrotransposon gene copy number, which is maintained at relatively constant levels in neoplastic and normal tissues (Kuo et al., 2009; Wang et al., 2002), using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Primer sequences are given in Table 3.2. A total of 30 serous, 1 mucinous and 2 tumors of undetermined histology with clinical characteristics similar to the entire cohort were analyzed. Pooled human placental DNA served as a wild type control (Bioline, Taunton, MA).

Methylation-specific PCR analysis. Genomic DNA isolated as described above was treated with bisulfite using the EZ DNA methylation kit (Zymo Research, Orange, CA). A total of 27 serous, 1 mucinous and 2 undetermined EOC tumors with clinical characteristics similar to the entire cohort were analyzed. The modified DNA was eluted with a final volume of 16 µl, and 1 µl to 2 µl were used for the methylation specific polymerase chain reactions (MSP). In case of *mir-34a*, amplification conditions were 5 min at 95°C; 2 cycles of 20 sec at 95°C, 30 sec at 68°C, and 30 sec at 72°C; followed by 2 cycles with 66°C annealing temperature, then 34 cycles with 65°C annealing temperature and a 4 min final extension at 72°C. For *mir-34b*/c*, amplification conditions were 37 cycles of 20 sec at 95°C, 30 sec at 61°C and 30 sec at

72°C, 4 minutes final extension. Primers used were previously reported (Lodygin et al., 2008; Lujambio et al., 2008) and are listed in Table 3.2. PCR products were separated by electrophoresis on an 8% polyacrylamide gel and band intensities visually scored.

Cell culture experiments. Human *p53* mutant ovarian adenocarcinoma cell line SKOV-3 was obtained from the American Type Culture Collection and maintained according to the supplier's directions. For proliferation and apoptosis analysis, cells were seeded in triplicate in 24-well plates and the next day transfected with Pre-miR synthetic miRNA molecules (Ambion, Austin, TX) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Proliferation was assessed by BrdU incorporation and apoptosis assessed by cleaved caspase-3 staining 48 hours post-transfection as previously described (Nikitin and Lee, 1996; Zhou et al., 2006). For the apoptosis assay, cells were treated with 5 μM camptothecin for 17 hours to induce apoptosis. Invasion assays were performed in a chamber containing an 8 μm pore-size PET membrane coated with a uniform layer of BD Matrigel™ Basement Membrane (BD Bioscience, San Jose, CA). For MET knock down experiments, 2 μmol/mL of MET siRNA (sc-29397, Santa Cruz, Santa Cruz, CA) was transfected. 24 hours after transfection, 5 x 10^3 SKOV-3 cells per each well were seeded in a control insert or Matrigel insert in serum free media and translocated toward complete growth media containing 20 ng/μl hepatocyte growth factor (HGF). 20 hours after additional culture, invading cells were stained by Multiple Staining Solution (Polysciences, Inc., Warrington, PA) and counted under the microscope. All experiments were performed in triplicate and three different fields of each well were counted.

Western blotting. Cells were lysed with RIPA buffer (25mM Tris, pH 8.2, 50mM NaCl, 0.1% SDS, 0.5% Nonidet P-40, 0.5% deoxycholate) containing protease inhibitor cocktail (Roche, Indianapolis, IN). Lysates were resolved by 8% SDS-PAGE, transferred to PVDF membrane, and probed with antibodies against MET (clone C-28, Santa Cruz, Santa Cruz, CA), CDK4 (clone C-22, Santa Cruz, Santa Cruz, CA) and GAPDH (clone 6C5, Advanced Immunochemical, Long Beach, CA).

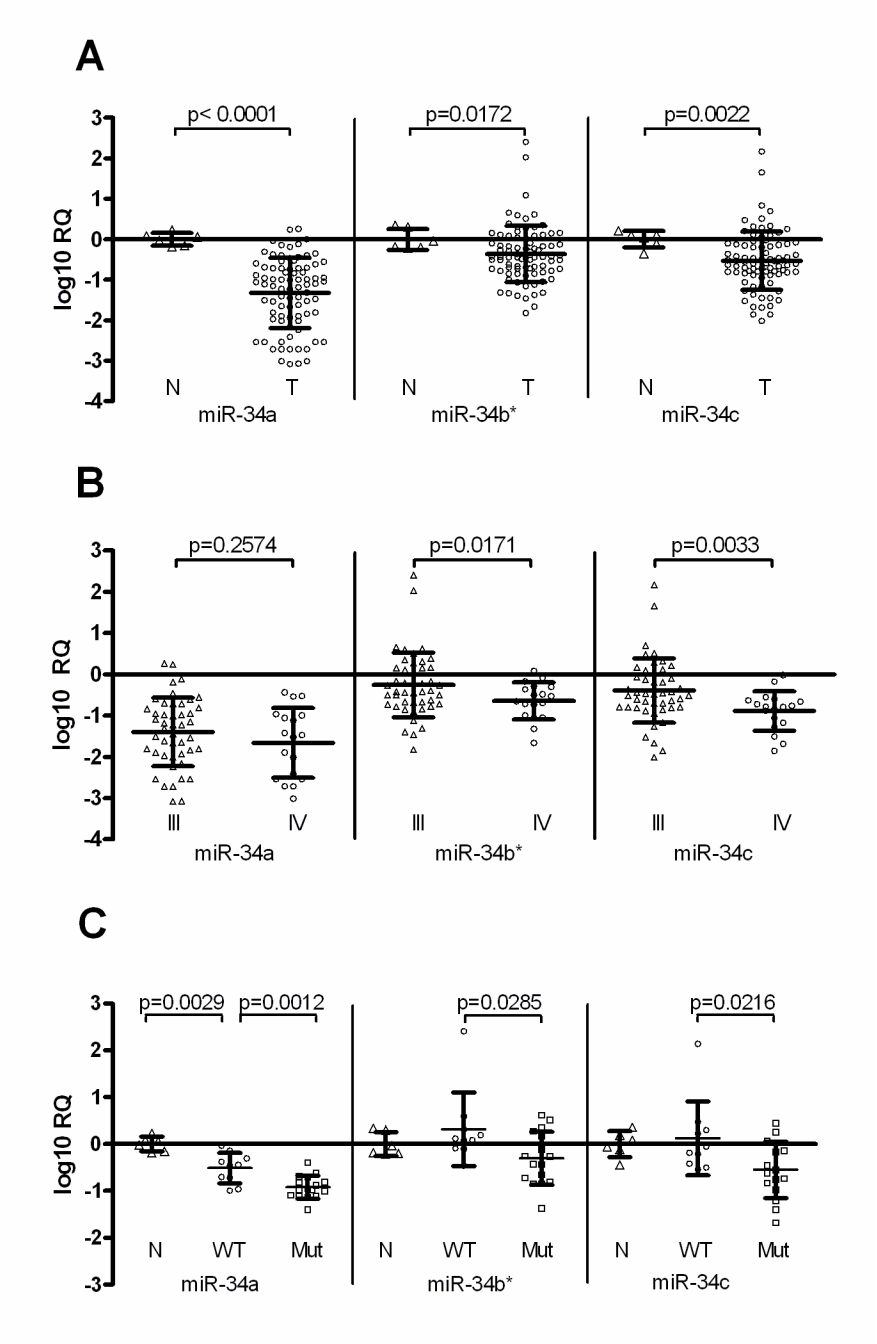
Statistical analysis. Statistical tests used were two-sided Student's t tests, with Welch's correction for unequal variance where appropriate, using InStat 3.05 and Prism 4.03 software (Graphpad, Inc., La Jolla, CA).

3.5 Results

3.5.1 miR-34 expression is reduced in EOC and is correlated with metastatic stage.

To determine miR-34 family expression in EOC, we isolated total RNA from 83 EOC samples and compared expression levels to that in six wild type OSE primary cell samples (Table 3.1). We observed significantly reduced expression for all three family members in EOC compared to wild type (Figure 3.2A), with miR-34a most significantly reduced by 21.2-fold ($p< 0.0001$), while miR-34b* and miR-34c were reduced 2.3-fold ($p= 0.0172$) and 3.4-fold ($p= 0.0002$), respectively. It has been recently reported that Drosha and Dicer

Figure 3.2 miR-34 is downregulated in EOC and is associated with metastatic clinical stage. A, qRT-PCR analysis reveals significantly reduced miR-34a, miR-34b*, and miR-34c expression in a panel of EOC samples (n=83; ○) relative to normal OSE samples (n=6; $P < 0.0001$, $P= 0.0172$, and $P= 0.0022$, respectively; Δ). B, ovarian cancer stage was determined by surgical evaluation during cryoreduction/debulking surgery. miR-34a, miR-34b*, and miR-34c expression is shown for stage III (n=46; Δ) and stage IV (n=18; ○) tumors. miR-34b* and miR-34c expression is significantly decreased in stage IV (distant metastasis) compared with stage III (localized to peritoneum; $P= 0.0171$ and 0.0033, respectively). C, adenocarcinomas (serous type, n=26) and wild-type OSE samples (n=6) were analyzed for *p53* mutational status by direct sequencing and correlated with miR-34 expression. Whereas both *p53* wild-type (WT; ○) and *p53* mutant (Mut; □) EOC samples show significantly reduced miR-34a expression compared with wild-type OSE (N; Δ), EOC samples with mutant *p53* show most downregulated expression for miR-34a, miR-34b*, and miR-34c ($P= 0.0012$, 0.0285, and 0.0216, respectively). Bars, SD.


A
p< 0.0001
p=0.0172
p=0.0022
log10 RQ
N
T
miR-34a
miR-34b*
miR-34c
B
p=0.2574
p=0.0171
p=0.0033
log10 RQ
III
IV
miR-34a
miR-34b*
miR-34c
C
p=0.0029
p=0.0012
p=0.0285
p=0.0216
log10 RQ
N
WT
Mut
miR-34a
miR-34b*
miR-34c

expression is deregulated in EOC (Merritt et al., 2008; Zhang et al., 2008). To test whether alteration in miR-34 expression can be explained by Drosha/Dicer-mediated global changes in miRNA processing, we determined miR-199a* expression, which has been shown to be deregulated in EOC (Iorio et al., 2007; Yang et al., 2008). miR-199a* expression appears to be elevated, although this is not statistically significant (Figure 3.3). No significant differences in expression of miR-34 family members were detected among different histological types of EOC, with an exception of reduced miR-34a expression in endometrioid compared to serous adenocarcinoma (Figure 3.4). Tumor staging is linked to survival, with stage III tumors having tumor cell dissemination in the peritoneum, while stage IV tumors have distant metastasis, commonly to liver, and is indicative of poor prognosis. We compared gene expression in stage III and stage IV tumors, and observed significantly reduced miR-34b* and miR-34c expression in stage IV tumors ($p= 0.0171$ and $p= 0.0033$, respectively) (Figure 3.2B), suggesting that miR-34b* and miR-34c may be involved in metastatic progression. Interestingly however, change in miR-34a expression was not statistically significant ($p= 0.2574$).

3.5.2 Decreased miR-34 expression is associated with *p53* mutation.

Mutation of *p53* is a common event in many human cancers but is particularly common in high-grade serous EOC (Corney et al., 2008). We therefore took a subset of our serous EOC samples (n= 26) and sequenced *p53* exons 4-11, where over 99% of *p53* mutations are located (Table 3.3). We have found that while miR-34a expression is reduced in both in samples with

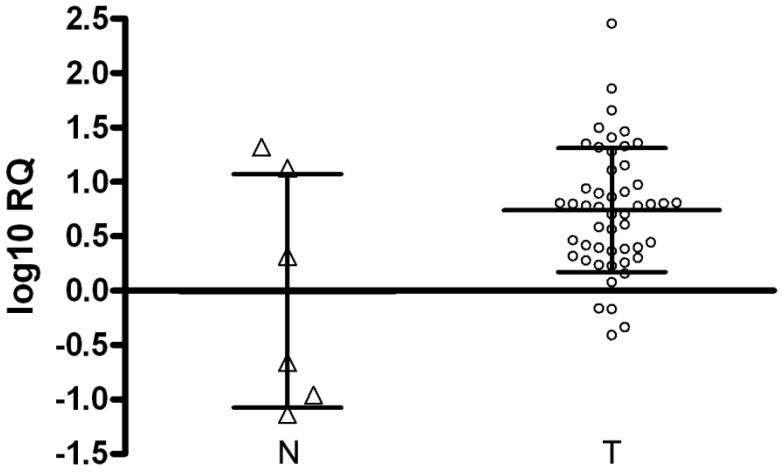


Figure 3.3 miR-199a* is not significantly altered in EOC. qRT-PCR profiling demonstrates no significant alteration in miR-199a* expression between EOC samples (n= 50, open circles) and normal OSE (n= 6, open triangles) (p= 0.1578). Error bars represent standard deviation.

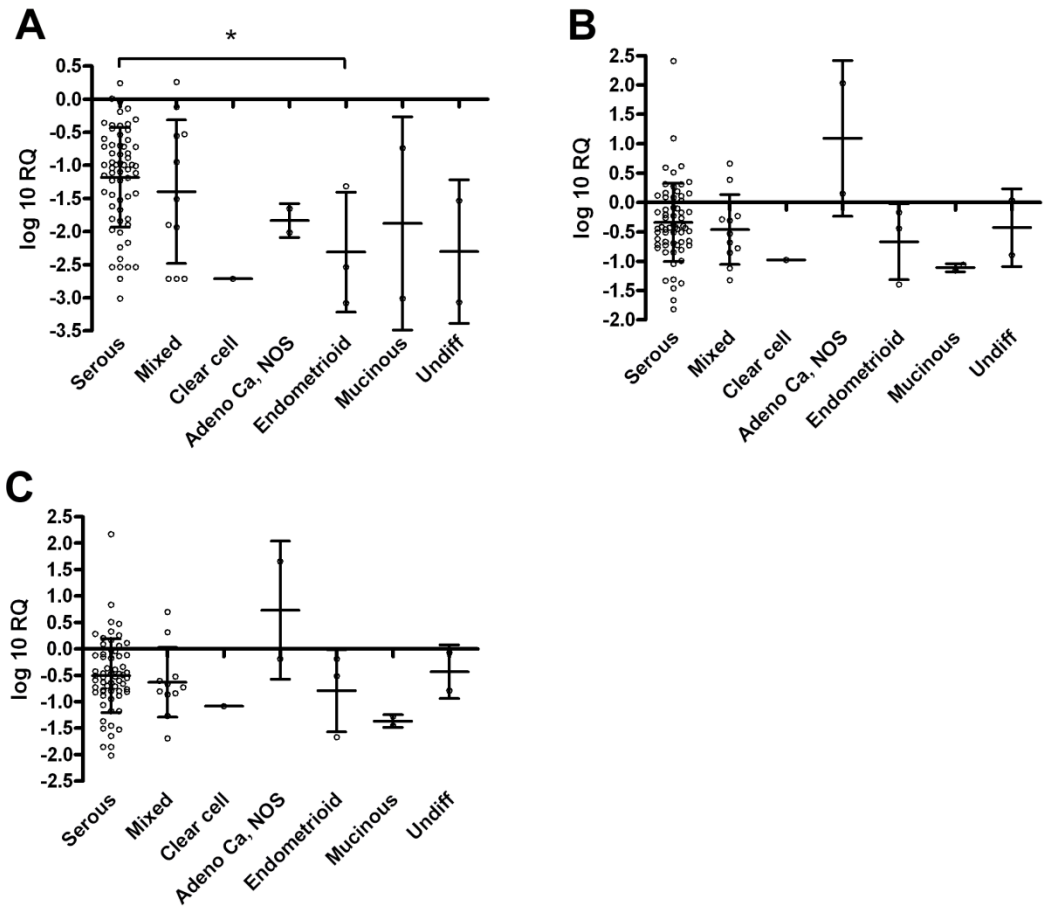


Figure 3.4 miR-34 family expression in different EOC subtypes. qRT-PCR analysis reveals significantly reduced miR-34a expression in endometrioid compared to serous EOC ($p= 0.0144$) (A), while no significant alteration in miR-34b* (B) or miR-34c (C) expression is observed. Adeno Ca, adenocarcinoma. Error bars represent standard deviation.

Table 3.3 *p53* mutation status of human samples.

Case no.	Mutation type	exon	codon	Change	Wild-type	Mutated	Wild-type AA	Mutated AA
OvCa 1	Wild-type							
OvCa 2	Wild-type							
OvCa 6	Wild-type							
OvCa 11	Wild-type							
OvCa 12	Wild-type							
OvCa 21	Wild-type							
OvCa 24	Wild-type							
OvCa 27	Wild-type							
OvCa 28	Wild-type							
OvCa 31	Wild-type							
OvCa 4	Missense	7	237	G>A	ATG	ATA	Met	Ile
OvCa 7	Missense	6/7	220/229	A>G / T>C	TAT/TGT	TGT/CGT	Tyr/Cys	Cys/Arg
OvCa 8	Missense	5	176	G>T	TGC	TTC	Cys	Phe
OvCa 10	Missense	11	375	A>G	CAG	CGG	Gln	Arg
OvCa 14	Missense	7	244	G>A	GGC	GAC	Gly	Asp
OvCa 15	Missense	7	237	G>T	ATG	ATT	Met	Ile
OvCa 17	Missense	8	286	G>A	GAA	AAA	Glu	Lys
OvCa 18	Missense	5	151	C>T	CCC	TCC	Pro	Ser
OvCa 25	Missense	7	248	G>A	CGG	CAG	Arg	Glu
OvCa 30	Missense	11	385	T>C	TTC	TCC	Phe	Ser
OvCa 32	Missense	7	241	C>G	TCC	TGC	Ser	Cys
OvCa 13	Nonsense	5	144	C>T	CAG	TAG	Gln	STOP
OvCa 33	Nonsense	4	107	C>A	TAC	TAA	Tyr	STOP
OvCa 16	Insertion	5	169	+ T	ATG			
OvCa 22	Insertion	4	99	+ C	TCC			
OvCa 34	Deletion	7	241	- T	TCC			

wild type or mutant *p53*, patients with mutant *p53* demonstrate significantly lower expression of all miR-34 family members than patients with wild type *p53* (p= 0.0012, p= 0.0285 and p= 0.0216 for miR-34a, miR-34b* and miR-34c, respectively) (Figure 3.2C).

3.5.3 Regulation of *miR-34* by promoter methylation and copy number alterations.

Promoters of both *mir-34a* and *mir-34b*/c* are located in CpG islands and methylation has been reported to regulate miR-34a expression in several cancer cell lines and primary prostate tumors and melanomas, while miR-34b*/c expression in colorectal cancer is also epigenetically regulated (Lodygin et al., 2008; Toyota et al., 2008). Such methylation has not been reported in ovarian cancer, so we set out to determine the frequency of such methylation by methylation-specific PCR analysis. Methylation at the *mir-34a* and *mir-34b*/c* loci was observed in 27% (8/30) and 47% (14/30) EOC samples, respectively (Figure 3.4A). All samples (8/8) with *mir-34a* methylation demonstrate reduced miR-34a expression, while 57% (8/14) of samples with methylation at *mir-34b*/c* show reduced miR-34b*/c expression. Classification of samples based upon *p53* mutation status revealed that 21% (3/14) and 50% (7/14) mutant *p53* samples show promoter methylation at *mir-34a* and *mir-34b*/c*, respectively, while for samples with wild type *p53*, 38% (5/13) and 46% (6/13) show methylation, respectively.

We next raised the question of whether loss of heterozygosity (LOH) or copy number alterations could be responsible for reduced miR-34a expression and designed custom TaqMan primer and probes to amplify the *mir-34a* locus

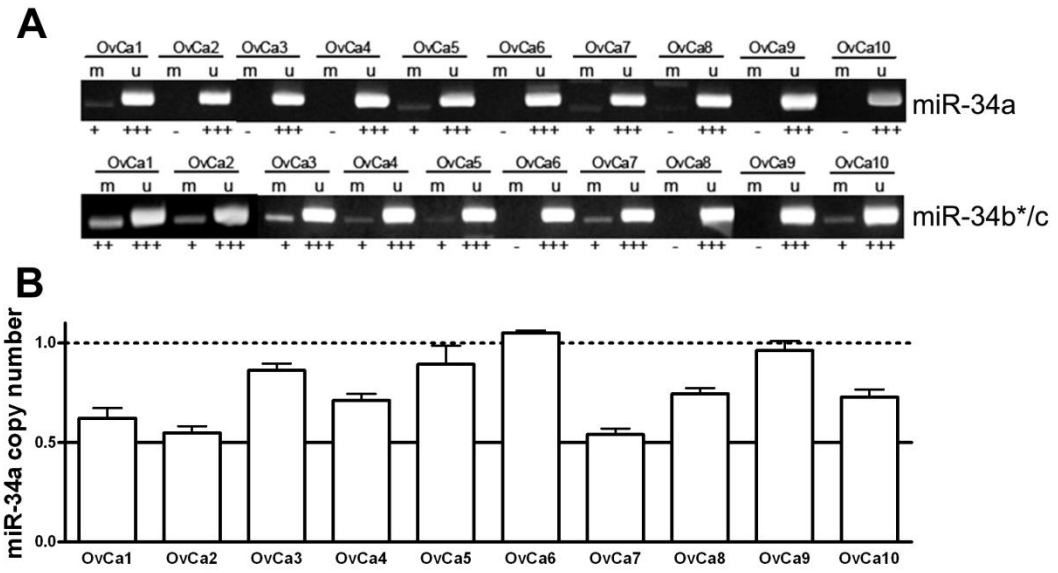


Figure 3.5 *mir-34* promoter methylation and copy number variations are common in EOC. A and B, representative examples of *mir-34a* and *mir-34b*/c* promoter methylation determined by methylation-specific PCR with primers specific for methylated (m) and unmethylated (u) DNA (A) and copy number changes at the *mir-34a* locus determined by qPCR (B). Bars, SD. Panel A kindly provided by Dr. Heiko Hermeking and Markus Vogt.

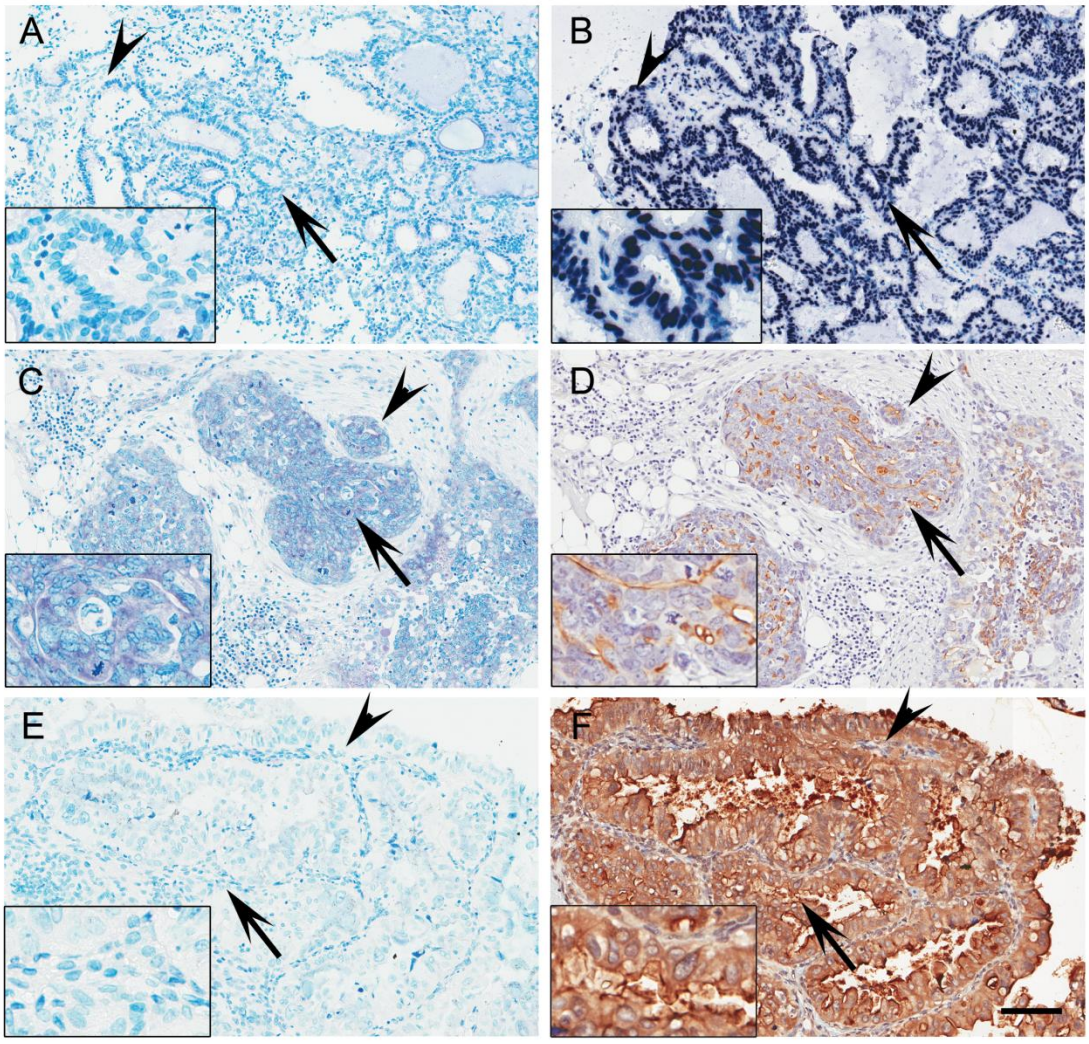
in a qPCR assay. Reduced copy number at the *mir-34a* locus was observed in 39% (13/33) of EOC samples (Figure 3.4B), of which 92% (12/13) had reduced miR-34a expression. Three of these 12 samples (25%) with reduced *mir-34a* copy number and expression show no *p53* mutation or promoter methylation. Taken together, reduced miR-34a expression is associated with *p53* mutation, *mir-34a* promoter methylation and/or copy number variation in 82% (27/ 33) of EOC samples.

3.5.4 miR-34 and MET expression in EOC paraffin sections.

To explore miR-34 expression in EOC tissue, we performed miRNA *in situ* hybridization with paraffin-embedded tissue sections of human ovarian cancer. In the case of miR-34a probe, we observed positive signal in cytoplasm compared to control probe, whereas U6 snRNA was exclusively expressed in nucleus as expected (Figure 3.5). We tested a total of 21 cases of serous EOC with LNA-miR-34a probe. Consistent with qRT-PCR data, 85.7% (18/21) of cases have weak or undetectable miR-34a expression.

One of the shared targets of miR-34 family is the receptor tyrosine kinase MET according to bioinformatic assessment and luciferase assays (He et al., 2007; Migliore et al., 2008). Furthermore, the majority of EOC express elevated levels of MET (Auersperg et al., 2001). Thus, we decided to compare MET and miR-34a expression level in parallel sections of 17 cases using semi-quantitative immunohistochemistry analysis. Staining intensity was scored from 0 (no staining) to 4 (strong staining). EOC cases expressing moderate to strong miR-34a had a relatively weak expression of MET. On the contrary, low miR-34a expressing EOC had strong expression of MET (Figure

Figure 3.6 miR-34a expression is inversely associated with MET expression in EOC. Sections of formalin-fixed, paraffin-embedded human EOC specimens were hybridized with DIG-labeled LNA control probe (A), U6 small nuclear RNA probe (B), and miR-34a probe (C and E) or immunostained with MET antibody (D and F). C and D, EOC with strong miR-34a expression shows relatively low level of MET expression. E and F, on the contrary, EOC with weak miR-34a expression has strong staining for MET. Insets show high magnification of areas indicated with arrows. Similar structures of parallel sections (A and B, C and D, and E and F) are indicated with arrowheads. Methyl green and hematoxylin were used for counterstaining of in situ hybridization and immunostaining, respectively. Scale bar, 100 μm. Kindly provided by Chang-il Hwang.


A
B
C
D
E
F

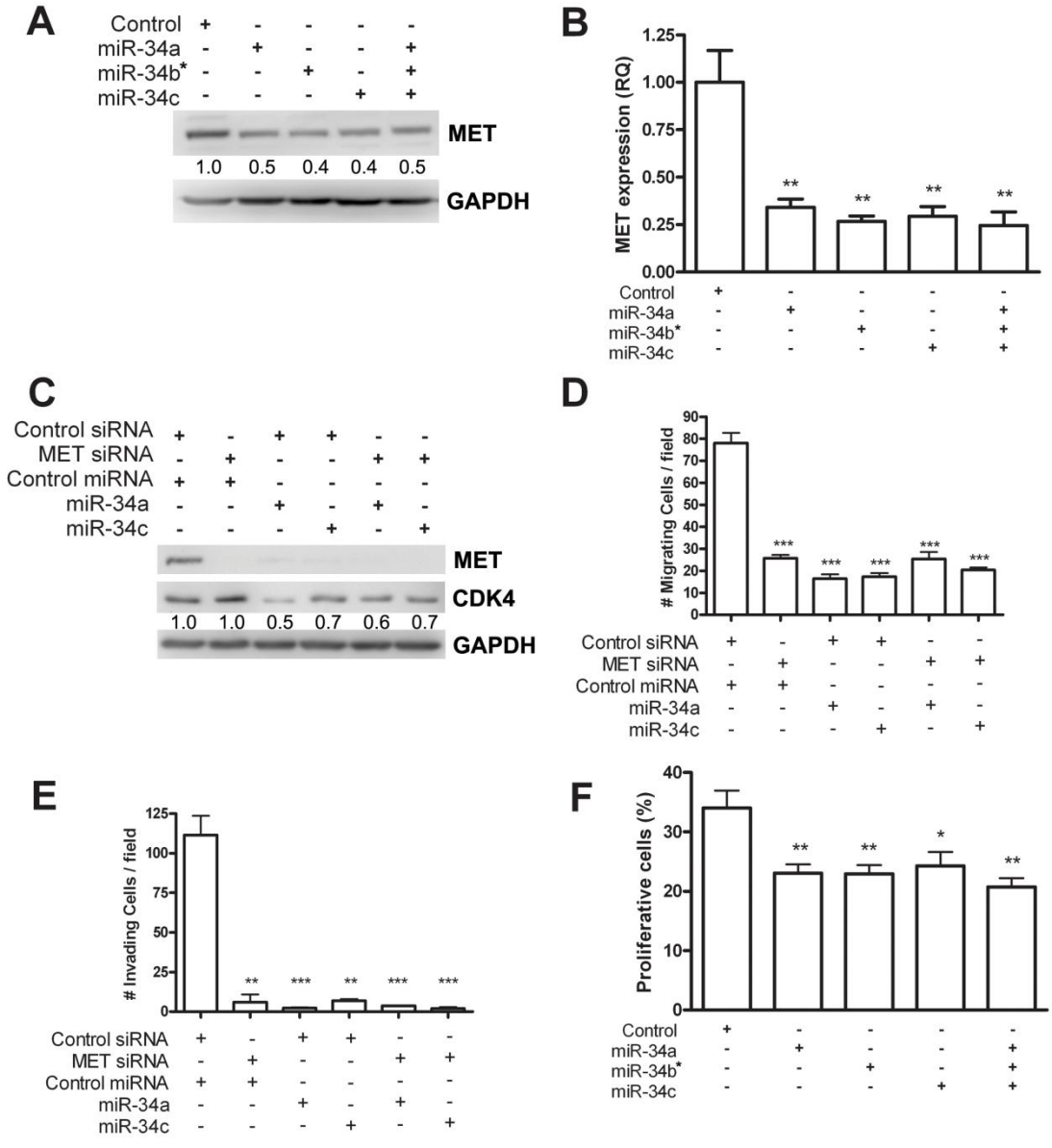
3.5). MET and miR-34a staining was found to be inversely correlated (r=-0.5898, p= 0.0162), confirming that miR-34a might play a role in regulating MET expression in EOC.

3.5.5 miR-34 reduces migration, invasion and proliferation in EOC cells.

To determine the role of miR-34 in human ovarian cancer, we transfected synthetic miR-34 molecules either separately or in combination into SKOV-3, *p53* null human ovarian adenocarcinoma cells (Figure 3.6). SKOV-3 cells express low endogenous levels of all three miR-34 family members (Corney et al., 2007; Zhang et al., 2008) and is therefore well suited to test functions of miR-34. We transfected SKOV-3 cells with 15 nM miR-34 individually or 5 nM combined and observed reduced amounts of MET protein and mRNA (Figure 3.6). Even more significant reduction of MET levels was observed after transfection with 30 nM of miR-34 (Figure 3.6C).

The miR-34 family has been shown to reduce cell invasion in gastric and hepatocellular carcinoma cells, at least partially through downregulation of MET (Li et al., 2009b; Migliore et al., 2008). To examine the role of miR-34 family in invasion and motility in ovarian cancer, we performed transwell motility and Matrigel invasion assays with miR-34 family and/or MET siRNA transfected SKOV-3 cells. Notably, while MET knock down was observed after MET siRNA treatment, CDK4, which is another target of miR-34 family, was not affected by MET siRNA but only after miR-34 family transfection (Figure 3.6C). As expected, individual miR-34 reconstitution by transfection caused significant reduction in motility and invasion in the presence of the MET ligand, HGF (Figure 3.7D, E and Figure 3.8). However, when miR-34 and MET siRNA

Figure 3.7 miR-34 reconstitution decreases migration, invasion, and proliferation in EOC cells. A and B, individual miR-34 family member (15 nmol/L) or entire miR-34 family (5 nmol/L each) was transfected, and statistically significant reduction in mRNA was observed for each treatment (miR-34a, P= 0.028; miR-34b*, P= 0.0017; miR-34c, P= 0.0022; entire miR-34 family, P= 0.0020), although reconstitution of entire miR-34 family does not further downregulate MET expression. C, MET siRNA and/or indicated miR-34 precursor molecule (30 nmol/L) was transfected in SKOV-3 cell line. Transfected cell lysates were probed against MET, CDK4, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in Western blot analysis. MET siRNA induced strong knockdown of MET protein with no effect on CDK4, whereas miR-34 downregulated CDK4 as well as MET. D and E, MET siRNA and/or miR-34 transfection induced significant reduction of cell migration (MET siRNA, P= 0.0005; miR-34a, P= 0.0003; miR-34c, P= 0.0003; MET siRNA and miR-34a, P= 0.0008; MET siRNA and miR-34c, P= 0.0003) and invasion (MET siRNA, P= 0.0013; miR-34a, P= 0.0009; miR-34c, P= 0.0011; MET siRNA and miR-34a, P= 0.0009; MET siRNA and miR-34c, P= 0.0009). F, quantitative assessment of proliferation by bromodeoxyuridine incorporation 48 h after transfection of SKOV-3 cells with either 15 nmol/L synthetic miR-34a, miR-34b*, or miR-34c pre-miR individually or 5 nmol/L of each pre-miR concurrently (miR-34a, P= 0.0044; miR-34b*, P= 0.0042; miR-34c, P= 0.0106; miR-34 in combination, P= 0.0021). Bars, SD. Parts A-E kindly provided by Chang-il Hwang.


A
Control + - - - -
miR-34a - + - - +
miR-34b* - - + - +
miR-34c - - - + +
MET
1.0 0.5 0.4 0.4 0.5
GAPDH
B
MET expression (RQ)
1.25
1.00
0.75
0.50
0.25
0.00
**
Control + - - - -
miR-34a - + - - +
miR-34b* - - + - +
miR-34c - - - + +
C
Control siRNA + - + + - -
MET siRNA - + - - + +
Control miRNA + + - - - -
miR-34a - - + - + -
miR-34c - - - + - +
MET
CDK4
1.0 1.0 0.5 0.7 0.6 0.7
GAPDH
D
Migrating Cells / field
90
80
70
60
50
40
30
20
10
0
***
Control siRNA + - + + - -
MET siRNA - + - - + +
Control miRNA + + - - - -
miR-34a - - + - + -
miR-34c - - - + - +
E
Invading Cells / field
125
100
75
50
25
0
**
***
Control siRNA + - + + - -
MET siRNA - + - - + +
Control miRNA + + - - - -
miR-34a - - + - + -
miR-34c - - - + - +
F
Proliferative cells (%)
40
30
20
10
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Control + - - - -
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miR-34b* - - + - +
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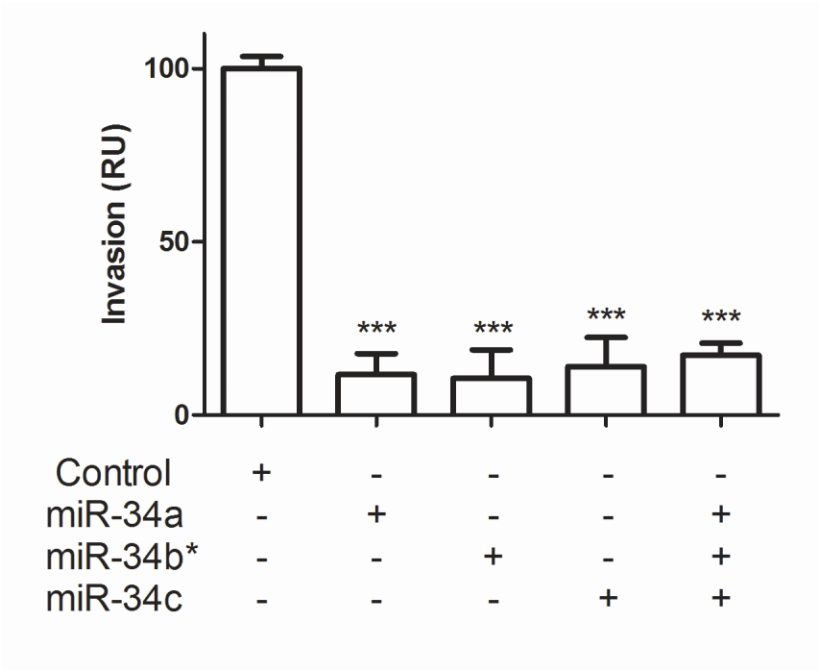

Figure 3.8 miR-34 family reduces invasion. Individual miR-34 family member (30 nM) or entire miR-34 family (10 nM each) were transfected in SKOV-3 cell line. miR-34 family transfection induced significant reduction of invasion (miR-34a, $p<0.0001$; miR-34b*, $p<0.0001$; miR-34c, $p<0.0001$; all three family members, $p<0.0001$). Error bars represent standard deviation. Kindly provided by Chang-il Hwang.

were transfected together no further reduction was observed, demonstrating that MET downregulation by miR-34 is largely responsible for the reduced invasion.

Next, we asked whether miR-34 family reconstitution reduces cell proliferation, since miR-34 family also can target cell cycle related genes such as CDK4 (Figure 3.6C). Transfection of SKOV-3 cells with either 15 nM miR-34a, miR-34b* or miR-34c reduced proliferation by approximately 30% compared to control transfected cells ($p= 0.0044$, $p= 0.0042$ and $p= 0.0106$, respectively) (Figure 3.6F). We next treated cells with a combination of 5 nM of each miR-34 family member to determine whether additional suppression could be achieved due to sequence, and presumably target, differences of miR-34 family members. Although the percentage of proliferative cells was reduced to 20.7% ($p= 0.0021$ compared to control), the difference in reduction compared to miR-34c transfection individually was not statistically significant ($p= 0.0876$). Additionally, we assessed the amount of apoptosis in miR-34 transfected cells by determination of cleaved caspase 3 staining (Figure 3.8). No significant change in number of apoptotic cells was observed in *p53* mutant SKOV-3 cells, consistent with miR-34-induced apoptosis being p53-depenedent (Yamakuchi et al., 2008).

3.6 Discussion

Previously we showed that miR-34b-5p and miR-34c expression is reduced in a p53-dependent manner in a mouse model of EOC, while others also reported reduced miR-34 expression in a variety of cell lines and mouse models (Bommer et al., 2007; Chang et al., 2007; Corney et al., 2007; He et

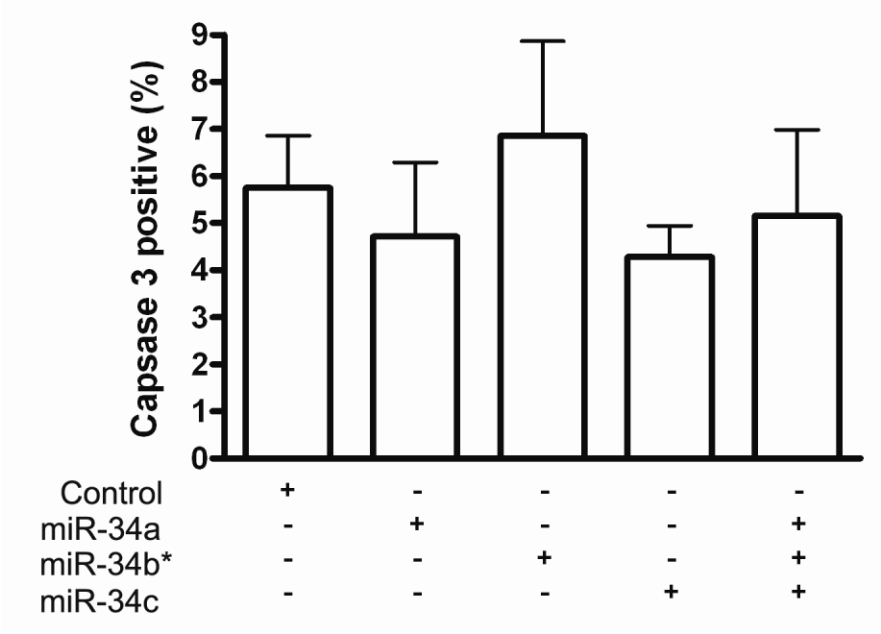

Figure 3.9 Apoptosis is unaffected by miR-34 reconstitution. SKOV-3 cells were transfected with either 15 nM miR-34a, miR-34b* or miR-34c Pre-miR individually, or 5 nM of each concurrently. Number of apoptotic cells was determined by cleaved caspase 3 immunostaining 48 hours after transfection and 17 hours after treatment with 5 µM camptothecin to induce apoptosis. Compared to cells transfected with 15 nM non-targeting control Pre-miR, no significant alteration in number of cells undergoing apoptosis was observed (miR-34a, $p= 0.4066$; miR-34b*, $p= 0.4513$; miR-34c, $p= 0.1209$; all three family members, $p= 0.6554$). Error bars represent standard deviation.

al., 2007; Raver-Shapira et al., 2007; Tarasov et al., 2007) These results led us to question the involvement of miR-34 in human EOC and we show here that miR-34 family expression is also significantly reduced in human EOC, particularly in patients with *p53* mutations.

Recently, the involvement of Drosha and Dicer in EOC has been reported, linking reduced expression of these proteins to poor outcome (Merritt et al., 2008). Yet while reduced Drosha/Dicer processing might be expected to lead to globally decreased miRNA expression, this does not appear to account for all miRNA expression defects, given that miR-199a* expression is modestly increased (Figure 3.3). Moreover, unlike miR-34a, miR-34b*/c expression is not reduced in tumors with wild type *p53*. This discrepancy between miR-34a and miR-34b*/c expression suggests that, in addition to shared p53-dependent transactivation, control mechanisms unique to miR-34a are altered in tumors with wild type *p53*. Underlining differences between the two *mir-34* loci is our observation that miR-34b*/c, but not miR-34a, is significantly associated with stage IV distant metastatic disease (Figure 3.2B). To understand the cause of these differences we investigated the role of promoter methylation and copy number variations of *mir-34*. *mir-34* promoter methylation has been reported in several tumor types (Lodygin et al., 2008; Toyota et al., 2008), while megabase pair deletions at chromosome 1p36 containing the *mir-34a* locus have been identified in 7 low-grade serous carcinomas (Kuo et al., 2009) and in neuroblastoma (Welch et al., 2007). Indeed, promoter methylation was observed at *mir-34a* and *mir-34b*/c* in 27% and 47% of EOC samples, respectively. Furthermore, reduced *mir-34a* copy number was observed in 39% of samples. However, there was no direct correlation between methylation or copy number and miR-34 expression

levels. There are several possible explanations that may account for these data. Firstly, although our p53 sequencing data identified *p53* mutations with high confidence, mutation in genes that regulate p53 may be involved, such as MDM2 which post-translationally silences p53 through ubiquitinylation (Haupt et al., 1997; Kubbutat et al., 1997), while *p53* may itself be epigenetically silenced (Kang et al., 2001). Secondly, many miRNAs, including miR-34a, have been shown by Chang et al. to be suppressed by c-Myc (Chang et al., 2008), an oncogene frequently overexpressed in multiple tumor types, including in EOC. Additionally, it is likely that additional transcription factors regulate miR-34 expression. Very recently, Christoffersen et al. studied human primary hTERT-immortalized TIG3 fibroblasts and observed p53-independent transcription of miR-34a (Christoffersen et al., 2010). Oncogene-induced senescence (OIS) mediated through B-RAF activation induces miR-34a expression in cells treated with a p53 siRNA or a p53 dominant negative variant. Through chromatin immunoprecipitation experiments, the authors demonstrate that the ETS oncogene family member and transcription factor ELK1 binds a conserved region in the *mir-34a* promoter.

Clearly, future studies are required to obtain a more complete understanding of the regulation of miR-34 expression in both normal development and in disease. However, the observation of B-RAF induced miR-34a expression through OIS raises an important question regarding the etiology of EOC. It has been hypothesized that low-grade and high-grade serous tumors have distinct precursor lesions (Corney et al., 2008; Singer et al., 2005), with *p53* mutations rarely found in low-grade tumors but common in high-grade. Interestingly, and further emphasizing the differing molecular defects between the two tumor types, activating *B-RAF* mutations are found

exclusively in low-grade serous EOC (Shih Ie and Kurman, 2004; Singer et al., 2003a). Together, this suggests that activation of miR-34a transcription by B-RAF/ELK1 and p53 in low-grade serous EOC induces senescence and prevents progression to high-grade disease. In contrast, a lack of *B-RAF* mutations combined with frequent *p53* mutation in high-grade serous EOC would appear to largely eliminate miR-34 expression and subsequently tumor suppression capability is lost. Additionally, while *p53* mutation has no impact on *mir-34b*/c* methylation, *mir-34a* methylation is more common in samples with wild type *p53*, consistent with a requirement for diminished p53-miR-34 activity in order to progress to carcinoma. Together, these observations suggest that high-grade tumors arise from a population of cells with mutated *p53* but wild type *B-RAF*.

These data demonstrating correlation of miR-34b*/c expression with metastatic disease suggests that these two miRNAs will be useful as a prognostic marker. This observation is in a good agreement with recent report that low miR-34a levels are correlated with increased probability of relapse in non-small cell lung carcinoma (Gallardo et al., 2002) and reinforces the importance of decreased miR-34 expression. Future studies based on complete follow-up data will determine if miR-34 expression correlates with survival of EOC patients.

The functional studies of miR-34 reconstitution suggest therapeutic applications too. miRNAs represent attractive candidates for gene therapy approaches for several reasons. Computationally, individual miRNAs have been predicted to target tens or hundreds of mRNAs for translational repression. Indeed, one miRNA may regulate many targets. For example, in the case of miR-223 in neutrophils, hundreds of proteins are directly repressed

which has a significant impact on phenotype, despite protein repression being relatively modest (Baek et al., 2008; Johnnidis et al., 2008). It is noteworthy that microarray experiments performed after miR-34 reconstitution in cancer cell lines revealed highly significant alterations that are clustered to important biological pathways, for example cell cycle pathway genes (Bommer et al., 2007; He et al., 2007). These data suggest that reconstituted expression of a downregulated miR-34 might reset or otherwise induce normal function of these gene networks.

Due to its large number of transcriptional targets p53 has also been an attractive candidate for gene therapy. It was therefore disappointing that p53 gene therapy in EOC failed in phase II/III clinical trials (Zeimet and Marth, 2003). Yet while p53 therapy may have failed due to p53 degradation through MDM2 and dominant negative tetramer formation, gene therapy with a p53-independent miR-34 transgene under control of a strong promoter would not be expected to face either of these problems. Furthermore, miR-34 may be more attractive than p53 due to its small size, making it more amenable to packaging in viral and non-viral technologies. No less of importance, a number of EOCs do not harbor *p53* mutations but express low levels of miR-34 which could be corrected. Thus, it would be interesting to study the consequence of miR-34 delivery to EOC cells with wild type *p53*. However, currently characterized EOC cell lines carrying wild type *p53*, such as the serous adenocarcinoma cell line A2780 and clear cell carcinoma cell line TOV-21G (He et al., 2007; Migliore et al., 2008), demonstrate high levels of expression of endogenous miR-34.

MET is one of few common targets for all three miR-34 family members. Taking into account that the majority of EOC express elevated

levels of MET (Auersperg et al., 2001) we have tested the role of all miR-34 family members in repression of MET in EOC cells by using Western blotting, invasion and motility assays, finding that all three members inhibit MET. Small-molecule MET inhibitors are now under clinical trials for several cancers (Comoglio et al., 2008), and Sawada et al. showed that MET siRNA could reduce adhesion, invasion, metastasis and tumor burden in intraperitoneal ovarian cancer xenograft model, but did not affect proliferation (Sawada et al., 2007). On the contrary, miR-34 therapeutics may have advantages compared to siRNA approach, since miR-34 is capable of regulating cell proliferation as well as invasion through targeting several target genes in addition to MET, such as MYC, E2F3 and others. This is underscored by our experiment demonstrating reduced CDK4 protein after miR-34 treatment, but not MET siRNA (Figure 3.7C).

Taken together, our data demonstrates the frequent reduction of miR-34 family expression in EOC and its functional properties as an inhibitor of proliferation and invasion. This miRNA family is therefore an attractive candidate gene for further studies aimed at better understanding of disease pathogenesis and development of novel therapeutic approaches.

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CHAPTER 4

GENERATION OF MIR-34B/C CONDITIONAL KNOCKOUT MICE

4.1 Abstract

It was recently shown that the transcription factor p53 directly transactivates the miR-34 family of microRNAs. By down regulating mRNA targets such as Cyclin D1, Cdk4/6, Bcl-2, Sirt1 and Met, which are involved in diverse cellular processes such as proliferation, apoptosis, senescence and invasion, miR-34 appear to fulfill at least part of the p53 response. Whether the miR-34 family functions as tumor suppressors in a similar manner to p53 has not been characterized. However, reduced proliferation and invasion following reconstitution of miR-34 expression, and our observations of decreased miR-34 expression in human epithelial ovarian cancer patients, are consistent with such a hypothesis. To address this question, ES cells and mice carrying conditional alleles of *mir-34b/c* were prepared. Expression profiling revealed that miR-34 is differentially expressed during ES cell differentiation and that miR-34 is most strongly expressed during G0/1 phase of the cell cycle. Although no obvious phenotype was observed following *mir-34b/c* inactivation in pluripotent ES cells, mutant embryoid bodies demonstrated a plastic adherence defect and dissociated embryoid body cells underwent greater population doublings compared to wild type cells. Future studies will take advantage of *mir-34b/c*$^{loxP/loxP}$ mice to study the role of these miRNAs during cancer initiation and progression.

4.2 Introduction

The miR-34 family of microRNAs (miRNA), comprising miR-34a, miR-34b and miR-34c, were shown independently by several groups to be direct targets of the transcription factor and tumor suppressor p53 (Bommer et al., 2007; Chang et al., 2007; Corney et al., 2007; He et al., 2007; Raver-Shapira et al., 2007; Tarasov et al., 2007). Studies in cell cultures have revealed that the miR-34 family is involved in control of many of the processes that are dysregulated in cancer, including apoptosis, proliferation, senescence, motility, invasion and adhesion-independent growth in soft agar (Bommer et al., 2007; Chang et al., 2007; Christoffersen et al., 2010; Cole et al., 2008; Corney et al., 2007; Corney et al., 2010; He et al., 2007; Hermeking, 2010; Kumamoto et al., 2008; Li et al., 2009b; Migliore et al., 2008; Raver-Shapira et al., 2007; Rokhlin et al., 2008; Sun et al., 2008; Tarasov et al., 2007; Tazawa et al., 2007; Wei et al., 2008; Yamakuchi et al., 2008). Our understanding of the role of miR-34 in control of these processes is aided by the research describing validated mRNA targets of this miRNA family. Validated targets include Bcl-2, Cyclin D1 and E2, Cdk4/6, c-Myc, E2f3, Notch1/2 and Met (reviewed by Hermeking, 2010). Interestingly, the majority of miR-34 anti-invasion and migratory effect can be accounted for by downregulation of the Met oncogene (Corney et al., 2010; Migliore et al., 2008), despite the fact that in addition to the genes listed above, hundreds of targets have been predicted computationally to be targeted by miR-34 (John et al., 2004). These data suggest that the miR-34 family act as tumor suppressors and consistent with this hypothesis, miR-34 expression is reduced in a variety of human cancers via multiple mechanisms. Mutation of *p53* has strong correlation with reduced miR-34 expression in

serous epithelial ovarian cancer (EOC) (Corney et al., 2010) and chronic lymphocytic leukemia (CLL) (Mraz et al., 2009; Zenz et al., 2009), while copy number variation at the *mir-34a* genomic locus has been identified in serous EOC (Corney et al., 2010; Kuo et al., 2009; Zhang et al., 2008), CLL (Calin et al., 2004) and neuroblastoma (Wei et al., 2008; Welch et al., 2007). The three members of the miR-34 family are transcribed as two transcripts; *mir-34a* is located on chromosome 1 (mouse chromosome 4) and is transcribed by itself, while *mir-34b* and *mir-34c*, located 417 bp apart (435 bp in mice) on chromosome 11 (mouse chromosome 9), are co-transcribed and processed from a single transcript. Both *mir-34a* and *mir-34b/c* promoters are enclosed in CpG islands that are heavily methylated in various cancers including lung, breast, colon, pancreatic and EOC (Corney et al., 2010; Lodygin et al., 2008; Toyota et al., 2008). Finally, miRNA biogenesis machinery components Drosha and Dicer are frequently dysregulated in cancer, resulting in altered miRNA expression (Karube et al., 2005; Merritt et al., 2008; Zhang et al., 2006), while Dicer inactivation also drives tumorigenesis (Kumar et al., 2007; Kumar et al., 2009).

The miR-34 family is extremely well conserved among metazoans. One copy of the miR-34 gene is found in most animals, with up to five copies in higher vertebrates (Table 4.1). While miRNA sequence is evolutionarily well conserved, expression pattern might not necessarily be the same between species. One such example is miR-9 which, despite 100% sequence conservation, is differentially expressed in fruit fly, zebrafish, rats and humans (Delaloy et al., 2010; Leucht et al., 2008; Li et al., 2006). In *Caenorhabditis elegans,* a *mir-34:gfp* fusion transgene is expressed in most somatic tissues, such as head neurons, intestine and excretory canal, in addition to mature

Table 4.1 *mir-34* gene copy number in metazoans.

1 copy	**2 copies**	**3 copies**
Anopheles gambiae	*Branchiostoma floridae*	*Bos taurus*
Apis mellifera	*Xenopus tropicalis**	*Canis familiaris*
Ateles geoffroyi		*Danio rerio*
Bombyx mori		*Gallus gallus*
Caenorhabditis briggsae		*Homo sapiens*
Caenorhabditis elegans		*Macaca mulatta*
Capitella sp.		*Mus musculus*
Ciona intestinalis		*Pan troglodytes*
Ciona savignyi		*Rattus norvegicus*
Daphnia pulex		
Drosophila sp.		
Equus caballus		
Lagothrix lagotricha		
Lottia gigantea		
Macaca nemestrina		
Monodelphis domestica		
Ornithorhynchus anatinus		
Pan paniscus		
Pongo pygmaeus		
Saguinus labiatus		
Strongylocentrotus purpuratus		
Sus scrofa		
Tribolium castaneum		

* *X. tropicalis* genome contains two unique *mir-34* genes with 5 copies in total (1 *mir-34a* locus and 4 *mir-34b* loci). Table is current to miRBase release 14.

vulva cells (Kato et al., 2009). As determined by *in situ* hybridization, miR-34 is also expressed in neural tissue, such as hindbrain, but not forebrain or midbrain, of larval and adult *Danio rerio*, while miR-34b is expressed in habenular nuclei of the thalamus (Kapsimali et al., 2007). Neural miR-34a expression is conserved in *Gallus gallus* embryos, while localization is additionally observed in the atria (Darnell et al., 2006). miR-34b and miR-34c localization has not been reported in either *D. rerio* or *G. gallus*. Localization of miR-34a, but not miR-34b or miR-34c, has been observed in neural tissue and gastro-intestinal tract of mouse embryos (Lodygin et al., 2008). In the adult mouse, intense miR-34c staining was observed specifically in pachytene spermatocytes and round spermatids but not in interstitial tissues (Bouhallier et al., 2010).

Despite observations which strongly suggest that the miR-34 family acts as tumor suppressors, no controlled *in vivo* studies have been performed to directly test this hypothesis. The gold standard to test such a hypothesis is to knockout the gene in a model organism, most frequently in the mouse, followed by detailed phenotypic characterization focusing on observation of tumor incidence. Therefore, we have prepared embryonic stem (ES) cells and mice carrying conditional alleles of *mir-34a* and *mir-34b/c* gene. Characterization of *mir-34a* conditional mice will be reported elsewhere (Chang-Il Hwang and Alexander Nikitin, personal communication). Here, the generation of *mir-34b/c*$^{loxP/loxP}$ mice is described.

4.3 Methods

MicroRNA in situ hybridization. In situ hybridization was performed as

previously described (Corney et al., 2010; Nelson et al., 2006). Briefly, 6 µm-thick paraffin sections of 13.5-day old FVB/N mouse embryos were deparaffinized and dehydrated, followed by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) fixation (Pena et al., 2009). Following pre-hybridization blocking, sections were incubated overnight at 55-56°C with digoxigenin-labeled locked nucleic acid- (LNA) modified miR-34a or miR-34b oligo probe (Eqixon, Woburn, MA). After stringency washes, sections were incubated with anti-digoxigenin alkaline phosphatase-labeled secondary antibody (Roche, Indianapolis, IN) overnight at 4°C followed by development of stain with NBT/BCIP (Promega, Fitchburg, WI). Finally, sections were hydrated, cleared in xylene, mounted and coverslipped and digital images prepared using ScanScope CS slide scanner (Aperio, Vista, CA).

Embryonic stem cell culture. The v6.5 mouse embryonic stem cell line (derived from F1 blastocysts from C57BL/6 x 129/sv cross (Eggan et al., 2001)) was chosen due to the advantages of allowing for targeting with isogenic DNA and providing hybrid vigor (Eggan et al., 2001). Cells were cultured in undifferentiating conditions on mitotically inactivated feeder cells and in the presence of LIF at 37°C in 5% CO_2. Briefly, medium used was Dulbecco's Modified Eagle Medium (high glucose) containing 15% heat-inactivated ES cell qualified fetal bovine serum, 6 mM L-glutamine, 1 mM sodium pyruvate, 1x Dulbecco's non-essential amino acids (all purchased from Invitrogen, Carlsbad, CA) plus 10^{-4} M 2-mercaptoethanol (Sigma, St. Louis, MO), and 10^3 units/ml LIF (Millipore, Billerica, MA). For positive neomycin selection, cells were cultured in medium containing 200 µg/ml G418. Mouse embryonic fibroblast (MEF) cells were prepared from 13.5-15.5-day old

C57Bl/6 embryos carrying neomycin cassette under control of PGK promoter (C57BL/6J-Tg*(pPGKneobpA)*3Ems/J, The Jackson Laboratory, Bar Harbor, ME; stock number 002356) and prior to use as feeder cells mitotically inactivated by exposure to 10 µg/ml mitomycin C for one hour at 37°C followed by three washes with phosphate-buffered saline (PBS). ES cells were routinely passaged using 0.25% trypsin/EDTA prior to reaching confluence. Feeder cells were separated from ES cells by differential attachment to gelatinized plates for one hour at 37°C.

Embryonic stem cell differentiation. Recently thawed undifferentiated ES cells were trypsinized, depleted of feeder cells and counted. Embryoid bodies were initially formed using the hanging drop method (McKinney-Freeman and Daley, 2007). Briefly, cells were resuspended at a concentration of $1.5x10^4$ cells per ml in ES medium without LIF and, using a multichannel pipette, rows of 20 µl drops prepared on the lids of culture dishes. Lids were carefully inverted, placed on to culture dish bottoms containing 5 ml PBS and incubated undisturbed for two days at 37°C, 5% CO_2. Embryoid bodies were subsequently transferred to bacterial-grade plastic petri dishes and maintained in suspension culture. After four days of suspension culture, medium was changed to fresh ES medium without LIF either with or without 1.5 µg/ml retinoic acid and cultured for a further four days.

DNA content analysis. One day prior to FACS, cells were passed such that they were 50% confluent the following day. Following trypsinization and feeder cell depletion, cells were resuspended to a concentration of $2x10^6$ cells per ml in pre-warmed ES cell medium, 100 µl 1 mM Hoechst 33342 added to 2

ml of cell suspension and incubated at 37°C for 30 minutes followed by filtration through a 70 µm nylon mesh. Cells were maintained in Hoeschst 33342-containing medium on ice until analyzed by FACS Aria machine (BD Biosciences, San Jose, CA). Unstained cells were used for setting of gates. Following sorting of cells into G0/1, S and G2/M phases, total RNA was isolated using mirVana total RNA Isolation Kit (Ambion, Austin, TX) according to the manufacturer's instructions.

Quantitative reverse transcription PCR. qRT-PCR was performed as previously described (Corney et al., 2007; Corney et al., 2010) using *Rnu6b* amplification as housekeeping control.

4.4 Results

4.4.1 miR-34a and miR-34b are widely expressed in the mouse embryo

While miR-34a *in situ* hybridization staining is observed in neural tissue and the gastro-intestinal tract of the mouse embryo, poor resolution and clarity did not permit a thorough assessment of miR-34a localization at the cellular level (Lodygin et al., 2008). Therefore, we performed *in situ* hybridization experiments in the mouse embryo to localize miR-34a and miR-34b/c expression to both increase our understanding of miR-34 biology and provide a basis for tissue-specific inactivation in later experiments.

Thirteen point five day old mouse embryo sections incubated with a non-targeting oligo resulted in undetectable staining and the U6 positive control was expressed in all cells as expected, while miR-34a staining was

also observed, albeit at levels far lower than that observed for U6 (Figure 4.1). miR-34a staining was most prominently observed in lung and gastro-intestinal epithelium, ventricle and atrium of heart, and cartilage of developing ribcage and spine and dorsal root ganglion (DRG) and trigeminal ganglion (Figure 4.2). While miR-34a was mostly expressed in the epithelial cells of lung and intestine, fainter staining was also observed in surrounding stroma (Figure 4.2A, E). miR-34b had a similar expression pattern, with strong staining in lung epithelium, ventricle of heart, cartilage and DRG but additionally staining was observed in pancreas and male gonads (Figure 4.3). While miR-34a and miR-34b expression is observed in DRG of the developing mouse embryo, staining in the brain was not observed. Given data from other model organisms showing expression in the brain, we hypothesized that in addition to spatial restriction, expression is also temporally restricted and we therefore determined whether either miRNA is expressed in the adult mouse brain. Towards this, sagittal sections of adult mouse brain were hybridized with miR-34a or miR-34b probes. miR-34a is strongly expressed in layers II and III of the cerebral cortex which are mostly stellate, Martinotti and pyramidal cells (Figure 4.2 F). Staining is notably absent in the horizontal cell-containing layer I of the cortex. Strong staining is also observed in dentate gyrus of hippocampus (Figure 4.2 G) and Purkinje cells of cerebellum (Figure 4.2 H). Similarly, miR-34b is expressed in Purkinje cells although less pronounced. Rather, miR-34b is more widely expressed throughout the molecular layer of cerebellum, and the cerebral cortex (Figure 4.3 G, H). Expression of miR-34 in embryonic and adult ovary is currently being determined.

Figure 4.1 miR-34 *in situ* hybridization in the mouse embryo. Mouse embryo paraffin sections were incubated with miR-34a probe (A), U6 probe as positive control (B) or a non-targeting control probe (C). Dark blue/purple, Digoxigenin staining; light green, methyl green counterstain. Calibration bar is 500 µm.

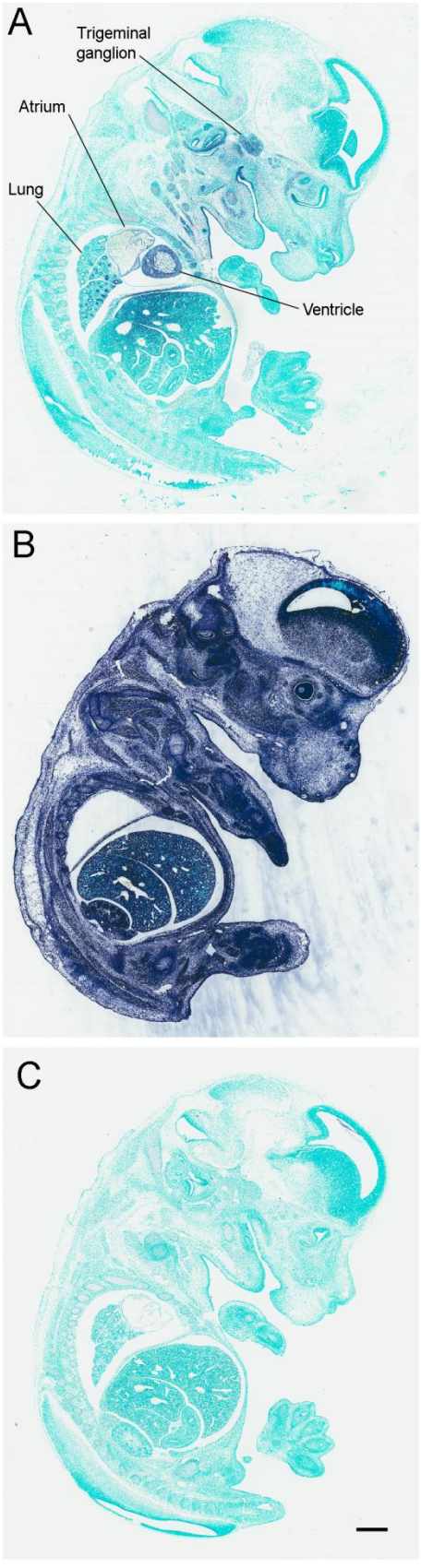

A
Trigeminal ganglion
Atrium
Lung
Ventricle
B
C

Figure 4.2 miR-34a expression in mouse embryo and adult brain. miR-34a localization was interrogated by *in situ* hybridization in mouse embryo and revealed strong staining in lung epithelium (A; arrow), ventricle (arrow) and atrium (arrowhead) of heart (B), trigeminal ganglion (C; arrow), dorsal root ganglion (arrow) and cartilage of developing ribcage (arrowheads) (D) and intestinal epithelium (E; arrow). In the adult brain, miR-34a is expressed in layers II/III of the cortex, but not layer I (F), dentate gyrus of hippocampus (G; arrow). Particularly strong staining is observed in Purkinje cells (H; arrow). Dark blue/purple, Digoxigenin staining; light green, methyl green counterstain. Calibration bar is 50 µm (A, D, E and H), 95 µm (C, F and G) and 165 µm (B).

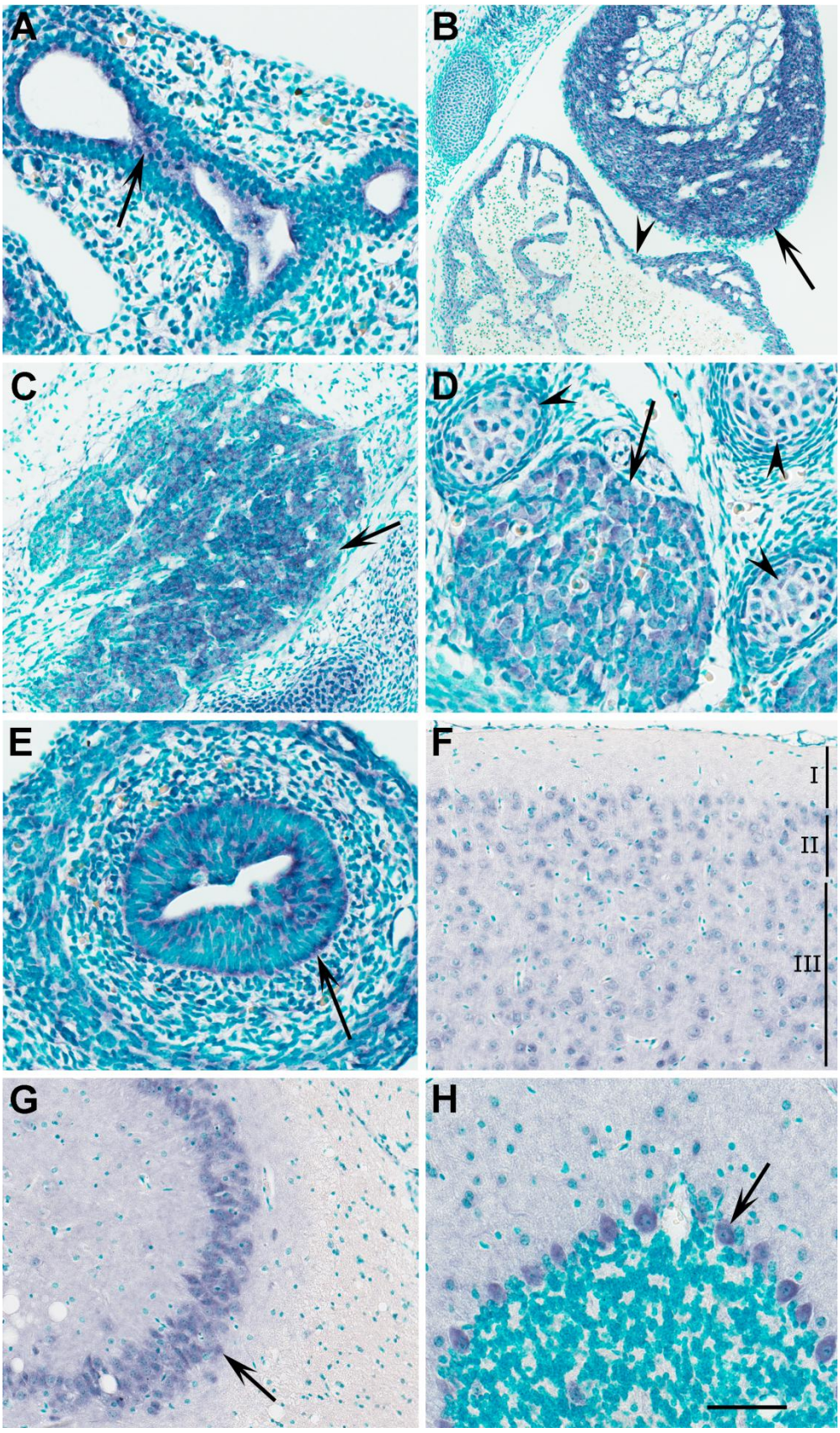
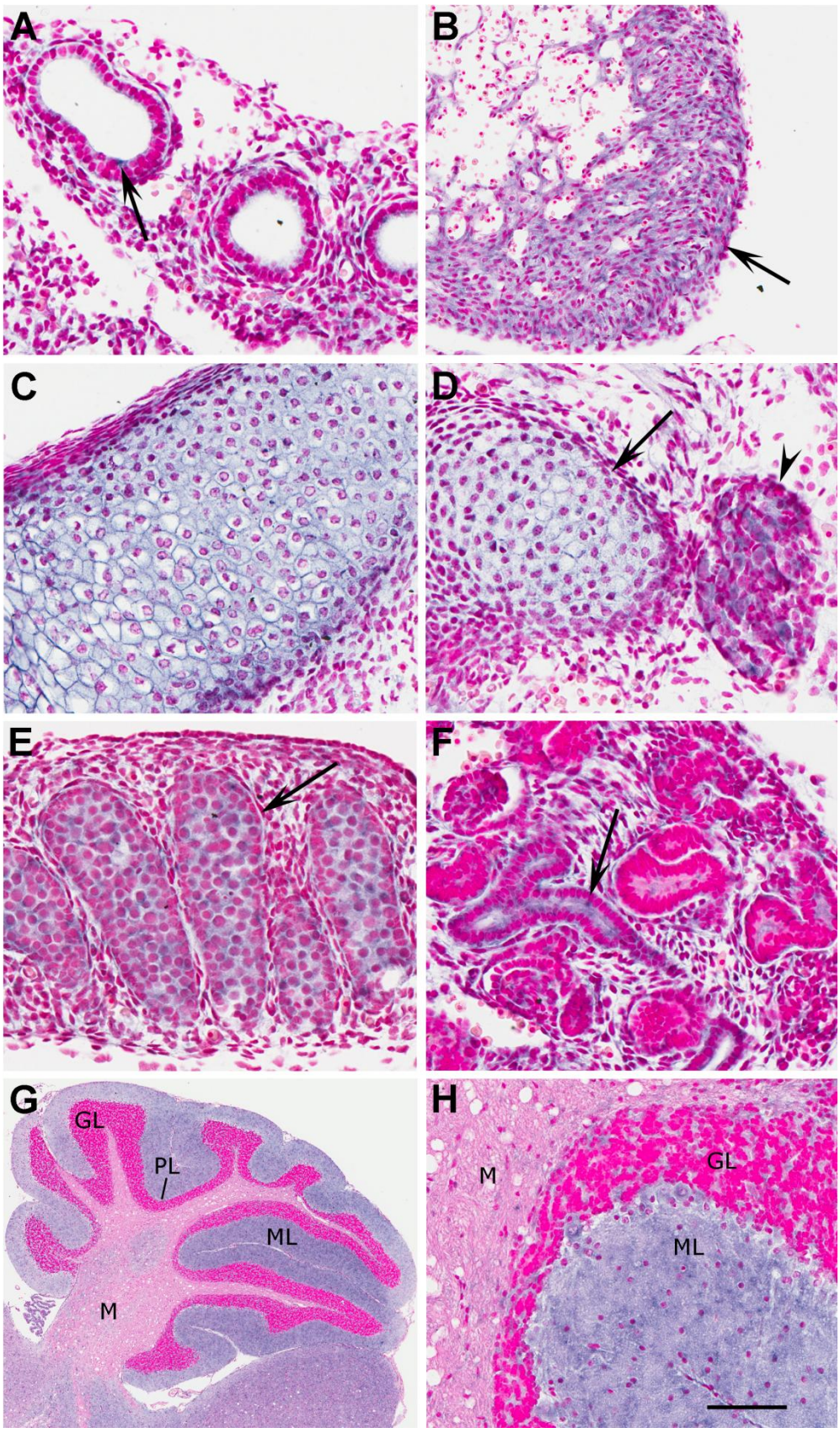

A
B
C
D
E
F
I
II
III
G
H

Figure 4.3 miR-34b expression in mouse embryo and adult brain. miR-34b localization was interrogated by *in situ* hybridization in mouse embryo and revealed strong staining in lung epithelium (A; arrow), ventricle of heart (B; arrow), cartilage (C), cartilage of spine (arrow) and dorsal root ganglion (D; arrowhead), testis (E; arrow) and pancreas (F; arrow). In the adult brain, miR-34b is expressed throughout the cortex but most prominently in the Purkinje layer (PL) and molecular layer (ML) of the cerebellum (G, H). Dark blue/purple, Digoxigenin staining; pink, nuclear fast red counterstain. GL, granular layer; M, medulla. Calibration bar is 50 µm (A, D, E, and F), 55 µm (G), 70 µm (H) and 95 µm (B).


A
B
C
D
E
F
G
GL
PL
ML
M
H
M
GL
ML

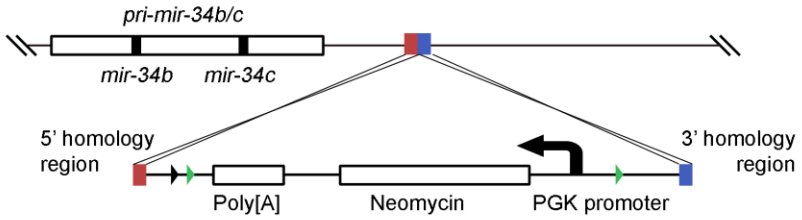
4.4.2 Use of BAC recombineering to prepare mir-34b/c$^{loxP/loxP}$ targeting construct

Bacterial artificial chromosome (BAC) recombineering relies on the controlled expression of lambda Red proteins resulting in recombination between short (approximately 500 bp) regions of homology between donor and recipient pieces of DNA in modified *Escherichia coli* cells (reviewed in Copeland et al., 2001). Advances in utilizing phage-based recombination systems, however, have reduced the length of homologies ten-fold to 40-50 bp (Zhang et al., 1998). The main significance of this development is the ability to PCR-amplify any genetic material to be inserted into a BAC using primers containing the required 40-50 bp homologies, which greatly simplifies recombineering experiments. The most recent generation of BAC recombineering has utilized dual negative and positive selection through the *galK* gene (Warming et al., 2005). This modification allows precise control of location of genetic modification through PCR-based amplification but with the significant advantage of allowing introduction of point mutations and *loxP* sites and an unlimited number of targeting steps, opposed to previous 2-3 steps.

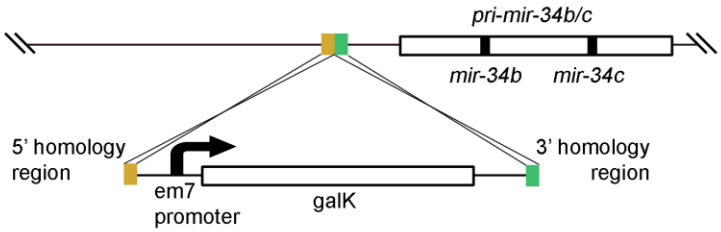
To generate a *mir-34b/c* targeting construct, the galK recombineering method was used to flank the *mir-34b/c* primary transcript with *loxP* sites and insert a *FRT*-flanked neomycin/kanamycin cassette for prokaryotic and eukaryotic selection (Figure 4.4). The bacteriophage P1 protein Cre recombinase recognizes the 34 bp *loxP* sequence and causes deletion or inversion of flanked sequence, depending on orientation of the pair of *loxP* sites (reviewed by Nagy, 2000), while *FRT* sites function in a similar manner

Figure 4.4 *mir-34b/c* targeting strategy. The *mir-34b/c* allele was conditionally targeted by homologous recombination to introduce *loxP* sites flanking the primary transcript. In the first step (A), a neomycin cassette flanked by *FRT* sites (green triangles) and one *loxP* site (black triangle) was PCR-amplified using primers with areas homologous to a region 3' of the *mir-34b/c* primary transcript (red and blue boxes) and homologously recombined. The neomycin cassette was inserted in an opposite orientation to *mir-34b/c* to allow potential generation of hypomorphic allele. The neomycin cassette also contains its own *em7* promoter for prokaryotic expression of kanamycin. Following a similar strategy, a PCR-amplified galK cassette was inserted 5' of the *mir-34b/c* primary transcript (yellow and green boxes indicate homologous DNA) (B). Following this, the galK cassette was substituted for a single *loxP* site (black triangle) flanked by homologous DNA sequences (yellow and green boxes). The final targeting construct is shown in (C). *FRT*- and *loxP*-mediated recombination events are depicted by dotted lines. PCR primer binding sites are indicated by arrows, with the following abbreviations: A, mirn34bcA; B, mirn34bcB; C, mirn34bcC; D, mirn34bcD; E, mirn34bcE. Expected PCR product sizes are given in Table 4.4.

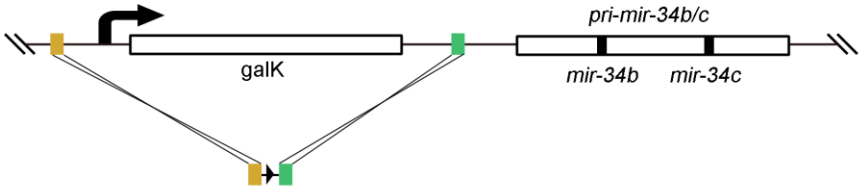
A Stage 1: insertion of FRT-flanked neomycin cassette



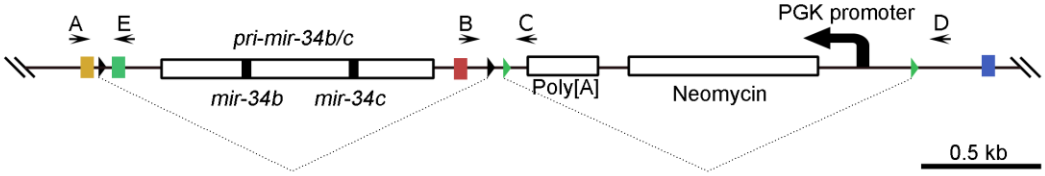
B Stage 2a: insertion of galK cassette



Stage 2b: Replacement of galK cassette with loxP Ultramer



C Final targeting construct



after recognition by the Flpe protein. First, BAC RP23-318E9 (C57Bl/6 genomic DNA; BACPAC Resource Center at Children's Hospital Oakland Research Institute, California) containing the complete *mir-34b/c* locus was electroporated into SW102 *E. coli* cells (Warming et al., 2005) and BAC identity confirmed by PCR and RFLP analysis. Subsequently, a neomycin/kanamycin cassette, flanked by two *FRT* sites and one *loxP* site, was PCR amplified from plasmid pL451 with primers "Neo_F" and "Neo_R" (primer sequences are given in Table 4.2), each containing 66-70 bp of homology to a region 5' of *mir-34c*, using Herculase II Fusion Polymerase (Stratagene, La Jolla, CA) with conditions 95°C 2 minutes followed by 10 cycles of 95°C, 20 seconds; 55°C +1°C per cycle, 20 seconds; 72°C, 1 minute, followed by 20 additional cycles of 95°C, 20 seconds; 65°C, 20 seconds; 72°C, 1 minute and a final 3 minute extension at 72°C. Before electroporation into SW102 cells, the PCR reaction was digested with DnpI, which cleaves its recognition site only when *dam*-methylated, and PvuI and ScaI which cut outside the PCR product, to digest pL451 template present in PCR reaction. Subsequently, the digested PCR reaction was run on 1% agarose gel and the 1.95 kb product excised under long wave UV radiation and purified using Qiaex II Gel Extraction Kit (Qiagen, Valencia, CA). Purified neomycin cassette was electroporated in to heat shocked SW102 bacteria containing RP23-318E9 BAC and plated out on to 23.5 µg/ml chloramphenicol and 25 µg/ml kanamycin LB plates at 32°C as previously described (Warming et al., 2005). 36 hours later, 23 colonies from electroporated heat shocked SW102 cells were picked for colony-PCR to screen for correct insert of Neo cassette. Fifteen of twenty three (65%) picked colonies had a correctly inserted neomycin cassette as evidenced by diagnostic 251 bp PCR product

Table 4.2 Primers used during generation of *mir-34b/c* conditional knockout mice.

Primer name	**Primer sequence (5' – 3')**
mirn34bcA	ctgcgcttctttcttcgatgtagc
mirn34bcB	tggctttaggatctccatttcagc
mirn34bcC	gactagagcttgcggaacccttc
mirn34bcD	acctggttaagtgggctgagttcc
mirn34bcE	acaacttcttcacggcttccagac
Neo_F	ttaaatggttagttgctatacagacaaaatctccagggaaacgtctcacacatg attgtgacctggcggccgctctagaactagtgga*
Neo_R	aatatacctggttaagtgggctgagttcccttgccccatggaaaattatgaaaa catttaacgttcccttccctcgaggtcgacggtatc*
galK+5'loxP_F	gatggatgtaggtctctgcgcttctttcttcgatgtagcagtaactacactgtgaa gataaacttccctgttgacaattaatcatcggca**
galK+5'loxP_R	aggggcaatgggagggctcctgcagagcctcctgggaaggggccggggga gatggagagacagattcttgatcagcactgtcctgctcctt**
BAC1_F	gacgatttccggcagtttctacac
BAC1_R	attaagcattctgccgacatggaa
BAC2_F	taatgcagccatctgctcatcatc
BAC2_R	gaccaggagctgcttactgaggac
BAC3_F	cgtctctcatattacacgccatga
BAC3_R	tttccagctgtcgattgagttgtc

* Underlined sequence is homologous to 3' of *mir-34b/c* primary transcript.

** Underlined sequence is homologous to 5' of *mir-34b/c* primary transcript.

with primers “mirn34bcB” and “mirn34bcC” in addition to wild type BAC as evidenced by 249 bp product from amplification with primers “mirn34bcB” and “mirn34bcD” (Table 4.3 and Figure 4.5), suggesting multiple copies of BAC. Therefore, BAC DNA was isolated from neomycin-inserted colony number 3 and re-electroporated into wild type SW102 cells and selection on 23.5 µg/ml chloramphenicol and 25 µg/ml kanamycin LB plates. Since electroporation is expected to result in uptake of a single BAC molecule by each *E. coli* cell, this re-electroporation step eliminates mosaicism since the unmodified BAC provides no kanamycin resistance. Colony-PCR of 10 randomly picked colonies demonstrated amplification of neomycin cassette but no amplification of wild type BAC. Colony number 6 was used for subsequent manipulations. In the targeting second step, a Herculase II Fusion Polymerase PCR-amplified galK cassette was amplified from pGalK (Warming et al., 2005) with primers “galK+5’loxP_F” and “galK+5’loxP_R” containing 66-70 bp homology to a region 5’ of the *mir-34b* primary transcript, with same conditions as used for neomycin amplification. PCR reaction was digested with DnpI, ScaI and PvuII and then run on a 1% agarose gel followed by purification with Qiaex II Gel Extraction Kit. GalK PCR product was electroporated into SW102 colony 6 and grown on M63 minimal plates supplemented with 0.2% galactose, 0.1 mg/ml L-leucine, 40 ng/ml d-biotin, 23.5 µg/ml chloramphenicol and 25 µg/ml kanamycin at 32°C for 3 days as previously described (Warming et al., 2005). BAC DNA was isolated from colonies appearing after 3 days growth on minimal plates and electroporated into wild type SW102 cells to eliminate any contaminating galK$^-$ BAC molecules in SW102 cells. Culture on MacConkey agar plates confirmed insertion of the galK cassette. In the final step, the galK cassette was replaced with a single *loxP* site. Two complementary 134 bp

Table 4.3 Expected PCR genotyping product sizes. PCR product sizes for genotyping of knockout targeting construct, ES cells and of animals prior to Flpe-mediated neomycin excision are shown. Primer sequences are given in Table 4.2.

<table>
<tr><th></th><th>mirn34bcC</th><th>mirn34bcD</th><th>mirn34bcE</th></tr>
<tr><td rowspan="2">mirn34bcA</td><td>Wild type: N/A</td><td rowspan="2">N/A</td><td>Wild type: 289 bp</td></tr>
<tr><td>loxP recombined (null allele): 160 bp</td><td>Floxed: 323 bp</td></tr>
<tr><td rowspan="2">mirn34bcB</td><td rowspan="2">Floxed: 251 bp</td><td>Wild type: 177 bp</td><td rowspan="2">N/A</td></tr>
<tr><td>FRT recombined: 335 bp</td></tr>
</table>

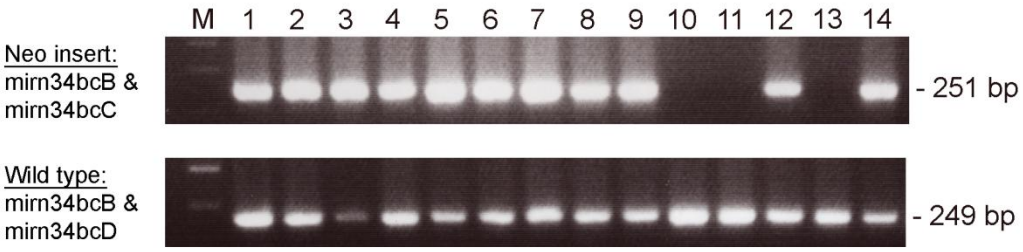


Figure 4.5 Identification of correctly inserted neomycin cassette in *mir-34b/c* locus. Colony PCR was performed on SW102 *E. coli* electroporated with neomycin cassette using primers mirn34bcB and mirn34bcC to amplify correctly inserted neomycin cassette and primers mirn34bcB and mirn34bcD to amplify the wild type locus.

Table 4.4 Ultramer oligos used during generation of *mir-34b/c* conditional knockout mice.

Ultramer name	**Ultramer sequence (5' – 3')**
loxP_ultramer_F	tgcgcttctttcttcgatgtagcagtaactacactgtgaagataaacttcataacttcgtatagcatacattatacgaagttattcaagaatctgtctctccatctccccggcccttcccaggaggctctgca*
loxP_ultramer_R	tgcagagcctcctgggaaggggccggggagatggagagacagattcttgaataacttcgtataatgtatgctatacgaagttatgaagtttatcttcacagtgtagttactgctacatcgaagaaagaagcgca*

* Underlined sequence is homologous to 5' of *mir-34b/c* primary transcript.

Ultramer Oligos (Integrated DNA Technologies, Inc., Coralville, IA) containing *loxP* site flanked by 50 bp regions of homology were synthesized (Table 4.4) and annealed by heating to 95°C in heating block for 4 minutes followed by turning off heating block and letting cool to room temperature over approximately 3 hours. Annealed *loxP* Ultramer duplex was electroporated into galK$^+$ SW102 cells and cultured for 3 days at 32°C on M63 plates supplemented with 0.2% 2-deoxy-galactose (DOG), 0.1 mg/ml L-leucine, 0.2% glycerol, 40 ng/ml d-biotin, 23.5 µg/ml chloramphenicol and 25 µg/ml kanamycin. Finally, colonies were picked and screened by colony-PCR for correct insertion of the 5' *loxP* site followed by direct sequencing of the regions of BAC that were modified.

4.4.3 A novel quantitative PCR assay to identify targeted ES cells

Towards generation of *mir-34b/c* conditional mice, v6.5 ES cells were targeted with the *mir-34b/c* targeting BAC. Our initial targeting experiments with an 11.2 kb fragment of BAC (with 5 kb and 3 kb arms of homology at 5' and 3', respectively) were unsuccessful with no homologous recombination events identified (data not shown). We therefore sought an alternative approach and utilized the full-length BAC construct to considerably increase the arms of homology to 114 kb and 83 kb at 5' and 3', respectively. $1x10^7$ v6.5 cells were electroporated with 25 µg PI-SceI-linearized modified BAC using Gene Pulser II (Bio-Rad, Hercules, CA) at settings of 320 V and 250 µF and, after a 20 minute incubation on ice, divided equally between 8-10 10 cm dishes containing mitotically inactivated G418-resistant MEF feeder cells. 24 hours later, 200 µg/ml G418 was added to ES culture medium and thereafter

medium changed daily. 10 days post electroporation, 333 distinct ES cell colonies were manually picked and transferred to individual wells of 96-well plates with mitotically inactive MEF feeder cells and expanded in triplicate with two plates for storage at -80°C and one plate without feeder cells for DNA isolation.

DNA was isolated in 96-well plate format as described (Ramirez-Solis et al., 1992). Briefly, 50 µl lysis buffer (10 mM Tris (pH7.5), 10 mM EDTA (pH 8), 10 mM NaCl, 0.5% (w/v) sarcosyl and 1 mg/ml Proteinase K) was added to each well, incubated at 60°C overnight followed by adding 100 µl of ice cold slurry of 75 mM NaCl in 100% ethanol to precipitate DNA. Plates were carefully inverted to discard NaCl/ethanol and washed twice with 70% ethanol, air dried and resuspended in 50 µl 10 mM Tris (pH 8.5).

Use of extremely long arms of homology necessitated a screening approach other than Southern blotting or long-range PCR. Therefore, to distinguish between homologous and non-homologous recombination events, two screening steps were performed. In the first step, endpoint PCR was used to amplify the BAC backbone with three primer sets BAC1 (product size 288 bp), BAC2 (product size 207 bp) and BAC3 (product size 286 bp) (Table 4.2 and Figure 4.6). PCR conditions were 95°C for 2 minutes followed by 30 cycles of 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 1 minute followed by a final 72°C extension for 3 minutes on the final cycle. Forty-six percent (107/230) of colonies had no evidence of any BAC backbone amplification and were screened in a second step; colonies with amplification were discarded. To identify homologous recombination during generation of conventional knockout ES cells, Valenzuela et al. described a loss of native allele assay, wherein a neomycin reporter replaces one allele of an

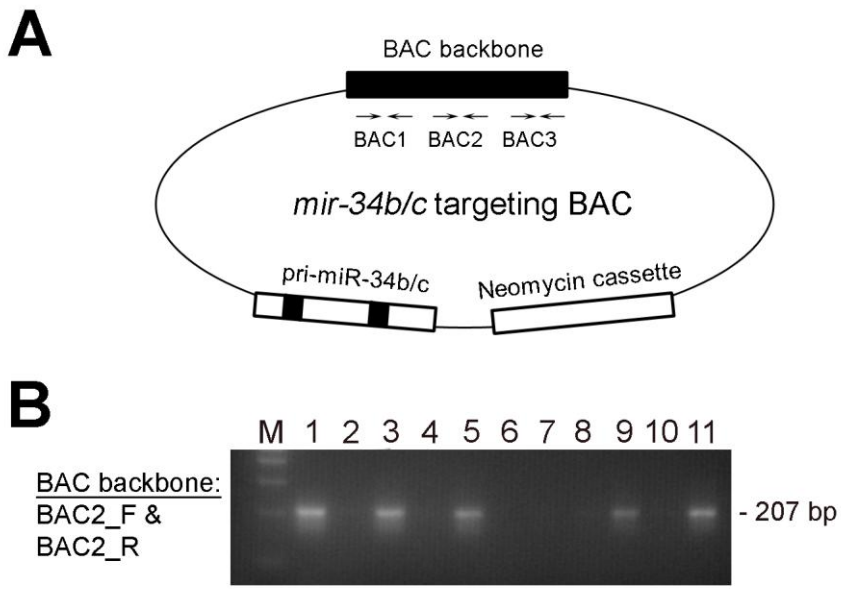
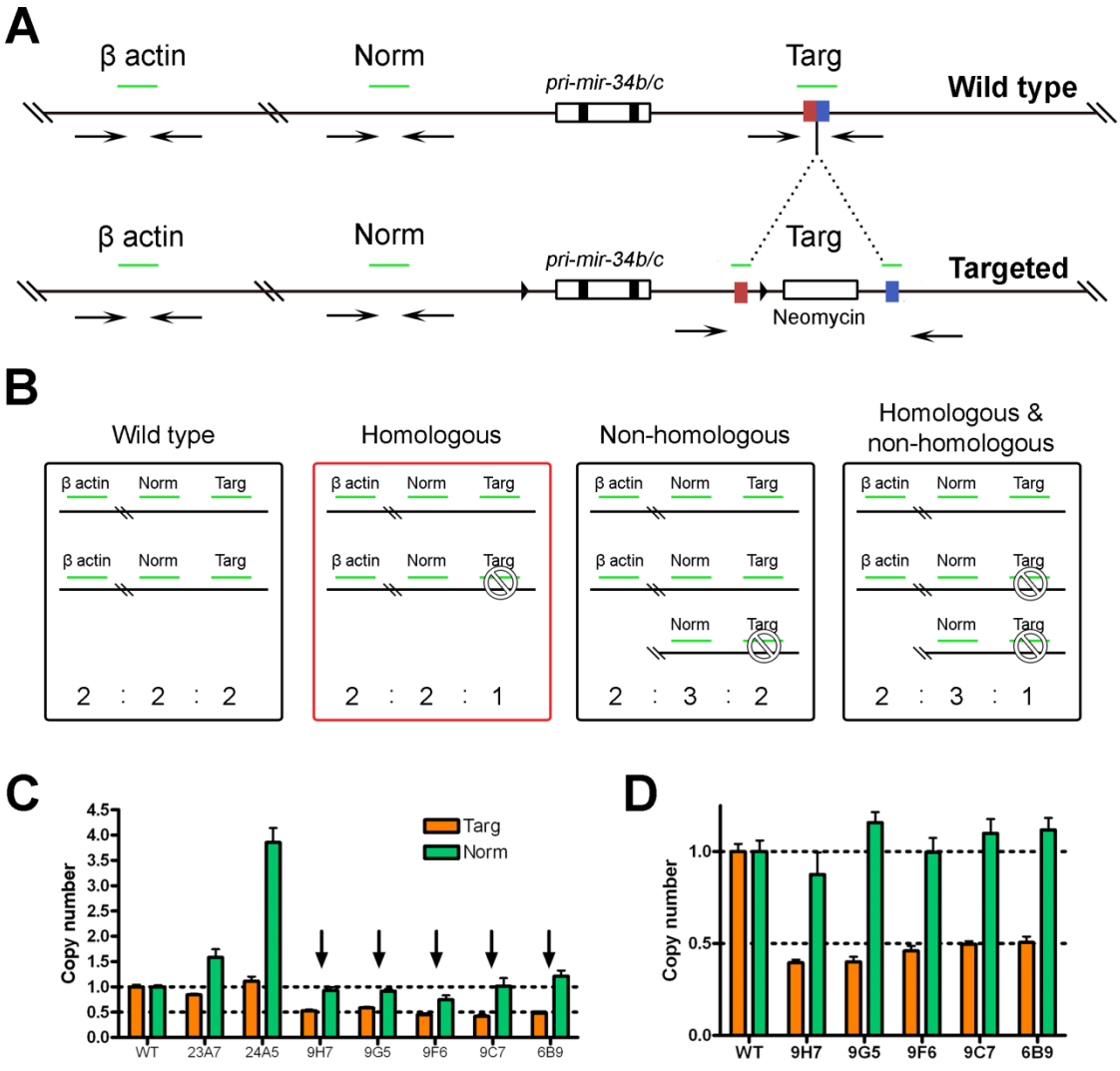


Figure 4.6 First round of screening for homologous recombination in ES cells. A, Schematic of conventional endpoint PCR amplification for BAC backbone DNA (black rectangle) with primer sets BAC1, BAC2 and BAC3. B, Representative screening results for 11 ES cell colonies amplified with BAC2 primers with diagnostic 207 bp PCR product. Colonies in lanes 1, 3, 5, 9 and 11 were discarded due to BAC2 amplification, while negative lanes were subsequently screened for BAC1 and BAC3 amplification. Schematic is not to scale.

endogenous gene (Valenzuela et al., 2003). In our conditional knockout approach, however, no endogenous material is lost. Therefore, an assay, termed here a “loss of homozygosity assay”, was designed so that carefully designed custom TaqMan probes amplify the endogenous region precisely where the *FRT*-flanked neomycin cassette is inserted: PCR amplification at “Targ” region of wild type DNA will give an expected product size of 73 bp, while the locus with the neomycin insert will not be amplified due to the 2 kb product size (Figure 4.7). Clones having undergone homologous recombination (and are consequently heterozygous at the *mir-34b/c locus*) have 50% reduction in “Targ” copy number at this locus relative to both β actin control on chromosome 5 and “Norm” located at an unmodified region of the BAC. In contrast, clones having undergone non-homologous recombination have increased “Norm” copy number and wild type “Targ” copy number. Rare cases of both homologous and non-homologous recombination occurring in the same cell can be identified by 50% reduction in “Targ” copy number concomitant with increased “Norm” copy number. In total, 5 clones with homologous recombination were identified (5/230 (2.2%) of all picked clones analyzed; 5/68 (7.4%) of analyzed BAC backbone-negative clones) and expanded from frozen 96-well plates. Two clones, 9F6 and 9G5, were chosen for further expansion, karyotyping and mycoplasma testing, the results of which showed normal euploid karyotype and no mycoplasma contamination. Based upon colony morphology, clone 9F6 was chosen for microinjection into C57BL/6 blastocysts which were subsequently transferred to pseudopregnant females. Seven high ES cell-derived (80-100%) chimeras were identified based upon agouti coat color and, at sexual maturity, were mated with Rosa26-Flpe transgenic mice (Farley et al., 2000), resulting in simultaneous

Figure 4.7 Loss of homozygosity assay identifies homologous recombination. A, Schematic outlining *mir-34b/c* locus and location of TaqMan qPCR primers (arrows) and probes (green lines). Amplification at three unique loci allows one to distinguish between wild type, homologous and non-homologous recombination. First, β actin copy number should be constant across all samples, while “Norm” and “Targ” loci are contained on both wild type genomic DNA and modified BAC DNA. Importantly, while “Norm” amplification occurs efficiently in both wild type and BAC-derived DNA, “Targ” amplification can only occur in wild type DNA due to insertion of a neomycin cassette at the “Targ” locus in BAC DNA. As shown in B, wild type colonies have equivalent copy number at all three loci, while colonies that have undergone homozygous recombination have equal β actin and “Norm” but reduced “Targ” copy number. Increased “Norm” copy number is diagnostic of a non-homologous recombination event, which might occur with or without concomitant homologous recombination event. C, Compared to wild type parental v6.5 genomic DNA (WT), the majority of colonies demonstrated evidence of a single non-homologous recombination (e.g. 23A7), while rare colonies had undergone multiple non-homologous recombination events (e.g. 24A5). A total of 5 colonies that had undergone homologous recombination were identified (arrows) which was validated by replicating qPCR with a new preparation of genomic DNA (D).


A
β actin
Norm
pri-mir-34b/c
Targ
Wild type
β actin
Norm
pri-mir-34b/c
Targ
Targeted
Neomycin
B
Wild type
Homologous
Non-homologous
Homologous & non-homologous
β actin
Norm
Targ
2 : 2 : 2
2 : 2 : 1
2 : 3 : 2
2 : 3 : 1
C
Copy number
Targ
Norm
WT
23A7
24A5
9H7
9G5
9F6
9C7
6B9
D
Copy number
WT
9H7
9G5
9F6
9C7
6B9

germline transmission and *FRT*-mediated deletion of neomycin cassette.

4.4.4 Inactivation of mir-34b/c in cell culture

To assess the role of miR-34b/c in ES cells and their differentiated progeny, conventional null ES cells were prepared. Firstly, cells homozygous for the conditional, floxed, allele were prepared by culturing cells in high concentrations of G418 as previously described (Mortensen et al., 1992). After 10 days culture in ES cell medium containing 4.38-5.25 µg/ml G418, individual colonies were picked and expanded in 96-well plates of mitotically inactivated feeder cells. Genomic DNA was isolated as described earlier and PCR genotyping performed. Two of eighty one (2.5%) colonies had become homozygous for the conditional allele (Figure 4.8 A). Importantly, homozygous floxed/null ES cells demonstrate no wild type locus by PCR amplification, confirming generation of true homologous recombinants and not a transgenic insert. One of these colonies, 9F6C1, was expanded and $4.5x10^6$ cells electroporated with 40 µg circular pPGK-Cre-bpA plasmid (kindly provided by Dr. Klaus Rajewsky; Addgene plasmid 11543). Electroporated cells were grown on mitotically inactivated feeder cells for 48 hours, after which time cells were trypsinized and re-plated at low density prior to picking of individual colonies. The re-plating step allows Cre expression and avoids mixed colonies should Cre/*loxP*-mediated recombination occur after the cell divides. Thirty three percent (32/96) of picked ES cell colonies had undergone Cre/*loxP*-mediated recombination as demonstrated by PCR (Figure 4.8 B).

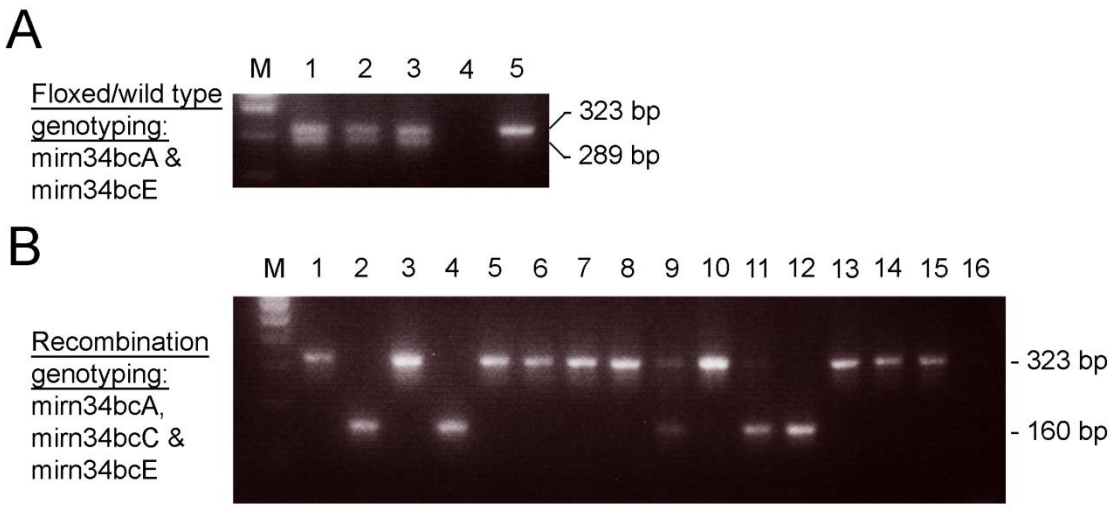


Figure 4.8 Cre/*loxP*-mediated *mir-34b/c* inactivation in homozygous ES cells. A, 9F6 *mir-34b/c*$^{loxP/+}$ ES cells were cultured in high concentrations of G418 and rare instances of gene conversion to homozygosity was identified. PCR genotyping with PCR primers mirn34bcA and mirn34bcE was performed with 289 bp diagnostic for wild type allele and 323 bp diagnostic for floxed allele. Lane 5, homozygous colony 9F6C1; lane 4, no template control. B, 9F6C1 *mir34bc*$^{loxP/loxP}$ ES cells were electroporated with pPGK-Cre-bpA plasmid as described in the text. Recombination between *loxP* sites was identified by PCR with primers mirn34bcA, mirn34bcC and mirn34bcE, with 323 bp amplicon diagnostic of floxed allele and 160 bp diagnostic of *mir-34b/c* null allele. 100% recombination was observed in approximately 30% of colonies (e.g. lanes 2, 4 and 12), while less efficient recombination was not as frequent (e.g. lanes 9 and 11). Lane 16, no template control.

No obvious changes in morphology or growth characteristics were observed in *mir-34b/c*-null ES cells in undifferentiated growth conditions, although whether proliferation defects are present in long-term culture is currently being determined by cell doubling time and BrdU incorporation. However, during the generation of embryoid bodies, overt changes in adhesion characteristics were observed. When embryoid bodies were transferred from hanging drops to suspension culture on bacterial-grade plastic petri dishes almost all *mir-34b/c*$^{loxP/+}$ 9F6 embryoid bodies attached to the plate and flattened out, while few *mir-34b/c*$^{-/-}$ embryoid bodies attached and instead remained in suspension (Figure 4.9). These observations, together with reduced soft agar colony formation in neoplastic OSE cells after miR-34b/c reconstitution (Corney et al., 2007), indicate a role for miR-34b/c in cell adhesion and/or epithelial-to-mesenchymal transition (EMT). When embryoid bodies are dissociated and single cells cultured in adherent culture conditions, *mir-34b/c*$^{-/-}$ cells have significantly increased population doubling (Figure 4.9) and are consistent with decreased proliferation following miR-34 reconstitution.

4.4.5 miR-34 expression in ES cells

Cell proliferation and differentiation are tightly controlled processes. p53 has well-known roles in controlling proliferation, in part through transactivation of its targets *p21* and *mir-34* family. Interestingly, p53 has also been implicated in control of cell differentiation through repression of *Nanog*, which results in loss of pluripotency in mouse ES cells (Lin et al., 2005). Consistent with this, multiple groups recently demonstrated an important role for p53 in induced

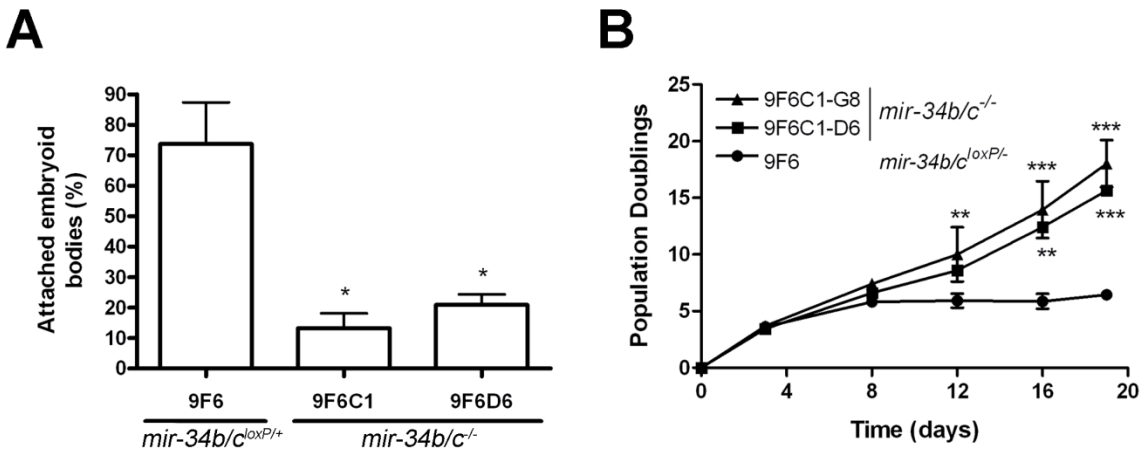


Figure 4.9 *mir-34b/c* inactivation in embryoid bodies results in plastic adherence defect and increased population doubling. A, *mir-34b/c*$^{-/-}$ embryoid bodies have plastic adherence defect Following formation in hanging drop culture, embryoid bodies were transferred to suspension culture on bacterial-grade plastic petri dishes. While 73.67% *mir-34b/c* heterozygous floxed embryoid bodies attached, the majority of *mir-34b/c*-null embryoid bodies derived from two independent null colonies remained in suspension and unattached to the plastic (p= 0.0276 and 0.0339, for *mir-34b/c*-null colonies 9F6C1D6 and 9F6C1-G8. B, *mir-34b/c*-null dissociated embryoid body-derived differentiated ES cells show continued and increased population doubling compared to mir-34b/c$^{loxP/-}$ cells. Error bars represent standard deviation.

pluripotent stem (iPS) cell reprogramming to an ES cell-like state (Hong et al., 2009; Kawamura et al., 2009; Li et al., 2009a; Marion et al., 2009; Utikal et al., 2009), although this may be a function of p53 role in proliferation rather than differentiation (Hanna et al., 2009). We performed miR-34 expression analysis in wild type ES cells undergoing controlled differentiation in embryoid bodies (Figure 4.10). Compared to pluripotent ES cells, miR-34b and miR-34c expression is significantly reduced ($p < 0.05$) at every stage of embryoid body formation. The picture for miR-34a expression, however, is less clear. An initial reduction in miR-34a expression is observed in 2-day old embryoid bodies ($p= 0.0162$) but is followed by an increase in expression in older embryoid bodies to levels not significantly different to that in ES cells. One explanation for this observation is that *mir-34a* and *mir-34b/c* loci are differentially regulated at the transcriptional level during the differentiation process, for example to suppress, but not eliminate, miR-34-mediated inhibition of proliferation. Consistent with this, multiple members of the Notch signaling pathway, such as receptors Notch 1 and Notch 2 and their ligands Delta-like 1 and Jagged 1, are validated miR-34 targets (Bommer et al., 2007; Ji et al., 2009; Lewis et al., 2003; Pang et al., 2010). In addition to a role in proliferation, Notch signaling plays a role in differentiation and cell fate decisions. While its function may be cell-type dependent, tight control over Notch signaling is important in mammary gland stem cells and keratinocytes for controlled differentiation (Bouras et al., 2008; Rangarajan et al., 2001).

Reconstitution or overexpression of miR-34 has been shown to result in cell cycle arrest at G1 phase (He et al., 2007; Ji et al., 2009; Lodygin et al., 2008; Tarasov et al., 2007). Interestingly, ES cells have considerably different cell cycle structure compared to differentiated cells with a short G1 phase;

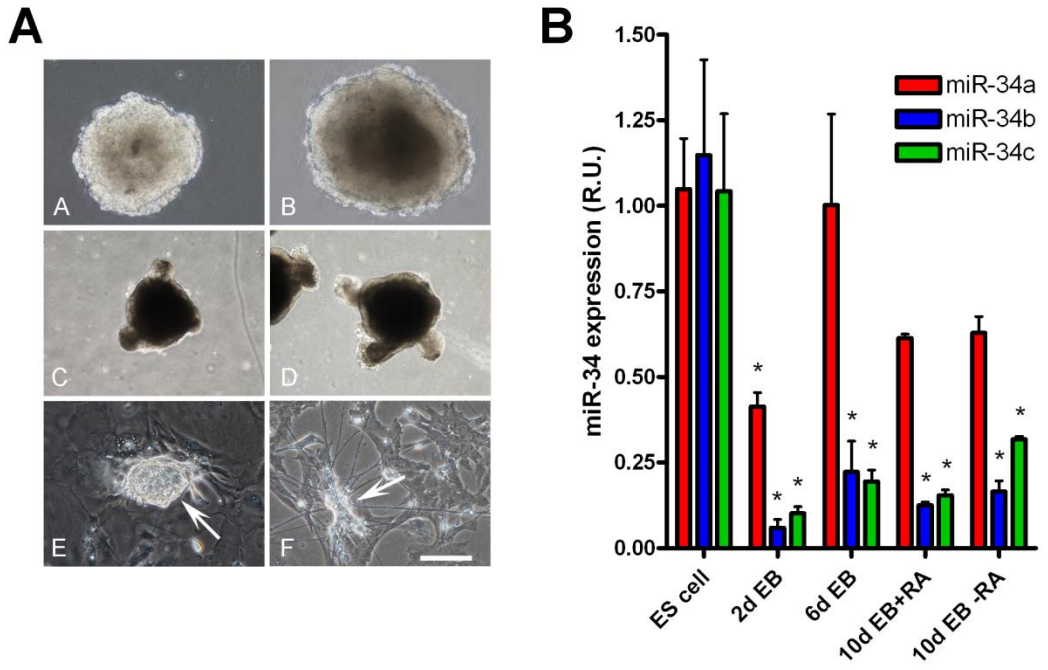


Figure 4.10 The miR-34 family is differentially expressed during ES cell differentiation. ES cells were induced to undergo differentiation in embryoid bodies (A): 2-day old EB (inset A); 6-day old EB (inset B); 10-day old EB incubated with retinoic acid (inset C) or without retinoic acid (inset D); myocardiocytes (arrow) observed spontaneously beating 5 days after enzymatic digestion of 10-day EBs and plating onto gelatinized cell culture dishes (inset E); F, Neuronal cells (arrow) observed 5 days after enzymatic digestion of 10-day EBs (inset F). Bar= 112.5 µm (A, B), 500 µm (C, D) and 100 µm (E, F). B, Total RNA isolated from EBs at various stages of differentiation was used in qRT-PCR reactions for miR-34a (red), miR-34b (blue) and miR-34c (green). Error bars represent standard error of the mean. *, $p < 0.05$.

cells spend most of their time in S phase (Stead et al., 2002; reviewed in White and Dalton, 2005). This has been shown to be due to a lack of Cdk4/Cdk6-cyclin D1 activity at G1 phase and precocious cell cycle-independent Cdk2 and cyclin A/E activity (Savatier et al., 1996; Stead et al., 2002). In light of the fact that miR-34 targets Cdk4/Cdk6 and cyclin D1 (Bommer et al., 2007; Corney et al., 2010; Fujita et al., 2008; He et al., 2007; Lodygin et al., 2008; Sun et al., 2008) and that miR-34 is differentially expressed during HeLa cell cycle (Zhou et al., 2009), we investigated the expression of miR-34 in each phase of the cell cycle of wild type ES cells. ES cells were incubated with the cell membrane-permeable fluorophore Hoechst 33342, fluorescence-activated cell sorted (FACS) based upon DNA content using FACS Aria (BD Biosciences, San Jose, CA) and total RNA isolated was used in qRT-PCR reactions. While only 8% of cells were in G0/1 phase, miR-34 expression was highest in this population and expression during S and G2/M phase was 38-fold to 167-fold lower than during G0/1 (Figure 4.11). We next sorted primary cultured wild type MEF cells and a similar trend was observed, albeit with overall 4- to 8-fold reduced expression, consistent with data presented in Figure 4.10. These data support the hypothesis that miR-34 has a function in controlling Cdk4/Cdk6-cyclin D1 activity and proliferation in ES cells.

4.5 Discussion

As shown in Table 4.1, one copy of the *mir-34* gene is found in most animals, with more copies in higher vertebrates. *Branchiostoma floridae*, the Florida lancet, has two identified copies of *mir-34* in its genome and sits between the

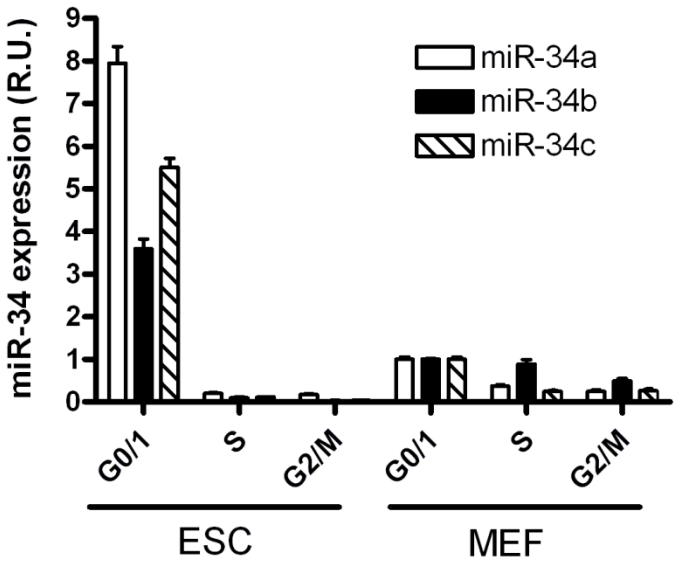


Figure 4.11 miR-34 is preferentially expressed during G0/1 phase of the cell cycle. ES cells (ESC) or MEF cells were sorted based upon DNA content and total RNA isolated. qRT-PCR expression analysis reveals 38-fold, 48-fold and 53-fold reduced expression in S phase compared to G0/1 phase for miR-34a, miR-34b and miR-34c, respectively. During G2/M phase, expression is reduced 38-fold and 167-fold for miR-34a and miR-34b/c, respectively. A similar relationship, although less pronounced, is observed for MEF cells.

invertebrates, with one copy, and most vertebrates that have three. Interestingly, *B. floridae* might be considered a proto-vertebrate and is widely regarded to be a close relative to the chordate ancestor that eventually gave rise to higher vertebrates 500 million years ago (Putnam et al., 2008). It therefore appears that the progression from one to three *mir-34* copies has been selected during evolution.

Mechanisms of tumor suppression have become more complex simultaneously with the increasing complexity of multicellular organisms. For example, the ancestral *p53* gene, *nvp-63*, found in the sea anemone *Nematostella vectensis* functions in protecting the germline, but not somatic cells, from UV irradiation-induced DNA damage (Pankow and Bamberger, 2007). Over hundreds of millions of years, *nvp-63* has evolved into a three-member family, comprising *p53*, *p63* and *p73,* which protect both germ and somatic cell lineages from a diverse range of oncogenic stimuli (Belyi and Levine, 2009). Given our current understanding of the role of miR-34 in tumor suppression, an increase in number of *mir-34* genes is consistent with an important role in evolutionary fitness.

Our *in situ* hybridization experiments reveal that miR-34a is expressed in the adult, but not embryonic, mouse brain consistent with expression in *C. elegans*, *D. rerio* and *G. gallus* (Darnell et al., 2006; Kapsimali et al., 2007; Kato et al., 2009). Additionally, however, expression is observed in cardiac muscle, epithelial tissues of lung and intestine and cartilage and peripheral nervous tissue. miR-34b, and by extension miR-34c due to sharing of promoter, is expressed in the same tissues as miR-34a with the exception of loss of staining in intestine and gain in pancreas and testis. The strong miR-34b expression and overlap in expression with miR-34a is surprising given

qRT-PCR data suggesting little co-expression (Bommer et al., 2007). However, there is an important caveat that might explain these differences. Bommer et al. determined miR-34 expression in the adult mouse and, as observed in brain, miR-34a and miR-34b expression appears to be developmentally regulated in this tissue. Evaluation of miR-34a and miR-34b expression by *in situ* hybridization at later stages of embryonic development will be required to fully understand expression pattern. However, the possibility remains that, due to high sequence similarity, miR-34a and miR-34b probes are not completely specific. This can be best addressed by *in situ* hybridization experiments using miR-34a and miR-34b probes in *mir-34b/c*-null embryos.

To study the role of miR-34b/c in the mouse, conditional knockout mice were prepared. To facilitate this, a recombineering approach was used, which takes advantage of the high efficiency of homologous recombination between short regions of homology in an *E. coli* system. The targeting construct, with a total of 8 kb arms of homology, was electroporated into E14.1 ES cells, but no homologous recombination events were identified after screening over 300 colonies. We therefore used BAC targeting in ES cells, taking advantage of the large arms of homology that this approach affords. Using a novel qPCR screening approach, 2.2% (5/230) clones were identified as having undergone homologous recombination. Valenzuela et al. prepared 200 conventional knockout ES cells using BAC electroporation and calculated an average targeting frequency of 3.8% using a related qPCR assay (Valenzuela et al., 2003). Since cells are grown and screened in 96-well plates, rather than 24-well plates used for traditional Southern blotting approaches, BAC recombineering combined with a BAC qPCR assay results in a relatively high throughput method well suited to rapid creation of conditional knockout ES

cells ready for blastocyst injections.

mir-34b/c$^{-/-}$ embryoid bodies appear to have reduced propensity to attach to bacterial plastic compared to wild type embryoid bodies. This observation suggests that miR-34b/c functions in cell adhesion. EMT is a process whereby epithelial cells lose their characteristic apical-basal polarity, reorganize their cytoskeleton and acquire migratory and invasiveness properties typical of mesenchymal cells (Acloque et al., 2009). This process is critical for normal development, for example neuroepithelial cells of the neural tube undergo EMT resulting in migratory neural crest cells that navigate away from the neural tube to generate peripheral ganglia (Acloque et al., 2009). Yet it is becoming increasingly accepted that an EMT-like process may play a role in carcinogenesis, since metastatic cells share similar traits to normal cells that have undergone EMT, such as the ability to migrate and invade (Gregory et al., 2008; Mani et al., 2008; Morel et al., 2008; Polyak and Weinberg, 2009; Thiery, 2002). Given these observations, it is noteworthy that multiple members of the Notch pathway are predicted or validated targets of miR-34, including receptors Notch 1, Notch 2, Notch 3, and their ligands Delta-like 1, Jagged 1 (Hermeking, 2010; Ji et al., 2008; Li et al., 2009c; Pang et al., 2010; Tsang et al., 2010). Notch is critical for EMT to proceed correctly in the developing zebrafish, in part through activation of Snail signaling (Timmerman et al., 2004). In addition to regulating EMT, differentiation and cell fate decisions (reviewed by Bolos et al., 2007), Notch signaling is frequently altered in cancer and is most well understood to be oncogenic, although depending on cell type and mutation type can be tumor suppressive (reviewed by Radtke and Raj, 2003). Notch has also been shown to play a potential role in EMT during carcinogenesis and its overexpression is correlated with EMT-

like characteristics in cells grown in culture, such as increased E-cadherin expression (Sahlgren et al., 2008; Timmerman et al., 2004), while inhibition of Notch signaling increases E-cadherin expression *in vivo* (Leong et al., 2007). Additionally, the hepatocyte growth factor receptor, c-Met, has pro-migratory and EMT function and is a critical target in miR-34-dependent inhibition of invasion and motility (Corney et al., 2010; He et al., 2007; Migliore et al., 2008). Together, these observations suggest that miR-34b/c may control EMT processes during embryonic development and cancer progression. Clearly it should be stressed, however, that given the preliminary nature of these results detailed follow up and experimental interrogation is required.

In addition to targeting of numerous members of the Notch signaling pathway, miR-34 family has been shown to regulate expression of multiple proteins involved in cell cycle progression such as Cdk4, Cdk6 and Cyclin D1 (Bommer et al., 2007; Corney et al., 2010; Fujita et al., 2008; He et al., 2007; Lodygin et al., 2008; Sun et al., 2008). The Cdk4/Cdk6/Cyclin D complexes are important for progression through the G1/S phase transition and their inactivation results in reduced proliferation, although only of certain cell lineages (Malumbres and Barbacid, 2005). While these proteins are active at G1/S transition, ES cells have a short G1 phase due to low Cdk4/Cdk6-Cyclin D1 activity. We therefore hypothesized that miR-34 expression would be highest at G0/1 in ES cells and our observations support this (Figure 4.10). On the other hand, Cdk4/Cdk6-Cyclin D1 is active in differentiated cells and consistent with this miR-34 levels are reduced 4- to 8-fold during G0/1 phase compared to ES cells.

In conclusion, there is evidence to support a role for miR-34 family in both normal development and in carcinogenesis, potentially though control of

Notch signaling and cell cycle progression. The *mir-34b/c*$^{loxP/loxP}$ conditional knockout ES cells, and mice, that have been generated will allow for a more complete understanding of miR-34b/c function in normal development and disease.

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CHAPTER 5

SUMMARY AND FUTURE DIRECTIONS

5.1 Summary

Given that mutation of the transcription factor *p53* is one of the most common genetic alterations observed in EOC, particularly in high-grade serous tumors (Chapter 1 and reviewed in Corney et al., 2008), a study was initiated to determine whether inactivation of *p53* would result in miRNAome alterations (Chapter 2 and Corney et al., 2007). Towards this aim we took advantage of mice carrying conditional alleles of *p53*, such that *p53* is inactivated by Cre/*loxP*-mediated recombination. Previously, we demonstrated that adenovirus mediated delivery of Cre (Ad*Cre*) to the OSE of mice carrying conditional alleles of both *p53* and *Rb* results in frequent serous adenocarcinomas (Flesken-Nikitin et al., 2003). However, to identify alterations in miRNA expression immediately after acute *p53* inactivation, we decided to use a cell culture approach, whereby the ovary from $p53^{loxP/loxP}$ mice is enzymatically digested to release the OSE which is briefly cultured prior to Ad*Cre* treatment. Compared to non-floxed wild type OSE, 84 miRNAs were significantly up/downregulated after *p53* inactivation, demonstrating that p53 does indeed control miRNA expression, at least indirectly. We observed that miR-34b and miR-34c, which are encoded by genes located 417 bp apart on human chromosome 11q23 and together form the miR-34b/c cluster, were two of the most down regulated miRNAs, with a 12-fold reduction in their expression as determined by qRT-PCR. Through a bioinformatics approach, a

conserved p53 responsive element (p53RE) was identified 2.4 kb upstream of mouse *mir-34b/c* and 3.8 kb upstream of human *mir-34b/c*. We observed that expression of both miRNAs after treatment with the DNA damaging agent doxorubicin was p53-dependent and, taken together with ChIP-PET data (Wei et al., 2006) and ChIP-PCR performed by other research groups (Bommer et al., 2007; He et al., 2007), demonstrates that p53 directly transactivates miR-34b and miR-34c. In addition, several other groups concomitantly identified another miR-34 family member, miR-34a, as being directly regulated by p53 through the presence of a p53RE 30 kb upstream of the miRNA mature sequence (Bommer et al., 2007; Chang et al., 2007; He et al., 2007; Raver-Shapira et al., 2007; Tarasov et al., 2007).

The data shown here, as well as those of others, demonstrates that members of the miR-34 family control important processes that are altered in cancer, including proliferation, apoptosis and senescence in various cell types (Chang et al., 2007; Corney et al., 2007; He et al., 2007; Raver-Shapira et al., 2007; Tarasov et al., 2007). Importantly, however, we have demonstrated that miR-34b and miR-34c cooperate in the suppression of proliferation and adhesion-independent growth in soft agar, and that maximal suppression is achieved when both miRNAs are expressed (Chapter 2 and Corney et al., 2007). This observation may be due to the fact that each miRNA is predicted to target a separate pool of mRNAs in addition to a conserved pool. Predicted targets include *Cdk4*, *Met*, *Ezh2*, *Cyclin D1*, *Myc* and *Bcl-2, Notch 2*, *Jagged 1* and *Sirt1* and a number of these have been validated as genuine targets (Hermeking, 2010; Tsang et al., 2010).

Given the strong evidence that members of the miR-34 family are directly transactivated by p53 and function in several pathways controlled by

p53 (i.e. proliferation, apoptosis and senescence), we decided to initiate a follow-on study to elucidate the role of miR-34 in human EOC (Chapter 3 and Corney et al., 2010). First, expression profiling of a panel of human EOC specimens revealed that expression of all three miR-34 family members are frequently reduced compared to wild type OSE cells. Reduced miR-34a and miR-34b/c expression was strongly correlated with presence of mutant *p53*, yet interestingly, miR-34a was reduced in patients with wild type *p53* too. We interrogated two potential p53-independent mechanisms of miR-34a regulation: promoter methylation and gene copy number variation. Methylation-specific PCR analysis shows that promoter region of *mir-34a*, as well as *mir-34b/c*, are methylated in 27% (8/30) and 47% (14/30) of EOC, in agreement with methylation observed in other neoplastic tissues (Lodygin et al., 2008; Lujambio et al., 2008; Toyota et al., 2008). Additionally, reduced *mir-34a* gene copy number was observed in 39% (13/33) of EOC specimens analyzed, consistent with deletion in neuroblastoma and low grade serous EOC tumors (Cole et al., 2008; Kuo et al., 2009; Welch et al., 2007). These observations strongly suggest that loss of miR-34 expression can occur via a p53-independent mechanism in some cases. Recently, Christoffersen et al. presented data suggesting that an ETS family transcription factor, ELK1, is capable of inducing transcription of miR-34a (Christoffersen et al., 2010). Whether this is also the case for miR-34b/c remains to be determined.

To enable comprehensive analysis of miR-34b/c in a natural context, a conditional *mir-34b/c* knockout mouse strain was prepared (Chapter 4). While gene reconstitution, overexpression and knockdown studies performed by us and others have given noteworthy insight to the role of these miRNAs in disease, such approaches may result in non-physiological levels of

expression, off-site effects and nonspecific alterations due to random transgene integration site. To the contrary, targeted mutation approaches, such as gene knockout or knockdown, do not suffer from these limitations and are considered the gold standard for evaluation of gene function in physiological context.

5.2 Future directions

5.2.1 Control of mir-34 transcription

The p53 protein has been shown to bind promoter regions of both *mir-34* loci resulting in their transactivation, while reduced miR-34 expression is strongly correlated with *p53* mutation in human cancers (Corney et al., 2010; Mraz et al., 2009; Zenz et al., 2009). Recently, alternative mechanisms of *mir-34* regulation have been studied, with a focus on promoter methylation and copy number variation (Cole et al., 2008; Corney et al., 2010; Kuo et al., 2009; Lodygin et al., 2008; Lujambio et al., 2008; Toyota et al., 2008; Welch et al., 2007). However, a complete understanding of transcriptional control over *mir-34* loci has not been achieved. miR-34c expression in testis has been shown to be p53-independent (Bouhallier et al., 2010), demonstrating that transcription factors other than p53 are also involved in *mir-34b/c* regulation. Two transcription factors have recently been proposed to regulate miR-34a expression with strong evidence of directly binding the *mir-34a* promoter from chromatin-immunoprecipitation experiments. First, ELK1 directly binds *mir-34a* in response to B-RAF-mediated oncogene-induced senescence (OIS) in primary human fibroblasts (Christoffersen et al., 2010). Meanwhile, *mir-34a* is

transactivated by AP-1 in the chronic myelogenous leukemia (CML) cell line K562 following phorbol ester-directed differentiation (Ichimura et al., 2010). The AP-1 transcription factor complex, comprised of c-Fos and c-Jun family proteins, is activated by external stimuli via MAPK signaling and is strongly implicated in oncogenic signaling, although some tumor suppressive role has also been reported (reviewed by Eferl and Wagner, 2003). While phorbol ester appears to induce highest transcriptional activity, other stimuli include UV irradiation, TNF α, TGF β, interleukin-1 and mitomycin C (reviewed by Angel and Karin, 1991). Perhaps AP-1-mediated *mir-34a* transcription in response to mitogenic stimuli, and ELK1-mediated *mir-34a* transcription following OIS, functions as a fail-safe mechanism to halt neoplastic growth. In theory, this makes a great deal of sense given that *p53* mutation is a potentially early event in carcinogenesis and without p53-independent transactivation miR-34 function would be completely ablated. Such mitogen-stimulated expression of an antimitogenic gene results in an incoherent feed-forward loop and is typical of the general role of miRNAs in dampening signaling pathways (Hornstein and Shomron, 2006; O'Donnell et al., 2005; Sylvestre et al., 2007).

Certainly, similar currently unidentified pathways might be expected to control miR-34b/c following mitogenic stimulation. Indeed, MAPK signaling was recently implicated in control over miR-34b/c expression following DNA damage (Cannell et al., 2010). Taken together, knowledge of these transcription pathways might go some way to explaining our observations of frequent promoter methylation and copy number variation in human EOC, since both methylation and reduced copy number would be expected to largely eradicate both p53-dependent and independent transcription in response to oncogenic stimuli and might be a critical step in carcinogenesis (Figure 5.1).

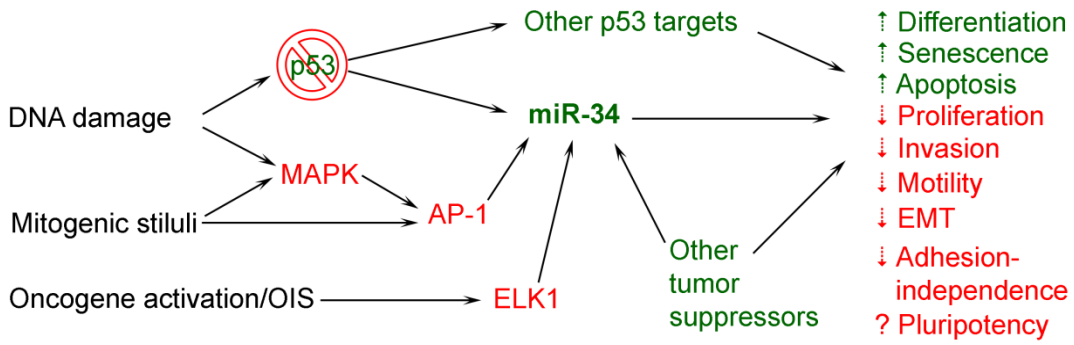

Figure 5.1 A model of known transcriptional regulation of miR-34 and their function. Among other stimuli, DNA damage posttranslational activates p53, resulting in increased transcriptional activity of *mir-34a*, *mir-34b/c* and other p53 targets. However, *p53* mutation is thought to be an early event during carcinogenesis. Yet, through an incoherent feed-forward loop, potentially oncogenic signaling transcriptionally activates miR-34 and dampens the oncogenic signal. Inactivation of the *mir-34* loci, by either methylation or reduced copy number, would largely eliminate miR-34 control over carcinogenesis. Potentially oncogenic genes and processes are indicated in red, while green text indicates tumor suppressive genes/processes.

5.2.2 miR-34 role in carcinogenesis

Experiments to understand the role of miR-34 during cancer initiation will rely heavily on availability of *mir-34b/c* knockout mice. A first aim will be to cross *mir-34b/c* mice with *mir-34a* conditional knockout mice and EIIA-Cre transgenic mice (Lakso et al., 1996), to generate *mir-34a*, *mir-34b/c* and *mir-34a*; *mir-34b/c* compound conventional knockout mice to determine phenotype in regards to embryonic and adult development and cancer susceptibility. Characterizing the effect of *mir-34* inactivation in mouse embryonic fibroblasts (MEFs) and ovarian surface epithelial cells (OSE) will give a rapid indication of p53-independent miR-34 control of proliferation, apoptosis, senescence, colony formation and transformation potential.

Towards cancer susceptibility *in vivo*, wild type and knockout mice exposed to mutagenic treatments, such as whole body gamma irradiation, topical administration with 7,12-dimethylbenz[a]anthracene (DMBA)/phorbol 12-myristate 13-acetate (TPA) or intraperitoneal administration of N-nitroso-N-methylurea (MNU) will allow analysis of DNA damage response in the absence of miR-34, while spontaneous tumor formation can be assessed in untreated knockout mice. *p53*-null mice have increased tumor susceptibility, especially when challenged with genotoxic stimuli (Donehower et al., 1992; Jacks et al., 1994; Kemp et al., 1993; Kemp et al., 1994); a similar or slightly less severe phenotype after *mir-34* inactivation is anticipated.

To build upon characterization of conventional *mir-34* knockout mice, in particular in the event of embryonic lethality, *mir-34a* and/or *mir-34b/c*$^{loxP/loxP}$ mice can be crossed with tissue-specific Cre transgenic mice, such as PB4-Cre to induce gene inactivation in prostate epithelium (Wu et al., 2001),

MMTV-Cre to induce gene inactivation in mammary epithelium (Cheng et al., submitted) and Stra8-Cre to inactivate *mir-34* in spermatocytes (Sadate-Ngatchou et al., 2008). Characterization of phenotype following inactivation in testis will be of particular interest given strong expression in this tissue (Bouhallier et al., 2010 and chapter 4).

Our initial identification of miR-34b/c was in OSE cells and, given our findings of frequent loss of miR-34 expression in human ovarian cancers, *mir-34* conditional mice will be useful to identify what role these genes play in ovarian cancer pathogenesis. Unfortunately, however, no suitable Cre-expressing transgenic line exists due to an absence of OSE-specific promoter (Corney et al., 2008; Nikitin and Hamilton, 2005). To circumvent this quandary, the enclosed anatomical location of the mouse ovary within the ovarian bursa can be used to selectively expose the OSE to Ad*Cre* (Flesken-Nikitin et al., 2003). This approach has a number of advantages over other methods to model EOC in the mouse. Firstly, intrabursal administration of Ad*Cre* removes the requirement for an OSE-specific promoter, while gene inactivation is accomplished in adult immunocompetent mice. The approach also allows conditional and temporal control of the initiating events, which is particularly useful for modeling the early stages of EOC initiation (Corney et al., 2008; Nikitin and Hamilton, 2005). Secondly, a low dose of Cre mimics random low frequency somatic mutation which can result in a more accurate model of tumor initiation (Jackson et al., 2001). Given that inactivation of *p53* in the OSE results in tumor formation in only 6% of mice treated with Ad*Cre* (Flesken-Nikitin et al., 2003), one might anticipate a similar low frequency of tumor formation after *mir-34* inactivation. However, this might not be the case, particularly in *mir-34a*; *mir-34b/c* compound conditional knockout mice, since

targets of miR-34 are vast, numbering in the hundreds, and function in multiple signaling pathways and cellular processes.

Our analysis of miR-34 expression in human EOC specimens revealed that miR-34b and miR-34c, but not miR-34a, is downregulated to a greater extent in stage IV tumors than stage III tumors. While the possibility remains that reduced miR-34b/c is a passenger alteration, reconstitution of miR-34b or miR-34c reduces cell motility and invasion (Chapter 3 and Corney et al., 2010; Migliore et al., 2008) and suggests their direct involvement in EOC progression. Our preliminary experiments have shown that miR-34 reconstitution in neoplastic EOC cells reduces tumor formation in a mouse model of peritoneal spreading (David Corney and Alexander Nikitin, unpublished observations), while tail-vein injection experiments of miR-34b/c transfected head and neck cancer cell line SIHN-011B show reduced lung metastasis formation (Lujambio et al., 2008).

These observations suggest that miR-34b/c is involved in metastatic progression. It has been shown that when p53 is ectopically expressed in *p53*-deficient tumors *in vivo*, tumors rapidly regress through induction of apoptosis or senescence depending on cell type (Martins et al., 2006; Ventura et al., 2007; Xue et al., 2007). Based upon the diverse range of miR-34 targets, one might expect that inducing miR-34 in neoplastic cells deficient in the p53 pathway will have therapeutic benefit. Such a hypothesis might be tested by intraperitoneal injection of *p53*-null and/or *mir-34*-null neoplastic cell lines carrying into mice followed by activation of miR-34 expression from a tet-on based lentiviral system (Shin et al., 2006).

One of the signaling pathways known to play a critical role in invasion and metastasis is the hepatocyte growth factor (HGF)/MET pathway (Mazzone

and Comoglio, 2006). HGF is a pleiotropic factor capable of controlling the proliferation, survival, motility and morphology of a wide spectrum of tissues including epithelial cells through activation of Met tyrosine kinase activity (Bottaro et al., 1991);(Naldini et al., 1991). MET expression has been shown to be a poor prognostic indicator of patients with cancer and knock down of MET by siRNA decreased tumor growth of SKOV-3 ovarian cancer cells injected intraperitoneally into mice (Sawada et al., 2007). Since Met is a well established miR-34 target that has been shown to be required for miR-34 anti-invasion and motility activity in cell culture, it will be interesting to see whether Met is also required for metastatic progression after *mir-34* inactivation *in vivo*.

Currently, strand-specific function of miR-34 (i.e. miR-34a versus miR-34a*) is unaddressed, and the targeting strategy currently reported results in inactivation of both strands of miR-34b and miR-34c. Until recently, only concurrent inactivation or overexpression of both miRNAs was possible, since insertion of a *loxP* site in the hairpin region of a pre-miRNA would likely result in little or no processing by Dicer, while targeted mutation of the seed region of one strand may result in loss of secondary hairpin structure and consequently reduced processing. Work in the Hannon lab has identified processing of a miR-451, an endogenous non-canonical Dicer-independent miRNA containing a single, mature, miRNA localized to the loop of the hairpin with no passenger strand (Cheloufi et al., 2010). As a result, one could envision designing a lentivirus containing an artificial Dicer-independent miRNA to allow expression of one miRNA per hairpin without production of any passenger strand. Furthermore, such a system could be optimized for conditional strand inactivation *in vivo*, as outlined in Figure 5.2.

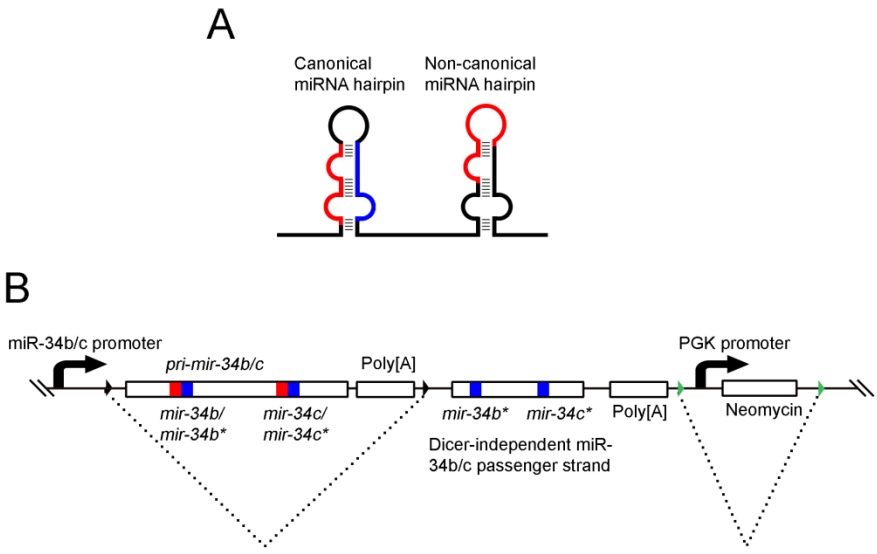

Figure 5.2 A strategy for strand-specific miRNA inactivation. A, Schematic of canonical, Dicer-dependent miRNA hairpin (left) and non-canonical, miR-451-based Dicer-independent miRNA hairpin (right). Guide strand shown in red and passenger strand shown in blue. B, A strategy for guide strand-specific inactivation of miR-34b and miR-34c. *pri-mir-34b/c*, under control of its endogenous promoter, is flanked by *loxP* sites (black triangles). An endogenous poly[A] site prevents polymerase II read-through to a Dicer-independent miR-34b* and miR-34c* cassette that has been inserted 3' of *pri-mir-34b/c*. Following Cre-*loxP*-mediated inactivation of *pri-mir-34b/c* and subsequent loss of poly[A] site, non-canonical miR-34b* and miR-34c* are transcribed under control of the endogenous promoter. A neomycin cassette flanked by FRT sites (green triangles) allows for positive selection in ES cells. Recombination between *loxP* and *FRT* sites indicated by dotted lines.

5.2.3 Role of miR-34 in cell reprogramming and normal development

Cell fusion and nuclear transfer approaches have been successful in reprogramming somatic cells to a pluripotent state, although the mechanism of this reprogramming process is unclear (Cowan et al., 2005; Wakayama et al., 1998; Wilmut et al., 1997). In 2006, however, Takahashi and Yamanaka reported that ectopic expression of just 4 genes, Oct3/4, Sox2, c-Myc and Klf4, followed by selection for *Fbx15* expression, is sufficient to reprogram adult and embryonic mouse somatic cells into a pluripotent state (Takahashi and Yamanaka, 2006). With the exception of being able to generate adult chimaeric mice, such cells, termed induced pluripotent stem (iPS) cells, are analogous to ES cells by all functional assays. However, at the molecular level, such iPS cells are not comparable to ES cells, having different mRNA gene expression and methylation profiles, suggesting that reprogramming is not complete. The same group and others went on to show that using the same four factors followed by selection for *Nanog* expression, instead of *Fbx15*, results in iPS cells with an almost identical DNA methylation pattern of ES-cell specific genes, greater identity to ES cells by microarray analysis compared to *Fbx15* iPS cells and, most importantly, generation of germline transmitting chimeras (Okita et al., 2007; Wernig et al., 2007).

The most recent studies show that expression of the four reprogramming factors is only required for 10-12 days, after which iPS cells enter a self-sustaining pluripotent state and the ectopic genes are silenced (Brambrink et al., 2008; Stadtfeld et al., 2008a) can be completely removed (Kaji et al., 2009; Stadtfeld et al., 2008b; Woltjen et al., 2009). Lack of viral insertion and excision of transgene is important to reduce risk of tumor

formation, since tumors have been observed after c-Myc reactivation (Okita et al., 2007). Although the precise mechanism is yet to be clearly defined, reprogramming appears to be a stochastic process that can be accelerated by elimination of the p53 pathway (Hanna et al., 2009; Hong et al., 2009; Kawamura et al., 2009; Li et al., 2009a; Marion et al., 2009; Utikal et al., 2009). Whether this effect is due to increased proliferation following p53 pathway inactivation or a role of p53 in promoting differentiation, i.e. through inhibition of *Nanog* (Lin et al., 2005), remains to be determined, although Hanna et al. suggested the former. However, I hypothesize that miR-34 also plays a role in inhibiting reprogramming and that this inhibition is not necessarily dependent on its anti-proliferative function. Among the known targets of miR-34 are two of the original reprogramming factors, Myc and Klf4 (Christoffersen et al., 2010; Kong et al., 2008; Lujambio et al., 2008; Tazawa et al., 2007; Klf4 validation by our unpublished observations, David Corney, Chang-il Hwang, Kirsten Elzer and Alexander Nikitin). Therefore, miR-34 inactivation should relieve c-Myc and Klf4 repression and result in increased reprogramming efficiency. Given recent data showing p53-independent miR-34a transcription (Christoffersen et al., 2010), this outcome might be independent of p53 and so it would be interesting to observe reprogramming efficiency 1) in *p53* wild type, *mir-34*-null cells and 2) *p53*-null cells with miR-34 reconstitution via p53-independent lentiviral transgene expression, such as the lentivirus used in Chapter 2. These experiments would determine whether the observed involvement of p53 in inhibiting reprogramming is dependent on intact miR-34 function.

A role for miR-34 family in cell reprogramming leads one to question their potential function normal stem cells and during differentiation. One well-

characterized model of stem cell differentiation is that of the hematopoietic system, where multipotent hematopoietic stem cells (HSC) give rise to all blood cells (reviewed by Orkin and Zon, 2008). As previously mentioned, miR-34a expression is induced following phorbol ester-directed differentiation of K562 cell line towards megakaryocytic lineage (Ichimura et al., 2010; Navarro et al., 2009). miR-34a reconstitution in the absence of phorbol ester reduced proliferation as expected, but also induced differentiation as measured by CD41 expression. The mechanism appears to involve direct repression of MEK1 and Myb, although Navarro et al. report that Myb expression is reduced prior to miR-34a expression, leading the authors to suggest that miR-34a is required to maintain, but not establish, Myb repression prior to differentiation. Further supporting a role in stem cell differentiation, miR-34c is reported to be expressed most strongly in more differentiated late pachytene spermatocytes and round spermatids than early pachytene spermatocytes (Bouhallier et al., 2010). It is worth noting, however, that miR-34 function may be cell-type specific, for example miR-34 expression is decreased during early ES cell differentiation (Chapter 4).

In conclusion, the mouse studies presented here identified p53-dependent regulation miR-34b/c in OSE and provided evidence to suggest a role in tumor suppression through inhibition of proliferation and adhesion-independent growth. Our follow-up study in human EOC further supports a role in tumor suppression, firstly by providing evidence that miR-34 expression is reduced in EOC via multiple mechanisms and secondly showing that parameters of advanced disease, such as invasion and motility, are also controlled by miR-34 family. To provide the gold standard evidence to support a hypothesis that miR-34 family represent tumor suppressors, gene targeting

experiments must be performed. Therefore our most recent work has focused on the generation of *mir-34b/c* conditional knockout mice. Characterization of these *mir-34b/c*, and *mir-34a*, knockout mice will allow for a more complete understanding of their role *in vivo* and their potential therapeutic use in humans.

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