The Oncogenic Role of miR-155

Samuel Mattiske

B. Medical and Pharmaceutical Biotechnology (Honours)

Thesis submitted for the Degree of Masters of Philosophy (Medical Science)

Cancer Therapeutics Laboratory,

Faculty of Health Sciences,

Discipline of Medicine,

University of Adelaide, South Australia

August 2013

Contents

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree. I give consent to this copy of my thesis when deposited in the University Library, being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968. The author acknowledges that copyright of published works contained within this thesis resides with the copyright holder(s) of those works. I also give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library catalogue and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

Signed _____ Date _____

List of Publications

Sam Mattiske, Rachel J Suetani, Paul M Neilsen, David F Callen (2012) The Oncogenic Role of miR-155 in Breast Cancer. *Cancer Epidemiology, Biomarkers and Prevention*. Published.

Paul M Neilsen*, Jacqueline E Noll*, Sam Mattiske*, Cameron P Bracken, Philip A Gregory, Renee B Schulz, Sue P Lim, Raman Kumar, Rachel J Suetani, Gregory J Goodall and David F Callen. (2012) Mutant p53 drives invasion in breast tumors through up-regulation of miR-155. *Oncogene*. Published

Sam Mattiske, Kristen Ho, Paul M. Neilsen, Jacqueline E. Noll, David F. Callen and Rachel J. Suetani. (2013) TAp63 regulates oncogenic miR-155 to mediate migration and tumour growth. *Oncotarget*. Manuscript submitted

* These authors contributed equally to this work

Acknowledgements

I would like to firstly acknowledge my supervisors Rachel Suetani, Paul Neilsen and David Callen. Thankyou David for offering me the scholarship that gave me the opportunity to undertake this work. Thankyou Paul for your assistance interpreting data, and also for graciously offering me joint first author on the oncogene paper. Most of all I would like to thank Rachel for her guidance and support. Rachel was there for me on a daily basis to assist me and keep me on track, and was always understanding of the issues I was having. She was always supportive and encouraging even when under stress from her own project. Thankyou Rachel.

I would also like to acknowledge the other members of the Cancer Therapeutics Laboratory. Thankyou for being helpful and friendly, and making the lab an enjoyable workplace. In particular I thank the lab manager Renee Schulz, who was always happy to help teach new techniques and was patient even when asked stupid questions. Especially when I first started my candidature, without Renee's assistance I would have been lost in the lab.

Finally I would like to thank my family and friends, but most of all my loving fiancée Eleanor Kelly, for supporting me throughout my postgraduate studies. Thankyou Eleanor for taking care of me and always being caring and understanding. Without having you there for me, I don't know what I would have done. And similarly, thankyou to me friends, being able to relax and unwind with such good friends after stressful weeks and failed experiments helped keep me sane.

Overview

MicroRNAs (miRs) are regulatory small noncoding RNAs that control expression of target genes by inhibiting translation and directly targeting messenger RNA (mRNA) transcripts for degradation [1]. The mature miR binds to its target by partial complementarity, usually in the 3`UTR of target mRNA. Each miR has a specific complementary seed sequence, around 7 or 8 nucleotides long. By binding to the seed sequence on the mRNA, the miR can either cause the target mRNA to be destroyed, or merely inhibit subsequent translation of the mRNA [2-4]. A single miR can regulate multiple targets [5].

miR expression profiles have been used to classify cancers, reviewed in [6], and investigations into breast cancer expression profiles have discovered abnormally high levels of particular miRs [7-10]. Studies are underway to identify the mechanisms underlying the deregulation of miRs and their association with cancer [11]. In breast cancer a small number of miRs have been found to be significantly deregulated in breast cancer tissue compared with non-malignant breast tissue [7, 9, 10, 12].

Expression profiling of miRs comparing normal breast tissue and breast tumours have found that miR-155 is upregulated in breast cancer and can act as an oncomir [9-11]. Since miRs operate by inhibiting the translation of their target mRNA, one could speculate that miR-155 targets might be critical in breast tumour progression and metastasis.

The aim of this work was to investigate the oncogenic role of miR-155 in breast cancer.

Chapter 1 is a literature review focussed on miR-155 in breast cancer, including the clinical relevance of miR-155, functional characterisation, regulation of miR-155 and target genes of miR-155. In the review, a comprehensive list of all confirmed miR-155 target genes was compiled, in order to act as a resource for future researchers investigating the functional

significance of miR-155 dysregulation. The review also encompasses the origin of this miR and subsequent processing. The main aim of the literature review was to establish the field of knowledge, in order to identify areas of interest for future research: areas involving miR-155 in breast cancer that had not been fully explored.

In Chapter 2 the upregulation of miR-155 by p63 and mutant p53 in breast cancer is investigated, as well as the novel downstream target of miR-155, ZNF652. ZNF652 was an appealing target gene to investigate, as it was found to repress drivers of invasion and metastasis and could be a key downstream target of miR-155 and thus be the basis for miR-155's oncogenic effects in breast cancer. The discovery that miR-155 was upregulated by p63 and mutant p53 was exciting, as the regulation of miR-155 is an area that has not previously been thoroughly researched (as revealed by the literature review in Chapter 1).

The regulation of miR-155 is a theme that is continued in Chapter 3, which investigates which p63 isoform is responsible for the regulation of miR-155. The scope of this work was broader and not limited to breast cancer alone, as the mechanism of miR-155 regulation could be relevant to any of the cancer types in which miR-155 is upregulated. The TAp63 and ΔNp63 isoforms have opposing effects in cancer, and understanding the mechanism of regulation of miR-155 could aid our understanding of how miR-155 becomes highly upregulated in invasive breast cancers. Furthermore, this understanding could be used to better diagnose or treat patients with invasive breast cancer.

Finally, Chapter 4 summarises the results and implications of this research.

References

1. Thiery JP, Acloque H, Huang RY, Nieto MA. Epithelial-mesenchymal transitions in development and disease. Cell. 2009; 139: 871-90.
2. Llave C, Xie Z, Kasschau KD, Carrington JC. Cleavage of Scarecrow-like mRNA targets directed by a class of Arabidopsis miRNA. Science. 2002; 297: 2053-6.
3. Rhoades MW, Reinhart BJ, Lim LP, Burge CB, Bartel B, Bartel DP. Prediction of plant microRNA targets. Cell. 2002; 110: 513-20.
4. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell. 2004; 116: 281-97.
5. Krek A, Grun D, Poy MN, Wolf R, Rosenberg L, Epstein EJ, MacMenamin P, da Piedade I, Gunsalus KC, Stoffel M, Rajewsky N. Combinatorial microRNA target predictions. Nat Genet. 2005; 37: 495-500. Epub 2005 Apr 3.
6. Lu J, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, Peck D, Sweet-Cordero A, Ebert BL, Mak RH, Ferrando AA, Downing JR, Jacks T, Horvitz HR, Golub TR. MicroRNA expression profiles classify human cancers. Nature. 2005; 435: 834-8.
7. Volinia S, et al. A microRNA expression signature of human solid tumors defines cancer gene targets. Proc Natl Acad Sci U S A. 2006; 103: 2257-61.
8. Blenkiron C, Goldstein LD, Thorne NP, Spiteri I, Chin SF, Dunning MJ, Barbosa-Morais NL, Teschendorff AE, Green AR, Ellis IO, Tavare S, Caldas C, Miska EA. MicroRNA expression profiling of human breast cancer identifies new markers of tumor subtype. Genome Biol. 2007; 8: R214.
9. Iorio MV, et al. MicroRNA gene expression deregulation in human breast cancer. Cancer Res. 2005; 65: 7065-70.
10. Hannafon BN, Sebastiani P, de Las Morenas A, Lu J, Rosenberg CL. Expression of microRNA and their gene targets are dysregulated in preinvasive breast cancer. Breast. 2011; 13: R24.
11. O'Day E, Lal A. MicroRNAs and their target gene networks in breast cancer. Breast. 2010; 12: 201. Epub 2010 Mar 19.
12. Kong W, He L, Coppola M, Guo J, Esposito NN, Coppola D, Cheng JQ. MicroRNA-155 regulates cell survival, growth, and chemosensitivity by targeting FOXO3a in breast cancer. J Biol Chem. 2010; 285: 17869-79.

Chapter 1 – The Oncogenic Role of miR-155 in Breast Cancer

Sam Mattiske, Rachel J Suetani, Paul M Neilsen, David F Callen

Cancer Therapeutics Laboratory

Discipline of Medicine

University of Adelaide, SA, Australia

Cancer Epidemiology, Biomarkers and Prevention 2012; 21(8): 1236-43

Statement of Authorship

Statement of Authorship

Title of Paper	The oncogenic role of miR-155 in breast cancer
Publication Status	☑ Published, ○ Accepted for Publication, ○ Submitted for Publication, ○ Publication style
Publication Details	

Author Contributions

By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

Name of Principal Author (Candidate)	Sam Mathise		
Contribution to the Paper	Conception and design, development of methodology, acquisition of data, analysis and interpretation, writing/review/revision		
Signature		Date	1-7-13

Name of Co-Author	Rachel Smeltzer		
Contribution to the Paper	Conception and design, analysis and interpretation, writing/review/revision, study supervision		
Signature		Date	16.7.13 (signed electronically)

Name of Co-Author	Paul Neilsen		
Contribution to the Paper	writing/review/revision, study supervision		
Signature		Date	22/7/13

Name of Co-Author	David Callen		
Contribution to the Paper	writing/review/revision, study supervision		
Signature		Date	22/7/13

Abstract

miR-155 is an oncogenic microRNA with well described roles in leukemia. However, additional roles of miR-155 in breast cancer progression have recently been described.

A thorough literature search was conducted to review all published data to date examining the role of miR-155 in breast cancer. Data on all validated miR-155 target genes was collated to identify biological pathways relevant to miR-155 and breast cancer progression. Publications describing the clinical relevance, functional characterisation, and regulation of expression of miR-155 in the context of breast cancer are reviewed. 147 validated miR-155 target genes were identified from the literature. Pathway analysis of these genes identified likely roles in apoptosis, differentiation, angiogenesis, proliferation and EMT. The large number of validated miR-155 targets presented here provide many avenues of interest as to the clinical potential of miR-155. Further investigation of these target genes will be required to elucidate the specific mechanisms and functions of miR-155 in breast cancer. This is the first review examining the role of miR-155 in breast cancer progression. The collated data of target genes and biological pathways of miR-155 identified in this review suggest new avenues of research for this oncogenic microRNA.

Introduction

MicroRNAs (miRNAs) are small noncoding RNAs which control expression of target genes by either inhibiting protein translation or directly targeting mRNA transcripts of target genes for degradation (1). Each miRNA has a specific seed sequence 7-8 nucleotides long, which directly binds to complementary sequences in regulatory regions of target genes. These binding regions are often in the 3' UTR of target genes, but increasingly are being reported in other non-coding regions such as promoter or intronic regions (2). The short length of the seed sequence facilitates the targeting of many transcripts by a single miRNA (3). Some estimates suggest that 30% of all eukaryotic genes are regulated by miRNAs (4; 5). miR-155, a miRNA widely reported to be involved in lymphoma, is also now emerging to have a role in the progression of solid cancers (6). This review will focus on the microRNA miR-155, and its role in breast cancer.

miRNAs were discovered in 1993 when the *C.elegans* lin-4 gene, which is transcribed but not translated, was found to regulate levels of LIN-14 protein (7; 8). Since this discovery there have been over 500 miRNAs described, regulating a wide range of genes and cellular processes, although the total predicted number of unique miRNAs encoded by the human genome is estimated to be over 1000 (9). Many of these miRNAs are organised as gene clusters and transcribed as multicistronic messages – for example, the *MIRH1* gene encodes 6 different miRNAs (10). The transcription and processing of miRNAs has been well characterised, and is depicted in Figure 1 using miR-155 as an example. miRNAs originate from a ~70 nucleotide RNA hairpin pre-miRNA processed from the RNA transcript of the host gene (11) (in the case of miR-155, the host gene *BIC*). The pre-miRNA is typically cleaved by the Drosha and Dicer exonucleases into a ~22 nucleotide RNA duplex. One strand

of the duplex becomes the mature miRNA and is usually the functional, regulatory unit (12; 13) while the other is designated miR* and is usually degraded. The mature miRNA is loaded into Argonaute proteins, forming the RNA Induced Silencing Complex (RISC). The mature miRNA may then bind to its target by partial complementarity of target gene mRNA and either inhibit translation or cause degradation of the mRNA.

The miR-155 host gene, *BIC*, was first described in 1989 and postulated to be involved in the progression of lymphoma (14). In 2002, Lagos-Quintana *et al* identified miR-155 as a regulatory RNA (15). Subsequently, studies have focussed on the roles of miR-155 in lymphoma (16-19), and also in viral infection, cardiovascular disease and solid cancers (6; 20-22). miR-155 has over 400 predicted gene targets (23) and more than 100 confirmed *bona fide* targets . There is now an emerging role of miR-155 in breast cancer progression (20; 21; 24) which is the focus of this review.

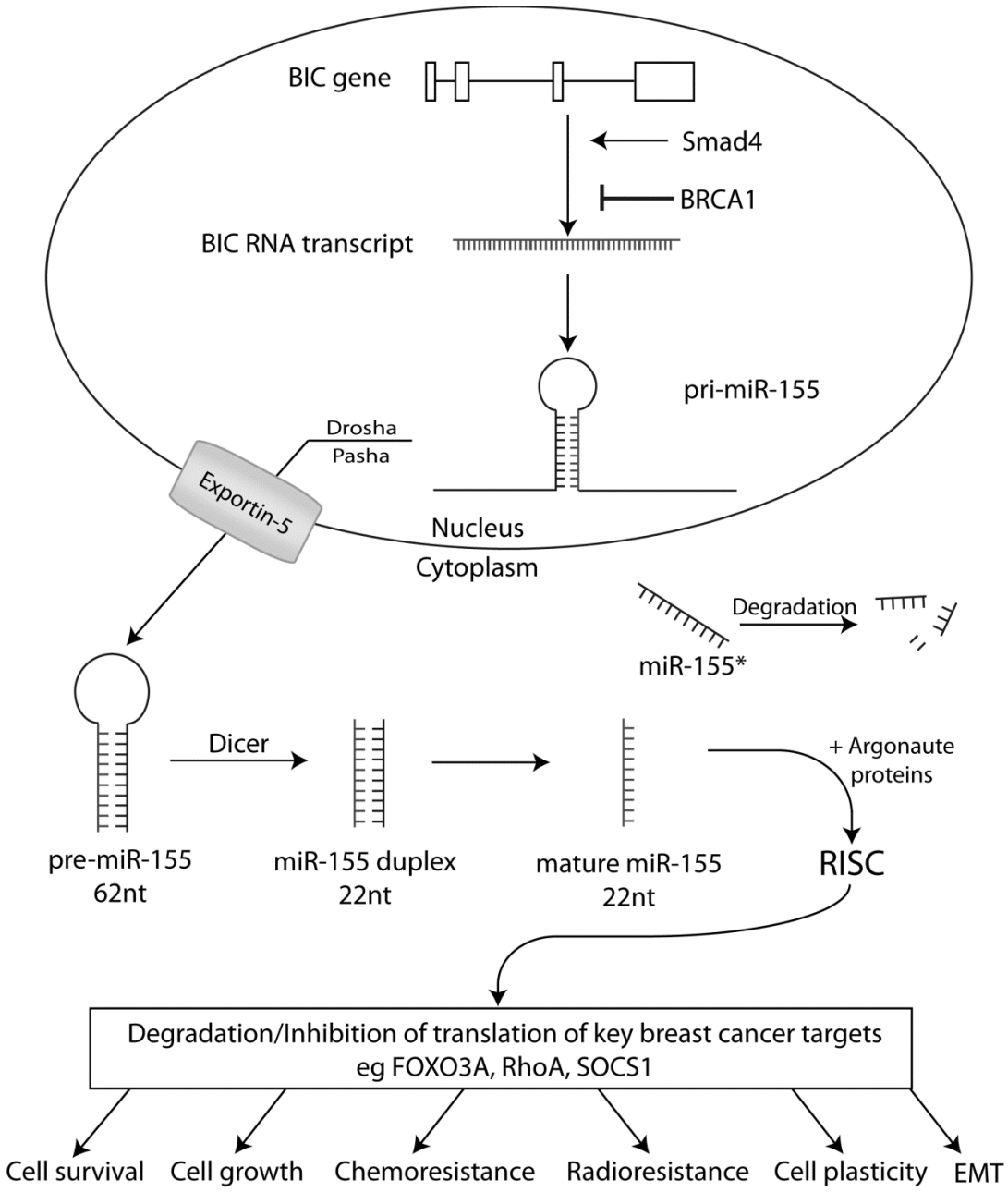


Figure 1: Cellular processing and downstream effects of miR-155 in breast cancer

The pri-miR-155 RNA hairpin transcript is processed from the RNA transcript of the *BIC* gene. Transcription of BIC is promoted by Smad4, and inhibited by BRCA1. After processing by Drosha, Pasha, Exportin-5 and Dicer, the mature miR-155 forms a complex with Argonaute proteins called the RNA Induced Silencing Complex (RISC), in order to inhibit the translation of miR-155 target mRNAs, such as RhoA, FOXO3A and SOCS1. The

inhibition of target genes by miR-155 in breast cancer can cause such effects as an increase in EMT, cell plasticity, cell survival, growth, chemoresistance and radioresistance.

Clinical Relevance of miR-155 in Breast Cancer

Studies show the expression level of miR-155 is upregulated in breast cancer with high levels of miR-155 associated with clinicopathological markers, tumour subtype and poor survival rates, summarised in Table 1 (21, 25-34).

miR-155	Tissue type	Ref
↑ in breast cancer	76 Breast tumour 10 Normal breast	(25)
↑ in breast cancer	363 Breast tumour 177 Normal breast	(26)
↑ in ER- tumours	93 Breast tumour 5 Normal breast	(27)
↑ in malignant breast tissue	34 Breast tumour 6 Normal Breast	(28)
↑ in PR+ tumours	Serum – 13 breast cancer patients, 8 healthy patients	(29)
↑ in grade II and III tumours ↑ in ER- PR- tumours	Tumour, normal adjacent tissue and serum from 68 breast cancer patients Tissue and serum from 40 healthy patients	(30)
Associated with higher tumour grade, advanced tumour stage, lymph node	92 Breast tumour and normal adjacent tissue	(31)

metastasis		
↑ in 41 of 45 invasive ↑ 2 of 17 noninvasive tumours	45 Invasive breast tumour 17 Noninvasive breast tumour	(32)
↑ in 55 breast tumours ↑ 31 recurrent tumours	77 breast tumour 11 Normal breast 38 Recurrent breast tumour	(21)
↑ in breast metastases	13 Breast tumour and paired metastasis	(33)
↑ in tumours	8 Breast tumour and normal adjacent tissue	(34)

Table 1 – Summary studies examining miR-155 expression in breast cancer

Of 29 miRNAs found to be dysregulated in breast cancer, the majority were downregulated, with only miR-155 and miR-21 significantly upregulated (25). Expression levels of 15 of these dysregulated miRNAs independently predict the invasive potential of breast tissue samples (25). A small microarray study of 8 fresh breast tumour samples found miR-155 was upregulated in the breast tumours compared to normal adjacent tissue (34). In a larger study, 62 breast carcinomas were analysed to determine miR-155 levels. Out of 17 non-invasive tumours, only 2 (12%) exhibited a high level of miR-155 expression. Conversely, 41 of the 45 invasive tumours (91%) displayed miR-155 upregulation (32). In a further study expression levels of FOXO3A, a miR-155 target gene, was determined in 77 primary breast tumours, 38 recurrent tumours and 11 normal tissue samples. Results showed miR-155 was upregulated and FOXO3A downregulated in a majority of primary tumours, and also that high miR-155 and low FOXO3A expression was associated with recurrent tumours after

radiotherapy or chemotherapy (21). These studies linked miR-155 expression to both invasiveness and recurrence of breast tumours, and demonstrated that expression levels of miR-155 and its specific target genes are of potential clinical prognostic value.

In a robust study of lung, stomach, prostate, colon, pancreatic tumours and 363 breast tumours, Volinia *et al.* globally compared miRNA expression levels in multiple tumour and pooled normal tissue samples to identify dysregulated miRNAs in tumour samples. Comparisons of normal and tumour tissue derived from each individual tissue showed that miR-155 expression was upregulated in breast, colon and lung cancers. Interestingly, miR-155 was one of only two miRNAs (the other being the miR-200 family) found to be upregulated in both breast and lung cancer, implying that these microRNAs may be part of a common mechanism in the development of cancer in these organs (26).

miR-155 expression levels have been shown to be associated with metastasis events and invasive properties of breast cancer. In one study, increased miR-155 expression was associated with high tumour grade, advanced stage and lymph node metastasis (31). Disease free and overall survival were also negatively correlated with miR-155 levels, further showing the potential of miR-155 as a miRNA of clinical interest. These findings were further supported by two studies involving microarray analyses of FFPE breast cancer samples, which found that miR-155 expression was upregulated in metastases (28, 33).

Since miR-155 is associated with poor prognosis and/or metastasis, a correlation of miR-155 levels with breast cancer clinicopathological markers would be expected. Analysis of 93 breast cancers for both miRNA levels alongside mRNA levels, to classify tumour subtypes, showed miR-155 levels were significantly upregulated in basal-like tumours and in estrogen

receptor negative (ER-) tumours (27). The correlation with basal-like tumours has particular clinical relevance due to the poor prognosis of this tumour subtype.

Studies have investigated whether serum samples could be used to identify aberrant miRNA expression levels in breast cancer patients. In a small study of 21 patients Zhu *et al.* found that multiple miRNAs could be detected in sera and the miRNA levels correlated with the levels in tissue samples (29). The expression of miR-155 was higher in the serum of PR+ breast cancer patients than in the serum of PR- patients (29). Further studies confirmed these findings, with a significant correlation (R^2=0.853) between miRNA levels in fresh breast cancer tissue and matched serum samples (30). They confirmed that miR-155 was upregulated in breast cancer, and also that high miR-155 was associated with grade II and III tumours and ER- and PR- tumours (30). The detection of miR-155 expression levels in serum is a potential clinical prognostic indicator of tumour grade and hormone receptor status. The relationship of PR status and miR-155 expression is unresolved with two studies reporting contradictory results (27, 29). The topic of serum miRNAs is also somewhat controversial, with some studies suggesting that serum miRNA levels are robust (35; 36), and others claiming that the miRNAs often used as normalisation controls are highly variable in sera samples, and thus miRNA quantification in sera is not reproducible (37). This suggests analysis of serum alone is not sufficient to determine whether miR-155 is differentially expressed. Since the number of samples in these studies is generally low, resolution requires a more robust study.

Taken together, these studies show miR-155 expression is upregulated in breast cancer, consistent with its status as an oncomiR, and is associated with more aggressive breast tumours. However, the relationships between miR-155 and clinicopathological markers, such

as ER and PR status and tumour subtype, is inconsistent, probably due to small sample sizes and methodological aspects. For example, the upregulation of miR-155 expression in PR+ tumours was only identified in one study of a small number of samples (29). Further studies are required to confirm, and elucidate the basis, of the relationship between miR-155 and hormone receptor status.

Functional characterisation of miR-155 oncogenic activities in breast cancer

An important step in determining the clinical significance of miR-155, is to determine whether high expression levels are causally related to the development of breast cancer. *In vitro* effects of altering miR-155 expression levels were assessed in a panel of breast cancer cell lines (21). miR-155 expression was inhibited by anti-miR in HS578T cells. An anti-miR is a 2′-O-methyl oligoribonucleotide that inhibits the action of a miRNA. One proposed mechanism for anti-miR action is antisense binding to the mature miRNA positioned in the RISC (38). The HS578T cell line expresses high levels of endogenous miR-155, and anti-miR-155 application resulted in cell cycle arrest and induction of apoptosis, implicating miR-155 in these processes (21). Conversely, ectopic overexpression of miR-155 in BT474 cells, which express very low levels of endogenous miR-155, promoted cell proliferation and survival and also improved chemoresistance (21). Taken together, these findings demonstrate that miR-155 has a role in cell proliferation and apoptosis, two cellular processes frequently aberrant in cancer. Similar results have also been reported in breast cancer cell lines MDA-MB-231 and MCF-7 where ectopic miR-155 overexpression increased proliferation, while inhibition of miR-155 expression by a specific anti-miR inhibits proliferation and increases radio-sensitivity of cells *in vitro* (20; 31).

Xenografted human breast cancer cells in immunodeficient mice provide *in vivo* confirmation of miR-155 as an oncomiR. Xenografts of MDA-MB-231 cells showed reduced tumour volumes compared to control xenografts when anti-miR-155 is expressed, while overexpression of miR-155 accelerated tumour growth (20). Similar, a xenograft of MDA-MB-468 cells, with low endogenous miR-155 expression, showed accelerated tumour growth when miR-155 was overexpressed (24). In the same study, knockdown of miR-155 in an orthotopically transplantated mouse tumour cell line inhibited tumour growth (24). Contrary to this, a recent study using the 4T1 mouse mammary model showed that miR-155 had no effect on growth of the primary tumour (39).

Although numerous studies show miR-155 is upregulated in human breast cancer, the cause of aberrant miR-155 levels is not well characterised. TGFβ treatment of NMuMG (normal murine mammary gland) cells results in significant upregulation of miR-155 and an epithelial to mesenchymal transition (EMT) (32). TGFβ is known to drive EMT, where immobile epithelial cells alter their morphology to become motile mesenchymal cells to promote invasion (40) and consequently cancer progression (41; 42). InNMuMG cells, Smad4, a key signalling molecule in the TGFβ pathway, can bind to the *BIC* promoter and enrich miR-155 expression levels, thereby augmenting the TGFβ EMT process (32). Knockdown of miR-155 in NMuMG cells by anti-miR suppressed, and ectopic overexpression of miR-155 enhanced TGFβ-mediated EMT (32). Furthermore a key molecule in EMT, RhoA, is a target of miR-155, and expression of RhoA is reduced when miR-155 is ectopically expressed. When RhoA was expressed without its 3`UTR (containing the miR-155 seed sequence) the EMT phenotype caused by miR-155 was abrogated (32). The ability to reverse a severe phenotypical change by reexpressing just one of the targets of miR-155 alludes to a potential therapeutic approach. Many miRNAs are known to have a role in metastasis and EMT (43), so in light of these findings it is plausible the basis of miR-155 in promoting breast cancer, in

particular the higher grade invasive breast cancers, is from the promotion of EMT. However, the findings from the 4T1 mouse model (39) contradict the findings in the NMuMG cell line (32). Unfortunately, both of the cell lines are of mouse origin. A miR-155 target gene in a mouse model will not necessarily be a target gene in humans, as the 3`UTR region of transcripts is a common location for miRNA seed sequences, and is not highly conserved between mice and humans. These conflicting results call into question the suitability of using a mouse-specific model for a miRNA study.

Regulation of miR-155 expression

Perhaps the most remarkable recent finding in relation to the role of miR-155 in breast cancer is the involvement with BRCA1. BRCA1, the breast cancer susceptibility gene, is involved in DNA damage repair and cell cycle progression. Mutations of BRCA1 are associated with a high risk of developing breast cancer (24). In a recent study, mouse embryonic stem cells expressing the R1699Q BRCA1 underwent spontaneous differentiation. The mutant cells displayed high levels of miR-155, and overexpression of miR-155 in BRCA1 wild type cells gave a similar phenotype to the mutant, indicating that BRCA1 was acting through miR-155 (24). In mice, a loss of functional BRCA1 results in miR-155 upregulation. These results were recapitulated in human cell lines, where deficient BRCA1 cells have 50-fold higher miR-155 levels compared to those with functional BRCA1 (24). Furthermore, the transient overexpression of BRCA1 reduces expression of miR-155. In clinical samples it was found that miR-155 levels were two to six fold higher in BRCA1 mutant tumours (24). The mechanism of BRCA1 regulation of miR-155 was through direct binding of BRCA1 protein to the miR-155 promoter. This in turn recruits histone deacetylase (HDAC) to repress the

expression of BIC and thus miR-155 (24). This close association with the breast cancer susceptibility gene reinforces the importance of miR-155 in breast cancer.

Target genes of miR-155

The function of microRNAs are limited to inhibition of their target mRNA and consequent effects on cellular processes. miR-155 clearly has a role in breast cancer, and understanding this role requires the identification of critical miR-155 target genes.

Targetscan is an *in silico* prediction software commonly used to identify putative target genes of particular miRNAs by alignment of the 7 or 8 nucleotide seed sequence with the 3' UTR of 30,858 human transcripts based on conservation between human and mouse sequences (23). Targetscan version 6 predicts 440 miR-155 targets (23; 44) based on sequence homology and conservation. Confirmation of these potential targets requires validation *in vitro*. To this end, we conducted literature search to identify published validated miR-155 target genes. A target was defined as validated when there was a specific luciferase 3`UTR reporter assay, which defines if miR-155 directly targets the transcript, together with at least one other quantitative method, such as qRT-PCR or Western blot analysis, to assess the repression of the expression levels of the endogenous target gene.

Supplementary Table 1 displays a comprehensive list of 147 validated target genes identified in a wide range of miR-155 studies (45-87), and their prediction status by Targetscan. 103 target genes (including 11 target genes validated in other studies) were identified in a single high throughput next generation sequencing (NGS) study and validated by luciferase reporter assay (50). The remaining 44 target genes and their method of validation are displayed in

Supplementary Table 2. Of the validated miR-155 target genes, approximately half (48%) were predicted by Targetscan software (23; 44). This highlights the drawbacks in relying on *in silico* prediction tools to investigate potential miRNA targets. The discrepancy between predicted and observed miR-155 binding sites is affected by miR-155 targeting non-conserved sites in target genes, as Targetscan by default searches for seed sequences conserved between human and mouse. Performing a Targetscan search irrespective of site conservation predicts 2390 potential miR-155 targets, and encompasses all but 9 validated target genes. This is the first comprehensive collation of all known miR-155 target genes, and will be a valuable resource for future reference and research.

Although only a fraction of validated miR-155 target genes have a confirmed role in breast cancer, a number of the targets are involved in cancer-related pathways such as apoptosis, proliferation and EMT (20; 21; 32; 88; 89), as shown in Figure 2. The presence of validated miR-155 targets in these pathways highlight the importance of miR-155 in cancer progression.

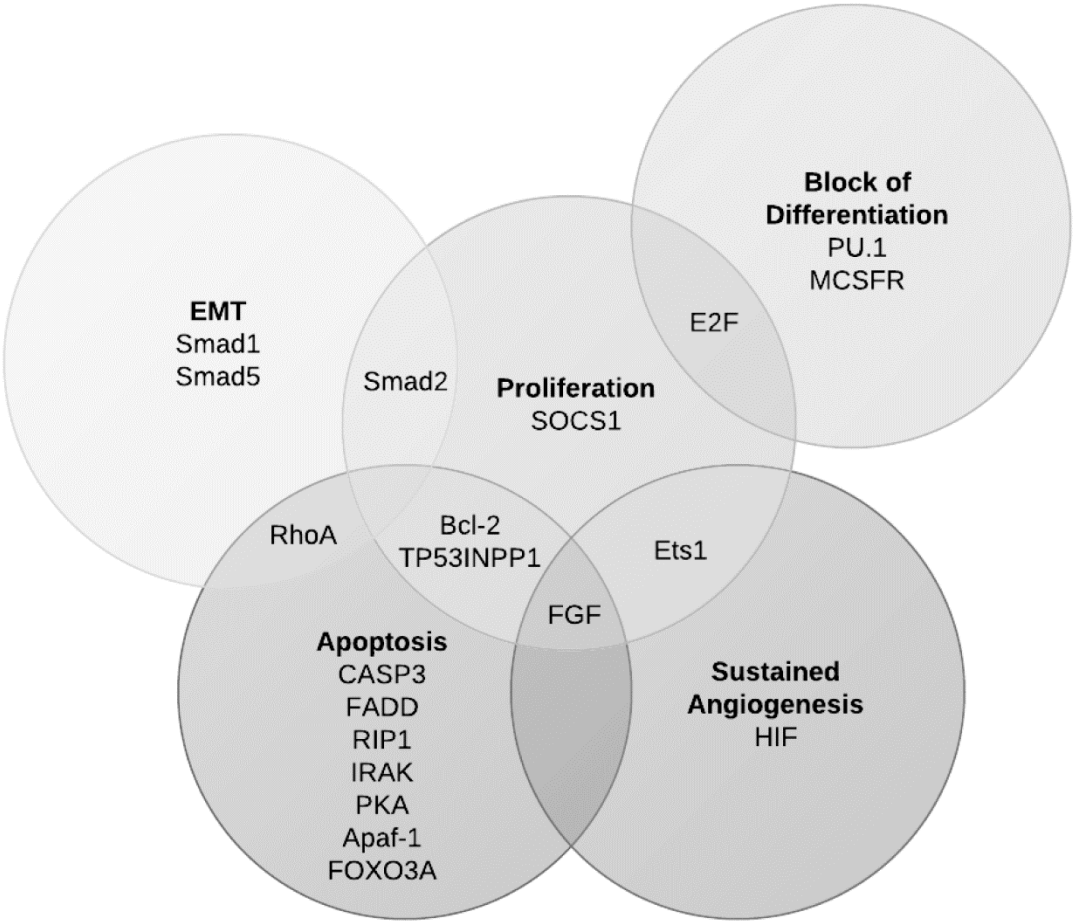


Figure 2 - miR-155 target genes involved in cancer-related pathways

Validated miR-155 target genes are present in multiple pathways associated with cancer and cancer progression, including but not limited to: EMT, proliferation, block of differentiation, apoptosis, sustained angiogenesis. Pathway analysis was completed using DAVID bioinformatics resource (v 6.7).

Conclusion

As an oncomiR, expression levels of miR-155 are consistently upregulated in breast tumour samples. Studies have defined the clinical significance of miR-155 in breast cancer with an association with clinical markers, more aggressive tumours and decreased survival. However, there are some contradictory findings reported, for instance the varied association of miR-155 with hormone receptor status. It is also unclear as to whether miR-155 functions initiate cancer, or predominantly promotes tumour progression. In a mouse model, miR-155 has been shown to transform B-cells (90) but in breast cells has only been shown to enhance cancerous properties of tumour cells. More investigation is required to fully understand the significance of aberrantly high levels of miR-155 in breast cancer.

Exploration of the function of miR-155 in breast cancer cell lines and xenograft models shows that miR-155 enhances tumour growth, promotes cell proliferation, inhibits apoptosis and acts as a mediator of TGFβ-driven EMT. In particular, the role of miR-155 in EMT has promising therapeutic potential, given that miR-155 levels have been shown to be elevated in invasive tumours and in breast tumour metastases. The large number of validated miR-155 targets presented in Supplementary Table 1 provide many avenues of further investigation as to the clinical potential of miR-155. The further investigation of these targets will be required to confirm the mechanistic and regulatory actions of miR-155, and their contribution to breast cancer.

Supplementary Tables

Supplementary Table 1 – Validated miR-155 target genes and prediction status by Targetscan

An extensive literature search identified published validated miR-155 target genes. Validation was defined as luciferase reporter assay and at least one other method. Prediction of miR-155 targeting was determined using Targetscan (v 6.0), searching for conserved sites between human and mouse. Alternative gene names are given in bracket

miR-155 Target Gene	Accession No.	Predicted	Not predicted	Not predicted (nonconserved)	Reference
AGTR1 (AT1R)	NM_000685		X		(45-48)
AICDA (AID)	NM_020661.2	X			(49)
ANAPC16 (C10orf104)	NM_173473.2		X		(50)
APAF1	NM_013229.2		X		(50)
ARID2	NM_152641	X			(50; 51)
ARL15	NM_019087.2		X		(50)
ARL6IP5	NM_006407.3		X		(50)
ARMC2	NM_032131.4		X		(50)
ARNT	NM_001668		X	X	(52)
BACH1	NM_206866	X			(50-52)
BCL2	NM_000633.2		X		(53)
BCORL1	NM_021946.4	X			(50)
C10orf26	NM_001083913.1	X			(50)

C16orf62	NM_020314.4		X		(50)
C3orf18	NM_016210.4	X			(50)
C5orf44	NM_001093755.1		X		(50)
CARD11	NM_032415.3	X			(50)
CARHSP1	NM_014316.2	X			(50)
CASP3	NM_004346		X	X	(54)
CCDC41	NM_016122.2		X		(50)
CDC40	NM_015891.2		X		(50)
CEBPB	NM_005194	X			(51; 52; 55-57)
CHD9	NM_025134.4	X			(50)
CIAPIN1	NM_020313.2		X		(50)
CKAP5	NM_001008938.1		X		(58)
CLUAP1	NM_015041.1		X		(50)
CREB1 (CREB)	NM_004379.3	X			(59)
CSF1R (CD115)	NM_005211	X			(52)
CSNK1A1	NM_001025105.1		X		(50)
CTLA4	NM_005214	X			(60)
CUX1 (CUTL1)	NM_181552	X			(52)
CYP2U1	NP_898898.1		X		(50)
DCUN1D2	NM_001014283.1		X		(50)
DET1	NM_017996.3	X			(50)
DHX40	NM_024612.4	X			(50)

E2F2	NM_004091.2	X			(50)
EIF2C4	NM_017629.2	X			(50)
ETS1	NM_001143820	X			(48; 50)
EXOSC2	NM_014285.5		X		(50)
FADD	NM_003824		X	X	(54; 61)
FAM135A	NM_001105531.2	X			(50)
FAM177A1	NM_173607.3		X		(50)
FAM199X (CXorf39)	NM_207318.2		X		(50)
FAM91A1	NM_144963.2		X		(50)
FGF7 (KGF)	NM_002009	X			(62)
FOXO3 (FOXO3A)	NM_001455	X			(21; 63)
GABARAPL1	NM_031412.2		X		(50)
GATM	NM_001482.2		X		(50)
GCET2 (HGAL)	NM_152785.4		X		(64)
GCFC1 (C21orf66)	NM_016631.3	X			(50)
GNAS	NM_016592.2	X			(50)
GOLT1B	NM_016072.4		X		(50)
HBP1	NM_012257.3	X			(50)
HIF1A	NM_001530		X		(52)
HIVEP2	NM_006734	X			(50; 51; 57)
IGJ	NM_144646.3		X		(50)
IKBKE (IKKE)	NM_014002	X			(61; 65)

IL13RA1	NM_001560		X	X	(66)
IL17RB	NM_018725.3		X		(50)
INPP5D (SHIP1)	NM_001017915	X			(67-71)
INPP5F	NM_014937.2		X		(50)
INTS6	NM_012141.2		X		(50)
IRAK3 (IRAKM)	NM_007199		X	X	(72)
JARID2	NM_004973	X			(50; 52; 73)
KBTBD2	NM_015483.2		X		(50)
KIAA0430	NM_014647.3		X		(50)
KIAA1274	NM_014431.2	X			(50)
KLHL5	NM_015990.4		X		(50)
LCORL	NM_153686.7		X		(50)
LNX2	NM_015093	X			(50)
LRIF3 (C1orf103)	NM_018372.3		X	X	(50)
LRRC59	NM_018509.3	X			(50)
LSM14A	NM_001114093.1	X			(50)
MAP3K10	NM_002446.3	X			(50)
MAP3K14	NM_003954.2	X			(50)
MASTL	NM_001172303.1		X		(50)
MBNL3	NM_018388.3	X			(50)
MCM8	NM_032485.4		X		(50)
MED13L	NM_015335.4		X		(50)

MEF2A	NM_005587.2	X			(50)
MORC3	NM_015358.2		X		(50)
MPP5	NM_022474.2		X		(50)
MRPL18	NM_014161.3		X		(50)
MRPS27	NM_015084.2		X		(50)
MYBL1	NM_001080416.2	X			(50)
MYD88	NM_001172567		X		(74)
MYO10	NM_012334	X			(57)
MYO1D	NM_015194.1	X			(50)
MYST3	NM_001099412.1		X	X	(50)
NARS	NM_004539.3		X		(50)
PAK2	NM_002577.4	X			(50)
PCDH9	NM_203487.2	X			(50)
PDCD4	NM_014456.3		X		(50)
PHC2	NM_198040.2	X			(50)
PHF14	NM_014660.3		X		(50)
PHF17	NM_199320.2	X			(50)
PICALM	NM_007166		X		(50; 52)
PKN2	NM_006256.2	X			(50)
POLE3	NM_017443.4		X		(50)
PRKAR1A	NM_002734.3	X			(50)
RAB11FIP2	NM_014904.2	X			(50)

RAC1	NM_006908.4		X		(50)
RAPGEF2	NM_014247.2		X		(50)
RHEB	NM_005614.3	X			(50)
RHOA	NM_001664		X	X	(32)
RIPK1	NM_003804		X	X	(61)
RNF123 (KPC1)	NM_022064	X			(75)
SAP30L	NM_024632.5	X			(50)
SATB1	NM_002971.3	X			(76)
SDCBP	NM_005625.3	X			(50)
SECISBP2	NM_024077.3		X		(50)
SKI	NM_003036	X			(77)
SLA	NM_001045556	X			(52)
SLC33A1	NM_004733.3	X			(50)
SLC35F2	NM_017515.4		X		(50)
SMAD1	NM_005900	X			(57)
SMAD2	NM_005901	X			(50; 78)
SMAD5	NM_005903		X		(51; 57; 79)
SMARCA4	NM_001128849.1	X			(50)
SOCS1	NM_003745	X			(20; 75; 80)
SOX6	NM_017508.2		X		(81)
SPI1 (PU.1)	NM_001080547		X		(52; 82)
TAB 2 (MAP3K7IP2)	NM_015093	X			(50; 72; 83; 84)

TAF5L	NM_014409.3		X		(50)
TBC1D14	NM_020773.2		X		(50)
TBCA	NM_004607.2		X		(50)
TCF12	NM_207036.1		X		(50)
TLE4	NM_007005.3	X			(50)
TOMM20	NM_014765.2	X			(50)
TP53INP1	NM_033285	X			(50; 85; 86)
TRAK1	NM_001042646.1		X		(50)
TRIP13	NM_004237.3		X		(50)
TSGA14	NM_018718.1	X			(50)
TSPAN14	NM_030927.2	X			(50)
UBQLN1	NM_013438.4		X		(50)
VPS18	NM_020857.2	X			(50)
WEE1	NM_003390	X			(50; 87)
WHSC1L1	NM_023034.1		X		(50)
WWC1	NM_001161661.1	X			(50)
ZKSCAN5	NM_014569.3		X		(50)
ZNF248	NM_021045.1	X			(50)
ZNF254	NM_203282.2		X		(50)
ZNF273	NM_021148.2		X		(50)
ZNF28	NM_006969.2		X		(50)
ZNF611	NM_030972.3		X		(50)

ZNF652	NM_001145365.1	X			(50; 51)
ZNF83	NM_001105549.1		X		(50)

Supplementary Table 2 – Validated miR-155 target genes and validation method

Predicted miRNA target genes were validated by luciferase 3' UTR reporter assay and at least one other method. * next generation sequencing, † immunohistochemistry, ‡ stable isotope labelling by amino acids in cell culture, § enzyme-linked immunosorbent assay, || fluorescence activated cell sorting.

Target gene	Accession No.	3' UTR luciferase assay	Western blot	qRT-PCR	Other	Reference
AGTR1 (AT1R)	NM_000685	X	X	X	Radioreceptor binding	(45-48)
AICDA (AID)	NM_020661.2	X	X			(49)
ARID2	NM_152641	X			NGS*	(50; 51)
ARNT	NM_001668	X	X	X	mRNA microarray	(52)
BACH1	NM_206866	X	X	X	NGS, mRNA microarray	(50-52)
BCL2	NM_000633.2	X			Anti-miR	(53)
CASP3	NM_004346	X	X		Flow cytometry	(54)

CEBPB	NM_005194	X	X	X	IHC†, mRNA microarray	(51; 52; 55-57)
CKAP5	NM_001008938.1	X	X		SILAC‡	(58)
CREB1 (CREB)	NM_004379.3	X	X			(59)
CSF1R (CD115)	NM_005211	X	X	X	Flow cytometry, mRNA microarray	(52)
CTLA4	NM_005214	X			Flow cytometry	(60)
CUX1 (CUTL1)	NM_181552	X	X	X	mRNA microarray	(52)
ETS1	NM_001143820	X	X		NGS	(48; 50)
FADD	NM_003824	X	X		Flow cytometry	(54; 61)
FGF7 (KGF)	NM_002009	X			ELISA§, FACS‖	(62)
FOXO3 (FOXO3A)	NM_001455	X	X			(21; 63)
GCET2 (HGAL)	NM_152785.4	X	X	X		(64)
HIF1A	NM_001530	X	X	X	mRNA microarray	(52)
HIVEP2	NM_006734	X		X	NGS	(50; 51; 57)
IKBKE (IKKE)	NM_014002	X	X	X	miR-155 inhibitor	(61; 65)

IL13RA1	NM_001560	X	X			(66)
INPP5D (SHIP1)	NM_001017915	X	X	X	Bead chip array, anti-miR, IHC	(67-71)
IRAK3 (IRAKM)	NM_007199	X	X	X		(72)
JARID2	NM_004973	X	X	X	miRNA sponge (+luciferase and RT-PCR), mRNA microarray, NGS	(50; 52; 73)
MYD88	NM_001172567	X	X			(74)
MYO10	NM_012334	X		X		(57)
PICALM	NM_007166	X	X	X	mRNA microarray, NGS	(50; 52)
RHOA	NM_001664	X	X	X		(32)
RIPK1	NM_003804	X	X			(61)
RNF123 (KPC1)	NM_022064	X	X			(75)
SATB1	NM_002971.3	X	X		miR-155 inhibitor	(76)
SKI	NM_003036	X	X	X	Anti-miR	(77)
SLA	NM_001045556	X	X	X	mRNA microarray	(52)
SMAD1	NM_005900	X	X	X		(57)

SMAD2	NM_005901	X	X		NGS	(50; 78)
SMAD5	NM_005903	X	X	X		(51; 57; 79)
SOCS1	NM_003745	X	X	X	IHC	(20; 75; 80)
SOX6	NM_017508.2	X	X	X		(81)
SPI1 (PU.1)	NM_001080547	X	X	X	mRNA microarray	(52; 82)
TAB2	NM_015093	X	X	X	NGS	(50; 72; 83; 84)
TP53INP1	NM_033285	X	X	X	IHC, NGS	(50; 85; 86)
WEE1	NM_003390	X	X		NGS	(50; 87)
ZNF652	NM_001145365.1	X	X	X	NGS	(50; 51)

References

1. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell.* 2004;**116**:281-297.
2. Bartel DP. MicroRNAs: target recognition and regulatory functions. *Cell.* 2009 **136**:215-233.
3. Krek A, Grun D, Poy MN, Wolf R, Rosenberg L, Epstein EJ, et al. Combinatorial microRNA target predictions. *Nat Genet.* 2005;**37**:495-500.
4. Lewis BP, Shih IH, Jones-Rhoades MW, Bartel DP, Burge CB. Prediction of mammalian microRNA targets. *Cell.* 2003;**115**:787-798.
5. Yu Z, Jian Z, Shen SH, Purisima E, Wang E. Global analysis of microRNA target gene expression reveals that miRNA targets are lower expressed in mature mouse and Drosophila tissues than in the embryos. *Nucleic Acids Res.* 2007;**35**:152-164.
6. Faraoni I, Antonetti FR, Cardone J, Bonmassar E. miR-155 gene: a typical multifunctional microRNA. *Biochim Biophys Acta.* 2009;**1792**:497-505.
7. Wightman B, Ha I, Ruvkun G. Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in C. elegans. *Cell.* 1993;**75**:855-862.
8. Lee RC, Feinbaum RL, Ambros V. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell.* 1993;**75**:843-854.
9. Berezikov E, Guryev V, van de Belt J, Wienholds E, Plasterk RH, Cuppen E. Phylogenetic shadowing and computational identification of human microRNA genes. *Cell.* 2005;**120**:21-24.
10. Rao E, Jiang C, Ji M, Huang X, Iqbal J, Lenz G, et al. The miRNA-17 approximately 92 cluster mediates chemoresistance and enhances tumor growth in mantle cell lymphoma via PI3K/AKT pathway activation. *Leukemia*. 2011 In press
11. Lee Y, Jeon K, Lee JT, Kim S, Kim VN. MicroRNA maturation: stepwise processing and subcellular localization. *Embo J.* 2002;**21**:4663-4670.
12. Hutvagner G, Zamore PD. A microRNA in a multiple-turnover RNAi enzyme complex. *Science.* 2002;**297**:2056-2060.
13. Pratt AJ, MacRae IJ. The RNA-induced silencing complex: a versatile gene-silencing machine. *J Biol Chem.* 2009;**284**:17897-17901.
14. Clurman BE, Hayward WS. Multiple proto-oncogene activations in avian leukosis virus-induced lymphomas: evidence for stage-specific events. *Mol Cell Biol.* 1989;**9**:2657-2664.
15. Lagos-Quintana M, Rauhut R, Yalcin A, Meyer J, Lendeckel W, Tuschl T. Identification of tissue-specific microRNAs from mouse. *Curr Biol.* 2002;**12**:735-739.
16. Eis PS, Tam W, Sun L, Chadburn A, Li Z, Gomez MF, et al. Accumulation of miR-155 and BIC RNA in human B cell lymphomas. *Proc Natl Acad Sci USA.* 2005;**102**:3627-3632.
17. Kluiver J, Poppema S, de Jong D, Blokzijl T, Harms G, Jacobs S, et al. BIC and miR-155 are highly expressed in Hodgkin, primary mediastinal and diffuse large B cell lymphomas. *J Pathol.* 2005;**207**:243-249.
18. Kluiver J, Haralambieva E, de Jong D, Blokzijl T, Jacobs S, Kroesen BJ, et al. Lack of BIC and microRNA miR-155 expression in primary cases of Burkitt lymphoma. *Genes Chromosomes Cancer.* 2006;**45**:147-153.
19. Kluiver J, van den Berg A, de Jong D, Blokzijl T, Harms G, Bouwman E, et al. Regulation of pri-microRNA BIC transcription and processing in Burkitt lymphoma. *Oncogene.* 2007;**26**:3769-3776.

20. Jiang S, Zhang HW, Lu MH, He XH, Li Y, Gu H, et al. MicroRNA-155 functions as an OncomiR in breast cancer by targeting the suppressor of cytokine signaling 1 gene. *Cancer*. 2010;**70**:3119-3127.
21. Kong W, He L, Coppola M, Guo J, Esposito NN, Coppola D, et al. MicroRNA-155 regulates cell survival, growth, and chemosensitivity by targeting FOXO3a in breast cancer. *J Biol Chem*. 2010;**285**:17869-17879.
22. O'Day E, Lal A. MicroRNAs and their target gene networks in breast cancer. *Breast*. 2010;**12**:201.
23. Lewis BP, Burge CB, Bartel DP. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell.* 2005;**120**:15-20.
24. Chang S, Wang RH, Akagi K, Kim KA, Martin BK, Cavallone L, et al. Tumor suppressor BRCA1 epigenetically controls oncogenic microRNA-155. *Nat Med*. 2011;**17**:1275-82.
25. Iorio MV, Ferracin M, Liu CG, Veronese A, Spizzo R, Sabbioni S, et al. MicroRNA gene expression deregulation in human breast cancer. *Cancer Res.* 2005;**65**:7065-7070.
26. Volinia S, Calin GA, Liu CG, Ambs S, Cimmino A, Petrocca F, et al. A microRNA expression signature of human solid tumors defines cancer gene targets. *Proc Natl Acad Sci USA.* 2006;**103**:2257-2261.
27. Blenkiron C, Goldstein LD, Thorne NP, Spiteri I, Chin SF, Dunning MJ, et al. MicroRNA expression profiling of human breast cancer identifies new markers of tumor subtype. *Genome Biol.* 2007;**8**:R214.
28. Hui AB, Shi W, Boutros PC, Miller N, Pintilie M, Fyles T, et al. Robust global micro-RNA profiling with formalin-fixed paraffin-embedded breast cancer tissues. *Lab Invest.* 2009;**89**:597-606.
29. Zhu W, Qin W, Atasoy U, Sauter ER. Circulating microRNAs in breast cancer and healthy subjects. *BMC Res Notes.* 2009;**2**:89.
30. Wang F, Zheng Z, Guo J, Ding X. Correlation and quantitation of microRNA aberrant expression in tissues and sera from patients with breast tumor. *Gynecol*. 2010**;119**:586-593.
31. Chen J, Wang BC, Tang JH. Clinical significance of MicoRNA-155 expression in human breast cancer. *J Surg Oncol*. 2011 In press
32. Kong W, Yang H, He L, Zhao JJ, Coppola D, Dalton WS, et al. MicroRNA-155 is regulated by the transforming growth factor beta/Smad pathway and contributes to epithelial cell plasticity by targeting RhoA. *Mol Cell Biol.* 2008;**28**:6773-6784.
33. Baffa R, Fassan M, Volinia S, O'Hara B, Liu CG, Palazzo JP, et al. MicroRNA expression profiling of human metastatic cancers identifies cancer gene targets. *J Pathol.* 2009;**219**:214-221.
34. Yan LX, Huang XF, Shao Q, Huang MY, Deng L, Wu QL, et al. MicroRNA miR-21 overexpression in human breast cancer is associated with advanced clinical stage, lymph node metastasis and patient poor prognosis. *RNA.* 2008;**14**:2348-2360.
35. Gilad S, Meiri E, Yogev Y, Benjamin S, Lebanony D, Yerushalmi N, et al. Serum microRNAs are promising novel biomarkers. *PLoS One.* 2008;**3**:e3148.
36. Fang C, Zhu DX, Dong HJ, Zhou ZJ, Wang YH, Liu L, et al. Serum microRNAs are promising novel biomarkers for diffuse large B cell lymphoma. *Ann Hematol*. 2011;**91**:553-9
37. Appaiah HN, Goswami CP, Mina LA, Badve S, Sledge GW Jr, Liu Y, et al. Persistent upregulation of U6:SNORD44 small RNA ratio in the serum of breast cancer patients. *Breast Cancer Res.* 2011;**13**:R86.
38. Meister G, Landthaler M, Dorsett Y, Tuschl T. Sequence-specific inhibition of microRNA- and siRNA-induced RNA silencing. *RNA.* 2004;**10**:544-550.

39. Xiang X, Zhuang X, Ju S, Zhang S, Jiang H, Mu J, et al. miR-155 promotes macroscopic tumor formation yet inhibits tumor dissemination from mammary fat pads to the lung by preventing EMT. *Oncogene*. 2011;**30**:3440-53
40. Wendt MK, Allington TM, Schiemann WP. Mechanisms of the epithelial-mesenchymal transition by TGF-beta. *Future Oncol.* 2009;**5**:1145-1168.
41. Yang J, Weinberg RA. Epithelial-mesenchymal transition: at the crossroads of development and tumor metastasis. *Dev Cell.* 2008;**14**:818-829.
42. Trimboli AJ, Fukino K, de Bruin A, Wei G, Shen L, Tanner SM, et al. Direct evidence for epithelial-mesenchymal transitions in breast cancer. *Cancer Res.* 2008;**68**:937-945.
43. Bracken CP, Gregory PA, Khew-Goodall Y, Goodall GJ. The role of microRNAs in metastasis and epithelial-mesenchymal transition. *Cell Mol Life Sci.* 2009;**66**:1682-1699.
44. Friedman RC, Farh KK, Burge CB, Bartel DP. Most mammalian mRNAs are conserved targets of microRNAs. *Genome Res.* 2009;**19**:92-105.
45. Martin MM, Lee EJ, Buckenberger JA, Schmittgen TD, Elton TS. MicroRNA-155 regulates human angiotensin II type 1 receptor expression in fibroblasts. *J Biol Chem.* 2006;**281**:18277-18284.
46. Cheng W, Liu T, Jiang F, Liu C, Zhao X, Gao Y, et al. microRNA-155 regulates angiotensin II type 1 receptor expression in umbilical vein endothelial cells from severely pre-eclamptic pregnant women. *Int J Mol Med*. 2011;**27**:393-399.
47. Zheng L, Xu CC, Chen WD, Shen WL, Ruan CC, Zhu LM, et al. MicroRNA-155 regulates angiotensin II type 1 receptor expression and phenotypic differentiation in vascular adventitial fibroblasts. *Biochem*. 2010;**400**:483-488.
48. Zhu N, Zhang D, Chen S, Liu X, Lin L, Huang X, et al. Endothelial enriched microRNAs regulate angiotensin II-induced endothelial inflammation and migration. *Atherosclerosis*. 2011;**215**:286-93
49. Borchert GM, Holton NW, Larson ED. Repression of human activation induced cytidine deaminase by miR-93 and miR-155. *BMC Cancer.* 2011;**11**:347.
50. Xu G, Fewell C, Taylor C, Deng N, Hedges D, Wang X, et al. Transcriptome and targetome analysis in MIR155 expressing cells using RNA-seq. *RNA.* 2010;**16**:1610-1622.
51. Yin Q, McBride J, Fewell C, Lacey M, Wang X, Lin Z, et al. MicroRNA-155 is an Epstein-Barr virus-induced gene that modulates Epstein-Barr virus-regulated gene expression pathways. *J Virol.* 2008;**82**:5295-5306.
52. O'Connell RM, Rao DS, Chaudhuri AA, Boldin MP, Taganov KD, Nicoll J, et al. Sustained expression of microRNA-155 in hematopoietic stem cells causes a myeloproliferative disorder. *J Exp Med.* 2008;**205**:585-594.
53. Willimott S, Wagner SD. MiR-125b and miR-155 contribute to BCL2 repression and proliferation in response to CD40 ligand (CD154) in human leukemic B-cells. *J Biol Chem*. 2011;**287:**2608-17
54. Wang HQ, Yu XD, Liu ZH, Cheng X, Samartzis D, Jia LT, et al. Deregulated miR-155 promotes Fas-mediated apoptosis in human intervertebral disc degeneration by targeting FADD and caspase-3. *J Pathol*. 2011;**225**:232-42.
55. He M, Xu Z, Ding T, Kuang DM, Zheng L. MicroRNA-155 regulates inflammatory cytokine production in tumor-associated macrophages via targeting C/EBPbeta. *Cell Mol Immunol.* 2009;**6**:343-352.
56. Worm J, Stenvang J, Petri A, Frederiksen KS, Obad S, Elmen J, et al. Silencing of microRNA-155 in mice during acute inflammatory response leads to derepression of c/ebp Beta and down-regulation of G-CSF. *Nucleic Acids Res.* 2009;**37**:5784-5792.

57. Yin Q, Wang X, Fewell C, Cameron J, Zhu H, Baddoo M, et al. MicroRNA miR-155 inhibits bone morphogenetic protein (BMP) signaling and BMP-mediated Epstein-Barr virus reactivation. *J Virol*. 2010;**84**:6318-6327.
58. Lossner C, Meier J, Warnken U, Rogers MA, Lichter P, Pscherer A, et al. Quantitative Proteomics Identify Novel miR-155 Target Proteins. *PLoS One*. 2011;**6**:e22146.
59. Liu S, Yang Y, Wu J. TNFalpha-induced up-regulation of miR-155 inhibits adipogenesis by down-regulating early adipogenic transcription factors. *Biochem*. 2011;**414**:618-624.
60. Sonkoly E, Janson P, Majuri ML, Savinko T, Fyhrquist N, Eidsmo L, et al. MiR-155 is overexpressed in patients with atopic dermatitis and modulates T-cell proliferative responses by targeting cytotoxic T lymphocyte-associated antigen 4. *J Allergy Clin Immunol*. 2010;**126**:581-9.e1-20.
61. Tili E, Michaille JJ, Cimino A, Costinean S, Dumitru CD, Adair B, et al. Modulation of miR-155 and miR-125b levels following lipopolysaccharide/TNF-alpha stimulation and their possible roles in regulating the response to endotoxin shock. *J Immunol.* 2007;**179**:5082-5089.
62. Pottier N, Maurin T, Chevalier B, Puissegur MP, Lebrigand K, Robbe-Sermesant K, et al. Identification of keratinocyte growth factor as a target of microRNA-155 in lung fibroblasts: implication in epithelial-mesenchymal interactions. *PLoS One.* 2009;**4**:e6718.
63. Yamamoto M, Kondo E, Takeuchi M, Harashima A, Otani T, Tsuji-Takayama K, et al. miR-155, a Modulator of FOXO3a Protein Expression, Is Underexpressed and Cannot Be Upregulated by Stimulation of HOZOT, a Line of Multifunctional Treg. *PLoS One*. 2011;**6**:e16841.
64. Dagan LN, Jiang X, Bhatt S, Cubedo E, Rajewsky K, Lossos IS. miR-155 regulates HGAL expression and increases lymphoma cell motility. *Blood*. 2011;**119**:513-20.
65. Lu F, Weidmer A, Liu CG, Volinia S, Croce CM, Lieberman PM. Epstein-Barr virus-induced miR-155 attenuates NF-kappaB signaling and stabilizes latent virus persistence. *J Virol.* 2008;**82**:10436-10443.
66. Martinez-Nunez RT, Louafi F, Sanchez-Elsner T. The interleukin 13 (IL-13) pathway in human macrophages is modulated by microRNA-155 via direct targeting of interleukin 13 receptor alpha1 (IL13Ralpha1). *J Biol Chem*. 2011;**286**:1786-1794.
67. Bhattacharyya S, Balakathiresan NS, Dalgard C, Gutti U, Armistead D, Jozwik C, et al. Elevated miR-155 promotes inflammation in cystic fibrosis by driving hyper-expression of interleukin-8. *J Biol Chem.* 2011;**286**:11604-15**.**
68. O'Connell RM, Chaudhuri AA, Rao DS, Baltimore D. Inositol phosphatase SHIP1 is a primary target of miR-155. *Proc Natl Acad Sci USA.* 2009;**106**:7113-7118.
69. Cremer TJ, Ravneberg DH, Clay CD, Piper-Hunter MG, Marsh CB, Elton TS, et al. MiR-155 induction by F. novicida but not the virulent F. tularensis results in SHIP down-regulation and enhanced pro-inflammatory cytokine response. *PLoS One.* 2009;**4**:e8508.
70. Pedersen IM, Otero D, Kao E, Miletic AV, Hother C, Ralfkiaer E, et al. Onco-miR-155 targets SHIP1 to promote TNFalpha-dependent growth of B cell lymphomas. *EMBO Mol Med.* 2009;**1**:288-295.
71. Costinean S, Sandhu SK, Pedersen IM, Tili E, Trotta R, Perrotti D, et al. Src homology 2 domain-containing inositol-5-phosphatase and CCAAT enhancer-binding protein beta are targeted by miR-155 in B cells of Emicro-MiR-155 transgenic mice. *Blood.* 2009;**114**:1374-1382.

72. Zhou H, Huang X, Cui H, Luo X, Tang Y, Chen S, et al. miR-155 and its star-form partner miR-155* cooperatively regulate type I interferon production by human plasmacytoid dendritic cells. *Blood.* 2010;**116**:5885-5894.
73. Bolisetty MT, Dy G, Tam W, Beemon KL. Reticuloendotheliosis virus strain T induces miR-155, which targets JARID2 and promotes cell survival. *J Virol.* 2009;**83**:12009-12017.
74. Tang B, Xiao B, Liu Z, Li N, Zhu ED, Li BS, et al. Identification of MyD88 as a novel target of miR-155, involved in negative regulation of Helicobacter pylori-induced inflammation. *FEBS Lett*. 2010;**584**:1481-6.
75. Lu C, Huang X, Zhang X, Roensch K, Cao Q, Nakayama KI, et al. MiR-221 and miR-155 regulate human dendritic cell development, apoptosis and IL-12 production through targeting of p27kip1, KPC1 and SOCS-1. *Blood*. 2011;**117**:4293-303.
76. McInnes N, Sadlon TJ, Brown CY, Pederson S, Beyer M, Schultze JL, et al. FOXP3 and FOXP3-regulated microRNAs suppress SATB1 in breast cancer cells. *Oncogene*. 2011;**31**:1045-54
77. Levati L, Pagani E, Romani S, Castiglia D, Piccinni E, Covaciu C, et al. MicroRNA-155 Targets the SKI Gene in Human Melanoma Cell Lines. *Pigment Cell Melanoma Res*. 2011;**24**:538-50.
78. Louafi F, Martinez-Nunez RT, Sanchez-Elsner T. MicroRNA-155 targets SMAD2 and modulates the response of macrophages to transforming growth factor-β. *J Biol Chem.* 2010;**285**:41328-41336.
79. Rai D, Kim SW, McKeller MR, Dahia PL, Aguiar RC. Targeting of SMAD5 links microRNA-155 to the TGF-beta pathway and lymphomagenesis. *Proc Natl Acad Sci USA.* 2010;**107**:3111-3116.
80. Lu LF, Thai TH, Calado DP, Chaudhry A, Kubo M, Tanaka K, et al. Foxp3-dependent microRNA155 confers competitive fitness to regulatory T cells by targeting SOCS1 protein. *Immunity.* 2009;**30**:80-91.
81. Xie Q, Chen X, Lu F, Zhang T, Hao M, Wang Y, et al. Aberrant expression of microRNA 155 may accelerate cell proliferation by targeting sex-determining region Y box 6 in hepatocellular carcinoma. *Cancer*. 2012;**118**:2431-42
82. Martinez-Nunez RT, Louafi F, Friedmann PS, Sanchez-Elsner T. MicroRNA-155 modulates the pathogen binding ability of dendritic cells (DCs) by down-regulation of DC-specific intercellular adhesion molecule-3 grabbing non-integrin (DC-SIGN). *J Biol Chem.* 2009;**284**:16334-16342.
83. Imaizumi T, Tanaka H, Tajima A, Yokono Y, Matsumiya T, Yoshida H, et al. IFN-gamma and TNF-alpha synergistically induce microRNA-155 which regulates TAB2/IP-10 expression in human mesangial cells. *Am J Nephrol*. 2010;**32**:462-468.
84. Ceppi M, Pereira PM, Dunand-Sauthier I, Barras E, Reith W, Santos MA, et al. MicroRNA-155 modulates the interleukin-1 signaling pathway in activated human monocyte-derived dendritic cells. *Proc Natl Acad Sci USA.* 2009;**106**:2735-2740.
85. Wang Y, Scheiber MN, Neumann C, Calin GA, Zhou D. MicroRNA Regulation of Ionizing Radiation-Induced Premature Senescence. *Int J Radiat Oncol Biol Phys*. 2010;**81**:839-48
86. Gironella M, Seux M, Xie MJ, Cano C, Tomasini R, Gommeaux J, et al. Tumor protein 53-induced nuclear protein 1 expression is repressed by miR-155, and its restoration inhibits pancreatic tumor development. *Proc Natl Acad Sci USA.* 2007;**104**:16170-16175.

87. Butz H, Liko I, Czirjak S, Igaz P, Khan MM, Zivkovic V, et al. Down-regulation of Wee1 kinase by a specific subset of microRNA in human sporadic pituitary adenomas. *J Clin Endocrinol Metab*. 2010;**95**:E181-191.
88. Huang da W, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc.* 2009;**4**:44-57.
89. Huang da W, Sherman BT, Lempicki RA. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. *Nucleic Acids Res.* 2009;**37**:1-13.
90. Costinean S, Zanesi N, Pekarsky Y, Tili E, Volinia S, Heerema N, et al. Pre-B cell proliferation and lymphoblastic leukemia/high-grade lymphoma in E(mu)-miR155 transgenic mice. *Proc Natl Acad Sci USA.* 2006;**103**:7024-7029.

Chapter 2 – Mutant p53 drives invasion in breast tumors through up-regulation of miR-155

Paul M Neilsen[1,2]*, Jacqueline E Noll[1,2]*, Sam Mattiske[1,2]*, Cameron P Bracken[2,3], Philip A Gregory[2,3], Renee B Schulz[1,2], Sue P Lim[1,2], Raman Kumar[1,2], Rachel J Suetani[1,2], Gregory J Goodall[2,3,4] and David F Callen[1,2]

[1]Centre for Personalised Cancer Medicine, University of Adelaide, Adelaide, SA, Australia

[2]Discipline of Medicine, University of Adelaide, Adelaide, SA, Australia

[3]Centre for Cancer Biology, SA Pathology, Adelaide, SA, Australia

[4]School of Molecular and Biomedical Science, University of Adelaide, Adelaide, SA, Australia

*These authors contributed equally to this work.

Oncogene 2012; 16(10): 305

Statement of Authorship

Statement of Authorship

Title of Paper	Mutant p53 drives invasion in breast tumours through up-regulation of miR-155
Publication Status	☑ Published, ○ Accepted for Publication, ○ Submitted for Publication, ○ Publication style
Publication Details	

Author Contributions

By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

Name of Principal Author (Candidate)	Sam Mattiske		
Contribution to the Paper	Conception and design, development of methodology, acquisition of data, analysis/interpretation, writing/review/revision		
Signature		Date	1-7-13

Name of Co-Author	Rachel Suetani		
Contribution to the Paper	Analysis/interpretation, writing/review/revision, study supervision		
Signature		Date	16.7.13 (signed electronically)

Name of Co-Author	Paul Neilsen		
Contribution to the Paper	Conception and design, development of methodology, acquisition of data, writing/review/revision, study supervision		
Signature		Date	22/7/13

Name of Co-Author	David Callen		
Contribution to the Paper	Conception and design, writing/review/revision, study supervision		
Signature		Date	22/7/13

Statement of Authorship

Title of Paper	Mutant p53 drives invasion in breast tumours through up-regulation of miR-155
Publication Status	☑ Published, ○ Accepted for Publication, ○ Submitted for Publication, ○ Publication style
Publication Details	

Author Contributions

By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

Name of Principal Author (Candidate)	Sam Mattiske		
Contribution to the Paper			
Signature		Date	1-7-13

Name of Co-Author	Jacqueline Noll		
Contribution to the Paper	Conception and design, development of methodology, acquisition of data, analysis/interpretation, writing/review/revision		
Signature		Date	18.7/13 (signed electronically)

Name of Co-Author	Cameron Bracken		
Contribution to the Paper	Acquisition of data, writing/review/revision		
Signature		Date	22/7/13

Name of Co-Author	Phil Gregory		
Contribution to the Paper	Acquisition of data, writing/review/revision		
Signature		Date	22/7/13

Statement of Authorship

Title of Paper	Mutant p53 drives invasion in breast tumour through up-regulation of miR-155
Publication Status	☑ Published, ○ Accepted for Publication, ○ Submitted for Publication, ○ Publication style
Publication Details	

Author Contributions

By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

Name of Principal Author (Candidate)	Sam Mattiske		
Contribution to the Paper			
Signature		Date	1-7-13

Name of Co-Author	Renee Schultz		
Contribution to the Paper	Acquisition of data		
Signature		Date	27/6/2013

Name of Co-Author	Sue Ping Lim		
Contribution to the Paper	Acquisition of data		
Signature		Date	1.6.2013

Name of Co-Author	Raman Kumar		
Contribution to the Paper	Acquisition of data, analysis/interpretation		
Signature		Date	22.7.2013

Statement of Authorship

Title of Paper	Mutant p53 drives invasion in breast tumours through up-regulation of miR-155
Publication Status	☑ Published, ○ Accepted for Publication, ○ Submitted for Publication, ○ Publication style
Publication Details	

Author Contributions

By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

Name of Principal Author (Candidate)	Sam Mattiske		
Contribution to the Paper			
Signature		Date	1.7.13

Name of Co-Author	Greg Goodall		
Contribution to the Paper	Study supervision		
Signature		Date	22/7/13

Name of Co-Author			
Contribution to the Paper			
Signature		Date	

Name of Co-Author			
Contribution to the Paper			
Signature		Date	

Abstract

Loss of p53 function is a critical event during tumorigenesis, with half of all cancers harbouring mutations within the *TP53* gene. Such events frequently result in the expression of a mutated p53 protein with gain-of-function properties that drive invasion and metastasis. Here, we show that the expression of miR-155 was up-regulated by mutant p53 to drive invasion. The miR-155 host gene was directly repressed by p63, providing the molecular basis for mutant p53 to drive miR-155 expression. Significant overlap was observed between miR-155 targets and the molecular profile of mutant p53-expressing breast tumors *in vivo*. A search for cancer-related target genes of miR-155 revealed ZNF652, a novel zinc finger transcriptional repressor. ZNF652 directly repressed key drivers of invasion and metastasis, such as *TGFB1*, *TGFB2, TGFBR2, EGFR, SMAD2* and *VIM*. Furthermore, silencing of ZNF652 in epithelial cancer cell lines promoted invasion into matrigel. Importantly, loss of ZNF652 expression in primary breast tumors was significantly correlated with increased local invasion and defined a population of breast cancer patients with metastatic tumors. Collectively, these findings suggest that miR-155 targeted therapies may provide an attractive approach to treat mutant p53-expressing tumors.

Introduction

The p53 tumor suppressor is a transcription factor that plays a pivotal role in the prevention of neoplastic transformation. Disruption of the p53 pathway is arguably a universal requirement for the development of cancer and is ultimately achieved through either direct mutation of the *TP53* gene or indirect inactivation of downstream p53 signalling pathways. However, inactivation of the tumor suppressor function of p53 is not the only outcome of a *TP53* mutation. Increasing evidence suggests that the mutated p53 protein exhibits traits consistent with the acquisition of an oncogenic gain-of-function (1, 2). For example, mutations of *TP53* gene are frequently missense, involve similar codons within the p53 DNA binding domain and are expressed at remarkably high levels in tumor cells (1). Somatic *TP53* mutations occur in approximately 20% of breast tumors, which in turn define a population of particularly aggressive breast lesions associated with a poor prognosis (3, 4).

It is postulated that mutant p53 acquires new oncogenic properties as a result of local or global distortion of the protein structure, exposing previously hidden binding interfaces to facilitate gain-of-function interactions with novel binding partners. In particular, mutant p53 has been shown to acquire the ability to sequester p63 and p73, and this is thought to promote tumor invasion and metastasis (5, 6). So what are the key downstream target genes of p63 and p73 that are deregulated to promote tumor progression in cancer cells expressing mutant p53? Recent studies have identified the metastasis suppressors SHARP1 and cyclin G2 as critical mutant p53 targets that are deregulated through loss of p63 signalling (6). The expression of these two genes alone defined a subset of particularly aggressive breast tumors associated with poor patient outcome (6). Such examples demonstrate the potential utility of targets of mutant p53 as robust clinical prognostic tools and therapeutic targets in mutant p53

expressing tumors, however a complete understanding of the mechanisms and pathways exploited by mutant p53 to drive tumorigenesis remain poorly understood. A major aspect of the anti-metastatic activity of p63 resides in its ability to regulate non-coding RNAs (7) and microRNA processing complexes (8), yet the ability of mutant p53 to dysregulate these pathways remains unexplored. We report here that mutant p53 inactivates the p63 signalling pathway to up-regulate the expression of an oncogenic microRNA, miR-155. In turn, elevated miR-155 levels promote cellular transformation and invasion through the targeting of key suppressors of metastasis.

Results

miR-155 promotes migration, invasion and amoeboid transformation

Several microRNAs have been recently implicated in the promotion of tumorigenesis and have emerged as potential therapeutic targets. In particular, miR-155 is considered as an oncomiR as it is up-regulated in breast tumors (9) and this increased expression is associated with the promotion of tumorigenesis (10). To gain a wider appreciation for the role of miR-155 during breast tumorigenesis, we measured miR-155 levels in established breast cancer cell lines of epithelial (non-invasive) and mesenchymal (invasive) origin (11). Indeed, miR-155 was undetectable in epithelial cell lines, but was highly expressed in 4 out of 5 of mesenchymal cell lines (Supplementary Figure 1), suggesting an upregulation of miR-155 during epithelial to mesenchymal transition (EMT) of breast cancers. Importantly, this observation was not restricted to breast cancer cell lines, as miR-155 levels were absent in the majority of epithelial lines from the NCI-60 panel of cancer cell lines whilst showing significantly higher expression in the mesenchymal lines (Figure 1A). To assess whether miR-155 can drive EMT in breast tumors, we stably expressed miR-155 in the ZR-75-1 epithelial breast cancer cell line. Although present at only modest levels as compared to some mesenchymal breast cancer lines (Supplementary Figure 2), exogenous miR-155 dramatically enhanced the ability of ZR-75-1 cells to migrate in a scratch-wound assay (Figure 1B). Expression of miR-155 also endowed ZR-75-1 cells with a newly acquired ability to invade through matrigel (Figure 1C). Interestingly, miR-155 expression also induced an amoeboid transformation of the ZR-75-1 epithelial cell line, as characterised by the enlarged filamentous cytoplasm with frequent protrusion of lamellipodia from these ZR-75-1 cells expressing miR-155 (Figure 1D).

Figure 1

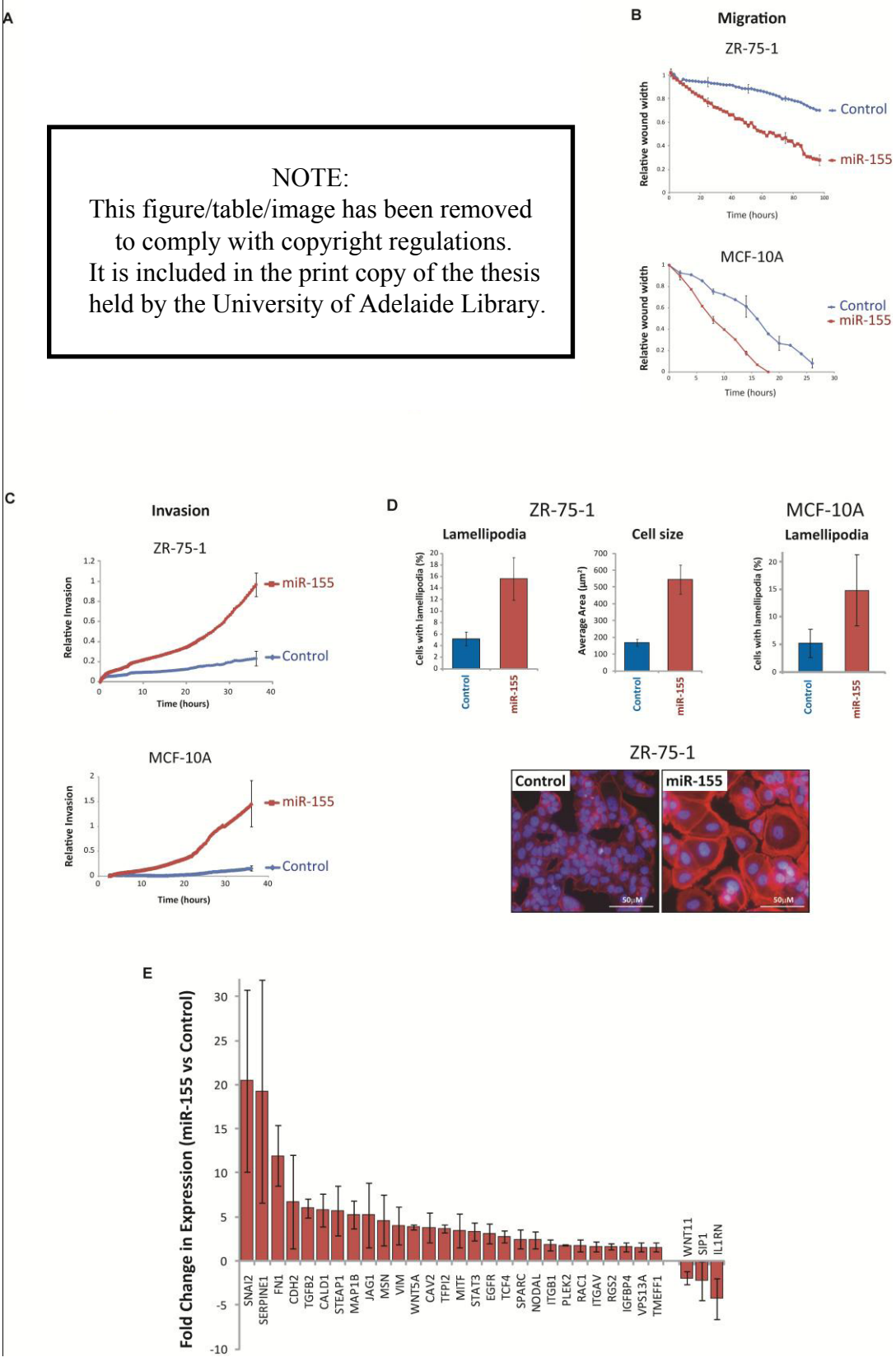

A
NOTE:
This figure/table/image has been removed
to comply with copyright regulations.
It is included in the print copy of the thesis
held by the University of Adelaide Library.
B
Migration
ZR-75-1
Relative wound width
Time (hours)
Control
miR-155
MCF-10A
Relative wound width
Time (hours)
Control
miR-155
C
Invasion
ZR-75-1
Relative Invasion
Time (hours)
miR-155
Control
MCF-10A
Relative Invasion
Time (hours)
miR-155
Control
D
ZR-75-1
Lamellipodia
Cells with lamellipodia (%)
Control
miR-155
Cell size
Average Area (µm²)
Control
miR-155
MCF-10A
Lamellipodia
Cells with lamellipodia (%)
Control
miR-155
ZR-75-1
Control
miR-155
50µM
50µM
E
Fold Change in Expression (miR-155 vs Control)
SNAI2
SERPINE1
FN1
CDH2
TGFB2
CALD1
STEAP1
MAP1B
JAG1
MSN
VIM
WNT5A
CAV2
TFPI2
MITF
STAT3
EGFR
TCF4
SPARC
NODAL
ITGB1
PLEK2
RAC1
ITGAV
RGS2
IGFBP4
VPS13A
TMEFF1
WNT11
SIP1
IL1RN

Figure 1. miR-155 expression drives invasion in ZR-75-1 and MCF-10A cells

A. The relative expression levels of miR-155 were determined in the NCI-60 panel. Cell lines were classified as epithelial and mesenchymal based on the E-cadherin / vimentin protein ratio (38).

B. The ability of ZR-75-1 cells (control or miR-155) or MCF-10A cells (control or miR-155) to migrate was determined by a scratch-wound assay using Incucyte (Essen). Phase images were taken every 120 minutes and relative wound width was calculated in real-time using Incucyte software.

C. ZR-75-1 cells (control or miR-155) or MCF-10A cells (control or miR-155) were plated in the upper chamber of a CIM-16 plate coated with 5% matrigel. Invasion was measured in real-time using an xCelligence RTCA DP analyser.

D. ZR-75-1 cells (control or miR-155) or MCF-10A cells (control or miR-155) were seeded at 10% confluence and grown for 12 days. Cells were stained for F-actin using a phalloidin antibody, with lamellopodia and cell size scored as described in Materials and Methods. Representative images of ZR-75-1 cells (control or miR-155) are shown.

E. The expression of EMT related genes in ZR-75-1 cells expressing miR-155 was determined using a low density PCR array. Data are presented as a fold change in expression relative to ZR-75-1 expressing a scrambled RNA sequence (control). Data presented is an average of three independent biological replicates.

To ensure that these observations were not restricted to the ZR-75-1 cell line, miR-155 was also stably expressed in the non-malignant breast epithelial cell line, MCF-10A (Supplementary Figure 2B). Exogenous miR-155 enhanced the ability of MCF-10A cells to migrate (Figure 1B) or invade through matrigel (Figure 1C) to levels similar to those observed in ZR-75-1 cells. Although miR-155 was able to enhance the frequency of lamellipodia protrusions from MCF-10A cells (Figure 1D), it did not alter the size of the MCF-10A cells (data not shown). These findings indicate that miR-155 has general pro-migratory and pro-invasive characteristics in epithelial breast cell lines, although the mode of transformation (i.e. amoeboid) may be specific to cell lines.

To examine the altered transcriptional events associated with the miR-155-mediated amoeboid transformation of ZR-75-1 cells and accompanying pro-migratory and pro-invasive characteristics, the expression levels of 84 EMT-related genes (see Materials and Methods) were assessed by real-time PCR (Figure 1E). Indeed, expression of miR-155 in ZR-75-1 cells was associated with a significant up-regulation of numerous mesenchymal markers, with the major transcriptional reprogramming by miR-155 converging upon the TGF-β signalling pathway. Therefore, we proposed that miR-155 may be aberrantly driving the TGF-β signalling pathway in the absence of an exogenous TGF-β ligand. Indeed, the addition of exogenous TGF-β to ZR-75-1 cells phenocopied the effects of miR-155 expression, as characterised by an increased invasive capacity and an amoeboid transformation involving enlarged cytoplasm and increased frequency of lamellipodia (Supplementary Figure 3). Thus, our findings demonstrate that miR-155 aberrantly stimulates the TGF-β pathway in the absence of exogenous TGF-β.

miR-155 is a target microRNA of mutant p53

Mutant p53 and miR-155 expression are both associated with invasive properties *in vivo* (2, 10), which prompted us to investigate whether they promote malignant transformation through a similar oncogenic axis. We initially determined if miR-155 can phenocopy mutant p53 in its ability to drive invasion and mensenchymal transformation when expressed in the same genetic background. Expression of miR-155 in H1299 cells significantly enhanced their invasive capabilities and drove a mesenchymal phenotype with close resemblance to that observed upon induced mutant p53 expression (Supplementary Figure 4). These observations indicate that miR-155 and mutant p53 may drive overlapping oncogenic pathways. Since mutation of *TP53* is considered an early event during breast tumorigenesis (12, 13), we speculated that miR-155 may be a down-stream target of mutant p53 that reprograms the cancer transcriptome to drive invasion and metastasis. Indeed, induced expression of either the p53 mutant R248Q or R282W in the p53-null H1299 background was associated with a dose-dependent increase in MIR155HG (the precursor transcript for miR-155) expression or mature miR-155 levels (Figure 2A). We next examined if endogenous mutant p53 can constitutively regulate the expression of miR-155 levels. The mesenchymal breast cancer cell line BT549 possesses the highest miR-155 levels of those tested (Supplementary Figure 1) and also expresses an endogenous p53 mutant, R249S. Knockdown of this endogenous p53 mutant in BT549 cells resulted in a significantly reduced level of miR-155 (Figure 2B), confirming a role for mutant p53 in the aberrant activation of miR-155.

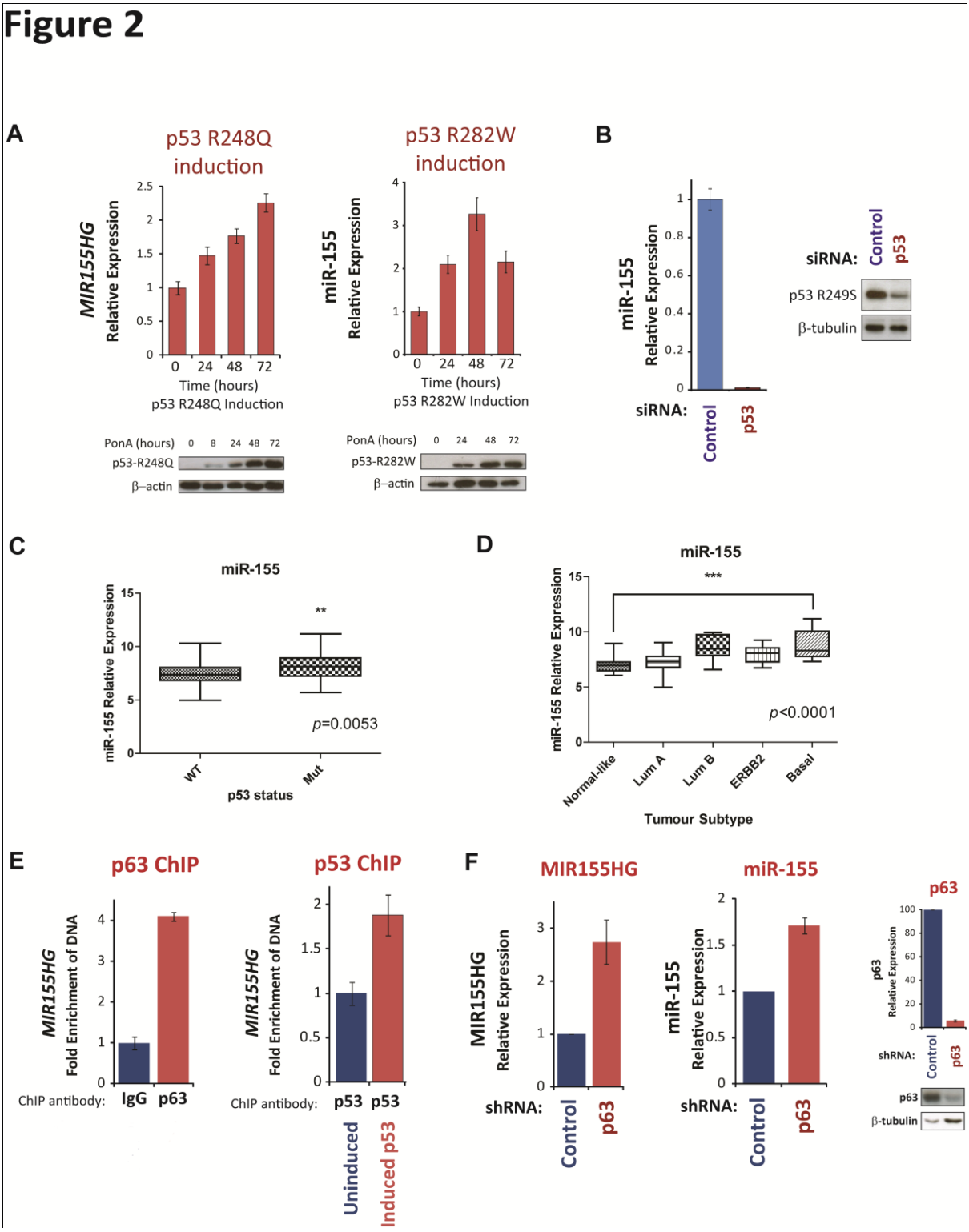


Figure 2. miR-155 expression is regulated by p63

A. H1299 inducible p53-R248Q or p53-R282W cells were treated with 2.5 µg/mL PonA for 0, 24, 48 or 72 hours to induce either p53 R248Q or p53 R282W protein

expression. Relative *MIR155HG* expression levels or mature miR-155 levels were determined by specific RT-PCR. Induction of p53 R248Q and p53 R282W proteins were determined by Western blot analysis.

B. Endogenous mutant p53 (R249S) expression was silenced by transient transfection of a specific siRNA targeting p53 (see Materials and Methods). Relative miR-155 expression was determined by an ABI Taqman miRNA assay. Knockdown of endogenous p53 R249S was confirmed by Western blot analysis.

C. The expression levels of miR-155 and p53 status of a cohort of 100 breast tumors were derived from a publically available dataset (GSE19536).

D. Publically available gene expression profiling of the cohort of 100 breast tumors from (C) was used to segregate the cases into breast cancer subtypes. The expression levels of miR-155 were significantly different across the subtypes, as determined using a 1-way ANOVA.

E. DNA-p63 complexes were immunoprecipitated from MCF-10A cell lysates using a p63 antibody (or IgG control) and normalised against two unrelated genomic regions (see Materials and Methods). Fold enrichment of a putative p63-RE within the *MIR155HG* gene (as compared to IgG control) was determined. The anti-p63 antibody (H-129) detects both the TAp63 and ΔNp63 isoforms. The ability of p53 to bind this response element was determined in H1299 cells upon induction of wild-type p53 protein. Fold enrichment of p53 within this region of the *MIR155HG* gene was determined by ChIP analysis following induction of p53-WT in EI H1299 cells for 24 hours.

F. Endogenous p63 was silenced in MCF-10A cells through constitutive lentiviral-mediated expression of a specific shRNA targeting the p63 transcript. Relative expression levels of *MIR155HG* or mature miR-155 were determined by specific RT-PCR in MCF10A cells expressing p63-specific shRNA (which targets both the TAp63 and ΔNp63 isoforms) or a non-targeting control shRNA (as above). Knockdown of p63 expression was confirmed by real-time PCR (using primers that detect both the TAp63 and ΔNp63 isoforms) or through Western blot analysis (using the H-129 antibody that detects both TAp63 and ΔNp63 isoforms).

We next examined if miR-155 was up-regulated by mutant p53 *in vivo*, which was achieved through the analysis of a publically available cohort of 100 breast tumors in which both the global microRNA expression levels had been profiled and the *TP53* gene sequenced. Indeed, miR-155 expression levels were significantly higher in breast tumors with p53 mutations as compared to those with wild-type p53 (Figure 2C). Furthermore, miR-155 expression was most elevated in the basal breast tumors, the most aggressive breast cancer subtype frequently associated with p53 mutations (Figure 2D). Collectively, these observations support the notion of miR-155 as a mutant p53 target gene in breast tumors.

miR-155 is directly repressed by p63

Mutant p53 drives invasion and metastasis through inactivation of targets such as p63 (5, 6), ablating the p63 signalling pathways responsible for the suppression of invasion and metastasis. Therefore, we examined a role for p63 in the transcriptional control of *MIR155HG*. *In silico* analysis using the p63Scan software (14) identified a putative p63 response element (RE) in the 3rd exon of the *MIR155HG* gene. Chromatin immunoprecipitation (ChIP) experiments were used to subsequently demonstrate that endogenous p63 was directly recruited to this consensus p63-RE in MCF10A cells (Figure 2E). Knockdown of p63 also resulted in a significantly increased expression level of both the MIR155HG transcript and the mature miR-155 (Figure 2F). Collectively, these findings indicate that p63 functions as a direct transcriptional repressor of miR-155. Previous observations have also shown that miR-155 was repressed by wild-type p53, albeit through an unknown mechanism (15). Indeed, induction of wild-type p53 in H1299 cells repressed MIR155HG expression (Supplementary Figure 5). Our studies provide mechanistic insight into this repression, as ChIP analysis demonstrates that this induced wild-type p53 protein is also recruited to this newly identified p63-RE located within the *MIR155HG* gene (Figure

2E). These findings suggest a redundancy of function between wild-type p53 and p63 in the regulation of miR-155 expression via transcriptional repression of the precursor MIR155HG transcript.

Identification of downstream targets of the miR-155 • mutant p53 axis

Having established miR-155 as a downstream oncomiR of mutant p53, we sought to identify the specific targets of the miR-155 • mutant p53 axis that drive invasion in breast tumors. To obtain an understanding of the landscape of miR-155 targets, a comprehensive list of *bone fide* miR-155 targets was collated from the literature (Supplementary Table 1). Selection criteria for these published miR-155 target genes included both the demonstration of endogenous transcript regulation by miR-155 and validation of the miR-155 seed sequence through a reporter assay. This list contains 140 genes and is enriched with known tumor suppressors. Therefore, we speculated that mutant p53 may indirectly down-regulate the expression of these genes through aberrant up-regulation of miR-155. A relationship between the defined miR-155 targets and mutant p53 in breast cancer was examined through analysis of publically available transcript profiles of 251 p53-sequenced primary breast tumors (4). Remarkably, 42 of these 140 miR-155 targets (30%) showed a significantly lower expression in breast tumors with mutant p53 compared with tumors with wild type p53 (Figure 3A; Supplementary Table 2). These 42 genes can be considered as the key downstream targets of the mutant p53 • miR-155 axis and are therefore likely to play critical roles in the suppression of breast cancer invasion and metastasis.

Figure 3. Four genes commonly regulated by the mutant p53•miR-155 axis are associated with poor metastasis-free survival

A. Of the 140 published miR-155 target genes (see Supplementary Table 1), the expression of 42 of these genes was significantly correlated with mutant p53 status *in vivo* (4). Loss of expression of 4 of these 42 genes predict metastasis *in vivo*, as determined from a publicly available dataset of 78 breast tumors (16).

B. Kaplan-Meier plots derived from publically available survival data associated with a cohort of 78 breast tumors with metastasis-free survival data (16). Expression of ZNF652, PCDC4, TCF12 and IL7RB was sourced from expression microarray analyses. Tumors were ranked for gene expression, with those tumors below the median expression level of the cohort defined as 'Low Expression' and those tumors above the median expression level of the cohort defined as 'High Expression'.

The potential of the expression of these 42 genes to predict breast cancer metastasis was evaluated through the analysis of a publically available dataset of expression profiling in

78 breast tumors (16). Low expression of four genes (*ZNF652*, *PDCD4*, *TCF12* or *IL17RB*) was associated with a significantly higher frequency of metastasis-related poor outcomes (Figure 3B). This suggests that these four miR-155 • mutant p53 targets drive pathways that prevent breast cancer invasion and metastasis. ZNF652 was considered an excellent candidate for further investigation as this gene encodes a classical zinc-finger DNA binding transcription factor that functions as a transcriptional repressor (17, 18), potentially orchestrating downstream pathways of the miR-155 • mutant p53 axis.

ZNF652 is an epithelial marker and suppresses tumor cell invasion

Initially we measured ZNF652 transcript and protein levels in breast cancer cell lines, and found they are highly expressed in some epithelial cell lines, but are significantly down-regulated in mesenchymal cell lines (Figure 4A and B). The expression profile of ZNF652 in these cell lines resembles an inverse reflection of their miR-155 expression profiles (see Supplementary Figure 2A), which is consistent with previous reports demonstrating that miR-155 targets the ZNF652 transcript through a specific seed sequence in the 3'UTR (19, 20). Indeed, over-expression of miR-155 markedly reduced endogenous ZNF652 protein levels in the H1299 epithelial lung carcinoma cell line (Figure 4C), further demonstrating ZNF652 as a miR-155 target. In a wider survey of ZNF652 expression in breast cell lines using gene expression microarray data of 51 breast cancer cell lines (21) classified into luminal (epithelial) and basal B (mesenchymal) (11), the expression of ZNF652 is highly negatively correlated with the expression of vimentin, a well-known mesenchymal marker (r^2=0.33, p<0.0001) (Supplementary Figure 6). The luminal (epithelial) cell lines have a significantly higher expression of ZNF652 compared with the basal B (mesenchymal) cell lines (p<0.0001) that express high levels of vimentin. These data define ZNF652 as a novel marker for breast epithelial cells and indicate that ZNF652 expression is down-regulated during

mesenchymal transformation, presumably in response to up-regulation of miR-155. We further examined if the loss of ZNF652 alone is sufficient to drive invasion. ZNF652 expression was silenced in H1299 cells using a mixture of two specific siRNAs, and their subsequent capacity to invade through matrigel assessed (Figure 4D). Ablation of ZNF652 expression resulted in a significant increase in the invasive potential of H1299 cells. These data indicate that ZNF652 is a key downstream target of the miR-155 • mutant p53 axis that regulates the invasive properties of tumor cells.

Figure 4

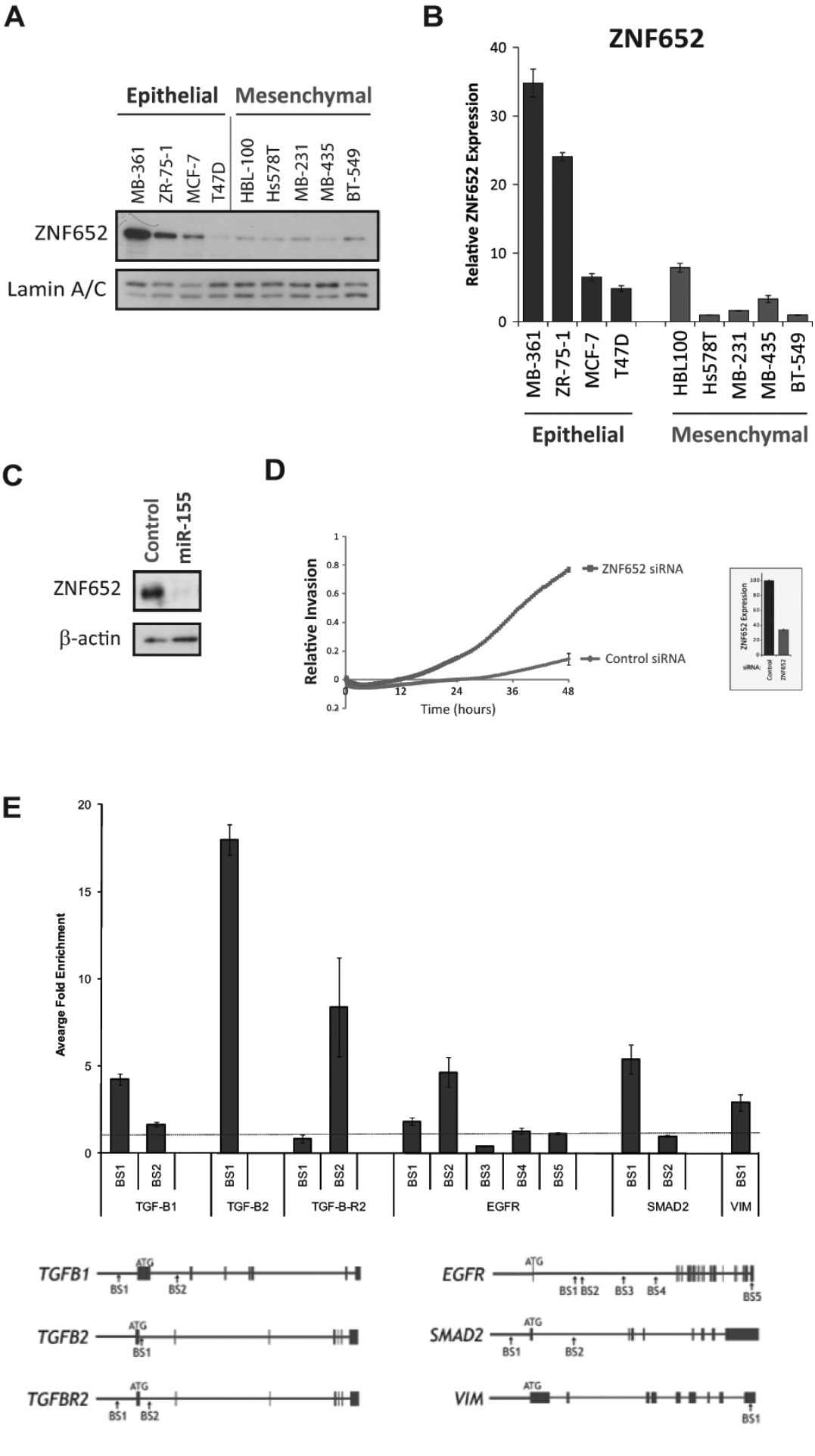

A
Epithelial
Mesenchymal
MB-361
ZR-75-1
MCF-7
T47D
HBL-100
Hs578T
MB-231
MB-435
BT-549
ZNF652
Lamin A/C
B
ZNF652
Relative ZNF652 Expression
40
30
20
10
0
MB-361
ZR-75-1
MCF-7
T47D
HBL100
Hs578T
MB-231
MB-435
BT-549
Epithelial
Mesenchymal
C
Control
miR-155
ZNF652
β-actin
D
Relative Invasion
1
0.8
0.6
0.4
0.2
0
0.2
0
12
24
36
48
Time (hours)
ZNF652 siRNA
Control siRNA
ZNF652 Expression
siRNA:
Control
ZNF652
E
Avearge Fold Enrichment
20
15
10
5
0
BS1
BS2
BS1
BS1
BS2
BS1
BS2
BS3
BS4
BS5
BS1
BS2
BS1
TGF-B1
TGF-B2
TGF-B-R2
EGFR
SMAD2
VIM
TGFB1
ATG
BS1
BS2
TGFB2
ATG
BS1
TGFBR2
ATG
BS1
BS2
EGFR
ATG
BS1 BS2
BS3
BS4
BS5
SMAD2
ATG
BS1
BS2
VIM
ATG
BS1

Figure 4. ZNF652 is an epithelial cell marker which is down-regulated in EMT by miR-155

A. Nuclear extracts from a panel of epithelial and mesenchymal breast cancer cell lines were screened for ZNF652 protein expression with a specific polyclonal anti-ZNF652 antibody (17). Equal loading was determined by Lamin A/C.

B. Relative mRNA expression of ZNF652 was determined in a panel of cell lines by specific RT-PCR.

C. ZNF652 protein levels were determined in H1299 cells following constitutive expression of miR-155 (described and characterised in Supplementary Figure 4).

D. Knockdown of ZNF652 expression using a 50:50 mixture of two specific siRNA oligonucleotides in H1299 resulted in an enhanced ability of H1299 cells to invade into matrigel, as determined in real-time using an xCelligence RTCA DP analyser. ZNF652 transcript levels were determined by real-time PCR following siRNA-mediated knockdown for 48 hours.

E. *In silico* screening of the classical promoter regions (10kB upstream), first introns and 3'UTRs of *TGFB1*, *TGFB2*, *TGFBR2*, *EGFR*, *SMAD2* and *VIM* identified multiple putative ZNF652 binding sites (BS), as illustrated in schematic diagram. ChIP analysis was performed in ZR-75-1 cells using a specific ZNF652 antibody, with the fold-enrichment of ZNF652 bound at each putative ZNF652 binding site determined relative to an IgG control and normalised against two unrelated genomic regions (see Materials and Methods).

ZNF652 is a master regulator of the EMT gene network

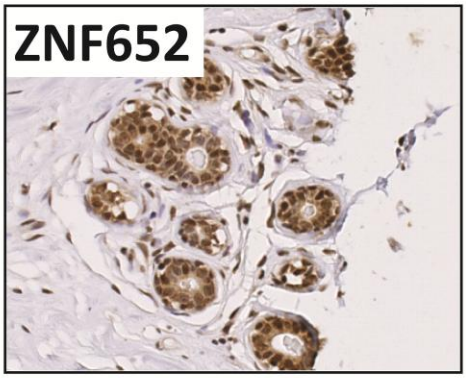
ZNF652 is a repressor of gene transcription (17), and we hypothesised that this transcription factor may suppress invasion and metastasis through constitutive repression of key drivers of mesenchymal transformation. Identification of the key ZNF6562 targets will further elucidate the downstream regulatory network responsible for metastasis in mutant p53-miR-155 expressing breast tumors. We identified putative consensus ZNF652 DNA binding sequences (AnGGGTTAA) (18) in the classical promoter region, first intron or 3' UTR of *TGFB1*, *TGFB2, TGFBR2, EGFR, SMAD2* and *VIM* (Figure 4E). Direct recruitment of endogenous ZNF652 to these gene regulatory elements within *TGFB1, TGFB2, TGFBR2, EGFR, SMAD2* and *VIM* was demonstrated through ChIP analysis in ZR-75-1 cells (Figure 4E), confirming these genes as direct targets of ZNF652. Silencing of ZNF652 expression in the ZR-75-1 cell line also resulted in a de-repression of several of these target genes, including *TGFB1*, *TGFBR2, EGFR* and *VIM* (Supplementary Figure 7). EMT gene expression profiling (as described in Figure 1E) was subsequently performed to assess the global influence of loss of ZNF652 expression on ZR-75-1 cells. There was considerable overlap between the EMT gene expression profiles in response to loss of ZNF652 expression or over-expression of miR-155 (Supplementary Figure 8), suggesting that a large proportion of EMT gene regulation by miR-155 may be facilitated through its ability to target ZNF652. The identified ZNF652 targets converge upon the TGF-β signalling pathway, as was observed for miR-155. Thus, we hypothesize that ZNF652 may suppress invasion through repression of the TGF-β signalling pathway. Indeed, forced expression of ZNF652 ablated the ability of exogenous TGF-β to activate SMAD proteins, as demonstrated using a SMAD reporter assay (Supplementary Figure 9). Collectively, the data suggest that ZNF652 is a master repressor of the EMT gene network.

ZNF652 suppresses invasion *in vivo*

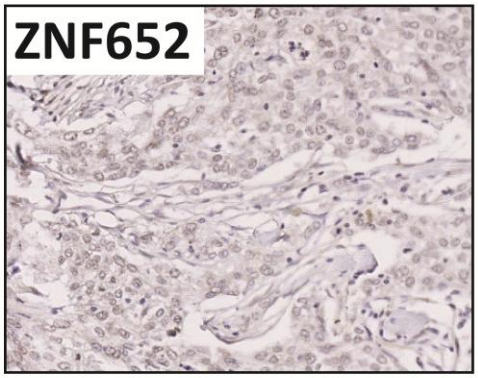
The potential of ZNF652 to suppress invasion *in vivo* was evaluated in human breast tumors by staining tumor microarrays of paraffin embedded formalin fixed tissues with our affinity purified polyclonal anti-ZNF652 antibody (Figure 5A). ZNF652 protein levels were assessed in 112 breast tumors of known invasive potential, as assessed by the TNM scoring available for each tumor. There is a highly significant association of low ZNF652 levels and increased propensity for tumor invasion in distant organs or structures ($p < 0.001$; Fisher exact test), thus demonstrating that ZNF652 is a *bone fide* suppressor of tumor cell dissemination and invasion.

Figure 5

A



Normal Breast Tissue



Malignant Breast Tissue

INVASION

	Low	Medium	High *
Low ZNF652	25	17	8
High ZNF652	49	13	0

B

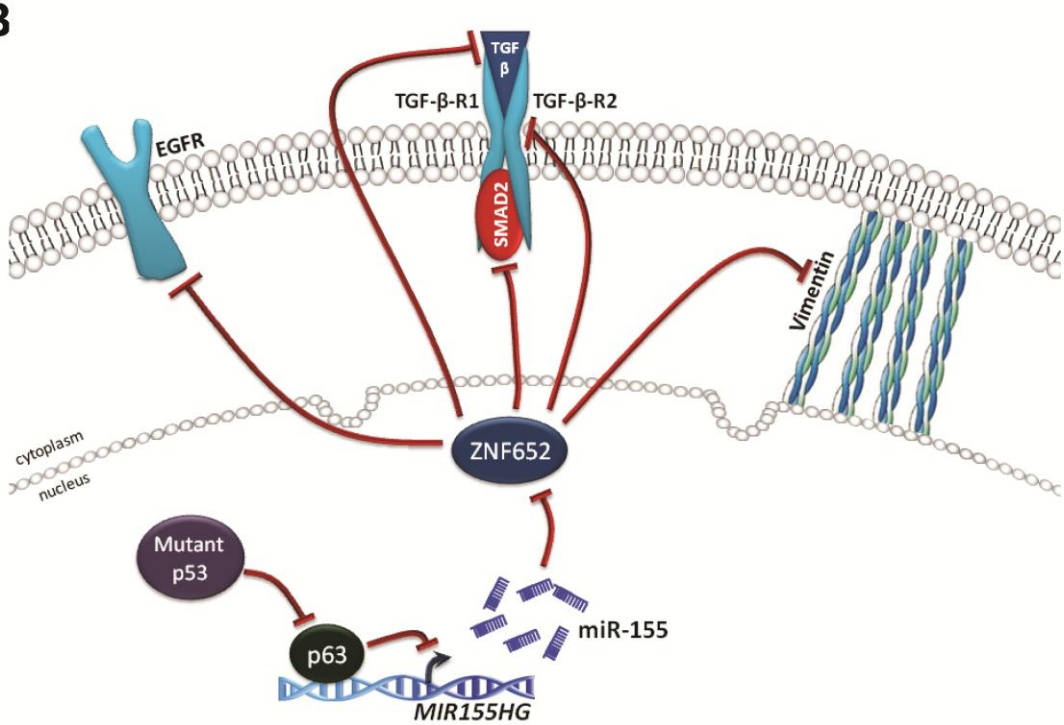


Figure 5. Loss of ZNF652 expression is associated with increased tumor invasion *in vivo*

A. Immunohistochemistry was performed on breast tissue microarrays to determine ZNF652 expression *in vivo*. ZNF652 expression was significantly associated (* indicates $p<0.001$, Fisher exact test) with tissue invasion as assessed by the TNM staging (where 'low', 'medium' or 'high' indicates T2, T3 or T4 classifications, respectively) as defined by the American Joint Committee on Cancer. Tissues were classified as low ZNF652 expression when there was negligible staining of 95% of the breast tumor nuclei.

B. Schematic diagram representing the proposed mechanism of mutant p53 driving EMT and tumor cell invasion through a pathway involving miR-155 and ZNF652.

Based on our evidence, we propose that epithelial cancers expressing mutant p53 acquire enhanced invasive and metastatic potential through up-regulation of miR-155. This oncomiR drives invasion by directly repressing the target transcript ZNF652, which as a consequence causes the de-repression of a network of EMT genes and acquisition of an invasive cell phenotype (summarised diagrammatically in Figure 5B). Collectively, this study provides evidence that miR-155 targeted therapies may provide an attractive approach to treat mutant p53 expressing breast tumors. Furthermore, histological assessment of the key targets of the mutant p53 • miR-155 axis may have prognostic implications in the clinical management of breast cancer patients.

Discussion

miR-155 drives breast cancer cell transformation and invasion

This study implicates a role for miR-155 in the transformation of the ZR-75-1 and MCF-10A epithelial breast cells (Figure 1). It is of particular interest that miR-155 could impart pro-migratory and pro-invasive properties to the non-malignant MCF-10A cell line, as these findings potentially indicate that miR-155 possesses transforming capabilities in the breast epithelium. In addition, miR-155 drove an amoeboid transformation of ZR-75-1 cells. The amoeboid transition of epithelial tumor cells represents a major mode of migration and invasion (22). This pro-invasive amoeboid transformation was associated with the formation of lamellipodia, which are typically restricted to highly mobile and invasive cells (23), properties not frequently associated with the epithelial ZR-75-1 cell line. Our findings are consistent with the pre-existing notion of miR-155 as a breast cancer oncomiR. Previous studies have shown that ectopic expression of miR-155 mimics in the MCF-7 or BT-474 breast cancer cell lines was associated with increased proliferation rates (10, 24). Furthermore, the ectopic expression of miR-155 mimics in the MDA-MB-231 breast cancer cell line accelerated the growth of mammary fat pad tumor xenograft tumors *in vivo* (10). Our findings suggest that these previously observed oncogenic activities of miR-155 in breast cancer cell lines result from its ability to rearrange the actin cytoskeleton to reconfigure the cell with a pro-invasive morphology (Figure 1).

In contrast to our findings, a previous report suggests that ectopic expression of miR-155 did not drive a mesenchymal phenotype in the NMuMG murine mammary epithelial cell line (25). Furthermore, ectopic expression of miR-155 had no influence on the proliferation or primary tumor growth of 4T1 murine mammary cell lines *(26)*. In fact, findings from

Xiang et al suggest that miR-155 suppresses the ability of 4T1 cells to undergo EMT. Indeed, it appears that the biological activity of miR-155 in human cell lines (10, 24) is vastly different to that observed upon miR-155 expression in mammary cell lines that originate from mice (25, 26), suggesting a lack of conservation of miR-155 seed sequences in its target 3'UTRs across these two species.

The complex regulation of miR-155 expression

Herein, we demonstrated that either p53 or p63 can directly repress miR-155 levels through direct association with the promoter of *MIR155HG*. As such, a gain-of-function mutation in the *TP53* gene would act as a double-edged sword to activate miR-155 levels through both a loss of wild-type p53-mediated repression and also through the inactivation of p63-mediated repression as a result of the oncogenic activities of mutant p53 (5, 6). Findings from Adorno *et al* suggest that the presence of TGF-β is a critical factor for mutant p53 to sequester p63 from its target genes. Therefore, it is tempting to speculate a role for TGF-β in the regulation of miR-155 expression. This is indeed the case, as miR-155 has been previously shown to be upregulated upon TGF-β treatment (25). The nature of the relationship between miR-155 and TGF-β is quite complex, as we demonstrated that the major transcriptional reprogramming by miR-155 converged upon the TGF-β pathway and ectopic expression of miR-155 aberrantly sensitised cells to exogenous TGF-β (Figure 1 and Supplementary Figure 3). Collectively, these observations suggest that miR-155 and TGF-β exist in a positive feedback loop.

Targets of the mutant p53 • miR-155 axis

We suggest that *ZNF652*, *PDCD4*, *TCF12* and *IL17RB* are critical targets of the mutant p53 • miR-155 axis in breast cancer (Figure 3), thus implicating them as suppressors of breast

cancer invasion and metastasis. Consistent with this hypothesis, PDCD4 has been previously shown to inhibit motility and invasion of breast cancer cell lines *in vitro* (27, 28) and is down-regulated in invasive breast carcinomas *in vivo* (29). Likewise, loss of IL17RB expression is a strong biomarker for clinical outcome in breast cancer patients receiving tamoxifen monotherapy (30). Our findings are consistent with a similar role for ZNF652 as a suppressor of breast cancer cell invasion. We demonstrate that ZNF652 directly represses *TGFB1, TGFB2, TGFBR2, EGFR, SMAD2* and *VIM*; genes that have all been previously implicated in the promotion of mesenchymal transformation and invasion (6, 31-34). As such, we have defined a new role for ZNF652 as a master regulator of the EMT gene network.

In conclusion, we have identified miR-155 as a novel target of mutant p53 • p63 axis. Elevated miR-155 levels promote cellular transformation and invasion through the targeting of key suppressors of metastasis, such as ZNF652. Collectively, these findings suggest that miR-155 targeted therapies may provide an attractive approach to treat mutant p53-expressing tumors.

Materials and Methods

Cell culture, plasmids and treatments

H1299 cells were maintained in DMEM supplemented with 10% FBS. ZR-75-1 and BT549 cells were maintained in RPMI supplemented with 10% FBS and 1mM sodium pyruvate. H1299 cells with inducible expression of wild-type or mutant p53 are as described previously (35). H1299 and ZR-75-1 were engineered to express either miR-155 or a non-targeting SCR control through retroviral mediated transduction with viruses generated by the pMSCV-Puro-GFP-miR-155 or pGIPZ-non-targeting (Open Biosystems) vectors, followed by selection in 500ng/mL puromycin (Sigma Aldrich, Castle Hill, NSW). To silence gene expression in cell lines, cells were transfected with 10 μM of various specific siRNA molecules (Qiagen, Doncaster, VIC; listed in Supplementary Table 3) and transfected using lipitoid transfection reagent following manufacturer's protocol (36). For constitutive knockdown, the pGIPZ lentiviral shRNAmir system expressing two independent ZNF652-specific shRNA oligonucleotides (V3LHS_368392 or V2LHS_229362) or a p63-specific shRNA oligonucleotides (V2LHS_24250) was used in accordance with the manufacturer's protocol (Open Biosystems, Huntsville, AL).

Immunofluorescence

Cells were plated at 10% confluence on glass cover slips and grown for 12 days. Where indicated, cells were treated with 2ng/mL TGF-β. Media was refreshed every 3 days. For actin staining, media was removed from cells followed by 1 × wash with warmed PBS. Cells were fixed with warmed 4% PFA for 15 minutes and blocked in 1% BSA/PBS for 20 minutes. Cells were washed in PBS and permeabilised for 5 minutes with 0.1% Triton-X/PBS. Phalloidin antibody (diluted 1:500 in 0.1% Triton-X/PBS) was added and incubated for 1 hour at room temperature in the dark. Cells were washed 2 × with PBS and mounted in

Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA). Cells were imaged using Olympus IX70 inverted microscope.

To score cell size, the major and minor axis of the cells was measured. To score lamellopodia, cells with lamellopodia (visualised with actin stain) were counted. Total cell numbers were determined by DAPI stain. Analysis was performed using ImageJ software over ≥4 fields of view. A minimum of 100 cells were counted per condition.

Isolation of RNA, RT-PCR and microRNA analysis

Briefly, total RNA was extracted from cells using RNeasy mini kit (Qiagen), with quantitative real-time PCR performed as previously described (37). Specific primers for real-time PCR are listed in Supplementary Table 3. Low density PCR arrays of 84 EMT-related genes were performed using the Human Epithelial to Mesenchymal Transition RT^{2}Profiler PCR Array System (SABiosciences, Frederick, MD) as per manufacturer's protocol. MiR-155 analysis was performed using the ABI Taqman miRNA assay for miR-155, normalising levels to U6, U48 and miR-16 according to the standard protocol.

Western blot analysis and Chromatin Immunoprecipitation (ChIP)

Western blot and ChIP analysis are described Supplementary Materials and Methods.

Invasion/Migration Assays

Real-time invasion assays were performed using the xCelligence Real-Time Cell Analyzer (RTCA) DP (Roche, Castle Hill, NSW), as per the manufacturer's protocol. Briefly, sub-confluent cell cultures were collected in serum free media and plated at 2×10^4 cells per well in the top chamber of a CIM-Plate 16 pre-coated with 5% matrigel (BD Biosciences). DMEM containing 10% FCS was used as a chemo-attractant. Real-time migration assays were

performed using Incucyte (Essen, Michigan, USA). Phase images were taken every 15 minutes and wound closure and cell confluence calculated using specific Incucyte software.

Breast tissue microarrays and immunohistochemistry

Breast tissue microarrays BR951, BR961 and BR963 were sourced from US Biomax Inc. Immunohistochemistry was performed to determine ZNF652 expression. Briefly, slides were heated at 50˚C for 2 hours followed by dewaxing 3 × 5 min with xylene, rehydration 3 × 5 min with 100% ethanol and 2 × 3 min with PBS. Slides were subsequently treated with 1:100 dilution H_2O_2 in PBS for 5 min at RT followed by 2 × 3 min wash in PBS. Slides were immersed in citrate buffer and microwaved on high for 3 min (until boiling) followed by 15 min on low. Cooled slides were washed 2 × 3 min in PBS. Slides were blocked in 5% rabbit serum in PBS at RT for 30 min and incubated with an affinity purified rabbit polyclonal antibody against ZNF652 (17) overnight at 4˚C. Slides were washed 2 × 3 min in PBS followed by addition of biotinylated rabbit specific secondary antibody (Dako Australia, Cambellfield, VIC) at 1:400 in blocking solution and incubated for 1 hr at RT in a humid chamber. Streptavidin (1:500 dilution in PBS) was added and incubated for 1 hr at RT in a humid chamber. DAB and H_2O_2 solutions were added and incubated at RT for 6 min. Sections were counterstained with Lillie-Mayer haematoxylin for 15-30 sec, washed once with water and dehydrated through 3 × 5 min washes 100% ethanol and 3 × 5 min washes with xylene. Slides were subsequently mounted in DPX.

Acknowledgements

We thank Dr Erik Flemington for his kind gift of the pMSCV-Puro-GFP-miR-155 plasmid. We also thank Dr Emily Paterson and Bee Suan Tay for technical assistance.

Grant Support

This study was supported by the National Health and Medical Research Council (NHMRC) of Australia (project #1009447).

Conflict of Interest

The authors declare no conflict of interest.

Supplementary Materials and Methods

Western blot analysis

Briefly, cells were resuspended in Buffer A (10mM Hepes, 10mM KCl, 1.5mM $MgCl_2$, 0.34M sucrose, 10% glycerol) supplemented with 25 × protease inhibitor (Roche). Triton X-100 was added to a final concentration of 0.1% followed by incubation on ice for 8 minutes. Nuclei were pelleted by centrifugation for 5 minutes (1,300 × *g*, 4°C). Nuclear pellets were subsequently lysed in lysis buffer (50mM Tris-HCl (pH 7.5), 250mM NaCl, 1mM EDTA, 50mM NaF, 0.5% Triton-X 100, 0.1mM Na_3VO_4) with 1× protease inhibitor cocktail. Lysates were sonicated 1× 15 seconds at 25% amplitude and clarified (13,200 rpm, 4°C) for 15 min. Western blot analysis was performed as previously described (Kumar *et al.*, 2005). Antibodies used for western blot were: mouse α-p53 DO-1 (Santa Cruz Biotechnology, Santa Cruz, CA), mouse α-β actin (Sigma Aldrich), mouse α-Lamin A/C (BD Biosciences, San Jose, CA), rabbit α-ZNF652 (Kumar *et al.*, 2006), α-mouse IgG HRP linked (GE Healthcare, Munich, Germany), α-rabbit IgG HRP linked (GE Healthcare).

Chromatin Immunoprecipitation (ChIP)

Cell lines were collected and DNA and proteins were cross-linked by addition of 1% formaldehyde for 9 min with rotation at RT. To stop cross-linking, 625mM cold glycine was added, mixed and centrifuged for 5 minutes at 300 *g*. Cells were subsequently washed twice with 50 mL cold PBS. Cell pellets were lysed in 400 µL SDS Lysis buffer (1% SDS, 10mM EDTA, 50mM Tris-HCl pH 8.1) with protease inhibitors, followed by sonication (6 × 15 sec; 30% amplitude) using vibracell sonicator (Sonics and Materials Inc, Newtown, CT, USA). Following clarification, lysates were diluted 10-fold in dilution buffer (0.01% SDS, 1.1%

Triton X-100, 1.2mM EDTA, 16.7mM Tris-HCl pH 8.1, 167mM NaCl) and inputs collected. Lysates were precleared with Protein A sepharose beads with BSA and sonicated salmon sperm DNA (ssDNA) at 4°C with rotation for 2 hours. Lysates were subsequently incubated with 4 μg rabbit α-ZNF652, rabbit α-p63 H-129 (Santa Cruz) or rabbit IgG at 4°C with rotation overnight. Immune complexes were precipitated with Protein A sepharose with ssDNA at 4°C with rotation for 2 hours. Beads were washed once each with low salt immune complex wash buffer (20mM Tris-HCl pH 8, 150mM NaCl, 2mM EDTA, 1% Triton X-100, 0.1% SDS), high salt immune complex wash buffer (20mM Tris-HCl pH 8, 500mM NaCl, 2mM EDTA, 1% Triton X-100, 0.1% SDS), LiCl immune complex wash buffer (10mM Tris-HCl pH 8, 1mM EDTA, 0.25M LiCl, 1% NP-40, 1% sodium deoxycholate) and twice with TE buffer (10mM Tris-HCl pH 8, 1mM EDTA). Specific immune complexes were eluted in 250μL SDS elution buffer (1% SDS, 0.1M $NaHCO_3$). Cross-links were reversed by addition of 10μL 5M NaCl and heating at 65°C for 16 hours, followed by addition 10μL 0.5M EDTA, 20μL 1M Tris-HCl pH 6.5 and 4μL 10mg/mL Proteinase K and heating at 45°C for 1 hour. DNA was extracted using standard PCR purification kit (Qiagen). Levels of specific promoter DNAs were determined by real-time PCR using specific primers (Supplementary Table 3). Three independent biological replicates were performed.

Kumar R, Manning J, Spendlove HE, Kremmidiotis G, McKirdy R, Lee J *et al* (2006). ZNF652, a novel zinc finger protein, interacts with the putative breast tumor suppressor CBFA2T3 to repress transcription. *Mol Cancer Res* **4:** 655-65.

Kumar R, Neilsen PM, Crawford J, McKirdy R, Lee J, Powell JA *et al* (2005). FBXO31 is the chromosome 16q24.3 senescence gene, a candidate breast tumor suppressor, and a component of an SCF complex. *Cancer Res* **65:** 11304-13.

Supplementary Tables

Supplementary Table 1. Published miR-155 targets.

Accession Number	Gene Name	Reference
NM_013229.2	APAF1	(1)
NM_152641	ARID2	(1,2)
NM_019087.2	ARL15	(1)
NM_006407.3	ARL6IP5	(1)
NM_032131.4	ARMC2	(1)
NM_001668	ARNT	(3)
NM_000685	AT1R	(4-7)
NM_206866	(AGTR1)	(1-3)
NM_021946.4	BACH1	(1)
NM_173473.2	BC0RL1	(1)
NM_001083913.1	C10orf104	(1)
NM_020314.4	C10orf26	(1)
NM_018372.3	C16orf62	(1)
NM_016631.3	C1orf103	(1)
NM_016210.4	C21orf66	(1)
NM_001093755.1	C3orf18	(1)
NM_032415.3	C5orf44	(1)
NM_014316.2	CARD11	(1)
NM_004346.3	CARHSP1	(8)
NM_016122.2	CASP3	(1)
NM_015891.2	CCDC41	(1)
NM_005194	CDC40	(2,3,9-11)
NM_025134.4	CEBPB	(1)
NM_020313.2	CHD9	(1)
NM_015041.1	CIAPIN1	(1)
NM_005211	CLUAP1	(3,12)
NM_001025105.1	CSF1R	(1)
NM_005214	CSNK1A1	(12)
NM_181552	CTLA4	(3)

NM_207318.2	CUX1	(1)
NM_183075.2	CXorf39	(1)
NM_001014283.1	CYP2U1	(1)
NM_017996.3	DCUN1D2	(1)
NM_024612.4	DET1	(1)
NM_004091.2	DHX40	(1)
NM_017629.2	E2F2	(1)
NM_001143820	EIF2C4	(1,7)
NM_014285.5	ETS1	(1)
NM_003824	EXOSC2	(8,13)
NM_001105531.2	FADD	(1)
NM_173607.3	FAM135A	(1)
NM_144963.2	FAM177A1	(1)
NM_002009	FAM91A1	(20)
NM_001455	FGF7	(14,15)
NM_031412.2	FOXO3	(1)
NM_001482.2	GABARAPL1	(1)
NM_016592.2	GATM	(1)
NM_016072.4	GNAS	(1)
NM_012257.3	GOLT1B	(1)
NM_001530	HBP1	(3)
NM_006734	HIF1A	(1,2,11)
NM_144646.3	HIVEP2	(1)
NM_014002	IGJ	(13,16)
NM_001560	IKBKE	(18)
NM_018725.3	IL13RA1	(1)
NM_001017915	IL17RB	(25-29)
NM_014937.2	INPP5D	(1)
NM_012141.2	INPP5F	(1)
NM_007199	INTS6	(17)
NM_004973	IRAK3	(1,3,19)
NM_015483.2	JARID2	(1)
NM_014647.3	KBTBD2	(1)
NM_014431.2	KIAA0430	(1)
NM_015990.4	KIAA1274	(1)
NM_153686.7	KLHL5	(1)
NM_015093	LCORL	(1)
NM_018509.3	LNX2	(1)
NM_001114093.1	LRRC59	(1)

NM_002446.3	LSM14A	(1)
NM_003954.2	MAP3K10	(1)
NM_015093	MAP3K14	(1,17,35,36)
NM_001172303.1	MAP3K7IP2	(1)
NM_018388.3	MASTL	(1)
NM_032485.4	MBNL3	(1)
NM_015335.4	MCM8	(1)
NM_005587.2	MED13L	(1)
NM_015358.2	MEF2A	(1)
NM_022474.2	MORC3	(1)
NM_014161.3	MPP5	(1)
NM_015084.2	MRPL18	(1)
NM_001080416.2	MRPS27	(1)
NM_001172567	MYBL1	(22)
NM_012334	MYD88	(11)
NM_015194.1	MYO10	(1)
NM_001099412.1	MYO1D	(1)
NM_004539.3	MYST3	(1)
NM_002577.4	NARS	(1)
NM_203487.2	PAK2	(1)
NM_014456.3	PCDH9	(1)
NM_198040.2	PDCD4	(1)
NM_014660.3	PHC2	(1)
NM_199320.2	PHF14	(1)
NM_007166	PHF17	(1,3)
NM_006256.2	PICALM	(1)
NM_017443.4	PKN2	(1)
NM_002734.3	POLE3	(1)
NM_014904.2	PRKAR1A	(1)
NM_006908.4	RAB11FIP2	(1)
NM_014247.2	RAC1	(1)
NM_005614.3	RAPGEF2	(1)
NM_001664	RHEB	(24)
NM_003804	RHOA	(13)
NM_022064	RIPK1	(21)
NM_024632.5	RNF123	(1)
NM_005625.3	SAP30L	(1)
NM_024077.3	SDCBP	(1)
NM_003036	SECISBP2	(30)

NM_001045556	SKI	(3)
NM_004733.3	SLA	(1)
NM_017515.4	SLC33A1	(1)
NM_005900	SLC35F2	(11)
NM_005901	SMAD1	(1,31)
NM_005903	SMAD2	(2,11,32)
NM_001128849.1	SMAD5	(1)
NM_003745	SMARCA4	(21,33,34)
NM_001080547	SOCS1	(3,23)
NM_014409.3	SPI1	(1)
NM_020773.2	TAF5L	(1)
NM_004607.2	TBC1D14	(1)
NM_207036.1	TBCA	(1)
NM_007005.3	TCF12	(1)
NM_014765.2	TLE4	(1)
NM_033285	TOMM20	(1,37,38)
NM_001042646.1	TP53INP1	(1)
NM_004237.3	TRAK1	(1)
NM_018718.1	TRIP13	(1)
NM_030927.2	TSGA14	(1)
NM_013438.4	TSPAN14	(1)
NM_020857.2	UBQLN1	(1)
NM_003390	VPS18	(1,39)
NM_023034.1	WEE1	(1)
NM_001161661.1	WHSC1L1	(1)
NM_014569.3	WWC1	(1)
NM_021045.1	ZKSCAN5	(1)
NM_203282.2	ZNF248	(1)
NM_021148.2	ZNF254	(1)
NM_006969.2	ZNF273	(1)
NM_030972.3	ZNF28	(1)
NM_001145365.1	ZNF611	(1,2)
NM_001105549.1	ZNF652	(1)
	ZNF83	

Supplementary Table 2. Targets of the miR-155 • mutant p53 axis.

Accession Number	Gene Name	*p* value
NM_001083913.1	**C10orf26**	0.0000000000020
NM_014456.3	**PDCD4**	0.00000013
NM_005903	**SMAD5**	0.0000011
NM_024077.3	**SECISBP2**	0.0000022
NM_001042646.1	**TRAK1**	0.0000026
NM_015335.4	**MED13L**	0.0000033
NM_000685	**AGTR1**	0.0000060
NM_024632.5	**SAP30L**	0.000013
NM_016210.4	**C3orf18**	0.000017
NM_015084.2	**MRPS27**	0.000021
NM_033285	**TP53INP1**	0.000026
NM_019087.2	**ARL15**	0.000031
NM_001025105.1	**CSNK1A1**	0.000042
NM_015093	**LNX2**	0.00055
NM_001145365.1	**ZNF652**	0.00084
NM_001161661.1	**WWC1**	0.00095
NM_014904.2	**RAB11FIP2**	0.0010
NM_001482.2	**GATM**	0.0012
NM_012257.3	**HBP1**	0.0014
NM_015041.1	**CLUAP1**	0.0017
NM_020314.4	**C16orf62**	0.0017
NM_207036.1	**TCF12**	0.0018
NM_199320.2	**PHF17**	0.0026
NM_005900	**SMAD1**	0.0029
NM_014247.2	**RAPGEF2**	0.0036
NM_015990.4	**KLHL5**	0.0036
NM_025134.4	**CHD9**	0.0041
NM_007199	**IRAK3**	0.0063
NM_183075.2	**CYP2U1**	0.0074
NM_001668	**ARNT**	0.0076

NM_021946.4	**BCORL1**	0.0076
NM_001105549.1	**ZNF83**	0.0093
NM_002734.3	**PRKAR1A**	0.012
NM_018725.3	**IL17RB**	0.016
NM_002009	**FGF7**	0.017
NM_014647.3	**KIAA0430**	0.024
NM_012141.2	**INTS6**	0.036
NM_001099412.1	**MYST3**	0.044
NM_014431.2	**KIAA1274**	0.044
NM_153686.7	**LCORL**	0.046
NM_203487.2	**PCDH9**	0.048
NM_022474.2	**MPP5**	0.049

Supplementary Table 3. Sequences of oligonucleotides used throughout

Primer	Purpose	Sequence	Direction
Beta-actin-F	Real-time PCR	TACCTTCAACTCCATCATGAAGTG	Forward
Beta-actin-R	Real-time PCR	CCGGACTCGTCATACTCCTGCTTG	Reverse
Beta-Globin-ChIP-F	ChIP - neg control	GAAGAGCCAAGGACAGGTAC	Forward
Beta-Globin-ChIP-R	ChIP - neg control	CAACTTCATCCACGTTCACC	Reverse
CDC25B-ChIP-F	ChIP - neg control	GCTGTGGTGTGTAGGAAGCAGCAG	Forward
CDC25B-ChIP-R	ChIP - neg control	AAAGGAGGCCAAAGTCGGGCAATG	Reverse
Cyclophilin A-F	Real-time PCR	GGCCAGGCTCGTGCCGTTTTGC	Forward
Cyclophilin A-R	Real-time PCR	TGGCGTGTGAAGTCACCACCCTG	Reverse
EGFR-F	Real-time PCR	GGCTGTCCAACGAATGGGCCTA	Forward
EGFR-R	Real-time PCR	CGTGCGCTTCCGAACGATGTG	Reverse
EGFR-ZNF-BS1-F	ChIP	TAGAGACATGGAGGACTCCCGACC	Forward
EGFR-ZNF-BS1-R	ChIP	GCACATTTTTCCATGCAGATGAG	Reverse

		CAGC	
EGFR-ZNF-BS2-F	ChIP	ACAAGTGAGCTGCGCAGGGTG	Forward
EGFR-ZNF-BS2-R	ChIP	TCTTGAAAATGGGAAACTGGCT GTCA	Reverse
EGFR-ZNF-BS3-F	ChIP	GGCAGCTGCTTGCACCGAAAG	Forward
EGFR-ZNF-BS3-R	ChIP	ACGGGCCTGCATCTGGAAACT	Reverse
EGFR-ZNF-BS4-F	ChIP	GCTAAGCCATCCCCAAGTTTGTC G	Forward
EGFR-ZNF-BS4-R	ChIP	AGCCTGCTGGAAATGCAGCCC	Reverse
EGFR-ZNF-BS5-F	ChIP	GCGGGATATAGTTCTCTTTATGT AGCACTG	Forward
EGFR-ZNF-BS5-R	ChIP	ACCAAGCTAAATGTGCTTTCCAT CATCT	Reverse
MIR155HG-F	Real-time PCR	TCATGTCATTCTTAATTGCAGGT TTTGGC	Forward
MIR155HG-R	Real-time PCR	ACCTGGGGGAAAGTACCAGTTT CT	Reverse
MIR155-p63BS-F	ChIP	TCATGTCATTCTTAATTGCAGGT TTTGGC	Forward
MIR155-p63BS-R	ChIP	ACCTGGGGGAAAGTACCAGTTT CT	Reverse
PPIG-F	Real-time PCR	TGGACAAGTAATCTCTGGTCAA	Forward
PPIG-R	Real-time PCR	GTATCCGTACCTCCGCAAA	Reverse
SMAD2-ZNF-	ChIP	ACCCTCAGGCTCTTTCCAGAAGC	Forward

BS1-F			
SMAD2-ZNF-BS1-R	ChIP	AGGCACATAGAGCAGGGATTGCT	Reverse
SMAD2-ZNF-BS2-F	ChIP	ACGACCTACTGTTGACTGGAAGCCT	Forward
SMAD2-ZNF-BS2-R	ChIP	AGACCTTCATGATGATCCACTTCCACT	Reverse
TGFB1-F	Real-time PCR	GTACCTGAACCCGTGTTGCT	Forward
TGFB1-R	Real-time PCR	GTATCGCCAGGAATTGTTGC	Reverse
TGFB1-ZNF-BS1-F	ChIP	GGGTACCCTTGGCCCCATCTTA	Forward
TGFB1-ZNF-BS1-R	ChIP	AGCCACCACTTCCTCTCCCATGA	Reverse
TGFB1-ZNF-BS2-F	ChIP	CCTAACCAGCTGGGGAAGGGAGT	Forward
TGFB1-ZNF-BS2-R	ChIP	TGCTGTCTCATTCCAGAGACTGTGCT	Reverse
TGFB2-ZNF-BS1-F	ChIP	TTGCTCCGATTGGCGTGGAGC	Forward
TGFB2-ZNF-BS1-R	ChIP	CGGGTTTTGTCTCGGCGATGC	Reverse
TGFBR2-F	Real-time PCR	GGGGAAACAATACTGGCTGATCA	Forward
TGFBR2-R	Real-time PCR	GAGCTCTTGAGGTCCCTGTGCA	Reverse

TGFBR2-ZNF-BS1-F	ChIP	AGTACTGCAAGGACATGCTCCAAAAT	Forward
TGFBR2-ZNF-BS1-R	ChIP	TGTAGAACCACACTCCGTGCTCT	Reverse
TGFBR2-ZNF-BS2-F	ChIP	TCCACTGACGCCTTGGCATGGA	Forward
TGFBR2-ZNF-BS2-R	ChIP	AGACAGCGATCAGGTGGGTAGCCA	Reverse
VIM-F	Real-time PCR	TCCATTGGGTGGGAGAGCCAAGG	Forward
VIM-R	Real-time PCR	CCAGAGGGAGTGAATCCAGATTA	Reverse
VIM-ZNF-BS1-F	ChIP	TGCCACTGGGCTCTAAGCAGTGT	Forward
VIM-ZNF-BS1-R	ChIP	AGGAGTGGAGGCTTTGACAGAGT	Reverse
ZNF652-F	Real-time PCR	CTTCACCAGCAAACAGACTGTGAA	Forward
ZNF652-R	Real-time PCR	TTCTTTTCTGCATATCCATGGACG	Reverse
p53 siRNA	siRNA	GCAUGAACCGGAGGCCCAUdTdT	Sense
p53 siRNA	siRNA	AUGGGCCUCCGGUUCAUGCdTdT	Antisense
ZNF652 siRNA #1	siRNA	GUAGAGAAAGUCAGCGUUAUU	Sense

ZNF652 siRNA #1	siRNA	P-UAACGCUGACUUUCUCUACUU	Antisense
ZNF652 siRNA #2	siRNA	GAGAAGUGCUUUCGGGUGAUU	Sense
ZNF652 siRNA #2	siRNA	P-UCACCCGAAAGCACUUCUCUU	Antisense

Supplementary Table References

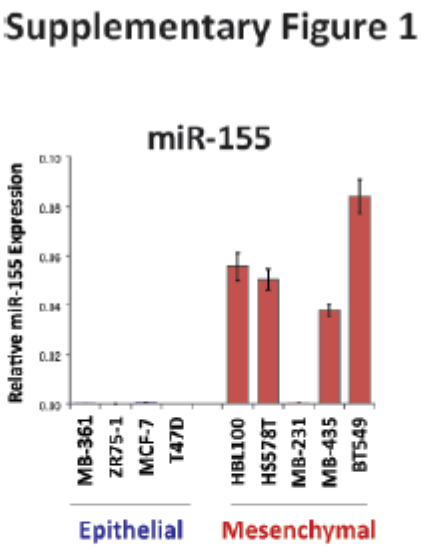
1. Xu, G., C. Fewell, C. Taylor, N. Deng, D. Hedges, X. Wang, K. Zhang, M. Lacey, H. Zhang, Q. Yin, J. Cameron, Z. Lin, D. Zhu, and E.K. Flemington. 2010. Transcriptome and targetome analysis in MIR155 expressing cells using RNA-seq. *Rna* 16:1610-1622.
2. Yin, Q., J. McBride, C. Fewell, M. Lacey, X. Wang, Z. Lin, J. Cameron, and E.K. Flemington. 2008. MicroRNA-155 is an Epstein-Barr virus-induced gene that modulates Epstein-Barr virus-regulated gene expression pathways. *J Virol.* 82:5295-5306. Epub 2008 Mar 5226.
3. O'Connell, R.M., D.S. Rao, A.A. Chaudhuri, M.P. Boldin, K.D. Taganov, J. Nicoll, R.L. Paquette, and D. Baltimore. 2008. Sustained expression of microRNA-155 in hematopoietic stem cells causes a myeloproliferative disorder. *J Exp Med.* 205:585-594. Epub 2008 Feb 2025.
4. Martin, M.M., E.J. Lee, J.A. Buckenberger, T.D. Schmittgen, and T.S. Elton. 2006. MicroRNA-155 regulates human angiotensin II type 1 receptor expression in fibroblasts. *J Biol Chem.* 281:18277-18284. Epub 12006 May 18274.
5. Cheng, W., T. Liu, F. Jiang, C. Liu, X. Zhao, Y. Gao, H. Wang, and Z. Liu. 2011. microRNA-155 regulates angiotensin II type 1 receptor expression in umbilical vein endothelial cells from severely pre-eclamptic pregnant women. *Int* 27:393-399. doi: 310.3892/ijmm.2011.3598. Epub 2011 Jan 3813.
6. Zheng, L., C.C. Xu, W.D. Chen, W.L. Shen, C.C. Ruan, L.M. Zhu, D.L. Zhu, and P.J. Gao. 2010. MicroRNA-155 regulates angiotensin II type 1 receptor expression and phenotypic differentiation in vascular adventitial fibroblasts. *Biochem* 400:483-488. Epub 2010 Aug 2022.
7. Zhu, N., D. Zhang, S. Chen, X. Liu, L. Lin, X. Huang, Z. Guo, J. Liu, Y. Wang, W. Yuan, and Y. Qin. 2011. Endothelial enriched microRNAs regulate angiotensin II-induced endothelial inflammation and migration. *Atherosclerosis* 2011:19.
8. Wang, H.Q., X.D. Yu, Z.H. Liu, X. Cheng, D. Samartzis, L.T. Jia, S.X. Wu, J. Huang, J. Chen, and Z.J. Luo. 2011. Deregulated miR-155 promotes Fas-mediated apoptosis in human intervertebral disc degeneration by targeting FADD and caspase-3. *J Pathol* 2011:2931.

9. He, M., Z. Xu, T. Ding, D.M. Kuang, and L. Zheng. 2009. MicroRNA-155 regulates inflammatory cytokine production in tumor-associated macrophages via targeting C/EBPbeta. *Cell Mol Immunol.* 6:343-352.
10. Worm, J., J. Stenvang, A. Petri, K.S. Frederiksen, S. Obad, J. Elmen, M. Hedtjarn, E.M. Straarup, J.B. Hansen, and S. Kauppinen. 2009. Silencing of microRNA-155 in mice during acute inflammatory response leads to derepression of c/ebp Beta and down-regulation of G-CSF. *Nucleic Acids Res.* 37:5784-5792. Epub 2009 Jul 5713.
11. Yin, Q., X. Wang, C. Fewell, J. Cameron, H. Zhu, M. Baddoo, Z. Lin, and E.K. Flemington. 2010. MicroRNA miR-155 inhibits bone morphogenetic protein (BMP) signaling and BMP-mediated Epstein-Barr virus reactivation. *J* 84:6318-6327. Epub 2010 Apr 6328.
12. Sonkoly, E., P. Janson, M.L. Majuri, T. Savinko, N. Fyhrquist, L. Eidsmo, N. Xu, F. Meisgen, T. Wei, M. Bradley, J. Stenvang, S. Kauppinen, H. Alenius, A. Lauerma, B. Homey, O. Winqvist, M. Stahle, and A. Pivarcsi. 2010. MiR-155 is overexpressed in patients with atopic dermatitis and modulates T-cell proliferative responses by targeting cytotoxic T lymphocyte-associated antigen 4. *J* 126:581-589.e581-520. Epub 2010 Jul 2031.
13. Tili, E., J.J. Michaille, A. Cimino, S. Costinean, C.D. Dumitru, B. Adair, M. Fabbri, H. Alder, C.G. Liu, G.A. Calin, and C.M. Croce. 2007. Modulation of miR-155 and miR-125b levels following lipopolysaccharide/TNF-alpha stimulation and their possible roles in regulating the response to endotoxin shock. *J Immunol.* 179:5082-5089.
14. Yamamoto, M., E. Kondo, M. Takeuchi, A. Harashima, T. Otani, K. Tsuji-Takayama, F. Yamasaki, H. Kumon, M. Kibata, and S. Nakamura. 2011. miR-155, a Modulator of FOXO3a Protein Expression, Is Underexpressed and Cannot Be Upregulated by Stimulation of HOZOT, a Line of Multifunctional Treg. *PLoS* 6:e16841.
15. Kong, W., L. He, M. Coppola, J. Guo, N.N. Esposito, D. Coppola, and J.Q. Cheng. 2010. MicroRNA-155 regulates cell survival, growth, and chemosensitivity by targeting FOXO3a in breast cancer. *J* 285:17869-17879. Epub 12010 Apr 17866.
16. Lu, F., A. Weidmer, C.G. Liu, S. Volinia, C.M. Croce, and P.M. Lieberman. 2008. Epstein-Barr virus-induced miR-155 attenuates NF-kappaB signaling and stabilizes latent virus persistence. *J Virol.* 82:10436-10443. Epub 12008 Aug 10427.
17. Zhou, H., X. Huang, H. Cui, X. Luo, Y. Tang, S. Chen, L. Wu, and N. Shen. 2010. miR-155 and its star-form partner miR-155* cooperatively regulate type I interferon production by human plasmacytoid dendritic cells. *Blood.* 116:5885-5894. Epub 2010 Sep 5817.
18. Martinez-Nunez, R.T., F. Louafi, and T. Sanchez-Elsner. 2011. The interleukin 13 (IL-13) pathway in human macrophages is modulated by microRNA-155 via direct targeting of interleukin 13 receptor alpha1 (IL13Ralpha1). *J Biol Chem* 286:1786-1794.
19. Bolisetty, M.T., G. Dy, W. Tam, and K.L. Beemon. 2009. Reticuloendotheliosis virus strain T induces miR-155, which targets JARID2 and promotes cell survival. *J Virol.* 83:12009-12017. Epub 12009 Sep 12016.
20. Pottier, N., T. Maurin, B. Chevalier, M.P. Puissegur, K. Lebrigand, K. Robbe-Sermesant, T. Bertero, C.L. Lino Cardenas, E. Courcot, G. Rios, S. Fourre, J.M. Lo-Guidice, B. Marcet, B. Cardinaud, P. Barbry, and B. Mari. 2009. Identification of keratinocyte growth factor as a target of microRNA-155 in lung fibroblasts: implication in epithelial-mesenchymal interactions. *PLoS One.* 4:e6718.

21. Lu, C., X. Huang, X. Zhang, K. Roensch, Q. Cao, K.I. Nakayama, B.R. Blazar, Y. Zeng, and X. Zhou. 2011. MiR-221 and miR-155 regulate human dendritic cell development, apoptosis and IL-12 production through targeting of p27kip1, KPC1 and SOCS-1. *Blood* 2011:25.
22. Tang, B., B. Xiao, Z. Liu, N. Li, E.D. Zhu, B.S. Li, Q.H. Xie, Y. Zhuang, Q.M. Zou, and X.H. Mao. 1481. Identification of MyD88 as a novel target of miR-155, involved in negative regulation of Helicobacter pylori-induced inflammation. *FEBS Lett* 584:1481-1486.
23. Martinez-Nunez, R.T., F. Louafi, P.S. Friedmann, and T. Sanchez-Elsner. 2009. MicroRNA-155 modulates the pathogen binding ability of dendritic cells (DCs) by down-regulation of DC-specific intercellular adhesion molecule-3 grabbing non-integrin (DC-SIGN). *J Biol Chem.* 284:16334-16342. Epub 12009 Apr 16322.
24. Kong, W., H. Yang, L. He, J.J. Zhao, D. Coppola, W.S. Dalton, and J.Q. Cheng. 2008. MicroRNA-155 is regulated by the transforming growth factor beta/Smad pathway and contributes to epithelial cell plasticity by targeting RhoA. *Mol Cell Biol.* 28:6773-6784. Epub 2008 Sep 6715.
25. Bhattacharyya, S., N.S. Balakathiresan, C. Dalgard, U. Gutti, D. Armistead, C. Jozwik, M. Srivastava, H.B. Pollard, and R. Biswas. 2011. Elevated miR-155 promotes inflammation in cystic fibrosis by driving hyper-expression of interleukin-8. *J Biol Chem* 2011:31.
26. O'Connell, R.M., A.A. Chaudhuri, D.S. Rao, and D. Baltimore. 2009. Inositol phosphatase SHIP1 is a primary target of miR-155. *Proc Natl Acad Sci U S A.* 106:7113-7118. Epub 2009 Apr 7119.
27. Cremer, T.J., D.H. Ravneberg, C.D. Clay, M.G. Piper-Hunter, C.B. Marsh, T.S. Elton, J.S. Gunn, A. Amer, T.D. Kanneganti, L.S. Schlesinger, J.P. Butchar, and S. Tridandapani. 2009. MiR-155 induction by F. novicida but not the virulent F. tularensis results in SHIP down-regulation and enhanced pro-inflammatory cytokine response. *PLoS One.* 4:e8508.
28. Pedersen, I.M., D. Otero, E. Kao, A.V. Miletic, C. Hother, E. Ralfkiaer, R.C. Rickert, K. Gronbaek, and M. David. 2009. Onco-miR-155 targets SHIP1 to promote TNFalpha-dependent growth of B cell lymphomas. *EMBO Mol Med.* 1:288-295.
29. Costinean, S., S.K. Sandhu, I.M. Pedersen, E. Tili, R. Trotta, D. Perrotti, D. Ciarlariello, P. Neviani, J. Harb, L.R. Kauffman, A. Shidham, and C.M. Croce. 2009. Src homology 2 domain-containing inositol-5-phosphatase and CCAAT enhancer-binding protein beta are targeted by miR-155 in B cells of Emicro-MiR-155 transgenic mice. *Blood.* 114:1374-1382. Epub 2009 Jun 1311.
30. Levati, L., E. Pagani, S. Romani, D. Castiglia, E. Piccinni, C. Covaciu, P. Caporaso, S. Bondanza, F.R. Antonetti, E. Bonmassar, F. Martelli, E. Alvino, and S. D'Atri. 1111. MicroRNA-155 Targets the SKI Gene in Human Melanoma Cell Lines. *Pigment Cell Melanoma Res* 2011:
31. Louafi, F., R.T. Martinez-Nunez, and T. Sanchez-Elsner. 2010. MicroRNA-155 targets SMAD2 and modulates the response of macrophages to transforming growth factor-{beta}. *J* 285:41328-41336. Epub 42010 Oct 41329.
32. Rai, D., S.W. Kim, M.R. McKeller, P.L. Dahia, and R.C. Aguiar. 2010. Targeting of SMAD5 links microRNA-155 to the TGF-beta pathway and lymphomagenesis. *Proc* 107:3111-3116. Epub 2010 Feb 3111.
33. Jiang, S., H.W. Zhang, M.H. Lu, X.H. He, Y. Li, H. Gu, M.F. Liu, and E.D. Wang. 2010. MicroRNA-155 functions as an OncomiR in breast cancer by targeting the suppressor of cytokine signaling 1 gene. *Cancer* 70:3119-3127. Epub 2010 Mar 3130.

34. Lu, L.F., T.H. Thai, D.P. Calado, A. Chaudhry, M. Kubo, K. Tanaka, G.B. Loeb, H. Lee, A. Yoshimura, K. Rajewsky, and A.Y. Rudensky. 2009. Foxp3-dependent microRNA155 confers competitive fitness to regulatory T cells by targeting SOCS1 protein. *Immunity.* 30:80-91.
35. Imaizumi, T., H. Tanaka, A. Tajima, Y. Yokono, T. Matsumiya, H. Yoshida, K. Tsuruga, T. Aizawa-Yashiro, R. Hayakari, I. Inoue, E. Ito, and K. Satoh. 2010. IFN-gamma and TNF-alpha synergistically induce microRNA-155 which regulates TAB2/IP-10 expression in human mesangial cells. *Am* 32:462-468. Epub 2010 Oct 2018.
36. Ceppi, M., P.M. Pereira, I. Dunand-Sauthier, E. Barras, W. Reith, M.A. Santos, and P. Pierre. 2009. MicroRNA-155 modulates the interleukin-1 signaling pathway in activated human monocyte-derived dendritic cells. *Proc Natl Acad Sci U S A.* 106:2735-2740. Epub 2009 Feb 2734.
37. Wang, Y., M.N. Scheiber, C. Neumann, G.A. Calin, and D. Zhou. 2010. MicroRNA Regulation of Ionizing Radiation-Induced Premature Senescence. *Int J Radiat Oncol Biol Phys* 2010:17.
38. Gironella, M., M. Seux, M.J. Xie, C. Cano, R. Tomasini, J. Gommeaux, S. Garcia, J. Nowak, M.L. Yeung, K.T. Jeang, A. Chaix, L. Fazli, Y. Motoo, Q. Wang, P. Rocchi, A. Russo, M. Gleave, J.C. Dagorn, J.L. Iovanna, A. Carrier, M.J. Pebusque, and N.J. Dusetti. 2007. Tumor protein 53-induced nuclear protein 1 expression is repressed by miR-155, and its restoration inhibits pancreatic tumor development. *Proc Natl Acad Sci U S A.* 104:16170-16175. Epub 12007 Oct 16172.
39. Butz, H., I. Liko, S. Czirjak, P. Igaz, M.M. Khan, V. Zivkovic, K. Balint, M. Korbonits, K. Racz, and A. Patocs. Down-regulation of Wee1 kinase by a specific subset of microRNA in human sporadic pituitary adenomas. *J* 95:E181-191. Epub 2010 Jul 2028.

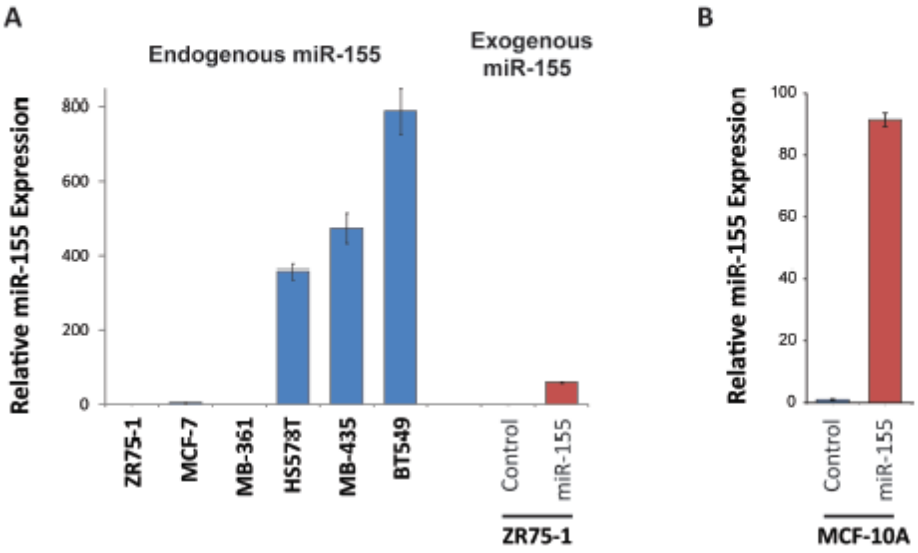
Supplementary Figures



Supplementary Figure 1. miR-155 expression in breast cancer cell lines

The relative expression levels of miR-155 were determined in a panel of epithelial and mesenchymal breast cancer cell lines (as indicated).

Supplementary Figure 2

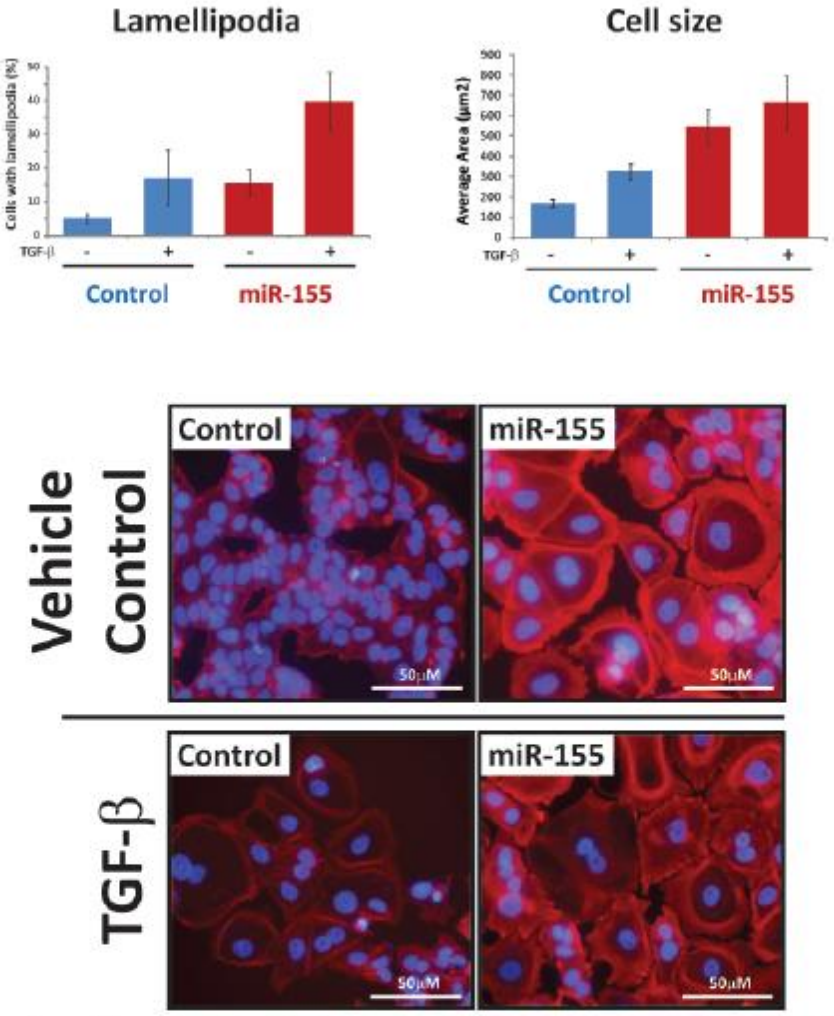


Supplementary Figure 2. Physiologically-relevant expression of miR-155 in ZR-75-1 cells

A. The relative expression levels of miR-155 were determined in a polyclonal population of ZR-75-1 cells stably expressing either retrovirally-delivered miR-155 or a scrambled non-targeting RNA, as compared with a panel of epithelial and mesenchymal breast cancer cell lines.

B. Relative expression of miR-155 was determined in polyclonal populations of MCF-10A cells stably expressing either retrovirally-delivered miR-155 or a scrambled non-targeting RNA.

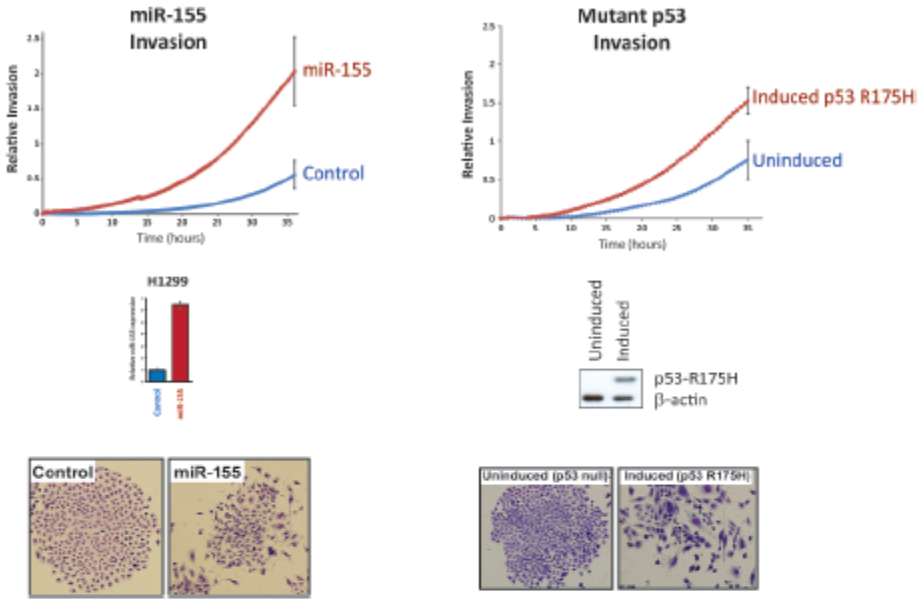
Supplementary Figure 3



Supplementary Figure 3. miR-155 expression phenocopies activation of the TGF-β pathway.

ZR-75-1 cells (control or miR-155) were seeded at 10% confluence and grown for 12 days in the presence of 2 ng/mL TGF-β (or vehicle control). Cells were stained for F-actin using a phalloidin antibody, with lamellopodia and cell size scored as described in Materials and Methods.

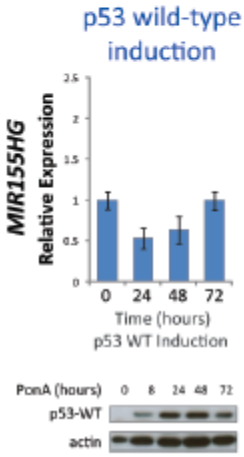
Supplementary Figure 4



Supplementary Figure 4. miR-155 expression phenocopies mutant p53 expression in H1299 cells.

H1299 cells expressing either miR-155 or a scrambled non-targeting RNA were plated in the upper chamber of a CIM-16 plate coated with 5% matrigel, with invasion assessed in real-time using an Xcelligence RTCA DP analyser. Similar experiments were performed using EI H1299 p53 R175H cells in the presence or absence of the inducing agent, PonA (2.5 µg/mL). The relative expression of miR-155 and p53 R175H were determined by real-time PCR and Western blot analysis, respectively. H1299 cells (as treated above) were plated in a 6-well format at 1000 cells/well and stained with Giesma after 10 days. Pictures show representative colonies.

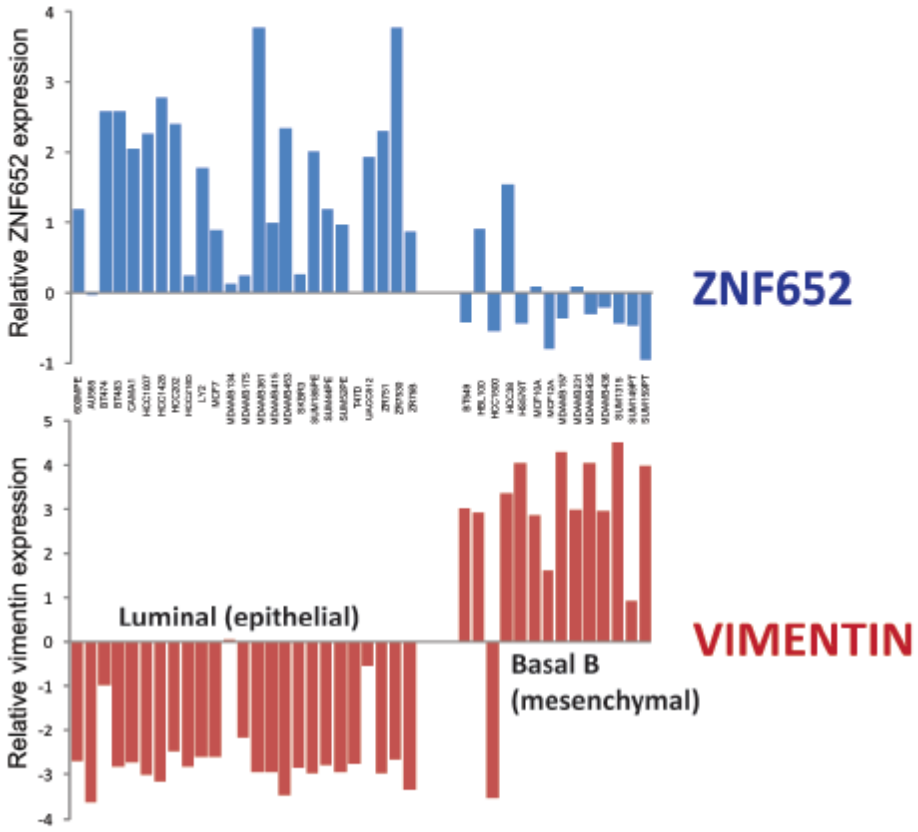
Supplementary Figure 5



Supplementary Figure 5. miR-155 is directly repressed by wild-type p53

EI p53-WT cells were treated with 2.5 µg/mL PonA for 0, 24, 48 or 72 hours to induce p53 protein expression. Relative *MIR155HG* expression and p53-WT protein expression were determined by specific RT-PCR or Western blot analysis, respectively.

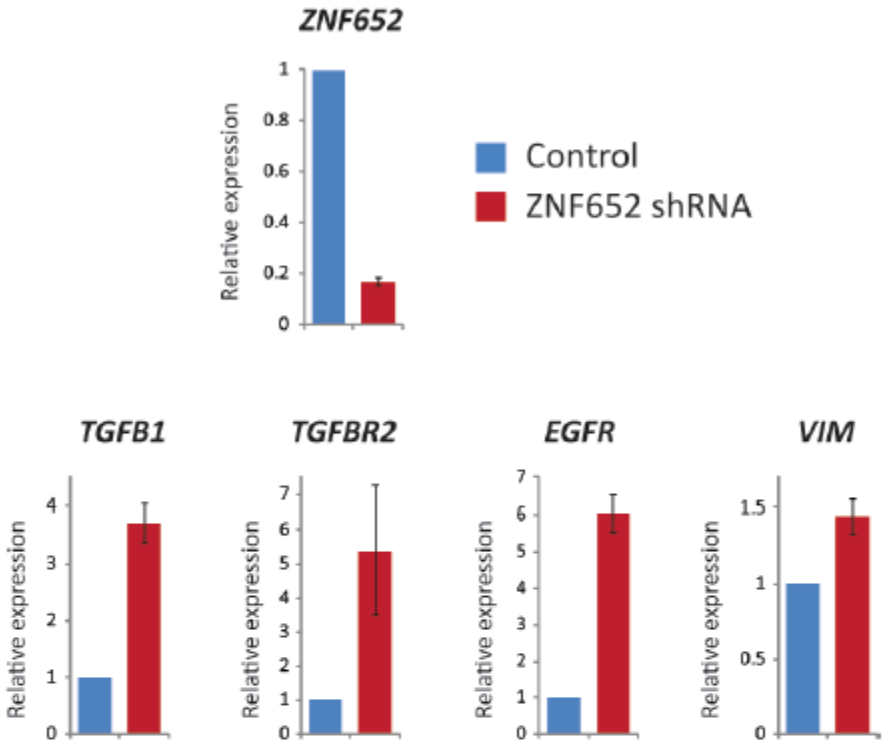
Supplementary Figure 6



Supplementary Figure 6. ZNF652 expression is inversely correlated with vimentin in breast cancer cell lines

Expression microarray data from a cohort of 51 breast cancer cell lines representing both luminal (epithelial) and basal b (mesenchymal) cell types (Neve, 2006) shows that ZNF652 expression is up-regulated in epithelial cell lines and inversely correlated with the expression of the mesenchymal marker, vimentin.

Supplementary Figure 7



Supplementary Figure 7. Knockdown of ZNF652 results in a de-repression of target genes

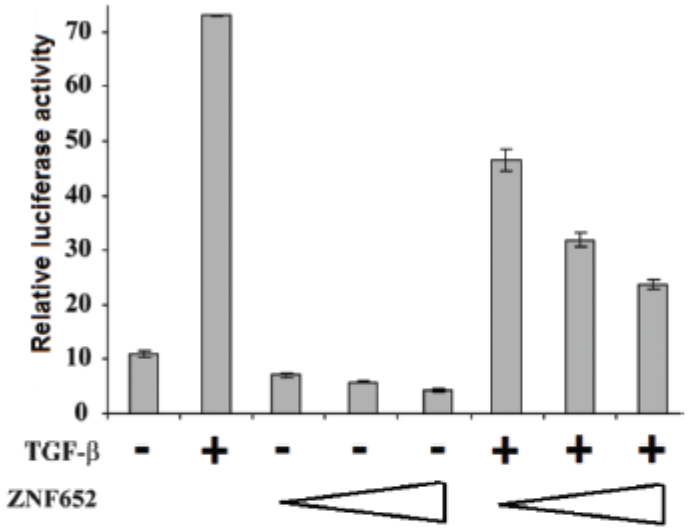
Polyclonal populations of ZR-75-1 cells expressing a ZNF652 shRNA (V3LHS_368392) or non-targeting (control) sequence were generated using the pGIPZ lentiviral shRNAmir system. The expression of ZNF652 and its target genes (*TGFB1, TGFBR2, EGFR and VIM*) were determined using real-time PCR. Similar results were observed using a second ZNF652-specific shRNAmir construct (V2LHS_229362) (data not shown).

Supplementary Figure 8

Supplementary Figure 8. miR-155 and ZNF652 regulate similar target genes within the EMT pathway.

The expression of 84 EMT related genes (listed in Supplementary Table 1) was determined in ZR-75-1 cells with silenced ZNF652 expression (from Supplementary Figure 7). Data presented is an average of three independent biological replicates. Heat maps show the comparison of these data with that of ZR-75-1 cells expressing miR-155 (from Figure 1E).

Supplementary Figure 9



Supplementary Figure 9. ZNF652 suppresses SMAD-mediated transactivation

H1299 cells were transiently-transfected with ZNF652 (25, 100 or 250 ug) and a firefly luciferase reporter construct driven by a canonical SMAD response element. Cells were incubated in the presence of 2 ng/mL TGF-β (or vehicle control) where indicated. Firefly luciferase activity was determined using a dual-luciferase reporter kit and normalised against a renilla luciferase internal transfection control as described in Materials and Methods.

References

1. Oren M, Rotter V. Mutant p53 gain-of-function in cancer. Cold Spring Harb Perspect Biol. 2010 Feb;2(2):a001107.
2. Brosh R, Rotter V. When mutants gain new powers: news from the mutant p53 field. Nat Rev Cancer. 2009 Oct;9(10):701-13.
3. Olivier M, Langerod A, Carrieri P, Bergh J, Klaar S, Eyfjord J, et al. The clinical value of somatic TP53 gene mutations in 1,794 patients with breast cancer. Clinical Cancer Research. 2006 February 15, 2006;12(4):1157-67.
4. Miller LD, Smeds J, George J, Vega VB, Vergara L, Ploner A, et al. An expression signature for p53 status in human breast cancer predicts mutation status, transcriptional effects, and patient survival. Proc Natl Acad Sci U S A. 2005 Sep 20;102(38):13550-5.
5. Muller PA, Caswell PT, Doyle B, Iwanicki MP, Tan EH, Karim S, et al. Mutant p53 drives invasion by promoting integrin recycling. Cell. 2009 Dec 24;139(7):1327-41.
6. Adorno M, Cordenonsi M, Montagner M, Dupont S, Wong C, Hann B, et al. A Mutant-p53/Smad Complex Opposes p63 to Empower TGF²-Induced Metastasis. Cell. 2009;137(1):87-98.
7. Huang Y, Chuang A, Hao H, Talbot C, Sen T, Trink B, et al. Phospho-[Delta]Np63[alpha] is a key regulator of the cisplatin-induced microRNAome in cancer cells. Cell Death Differ. 2011;18(7):1220-30.
8. Su X, Chakravarti D, Cho MS, Liu L, Gi YJ, Lin YL, et al. TAp63 suppresses metastasis through coordinate regulation of Dicer and miRNAs. Nature. 2010 Oct 21;467(7318):986-90.
9. Iorio MV, Ferracin M, Liu CG, Veronese A, Spizzo R, Sabbioni S, et al. MicroRNA gene expression deregulation in human breast cancer. Cancer Res. 2005 Aug 15;65(16):7065-70.
10. Jiang S, Zhang H-W, Lu M-H, He X-H, Li Y, Gu H, et al. MicroRNA-155 Functions as an OncomiR in Breast Cancer by Targeting the Suppressor of Cytokine Signaling 1 Gene. Cancer Research. 2010 April 15, 2010;70(8):3119-27.
11. Blick T, Widodo E, Hugo H, Waltham M, Lenburg ME, Neve RM, et al. Epithelial mesenchymal transition traits in human breast cancer cell lines. Clin Exp Metastasis. 2008;25(6):629-42.
12. Bergh J, Norberg T, Sjogren S, Lindgren A, Holmberg L. Complete sequencing of the p53 gene provides prognostic information in breast cancer patients, particularly in relation to adjuvant systemic therapy and radiotherapy. Nat Med. 1995 Oct;1(10):1029-34.
13. Davidoff AM, Kerns BJ, Pence JC, Marks JR, Iglehart JD. p53 alterations in all stages of breast cancer. J Surg Oncol. 1991 Dec;48(4):260-7.
14. Kouwenhoven EN, van Heeringen SJ, Tena JJ, Oti M, Dutilh BE, Alonso ME, et al. Genome-wide profiling of p63 DNA-binding sites identifies an element that regulates gene expression during limb development in the 7q21 SHFM1 locus. PLoS Genet. 2010 Aug;6(8):e1001065.
15. Brosh R, Shalgi R, Liran A, Landan G, Korotayev K, Nguyen GH, et al. p53-Repressed miRNAs are involved with E2F in a feed-forward loop promoting proliferation. Mol Syst Biol. 2008;4:229.
16. Minn AJ, Gupta GP, Siegel PM, Bos PD, Shu W, Giri DD, et al. Genes that mediate breast cancer metastasis to lung. Nature. 2005 Jul 28;436(7050):518-24.

17. Kumar R, Manning J, Spendlove HE, Kremmidiotis G, McKirdy R, Lee J, et al. ZNF652, a novel zinc finger protein, interacts with the putative breast tumor suppressor CBFA2T3 to repress transcription. Mol Cancer Res. 2006 Sep;4(9):655-65.
18. Kumar R, Cheney KM, McKirdy R, Neilsen PM, Schulz RB, Lee J, et al. CBFA2T3-ZNF652 corepressor complex regulates transcription of the E-box gene HEB. J Biol Chem. 2008 Jul 4;283(27):19026-38.
19. Yin Q, McBride J, Fewell C, Lacey M, Wang X, Lin Z, et al. MicroRNA-155 Is an Epstein-Barr Virus-Induced Gene That Modulates Epstein-Barr Virus-Regulated Gene Expression Pathways. J Virol. 2008 June 1, 2008;82(11):5295-306.
20. Xu G, Fewell C, Taylor C, Deng N, Hedges D, Wang X, et al. Transcriptome and targetome analysis in MIR155 expressing cells using RNA-seq. RNA. 2010 Aug;16(8):1610-22.
21. Neve RM, Chin K, Fridlyand J, Yeh J, Baehner FL, Fevr T, et al. A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes. Cancer Cell. 2006 Dec;10(6):515-27.
22. Pankova K, Rosel D, Novotny M, Brabek J. The molecular mechanisms of transition between mesenchymal and amoeboid invasiveness in tumor cells. Cellular and Molecular Life Sciences. 2010;67(1):63-71.
23. Machesky LM. Lamellipodia and filopodia in metastasis and invasion. FEBS Lett. 2008 Jun 18;582(14):2102-11.
24. Kong W, He L, Coppola M, Guo J, Esposito NN, Coppola D, et al. MicroRNA-155 Regulates Cell Survival, Growth, and Chemosensitivity by Targeting FOXO3a in Breast Cancer. Journal of Biological Chemistry. 2010 June 4, 2010;285(23):17869-79.
25. Kong W, Yang H, He L, Zhao JJ, Coppola D, Dalton WS, et al. MicroRNA-155 is regulated by the transforming growth factor beta/Smad pathway and contributes to epithelial cell plasticity by targeting RhoA. Mol Cell Biol. 2008 Nov;28(22):6773-84.
26. Xiang X, Zhuang X, Ju S, Zhang S, Jiang H, Mu J, et al. miR-155 promotes macroscopic tumor formation yet inhibits tumor dissemination from mammary fat pads to the lung by preventing EMT. Oncogene. 2011;30(31):3440-53.
27. Nieves-Alicea R, Colburn NH, Simeone AM, Tari AM. Programmed cell death 4 inhibits breast cancer cell invasion by increasing tissue inhibitor of metalloproteinases-2 expression. Breast Cancer Res Treat. 2009 Mar;114(2):203-9.
28. Santhanam AN, Baker AR, Hegamyer G, Kirschmann DA, Colburn NH. Pdcd4 repression of lysyl oxidase inhibits hypoxia-induced breast cancer cell invasion. Oncogene. 2010 Jul 8;29(27):3921-32.
29. Wen YH, Shi X, Chiriboga L, Matsahashi S, Yee H, Afonja O. Alterations in the expression of PDCD4 in ductal carcinoma of the breast. Oncol Rep. 2007 Dec;18(6):1387-93.
30. Ma XJ, Wang Z, Ryan PD, Isakoff SJ, Barmettler A, Fuller A, et al. A two-gene expression ratio predicts clinical outcome in breast cancer patients treated with tamoxifen. Cancer Cell. 2004 Jun;5(6):607-16.
31. Do TV, Kubba LA, Du H, Sturgis CD, Woodruff TK. Transforming growth factor-beta1, transforming growth factor-beta2, and transforming growth factor-beta3 enhance ovarian cancer metastatic potential by inducing a Smad3-dependent epithelial-to-mesenchymal transition. Mol Cancer Res. 2008 May;6(5):695-705.
32. Vuoriluoto K, Haugen H, Kiviluoto S, Mpindi JP, Nevo J, Gjerdrum C, et al. Vimentin regulates EMT induction by Slug and oncogenic H-Ras and migration by governing Axl expression in breast cancer. Oncogene. 2011 Mar 24;30(12):1436-48.

33. Yu Q, Stamenkovic I. Transforming growth factor-beta facilitates breast carcinoma metastasis by promoting tumor cell survival. Clin Exp Metastasis. 2004;21(3):235-42.
34. Akhurst RJ, Derynck R. TGF-beta signaling in cancer -- a double-edged sword. Trends in Cell Biology. 2001;11(11):S44-S51.
35. Noll JE, Jeffery J, Al-Ejeh F, Kumar R, Khanna KK, Callen DF, et al. Mutant p53 drives multinucleation and invasion through a process that is suppressed by ANKRD11. Oncogene. 2011;in press.
36. Utku Y, Dehan E, Ouerfelli O, Piano F, Zuckermann RN, Pagano M, et al. A peptidomimetic siRNA transfection reagent for highly effective gene silencing. Mol Biosyst. 2006 Jun;2(6-7):312-7.
37. Pishas KI, Al-Ejeh F, Zinonos I, Brown MP, Evdokiou A, Callen DF, et al. Nutlin-3a is a potential therapeutic for Ewing Sarcoma. Clinical Cancer Research. 2011;17(3):494-504.
38. Park SM, Gaur AB, Lengyel E, Peter ME. The miR-200 family determines the epithelial phenotype of cancer cells by targeting the E-cadherin repressors ZEB1 and ZEB2. Genes Dev. 2008 Apr 1;22(7):894-907.

Chapter 3 – TAp63 regulates oncogenic miR-155 to mediate migration and tumour growth

Sam Mattiske, Kristen Ho, Jacqueline E. Noll, Paul M. Neilsen, David F. Callen and Rachel J. Suetani

Cancer Therapeutics Laboratory, Centre for Personalised Cancer Medicine, University of Adelaide, Australia

Corresponding Authors: David Callen, Hanson Institute, University of Adelaide, Frome Road, SA 5005 Australia. Phone: 61-8-8222-3145; Fax: 61-8-8222-3217; E-mail: david.callen@adelaide.edu.au

Sam Mattiske: s.mattiske@live.com.au

Keywords: miR-155, TAp63, ΔNp63, cancer, tumorigenesis, migration

Statement of Authorship

Statement of Authorship

Title of Paper	TAp63 regulates oncogenic miR-155 to mediate migration and tumour growth
Publication Status	○ Published, ○ Accepted for Publication, ⊘ Submitted for Publication, ○ Publication style
Publication Details	

Author Contributions

By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

Name of Principal Author (Candidate)	Sam Mottishe		
Contribution to the Paper	Conception and design, development of methodology, acquisition of data, analysis/interpretation, writing/review/revision		
Signature		Date	1-7-13.

Name of Co-Author	Rachel Smeltzer		
Contribution to the Paper	Conception and design, development of methodology, analysis/interpretation, writing/review/revision, study supervision		
Signature		Date	

Name of Co-Author	Paul Neiken		
Contribution to the Paper	writing/review/revision, study supervision		
Signature		Date	22/7/13

Name of Co-Author	David Cotter		
Contribution to the Paper	writing/review/revision, study supervision		
Signature		Date	22/7/13

Statement of Authorship

Title of Paper	Tapes regulates oncogenic miR-155 in invasive migration and tumour growth.
Publication Status	○ Published, ○ Accepted for Publication, ◉ Submitted for Publication, ○ Publication style
Publication Details	

Author Contributions

By signing the Statement of Authorship, each author certifies that their stated contribution to the publication is accurate and that permission is granted for the publication to be included in the candidate's thesis.

Name of Principal Author (Candidate)	Sam Mathisen		
Contribution to the Paper			
Signature		Date	1-7-13

Name of Co-Author	Kristen Ho		
Contribution to the Paper	Acquisition of data		
Signature		Date	24/7/13

Name of Co-Author	Jacqueline Noll		
Contribution to the Paper	Acquisition of data		
Signature		Date	18.7.13 (signed electronically)

Name of Co-Author			
Contribution to the Paper			
Signature		Date	

Mattiske, S., Ho, K., Noll, J.E., Neilsen, P.M., Callen, D.F. & Suetani, R.J. (2013) TAp63 regulates oncogenic miR-155 to mediate migration and tumour growth.
Oncotarget, v. 4(11), pp. 1894-1903

Chapter 4 – Conclusion

Understanding the role of oncogenic miR-155 in cancer is important due to the prevalence of miR-155 upregulation in cancer. Investigating the mechanisms of regulation of miR-155 are particularly significant since the downstream targets of miR-155 and thus the action of miR-155 is well understood. Since miR-155 is upregulated in multiple types of cancer, understanding the mechanism of miR-155 regulation and its consequences could provide targets for future therapies, for example by targeting an upstream regulator and preventing miR-155 upregulation. Since miR-155 is associated with poor prognosis in some cancer types, the regulators of miR-155 could be valuable in early diagnosis or prognosis of aggressive cancers. miR-155 itself has been proposed as a potential biomarker for aggressive cancers.

Here we identify all confirmed targets of miR-155, identify miR-155 as downstream of the guardian of the genome, investigate the role of a novel miR-155 target and propose a novel mechanism of miR-155 regulation. The confirmed targets of miR-155 have never been presented together before this, and will be a valuable resource for future researchers. The tumour suppressor properties of the novel miR-155 target ZNF652 added to the list of targets associated with cancer, further shedding light on the oncogenic role of miR-155. The greatest contribution of this work is that regarding miR-155 regulation – an area that is still yet to be thoroughly investigated. First, we identify miR-155 as downstream of p53 and mutant p53, as a driver of invasion. Further, we elucidate a mechanism of direct miR-155 regulation by the p63 isoforms TAp63 and ΔNp63, where TAp63 inhibits ΔNp63 from driving miR-155 expression. Thus when TAp63 is absent, ΔNp63 can drive miR-155 expression and cause the high levels of miR-155 upregulation seen in multiple cancer types including breast cancer. In

addition, ΔNp63 is often the most expressed p63 isoform in cancer, giving this mechanism precedence in the context of cancer.

During the course of this work, some problems had to be overcome. Differentiating between the p63 isoforms was difficult both in terms of RNA and protein. The p63 antibody used (H-129) was not isoform specific, but this was overcome by using positive controls expressing only TAp63 or ΔNp63 in order to identify which bands corresponded to which isoforms. When carrying out quantitative realtime PCR, isoform-specific primers had to be designed and optimised, which proved time consuming but ultimately successful. The second major problem involved the knockdown cell lines used throughout this work. After numerous passages the cell lines had the tendency to lose the knockdown, where cells not carrying the knockdown were outgrowing those still carrying the knockdown. Solving this was accomplished by close monitoring of the fluorescence of the cells (as all knockdown cell lines also carried a fluorescent tag) and use of selective agent when appropriate to eliminate any cells not carrying the knockdown. Additionally, although we were able to show in Chapter 3 that ΔNp63 can bind and drive miR-155 expression (via ChIP and luciferase assays respectively), the p63 isoform expression did not show that ΔNp63 overexpression increased miR-155 expression levels. This was attributed to either the inhibitory presence of TAp63 or the cell line used (BT549) having a naturally very high level of miR-155 expression which could not be elevated more. Further work should utilise a cell line with lower endogenous miR-155 levels (and preferably an absence of TAp63) that could more reasonably respond to a stimulus such as exogenous ΔNp63 expression, in order to demonstrate the mechanism established using ChIP and luciferase.

In the future investigating the role of p53 in this pathway and also determining the clinical significance of the proposed mechanism would be valuable directions for research. In Chapter 2 we showed that mutant p53 was upstream of miR-155, but in light of the findings

regarding the p63 isoforms in Chapter 3 there is an opportunity to elucidate the specific mechanism of interaction between these 3 proteins that influences miR-155 expression. Does mutant p53 bind to TAp63 and sequester it, thus enabling ΔNp63 to drive miR-155 expression? Perhaps mutant p53 and ΔNp63 both bind to MIR155 and drive its expression. And what is the role of wildtype p53? Identifying interactions between these p53 family members and the resulting effects on miR-155 expression will be crucial to fully understanding how miR-155 becomes upregulated in cancer. Further, "rescue" experiments using anti-miR-155 similar to those in Chapter 3 would be valuable in conclusively establishing mutant variants of p53 as a driver of miR-155. Also of note is that only 42 out of 140 miR-155 targets decreased in response to mutant p53/miR-155 expression. This would indicate that mutant p53 is not the sole regulator of miR-155 and its downstream targets, and that miR-155 or more likely its targets are under multiple levels of control. Identifying the other mechanisms at work here could be valuable in prognosis or diagnosis, as a perturbation in miR-155 alone may not be sufficient to cause disease in some cases. It would also be valuable to confirm results from breast cancer cell lines in primary samples, as results from cell lines can be skewed due to the immortalisation of cell lines.

The novel tumour suppressor gene ZNF652 was one of the 42 miR-155 genes that showed lower expression in mutant p53 tumours and proved an interesting miR-155 target as it repressed key drivers of invasion and loss of ZNF652 correlated with invasion and metastasis. Investigation of ZNF652 function was valuable and revealed ZNF652 as a key regulator of EMT and suppressor of invasion, however the link between mutant p53 and ZNF652 was not fully explored. Future work on ZNF652 and mutant p53 should include validation of the mutant p53/miR-155 regulation of ZNF652 by modulating p53/mutant p53 in the presence or absence of anti-miR-155, which would indicate the importance of mutant p53 and miR-155 in ZNF652's regulation. Additionally, although the ZNF652 levels in

tumours supports ZNF652 as a tumour suppressor, any future work involving ZNF652 and primary tissue should be investigated for p63 mutation (in addition to p53 mutation) and also ER status. This will shed light on the mechanism of ZNF652 regulation at work in these tumours, in relation to the findings on p63 regulation of miR-155 as described in Chapter 3. Understanding the mechanism of miR-155 upregulation has clinical significance in cancer treatment due to the association of high levels of miR-155 with aggressive disease and poor prognosis. In particular the findings presented in Chapter 3 regarding p63 isoforms are significant since p63 immunohistochemistry is already used in the clinic. The p63 regulation of miR-155 mechanism not only provides insight useful for cancer prognosis, but also potentials for treatment. If the specific interactions between the p63 isoforms and mutant p53 can be determined, it is possible that a drug could block this interaction and thereby prevent upregulation of miR-155 downstream of mutant p53. Alternatively, cancers where a high level of ΔNp63 is identified (by the clinical test mentioned above) could potentially be targeted with a miR-155 specific drug, for example locked nucleic acids (LNAs), although LNA technology needs further development before it can become a therapeutic agent. Perhaps the most effective avenue for treating tumours with a high level of miR-155 would be to find a pathway that is inhibited by miR-155 and identify or develop a drug that kills cells lacking this pathway – a synthetic lethal strategy. Since the downstream targets and pathways of miR-155 are well documented, this would seem a promising approach.

References

1. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell. 2004; 116: 281-97.
2. Calin GA, et al. MicroRNA profiling reveals distinct signatures in B cell chronic lymphocytic leukemias. Proc Natl Acad Sci U S A. 2004; 101: 11755-60.
3. Tili E, Croce C, Michaille J. miR-155: on the crosstalk between inflammation and cancer. Int Rev Immunol. 2009; 28: 264-84.
4. Iorio MV, et al. MicroRNA gene expression deregulation in human breast cancer. Cancer Res. 2005; 65: 7065-70.
5. Volinia S, et al. A microRNA expression signature of human solid tumors defines cancer gene targets. Proc Natl Acad Sci U S A. 2006; 103: 2257-61.
6. Saito Y, Suzuki H, Matsuura M, Sato A, Kasai Y, Yamada K, Saito H, Hibi T. MicroRNAs in Hepatobiliary and Pancreatic Cancers. Front Genet. 2011; 2: 16.
7. Greither T, Grochola LF, Udelnow A, Lautenschläger C, Würl P, Taubert H. Elevated expression of microRNAs 155, 203, 210 and 222 in pancreatic tumors is associated with poorer survival. Int J Cancer. 2010; 126: 73-80.
8. Kong W, Yang H, He L, Zhao JJ, Coppola D, Dalton WS, Cheng JQ. MicroRNA-155 is regulated by the transforming growth factor beta/Smad pathway and contributes to epithelial cell plasticity by targeting RhoA. Mol Cell Biol. 2008; 28: 6773-84.
9. Neilsen PM, Noll JE, Mattiske S, Bracken CP, Gregory PA, Schulz RB, Lim SP, Kumar R, Suetani RJ, Goodall GJ, Callen DF. Mutant p53 drives invasion in breast tumors through up-regulation of miR-155. Oncogene. 2012; 16: 305.
10. Mattiske S, Suetani RJ, Neilsen PM, Callen DF. The Oncogenic Role of miR-155 in Breast Cancer. Cancer Epidemiol Biomarkers Prev. 2012; 21: 1236-43.
11. Bruning U, Cerone L, Neufeld Z, Fitzpatrick SF, Cheong A, Scholz CC, Simpson DA, Leonard MO, Tambuwala MM, Cummins EP, Taylor CT. MicroRNA-155 promotes resolution of hypoxia-inducible factor 1alpha activity during prolonged hypoxia. Mol Cell Biol. 2011; 31: 4087-96.
12. Wang HQ, Yu XD, Liu ZH, Cheng X, Samartzis D, Jia LT, Wu SX, Huang J, Chen J, Luo ZJ. Deregulated miR-155 promotes Fas-mediated apoptosis in human intervertebral disc degeneration by targeting FADD and caspase-3. J Pathol. 2011; 225: 232-42.
13. Yin Q, Wang X, McBride J, Fewell C, Flemington E. B-cell receptor activation induces BIC/miR-155 expression through a conserved AP-1 element. J Biol Chem. 2008; 283: 2654-62.
14. Louafi F, Martinez-Nunez RT, Sanchez-Elsner T. MicroRNA-155 targets SMAD2 and modulates the response of macrophages to transforming growth factor-{beta}. J Biol Chem. 2010; 285: 41328-36.
15. Xu J, Lamouille S, Derynck R. TGF-beta-induced epithelial to mesenchymal transition. Cell Res. 2009; 19: 156-72.
16. Lagos-Quintana M, Rauhut R, Yalcin A, Meyer J, Lendeckel W, Tuschl T. Identification of tissue-specific microRNAs from mouse. Curr Biol. 2002; 12: 735-9.
17. Eis PS, Tam W, Sun L, Chadburn A, Li Z, Gomez MF, Lund E, Dahlberg JE. Accumulation of miR-155 and BIC RNA in human B cell lymphomas. Proc Natl Acad Sci U S A. 2005; 102: 3627-32. Epub 2005 Feb 28.
18. Kutty R, Nagineni C, Samuel W, Vijayasarathy C, Hooks J, Redmond T. Inflammatory cytokines regulate microRNA-155 expression in human retinal pigment

epithelial cells by activating JAK/STAT pathway. Biochem Biophys Res Commun. 2010; 402: 390-5.
19. Chang S, et al. Tumor suppressor BRCA1 epigenetically controls oncogenic microRNA-155. Nat Med. 2011; 17: 1275-82.
20. Onyeagucha B, Mercado-Pimentel M, Hutchison J, Flemington E, Nelson M. S100P/RAGE signaling regulates microRNA-155 expression via AP-1 activation in colon cancer. Exp Cell Res. 2013; 18.
21. Mills A, Zheng B, Wang X, Voge lH, Roop D, Bradley A. p63 is a p53 homologue required for limb and epidermal morphogenesis. Nature. 1999; 398: 708-13.
22. Yang A, Schweitzer R, Sun D, Kaghad M, Walker N, Bronson R, Tabin C, Sharpe A, Caput D, Crum C, McKeon F. p63 is essential for regenerative proliferation in limb, craniofacial and epithelial development. Nature. 1999; 398: 714-8.
23. Yang A, Kaghad M, Wang Y, Gillett E, Fleming M, Dötsch V, Andrews N, Caput D, McKeon F. p63, a p53 homolog at 3q27-29, encodes multiple products with transactivating, death-inducing, and dominant-negative activities. Mol Cell. 1998; 2: 305-16.
24. Gressner O, Schilling T, Lorenz K, Schulze Schleithoff E, Koch A, Schulze-Bergkamen H, Lena A, Candi E, Terrinoni A, Catani M, Oren M, Melino G, Krammer P, Stremmel W, Müller M. TAp63alpha induces apoptosis by activating signaling via death receptors and mitochondria. Embo J. 2005; 24: 2458-71.
25. Su X, Chakravarti D, Cho M, Liu L, Gi Y, Lin Y, Leung M, El-Naggar A, Creighton C, Suraokar M, Wistuba I, Flores E. TAp63 suppresses metastasis through coordinate regulation of Dicer and miRNAs. Nature. 2010; 467: 986-90.
26. Sniezek J, Matheny K, Westfall M, Pietenpol J. Dominant negative p63 isoform expression in head and neck squamous cell carcinoma. Laryngoscope. 2004; 114: 2063-72.
27. Kurundkar D, Srivastava RK, Chaudhary SC, Ballestas ME, Kopelovich L, Elmets CA, Athar M. Vorinostat, an HDAC inhibitor attenuates epidermoid squamous cell carcinoma growth by dampening mTOR signaling pathway in a human xenograft murine model. Toxicol Appl Pharmacol. 2013; 266: 233-44.
28. Melino G. p63 is a suppressor of tumorigenesis and metastasis interacting with mutant p53. Cell Death Differ. 2011; 18: 1487-99.
29. Nylander K, Vojtesek B, Nenutil R, Lindgren B, Roos G, Zhanxiang W, Sjöström B, Dahlqvist A, Coates P. Differential expression of p63 isoforms in normal tissues and neoplastic cells. J Pathol. 2002; 198: 417-27.
30. Gu X, Coates P, Boldrup L, Nylander K. p63 contributes to cell invasion and migration in squamous cell carcinoma of the head and neck. Cancer Lett. 2008; 263: 26-34.
31. Tucci P, et al. Loss of p63 and its microRNA-205 target results in enhanced cell migration and metastasis in prostate cancer. Proc Natl Acad Sci U S A. 2012; 109: 15312-7.
32. Melino G, De Laurenzi V, Vousden K. p73: Friend or foe in tumorigenesis. Nat Rev Cancer. 2002; 2: 605-15.
33. Yang A, Kaghad M, Caput D, McKeon F. On the shoulders of giants: p63, p73 and the rise of p53. Trends Genet. 2002; 18: 90-5.
34. Adorno M, Cordenonsi M, Montagner M, Dupont S, Wong C, Hann B, Solari A, Bobisse S, Rondina MB, Guzzardo V, Parenti AR, Rosato A, Bicciato S, Balmain A, Piccolo S. A Mutant-p53/Smad complex opposes p63 to empower TGFbeta-induced metastasis. Cell. 2009; 137: 87-98.

35. Muller P, Vousden K, Norman J. p53 and its mutants in tumor cell migration and invasion. J Cell Biol. 2011; 192: 209-18.
36. Muller P, Caswell P, Doyle B, Iwanicki M, Tan E, Karim S, Lukashchuk N, Gillespie D, Ludwig R, Gosselin P, Cromer A, Brugge J, Sansom O, Norman J, Vousden K. Mutant p53 drives invasion by promoting integrin recycling. Cell. 2009; 139: 1327-41.
37. Utku Y, Dehan E, Ouerfelli O, Piano F, Zuckermann R, Pagano M, Kirshenbaum K. A peptidomimetic siRNA transfection reagent for highly effective gene silencing. Mol Biosyst. 2006; 2: 312-7.
38. Kumar R, Neilsen P, Crawford J, McKirdy R, Lee J, Powell J, Saif Z, Martin J, Lombaerts M, Cornelisse C, Cleton-Jansen A, Callen D. FBXO31 is the chromosome 16q24.3 senescence gene, a candidate breast tumor suppressor, and a component of an SCF complex. Cancer Res. 2005; 65: 11304-13.