Biochemistry and Biophysics Reports 25 (2021) 100920

Contents lists available at ScienceDirect

Biochemistry and Biophysics Reports

journal homepage: www.elsevier.com/locate/bbrep

P53 regulation of osteoblast differentiation is mediated through specific microRNAs

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ARTICLE INFO

Keywords:
p53
Osteoblast differentiation
microRNA
Bone

ABSTRACT

In order to understand the role of the p53 tumor suppressor gene in microRNA expression during osteoblast differentiation, we used a screen to identify microRNAs that were altered in a p53-dependent manner. MicroRNAs from MC3T3-E1 preosteoblasts were isolated from day 0 (undifferentiated) and day 4 (differentiating) and compared to a p53 deficient MC3T3-E1 line treated similarly. Overall, one fourth of all the microRNAs tested showed a reduction of 0.6 fold, and a similar number of them were increased 1.7 fold with differentiation. P53 deficiency caused 40% reduction in expression of microRNAs in differentiating cells, while a small percent (0.03%) showed an increase. Changes in microRNAs were validated using real-time PCR and two microRNAs were selected for further analysis (miR-34b and miR-140). These two microRNAs were increased significantly during differentiation but showed a dramatic reduction in expression in a p53 deficient state. Stable expression of miR-34b and miR-140 in MC3T3-E1 cells resulted in decreases in cell proliferation rates when compared to control cells. There was a 4-fold increase in p53 levels with miR-34b expression and a less dramatic increase with miR-140. Putative target binding sites for bone specific transcription factors, Runx2 and Osterix, were found for miR-34b, while Runx2, beta catenin and type 1 collagen were found to be miR-140 targets. Western blot analyses and functional assays for the transcription factors Runx2, Osterix and Beta-catenin confirmed microRNA specific interactions. These studies provide evidence that p53 mediated regulation of osteoblast differentiation can also occur through specific microRNAs such as miR-34b and miR-140 that also directly target important bone specific genes.

1. Introduction

Numerous pathways regulate osteoblast differentiation, and in the last decade a post transcriptional mechanism mediated by microRNAs has been actively explored in bone [1]. MicroRNAs (miRNAs) are small (~22 nucleotides) single-stranded, non-coding, RNAs that control the translation of mRNAs by annealing to the 3' UTR of the mRNA and degrading the messenger RNA (mRNA) target or by repressing translation [2]. Recent studies have shown a major role for miRNAs in a number of different biological and pathological processes including apoptosis, cell proliferation, and differentiation [3–7].

MicroRNAs are an important mechanism of regulation in bone since a deficiency or loss of DICER (a protein important in the processing of miRNAs) affects normal differentiation in bone [8]. This, in addition to research showing the importance of specific miRNAs in osteoblast differentiation suggests that miRNAs are important for the maintenance of normal bone homeostasis [9,10].

The tumor suppressor gene p53 plays a pivotal role in various cellular stresses such as DNA damage, hypoxia, senescence and other functions [11,12]. The biological response to these damages depend on the extent of the damage and results in either apoptosis or cell cycle arrest [12]. Its role in all these pathways also depend on p53's ability to regulate specific genes as a transcriptional regulator [13]. Aside from these numerous functions, our laboratory and others have shown a role for p53 in tissue differentiation [14,15]. P53 loss is commonly seen in osteosarcomas and results in the inability of these cells to express genes involved in terminal osteoblast differentiation and normal bone remodeling [16]. P53 expression and function within differentiating

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https://doi.org/10.1016/j.bbrep.2021.100920
Received 18 February 2020; Received in revised form 8 January 2021; Accepted 12 January 2021
Available online 1 February 2021

osteoblasts occur during a distinct window of time that is separate from its ability to influence cell cycle arrest [17]. We have also shown that p53 acts as a transcriptional activator for a number of bone specific genes especially osteocalcin [18]. Osteocalcin is a bone specific, differentiation related gene expressed late during differentiation. Interestingly a number of osteoblast specific genes such as osteopontin, vitamin D receptor, BMP2 and BMP4 have been shown to be direct p53 targets [19,20] and (unpublished observations). These studies suggest the importance of p53 in maintaining normal remodeling in postnatal bone.

As p53 plays a role in the regulation of microRNAs [21–23], we focused this study on p53 dependent miRNAs that are also differentiation related, to define p53's role in this process. To do this we performed gene array analyses using undifferentiated and differentiated osteoblasts and compared them to the changes seen in differentiating osteoblasts with loss of p53 expression. This allowed us to characterize p53 specific changes in miRNA expression.

2. Methods

2.1. Cell culture

The MC3T3 -E1 mouse osteoblast like cells originally obtained from mouse calvaria, were obtained from ATCC (ATCC-CRL- 2593) and used for experiments. In order to establish the role of p53 in the differentiation process we compared control MC3T3- E1 cells to one containing a stable p53 knockdown in MC3T3-E1 cells in a manner similar to that described earlier [24]. Single cell clones with an 80% reduction in endogenous p53 levels were used for these studies. The MC3T3-E1 lines were maintained in Alpha-MEM medium (Life Technologies) with 10% Fetal Bovine Serum (FBS) (Atlanta Pharmaceuticals).

2.2. Differentiation promotion treatment and RNA isolation

For studies exploring differentiation, cells were treated with the differentiating medium (DP media) consisting of basal Alpha -MEM medium containing 50 μg/mL ascorbic acid and 100 mM biglycerol phosphate. This treatment hastened the expression of genes involved in differentiation which can be seen within a week of exposure to the media [16–18]. We harvested the cells on days 2, 4, 6 and 8. Total RNA was isolated using Trizol (Thermo Scientific) and miRNA was isolated using the mirVana microRNA isolation kit (Applied Biosystem).

2.3. μParaflo™ microRNA microarray assay

Differential expression of mouse miRNAs (Sanger miRBase Release 18.0) was performed using μParaflo™ microfluidic chip technology (LC Sciences). For the microarray analysis, we selected the following samples: MC3T3E1 Day O control cells, MC3T3E1 Day 4 DP and p53KD –Day 4 DP media treated cells. We picked Day 4 after analyzing for p53 expression and found it to peak on that day. The methodology used for the μParaflo™ miRNA microarray asasay is previously described [25]. Data analysis was performed subtracting the background and then normalizing the signals using a LOWESS filter (Locally-weighted Regression), and statistical and clustering analysis was performed by LC Sciences using ANOVA to compare all three samples ($p < 0.01$).

2.4. Validation of MicroRNA expression with qRT-PCR analyses

For validation of the array we used ΔΔCT method for relative quantification of miRNAs.

mIR-x MIiRNA First Strand synthesis kit was used for cDNA isolation from microRNA (Takara Bio USA, 638313. Realtime PCR was performed using Mir-x miRNA qRT-PCR TB Green Kit (Takara Bio USA, 639676). The forward primer was the entire miRNA sequence of interest (IDT) and the reverse primer was supplied in the kit. ΔΔCT method was used for relative quantification of miRNAs and the fold difference in gene expression between a test sample and a calibrator sample (U6 miRNA) was calculated. To test statistical significance between the control and treatment cells, two-tailed Student's t-test or ANOVA statistical analyses was performed, with $p \leq 0.05$ considered statistically significant. Statistical analyses were performed with GraphPad Prism version 7.0 software (GraphPad Software, La Jolla, CA).

2.5. Creation of stable cell lines expressing miRNAs

MC3T3- E1 cells were transfected with pre-miR-140 and pre-miR-34b expression plasmids (Origene) with a neomycin selection marker and single cell clones were isolated and expanded to stable cell lines. A stable control line contained a similar construct with a scrambled sequence. Cells harboring p53 shRNA stably have previously been described [24].

2.6. Protein isolation & western blot assay

Protein changes were analyzed using western blots. MC3T3- E1 cells were lysed with sample buffer, consisting of 50 mM DTT, 2% SDS, 10% glycerol, 65 mM Tris-HCl pH 6.8, and cOmplete ULTRA Tablets Mini (Roche) protease inhibitor (1 tablet/10 mL). The primary antibodies used were p53, COL1A1, Beta Catenin (active), Runx2 (Cell Signaling), GAPDH (Proteintech) and Actin (Sigma) and the secondary antibody used were goat anti-mouse and anti-rabbit HRP (Thermo Scientific). Osterix and MDM2 antibodies were purchased from Santa Cruz Biotechnology.

2.7. Cell proliferation assay

Cells were plated at a density of 1×10^5 cells/mL in triplicate in a 6 well plate regular 10% serum containing basal media and grown to 80–90% confluency. They were then serum starved for 24 h before exposing them to serum containing medium and growth evaluated at three time points (24,48,72 h) using an automated cell counter. The experiment was repeated thrice in triplicate. A representative data is shown.

2.8. Luciferase reporter assays

For target validation: Reporter plasmids were created with synthetic oligonucleotide duplexes (IDT) containing the putative targets of miR-34b and miR-140 from the following mRNAs. For miR34b we used SP7 (osterix), Runx2, Mdm2, and for the putative targets of miR140: Runx2, Beta Catenin and Type 1 Collagen. The target sequences were cloned using pMIR-REPORT miRNA Expression Reporter System(ThermoFisher, USA). The luciferase assay was performed with and without miR 34- or mIR140-mimics (Ambion USA). Transfections of these constructs and mimics were done in triplicates using Effectene (Qiagen, USA).

For functional activity: As p53, Runx2, osterix and beta-catenin are transcription factors with specific DNA binding ability, we used reporter assays to monitor their functional activity which helped support our Western blot analyses of these proteins.

In order to evaluate the functional activity of p53 we transfected cells with, pG13-luc, a plasmid containing 13 canonical p53 binding site driving a luciferase reporter [26] Similar analyses were carried out for Runx2/Cbfa1 using Cbfa-luc [27], osterix (osterix-luc) [28] and Wnt/beta catenin pathway (TOPFLASH) [29]. Control cells received a scrambled sequence. All transfections were carried out for 48 h followed by measurements of luminescence as described previously [29].

3. Results

Analysis of microRNA expression during p53 dependent osteoblast differentiation: MC3T3-E1 cells cultured in osteogenic differentiation

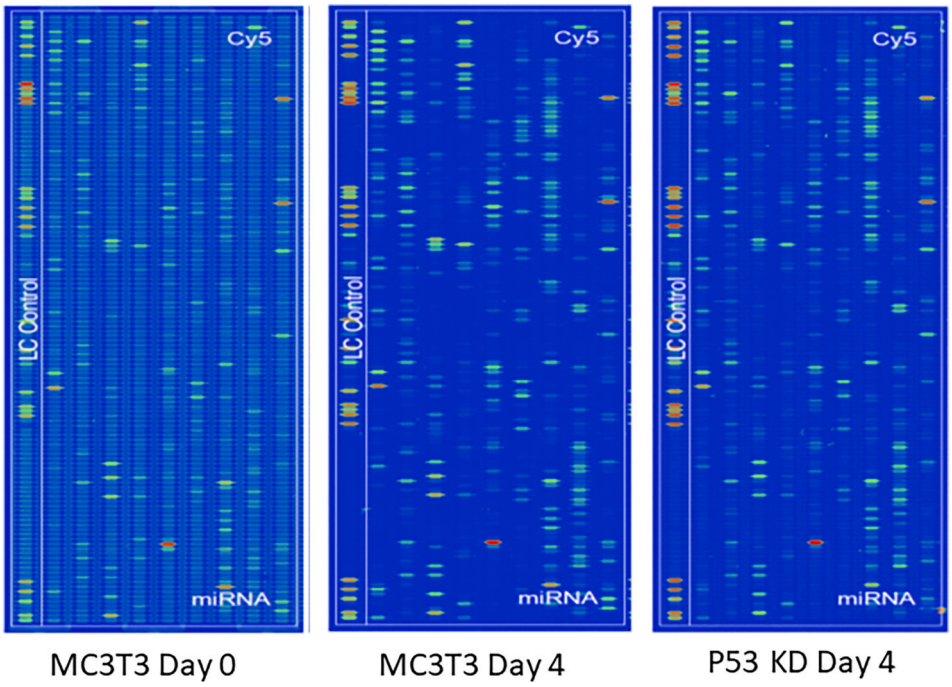

Fig. 1. MicroRNA expression profiles: Pre-osteoblast MC3T3- E1 were cultured in regular media (day 0) or after treatment with DP media (Day4). A similarly cultured p53 reduced (p53KD MC3T3-E1) cells were compared to determine p53 related and differentiation-related changes after 4day DP media exposure. A representative Cy5 image is shown.

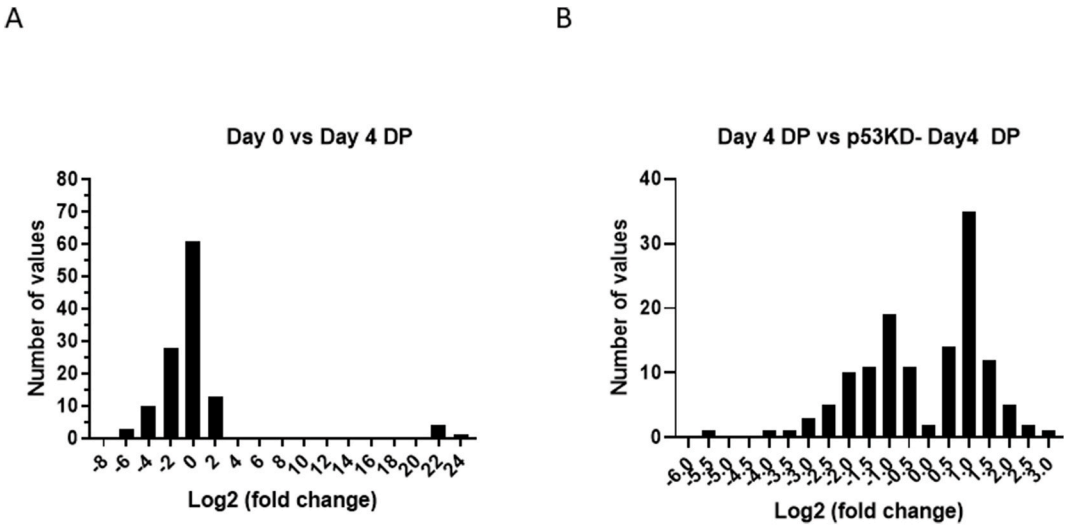

Fig. 2. Expression pattern of miRNAs during differentiation. Effect of p53 deficiency. Differentiation related miRNAs with statistically significant ($P < 0.001$) changes and high signals were compared to determine changes related to differentiation (A- Day 0 vs Day 4 DP)) and the effect of p53 deficiency during the same period (B- Day 4 DP vs p53KD-day 4 DP)).

promoting media (DP media) for different lengths of time ranging from 0, 2, 4, 6 and 8 days express differentiation markers related to osteogenesis [17,30]. We used cells where p53 levels were stably reduced to about 20% of the total (80% knockdown) and these cells have previously been described by our laboratory [24]. Control cells received a scrambled sequence. These cells were exposed to DP media for the same length of time as described for control MC3T3-E1 cells. In our previous work, we have established an increase in p53 expression during differentiation to be responsible for changes in osteoblast specific gene expression [31]. We have shown p53 levels to rise post –confluence, and after the addition of DP media. There is an increase in steady state levels of p53, which consistently reaches a peak 4–6 days after DP media addition [17]. The difference in timing of maximal increase is usually related to the degree of differentiation of the preosteoblasts during the start of the experiment. Therefore, it was necessary to first determine when the maximal p53 increase occurred before providing the sample for miRNA analysis. We exposed MC3T3-E1 control and MC3T3E1-p53shRNA cells (henceforth referred to as p53KD) to DP media and established the days when p53 levels were maximally elevated and used that sample (day 4-DP) along with control for analyses of microRNAs as described under methods. A sample Cy5 chip image of the experiment with corresponding changes in miRNA band intensities are shown in Fig. 1.

Overall, 1143 targets were tested in the array and when we compared both low and high copy number miRNAs with $P < 0.05$, we

Table 1A
MicroRNAs with significantly increased expression in the MC3T3 cells after 4 days of DP treatment as determined by microRNA microarray analysis.

MicroRNA	p-value	Control Day 0 Mean (RFS*)	Day 4 Mean (RFS*)	Log 2 (Day4/Control)
miR-7a-5p	2.33E-12	0.00020	1259	22.61
miR-301a-3p	6.63E-12	0.00022	966	22.04
miR-29b-2-5p	8.92E-11	0.00018	498	21.38
miR-539–5p	5.78E-10	0.00020	788	21.88
miR-466h-3p	4.87E-08	1280	2444	0.93
miR-466j	1.04E-07	328	750	1.19
miR-574–5p	1.04E-07	1327	2475	0.90
miR-29a-3p	2.67E-07	5299	10,710	1.2
miR-466f-3p	3.15E-07	1579	2755	0.80
miR-466a-3p	5.62E-07	282	570	1.01
miR-1187	5.67E-07	924	1791	0.96
miR-466f	7.13E-07	403	908	1.17
miR-669a-3p	7.77E-07	1457	2640	0.86
miR-669d-5p	9.62E-07	151	402	1.42
miR-468–3p	1.29E-06	152	617	2.02
miR-297a-5p	2.70E-06	153	377	1.30
miR-466h-5p	1.11E-05	245	690	1.49
miR-669e-5p	1.13E-05	168	461	1.46
miR-378–3p	1.19E-05	336	601	0.84
miR-672–5p	1.74E-05	216	607	1.49
miR-31–3p	2.36E-05	140	527	1.91
let-7i-5p	3.91E-05	4948	9807	0.99
miR-568	7.26E-05	415	759	0.87
miR-3097–5p	9.12E-05	330	756	1.19

* Relative Fluorescent Signal.

Table 1B
MicroRNAs with significantly decreased expression in the MC3T3 cells after 4 days of DP treatment as determined by microRNA microarray analysis.

MicroRNA	p-value	Control Day 0 Mean (RFS*)	Day 4 Mean (RFS*)	Log 2 (Day4/Control)
miR-342–3p	2.13E-10	4110	826	−2.32
miR-2861	5.32E-10	2398	467	−2.36
miR-3070b-3p	4.06E-09	1732	29	−5.90
miR-3075–5p	4.23E-09	1171	42	−4.80
miR-5115	8.29E-09	2923	492	−2.57
miR-1935	8.66E-09	1335	37	−5.17
miR-92b-3p	9.29E-09	1392	645	−1.11
miR-5099	9.46E-09	14,339	7830	−0.87
miR-5117–3p	1.08E-08	1274	104	−3.62
miR-346–5p	1.79E-08	2189	478	−2.19
miR-421–3p	2.59E-08	1150	103	−3.49
miR-3968	3.41E-08	645	149	−2.12
miR-290–5p	3.54E-08	716	53	−3.76
miR-3102–5p.2–5p	3.87E-08	2039	184	−3.47
miR-377–3p	5.82E-08	1414	19	−6.20
miR-665–5p	8.18E-08	2385	299	−3.00
miR-341–3p	1.57E-07	784	53	−3.90
miR-15a-3p	1.96E-07	1210	320	−1.92
miR-3072–5p	2.43E-07	1790	514	−1.80
miR-668–3p	2.45E-07	621	46	−3.75
miR-195–3p	2.99E-07	396	165	−1.26
miR-208a-5p	3.21E-07	516	83	−2.63
miR-714	3.27E-07	943	216	−2.12
miR-497–5p	3.53E-07	827	164	−2.33
miR-5122	4.30E-07	694	58	−3.57
miR-762	5.04E-07	5304	1525	−1.80
miR-365–3p	7.31E-07	2273	1053	−1.11
miR-5100	7.32E-07	2827	1300	−1.12
miR-1892	8.09E-07	1940	405	−2.26
miR-1195	8.19E-07	2409	753	−1.68
miR-652–5p	1.43E-06	488	91	−2.42
miR-720	1.73E-06	7805	4222	−0.89
miR-671–5p	1.75E-06	477	164	−1.54
miR-1894–3p	1.77E-06	973	298	−1.71
miR-705	2.79E-06	1746	685	−1.35
miR-1906	3.75E-06	485	129	−1.91
miR-2137	4.31E-06	10,038	4149	−1.27
miR-328–3p	5.22E-06	842	369	−1.19
miR-423–5p	6.14E-06	1814	512	−1.82
miR-328–5p	1.32E-05	1489	434	−1.78
miR-3077–5p	1.43E-05	1049	149	−2.82
miR-681	3.24E-05	497	52	−3.25
miR-700–3p	4.01E-05	353	124	−1.52

found 300 of them to increase with differentiation and a similar number (275) to decrease with differentiation (MC3T3E1 Day 0 versus Day 4DP). When the effect of p53 dosage was compared in differentiated osteoblasts (MC3T3E1 Day 4 DP and p53KD –Day 4 DP), 40% of the miRNAs were reduced by at least 0.6 fold. A small percentage (0.03%) of them were increased at least 1.7 fold with differentiation, about 49 of them were not expressed with p53 deficiency. Samples that were expressed highly with $P < 0.001$ were selected for further analysis.

A general reduction in miRNA expression occurs with osteoblast differentiation: When this set of highly expressed miRNAs were analyzed, there was a reduction in expression of several microRNAs with DP treatment. As shown in Fig. 2A we initially compared the changes seen on day 4 with day 0 untreated control sample to determine differentiation related changes in miRNA. Sixty-two showed no change while 43 were reduced with differentiation (<-1 in log 2 ratio). Eighteen were increased, ranging from over 2-fold to several fold. (>1 in log 2 ratio) (Fig. 2A.). When we compared differentiated cells with a normal complement of p53 to cells with reduced expression (Fig. 2B), we found 35 of them had doubled in expression (log2 ratio 1). Fifty-five miRNAs increased with p53 loss while 62 showed a decrease.

The list of statistically significant differentially expressed microRNAs with higher signal intensities as seen in the array are available in table (Table 1). We also validated the changes seen in some miRNA using real-time PCR. This included some miRNAs that had a high expression level as well as some with a lower signal. The miRNA tested were miR26a, 34b, 99a and 140. These miRNAs were tested at 0- time and 4 days after DP treatment of control and p53 KD cells (Fig. 2C-upper panel). The data from arrays is presented for comparison below (Fig. 2C-lower panel). 0-time data after p53 loss has been included in our PCR analysis. All the miRNA tested appeared to show slight reduction with p53 loss. The increase seen with differentiation in control miRNAs was not seen in p53 deficient cells. The array data shown below, mainly compared differentiation related changes after p53 expression loss. As seen in the figure, the changes closely followed what was seen in the array, thus providing validation of the array data (Fig. 2C).

As p53 is known to regulate the miR34 family, we compared their expression to p53 loss during differentiation and found that all of the three family members showed changes ranging from 1 to 2-fold increase during differentiation and expression was affected by p53 deficiency. We also chose to investigate mIR-140 further as it is an important chondrocyte and osteoblast differentiation factor [32,33]. These miRNAs belonged to the low signal group. (Fig. 3A). As shown in Fig. 3B, miR34b was increased more than the other 34 family members during differentiation. In the case of miR 140, there was a two-fold increase in miR140–5p with differentiation, and the loss of p53 caused a robust decrease in miR140–5p expression (Fig. 3C).

Identification of genes regulated by miR34b and miR140: To explore the possibility that some of the differentiation functions are perhaps mediated by these microRNAs, we searched for putative targets of mmu-mir −34b and mmu-mir −140 at microRNA.org. Several were identified as shown in figure with alignments of these sequences. Osterix (SP7) is a bone specific transcription factor that plays a major role along with Runx2 during osteoblast differentiation [34]. Recent studies have indicated a role for p53 in the regulation of osterix [35]. Mdm2 gene is transcriptionally regulated by p53 and we have found MDM2 protein to have bone specific functions [31,36]. The fragments of three potential targets of miR-34b and miR-140 were synthesized and cloned into luciferase vectors. Subsequently transfections were carried out with and without the corresponding mimics. As shown in Fig. 4A, transfection of the mimic significantly reduced the activity of all the putative targets of

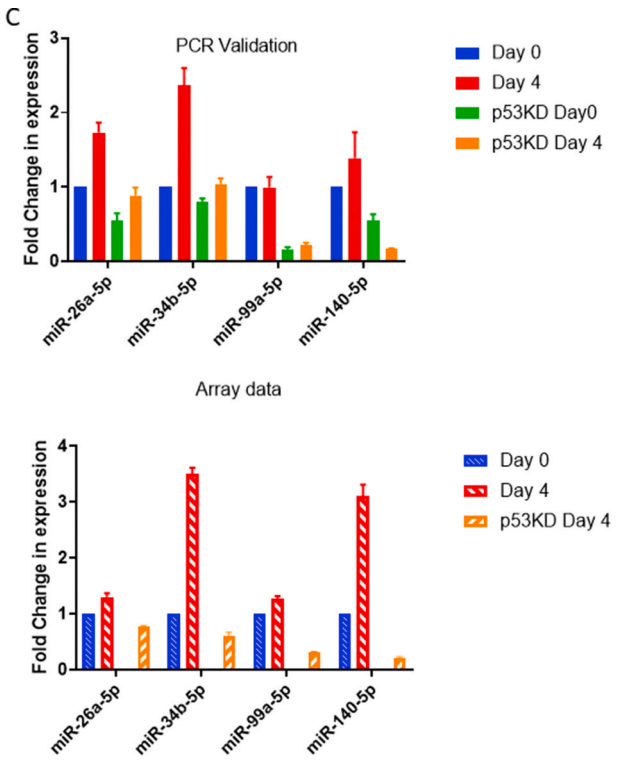

Fig. 2C. Realtime PCR validation of selected miRNAs. Several microRNA that underwent changes during differentiation were tested in MC3T3-E1 osteoblasts and p53 KD cells after exposure to DP media and compared to untreated controls. The comparative CT method was used to determine fold change (n = 3). Error bars indicate mean ± standard error of the mean. The array data is provided below in order to demonstrate reproducibility of the array data. The array did not include samples from 0- time p53 KD line.

miR34b that were studied. No change was seen when the targets for other miRNAs were used in the assay (non-specific target in Fig. 4A). To confirm reduction in target expression we used antibodies to Runx2 and osterix after transiently transfecting cells with miR34b mimic. As shown in Fig. 4B, both targets showed reduction in expression when the miR34b mimic was used. To demonstrate that the actual target (Runx2 and osterix) functions were reduced *in vitro*, we used a reporter assay to determine the impact on their transcription functions. Luciferase acitivity was measured using a construct that contained several copies of either the Runx2 (Cbfa1) DNA binding site or the osterix (SP7) DNA binding sites as described under methods. Transient transfection and overexpression of miR34b along with these reporter constructs produced 50% and 60% reduction in Runx2 and osterix function respectively. (Fig. 4B bar graphs). Mdm2 expression was also reduced by introduction of miR34b mimic (Fig. 4B).

In the case of miR-140, the targets chosen were Type 1 collagen, beta-catenin and Runx2. Type 1 collagen is expressed in osteoblasts during the matrix deposition phase during osteoblast differentiation [37]. Beta catenin as part of the Wnt canonical pathway is an important regulator of osteoblast differentiation [38]. To figure out if these putative targets are affected by miR-140, the target sequences were cloned as described above and transiently transfected with a miR-140 specific mimic to prove specificity (Fig. 4C). As seen in the figure, all three targets showed a statistically significant decrease in luciferase activity. To show specificity we used a target of a different miRNA (shown as a non-specific target in Fig. 4C), which showed no change. Further confirmation of protein expression and function of these targets were done using western blots and functional assays. Antibodies to active beta-catenin and runx2 was used in Western blot analyses to demonstrate loss of expression when cells were transfected with miR140 mimics Fig. 4D). As done for miR34b, we performed functional assays for Runx2 and Wnt/beta catenin acitivity with appropriate luciferase reporters. As shown in bar graphs in Fig. 4D there was a 90% reduction in both Runx2 and beta catenin activity when co-expressed with miR-140 showing that the mRNA of these genes are also likely targets of miR-140. Reduction in type1 collagen expression after transfection of cells with the miR140 mimic showed that COL1A1 is also a valid target for this miRNA.

Generation of stable cell lines expressing miR34b and miR140: To determine how these miRNAs may affect osteoblast function we generated cell lines stably overexpressing miR −34b and miR −140 in MC3T3-E1 cells by introducing the precursor miRNAs with a selection marker. Single cell clones of antibiotic resistant cells were selected and characterized. Quantitative measurement of miRNA levels in these stable lines showed a 1.6 and 1.5- fold significant increase ($p < 0.05$) in expression of mIR34b and 140 respectively in these cells. No appreciable change was noted the other miRNA of interest showing that the small but significant increase was specific to the miRNA transfected. (Fig. 5A and B).

Growth characteristics: As part of the characterization of these cell lines, we compared cell growth rates of the miRNA overexpressing cells when compared to control. It was interesting to note that the lines expressing exogenous miRNAs showed a significant decrease in cell numbers over time when compared to control, which stably expressed a scrambled sequence. While the increase in overexpression of the miRNA was modest, their effect on growth was profound suggesting that these microRNAs were behaving as tumor suppressors. The line overexpressing miR- 34b showed a greater reduction in cell proliferation when compared to the line overexpressing miR-140 (Fig. 5C).

P53 levels are increased in cells stably expressing microRNAs 34b: As the proliferation data suggested a significant inhibition in miR34b overexpressing cells, we wanted to determine if p53 levels were affected as miR-34b is known to be a p53 –regulated gene [39]. When the stable cell lines were analyzed for p53 expression, we found that the miR34b overexpressing line showed over a 3- fold increase in p53 levels when the blots were analyzed (quantitation not shown) (Fig. 6A). We also confirmed this increase in p53 in miR34b expressing cells equated to a 6-fold increase in p53 transcription activating function (Fig. 6B) when we transiently transfected a luciferase construct containing 13 copies of a p53 DNA binding sequence. It is well-known that p53 mediates the regulation of the 34b family of microRNAs but it was not clear why an increase in miR34b would increase p53 expression and function. In order to study this further we transiently transfected increasing amounts of miR-34b into cells and determined the levels of p53. As seen in Fig. 6C, there was a corresponding increase in p53 levels with increasing miR −34b suggesting either an increase in production of p53 or a stabilization of its expression was influenced by adding exogenous miR-34b. A similar effect was not seen when we introduced increasing amounts of miR-140 (data not shown). It is notable that our data shows mdm2 to be a putative target of miR34b (see Fig. 3A).

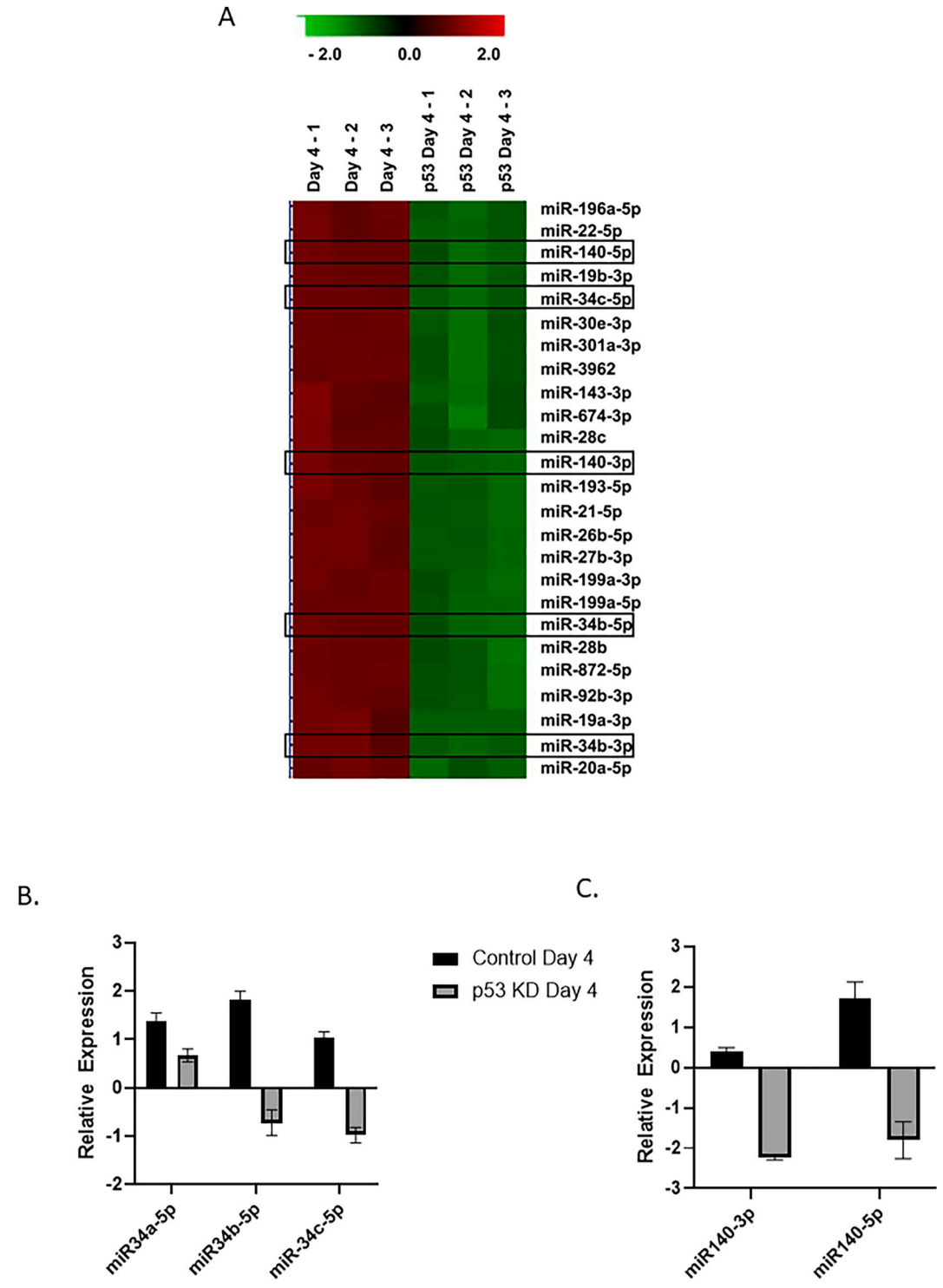

Fig. 3. AA. MicroRNA expression profile in differentiating osteoblasts with and without reduced p53 dosage as determined by microarray analysis. The miR34 and 140 family members are highlighted by a box. B. Fold changes in miR34 family members as determined by microarray analysis of samples in triplicate. C. Fold changes in miR140 as determined by microarray analysis (n = 3).

A.

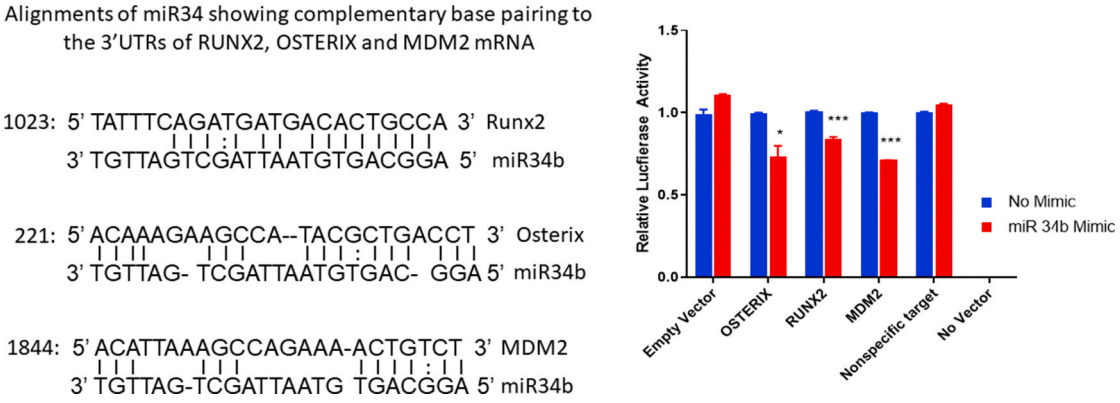

Fig. 4A. Bone specific targets Runx2 and Osterix are inhibited by miR34b: The RNA hybrid predicated binding site of miR34b to targets Runx2, Osterix and Mdm2 are indicated on the left. The wild type 3′UTR sequence of the respective target RNA was cloned downstream from the luciferase gene and the plasmid was transfected with and without siRNAs mimicking miR 34b or miR140 (non-specific target) inot MC3T3-E1 cells. Luciferase activities were normalized to transfections without siRNAs. *P < 0.05, **P < 0.01, ***P < 0.001.

B.

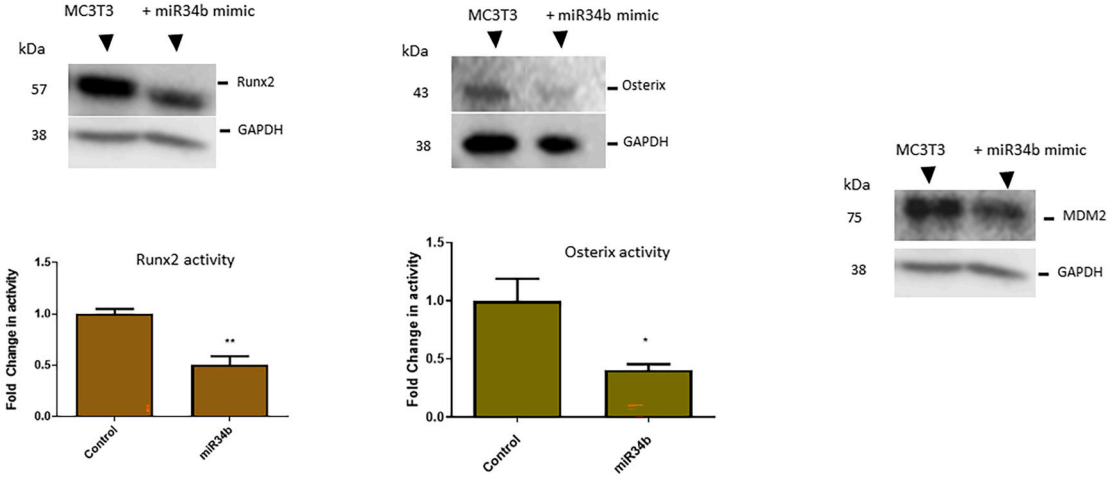

Fig. 4B. Target gene expressions are reduced by overexpression of miR34b: MC3T3 cells were transfected with miR34b mimics and maintained for 48 h. Western blots for indicated proteins were performed on total cell lysates. In order to demonstrate loss of function of Runx2 and Osterix, luciferase assays were carried out using Cbfa-luc a construct carrying the runx2 response element to determine runx2 function and an osterix construct carrying Osx/Sp7 response elements for osterix function. These constructs were transiently transfected with scrambled control or miR34b expression vector. (n = 3). *P < 0.05, **P < 0.01.

BMP-2 expression and function is increased in cells stably expressing miR140: MiR140 has been both implicated in the suppression of BMP2 expression directly [40,41] as well as indirectly by targeting the DNPEP gene to modulate BMP2 function [42]. When we tested our stable miRNA expressing lines for BMP-2 expression we found levels to be three-fold higher in miR-140 but not in miR34b expressing cells (Fig. 7A). The BMP-2 promoter was also activated in miR-140 cells (Fig. 7B).

MiRNAs targeting other bone specific factors are affected by p53 loss: While our results point to a role for p53 in regulating some of the bone specific transcription factors and other markers through miR-34b and miR-140, the mutual regulation of p53 and miR-34b suggests that this relationship between them is more complex. It was, however, obvious that p53 could directly regulate a number of bone specific genes through miRNAs. To generate a global view of how p53 might have altered several bone specific factors during differentiation we further analyzed the array. A quick survey of the literature for microRNAs that positively affect bone differentiation and are affected by loss of p53 was done. As shown in Fig. 8A, many of the miRNAs that are known to increase the Wnt pathway and activate beta-catenin function [43–47] were affected by p53 loss demonstrating that p53 positively affects this pathway. We also investigated miRNAs that affect BMP2 function and divided them according to the reported effect produced by different miRNAs in bone [44,48–54]. We found that irrespective of whether the miRNAs positively or negatively affected BMP-2 levels, p53 loss influenced their expression (Fig. 8B) suggesting that it might allow for stage

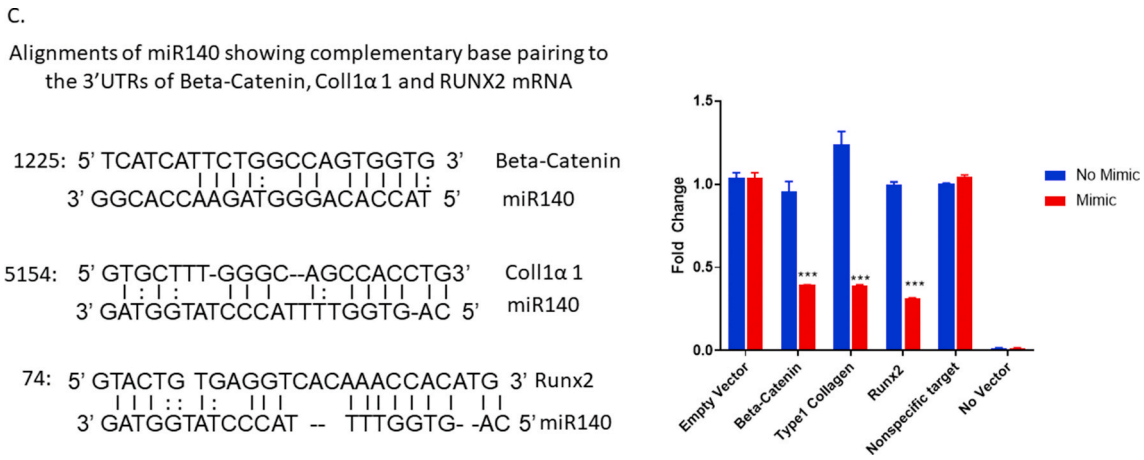

Fig. 4C. Bone specific targets Beta-Catenin, Cola1 and Runx2 are inhibited by miR-140. The RNA hybrid predicated binding site of miR140 to targets Beta-Catenin, Cola1 and Runx2 are indicated on the left. The wild type 3′UTR sequence of the respective target RNA was cloned downstream from the luciferase gene and this plasmid was transfected with and without siRNAs mimicking miR140 or miR34b (non-specific target) Luciferase activities were normalized to transfections without siRNAs (n = 3). *P < 0.05, **P < 0.01, ***P < 0.001.

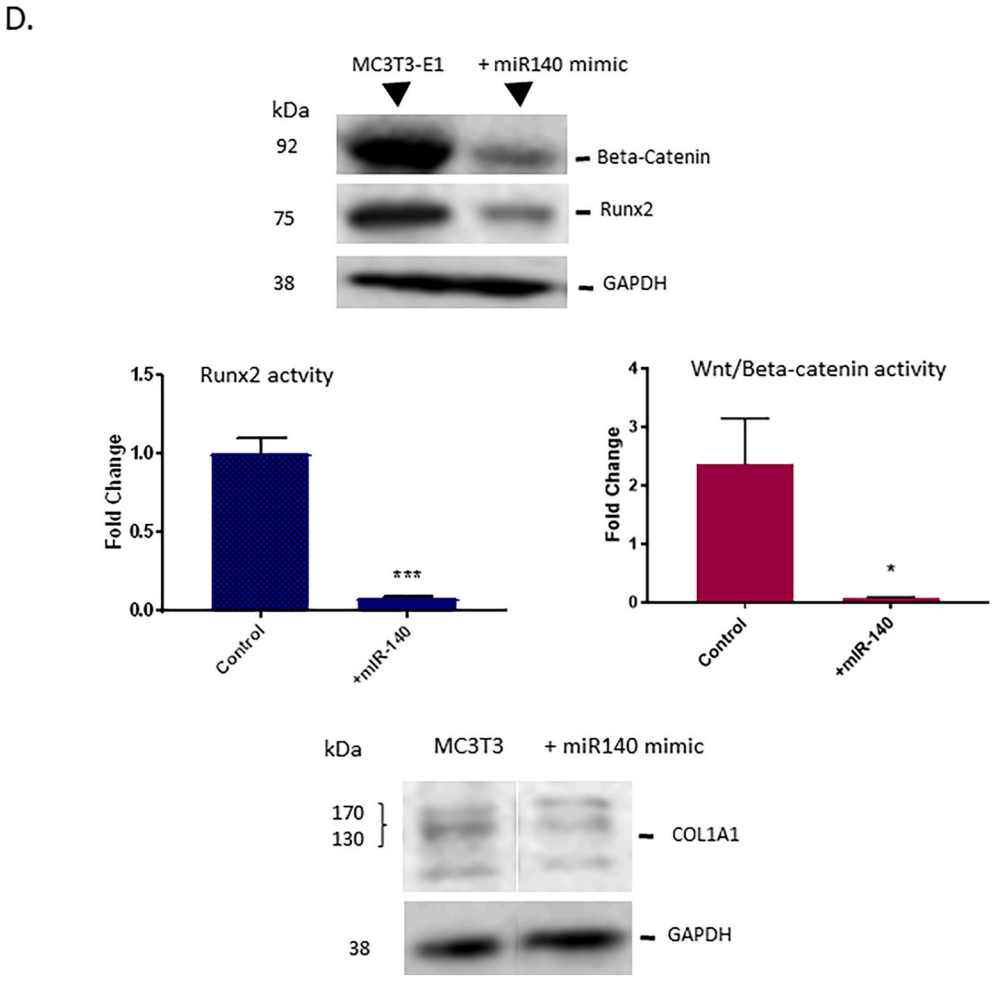

Fig. 4D. Target gene expressions are reduced by overexpression of miR140: MC3T3 cells were transfected with miR140 mimics and maintained for 48 h. Western blots for Runx2 and active beta-catenin proteins were performed on total cell lysates. In order to demonstrate loss of function of Runx2 luciferase assays were carried out using Cbfa-luc a construct carrying the runx2 response element to determine runx2 function. Wnt/Beta catenin function was monitored using TOPFLASH which contains the TCF/LEF response elements as described under methods. These constructs were transiently transfected with scrambled control or miR34b expression vector. (n = 3). *P < 0.05, ***P < 0.001. Western blot for Type1 collagen is provided below. Other details are as mentioned above for beta-catenin and Runx2 proteins.

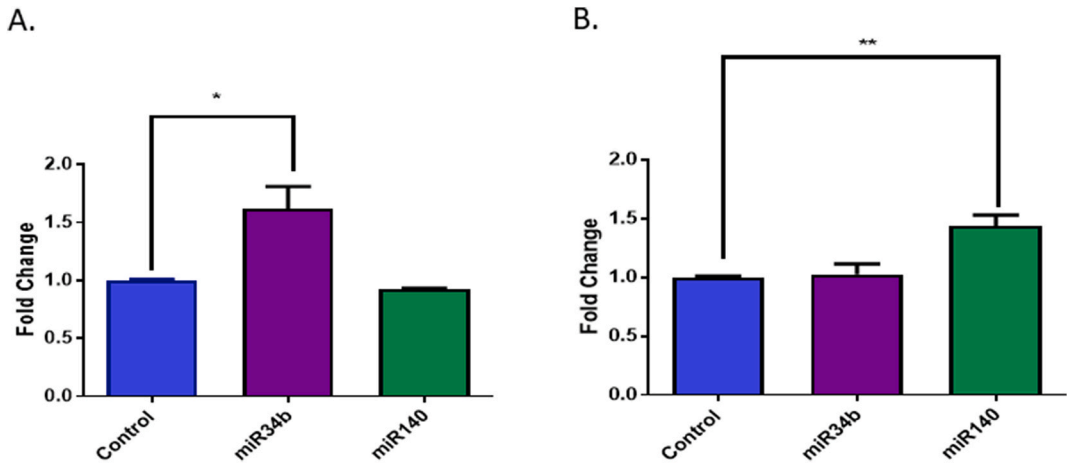

Fig. 5A & B. Generation of cell lines stably overexpressing miR34b and miR140. Stable lines overexpressing the respective miRNAs were created as described under methods. Antibiotic resistant single cell clones were isolated, expanded and the resulting RNA was characterized for changes in miR34b (A) and miR140 (B) gene expression by quantitative PCR. Experiments were run in triplicate and are presented as Mean ± SD. *P < 0.05, **P < 0.01.

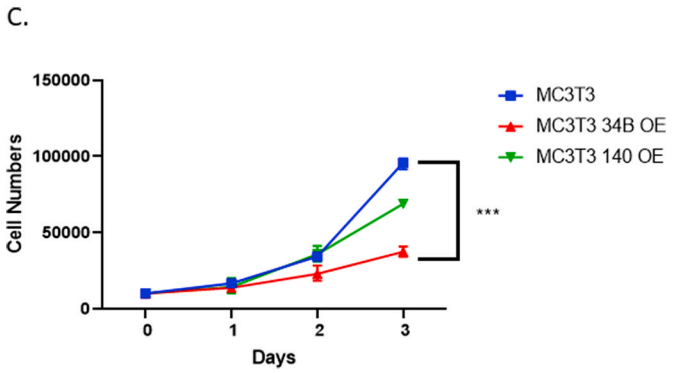

Fig. 5C. Growth rates of miR34b and miR140 overexpressing cells (OE)Equal numbers of cells were grown as described under methods and harvested after 1, 2 and 3 days: Cell growth rates were monitored using a cell counter. The asterisks indicate that the growth of cells overexpressing miR34b was significantly higher on day 3 than at other days.

specific expression of these bone anabolic factors during differentiation. We have observed similar regulation by p53 as previously documented in our studies [55].

4. Discussion

We have previously demonstrated a role for p53 in osteoblast differentiation where as a transcriptional factor, p53 regulates bone specific gene expression [56]. As p53 has been shown to participate in a number of different functions in normal cellular physiology, including microRNA regulation, we wanted to specifically determine how p53 might play a role in the regulation of differentiation related miRNAs. This study reports some of the data gathered from analyzing a miRNA array and the validation of two of the microRNAs that underwent changes with differentiation. We found that a large number of the highly

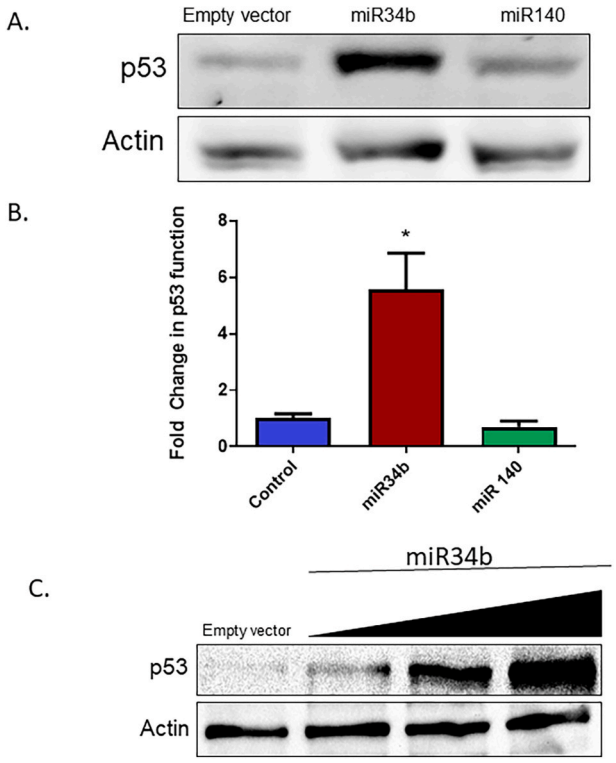

Fig. 6. Feedback positive activation of p53 by miR34b: A. p53 gene expression was estimated using Western blot analysis. B. P53 function was also monitored in these cell lines by transiently transfecting a luciferase vector with p53 response elements (PG-13-luc). *P < 0.05 when compared to control which received only the empty vector. C. Introduction of increasing amounts of miR-34b increased p53 expression. 2–8 μg of miR-34b expression vector was introduced into MC3T3E1 cells and western analysis was carried out after 48 h.

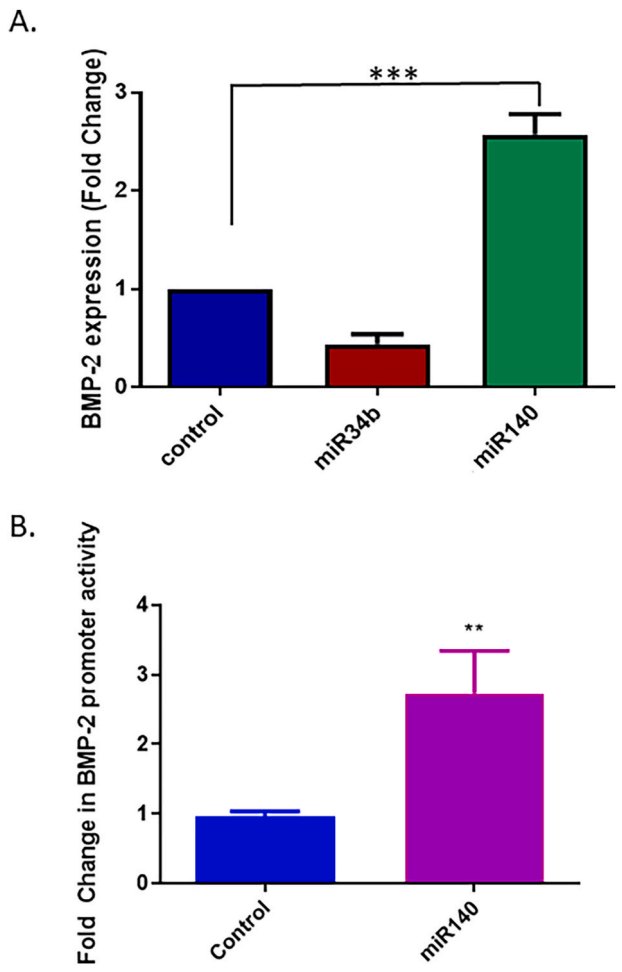

Fig. 7. Stable overexpression of miR140 increases BMP2 expression (A) and promoter activity (B). BMP2 levels were measured by realtime PCR in cells that stably expressed miR140 or miR 34b or control. Promoter activity was monitored 48 h following transient transfection of BMP-2 promoter reporter construct containing about 2000 nucleotides upstream of the start site.

expressed microRNAs were reduced with differentiation while a smaller percentage of them showed an increase. Irrespective of the change, a sizeable number of these miRNAs were affected by p53 deficiency. The dramatic effect of p53 reduction on miRNAs may likely be related to other functions in tissues such as apoptosis and cell cycle arrest and were not analyzed in this study. We focused our initial analyses on miRNAs that are important for osteoblast differentiation. Our results indicate that p53 directly or indirectly affects the expression of several of these miRNAs that underwent modulation during differentiation.

The results of conditional deletion of Dicer, an enzyme that is important for production of functional mature miRNA, has revealed the importance of miRNAs during all stages of bone development including prenatal, post-natal and bone modeling [8,9]. These studies show the need for miRNAs in the differentiation of progenitors to osteoblasts, and in the maintenance of bone remodeling, While this underscores the importance of studying miRNAs in bone, the fact that p53 can directly affect microRNA biogenesis through the enzyme Drosha [22] fuels the importance of studying p53's role in the process.

We chose two miRNAs to validate their roles in p53 mediated osteoblast differentiation, one a known p53 regulated miRNA (miR-34b) [23], and the other (mIR-140) which has an established function in mesenchymal cell differentiation [32]. Overexpression of both of these miRNAs resulted in an inhibition to growth suggesting that these microRNAs behave as tumor suppressors and is in line with the general functions of p53.

Among the miR-34 family, we found miR-34b to show significant changes during differentiation. While all the three members have some association with osteoblasts and osteosarcoma [57–59]. Both miR −34b and −34c affect osteoblast specific gene expression by targeting activities of Runx2 and Satb2 genes [57,59]. There is a need to regulate Runx2, which has both inhibitory and activating potential during differentiation [57]. Runx2 is also associated with aberrant cell proliferation and is present in high levels in tumors [48]. SATB2 is a nuclear matrix protein that promotes osteoblast differentiation by increasing Runx2 and other transcription factors [46]. It is regulated by miR-34b and c to maintain bone development and homeostasis in this tissue as shown in studies targeting this miRNA in vivo [57]. In this study, we show Runx2 and Osterix to be targets of miR-34b. This observation supports the general functions of p53 in antagonizing runx family of genes and osterix [35,60]. It is therefore likely that miRNA-mediated regulation provides an additional layer of control.

Our results also demonstrate a feed forward loop in which p53 and miR-34b activate each other. It is established that a p53 response element is present on miR-34 genes [39] and can explain the decrease seen during differentiation in cells lacking p53 expression. Since out results suggest an ability of miR-34b to increase p53 levels, there must a mutual regulation adding to the level of complexity. While we did not investigate specific mechanisms involved, it could likely represent stabilization of p53 [61]. However, this observation is novel for miR-34b, and may represent a tissue specific effect.

MiR140 is evolutionarily conserved and a known marker of chondrocytes with high expression during endochondral ossification [28,29,36]. However it has also been shown to be important to promote osteoblast differentiation in bone [42,62]. Our results show miR-140 to target Runx2 and beta-catenin/Wnt signaling pathway, which is not surprising given its essential role in chondrogenesis [32]. It is also interesting to note that in a miR-140 null mouse skeletal defects were found to be a result of reduced BMP signaling in vivo [42]. Our results showing an increase in BMP2 expression and function with increased miR-140 supports this observation.

Overall, our studies suggest that several miRNAs with known roles in skeletal biology are affected by a deficiency of p53. Some miRNAs such as the miR-34 family are directly regulated by p53 while others are indirect. As miRNAs have been reported to be important to attenuate osteoblast maturation in vivo and prevent a high bone mass phenotype [9], it is easy to expect p53 to have a role in this process. Therefore, the biological significance of p53-regulated miRNAs extends beyond their direct targeting of specific gene products shown here and might indicate a time and stage-dependent regulation of osteoblast gene expression that allows for homeostatic control of differentiation. This type of activity by p53 supplements other established roles as a guardian of the genome and epigenome [63].

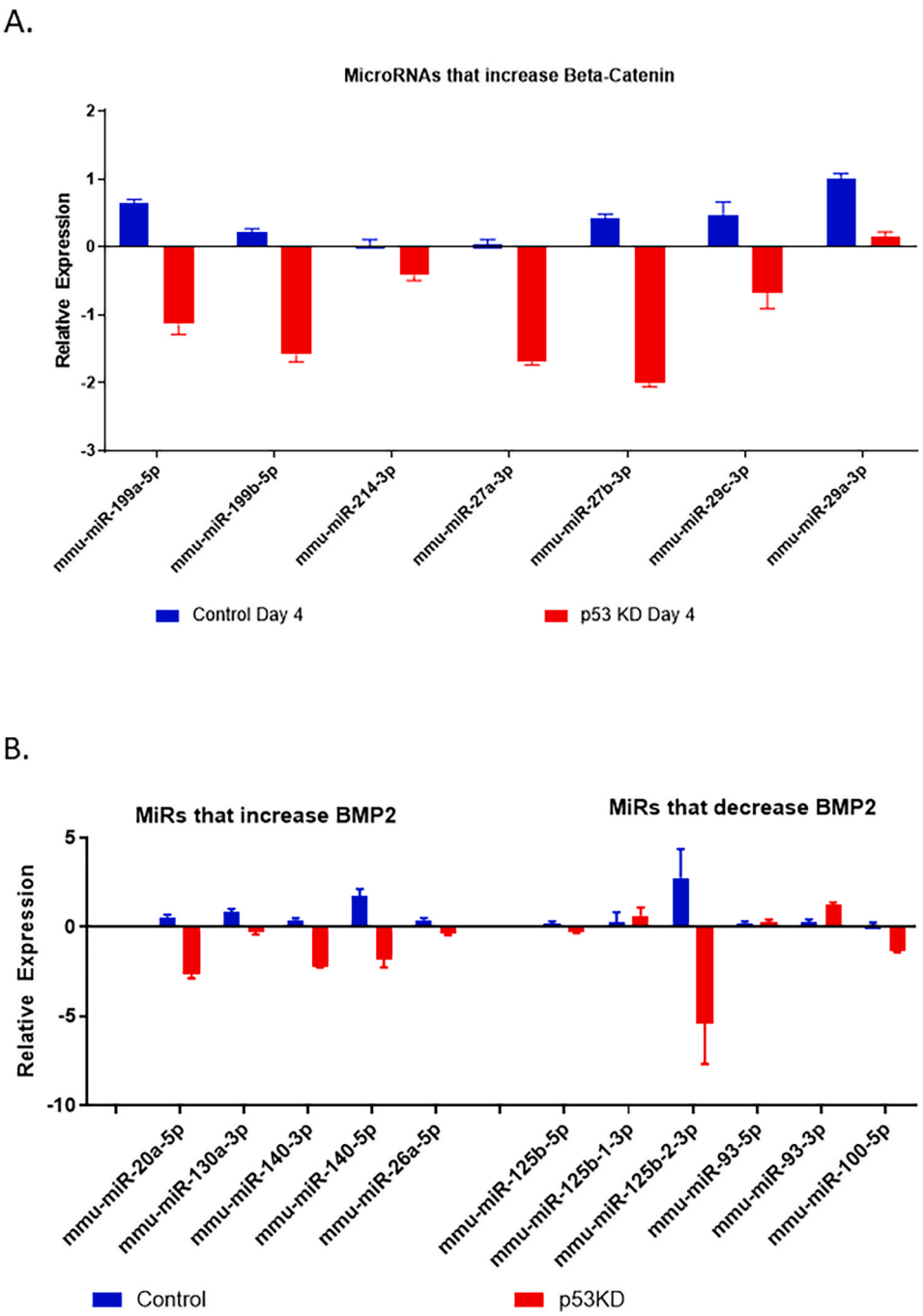

Fig. 8A and B. MicroRNAs that showed modulation during differentiation and were affected by p53 loss. We chose miRNAs that were reported for altering beta-catenin and BMP2 functions. These miRNAs were plotted based on their reported role in osteoblasts or mesenchymal cells as referenced in the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors gratefully acknowledge all gifts of reagents received from Drs. Vogelstein, Karsenty and Nanes. Some of the work described was done towards partial fulfillment of Master's thesis (SS and TK). Research funding from Midwestern University is also gratefully acknowledged.

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