miR-30b and miR-4454 *May* Reduce Melanoma Malignancy

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Abstract

MicroRNAs are small, non-coding RNAs that play a key role in regulating gene expression. Because MiR-30b and miR-4454 have been previously shown to exhibit tumor suppressor activity, we hypothesized that their upregulation will reduce the malignancy of SK-MEL-26 melanoma cells. SK-MEL-26 cells were seeded in a 6-well plate and incubated until they reached the desired confluency. The cells were transfected according to the Lipofectamine® 2000 DNA Transfection Reagent Protocol by Invitrogen. Two wells received SCR (control), two wells received miR-30b, and the last two were given miR-4454. Scratches were made in each well and images were taken of each well immediately after transfection, after 24 hours, and after 48 hours. The area of the scratches were found using ImageJ, and statistical analyses were performed with Excel. Within all experimental groups, the area significantly decreased from 0 to 24 hours, but the change was not significant from 24 to 48 hours, except for SCR ($p = 0.0058$). No significant results were observed between experimental groups. Both miR-30b and miR-4454 were able to slow down melanoma proliferation as there was no significant difference of the area between 24 and 48 hours, compared to the control. While the hypothesis is supported through the reduced growth rate in SK-MEL-26 cells transfected with MiR-30b and MiR-4454, the results may be inaccurate due to several limitations.

Introduction

Cancer is a disease that impacts many people worldwide. It is the uncontrollable growth of cells due to cell cycle dysfunction, and can develop from genetic mutations in proto-oncogenes or tumor suppressors. MicroRNAs can have different effects on different cells and their behavior. They are short, non-coding RNAs which support gene regulation by binding to mRNAs and preventing their translation (Lin and Gregory 2015). These miRNAs are transcribed as primary miRNAs or pri-miRNAs by RNA polymerase II in the nucleus. They are then cleaved by microprocessor to produce precursor miRNAs (pre- miRNAs). This microprocessor includes DROSHA and DiGeorge syndrome critical region 8 (DGCR8). Extropin 5 (XPO5) exports the pre miRNA into the cytoplasm and then goes to DICER1 to be processed further into a mature miRNA. A strand of the miRNA is loaded into the miRNA-induced silencing complex (miRISC) containing DICER1 and argonaute (AGO) proteins. The miRISC is then directed to target mRNAs to then go on to prevent their translation which in turn regulates genes (Lin and Gregory 2015). This is why they may also play a role in cancer progression by inhibiting translation of cancer-causing genes.

Upregulation of miR-30b in a breast cancer treatment known as Trastuzumab was shown to inhibit cell growth and proliferation by targeting CCNE2, a G1 cyclin, in the cancer cells (Ichickawa *et al*. 2012). Additionally, miR-30b has been found to reduce proliferation and increase apoptosis in endothelial cells (Li *et al.* 2015) and gastric cancer (Zhu *et al.* 2014). In gastric cancer, miR-30b targeted PAI-1, a protease inhibitor, which acted as a tumor suppressor gene and regulated the apoptosis of the gastric cancer cells (Zhu *et al.* 2014). Upregulation of miR-4454 in cartilage and lung cancer (Gu *et al.* 2021) also reduces proliferation and increases apoptosis. When upregulated in lung cancer, miR-4454 acted as a gene inhibitor which repressed the tumor phenotype by turning on 6 other genes as a result of an apoptotic signal (Gu *et al*. 2021). On the other hand, in hepatocellular carcinoma an increase in miR-4454 appeared to enhance cancer malignancy (Lin *et al.* 2021).

Melanoma is a type of skin cancer that develops in melanocytes generally due to a large accumulation of ultraviolet-signature mutations (Schadendorf *et al.* 2018). The role of miRNAs in melanoma is actively researched. A melanoma cell line known as SK-MEL-26 was transfected with SCR, miR-30b, and miR-4454 to determine whether the addition of the miRNAs impacted cancer malignancy. Based on other studies, the upregulation of both miR-30b and miR-4454 in SK-MEL-26 cells is hypothesized to decrease malignancy compared to non-transfected cells.

Methods

A 6cm dish with a monolayer of SK-MEL-26 cells in DMEM was provided. Under sterile conditions, the media was aspirated and the cells were washed twice with 3ml of PBS. The cells were trypsinized with 0.5ml trypsin and a 5 minute incubation at 37℃. The cells were then suspended in 5ml DMEM + Pen-Strep/10% FBS. 1ml of the cell suspension was transferred to a 50ml centrifuge tube and combined with 20ml DMEM + Pen-Strep/10% FBS. 3ml of the diluted suspension was dispensed into each well of a 6-well plate and left at 37℃ to reach 70-90% confluency.

The following week, the SK-MEL-26 cells were ready for transfection with SCR, miR-30b, and miR-4454. According to the Lipofectamine® 2000 DNA Transfection Reagent Protocol by Invitrogen, a 6-well plate requires 250µl of the DNA-lipid complex per well. As the transfections were performed in duplicate, the complexes were prepared in 3 microcentrifuge tubes with double the volume required. The following procedure was completed under sterile conditions. Following the suggested 1:2 ratio of microRNA to Lipofectamine, each tube received 2.2µg of the appropriate microRNA, 4.4µl Lipofectamine, and 550µl Opti-MEM (volumes adjusted for pipetting error with an additional 10% of reagent). The provided miR-30b was at a concentration of 392ng/ml, so 5.6µl was needed to reach

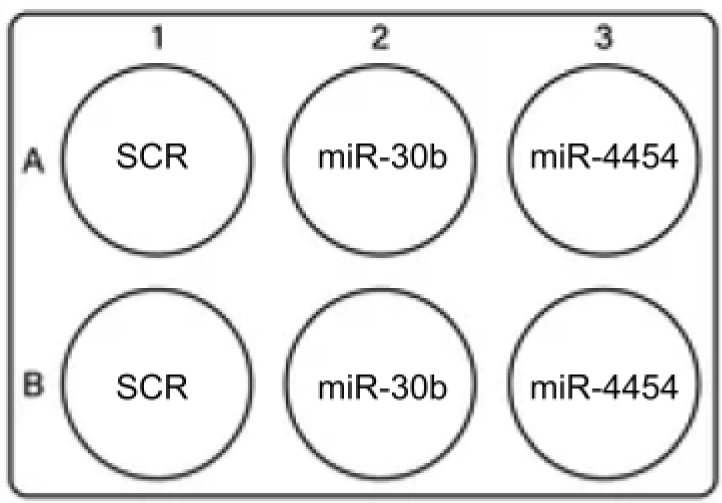

Figure 1. Transfection Setup. All wells contain SK-MEL-26 cells transfected with the indicated microRNA. SCR serves as a control.

the 2.2µg requirement. The miR-4454 was at 480.2ng/ml, so 4.6µl was needed. The appropriate volume of SCR was provided by Dr. B. 550µl Opti-MEM was added to each tube, followed by the appropriate microRNA, then Lipofectamine. Flicking was used to mix the solution after the addition of each reagent. The solutions were incubated for 20 minutes at room temperature with occasional flicking. 250µl was added to each of the 6 wells by slow dripping across the entire monolayer according to Figure 1.

Immediately following transfection, a P100 pipette tip was used to gently scratch a line in the center of each well through the SK-MEL-26 cells. A serological pipette was used as a guide to keep the scratches straight. A microscope was then used to take 3 images of each line (depicted in Figure 2), for a total of 18 images. Images were taken the same way after 24 hours and after 48 hours (54 images).

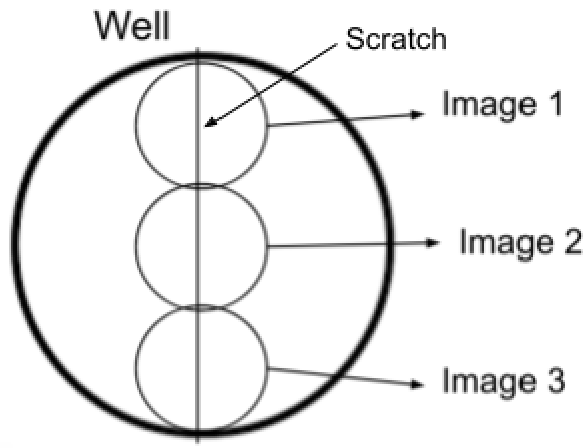

Figure 2. Three images were taken of each well at the top, middle, and bottom of the scratch at 0, 24, and 48 hours.

Images were analyzed with ImageJ software (See Figure 5 for more detailed image analysis). The areas of the scratch in each image were measured and were converted to percentages in Excel with the assumption that the areas obtained for zero hours were 100% (Figures 3 & 5). Excel was also used to calculate average area (Table 3), standard deviations, and perform two-tailed t-tests (Tables 1 and 2).

Results

By 24 hours, the scratches were almost completely closed in all 6 wells, and by 48 hours, the scratches were very difficult to identify due to a large accumulation of dead cells and cell debris. The area of the scratch in wells transfected with miR-30b and miR-4454 did not change significantly between 24 and 48 hours, however, SCR significantly decreased during that time period ($p = 0.0002$, Table 1, Figures 3 & 4). Relative to 0 hours, all areas obtained for 24 and 48 hours were significant (all $p < 0.00005$, Table 1, Figures 3 & 4). At 48 hours, the % area remaining in SCR wells was significantly less than that of both miR-30b and miR-4454 ($p = 0.015$, $p = 0.020$, respectively, Table 2, Figures 3 & 4). The average area for SCR, the control, decreased by over 90% after 48 hours, while the areas for miR-30b and miR-4454 respectively decreased by only 60.5% and 77.3% after the same amount of time (Table 3, Figures 3 & 4). It is also important to note that the average area of miR-30b and miR-4454 scratches *slightly* increased from 24 to 48 hours (Table 3). However, large standard deviations resulting from several outliers may have slightly skewed the data.

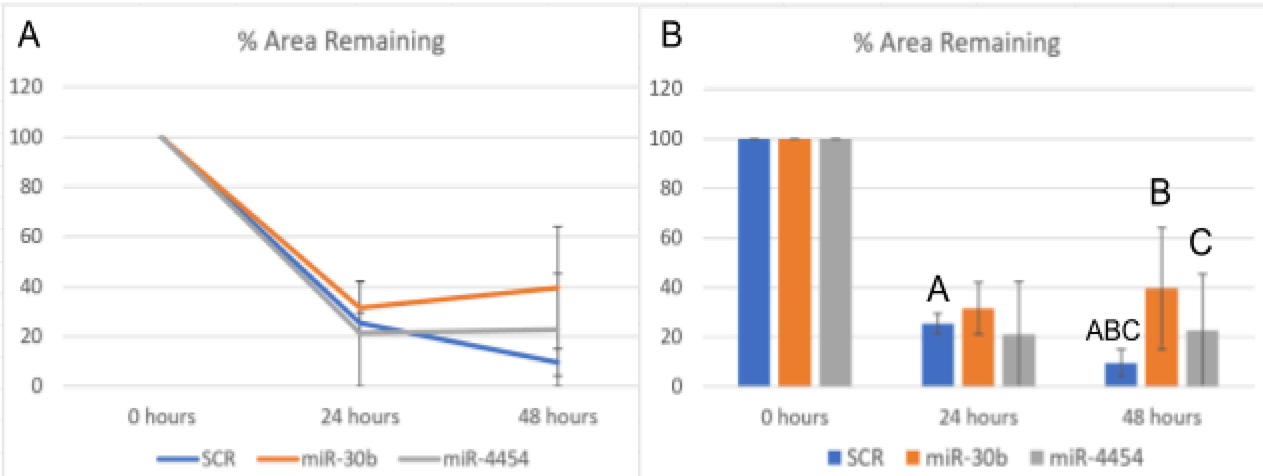

Figure 3. Mean % Area of Scratch Remaining 24 and 48 hours after transfection. Panels A and B both contain the same data. The different graphs are used for clarity. T-tests indicate a significant decrease in area for all experimental groups from 0 to 24 hours and 0 to 48 hours (p < 0.0000005). The decrease in area for SCR from 24 to 48 hours was significant (p = 0.002). The areas for miR-30b and miR-4454 were significantly different from SCR at 48 hours (p = 0.015, 0.020, respectively).

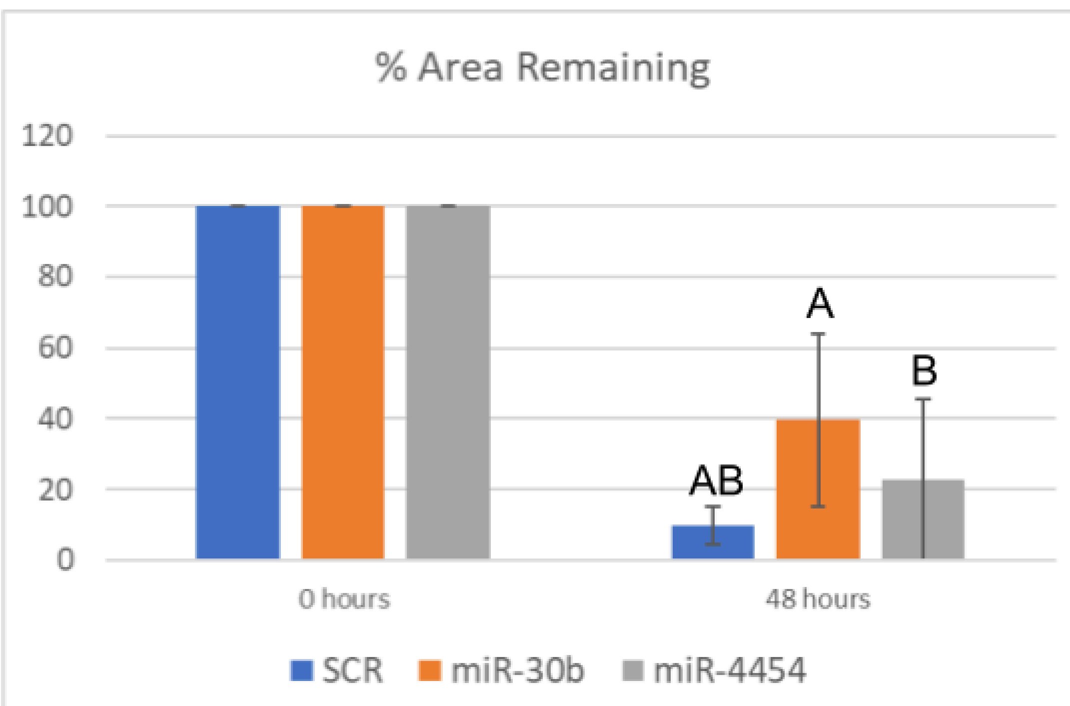

Figure 4. Average % area remaining after 48 hours. The difference is significant between 0 and 48 hours for all groups (p < 0.000005). At 48 hours, SCR was significantly less than both miR-30b and miR-4454 (p = 0.015 and 0.020, respectively).

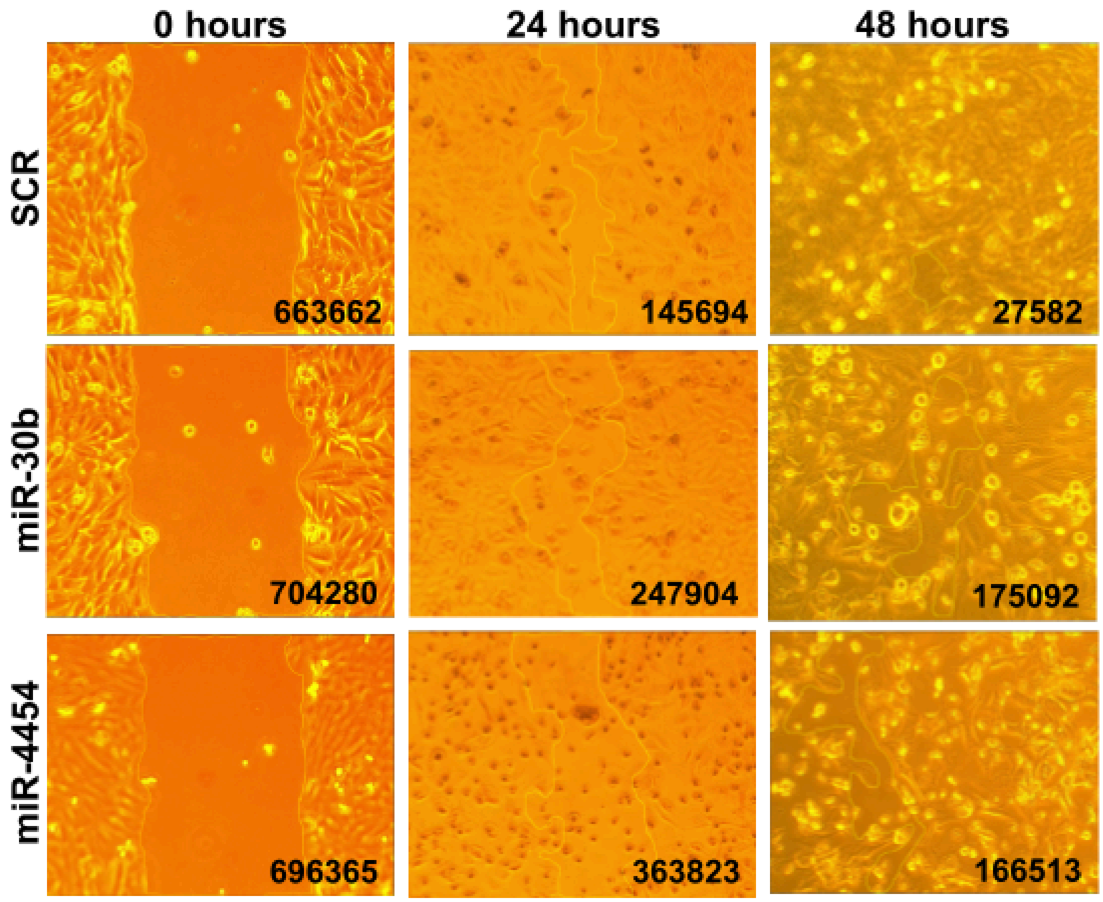

Figure 5. Images of each scratch for SCR, miR-30b, and miR-4454 at 0, 24, and 48 hours. Areas are shown in the bottom right corner.

Table 1. T-Test Results. P-values within the same experimental group.

	SCR	**miR-30b**	**miR-4454**
0 hours vs 24 hours	6.24×10^{-13}	1.78×10^{-8}	4.34×10^{-5}
24 hours vs 48 hours	*0.000176*	0.4841	0.7126
0 hours vs 48 hours	1.78×10^{-12}	0.000124	4.02×10^{-5}

Table 2. T-Test Results. P-values between experimental groups at 24 and 48 hours.		
	24 hours	**48 hours**
SCR vs 30b	0.20798	*0.01532*
SCR vs 4454	0.11574	*0.02021*
30b vs 4454	0.37129	0.79229

Table 3. Average % Area Remaining.			
	SCR	**miR-30b**	**miR-4454**
0 hours	100	100	100
24 hours	25.47	31.6	21.17
48 hours	9.58	39.51	22.67

Discussion

It was expected that after 0 hours the area would significantly decrease after the initial scratch as the cells began to invade the uninhibited area. This was seen in significant differences in all experimental groups from 0 to 24 hours and 0 to 48 hours. Additionally, it was hypothesized that transfection of SK-MEL-26 cells with miR-30b or miR-4454 would significantly reduce the aggressiveness of SCR by decreasing the rate of proliferation. This is shown by no significant difference in the scratch area from 24 hours to 48 hours in either of the microRNAs. Only SCR showed a significant difference from 24 hours to 48 hours, which served as a control by demonstrating the typical aggressive behavior of the melanoma cell line. Compared to the control, these results would indicate that the microRNAs were able to slow proliferation, and therefore reduce malignancy of the SK-MEL-26 cells. Additionally, while not significant, the *slight* increase in area from 24 to 48 hours for both microRNAs may indicate a combination of increased cell mortality and cell detachment compared to the control.

Previous studies have demonstrated comparable results. Ichikawa *et al.* 2012 found that upregulation of miR-30b led to decrease in cell proliferation of a cancer cell line. miR-4454 produced similar results in lung cancer according to Gu *et al.* 2021. The current results of this study would support these findings. The successful transfection of miR-30b or miR-4454 reduced cell proliferation in the melanoma cell line, SK-MEL-26. Inhibiting the growth of cancer cells is an important treatment in combating a hallmark trait of cancer, uncontrollable growth. While these results support the finding that miR-30b and miR-4454 can be useful in decreasing cell growth, there were several limitations to this experiment.

There were significant limitations that affected the accuracy of the results. The scratches on the plate were difficult to locate when the pictures were taken at each time mark. This meant when the area was measured it was likely inaccurate because the scratch was not located properly. There was also missing data for some of the images due detached cells covering the scratched area. The loss of data may have skewed the results. In future experiments, washing the SK-MEL-26 cells with a small volume of PBS to remove any dead or detached cells would help produce more accurate results by improving scratch visibility. Further research must be conducted in order to further validate this hypothesis by removing the aforementioned limitations.

Conclusion

Despite the limitations, the results show that upregulation of miR-30b and miR-4454 appear to reduce the aggressiveness of the SK-MEL-26 cells. Indicated by the significant growth of SCR transfected cells relative to the microRNA transfected cells, successful transfection of either microRNA led to slower invasion of the melanoma cells into the scratched area. It is important to note, however, that the accuracy of the results cannot be confirmed due to significant limitations.

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