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Mir-26a Overexpression Causes Increased Proliferation and Dysregulation of the Wnt Pathway in the Bipotential C2C12 Cell Line *In Vitro*

Josiah Eschbach[1], Elisha Pendleton[2], Phillip Rasnick[3], Nalini Chandar[1]

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Introduction

Micro RNA26a (miR-26a) is an miRNA that has been implicated in both the development of osteosarcoma and osteoblast differentiation but its specific roles in both are unclear and have shown seemingly conflicting results[4]. Our previous studies have shown that miR26a is induced by Menin (Men1) during osteoblast differentiation[5]. Menin is a tumor suppressor gene that is known to play important roles in cell fate, especially as it pertains to osteoblast differentiation[3]. It is also known that Men1 interacts with the protein Beta catenin which is necessary for osteoblast differentiation, however the mechanism is unclear[1]. This study was undertaken to better understand the mechanisms of how Men1 might mediate osteoblast differentiation through miR26a as well as to further elucidate miR26a's possible effects on development of osteosarcoma.

Materials and Methods

Cell Lines: C2C12 a bipotential mouse cell line was grown in D-MEM/F-12, with 10% Fetal Bovine serum in a modified atmosphere of 95% O2, 5% CO2 at 37°C. This line has the propensity to be induced to the osteoblast lineage when exposed to the bone anabolic agent BMP2. In addition we used two stably transfected C2C12 lines, previously created, that express two times and four times normal MiRNA-26a, termed 26a-1 and 26a- 2 respectively. (Figure 1)

Proliferation Assay: C2C12, 26a-1, and 26a-2 cells were transplanted to 10 centimeter plates in a manner that resulted in 3 plates for each line having 1.33*10^5 cells each, resulting in a total of 9 plates. The cells were then harvested from a single plate of each line at the times 24 hours, 48 hours, and 72 hours. At each time, cell counts for the plate were taken using an automated cell counter. This experiment was performed in triplicate.

Cell Cycle analysis: Each of the three cell lines were starved for 24 hours on serum free media then put back on DMEM/F12 for 24 hours or 48 Hours. Cells of each line and time parameter were then harvested, fixed, stained with propidium iodide, and processed using a flow cytometer.

qRT-PCR: Lab standard protocol was utilized for quantifying mRNA levels of Men1 and Beta-catenin for each of the three cell lines.

Western Blot: Standard western blot protocol was utilized for quantifying protein load of Men1, Beta-catenin, GSK-3B, and GAPDH (as a control)

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Results Overview

- The **C2C12** line is the control and has **normal** miR-26a levels. The **C2C12 miR-26a1** line has **double** normal expression of miR-26a and the **miR-26a2** line has **quadruple** normal expression of miR-26a.
- Proliferation assay demonstrates dose dependent **increase in proliferation rate** in relation to miR26a expression (Figure 2)
- Flow Cytometry Indicates **a higher proportion of 26a-1 and 26a-2 cells in the S and G2/M phases** of the cell cycle when compared to control C2C12 lines. (Figure 3)
- qRT-PCR analysis shows **increased** concentration of **Beta catenin** mRNA but **decreased** expression of **Menin (MEN1)** with increased expression of miR26a. (Figure 4)
- Western Blot analysis shows significantly **increased beta catenin** protein levels for the miR-26a2 line and significantly **decreased Men1** protein with both lines containing increased expression of miR-26a (Figure 5). Western Blot data also indicates a significant **decrease in GSK-3B** (Figure 6).

Figure 1: Demonstration of Stable Overexpression of miR26a in C2C12 cells

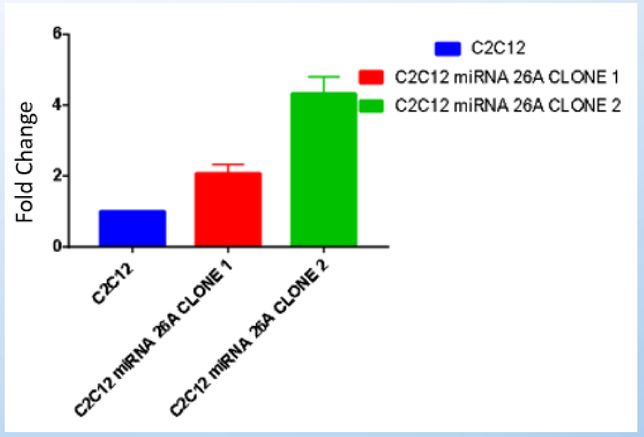


Figure 2: Proliferation Rate of C2C12 Cells Increases with miR26a Overexpression

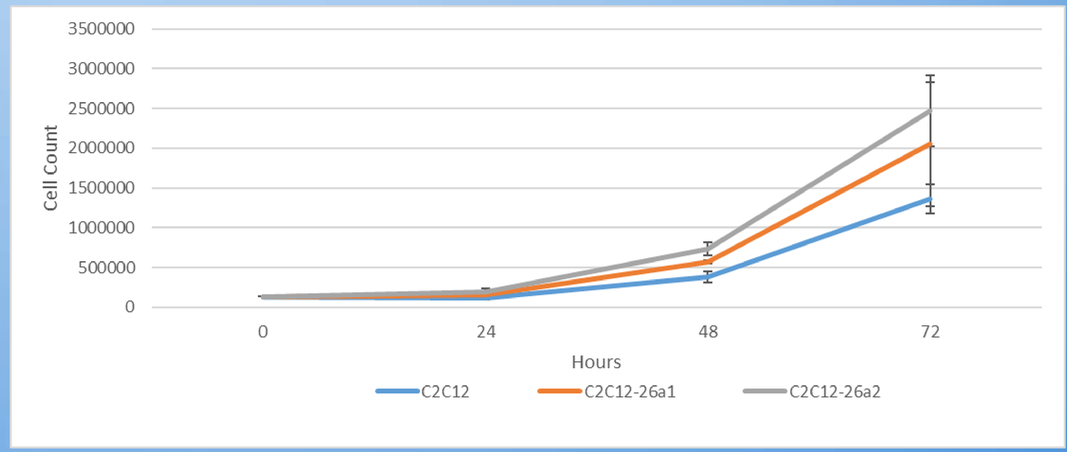


Figure 3: C2C12 Cells with Increased miR26a Expression are More Likely to be in the S or G2/M Phase of the cell cycle.

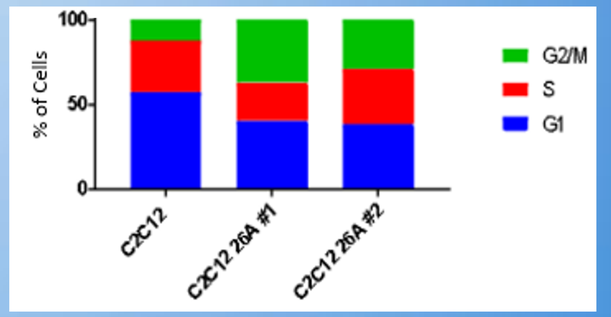
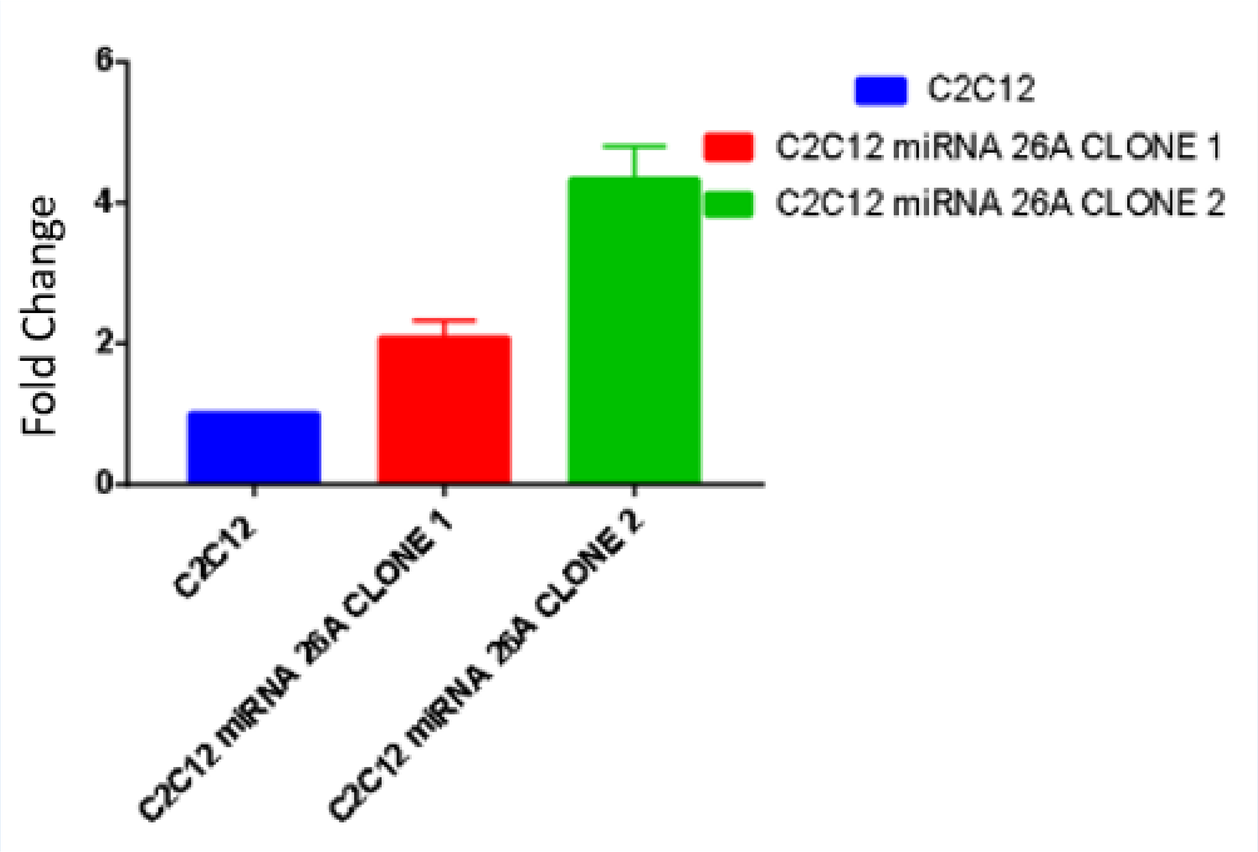


Figure 1: Stable **overexpression** of miR26a in C2C12 cells



An expression construct with miRNA-26a gene information was stably transfected into C2C12 pre-osteoblast cells and single cell clones of cells overexpressing the miRNA was isolated and characterized. The figure shows increased expression of miR26a in two such clones as measured by realtime PCR.

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Figure 3: C2C12 Cells with Increased miR26a Expression are More Likely to be in the S or G2/M Phase of the cell cycle.

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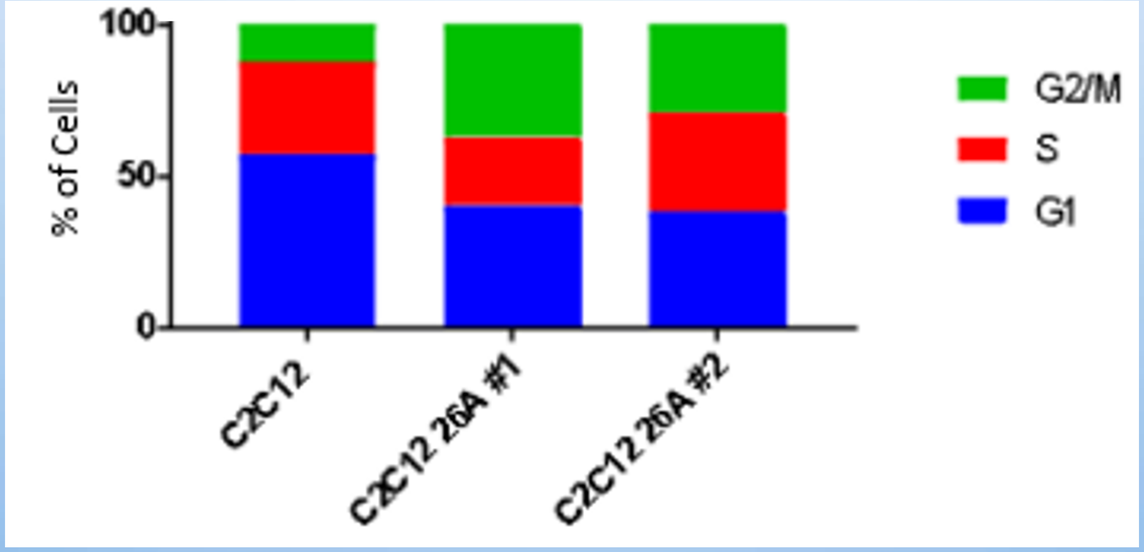

Cells were serum starved for 24hours and followed by an addition of media containing serum. Cells in on serum containing media for 24 hours before cell cycle analysis using a flow cytometer. Cells with miR26a overexpression were more likely to be in growth stages of the cell cycle as compared to the control C2C12 cells.

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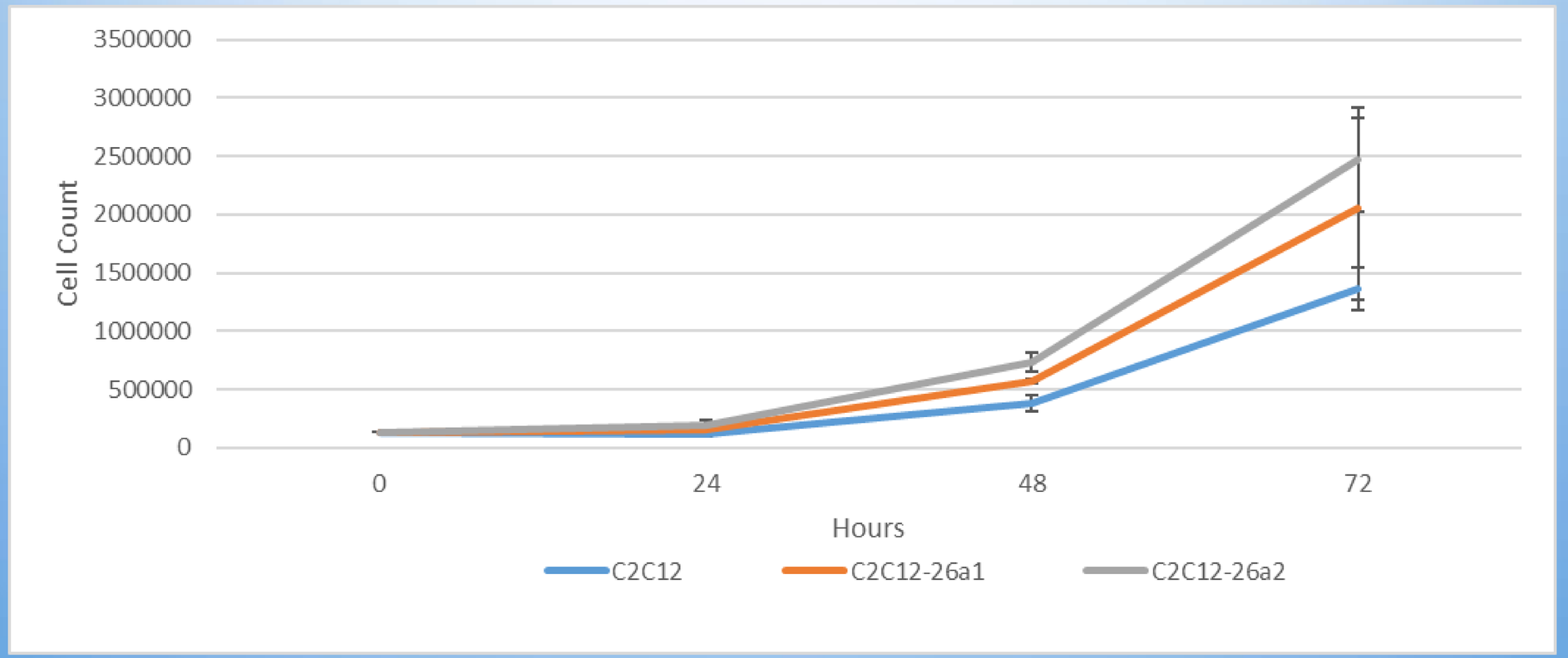
Figure 2: Proliferation Rate of C2C12 Cells Increases with miR26a Overexpression



Equal numbers of cells were serum starved for 24 hours followed by exposure to serum containing medium. Cell counts were obtained 24, 48 and 72 hrs following serum stimulation. The control C2C12 line proliferated the least, whereas the C2C12-26a2 line with the most miR-26a expression proliferated most.

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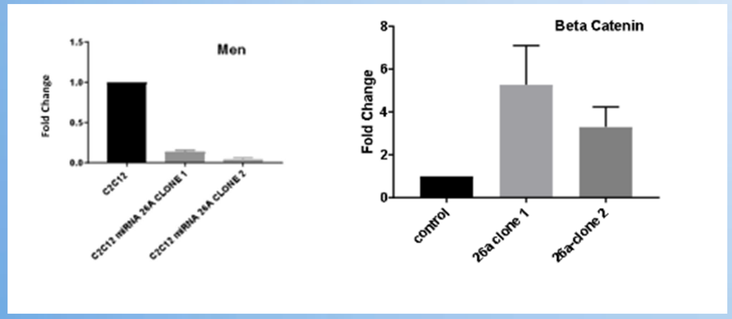
Figure 4: Men1 mRNA Levels Decrease with miR26a Overexpression. Beta Catenin mRNA Levels are Increased miR26a Overexpression.



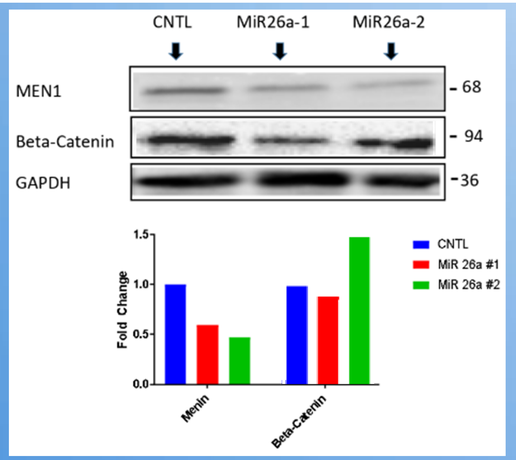
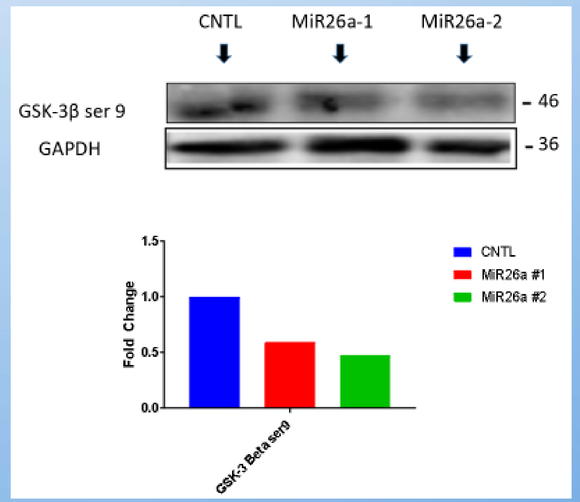


Figure 5: Men1 Protein Levels from Western Blot are Dramatically Reduced in miR26a Overexpression. Beta Catenin Protein Levels are not Significantly Changed in the miR-26a1 Line but is Significantly Increased in the miR-26a2 Line.

Figure 6: GSK-3B Levels are Significantly Decreased With miR-26a Overexpression and GSK-3B is Identified as a Possible Target of miR-26a



GSK-3B is a Potential Target of miR-26a

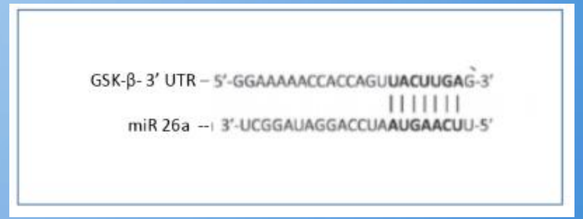


Figure 4: Men1 mRNA Levels Decrease with miR26a Overexpression. Beta Catenin mRNA Levels Increased miR26a Overexpression.

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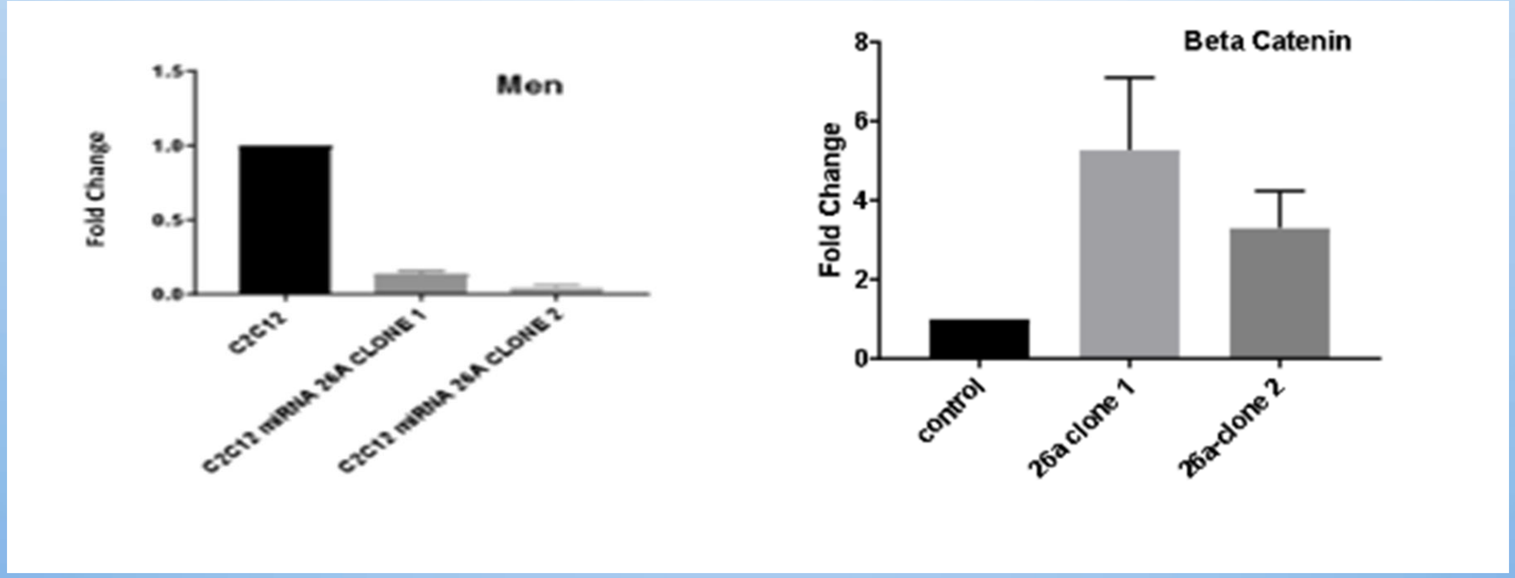
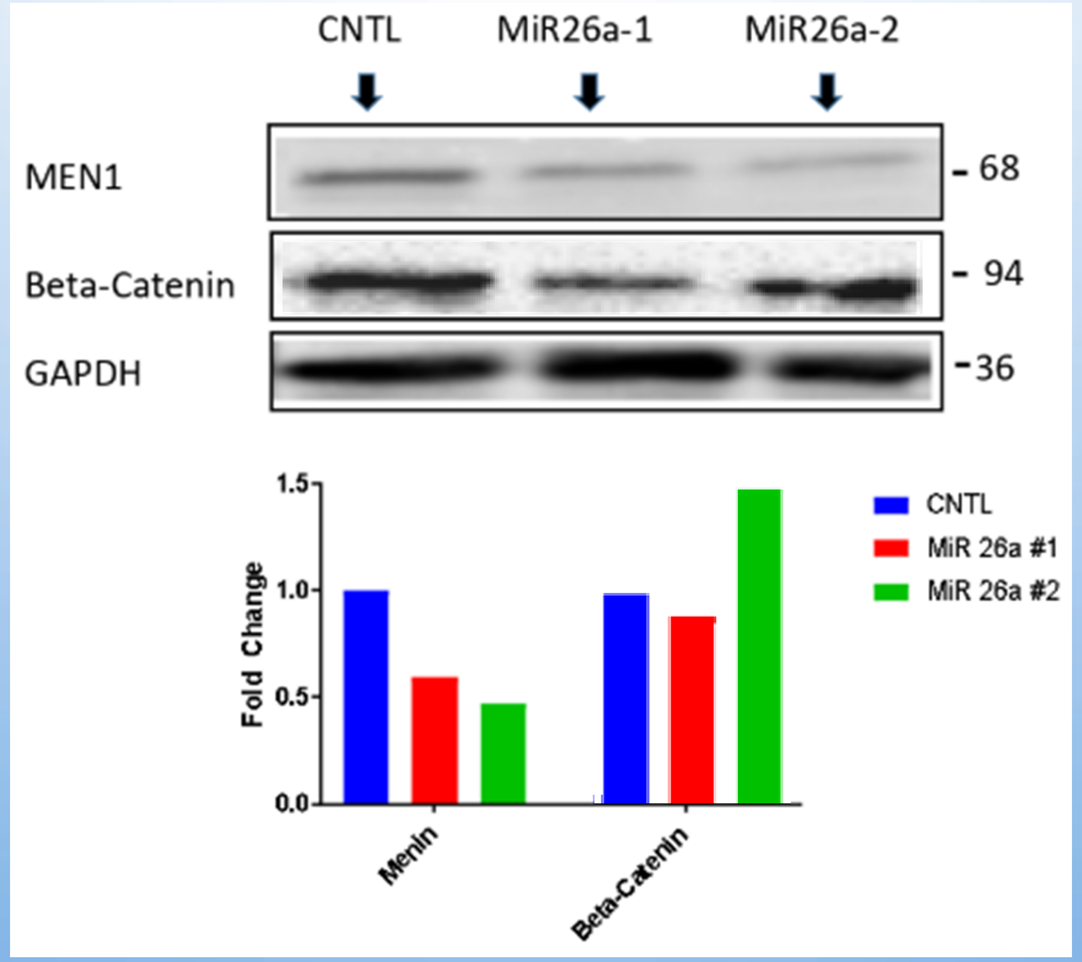


RNA expression was measured in cells using realtime PCR. A significant decrease in Menin is seen in the miR-26a overexpression lines. Beta Catenin is shown to be significantly increased in the overexpression lines when compared to the control C2C12 line
Beta catenin is known to be upregulated during osteoblast differentiation and menin has been implicated in this process. Here we show that it is miR26a that might mediate the increase in beta catenin necessary for osteoblast differentiation.

Figure 5: Men1 Protein Levels from Western Blot are Dramatically Reduced in miR26a Overexpression. Beta Catenin protein Levels are not Significantly Changed in the miR-26a1 Line but is Signficantly Increased in the miR-26a2 Line.



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Western blot analysis of these cells confirm a decrease in menin expression at the protein level. Beta catenin expression was protein levels were not significantly changed in the MiR-26a1 line but were significantly increased in the miR-26a2 line which has the highest miR-26a expression.

Figure 6: GSK-3B Levels are Significantly Decreased With miR-26a Overexpression and miR-26a is Identified as a Potential Direct Inhibitor

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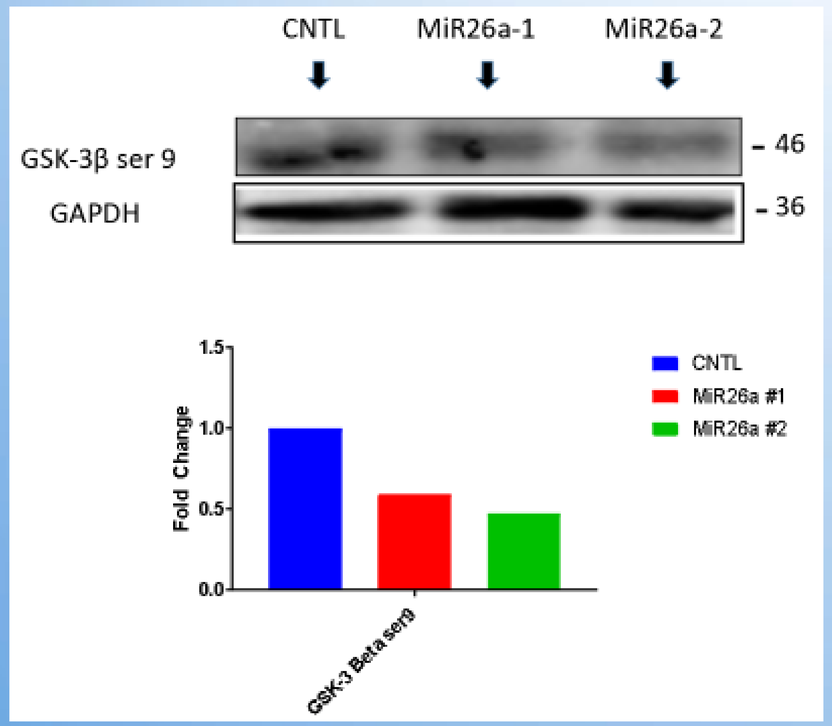
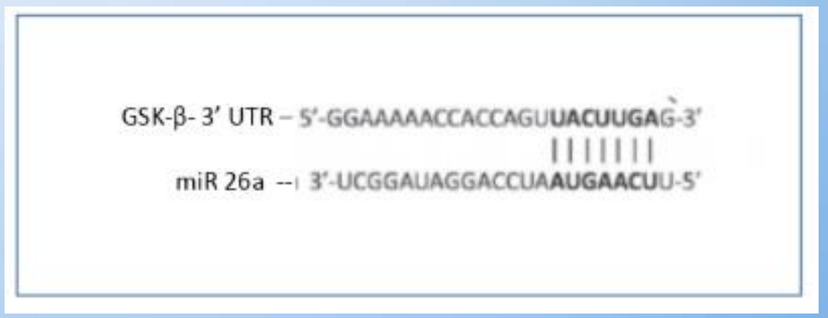


GSK-3B is a Potential Target of miR-26a



When the Wnt pathway is off, beta catenin is degraded by an activated GSK-3B complex. Here we show a decrease in GSK-3B expression due to an increase in miR 26a. In order to determine if this microRNA could represent a target for GSK-B, we searched the microRNA database and found a potential 3'UTR sequence that matches miR26a which suggests miRNA26a may be responsible for the decreased concentration of GSK-3B.

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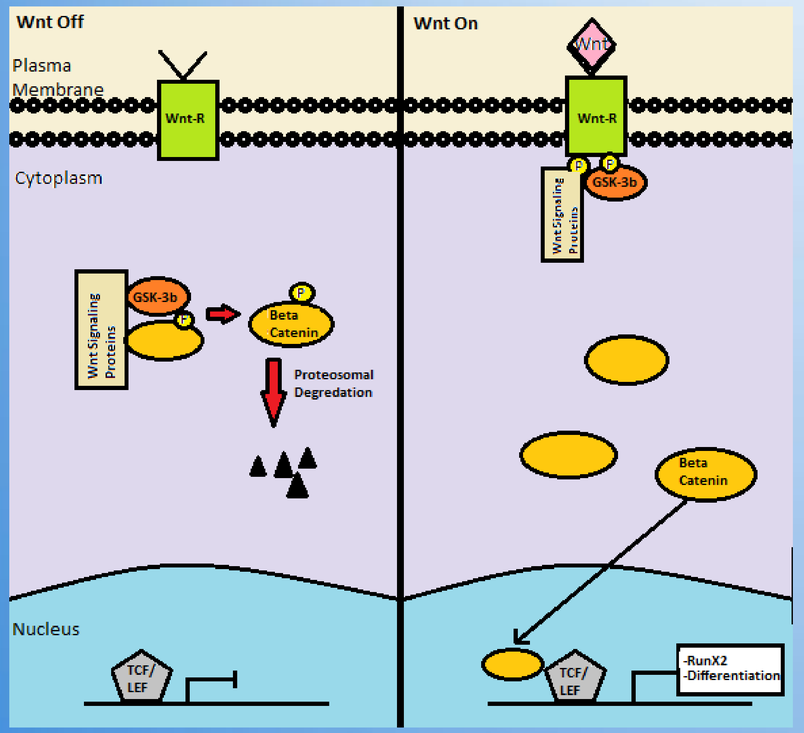

Figure 7: Wnt Signaling Pathway in Osteoblast Differentiation

Conclusions

- miR26a overexpression leads to higher proliferation rates and more cells actively dividing in vitro. This indicates that miR26a could act as an oncogene if constitutively overexpressed in osteosarcoma. A possible cause for this is the observed significant decrease in Men1 which is a tumor suppressor gene.
- miR26a overexpression increases beta catenin mRNA and protein levels while decreasing protein levels of GSK-3B. As levels of miR26a do increase at the beginning of osteoblast differentiation (via Menin), increased miR26a could explain increased activity of beta catenin necessary for osteoblast differentiation. Down regulation of GSK-3B, an inhibitor of Beta-catenin, via miR26a is a probable mechanism for these results.
- miR26a likely plays an integral role in the progression of osteoblast differentiation via increasing the availability of Beta-catenin which goes on to increase production of RunX2.
- Research should be conducted to determine if miR26a directly inhibits synthesis of GSK-3B.

Citations

1. Huang W, Yang S, Shao J, Li YP. Signaling and transcriptional regulation in osteoblast commitment and differentiation. *Front Biosci*. 2007;12:3068–3092.
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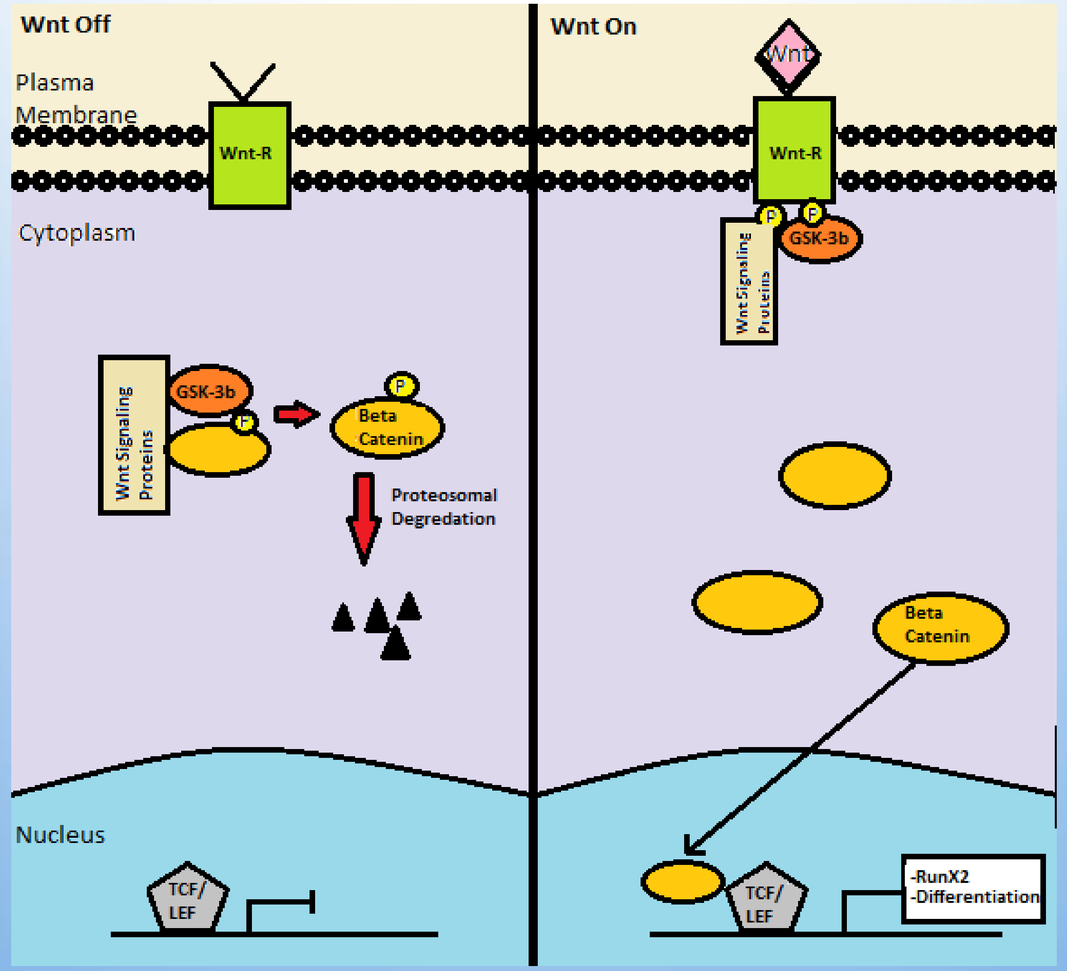
Figure 7: Wnt Signaling Pathway in Osteoblast Differentiation



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The Wnt pathway is integral to the progression of osteoblast differentiation. When Wnt is absent, the GSK-3B complex phosphorylates Beta-catenin, resulting in the destruction of Beta-catenin. However, when the Wnt receptor is activated, GSK-3B is inactivated and Beta catenin goes to the nucleus to increase RunX2 gene transcription which leads to osteoblast differentiation.