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The Effects of MicroRNA-21 on BMP Induced Dendritic Growth

Abstract

Dendrites are branched extensions that neurons put out in order to communicate to other neurons. Understanding dendritic growth would not only provide insight into a dynamic process that affects learning and memory, but it could also help us to understand diseases such as Alzheimer’s and Parkinson’s disease. Dendritic growth can be induced by a growth hormone from the Bone Morphogenetic Protein (BMP) family. Specifically, BMP-7 has been shown to induce dendritic growth in neurons, including sympathetic neurons. In this study, we examine the interaction between BMP signaling pathway and microRNAs for controlling dendritic growth. MicroRNAs are small, 20 base pair strands of RNA that regulate protein synthesis by attaching to mRNA and inactivating it or targeting it for destruction. Previous studies have shown that MIR-21 levels were decreased in sympathetic neurons upon exposure to BMP-7, however, the functional significance of this effect was not known. The results of this study show that overexpression of microRNA-21 (MIR-21) inhibits BMP-induced dendritic growth, whereas knockout of MIR-21 can stimulate dendritic growth to a small extent in control neurons. In addition, the data show that MIR-21’s effect on dendritic growth were not mediated by its effects on BMP receptor II (BMP-RII).
1. Introduction

Dendrites are one of two types of processes produced by neurons that are sites for synaptic formation between neurons. Dendritic growth is a dynamic process, where dendrites are grown and pruned on a daily basis, and very little is known about the exact mechanisms of this growth. Understanding this process is crucial for study of the communication between neurons. In Parkinson’s and Alzheimer’s disease, retraction of dendrites has been observed (Gue et. al., 1999). Dendritic retraction has also been observed in schizophrenia, Rett Syndrome, down syndrome, and autism spectrum disorder. Even chronic stress and anxiety cause retraction of dendrites (Kulkarni et. al., 2012). This common theme of dendritic retraction suggests that dendritic growth is a major component of neurodegenerative disease. Dendritic growth is common to both the peripheral and central nervous system.

Sympathetic neurons were chosen for this study. *In vivo*, these neurons show a multipolar arbor, with numerous, branched dendrites. However, in culture, in the absence of glial cells or growth factors, these cells will show little to no dendritic growth. This makes them ideal for studying the initiation of dendritic growth. One family of growth factors that induce dendritic growth are called (BMP) bone morphogenetic proteins (Lein et al., 1995). BMP’s are among the more prominent growth factors, in mammals, that induce neurons to grow dendrites. As a result, it is very important that we understand the molecular mechanisms of BMP’s, so that we may gain insight into dendritic growth within the human body.

BMP’s work through a SMAD secondary messenger system. SMAD transduces the BMP signal from the cytoplasm to the nucleus where it activates targets genes for
transcription (Nishimura et. al., 2003). BMP’s are not just active in the nervous system. They are found in many other parts of the mammalian body. For instance, BMP’s are involved in the regulation of follicle stimulating hormone sensitivity, thus possibly affecting infertility (Visser et. al., 2013). SMAD’s work through a variety of molecular mechanisms, including post-transcriptional regulation using microRNA’s (Davis et. al., 2010).

MicroRNA’s are small strands of RNA that are approximately 20 base pairs in length. These small inhibiting RNA’s post-transcriptionally regulate protein synthesis in cells. They work by attaching to complementary sequences located in messenger-RNA(mRNA), thereby inactivating said mRNA or targeting it for destruction. One microRNA can have multiple mRNA targets (Strickland et. al., 2011). This suggests a possible link between SMAD’s, BMP’s and dendritic growth. Preliminarily, it has been shown that, during BMP-induced dendritic growth, the levels of 20 different microRNA’s are increased or decreased (Thapa, 2012). One of the microRNA’s that were regulated by BMP was microRNA-21 (MIR-21). MIR-21 levels were decreased significantly during BMP induced dendritic growth, suggesting that it is involved in the suppression of dendritic growth. This summer, we investigated the role that MIR-21 plays in dendritic growth. Specifically, we wanted to find out if the up-regulation of MIR-21 results in decreased dendritic growth in BMP-7 treated cells and if the down-regulation of MIR-21 results in increased dendritic growth.
2. Materials and Methods

2.1 Materials

The embryonic day 21 rat pups for the Superior Cervical Ganglion dissections were obtained from the UC Davis Department of Animal Science through Dr. Pamela Lein. DMEM, Optimem and F12 base media were purchased from Invitrogen (San Diego, CA) and Gibco. BMP-7 was provided by Dr. Pamela Lein. Lipofectamine RNAi Max transfection reagent, anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) siRNA, GAPDH antibodies, MicroRNA-21 mimic, inhibitor, negative controls, neurofilament antibodies, goat anti-mouse fluorescent antibodies, goat anti-rabbit fluorescent antibodies, and Pro-long Gold were also purchased from Invitrogen. BMP-Receptor 2 antibodies were provided by Dr. Lein.

2.2 Primary Culture of Sympathetic Neurons

Sympathetic neurons were isolated from the superior cervical ganglion of embryonic day 21 rat pups according to previously described methods (Higgins et al., 1991). These cells were plated in 24 well plates, previously coated with poly-D-lysine (100µg/mL). Cells were kept in medium made of 0.5 mg/ml bovine serum albumin, 1.4 mM L-glutamine, 10 µg/ml insulin, 5.5 µg/ml transferin, 38.7 nM selenium and 0.1 µg/ml β-nerve growth factor (NGF). After one day in media, cells were treated with cytosine- β-D-arabinofuranoside (Ara-C, 1-2 µM) for 48 hours, thus making the cultures free of glial cells. Subsequently, the cells were treated under appropriate conditions.

2.3 Optimization of Transfection of Sympathetic Neurons with siRNA
Following the Ara-C, cells were transfected with 30 pmol and 75 pmol of fluorescently tagged siRNA. Cells were transfected according to the manufacturers protocol, and they were viewed under a fluorescent microscope. To further confirm the effectiveness of our transfections, transfected cells were fixed and immunostained for GAPDH enzyme, and examined for decreased levels of GAPDH compared to controls. The final concentrations used were 75 pmol of siRNA and 8% lipofectamine in Optimem mixed in a 1:1 ratio. 100µL of treatment was administered to each well. Cells were rinsed with culture media after 24 hours to minimize toxicity.

2.4 Transfection of Neurons with MIR-21 Inhibitor, Mimic, and Negative Controls

Using the optimized transfection procedure, cells were administered MIR-21 mimic, inhibitor, negative control for inhibitor, negative control for mimic, or cell media. The mimics and inhibitors were diluted to 50µM with RNAse-free water. After 24 hours, each condition was treated with either BMP-7, or cell media. Mimic and negative control mimic-treated cells were given 50 ng/mL of BMP, while inhibitor and negative control inhibitor-treated cells were given 5ng/mL of BMP. After an additional 48 hours, the cells were treated with the same amount of BMP or cell media. After 5 days, the cells were treated under the appropriate conditions.
2.5 Immunostaining

Treated cells were fixed with 4% paraformaldehyde for 20 minutes. After rinsing with PBS, cells were permeabilized with 0.1% Triton in PBS. Cells were subsequently blocked with 5% Bovine Serum Albumin (BSA) in PBS for 30 minutes. Cells were treated with primary antibodies diluted in BSA and left overnight at 4 °C. The primary antibodies used were mouse anti-GAPDH (1:800) and mouse anti-neurofilament, 8M132 (1:5000). The next day, cells were visualized using secondary antibodies diluted 1:1000 in BSA. After one hour, the coverslips were mounted onto microscope slides with Pro-long Gold, viewed with a Nikon microscope, and quantified using Image J and SPOT software. The staining for BMP-Receptor was slightly different. The cells were fixed with acetone and methanol, and the cells were not permeabilized. Cells were stained with mouse neurofilament primary antibodies at a 1:5000 dilution in order to visualize dendrites, cells were treated with BMP-Receptor 2 antibodies diluted 1:300 to visualize BMP-R levels, and cells were treated with GAPDH antibodies diluted 1:800 in order to visualize GAPDH.

2.6 Western Blot

Sympathetic neurons were treated with control media and BMP (50ng/mL) for 5 days. Cells were treated for 5 days, and then lysed in cell lysis buffer to obtain protein. Protein concentrations were estimated using a Bradford Assay and found to be 3.5µg/well. A 4-12 % Bis Tris gel was ran, with MES running buffer, for one hour, at 200V, and followed by nitrocellulose membrane that was blotted overnight at 4 °C. The membrane was blocked in 5% milk in PBS for one hour with BMP-RII primary antibodies (1:300). The membrane was treated with HRP-conjugated secondary antibodies and visualized
by chemiluminescence using Pierce ECL plus kit. A ChemiDoc Imager (Bio Rad) was used to visualize protein levels.

2.6 Statistical Analysis

All numerical data was tested for statistical significance using an ANOVA and Tukey’s test. The program that was used was SigmaPlot.

3. Results

3.1 Transfection Optimization

Though normally dividing cells have been successfully transfected with small RNAs to examine their effects (Khan et. al., 2009), a protocol has not been optimized for transfection of sympathetic neurons with small RNAs. Therefore, a protocol was developed and tested for this project. To test transfection efficiency, cells were treated with fluorescently-tagged GAPDH siRNA, at 75pmol and 30pmol, and compared against cells in control media. Control cells show no fluorescence (Figure 1b), however cells treated with 75pmol of siRNA show significant green fluorescence (Figure 1e), indicating that the cells were successfully transfected. To confirm that the transfection had produced an effect in the cells, cells were stained for GAPDH (Figure 1c,f). Not surprisingly, control cells showed no siRNA fluorescence and no changes in GAPDH levels (Figure 2). Cells treated with 30 pmol of siRNA showed lower percentages of neurons with siRNA insertion and lower percentages of decreased levels of GAPDH (43% and 75% respectively) than the cells that were treated with 75pmol of siRNA (89% and 99% respectively). Knowing that siRNA are similar in both size and function to microRNA, we decided to use the same amount of Lipofectamine RNAi max (4µg) with 75pmol of microRNA mimics and inhibitors.
3.2 MicroRNA-21 Mimic Causes Inhibition of Dendritic Growth in Sympathetic Neurons

To find out if MIR-21 inhibits dendritic growth, cells were transfected with MIR-21 mimic, administered 50ng/mL BMP-7 after 24 hours and their dendritic growth was compared to controls. After taking fluorescent images and counting the dendrites on neurons in all conditions, the average dendrite counts, with standard error, from cells treated with control media, mimic, negative control mimic, 50ng/mL of BMP-7, were obtained. Control media, mimic, and negative control mimic show no statistically significant difference in dendritic number, as their average dendrite counts are very close to zero (Figure 6, 3a). As predicted, BMP-7 treated cells had the largest average dendrite count of all the conditions (2.734 +/- 0.118) (Figure 6, 3b). Supporting our fluorescent data (Figure 3c), the mimic and BMP 50ng/mL treated cells showed some dendritic growth, but the average (1.867 +/- 0.0855) was lower than the BMP-7 treated cells. Negative Control + BMP had a lower average (2.287 +/- 0.107) than BMP treated cells, but was not statistically significant (Figure 6). After seeing the effects of MIR-21 mimic on dendrite numbers, we wanted to find out if MIR-21 has an effect on dendrite morphology. MIR-21 did not have any significant effect on dendrite length. There was a small decrease in average length of dendrites in neurons treated with MIR-21 mimic compared to negative control and BMP-7 treated cells, but it was very minor and not statistically significant.

3.3 MicroRNA-21 Inhibitor Induces a Small Increase in Dendritic Growth
Since over expression of MIR-21 inhibits dendritic growth, MIR-21 inhibitor was hypothesized to enhance dendritic growth. Cells were treated with control media, inhibitor, 5ng/mL of BMP-7 or inhibitor and 5ng/mL of BMP-7. Control media and negative control inhibitor had average dendrite counts very close to zero (Figure 7, 4a). BMP-7 treated cells had the largest average dendrite count of all the conditions (1.678 +/- 0.079) (Figure 7, 4b). The inhibitor treated cells had a higher amount of dendritic growth (0.723 +/- 0.058) than controls (Figure 7, 4c). The inhibitor and BMP 5ng/mL treated cells showed slightly less dendritic growth (1.435 +/- 0.079) than the BMP-7 treated cells, but the difference was not statistically significant (Figure 7, 4d). Negative Control + BMP had a lower average (1.459 +/- 0.108) than BMP treated cells, but was not statistically significant (Figure 7). Next, dendrite morphology was quantified. MIR-21 inhibitor did not have any significant effect on dendrite length. There was a small increase in average length of dendrites in neurons treated with MIR-21 inhibitor compared to negative control and BMP-7 treated cells, but it was very minor and not statistically significant.

3.4 BMP-Receptor II (BMP-RII) Levels Remain Unchanged in Cells Treated With 50ng/mL BMP Compared to Control Media

BMP receptor II is a transmembrane receptor that BMP-7 uses to induce the intercellular processes that lead to dendritic growth. In order to look at the effects of BMP-7 on BMP-RII, treated cells were fixed and stained with anti-BMP-RII antibodies and viewed using fluorescent microscopy. The levels of BMP-RII were determined, qualitatively, by judging the fluorescent intensities. Cells stained and treated with BMP-7 (Figure 10) do not show an obvious difference in BMP-RII levels compared to cells in control media (Figure 11).
In addition to immunofluorescent tagging, a Western blot was performed on lysed cells (Figure 16). There were bands corresponding to BMP-RII, around 60kD, observed in both control and BMP-7 treated cells. However, the levels of the BMP-RII were higher in controls compared to BMP-7 treated cells indicating that BMP-RII was not a target of MIR-21.
Figure 1. 40x pictures of cells treated with control media and 75pmol GAPDH siRNA: From left to right, the figure above shows a phase contrast image, fluorescent image showing levels of anti-GAPDH siRNA levels, and fluorescent image showing levels of GAPDH enzyme. The GAPDH were tagged using protein specific primary antibodies, and fluorescent-tagged secondary antibodies.
<table>
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<th>Conditions</th>
<th>% Neurons with siRNA inserted</th>
<th>% Neurons with decreased levels of GAPDH</th>
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<td>Control</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>30 pmol siRNA</td>
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<td>75</td>
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<td>75 pmol siRNA</td>
<td>89</td>
<td>99</td>
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**Figure 2.** Table showing numerical values of anti-GAPDH siRNA and GAPDH levels in sympathetic neurons: The figure above shows the levels of siRNA and GAPDH enzyme in treated and control neurons.
Figure 3. 40x Dendrite-stained fluorescent picture of neurons treated with control media (a), 50ng/mL BMP-7 (b), and MIR-21 mimic + 50ng/mL BMP-7 (c)
Figure 4. 40x Dendrite-stained fluorescent picture of neurons treated with control media (a), 5ng/mL BMP-7 (b), MIR-21 inhibitor (c), and MIR-21 Inhibitor + 5ng/mL BMP-7 (d)
Figure 5. 10x BMP-R-stained fluorescent picture of neurons treated with control media (a) and 50ng/mL BMP (b).
Figure 6. MIR-21 Mimic Treated Cells: Average Dendrites per Cell:
The average number of dendrites, with standard error bars, from cells treated with control media, mimic, negative control mimic, 50ng/mL of BMP-7, and mimic treatments + 50ng/mL BMP-7. Control media, mimic, and negative control mimic showed no statistically significant difference in dendritic number. Mimic + BMP 50ng/mL showed a statically significant decrease in average dendrite number. Negative Control + BMP had a slightly smaller average than BMP 50ng/mL, and was not shown to be statistically significant.
Figure 7. MIR-21 Inhibitor Treated Cells: Average Dendrites per Cell:
The average number of dendrites, with standard error bars, from cells treated with control media, inhibitor, negative control inhibitor, 5ng/mL of BMP-7, and inhibitor treatments + 5ng/mL BMP-7. BMP 5ng/mL, inhibitor + BMP, and negative control inhibitor + BMP showed no statistically significant difference in dendritic number. Inhibitor showed a statically significant increase in average dendrite number compared to control media and negative control.
Figure 8. MIR-21 Mimic Treated Cells: Average Dendrite Length per Neuron:
The average length of dendrites, with standard error bars, from cells treated with 50ng of BMP-7, and mimic treatments + 50ng/mL BMP-7. Cells showed slight decrease in dendritic length in mimic + 50ng/mL BMP compared to negative control + 50ng BMP, and in negative control compared to cells treated with 50ng/mL BMP. These differences were not statistically significant.
Figure 9. MiR-21 Inhibitor Treated Cells: Average Dendrite Length per Neuron: The average length of dendrites, with standard error bars, from cells treated with 50ng/mL of BMP-7, inhibitor treatments + 50ng/mL BMP-7, 5ng/mL of BMP-7, and inhibitor treatments + 5ng/mL BMP-7. Cells showed an increase in dendritic length in inhibitor + 5ng/mL BMP compared to negative control + 5ng/mL BMP and 5ng/mL BMP. Inhibitor treatments + BMP 50ng/mL and cells treated with 50ng/mL BMP showed very little difference in dendritic length. None of these differences were statistically significant.
Figure 16. Western Blot of cells treated with 50ng/mL of BMP-7 and Control Media and stained for BMP-RII: The right line corresponds to the cells treated with 50ng/mL BMP, the middle line corresponds to the cells in control media, and the left lines are the protein ladder.
4. Discussion:

Our research indicates that MIR-21 is involved in dendritic growth. Furthermore, MIR-21 is involved in the repression of dendritic growth, as shown by the reduction in dendritic growth in BMP plus mimic-treated cells. This is the first time that MIR-21 has been directly connected with dendritic growth in sympathetic neurons. Our data agrees with previous data that showed down-regulation of MIR-21 during BMP-induced dendritic growth (Thapa, 2012). Although other studies have found that MIR-21 enhances axonal growth in dorsal root ganglion neurons (Strickland et. al., 2011), our treatments did not have a noticeable effect on axonal growth. This suggests that the effects of MIR-21 on neurons are process specific. MIR-21’s effects on both dendrites and axons could have implications on future treatments using MIR-21. Due to MIR-21’s inhibition of dendrites and enhancement of axons, any treatments would need to consider the effects on both dendrites and axons.

The effects of MIR-21 on dendritic growth were modest. However, MIR-21 is not the only factor in this pathway. The BMP-7/SMAD pathway causes many transcriptional changes to induce dendritic growth (Garred, et. al., 2011). It is therefore likely that MIR-21 is inhibiting only a subset of the BMP targets. It would, however, be interesting to examine higher concentrations of MIR-21 mimic and inhibitor to determine if a larger effect on dendritic growth could be achieved. This will be especially important for the MIR-21 inhibitor since the increase in dendritic growth was small compared to the inhibition caused by the mimic.

MicroRNAs have an effect on processes through the degradation of proteins involved in the pathway (Strickland et. al., 2011). Therefore, in order to fully understand the effect of MIR-21 on BMP induced dendritic growth, it is necessary to identify the
targets of MIR-21 in this process. We looked at one potential target in this study, and we found that BMP-RII is not regulated by BMP-7. Because MIR-21 is down-regulated in cells treated with BMP-7, BMP-RII levels would need to have been increased in BMP treated cells, if BMP-RII were a target of MIR-21. Because our results did not show this, we can rule out BMP-RII as a target of MIR-21 in sympathetic neurons. There are a number of other targets of MIR-21 that are known to be interacting with the BMP signaling pathway in other systems. Sprouty-2 protein is a known target of MIR-21 in dorsal root ganglion neurons and is known to be downstream from the BMP/SMAD pathway (Strickland et. al., 2011). Jag1 is another potential target of the MIR-21/BMP-7 pathway. Jag1 is the ligand for Notch 1 cell surface receptor, and it has been identified as a potential target of MIR-21 (Buscaglia et. al., 2011). Jag1 is known to be up-regulated during BMP-induced dendritic growth (Garred, et. al., 2011). This makes Jag1 an excellent candidate for regulation by MIR-21.

In summary, we have shown that MIR-21 is involved in suppression of dendritic growth. Cells treated with MIR-21mimic showed significant decreases in dendritic growth. We also showed that the knock-out of MIR-21 does induce small amounts of dendritic growth. In addition, we succeeded in eliminating BMP-RII as a target of the SMAD/BMP-7 pathway.

5. Acknowledgments

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References


