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Short Communication

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Nano-topology guided neurite outgrowth in PC12 cells is mediated by miRNAs

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Abstract

MicroRNAs (miRNAs) are master regulators of gene expression at post-transcriptional level. The present study investigated the involvement of miRNAs in topological guidance of neurite outgrowth in an NGF treated PC12 cell model cultured on nano-patterned polyethylene terephthalate (PET) substrates fabricated with interference lithography. The expressions of 38 neuronal miRNAs were measured and 3 were found to be differentially regulated during topological guidance of neurite outgrowth. Altering the intracellular levels of these miRNAs disrupted the orderly growth of neurite along nano-patterned substrate. Our results showed miRNAs to be versatile regulators and their involvement should be thoroughly investigated for better understanding of biological processes.

From the Clinical Editor: In this basic science study, strong evidence was found that topological guidance is only one factor, and miRNA-s regulate axonal outgrowth from neurites. Nano-patterned polyethylene terephthalate substrates were used for the study, fabricated using interference lithography. Further studies of this biologically relevant process may pave the way to clinically useful axonal regrowth and axonal guidance methods.

Key words: Nano-topology; Neurite; PC12; MicroRNAs

Controlled neurite outgrowth guided by combinations of chemical and physical cues is important in regenerative medicine for the treatment of nerve injuries.[1,2] While chemo-attractants and repellents of neurite outgrowth have been extensively studied, the roles of physical cues such as micro- or nano-scale topologies in neurite guidance are less understood.[3] Many reported microgrooves induced alignment of neurites and indicated the importance of attachment of substrate on neurite outgrowth (see more discussion in S1 of Supplementary Document).[4-8] However, it is still being argued whether the effect is by topological guidance or simply confinement imposed by the micro structures. We showed[9] guided neurite growth of PC12 cells by nanogrooves smaller than the neurite itself. Such system minimized the complication of confinement and provided a great model to investigate the underlying mechanisms of topological neurite guidance.

MicroRNAs (miRNAs) are post-transcriptional regulators of gene expression and have been shown to be involved in a wide range of physiological processes. An increasing body of evidence has demonstrated the intimate role of microRNAs in regulating neurite initiation, outgrowth and patterning.[10-13]. In this study, we tested the hypothesis that miRNAs regulate topological neurite guidance and presented key evidence of its active involvement.

Methods

Polyethylene terephthalate (PET) substrate with nanogrooves with 100 nm in height, 300 nm in width with a 400 nm gap between consecutive grooves was produced as described.[9] PC12 cells were seeded on both grooved and flat substrates and treated with 50 ng/ml of NGF over 72 h. Total RNA was isolated using Cell-to-Ct reagent.[14] The expressions of 38 neuronal miRNAs

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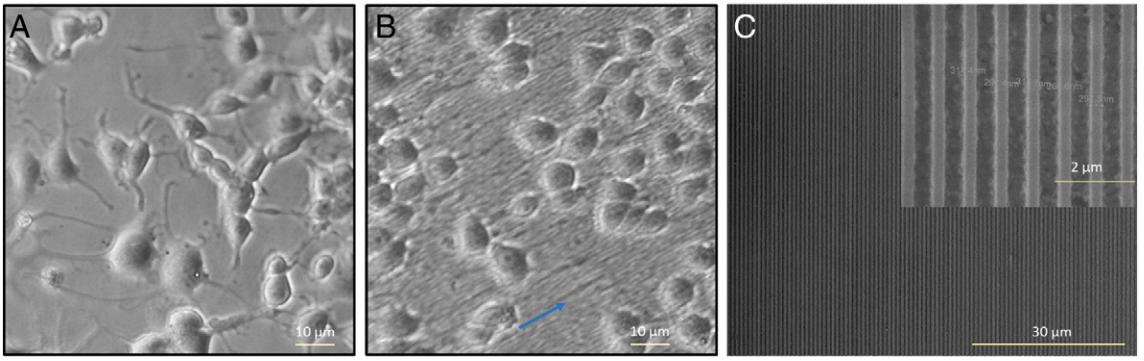

Figure 1. PC 12 on **(A)** flat and **(B)** nanogrooved PET substrates. The arrow indicates neurite alignment along nanogrooves. **(C)** SEM images of large area uniform grooves with inserted magnified view.

were measured using miR Φ qPCR assays (ETPL, A*STAR) on CFX-96 (BioRad). For functional study, PC12 cells were transfected with mimics and inhibitors of miR-221, miR-222 and of miR-124 (Qiagen) using Transfectin (BioRad) according to manufacturer's instructions.

Results

Upon NGF treatment, the neurites were found to extend in a direction parallel to the grooves (Figure 1, *B*) in contrast to the randomized neurite outgrowth on flat PET substrate (Figure 1, *A*). Previous work[15] showed nanostructures may have an effect on viability and we found that viability is not affected by nanogrooves (see S2 of Supplementary Document).

The expressions of 38 miRNAs involved in neurite outgrowth and morphology were measured (see S3 of Supplementary Document). Figure 2, *A* shows 6 miRNAs that were differentially regulated during NGF induced neurite outgrowth after normalization to reference miR-16 and miR-93. The time points were chosen to reflect the highest fold change in culture period to help to identify significant miRNA change (see S4 of Supplementary Document).

Among these miRNAs, miR-124, miR-221 and miR-222 were most significantly regulated between flat and grooved PET surface (see in Figure 2, *B-D*). miR-221 and miR-222 showed significantly lower expression on grooved surfaces whereas miR-124 showed significantly higher expression.

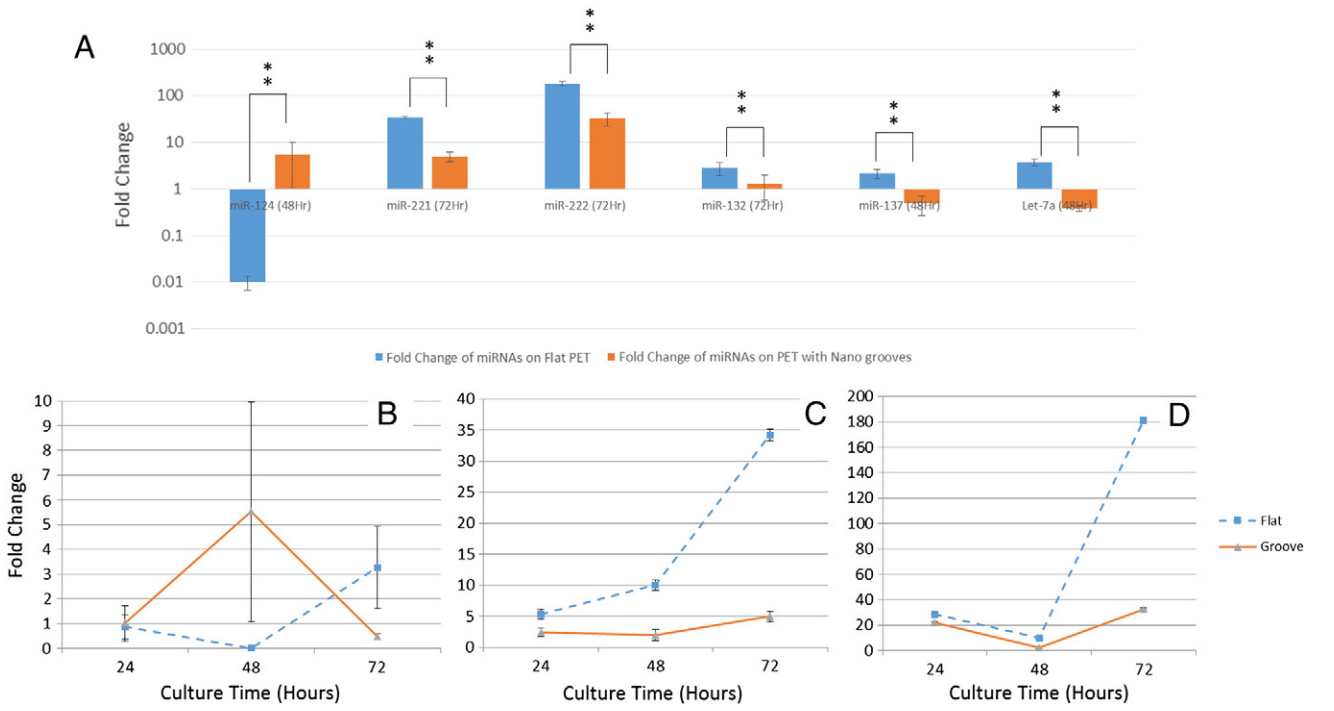

Figure 2. The most significant fold change in miRNA (NGF treated/untreated control) on flat and grooved PET surfaces during the 72 hours culture period **(A)**, fold changes of miR-124 **(B)**, miR-221 **(C)** and miR-222 **(D)** on flat and grooved PET surface over time.

The function of the three microRNAs was subsequently investigated by altering their intracellular levels. PC12 cells were transfected with inhibitor and mimics of miR-124, miR-221 and miR-222; and induced to differentiate on nanogrooved substrates. Transfection of miR-221 and miR-222 mimic resulted in approximately 800 fold overexpression of miR-221 and miR-222 compared to transfection controls whereas miR-124 inhibitor successfully attenuated miR-124 expression by more than 60%.

Figure 3 shows that when neurites were aligned, the FFT image contains sharp spikes orthogonal to the aligned neurites in spatial domain, whereas a spherical pattern with blurred boundary is indicative of less aligned neurites.[16] Both miR-124 inhibitor and miR-221 mimic transfection resulted in blurred FFT image, compared to the sharp spike in transfection control and negative control cells, indicating the increase in the intracellular levels of miR-221 and the decrease in miR-124 disrupted neurite alignment. However, miR-222 mimic transfected cells showed some spikes in the frequency domain.

We further examined neurite alignment by manually tracing neurite segments and measuring their deviation from the nanogrooves. The total length of neurite segments in each angle bracket was divided by total neurite length to produce the percentage of neurite length in each angle bracket (Figure 4).

Both miR-221 mimic and miR-124 inhibitor transfection led to high degree of deviation, with a marked decrease in the percentage of neurite length in angles between 0° and 10° to the nanogrooves. There was significant increase of neurite segments deviating more than 30° to grooves. The miR-222 mimic transfection resulted in a smaller degree of deviation with noticeable increase in neurite segments in between 30° and 70°.

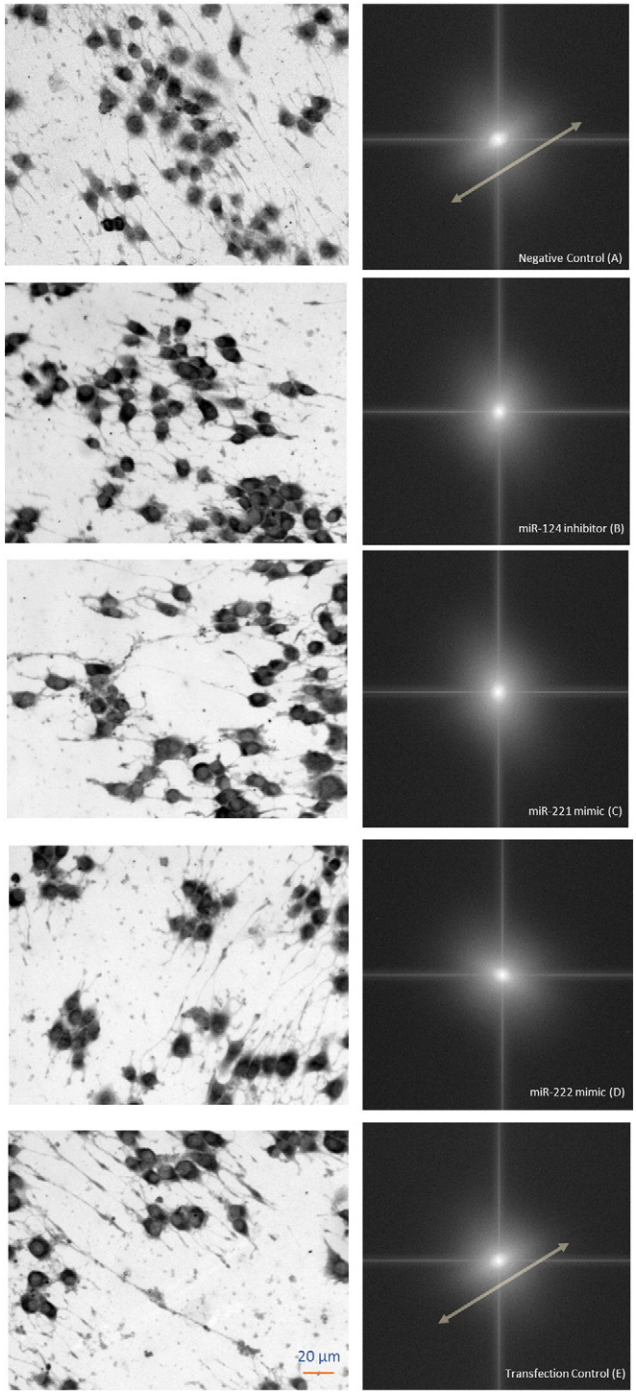

Figure 3. Left: Typical microscopic image of treatment conditions. Right: Averaged FFT images. From top to bottom: cells transfected with negative control **(A)**, miR-124 inhibitor **(B)**, miR-221 mimic **(C)**, miR-222 mimic **(D)**, transfection control **(E)**. White arrows show directions of spikes in FFT image.

Discussion

In this study, we showed nano-topological features led to aligned neurite outgrowth in PC12 cells. Interestingly, miR-221 and miR-222 expressions were lowered on PET nano-grooves, while miR-124 expression was shown to be higher. Altering of the miRNA levels was shown to reduce the alignment of neurites on nano-grooves. We suggest that neurite guidance may be linked to the transient up-regulation of miR-124 and down-regulation of miR-221 and miR-222 caused by nanostructured surface. There are also reports that found these miRNAs to regulate neurite growth in various cells. For example, the loss of miR-124 in Xenopus laevis retinal ganglion cells caused growth cone pathfinding errors,[17] and miR-124 was shown to alter neurite outgrowth in P19 cells.[18] In PC12 cells, miR-221 was reported to potentiate the formation of neurite network.[19] These reports provided further evidence of the involvement of the three miRNAs in neurite outgrowth and topological guidance.

Downstream targets of these miRNAs that could act as effectors in topological neurite guidance is being investigated. Due to our rudimentary understanding of miRNA-mRNA interaction and its cell context dependent nature, identification and validation of miRNA target genes is a challenge.[20] The expressions of 74 reported target genes were measured with qPCR but not found to be regulated by the miRNAs (S5(a) of Supplementary). Therefore more work needs to be done to identify potential targets in neurite directing (S5(b) of Supplementary). Further analysis using latest miRNA target prediction algorithm will be carried out. Nevertheless, current findings strongly support the conclusion that microRNAs participate in the outgrowth of neurites guided by topological cues.

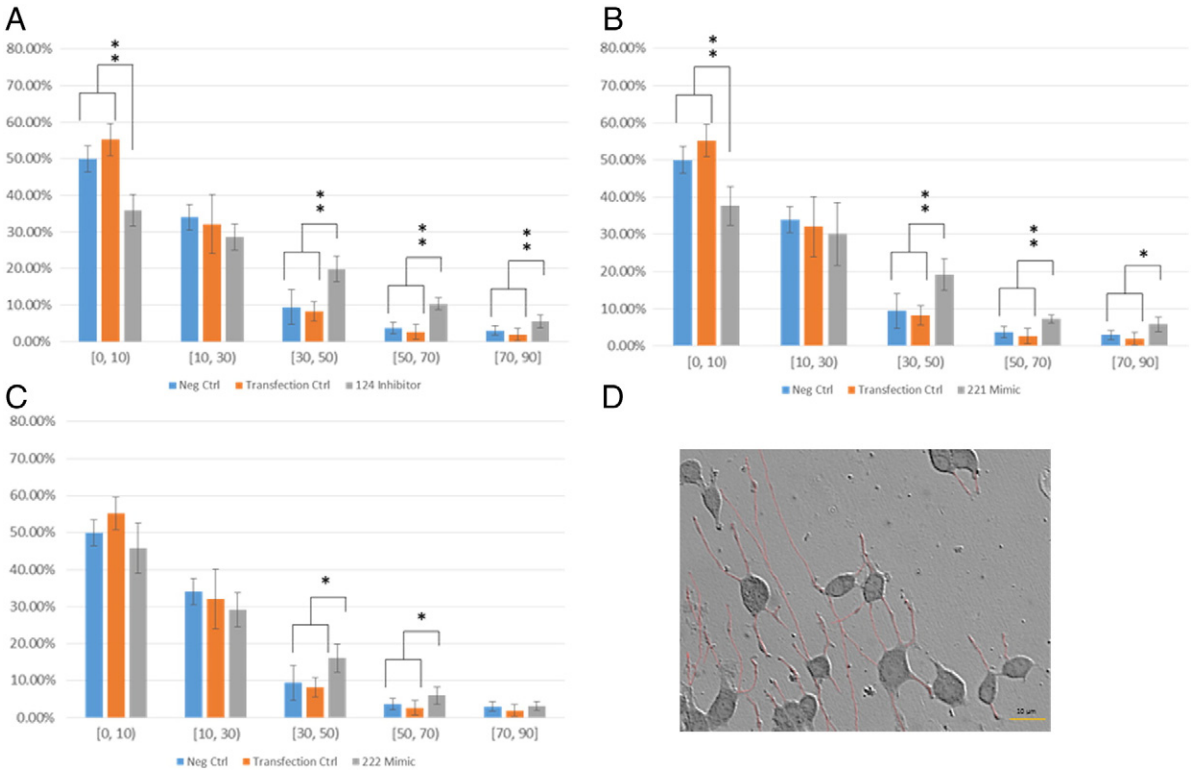

Figure 4. Percentage of neurite length in angle bracket for miR-124 inhibitor **(A)**, miR-221 mimic **(B)** and miR-222 mimic **(C)** and microscopic photo for neurite tracing **(D)**. A higher percentage of neurite length in larger angle brackets indicates more deviation of neurites in ordered alignment.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.nano.2014.07.011.

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