Published in final edited form as:

J Cell Physiol. 2015 July ; 230(7): 1515–1524. doi:10.1002/jcp.24897.

Exploration of molecular pathways mediating electric field-directed Schwann cell migration by RNA-Seq

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Abstract

In peripheral nervous systems, Schwann cells wrap around axons of motor and sensory neurons to form the myelin sheath. Following spinal cord injury, Schwann cells regenerate and migrate to the lesion and are involved in the spinal cord regeneration process. Transplantation of Schwann cells into injured neural tissue results in enhanced spinal axonal regeneration. Effective directional migration of Schwann cells is critical in the neural regeneration process. In this study, we report that Schwann cells migrate anodally in an applied electric field (EF). The directedness and displacement of anodal migration increased significantly when the strength of the EF increased from 50 mV/mm to 200 mV/mm. The EF did not significantly affect the cell migration speed. To explore the genes and signaling pathways that regulate cell migration in EFs, we performed a comparative analysis of differential gene expression between cells stimulated with an EF (100 mV/mm) and those without using next-generation RNA sequencing, verified by RT-qPCR. Based on the cut-off criteria ($FC > 1.2$, $q < 0.05$), we identified 1,045 up-regulated and 1,636 down-regulated genes in control cells versus EF-stimulated cells. A Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis found that compared to the control group, 21 pathways are down-regulated, while 10 pathways are up-regulated. Differentially expressed genes participate in multiple cellular signaling pathways involved in the regulation of cell migration, including pathways of regulation of actin cytoskeleton, focal adhesion, and PI3K-Akt.

Keywords

EF stimulation; anodal migration; regulation of actin cytoskeleton; focal adhesion

Introduction

In the peripheral nervous system, Schwann cells wrap around the axons of motor and sensory neurons to form the myelin sheath. Transplantation of Schwann cells into the lesions of injured peripheral and spinal cords may help to restore axonal myelination and promote the connection of neural circuitry leading to functional recovery. In spinal cord injury (SCI), mechanical trauma causes direct neurological damage to the spinal cord, which leads to

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neural tissue necrosis at the point of injury. Demyelination and degeneration of axons at the lesion cause functional loss of spinal cord tissue. It was reported that Schwann cells regenerated and migrated to the injury site post-SCI and were involved in the spinal cord regeneration process (Guest et al., 2005; Thuret et al., 2006). To promote injured spinal cord regeneration, Schwann cells were transplanted into the injured neural tissue. Results showed that Schwann cell implantation enhanced spinal axonal regeneration (Liu et al., 2014a; Iannotti et al., 2003).

Endogenous electric fields (EFs) have been detected in developing and regenerating tissues (Hotary and Robinson, 1990, 1991; McCaig et al., 2005). It was reported that the magnitude of direct current (DC) EFs that play physiological roles in development and regeneration is between 1 mV/mm and 100 mV/mm (McCaig et al., 2005). *In vitro*, studies have shown that many types of cells migrate in specific directions when exposed to a small applied EF (Nuccitelli, 2003; Meng et al., 2011; Zhao et al., 2011; Yao et al., 2009; Nishimura et al., 1996; Li et al., 2008). Recent studies have found that the migration of neural cells can be guided by EFs. Hippocampal neurons migrated to the cathode in EFs, and the EFs did not affect cell migration speed, while chicken Schwann cells migrated to the anode in EFs (McKasson et al., 2008). This raised the possibility that EFs may serve as an efficient cue to guide neural cell migration to a lesion to establish functional connections. The regulation of directional migration of cells in EFs involves cellular membrane receptors, intracellular signaling molecules, and cytoskeletons. Previous studies have shown that a number of molecules of intracellular signaling pathways may be involved in the regulation of EF-guided cell migration. Treated with inhibitors of PI3K or ROCK, the directional migration of hippocampal neurons in EFs was inhibited (Yao et al., 2008). The ROCK inhibitor Y27632 enhanced the viability of stem cells and inhibited EF-guided directional migration in induced pluripotent stem cells and neurons. However, it did not significantly affect the directionality of hNSC migration in an EF (Feng et al., 2012). Genetic disruption of PI3K γ decreased EF-induced signaling and abolished directional migration of healing epithelium in EFs. However, the deletion of the tumor suppressor PTEN, a repressor of PI3K signaling, enhanced the signaling and electrotactic responses of the cells to EFs (Zhao et al., 2006).

Because multiple signaling pathways may be involved in EF-guided cell migration, next-generation RNA sequencing provides an efficient approach to identify these pathways systemically. Next-generation sequencing is a high-throughput methodology and offers comprehensive analysis of gene expression profiling (Wang et al., 2009; Mortazavi et al., 2008; Morozova et al., 2009; Liu et al., 2014b). Compared to RT-qPCR arrays and microarray-based technologies, RNA-seq has several significant advantages, including high sensitivity for the detection of low abundant transcripts. In this study, we report the results of differentially expressed gene profiling between Schwann cells treated with EFs and cells without EF stimulation using RNA-seq and validated by RT-qPCR. Thus, we have generated a comprehensive dataset of gene expression to investigate the regulation of EF-guided cell migration.

Materials and Methods

Schwann cell culture

The procedure for isolation of Schwann cells from neonatal rats was approved by the Institutional Animal Care and Use Committee (IACUC) and completed at Wichita State University in Wichita, KS. Rat Schwann cells were isolated from the sciatic nerves of neonatal rats (postnatal day P1–3 rats), as described previously (Brookes et al., 1979; Chen et al., 2011). After the nerves were dissected from the neonates, connective tissue was separated and removed from the nerves. Then the nerves were cut into small pieces and digested with Hank's Balanced Salt Solution containing trypsin and collagenase. The dissociated cells were collected for culture by centrifugation. For routine culture, rat Schwann cells were cultured in cell culture dishes coated with Poly-D,L-ornithine (5 mg/ml in PBS, Sigma-Aldrich, St. Louis, MO). The cells were fed with Dulbecco's Modified Eagle's Medium (Lifetechnology, Grand Island, NY) with 10% fetal bovine serum supplemented with 10 ng/ml neuregulin (R&D Systems, Minneapolis, MN) and 5 μ M Forskolin (Sigma-Aldrich, St. Louis, MO) and cultured in a 37°C incubator with 5 % CO₂. Cells between passages 2 and 4 were used in all experiments.

Migration of Schwann cells in EFs and time-lapse imaging

To investigate the migration of Schwann cells in EFs, EFs were applied to the cultured cells as reported previously (Zhao et al., 1996; Yao et al., 2008). In brief, the Schwann cells were grown in a glass chamber made using coverslips. The final dimensions of the chambers were 30 mm $\times$ 0.8 mm $\times$ 0.15 mm. The cell culture chamber was coated with poly-L-lysine (100 μ g/ml, Sigma-Aldrich, St. Louis, MO) and laminin (20 mg/ml, Sigma-Aldrich, St. Louis, MO). To apply the EFs to the cultured cells in the chamber, agar-salt bridges (filled with Steinberg's solution gelled with 1% agar) were used to connect silver-silver chloride electrodes in beakers of Steinberg's solution and pools of cell culture medium. Culture conditions in controls were identical, except no EFs were applied.

To study the migration of Schwann cells in an applied EF, the cells were placed in the chamber and cultured for 36 to 72 hours. Then steady direct currents of 50 mV/mm, 100 mV/mm, and 200 mV/mm were applied to the cultured cells. Schwann cell migration was recorded by a time-lapse microscope (Zeiss Axio Observer microscope) placed in a plastic incubator with 5% CO₂ at 37°C. Sterile conditions were maintained throughout. Time-lapse image recording was performed to record cell migration using a ZEN 2011 imaging microscope software, and images were taken by digital camera (AxioCam MRm Rev.3 with FireWire). The migration of the cells was recorded for 1 or 2 hours, and then the EF power was switched on and the cell migration was recorded for an additional 1 or 2 hours, respectively. Cell migration was recorded by capturing images every 3 minutes during the recording period. In a separate experimental group, the cell migration was recorded for 2 hours in an EF (100 mV/mm), and then the EF polarity was reversed without changing the field strength and cell migration was recorded for 2 more hours.

Quantification of cell migration

The time-lapse images were analyzed by NIH ImageJ software (National Institutes of Health, Bethesda, MD). Cell migration was quantified using a previously reported method (Yao et al., 2008). The angle at which each cell moved with respect to the imposed EF direction was measured. The mean directedness of total cell movement was calculated from the equation $\sum i \cos \theta / n$, where θ is the angle between the field vector and the cellular translocation direction, and n is the total number of cells. The cosine of the angle would be equal to 1 if the cell moved directly along the field lines toward the cathode; 0 if the cell moved perpendicular to the field direction; and -1 if the cell moved directly toward the positive pole of the field. The net displacement was the distance of the cell migration along the field line. The cell migration velocity was calculated from the full distance of cell migration in a given time. To quantify the cell migration speed, directedness, and displacement, cells from four independent experiments were analyzed.

RNA sequencing

For RNA sequencing study, 100,000 cells were seeded in each cell culture chamber. Three independent experiments were performed for either the study of control cell migration or the cell migration study with EFs stimulation. Total RNA of the cells in the chamber without EF stimulation or with EF (100 mV/mm) stimulation for 2 hours was extracted using an RNeasy Mini Kit (Qiagen) according to the supplier's protocol. RNA purity and integrity were analyzed using Agilent 2100 Bioanalyzer. The Stranded mRNA-Seq was performed using the Illumina HiSeq2500 Sequencing System at the University of Kansas Medical Center—Genomics Core (Kansas City, KS). Total RNA (0.5 μ g) was used to initiate the Stranded mRNA-Seq library preparation protocol. The mRNA fraction was enriched with oligo dT capture, sized, reverse transcribed into cDNA, and ligated with appropriate indexed adaptors using the TruSeq Stranded mRNA Sample Preparation Kit (Illumina RS-122-2101/2102). Following Agilent Bioanalyzer QC of the library preparation and library quantification using the Roche Lightcycler96 with KAPA SYBR Fast Universal qPCR kit (KAPA Biosystems KK4601), the RNA-Seq libraries were adjusted to a 4 nM concentration and pooled for multiplexed sequencing. Libraries were denatured and diluted to the appropriate pM concentration (based on qPCR results) followed by clonal clustering onto the sequencing flow cell using the TruSeq Paired-End (PE) Cluster Kit v3-cBot-HS (Illumina PE401-3001). The clonal clustering procedure was automated using the Illumina cBOT Cluster Station. The clustered flow cell was sequenced on the Illumina HiSeq 2500 Sequencing System (Illumina, San Diego, CA) at a 2 $\times$ 100 bp paired-end resolution using the TruSeq SBS Kit v3-HS (Illumina FC401-3002). Following collection, sequence data was converted from the .bcl file format to fastQ files and de-multiplexed (if required) into individual sequences for further downstream analysis. Samples were analyzed in biological triplicates. The strand-specific sequenced reads were aligned to the rat genome (Rnor 5.0) using STAR (Dobin et al., 2013) (version 2.3). The differential gene expression was calculated using Cuffdiff (Trapnell et al., 2012) (version 2.1.1) with default parameters. Multiple hypotheses testing correction was performed using the Benjamini and Hochberg method (Benjamini and Hochberg, 1995). Genes with a false discovery rate less than or equal to 0.05 were considered significant.

Signaling pathway analysis

NCBI's FLink site was used to perform pathway analysis. NCBI gene ID's for either up-regulated or down-regulated genes were pasted into the gene database and then linked to the gene biosystems database. The resulting list was downloaded into Excel and filtered for KEGG pathways with at least 15 genes from our list of significant genes and comprised of more than 10% of the number of genes in the KEGG pathway (Kanehisa and Goto, 2000; Kanehisa et al., 2014). Then a hypergeometric test was applied with a $p = 0.05$ cutoff score. The list of significant EF genes in the significant pathways was downloaded from the FLink page. The PCA analysis, hierarchical clustering, MA plot, and FDR histogram were generated in EdgeR (Robinson et al., 2010). The gene counts used in edgeR were made by applying htseq-count script from HTSeq (Anders et al., 2014) to the STAR mapped reads.

qRT-PCR

Total RNA was extracted as described above, and cDNA was reverse-transcribed from total RNA using the High-Capacity cDNA Reverse Transcription Kit (Lifetechnology) according to the manufacturer's protocol. qPCR was performed using Power SYBR® Master Mix by the StepOnePlus™ qPCR System (Lifetechnology) at 95°C for 10 minutes, and then 40 cycles at 95°C for 15 seconds followed by 60°C for 60 seconds. Gene transcription was normalized in relation to transcription of the housekeeping rat GAPDH. The 2^{-Ct} method was used to calculate relative gene expression for each target gene. Primers used in RT-qPCR are provided in the supplemental materials in Table 1.

Statistics

Statistical analysis was conducted to assess the cell migration data using a paired t-test or an unpaired, two-tailed Student's t-test. A p value of 0.05 was considered to be statistically significant. Data are expressed as means $\pm$ standard deviation. At least three independent experiments were performed for each migration study.

Results

Schwann cells migrate toward the anode EFs

The Schwann cells showed random migration without EF stimulation (Figures 1A–D), while the cells showed a clear trend of migration toward the anode in an applied EF (100 mv/mm) (Figures 1E–H). In Figures 1I–L, each frame shows the superimposed migration tracks of 60–125 Schwann cells from three different experiments. The position of all cells at $t = 0$ minute is represented by the origin (0, 0). Each line represents the migration track of one single cell over a two-hour period. In the EF stimulation study, cells subjected to an applied EF (100 mV/mm and 200 mV/mm) showed clear anodal migration. A circular histogram (rose diagram) showed the distribution of migration cells in Figures 1E–H. The migrated cells showed clear biased distribution in the EFs (100 mV/mm and 200 mV/mm), and therefore, the graph indicates the anodal migration of the cells (Figures 1M–P).

EF-directed cell migration was dependent on voltage and time. In the EF group, after a one-hour exposure to an EF stimulation of 50 mV/mm, 100mV/mm, or 200mV/mm, the directedness was -0.10 ± 0.04 , -0.16 ± 0.06 , and -0.25 ± 0.05 , respectively (Figure 2A).

After a two-hour EF stimulation of 50 mV/mm, 100 mV/mm, and 200 mV/mm, the directedness was -0.05 ± 0.04 , -0.33 ± 0.03 , and -0.41 ± 0.05 , respectively (Figures 2B). The quantification of net displacement of the cells along the field line also showed an anodal migration pattern. For cells that were stimulated for one hour, the net displacement of cells in EFs of 50 mV/mm, 100 mV/mm, and 200 mV/mm was $-3.63 \pm 0.78 \mu\text{m}$, $-3.03 \pm 0.68 \mu\text{m}$, and $-3.92 \pm 1.11 \mu\text{m}$, respectively (Figure 6C). For cells that were stimulated for two hours, the net anodal displacement of the cell migration in EFs of 50 mV/mm, 100 mV/mm or 200 mV/mm were $-1.92 \pm 1.34 \mu\text{m}$, $-14.04 \pm 1.81 \mu\text{m}$, $-13.58 \pm 3.29 \mu\text{m}$, respectively (Figures 2D). These results demonstrate that the directedness and displacement of anodal migration increased when the EF strength or stimulation duration increased.

The cell migration velocities of cells before and after exposure to EFs were quantified and compared (Figure 2E, F). The migration speed of cells before EF stimulation was $1.13 \pm 0.01 \mu\text{m}/\text{min}$, which was decreased to $0.81 \pm 0.01 \mu\text{m}/\text{min}$ after the cells were subjected to an EF of 50 mV/mm for two hours ($p < 0.01$). However, the migration speed did not change significantly after the cells were subjected to EFs of 100 mV/mm and 200 mV/mm for two hours (Figures 2E, F).

Reversal of EFs poles reverses the migration direction of Schwann cells in EFs

To confirm the migration of Schwann cells to the anodal pole in EFs, cell migration was recorded before and after reversal of the EFs polarity. Schwann cells migrated toward the anode pole in an EF of 100 mV/mm (Figure 3A). After two hours, the EF polarity was reversed, and the cells showed the reversal of migration to the new anodal pole (Figure 3B). The tracking of cell migration and the circular histogram show the cell migration direction (Figures 3A–D). The quantification of migration directedness and the displacement along the field line also showed the reversal of migration induced by the reversal of EF polarity. The directedness of cell migration before and after EF stimulation (Figure 3E) was -0.31 ± 0.09 and 0.16 ± 0.06 , respectively. The displacement of cells along the field line before and after EF stimulation (Figure 3F) was $-0.884 \pm 2.24 \mu\text{m}$ and $3.17 \pm 1.75 \mu\text{m}$, respectively. The reversal of EF poles did not change the migration speed significantly.

Identification of differentially expressed genes in control and EF-treated Schwann cells

From the RNA-seq libraries, the total number of clean reads per library ranged from 28.7 to 36.1 million for control Schwann cells and from 29.3 to 32.8 million for Schwann cells treated with EFs. After mapping to the rat genome (Rnor 5.0), 25.4–31.9 and 25.8–29.0 million unique reads mapped to 14,521 and 14,546 Ensembl loci, with at least FPKM > 0.1 identified for the control cell and experimental cells, respectively. Based on the cut-off criteria ($FC > 1.2$, $q < 0.05$), we identified 1,045 up-regulated and 1,636 down-regulated genes in control cells versus EF-stimulated cells. A total of 7.54% reads were mapped to multiple locations, and 3.85% of the reads were unmapped overall. Only the uniquely mapped reads were considered in this analysis. Differential gene expression was calculated using Cufflinks. Based on the cut-off criteria ($FC > 1.2$, $p < 0.01$), we identified 1,045 up-regulated and 1,636 down-regulated genes in control cells versus EF-stimulated cells.

The principal components analysis (PCA) of the normalized expression values of the genes indicated a clear separation of control and EF-stimulated cell samples (Figure 4A). Similarly, an unsupervised two-dimensional hierarchical clustering of differentially expressed genes clearly separated the control and EF-stimulated cells (Figure 4B). An MA plot (Figure 4C) shows the mean expression across libraries compared to the log₂ fold change between conditions for all genes. Significantly deregulated genes are indicated in red. A histogram displaying significant FDR values suggests that about half of the genes are significant; however, only about half of those meet our fold change criteria (Figure 4D). To confirm the results of differentially expressed gene profiling, RT-qPCR validation was performed for 15 significantly changed genes composed of 7 up-regulated and 8 down-regulated genes (supplemental material in Table 2) for RT-qPCR validation. The expression of all genes tested in the RT-qPCR validation had the same trends as those tested in the RNA-seq (Figure 5). Spearman correlation of log₂ transformed data from RNA-seq and RT-qPCR showed a significant positive correlation between these two datasets ($R = 0.946$, $P < 0.001$). The data indicate that our RNA-seq results are reliable.

Differentially regulated KEGG pathways between control and EF-treated Schwann cells

To explore the genes and signaling pathways that regulate cell migration in EFs, we report a comparative analysis of differential gene expression between cells with and without EF stimulation using the RNA-seq approach with next-generation sequencing platforms, and verification by RT-qPCR. KEGG pathway analysis suggests the participation of differentially expressed genes in multiple cellular signaling pathways. Compared to the control group, 21 pathways are down-regulated, while 10 pathways are up-regulated (Figure 6). Among these pathways, focal adhesion and actin cytoskeleton pathways regulate actin function and are critical for cell motility. The genes in the focal adhesion pathway mediate actin polymerization for filopodia and lamellipodia formation (Figure 7). The genes in the actin cytoskeleton pathway mediate the activity of actin and myosin that regulate the function of filopodia and lamellipodia and focal adhesion (Figure 8). The analysis of our list of significant genes showed that 26 genes are down-regulated, while 17 genes are up-regulated compared with the control group in the focal adhesion pathway (supplemental material in Table 3). Additionally we have 25 down-regulated genes and 10 up-regulated genes compared with the control group in the actin cytoskeleton pathway (supplemental material in Table 4).

Among the significantly changed pathways, genes in the PI3K-Akt signaling pathway and MAPK pathway are involved in the regulation of cell motility, cell cycle, and cell proliferation and apoptosis. The PI3K-Akt signaling pathway has 51 down-regulated and 29 up-regulated genes (supplemental material in Table 5), and the MAPK pathway has 27 down-regulated and 31 up-regulated genes compared to the control group (supplemental material in Table 6).

Discussion

In this study, we investigated the migration of dissociated Schwann cells in EFs. Different from primary neurons or embryonic stem cell-derived neurons that migrated to the cathode

pole in EFs, Schwann cells migrated to the anodal pole in EFs (Yao et al., 2008; Li et al., 2014). Similar to the neurons, migration in EFs, directedness, and net displacement of directional migration increased with the EF strength and duration of stimulation. However, Schwann cells are less sensitive to EF guidance than neurons. The absolute value of Schwann cell migration directedness in EFs of 200 mV/mm for one hour stimulation was 0.25, compared to 0.43 of hippocampal neurons. When the cells were exposed to an EF of 200 mV/mm for two hours, the absolute value of the directedness increased to 0.41. Schwann cells showed a similar cell migration speed as that of the hippocampal neurons in an EF of 200 mV/mm.

In this study, we comprehensively examined the transcriptome of cells subjected to EFs and systemically identified signaling pathways using next-generation RNA sequencing. Differentially expressed genes discovered in this study target pathways important in cell migration, such as the actin cytoskeleton pathway and the focal adhesion pathway. These pathways regulate actin function, which is critical for cell motility. Previous studies reported that PI3K, PTEN, and small Rho GTPases mediated the migratory directedness, motility, and rate of cell movement undergoing chemotaxis and electrotaxis. PI3K is important for regulating F-actin assembly during chemotaxis. Neutrophils lacking PI3K have defects in both cell motility and directionality (Hannigan et al., 2002) in chemotaxis. The Inhibitor of PI3K or RhoA can decrease or even abolish the migration direction and orientation of several cell types in EFs (Zhao et al., 2004; Pu and Zhao, 2005). Genetic disruption of PI3K γ decreases EF-induced signaling and abolishes directed movements of healing epithelium in response to EFs. However, deletion of the tumor suppressor PTEN enhances signaling and electrotactic responses (Zhao et al., 2006). In this study, we observed a significant change in PI3K genes. We found that the expression of the PIK3R3 gene was down-regulated and that of the PIK3R5 gene was up-regulated in Schwann cells in EFs. These genes regulate both the actin cytoskeleton and focal adhesion pathways and may regulate EF-guided Schwann cell migration. In addition, the expression of PTEN, a negative regulator of focal adhesion and PI3K pathways, was down-regulated. This is consistent with a previous study that PTEN down-regulation is required for EF-guided cell migration. A previous study reported that the ROCK inhibitor Y27632 decreased the cathodal migration of hippocampal neuron migration in EFs (Yao et al., 2008). In addition, ROCK inhibition significantly increased the motility of iPS cells and reduced the directionality of iPS cells in an EF (Zhang et al., 2011a). Unexpectedly, in this study, we found that the gene expression of major small Rho GTPases members—RhoA, Cdc42, and Rac1—did not change significantly in Schwann cells treated with EFs. In the PI3K-Akt pathway, we also observed the up-regulation of the PKN3 gene. It has been reported that this gene appears to play a pivotal role in maintaining endothelial cell morphology, cell-cell junctions, and motility. The knockdown of PKN3 expression resulted in divergent cell morphology, impaired locomotion, disturbed adherens junction integrity, and irregular actin organization (Möpert et al., 2012).

In the focal adhesion pathway, a few integrin transmembrane receptors genes changed significantly. ITGA3 was up-regulated, while ITGA4, ITGA11, ITGB4, and ITGB8 were down-regulated in Schwann cells treated with EFs. On the ITGB-PKC-FAK-Paxillin-Parvin-Actin pathway, the PKC, Paxillin, and Parvin were up-regulated and, therefore,

regulated actin polymerization. Parvin is an adapter protein that interacts with other focal adhesion proteins such as Paxillin and ILK, leading to focal adhesion stabilization. Alpha-Parvin-deficient vascular smooth muscle cells fail to polarize their cytoskeleton, resulting in loss of persistent and directed migration (Montanez et al., 2009). Another study showed that knockdown of MLCK in Schwann cells induced plasma membrane expansion (Leitman et al., 2011). In this study, we detected the up-regulation of the MLCP gene (PPP1R12A gene) and down-regulation of the MLCK gene (MYLK gene). Previous studies have suggested that the ERK signaling cascade can regulate both cell motility (Hirotsu et al., 2000; Chen et al., 1994; Klemke et al., 1997; Wang et al., 1998; Nguyen et al., 1999; Zeigler et al., 1999) and cell growth and differentiation. Activation of MAP kinase ERK1/2 in the Ras-Raf-MEK-ERK1/2-MLCK pathway regulated the migration of epithelial cells (Zeigler et al., 1999; McCawley et al., 1999), fibroblasts [Klemke et al., 1997; Jeffers et al., 1998], and vascular smooth muscle cells (Graf et al., 1997). Further studies reported that when corneal epithelial cells were exposed to physiological electric fields, they demonstrated activation of ERK and accumulation of F-actin at the leading, cathodal-facing side of the cell (Zhao et al., 2002). In this study, we found that Ras was down-regulated. The other genes on this pathway, such as Raf, Mek ERK1/2, and MLCK, did not change significantly. However we detected the up-regulation of the PTPN5 gene and MKP genes (DUSP2, DUSP4, DUSP5, and DUSP10), and the down-regulation of the DUSP8 gene. The kinase ERK is negatively regulated by proteins PTP and MKP by dephosphorylation.

We found the expression of the EZR gene was up-regulated in the actin cytoskeleton pathway, while the expression of LIMK and SSH genes was down-regulated. The EZR protein serves as an intermediary between the plasma membrane and the actin cytoskeleton, and plays a key role in cell adhesion and migration. Cofilin binds to actin filaments and stimulates their disassembly. The SSH proteins dephosphorylate and activate cofilin (Wang et al., 2005), while LIMK phosphorylates and inactivates cofilin (Zhang et al., 2011b). LIMK1 may also be dephosphorylated and inactivated by SSH proteins. The gene expression of SSH and LIMK regulated the stability of the actin. However, we also found the down-regulation of IQGAP and APC genes. It was reported that in directionally migrating cells, IQGAP1 accumulates at the leading edge (Hart et al., 1996; Kuroda et al., 1996; Mataraza et al., 2003) and crosslinks actin filaments (Briggs and Sacks, 2003). IQGAP1 can also directly interact with APC at the leading edge. The down-regulation of IQGAP1 or APC by RNAi can result in the inhibition of the formation of an actin meshwork at the leading edge, cell migration, immobilization of the plus-ends of microtubules, and polarization of the MTOC (Noritake et al., 2005). In this study, the cell migration speed was not significantly affected by the EFs, and most cells showed directional migration. The findings in our study suggest that different pathways regulate Schwann cell migration in EFs.

In summary, in this study, we investigated the migration of Schwann cells in an applied electric field. We found that the directedness and displacement of anodal migration of Schwann cells increased significantly when the EF strength increased from 50 mV/mm to 200 mV/mm. The EF did not significantly affect the speed of cell migration. To explore the genes and signaling pathways that regulate the cell migration in EFs, we performed a comparative analysis of differential gene expression between cells with an EF (100 mV/mm)

stimulation and those without, using the next-generation RNA sequencing approach and verified by RT-qPCR. We discovered and validated the gene transcriptome of the control Schwann cells and the cells treated with EFs. We identified comprehensive genes that are involved in the regulation of directional migration and cell motility of Schwann cells in EFs. In future studies, the effect of the target genes on EF-guided cell migration will be determined further by pharmaceutical and genetic manipulation of individual genes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work was supported by Li Yao's start-up funding at Wichita State University, the National Center for Research Resources (P20 RR016475), the Kansas Intellectual and Developmental Disabilities Research Center, the National Institute of General Medical Sciences (P20 GM103418) from the National Institutes of Health, and an Institutional Development Award (IDeA) from the National Institute of General Medical Sciences of the National Institutes of Health under grant number P20 GM103418. The authors would acknowledge Clark Bloomer and Roseann Skinner of the KUMC Genomics Core for sequencing test.

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Figure 1.

Schwann cells migrate anodally in electric fields. (A–C) Random migration of cells during four-hour period without EFs stimulation. See also Supplementary Material Video 1. (E–G) Schwann cell migration in EFs (100 mV/mm). Cell migrated randomly without EFs stimulation in the first two hours. Cells migrated to the anode (left) with EF stimulation in the following two hours. See also Supplementary Material Video 2. Outlines of labeled cells in (B) and (F) highlight cell positions in (A) and (E), respectively. Outlines of labeled cells in (C) and (G) highlight cell positions in (B) and (F), respectively. Arrows show the directional migration of cells in EFs. Scale bar: 50 μ m. Figures (D) and (H) are schematics showing relative cell translocation and migration direction demonstrated in Figures (A–C) and (E–G), respectively. Blue lines indicate cell migration without EFs stimulation in first

two hours, red lines indicate cell migration with EFs stimulation in following two hours, dot represents cell center, and arrow represents cell migration direction. (I–L) Net translocation of Schwann cells during two-hour migration period in EFs. Migration paths determined by video monitor tracings. Position of all cells at $t = 0$ min is represented by origin position (center of frame), with migratory track of each cell at 120 min plotted as single line on graph. Each arm of axes represents 120 μm of translocation distance. (I) 0 mV/mm, (J) 50 mV/mm, (K) 100 mV/mm, (L) 200 mV/mm. (M–P) Circular histograms of cells in (I–L) correspondingly. Biased distribution of Schwann cells increased when EF increased from 0 mV/mm to 200 mV/mm. Interval range is 10 degrees. The tracks of anodal migration and cathodal migration of the cells are labeled with black and red colors respectively.

Figure 2.

Directedness, net displacement, and migration speed of Schwann cells in EFs. Figures (A), (B), and (C) show migration directedness, net displacement, and migration speed in a total period of two hours, which is composed of one-hour random migration and the following one-hour migration in EFs. Figures (D), (E), and (F) show migration directedness, net displacement, and migration speed in a total period of four hours, which is composed of two-hour random migration and the following two-hour migration in EFs. Average cosine (directedness), and net displacement increased toward the anode pole when field strength increased from 50 mV/mm to 200 mV/mm or when stimulation time was increased from one to two hours. The *, $p < 0.05$, is compared with cell migration before EF stimulation is switched on.

Figure 3.

Reversal of migration direction of Schwann cells with reversal of EF vectors. (A) Cell migration to anode pole from EF of 100 mV/mm under 2 hours of EF. (B) Reversed migration of same cells in EF of 100 mV/mm from 2 to 4 hours EF. (C) and (D) Circular histograms for cells in (A) and (B), respectively. Migrated Schwann cells show clear biased distribution toward the anode in EFs (100 mV/mm), indicating anodal migration of cells. Range of interval is 10 degrees. (E) Reversal of directedness and (F) reversal of net displacement of cell migration when EF pole is switched to opposite direction. (G) No significant change in cell migration rates before and after EF pole reversal. The tracks of anodal migration and cathodal migration of the cells are labeled with black and red colors respectively.

Figure 4.

Quality analysis of the RNA-seq results. (A) Principal components analysis of normalized expression values of genes for separation of control and EF-stimulated cell samples. (B) Unsupervised two-dimensional hierarchical clustering of differentially expressed genes showing the separation of control and EF-stimulated cell samples. (C) MA-plot showing mean expression across libraries compared to log2 fold change between conditions for all genes. Significantly deregulated genes shown as red dots. (D) Histogram showing significant FDR value. All plots generated with EdgeR.

Figure 5.

Verification of gene expression analysis of RNA-Seq by qRT-PCR. The genes (shown in supplemental materials table 2) selected for validation of RNA-Seq result were Areg, Cldn11, Errfi1, Nr4a3, Prrt2, Bcl3, Fam101a, Prdm1, Ptx3, Syt4, Serpinb2, Pik3r5, Pik3c2b, Map2k6, and Cdc42ep2.

Figure 6. KEGG pathway analysis of differentially expressed genes in multiple cellular signaling pathways involved in cell migration. (A) Up-regulated signaling pathways. (B) Down-regulated signaling pathways.

Figure 7.
Signaling pathway of focal adhesion. Red-color-labeled boxes show down-regulated genes. Blue-color-labeled boxes indicate up-regulated genes. Purple-color-labeled boxes contain both up- and down-regulated genes.

Figure 8.
 Signaling pathway of regulation of actin cytoskeleton. Red-color-labeled boxes show down-regulated genes. Blue-color-labeled boxes indicate up-regulated genes. Purple-color-labeled boxes contain both up- and down-regulated genes.