Robust temporal changes of cellular senescence and proliferation after sciatic nerve injury

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Abstract
Cellular senescence and proliferation are essential for wound healing and tissue remodeling. However, senescence-proliferation cell fate after peripheral nerve injury has not been clearly revealed. Here, post-injury gene expression patterns in rat sciatic nerve stumps (SRP113121) and L4–5 dorsal root ganglia (SRP200823) obtained from the National Center for Biotechnology Information were analyzed to decipher cellular senescence and proliferation-associated genetic changes. We first constructed a rat sciatic nerve crush model. Then, β-galactosidase activities were determined to indicate the existence of cellular senescence in the injured sciatic nerve. Ki67 and EdU immunostaining was performed to indicate cellular proliferation in the injured sciatic nerve. Both cellular senescence and proliferation were less vigorous in the dorsal root ganglia than in sciatic nerve stumps. These results reveal the dynamic changes of injury-induced cellular senescence and proliferation from both genetic and morphological aspects, and thus extend our understanding of the biological processes following peripheral nerve injury. The study was approved by the Animal Ethics Committee of Nantong University, China (approval No. 20190226-001) on February 26, 2019.

Key Words: bioinformatic analysis; cellular senescence; dorsal root ganglia; p16; peripheral nerve regeneration; peripheral nerve trauma; proliferation; rat sciatic nerves; sciatic nerve crush; β-galactosidase activities

Introduction
Peripheral nerve trauma is a universal public health issue that causes neuropathic pain and nerve dysfunction, and seriously affects patients’ quality of life. Following peripheral nerve injury, immune cells are recruited to damaged nerve stumps and dorsal root ganglia (Scholz and Woolf, 2007; Li et al., 2021; Zhang et al., 2021). Schwann cells and activated immune cells in the injured nerve stumps promote debris clearance, microenvironment reconstruction, and axon elongation (Chen et al., 2015). Injury signals that are dictated by the retrograde transport of calcium and signaling molecules activate the intrinsic growth capacity of neurons and advance nerve regeneration (Mahar and Cavalli, 2018). Emerging studies have demonstrated the importance of cell recruitment and activation during peripheral nerve regeneration (Jessen and Mirsky, 2016; Liu et al., 2019); however, the involvement of cellular senescence remains largely undetermined.

Cellular senescence is time-related cellular function degeneration (Schmeer et al., 2019). It is a stable cell cycle arrest state that is triggered by cellular insults, including telomere shortening, irradiation, oncogene activation, genmic or epigenomic damage, and tissue injury (He and Sharpless, 2017). Cellular senescence is considered as a driver and an important hallmark of aging (Hernandez-Segura et al., 2018). Notably, senescent cells secrete cytokines, chemokines, and proteinases, which are not only associated with the aging...
process, but also engaged in tissue remodeling and wound healing processes (Demaria et al., 2014; Guo et al., 2021). Senescence-associated secretory phenotype may stimulate the plasticity and stemness of cells. Therefore, transient senescence may have pro-regenerative functions (Calcino et al., 2019). However, persistent senescence may lead to chronic poorly healing wounds and be harmful for tissue remodeling and regeneration (Calcino et al., 2019; Wang and Shi, 2020). To discover the potential management of the regeneration of injured nerves, it is important to investigate cellular senescence status after peripheral nerve injury.

When the term was initially proposed, cellular senescence was described as the finite proliferative capacity of cells (Hayflick and Moorhead, 1961). Cellular proliferation, by the contrast, indicates the initiation of the cell cycle and increase of cell numbers. Cellular proliferation normally occurs within days after tissue injury and continues throughout the regeneration process. Proliferation of cells largely contributes to tissue augmentation and wound healing (Flanagan, 2000; Velnar and Gradinsnik, 2018). It has been demonstrated that, in the peripheral nervous system, molecules that modulate cellular proliferation can influence the regeneration and function recovery of injured peripheral nerves (Guseva et al., 2009; Li et al., 2015; Yi et al., 2019).

Considering the significant involvement of cellular senescence and proliferation in the wound healing process, in this study, we determined the genetic changes of cellular senescence and proliferation after sciatic nerve trauma by analyzing high-throughput data of sciatic nerves and dorsal root ganglia post-injury.

Materials and Methods

Bioinformatic analysis

High-throughput data of RNA in male Sprague-Dawley rat sciatic nerve stumps (0, 1, 4, 7, and 14 days post-injury) and L4–5 dorsal root ganglia (0, 3, 9 hours, 1, 4, and 7 days post-injury) were preserved in the National Center for Biotechnology Information with accession numbers SRP113121 (Zhao and Yi, 2019) and SRP200823 (Shen et al., 2020), respectively. Gene expressions were achieved based on the reads per kilobase transcriptome per million mapped reads method (Mortazavi et al., 2008). A schematic network of the cellular senescence pathway was built based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (map04218; https://www.genome.jp/pathway/map04218). Genes with an absolute fold change value of > 2 as well as a P-value < 0.05 were designated as differentially expressed as compared with uninjured control genes. The patterns of relative gene expression levels in differentially expressed as compared with uninjured control values of > 2 as well as a

Table 1 | List of antibodies used in this study

| Antibody | Source | Catalog number | Species | RRID | Dilution | Incubation time | Temperature |
|---------|--------|----------------|---------|------|----------|----------------|-------------|
| Ki67    | Abcam, Cambridge, MA, USA | ab16667 | Rabbit | AB_302459 | 1:500 | Overnight | 4°C |
| p16     | Signalway Antibody, College Park, MD, USA | 32050 | Rabbit | AB_302459 | 1:200 | Overnight | 4°C |
| CD68    | Abcam, Cambridge, MA, USA | ab31630 | Mouse | AB_1141557 | 1:100 | Overnight | 4°C |
| P4HB    | Abcam, Cambridge, MA, USA | ab2792 | Mouse | AB_303304 | 1:100 | Overnight | 4°C |
| S100B   | MilliporeSigma, St Louis, MO, USA | SA00009-2 | Mouse | AB_477499 | 1:400 | Overnight | 4°C |
| Cy3 goat anti-rabbit IgG (H+L) | Proteintech Group, Rosemont, IL, USA | SA10009-2 | Goat | AB_2890957 | 1:400 | 2 h | Room temperature |
| Alexa Fluor 488 goat anti-mouse IgG (H+L) | Abcam, Cambridge, USA | ab150117 | Goat | AB_2688012 | 1:400 | 2 h | Room temperature |

P4HB: Prolyl 4-hydroxylase subunit beta. 
5-Ethynyl-2′-deoxyuridine proliferation assay

5-Ethynyl-2′-deoxyuridine (EdU), a thymidine analogue that can incorporate into cellular DNA during DNA replication (Harris et al., 2018), was intraperitoneally injected into rats using an imaging kit (EdU Alexa Fluor® 647; Invitrogen, Carlsbad, CA, USA). EdU was injected at a concentration of 5 mg/kg 24 hours prior to sciatic nerve and dorsal root ganglion tissue collection. Collected tissue sections were exposed to rabbit anti-S100β (primary antibody, 1:400; Cat# ab52642, RRID AB_2890957, Proteintech Group, Rosemont, IL, USA) for 2 hours at room temperature. DAPI Fluoromount-G was applied to stain the nuclei. EdU visualization was performed using a fluorescence microscope.

Real-time polymerase chain reaction

Gene expression quantification was determined in rat sciatic nerves using SYBR Premix Ex Taq (TaKaRa, Dalian, China) using the 2(-ΔΔCT) method (Livak and Schmittgen, 2001) on an Applied Biosystems StepOne Real-Time system (Applied Biosystems, Foster City, CA, USA). Information of primers is shown in Table 2.

Table 2 | List of primer sequences used in this study

| Full name                          | Abbreviation | Forward | Reverse |
|------------------------------------|--------------|---------|---------|
| Cyclin dependent kinase inhibitor 1A | Cdkn1a       | 5′-GGG GAG TCC CGA TGT GTT CC-3′ | 5′-AGC CTC CCA GCC TGT ATC-3′ |
| Cyclin dependent kinase inhibitor 2A | Cdkn2a       | 5′-AAC ACT TCT GGT CGT ACC CC-3′ | 5′-CCC AGG GGA GGA GAG TAG AT-3′ |
| ATM serine/threonine kinase | Atm          | 5′-AGG TGT GAA ATG CAG ACG T-3′ | 5′-GAA CCG GGC TTA TGA GAG GA-3′ |
| Cyclin dependent kinase 1 | Cdk1         | 5′-AGG ACT CCA GCC TGT ATC TCA CTT-3′ | 5′-TAT CGG TAT TCC GAA CGG TCT-3′ |
| Minichromosome maintenance complex component 5 | Mcm5        | 5′-TGA GAC AAA AGG GGA GCA CA-3′ | 5′-GCT CCG CAG GAA ATG AA-3′ |

Cyclin F | Ccnf | 5′-CAC TGG TCT CTC CTA CAG CG-3′ | 5′-TCT CTC TCT TCG TCT TCC GTT TGC T-3′ |
Dihydropyrimidine reductase | Dhr | 5′-GCA AGA AGC GAG ACT TAC CCT-3′ | 5′-GTT TCC TCA CCC ATC ACC AG-3′ |
TIMP metalloproteinase inhibitor 1 | Timp1 | 5′-CTT CTG GCA TCC TCT TGT TG-3′ | 5′-CTG CGA ATC TCT TGA GCA TCT-3′ |
Glyceraldehyde-3-phosphate dehydrogenase | Gapdh | 5′-ACA GCA ACA GGG TGG TGG AC-3′ | 5′-TTT GAG GGT GGC GCA AAC AT-3′ |

Statistical analysis

The sample size was estimated from preliminary experiments. Data were summarized from three independent experiments and are presented as the mean ± standard error of the mean (SEM). One-way analysis of variance with Dunnett’s multiple comparisons post hoc test was performed to compare the differences among injured nerve stumps and the uninjured control (day 0) group using GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA). Significant differences were those with a P-value < 0.05.

Results

Characterization of cellular senescence in rat sciatic nerves post-injury

On investigating the involvement of cellular senescence post-injury, KEGG results showed that stress signals elicit the activation of various intracellular cascades, including mitogen-activated protein kinases and checkpoint kinases, suppress the activation of cyclin-dependent kinases, induce an irreversible cell-cycle arrest, and cause cellular senescence (Figure 1A). The abundances of genes involved in the cellular senescence pathway in intact or injured rat sciatic nerves were screened according to previously obtained sequencing data (0, 1, 4, 7, and 14 days post-injury; Yi et al., 2015) to identify significantly differentially expressed genes. MEK (MAP2K1, mitogen-activated protein kinase 1) and GADD45G (growth arrest and DNA damage inducible gamma) were increased at 1 day post-injury, CDKN1A p21 (cyclin-dependent kinase inhibitor 1A), CDKN2A p16 (cyclin-dependent kinase inhibitor 2A), ATM (ataxia-telangiectasia mutated), RAD1 (cell cycle checkpoint protein RAD1), and CHK1/2 (checkpoint kinase 1/2) were decreased post-injury. Increased amount of these genes might contribute to cellular senescence. On the contrary, FOXO3 (forkhead box O3), a gene that functions as a trigger for cellular apoptosis, was slightly decreased at day 1 post-injury. Down-regulated FOXO3 might exert an inhibition effect on cellular senescence. Moreover, many cell cycle-related genes, including CDC25A (cell division cycle 25A), CDK1/2/4 (cyclin-dependent kinase 1/2/4), CCND1 (cyclin D1), CCNA2 (cyclin A2), and CCNE1 (cyclin E1), showed elevated expressions, which indicates that cell division was robustly activated in the injured nerve stumps (Figure 1A).

The dynamic patterns of genes in KEGG cellular senescence showed that genes coding for essential factors of senescence, such as p16 and p21, were up-regulated post-injury. Genes coding for checkpoint kinases, cyclin-dependent kinases, and cyclin family members were also increased (Figure 1B).

Other than the identification of transcriptome signatures, sciatic nerve sections were subjected to immunostaining to visualize β-galactosidase activity. Some β-galactosidase signals could be observed in the day 0 control group. The signals of β-galactosidase seemed be attenuated at day 1 post-injury. However, much more intense signals were detected at later time points, especially at day 4 and day 7 post-injury (P < 0.05 at days 4 and 7; Figure 1C and D).

Gene expressions were additionally examined using real-time polymerase chain reaction (RT-PCR). RT-PCR results showed elevated mRNA expression levels of Cdkn1a, Cdkn2a, Atm, and Cdk1 in the injured sciatic nerves as compared with the day 0 group (P < 0.05; Figure 2). These observations, together with sequencing outcomes, indicated a significant involvement of senescence-associated genes post-injury.

Immunopositivity of cell senescence marker p16 (Uyar et al., 2020) in rat sciatic nerve stumps were further examined using immunostaining. Consistent with the β-galactosidase signals, the immunopositivity of p16 seemed to be robustly increased at multiple time points post-injury, particularly at days 4 and 7, as compared with day 0 (Figure 3A–C). Sciatic nerve stumps were further co-immunostained with p16 and Schwann cell marker S100β (Zhang et al., 2021a ) (Figure 3A), macrophage marker CD68 (Alves et al., 2018) (Figure 3B), as well as fibroblast marker P4HB (Schmid et al., 2020) (Figure 3C). Co-immunostaining of injured sciatic nerve stumps with p16 and S100β showed that the number of Schwann cells first decreased and then gradually increased post-injury, and the number of p16-positive Schwann cells was increased at days 4 and 7 post-injury as compared with the day 0 control (Figure 3A). Immunostaining with CD68 indicated that the number of macrophages was greatly increased immediately post-injury (day 1 post-injury), while the number of p16-positive macrophages seemed to be elevated at 4 and 7 days (Figure 3B). p16-positive fibroblasts also appeared to be increased post-injury (Figure 3C).
Characterization of cellular senescence in rat dorsal root ganglia post-injury

Cellular senescence status of dorsal root ganglia was also examined according to genetic and morphological aspects. Gene expression levels in rat dorsal root ganglia were screened according to previously obtained sequencing data (Gong et al., 2016). Gene changes were less robust in dorsal root ganglia than in injured sciatic nerves. Only GADD45α, p21, RAD9 (cell cycle checkpoint protein RAD9), and CycD (cyclin D1) were significantly altered post-injury (Figure 4A). In addition, many genes in dorsal root ganglia showed reduced expressions post-injury (Figure 4B).

The expression levels of genes involved in the cellular senescence-associated GO process (GO:0009398) post-injury were also analyzed based on sequencing data. The results demonstrated dysregulation of many cellular senescence-related genes in rat sciatic nerve stumps post-injury (Additional Table 1), but the expressions of only a few related genes were altered in dorsal root ganglia (Additional Table 2). Moreover, the abundance of senescence-associated secretory phenotype genes was further determined. Many genes were dysregulated, especially up-regulated, in sciatic nerves post-injury. The expression patterns of senescence-associated genes after sciatic nerve injury using real-time quantitative polymerase chain reaction. (A) The schematic diagram of cellular senescence pathway. The diagram was modified from the Kyoto Encyclopedia of Genes and Genomes (KEGG) map04218. Significantly increased gene levels are presented in red, while decreased gene levels are presented in green. (B) Patterns of genes in KEGG cellular senescence signaling post-injury. Relative highly-expressed genes are presented in blue, while relative weakly-expressed genes are presented in yellow. (C) β-galactosidase staining of sciatic nerve stumps (crush sites). Elevated β-galactosidase staining was observed after nerve injury. Scale bars: 50 μm (left) and 20 μm (right). (D) Relative positive areas of senescence-associated (SA)-β-galactosidase in sciatic nerve stumps. Relative positive areas were normalized by the day 0 group data. Data are expressed as the mean ± SEM (n = 3). *P < 0.05, vs. day 0 group (one-way analysis of variance followed by Dunnett’s multiple comparisons post hoc test). The experiments were repeated three times. ATM: ATM serine/threonine kinase; ATR: ATM serine/threonine kinase; AKT: AKT serine/threonine kinase; CCNE1: cyclin E1; CDCA5: cyclin dependent kinase 5A; CDK: cyclin dependent kinase; CHEK: CHK, checkpoint kinase; Ccna2, cyclin A2; CycB: CCNB1, cyclin B1; CycD: CCND2, CYCLIN D2; GADD45: growth arrest and DNA damage inducible; ERK: mitogen-activated protein kinase 1; Ets1: ETS proto-oncogene 1, transcription factor; FOXO3: forkhead box O3; HUS1: HUS1 checkpoint clamp component; MAPK1, mitogen-activated protein kinase 1; MAP2K: mitogen-activated protein kinase kinase; MEK: mitogen-activated protein kinase kinase 1; MKK3: mitogen-activated protein kinase kinase 3; MRE11: MRE11 homolog, double strand break repair nucleotide; mTOR: mechanistic target of rapamycin kinase; NBS1, nibrin; p16: CDKN2A, cyclin dependent kinase inhibitor 2A; p21: CDKN1A, cyclin dependent kinase inhibitor 1A; p38: mitogen-activated protein kinase 14; p53: tumor protein P53, PIK3CA, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha; PI3K: phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha; RAD1: RAD1, Rad related glycosylase inhibitor and calcium channel regulator; RAD9: RAD9 checkpoint clamp component; Raf: Raf-1 proto-oncogene, serine/threonine kinase; Ras: KRAS proto-oncogene, GTPase; RRAS2: RAS related 2; SIRT1: sirtuin 1. NEURAL REGENERATION RESEARCH

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Morphological immunostaining of β-galactosidase revealed the presence of cellular senescence in dorsal root ganglia of both uninjured and injured rats. Consistent with sequencing data that showed that the gene expressions of few cellular senescence-related genes were significantly changed in dorsal root ganglia, signals of β-galactosidase in dorsal root ganglia were not obviously altered post-injury (Figure 4C).

Characterization of cellular proliferation in rat sciatic nerves post-injury

Given that senescence is typically associated with the loss of replicative potential (Birch et al., 2018), we investigated the cellular senescence, we also determined changes of cellular proliferation using sequencing data of sciatic nerves after nerve injury. Here, the temporal expression profiles of a series of proliferation marker genes were investigated to determine cellular proliferation status (Whitfield et al., 2006). Many proliferation marker genes, including MKI-67, which is a gene coding for proliferating cell nuclear antigen Ki67, were elevated following peripheral nerve injury, especially at days 1 and 4 (Figure 5A).

Immunostaining of sciatic nerve stumps also revealed higher abundances of Ki67 than in uninjured nerves (P < 0.05 at day 7, Figure 5B and C). Sciatic nerves were co-immunostained with S100β, a marker of Schwann cells. Some co-localized S100β and Ki67 signals were detected in Ki67-positive cells at days 0-14 (Figure 5B and C).

To further visualize cell proliferation status, rats were injected with EdU. EdU incorporation was observed in day 0 sciatic nerve samples. Stronger EdU signals were detected after nerve injury. Summarized data showed that the relative number of EdU co-localized with DAPI increased at days 1, 4, and 7 post-injury (P < 0.05) and recovered to baseline levels at day 14 (Figure 5C-E). Immunostaining outcomes, consistent with genetic signatures, showed that peripheral nerve injury induced cellular proliferation at injured sites, especially at early time points.

Figure 4 | Cellular senescence signaling in rat dorsal root ganglia post-injury.

(A) A schematic diagram of the cellular senescence pathway. The cellular senescence pathway diagram was modified from the KEGG cellular senescence signaling (map04218). Increased gene levels are presented in red, while decreased gene levels are presented in green. (B) Expression patterns of genes in KEGG cellular senescence signaling in rat dorsal root ganglia. Relative highly-expressed genes are presented in blue, while relative weakly-expressed genes are presented in yellow. (C) β-galactosidase staining of dorsal root ganglia was presented. Cellular senescence in rat dorsal root ganglia were not obviously altered after trauma to peripheral nerves. Scale bars: 50 μm (left) and 20 μm (right). The experiments were repeated three times. ACT: AKT serine/threonine kinase; ATM: ATM serine/threonine kinase; ATR: ATR serine/threonine kinase; CCNE1: cyclin E1; CDCA5A: cell division cycle 25A; CDK: cyclin dependent kinase; CHEK: CHEK, checkpoint kinase; CYC: CCNB1, cyclin B1; CYCL: CCND2, cyclin D2; CYCA: CCNA2, cyclin A2; ERK: mitogen-activated protein kinase 1; ETS: ETS proto-oncogene 1, transcription factor; FGF3: fibroblast growth factor 3; GADD45A: growth arrest and DNA damage inducible; H352: H352, checkpoint clamp component; KEGG: Kyoto Encyclopedia of Genes and Genomes; MAPK2: mitogen-activated protein kinase; MAFK: mitogen-activated protein kinase 1; MEK: mitogen-activated protein kinase kinase; MKK3: mitogen-activated protein kinase kinase 3; MRE11: MRE11 homolog, double strand break repair nuclease; mTOR: mechanistic target of rapamycin kinase; NBN: NBS1, nibrin; p53: tumor protein P53; PI3K: phosphatidylinositol-4,5-bisphosphate 3-kinase; PI4K: phosphatidylinositol-4-kinase; RAF1: Raf-1 proto-oncogene, serine/threonine kinase; Ras: Ras; ROB1: RAD1, RAD1 homolog, double strand break repair protein; S100B: S100 beta protein; SPRY4: sprouty related evolutionarily conserved protein 4; TAF1: TAF1, TBP associated factor 1; TNF: tumor necrosis factor; TSC2: tuberous sclerosis complex 2; UNG: uracil DNA glycosylase.

Figure 5 | Cell proliferation in sciatic nerves post-injury.

(A) Expression patterns of proliferation marker genes in rat sciatic nerves post-injury. Relative highly-expressed genes are presented in blue, while relative weakly-expressed genes are presented in yellow. (B) Co-immunostaining of Ki67 and S100β in rat sciatic nerves. Ki67-positive cells are presented in red (stained by Cy3), while S100β-positive cells are presented in green (stained by Alexa Fluor 488). Co-immunostained cells are marked by arrowheads in white. The cell nucleus is labeled in blue. The number of Ki67-positive cells was increased after injury. (C) Relative numbers of Ki67 and DAPI co-localized cells. An increased number of Ki67 cells colocalized with S100β. (D) Number of EdU-positive cells are presented in green (stained by Alexa Fluor 488). Co-immunostained cells are marked by arrowheads in white. The cell nucleus is labeled in blue. The number of EdU-positive cells was increased after injury. Scale bars: 200 μm. (E) Relative numbers of EdU and DAPI co-localized cells. An increased number of EdU colocalized with DAPI was observed after nerve injury, as compared with day 0 (control). Data in C and E are expressed as the mean ± SEM (n = 3). *P < 0.05, **P < 0.01, ***P < 0.001, vs day 0 group (one-way analysis of variance with Dunnett's multiple comparisons post hoc test). The experiments were repeated three times. AURKB: Aurora kinase B; AURKA: Aurora kinase A; BIRC5: baculoviral IAP repeat containing 5; BUB1: BUB1 mitotic checkpoint serine/threonine kinase; CCNA2: cyclin A2; CCNB1: cyclin B1; CCNE1: cyclin E1; CCNF: cyclin F; CDC2: cell division cycle 2; CDC25A: cell division cycle 25A; CDK: cyclin dependent kinase; CHEK: CHEK, checkpoint kinase; CYC: CCNB1, cyclin B1; CYCL: CCND2, cyclin D2; CYCA: CCNA2, cyclin A2; ERK: mitogen-activated protein kinase 1; ETS: ETS proto-oncogene 1; FGF3: fibroblast growth factor 3; GADD45A: growth arrest and DNA damage inducible; H352: H352, checkpoint clamp component; KEGG: Kyoto Encyclopedia of Genes and Genomes; MAPK2: mitogen-activated protein kinase; MAFK: mitogen-activated protein kinase 1; MEK: mitogen-activated protein kinase kinase; MKK3: mitogen-activated protein kinase kinase 3; MRE11: MRE11 homolog, double strand break repair nuclease; mTOR: mechanistic target of rapamycin kinase; NBN: NBS1, nibrin; p53: tumor protein P53; PI3K: phosphatidylinositol-4,5-bisphosphate 3-kinase; PI4K: phosphatidylinositol-4-kinase; RAF1: Raf-1 proto-oncogene, serine/threonine kinase; Ras: Ras; ROB1: RAD1, RAD1 homolog, double strand break repair protein; SPRY4: sprouty related evolutionarily conserved protein 4; TAF1: TAF1, TBP associated factor 1; TNF: tumor necrosis factor; TSC2: tuberous sclerosis complex 2; UNG: uracil DNA glycosylase.
Similar to the senescence-associated genes, the temporal expressions of proliferation-associated genes were also validated using RT-PCR. RT-PCR showed that, consistent with RNA deep sequencing outcomes, mRNA expression levels of Mcm5 (minichromosome maintenance complex component 5), Ccnf (cyclin F), Dhfr (dihydrofolate reductase), and Timp1 (TIMP metalloproteinase inhibitor 1) were augmented in sciatic nerves post-injury (Figure 6).

Characterization of cellular proliferation in rat dorsal root ganglia post-injury

Cellular proliferation in rat dorsal root ganglia was assessed based on sequencing data of dorsal root ganglia at 0, 3, 9 hours, 1, 4, and 7 days after nerve injury (Gong et al., 2016). Unlike sciatic nerve stumps, the majority of proliferation marker genes, including Mki-67, were not significantly changed. Only UNG (uracil-DNA glycosylase) and Ccnf were elevated at 1, 4 and 7 days post-injury, Mcm5 and Dhfr were elevated at 4 and 7 days, and Timp1 was elevated at 4 days. Some proliferation marker genes even exhibited reduced expressions. For instance, in dorsal root ganglia, Plk1 (polo-like kinase 1) was down-regulated at 9 hours post-injury and Mapk13 (mitogen activated protein kinase 13) was down-regulated at 1 day post-injury (Figure 7A).

Ki67 and Edu staining showed that although a larger number of proliferating cells appeared to exist, changes of cellular proliferation in dorsal root ganglia were less noticeable than in sciatic nerves (Figure 7B and C).

Discussion

The degeneration and regeneration of peripheral nerves involve various sophisticated biological activities, including inflammatory and immune responses, as well as cellular growth and organ development (Yu et al., 2016; Yi et al., 2017). Together with strong and sustained inflammatory and immune responses, enhanced expressions of growth factors, proinflammatory cytokines, and matrix metalloproteinases have been observed (Xing et al., 2017; Zhang et al., 2019). These features are typical characteristics of cellular senescence (Hubackova et al., 2012; Pan et al., 2017). Observations of these senescence-associated phenotypes demonstrated the essential participation of cellular senescence post-injury. Using high-throughput data and KEGG pathway analysis, cellular senescence-associated gene changes were determined in this study. Sequencing data showed that Cdkn2a was elevated by nearly 4 fold at 4 and 7 days post-injury and remained highly expressed at around 2.5-fold versus its expression in uninjured nerve stumps at 14 days post-injury. Cdkn1a was also had a 2-fold increase from day 1 to day 7 post-injury. These findings suggest that p16 and p21 pathway-associated cellular senescence might be
induced by peripheral injury stress. β-Galactosidase activity was also found to increase at 4 and 7 days post-injury, which reveals the involvement of cellular senescence in the nerve regeneration process. Depressed β-galactosidase activity at 1 day post-injury might be due to reduced total cell populations at the injured site.

Meanwhile, many genes that promote cell cycle transition rather than cell cycle arrest, including CDK1, CDK2, and CDK4/6, were increased post-injury. The up-regulation of these cell cycle initiation-related genes indicates that, besides cell senescence, cell proliferation might be extensively involved in the peripheral nerve regeneration process. Consistent with this interpretation, the investigation of cell proliferation marker expressions revealed the importance of cell proliferation. These findings demonstrate the complexity of cellular transitions post-injury.

Other than genes in the canonical cellular senescence pathway, transcriptome profiles of consistently up-regulated or down-regulated senescence marker genes were also evaluated (Casella et al., 2019). A total of elevated 29 genes in senescence were discovered in rat sciatic nerve stumps. Only some of these genes, such as Slc5a7 (solute carrier family 9 member A7), Srp2 (sushi repeat-containing protein X-linked 2), Elmod1 (ELMO domain-containing protein 1), Stat1 (signal transducer and activator of transcription 1), Wdr63 (dyeaxen axonemal intermediate chain 3), Tap1 (transporter 1, ATP binding cassette subfamily B member), and Dio2 (iodothyronine deiodinase 2), showed an elevated expression post-injury, while the expressions of other genes, such as Dhrs7 (dehydrogenase/reductase 7), Pam (peptidylglycine alpha-amidating monooxygenase), Slc16a14 (solute carrier family 9 member A14), and Cldn1 (claudin 1), were decreased (Additional Figure 1A). The expression patterns of senescence marker genes that were down-regulated in senescence were also determined. However, many of these genes did not show expression reduction (Additional Figure 1B). A possible explanation could be that these senescence marker genes were identified in human cells (i.e., human umbilical vein endothelial cells, diploid fibroblasts, and aortic endothelial cells) and might not accurately reflect genetic changes in rats. The irregularity of senescence marker gene expressions could also be due to the mixed status of cellular senescence and proliferation at the injured site post-injury.

Cellular senescence/proliferation was also examined in dorsal root ganglia, because dorsal root ganglia receive retrograde transport of signals from the injury site and respond to injury signals (Abe and Cavalli, 2008; Allodi et al., 2012). Following peripheral nerve injury, senescence mainly occurred at the injured site, and the temporal dynamic changes of senescence/proliferation-associated genes in dorsal root ganglia were less robust than changes in sciatic nerves.

One limitation of the current study is that singles of β-galactosidase, Ki67, and EdU immunostaining in dorsal root ganglia were not quantified. Nonetheless, representative images also showed that changes of senescence-associated β-galactosidase immunostaining and proliferation-associated Ki67 and EdU immunostaining were not apparent in dorsal root ganglia.

Our results revealed that β-galactosidase signals decreased 7 days post-injury in sciatic nerves and dorsal root ganglia. The presence of transient senescent cells has been reported in the young adult rodents and acute senescent cells in adult rodents. These cells may be cleared via immunosurveillance after having executed their programmed function (Childs et al., 2014). In addition, following nerve injury, immune cells are quickly recruited to injured sites (Benowitz and Popovich, 2011; Chen et al., 2015). Therefore, the reduced signal of senescence cells may also be due to immune clearance.

Tissue sessions were co-immunostained with Ki67 and S100β to identify the proliferation status of Schwann cells. In the peripheral nervous system, Schwann cells are unique and essential glial cells that contribute to regeneration (Ma et al., 2016; Min et al., 2021). Indeed, transplantation of Schwann cells to injured sites has been successfully used to repair peripheral nerve defects (Hood et al., 2009). The colocalization of Ki67 and S100β at the injured site suggests that many Schwann cells undergo proliferation post-injury. Other than sciatic nerve stumps, Schwann cells are also important cell populations in dorsal root ganglia (Steffen et al., 2018). Perineuronal satellite cells of the dorsal root ganglia are generally considered as a subtype of Schwann cells (Armati and Mathey, 2013). Unlike sciatic nerve stumps, only a few Ki67-positive cells were identified in dorsal root ganglia, and the growth conditions of Schwann cells in dorsal root ganglia were not clearly observed. Combined with previous findings (Qian et al., 2018), our results further our understanding of the dynamic changes of major cell types in peripheral nerves, cellular senescence, and cell proliferation during sciatic nerve injury and regeneration. Overall, the current study reveals more about injury-induced senescence and proliferation cell fate, and contributes to our understanding of the cellular changes that occur following peripheral nerve injury.

Author contributions: Study conception and design: SYL, SY; experiment implementation and data analysis: YS, RRZ, QYL, SY; reagents/materials/analysis support: SYL, SY; manuscript writing: SYL, SY. All authors declare that they have no conflicts of interest.

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Additional files:

Additional file 1: Open peer review reports 1–3.

Additional Table 1: Expression levels of genes involved in cellular senescence process in rat sciatic nerve stumps post-injury.

Additional Table 2: Expression levels of genes involved in cellular senescence process in rat dorsal root ganglia post-injury.

Additional Table 3: Expression levels of senescence-associated secretory
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Additional Figure 1 Expression levels of senescence marker genes that were elevated (A) and reduced (B) in senescence models in rat sciatic nerves.

The experiments were repeated by three times. ANP32B: Acidic nuclear phosphoprotein 32 family member B; ARRD4: arrestin domain containing 4; BLCAP: BLCAP apoptosis inducing factor; CBS: cystathionine beta-synthase; CBX2: chromobox 2; CCND3: cyclin D3; CDCA7L: cell division cycle associated 7 like; CHPF2: chondroitin polymerizing factor 2; CLDN1: claudin 1; CLSTN2: calsyntenin 2; DHR7: dehydrogenase/reductase 7; DIO2: iodothyronine deiodinase 2; ELMOD1: ELMO domain containing 1; FFAM129A: Niban apoptosis regulator 1; BL: fibrillarin; GPR155: G protein-coupled receptor 155; H2AFJ: H2A.J histone; H1.1 linker histone, cluster member; HIST1H1D: H1.3 linker histone, cluster member; HIST2H2AB: H2A clustered histone 2A; FBR: lamin B receptor; LRPI0: LDL receptor related protein 10; NCSTN: nicastrin; PAM: peptidylglycine alpha-amidating monoxygenase; PARP1: poly(ADP-ribose) polymerase 1; PDLM1: PDZ and LIM domain 1; PLOD1: procollagen-lysine, 2-oxoglutarate 5-dioxygenase 1; POFUT2: protein O-fucosyltransferase 2; PTMA: prothymosin alpha; SLC9A7: solute carrier family 9 member A7; SLC16A14: solute carrier family 16 member 14; SLC02B1: solute carrier organic anion transporter family member 2B1; SRPX: sushi repeat containing protein X-linked; STAT1: signal transducer and activator of transcription 1; RND3: Rho family GTPase 3; SSRP1: structure specific recognition protein 1; TAP1: transporter 1, ATP binding cassette subfamily B member; TMEM30A: transmembrane protein 30A; TNFSF13B: TNF superfamily member 13B; WDR: WD repeat domain.
| Gene ID | Symbol | Description | Accession | Expression Level | GO Component | GO Function | GO Process | Blast nr |
|--------|--------|-------------|-----------|-----------------|---------------|-------------|------------|---------|
| 347.3430126 | Pnnpt1 | protein kinase C, delta | gi|18959250|ref|NP_579841.1 | [Rattus norvegicus] | GO:0007293//RNA binding | GO:0005740//mitochondrial nucleotide binding | [Rattus norvegicus] |
| 311860 | Cav1 | C-terminus of alpha isoform | gi|189083698|ref|NP_113744 | [Rattus norvegicus] | GO:0003797//cytoplasmic membrane fusion | GO:0005743//mitochondrial membrane | [Rattus norvegicus] |
| 306571 | Prkcd | protein kinase C, delta | gi|11693172|ref|NP_071794 | [Rattus norvegicus] | GO:0002373//mitochondrial membrane | GO:0005743//mitochondrial membrane | [Rattus norvegicus] |
| 17085 | Abl1 | C-terminus of alpha isoform | gi|189083698|ref|NP_113744 | [Rattus norvegicus] | GO:0002373//mitochondrial membrane | GO:0005743//mitochondrial membrane | [Rattus norvegicus] |
| 17051 | B2m | beta-2 microglobulin | gi|7549746|ref|NP_036644.1 | [Rattus norvegicus] | GO:0003797//cytoplasmic membrane fusion | GO:0005743//mitochondrial membrane | [Rattus norvegicus] |
| 25404 | Cav1 | C-terminus of alpha isoform | gi|189083698|ref|NP_113744 | [Rattus norvegicus] | GO:0002373//mitochondrial membrane | GO:0005743//mitochondrial membrane | [Rattus norvegicus] |
| 30503 | Abl1 | C-terminus of alpha isoform | gi|189083698|ref|NP_113744 | [Rattus norvegicus] | GO:0002373//mitochondrial membrane | GO:0005743//mitochondrial membrane | [Rattus norvegicus] |
| 30503 | Abl1 | C-terminus of alpha isoform | gi|189083698|ref|NP_113744 | [Rattus norvegicus] | GO:0002373//mitochondrial membrane | GO:0005743//mitochondrial membrane | [Rattus norvegicus] |
| Gene | Description | Entrez ID | E-value | Benjamini | FDR | q-value |
|------|-------------|-----------|---------|-----------|-----|---------|
| Smc6 | Structural maintenance of chromosomes 6 | 15.57706201 | 1.60E-09 | 1.62E-09 | 1.80E-09 | 2.01E-09 |
| Zmp82 | Zinc metalloendopeptidase STE24 | 27.01621477 | 2.23E-09 | 2.78E-09 | 2.56E-09 | 2.35E-09 |
| Bnip1 | Bim homolog protein receptor, type IA | 17.78948543 | 1.48E-08 | 1.58E-08 | 1.58E-08 | 1.61E-08 |
| Ybx1 | Y box binding protein 1 | 10.89426686 | 1.10E-08 | 1.18E-08 | 1.19E-08 | 1.23E-08 |
| Smc5 | Structural maintenance of chromosomes 5 | 8.76132077 | 1.07E-08 | 1.12E-08 | 1.13E-08 | 1.15E-08 |
| PaI2 | Polly-lyk2 | 16.41713708 | 1.32E-08 | 1.37E-08 | 1.38E-08 | 1.41E-08 |
| Meo1 | Mecosyn | 20.23571504 | 1.23E-08 | 1.26E-08 | 1.27E-08 | 1.29E-08 |
| Map3k3 | Map 3k3 | 7.56821054 | 8.75E-08 | 8.82E-08 | 8.86E-08 | 8.90E-08 |
| Smc5 | Structural maintenance of chromosomes 5 | 8.40696627 | 2.61E-08 | 2.69E-08 | 2.71E-08 | 2.73E-08 |
| Rbf1 | Ribosomal L1 domain containing 1 | 10.64002404 | 1.89E-08 | 1.94E-08 | 1.95E-08 | 1.97E-08 |
| Sife | Serine receptor (t-o-s) receptor binding transcription factor | 7.91431970 | 8.19E-08 | 8.26E-08 | 8.27E-08 | 8.31E-08 |
| Ear1 | Earpsi extension translation elongation factor 1 epsilon 1 | 20.80630244 | 2.31E-08 | 2.34E-08 | 2.35E-08 | 2.37E-08 |
| Nau1 | Nau1 family, SNF1-like kinase 1 | 8.62072605 | 5.02E-09 | 5.07E-09 | 5.11E-09 | 5.17E-09 |
| Bodl | B-cell CLL/lymphoma 6 | 6.51515369 | 8.46E-09 | 8.46E-09 | 8.46E-09 | 8.46E-09 |
| Vhr1 | V box binding protein 1 | 12.5388116 | 1.75E-08 | 1.78E-08 | 1.80E-08 | 1.82E-08 |
Up-regulated genes as compared with the uninjured control (0 hour) were labeled in red color while down-regulated genes were labeled in green color.
| Gene Symbol | Description | GO Process | GO Component | GO Term | Expression Level  |
|-------------|-------------|------------|--------------|---------|------------------|
| Fgf7 | Fibrillin growth factor 7 | GO:0003672//proteinaceous extracellular matrix; GO:0005737//extracellular matrix; GO:0007626//cell-cell adhesion | GO:0030840//cell-cell adhesion mediator activity; GO:0008230//cell adhesion | GO:0003820//intracellular region | 1.72649e-48 |
| Kitlg | Kit ligand | GO:0005125//cytokine activity | GO:0015255//angiogenesis; GO:0001935//endothelial cell proliferation; GO:0002548//monocyte; GO:0048608//reproductive structure development; GO:0050767//regulation of neurogenesis | GO:0001525//angiogenesis; GO:0001935//endothelial cell proliferation; GO:0002548//monocyte; GO:0048608//reproductive structure development; GO:0050767//regulation of neurogenesis | 1.72649e-48 |
| Timp1 | TIMP metalloproteinase inhibitor 1 | GO:0019722//calcium-mediated signaling; GO:0021987//cerebral cortex substance; GO:0010038//response to metal ion; GO:0010033//response to organic; GO:0017899//response to metal ion; GO:0010038//response to metal ion; GO:0010033//response to organic; GO:0017899//response to metal ion; GO:0010038//response to metal ion; GO:0010033//response to organic | GO:0001525//angiogenesis; GO:0001935//endothelial cell proliferation; GO:0002548//monocyte; GO:0048608//reproductive structure development; GO:0050767//regulation of neurogenesis | GO:0001525//angiogenesis; GO:0001935//endothelial cell proliferation; GO:0002548//monocyte; GO:0048608//reproductive structure development; GO:0050767//regulation of neurogenesis | 1.72649e-48 |
| Il6st | interleukin 6 signal transducer | GO:0019722//calcium-mediated signaling; GO:0021987//cerebral cortex substance; GO:0010038//response to metal ion; GO:0010033//response to organic; GO:0017899//response to metal ion; GO:0010038//response to metal ion; GO:0010033//response to organic | GO:0001525//angiogenesis; GO:0001935//endothelial cell proliferation; GO:0002548//monocyte; GO:0048608//reproductive structure development; GO:0050767//regulation of neurogenesis | GO:0001525//angiogenesis; GO:0001935//endothelial cell proliferation; GO:0002548//monocyte; GO:0048608//reproductive structure development; GO:0050767//regulation of neurogenesis | 1.72649e-48 |
| Gene | Product | Source | Description |
|------|---------|--------|-------------|
| Vegfa | vascular endothelial growth factor A | | |
| Mif | | | |
| Igfbp2 | insulin-like growth factor binding protein 2 | | |
| Nppa | | | |
| Nppb | | | |
| Rptor | | | |
| Gadd45a | | | |

**Note:** The table above lists genes and their corresponding descriptions. For a more detailed analysis, consult the original text for specific information on each gene's role in biological processes and functions.
### Table 1: Regulated Genes

| Gene Name | Description | Fold Change | p-value |
|-----------|-------------|-------------|---------|
| Cxcl11    | Chemokine (C-C motif) ligand 11 | 2.05| 0.0019 |
| J11       | Interleukin 11 | 1.00| 1.00 |
| Jfa       | Interleukin 1 alpha | 1.00| 1.00 |
| Cxcl6     | Chemokine (C-C motif) ligand 6 | 1.00| 1.00 |
| Cxcl12    | Chemokine (C-C motif) ligand 12 | 1.00| 1.00 |
| Cxcl3     | Chemokine (C-C motif) ligand 3 | 1.00| 1.00 |
| Cxcl5     | Chemokine (C-C motif) ligand 5 | 1.00| 1.00 |
| Cxcl20    | Chemokine (C-C motif) ligand 20 | 1.00| 1.00 |

**Up-regulated genes as compared with the uninfected control (3 day) were labeled in red color, while down-regulated genes were labeled in green color.**
Up-regulated genes as compared with the uninjured control (0 hour) were labeled in red color while down-regulated genes were labeled in green color.