Dvl3 Promotes the Phenotypic Transformation of RA-FLS and Aggravation of CIA Through Wnt Pathway by Exosome Intervention.

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Research article

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Abstract

ObjectiveThis study was performed to explore the expression pattern of Dvl3 in RA and investigate the function and mechanism of Dvl3 in RA by exosome intervention.

Methods: The expression pattern of Dvl3 was examined by IHC, WB, and qPCR. Modified exosomes obtained from culturing supernatant of RA-FLS infected with Dvl3 over expression (OE) lentivirus by ultracentrifugation were administrated to the target RA-FLS. The ability of survival, migration, and the production of inflammatory factor influenced by exosomal Dvl3 were detected by CKK8 kits, Tunel, migration test, qPCR, and enzyme-linked immunosorbent assay (ELISA) respectively; Pathological examination, ELISA, and behave revaluation were performed after injection of exosomes into the articular cavity of CIA mice. The possible downstream pathways of Dvl3 were screened by qPCR and WB, and verified by double luciferase reporter experiment.

Results

The expression level of Dvl3 was significantly increased in RA and CIA. Exosomes from the OE group could significantly promote cell proliferation activity, migration/invasion ability. The augment of TNF-α, IL-1β, IL-17, and IL-21 was observed in exosomal Dvl3-OE group. The deteriorated role of exosomal Dvl3 in collagen-induced arthritis model (CIA) has been fully demonstrated in terms of cartilage destruction, apoptosis, and inflammation. Over expression of Dvl3 was accompanied by the significant increase of β-catenin and RhoA activities.

Conclusion

This study discovered the high expression of Dvl3 of exosomes derived from RA patients which may possessed the ability to promote phenotypic transformation of RA-FLS and aggravation of CIA through Wnt pathway.

Significance

This is the first study on Wnt pathway in pathology of inflammatory arthritis including RA by exosome intervention.

Exosomal Dvl3 is demonstrated to promote the “semi-transformed” phenotype of FLS in RA.

This study endorses modified exosomes as a promising manipulation targeted on preventing joint destruction.

Introduction

Histological evaluations conducted by pioneering investigators revealed that there is a gross similarity between the hyperplastic synovium of rheumatoid arthritis (RA) and solid tumors of cancer patients[1, 2].

Recent advances efforts to explore the mechanism of "semi-transformation" of RA-FLS were mainly focused on the deregulation of proto-oncogene involved in cell cycle regulation caused by the early inflammatory events or that act as transcription factors expressed at high levels in RA-FLS related pathways[3]among which Wnt pathway performed as potential participants. Studies have shown an abnormal over-activation of Wnt signaling pathways in autoimmune diseases including RA[4, 5], and further research has also revealed the aberrant status of Wnt pathways which to some extend may response for RA deterioration[6].

Notably, as a principal component in both arms of Wnt signaling and governs several cellular processes, Dishevelled (Dvls) plays a key role in integrating and conductingupstream signals regardless of whether they are correct or abnormal[7].This propagation of information subsequently initiates a signaling cascade that ultimately leads to β-catenin stabilization or a β-catenin-independent effect [8, 9]. However, there are still many significant gaps in understanding the mechanism by which Dishevelled relays information to different extra- or intra-cellular compartments.

To ensure secreted factors can successfully transmit information, cells have evolved several mechanisms including some novel biomarkers identified by the latest research, for example, exosomes, which are considered to have diagnostic and therapeutic potentials in clinical[10].

Here, combined with one of the results of our previous research that Dvl3 mRNA was highly expressed in serum exosomes of RA patients, we speculated that Dvl3 may hold great promise in the comprehensive grasp of the pathogenesis. We performed this study to exam the expression pattern of Dvl3 in RA and evaluate the function of exosomal Dvl3 in RA as well as the possible downstream mechanism.

Materials And Methods

Seen in supplementary content.

Result

The expression of Dvl3 in the synovium of RA and CIA mice was significantly elevated

To determine whether Dvl3 expression is consistent with previous studies which indicated Wnt pathway is constitutively activated in a variety of inflammatory diseases including RA[4, 5], a total of 12 patients knee joint synovial tissue of RA and Trauma, and 12 joints of CIA and normal mice have been collected.

The result of IHC (Fig. 1D, I) showed brown or brown particles which indicated positive for Dvl3 was mainly located in the cytoplasm while parts of those can be seen in the nucleus. IHC (Fig. 1E, J) scores of Dvl3 were analyzed and showed significantly elevated Dvl3 expression in synovium of RA and CIA than that of Trauma patients and normal mice respectively. WB and qPCR showed a similar result (Fig. 1A, B, C).

Also, the expression of Dvl3 in RA-FLS and Trauma-FLS was further examined, and the results were consistent with synovial tissue. Compared with the trauma group, FLS cells extracted from the synovial membrane of RA patients have a higher level of fluorescence intensity which was further testified in WB and qPCR experiments (Fig. 1F, G). Similar to the expression of Dvl3 in synovium and FLS, exosomes extracted from RA-FLS contained a more abundant level of Dvl3 mRNA compared with those derived from trauma patients as shown in the qPCR result. (Fig. 1H).

Exosomal Dvl3 promotes the proliferation, invasion and inflammatory responses of RA-FLS

Combined with the highly activated level of Dvl3 in the exosomes of RA, we speculated a scenario that FLS of RA joint synovium could produce high levels of Dvl3 exosomes to modulate the biology of other FLS in a paracrine manner. To verify this hypothesis, the supernatant of RA-FLS infected with lentivirus *in vitro* was harvested to obtain exosomes with up-regulated Dvl3 of which administration to RA-FLS to explore its effects on the proliferation, apoptosis, migration, inflammation and bone erosion.

After exploring the conditions (S Fig. 1A-C), the MOI of lentivirus in the OE and the sham control was determined at 40. 48h after transfection, the supernatant of RA-FLS was collected after culturing for another 48 hours with the serum-free medium. After ultracentrifugation and resuspension, exosomes were identified by transmission electron microscopy and particle size analysis (S Fig. 2A-C). After condition exploration of the intervention of exosomes to targeted FLS (S. Figure 1. D), the number of infected FLS was confirmed at least $8X10^6$, the significant difference of Dvl3 mRNA level of target $3X10^4$ FLS could be detected. WB and the qPCR results verified the exosomes successfully alerted the Dvl3 expression level of the target cells (S Fig. 1E-G).

Up-taken of exosomes by RA-FLS can be observed under the high magnification (S Fig. 2D) by PKH67 Green Fluorescent Cell Linker Mini Kit. Interestingly, some of the exosomes taken by RA-FLS were distributed in colonies in the cytoplasm with a high density while some other RA-FLS has no obvious particles, it was assumed that the endocytosis ability of exosomes maybe related to the maturation and the instinct status of RA-FLS which acquired further investigation.

CKK-8 assay was used to detect the cell activity of FLS. The results (Fig. 2C) showed after 12 hours of intervention, the viability of the Lv-Dvl3 group was significantly increased, and this advantage remained at least 24h after exosomes intervention.

Previous studies suggested that the ability resistance to apoptosis and abnormal proliferation of RA-FLS make up the vital reasons that lead to the persistent destruction of joints[11].Thus, Tunel assay was performed to detect RA-FLS apoptosis influenced by exosomal Dvl3. According to the proportion of Tunel positive cells, intervention of exosome derived from Lv-Dvl3 significantly impaired the apoptosis of RA-FLS (Fig. 2.A, B). These data suggest the role of Dvl3 in promoting the proliferation and reducing apoptosis of RA-FLS.

Another characteristic of “semi-transformed” RA-FLS is the enhanced migration and invasion ability, therefore, healing assay and transwell cell migration test were performed. As the results showed (Fig. 2D-I), overexpression of Dvl3 by exosomes tends to promote the migration ability of cells and significantly promote the invasiveness of the cells and break through the matrix gel cells.

The expression of matrix metalloproteinases (MMPs) is the other factor closely correlated to FLS invasion. The results of WB and qPCR showed (Fig. 2J-L) the significantly enhanced expression levels of MMP3, MMP9, and MMP13 in the RA-FLS treated with Lv-Dvl3 related exosomes. Among them, MMP3 had the highest protein expression level.

The perturbation of inflammation plays a pivotal role in the pathology of RA. For example, TNF-α is one of the major targets of biological treatment for RA [16]. FLS in the synovium contain abundant rough endoplasmic reticulum and evidence of active secretory machinery thus can synthesize and secret a variety of inflammatory cytokines[12, 13]. Therefore, we used qPCR to detect the expression of inflammatory factors in RA-FLS cells after ingesting exosomes, and used ELISA for further verification.

The results (Fig. 2M, N) showed that exosmomal Dvl3 could significantly promote the transcription of TNF-α, IL-1β, IL-17, and IL-21. Afterward, the level of TNF-α, IL-1β, IL-17, and IL-21 secreted in the supernatant was detected by ELISA (Fig. 2N), and the results support the view that exosomal Dvl3 played a promotion role in various cytokines synthesis and secretion.

Exosomal Dvl3 mRNA aggravated the disease severity CIA

To further clarify the effectiveness of exosomes and the validity of the role of exosomal Dvl3 in mediating disease activity *in vivo*, collagen-induced arthritis (CIA) mouse model was constructed (Fig. 2A).

The exosomes obtained from $8X10^6$ cells of indicated treatment by LV were re-suspended in 10μl of PBS and then were injected into both of knee joint cavity of CIA model mice immediately separately. It can be seen (S Fig. 3A-C) that the expression of Dvl3 in the Lv-Dvl3 related exosome injection group was significantly higher than that of the control group at both the protein and mRNA level, which authenticated the effectiveness of exosomes intervention *in vivo*.

DBA/1 mice were immunized twice, and the evaluation of arthritis scores and the rating MPW was performed the day after the second injection on day22. Arthritis scores of both groups was observed to rise continually over time, and reached the peak around 4–5 weeks after the first immunization (Fig. 3B). As shown in the Fig. 3B, arthritis scores of mice in the Lv-Dvl3 derived exosome group climbed rapidly. Significant between two groups was detected on the 38th, 42nd, and 46th day.

The results of the MWT (Fig. 3C) showed that there was no significant difference in the early stage of the disease. Afterward, as the disease course continues, both groups of mice showed a downward trend in scores. At 34 days after the first immunization, compared with the control group, mice in the OE group showed an obvious decreasing in thread hold, and its decline was significantly faster than that in the

control group, this advantage was maintained till the recovery period. The above results were consistent with the arthritis score.

H&E staining of the knee joints was performed to evaluate various pathological indicators of the joints such as inflammatory cell infiltration, synovial hyperplasia, pannus formation, and joint space changes. The images (Fig. 3D) showed that the surface of the normal knee joint structure was clear and complete accompany with no obvious proliferation or damage and rare inflammatory cells. Pathological features such as inflammatory cell infiltration, synovial hyperplasia, and articular surface destruction could be observed in the CIA model. In terms of pathological score (Fig. 3G), Lv-Dvl3 derived exosome injection group was significantly higher than that of the control group, in which obvious synovial hyperplasia characteristic by mounts of inflammatory cell infiltration, and part of the joint cavity narrowing accompanied with subchondral bone erosion could be observed.

A typical pathological sign of RA is the formation of pannus and the subsequent erosion of subchondral bone. This process attribute to the excessive proliferation of FLS and the destruction of cartilage caused by enhanced pro-inflammatory and invasion ability. Therefore, the description of articular cartilage structure, subchondral bone, and bone tissue by Safranin O-Fast Green staining would help to define the role of Dvl3 in RA. As shown in figure (Fig. 3I), the articular surface of normal mice is attached with intact and smooth cartilage. The administration of exosomal Dvl3 was shown to impair the joints, for the thinner and accompanied by erosion or insect-like damage of the cartilage compared with that of the control group (Fig. 3H).

To further clarify the effect of exosomal Dvl3 on the invasion ability of RA-FLS, the expression level of matrix metalloproteinases (MMPs) in mouse knee joints was detected. It turned out that the exosome OE group significantly up-regulates all three MMPs which was consistent with results *in vitro* (Fig. 3J-L).

Tunel staining was used to detect the *in situ* apoptosis of the synovial membrane. The results (Fig. 3E, F) suggested a significant inhibition on cell apoptosis of exosomal Dvl3, for the aberrant lower percentage of positive cells in the OE group.

Finally, ELISA test was used to determine whether local administration of modified exosomes will affect systemic inflammatory levels. The results (Fig. 3M) showed that the increasing of Dvl3 in the knee joint could significantly up-regulate the level of serums TNF-α, IL-17 and IL-21, and has a slight up-regulated effect on IL-1β.

Exosomal Dvl3 up-regulated β-Catenin and RhoA pathway activity

To further explore the downstream of exosomal Dvl3 on the Wnt pathway, qPCR and WB experiments were performed to assess the influence of exosomal Dvl3 on the canonical and non-canonical Wnt pathways. qPCR results (Fig. 4A) showed that, compared with the control group, the over expression of Dvl3 by exosomes was accompanied by a significant increase in β-catenin and ROCK2, while no

significant changes were observed in the key molecular of Rac pathway of JNK and the protein involved in intercellular connectivity of PRK. Up-regulation of β-catenin and ROCK2 was validated by WB (Fig. 4B, C).

Dual luciferase reporter gene detection results (Fig. 4D, E) suggested that Dvl3 could activate both classical and non-classical Wnt signaling pathways. Verification of plasmid used in this experiment was performed by WB and qPCR (S Fig. 4)

Recent evidence has suggested the critical role of RhoA/ROCK pathway played in regulating the proliferation and differentiation of T cells[14, 15].An elegant study found Naive T cells from ROCK2-deficient mice showed impaired Th17 differentiation and decreased IL-17 and IL-21 secretion both of which were supposed to be involved in the pathogenesis of many autoimmune diseases.

Thus, the classic Wnt signaling pathway blocker of DDK1 was used to eliminate the influence of the Wnt/β-catenin pathway (Fig. 4F-J) to further examine the possibility of two arms of the Wnt pathway in conducting Dvl3 effect. The results (Fig. 4K-N) showed DKK1 inhibited the secretion of inflammatory factors, while the administration of DDK1 failed to alert the ability to produce IL-17 and IL-21 influenced by Dvl3 level. Interestingly, TNF-α and IL-1β seemed to get influenced by both Dkk1 and Dvl3. Taken together, we speculated that the mechanism contributing to Dvl3 function on FLS may be partly related to RhoA in the non-canonical Wnt/PCP pathway, and exosomes may play an essential role in coupling extra-cellular stimuli to multiple downstream signaling pathways.

Discussion

Upon activation, RA-FLS can establish its own autocrine or paracrine fashion and synthesis numbers of pro-inflammatory cytokines, growth factors, and matrix metalloproteinases (MMPs) thus further upgrade local inflammation response. An elegant study has demonstrated the migration of RA-FLS by implanted synovial cells with collagen into the joints of mice with severe combined immunodeficiency (SCID), which turned out that unlike FLS derived from healthy individuals or osteoarthritis patients, RA-FLS spontaneously invades and destroys cartilage [16]. All of the above underlines the urgent need for extra effort to uncover the mechanism hide under the phenotype of RA-FLS.

As an essential component in the Wnt signaling pathway, Dvls participate in the regulation of cell proliferation, survival, migration, differentiation, polarity, and stem cell regeneration. Studies have shown the levels of Dvl1 and 3 in lymph node metastases are significantly higher than those in the primary growths[17]. Khan et al.[18] reported that, when compared with Dvl1 which can be found in normal peripheral blood mononuclear cells (PBMC), Dvl2 and 3 are only expressed in chronic lymphocytic leukemia CLL cells. Our previous studies have shown that Dvl3 is significantly escalated in the peripheral blood exosomes of RA (upon publication). Combined with the important role in the Wnt signaling pathway, it is highly speculated that Dvl3 may play a crucial role in RA.

The expression of Dvl3 was elucidated by IHC, IF, qPCR, and Western Blot in synovial tissue and FLS which turned out that Dvl3 was significantly up-regulated in RA. Previous data have demonstrated that the Wnt signaling pathway was constitutively activated at a high level in numbers of inflammatory diseases including RA[4, 5]. It is speculated that in propagating Wnt signaling pathway Dvl3was induced to be up-regulated and play a known or potential physiological and pathological regulatory role.

Increasing evidences have confirmed that exosomes were involved in various biological processes including but not limited to cell-cell communication, signal transmitting, immune response modulating, viral packaging and release, as well as cancer progression[19–23] and has recently expanded to the pathogenesis of RA[24]. RA-FLS could promote destruction and inflammation in the pathogenesis of RA by producing a membrane form of TNF-α in exosomes that enhance the ability of activated T cells resistant to apoptosis[25]. The stable structure and long-distance transport ability endorse exosomes as an ideal platform for the integration and transmission of signaling molecules between cells, which promise them as novel administration applied in RA treatment.

In this study, mRNA level of the target molecule Dvl3 contained in the exosomes secreted by RA-FLS was successfully alerted through lentivirus transfection, and subsequently invention of exosomes to cells effectively mediated the expression level of Dvl3 in target RA-FLS. Further investigation revealed that over expression of Dvl3 significantly manipulated various biological processes of cells, including facilitating proliferation, inhibiting apoptosis, enhancing cell migration as well as invasion while promoting matrix metalloproteinase secretion, and a variety of cytokines such as TNF-α, IL-1β, IL-17, and IL-21 also exhibited to be profoundly elevated. Consistent with our study, a previous study has revealed the pro-inflammatory effect of the Wnt/β-catenin increased the production of matrix metalloproteinases in joint FLS and thereby deteriorated joint damage[26].

CIA model of mice has similar pathological features of inflammatory arthritis in synovial hyperplasia, inflammatory cell infiltration, and cartilage degradation[27]. Further studies on the effect of exosomal Dvl3 *in vivo* were performed which suggested that the increasing of Dvl3 level has an impairment effect on CIA demonstrated by exacerbation and aggravation of the disease course, escalated inflammation level of the system, and certain detrimental effects on synovial inflammation and cartilage erosion. This phenomenon indicated Dvl3 as a potential target of the interference mediated by exosomes for the treatment of RA.

Previous genetic evidences have shown that Disheveled protein acts as a branch-point and is an essential component of both arms of Wnt signaling, but the mechanism of how it manipulating the homeostasis of both pathways is unclear[28]. Given its critical involvement in propagating the Wnt signaling pathway and governing various development processes of a cell, Dvls can be called a master integrator of complex signals.

Severe key signal molecular in the classical and non-canonical Wnt pathways was detected by qPCR and it turned out that over-expression of Dvl3 significantly up-regulated the key molecular of classical Wnt pathway β-catenin, and the typical proteins of non-Classical Wnt pathway Rho GTPase and Rho-related

kinase2 (ROCK2), which was consistent with previous studies on Dvls[7]. Both of the ascent expressions of β-catenin and RhoA were verified by WB.

Dual luciferase reporter gene experiment was performed to further verify the possibility of Dvl3/β-catenin/TCF and Dvl3/Rho/ROCK signaling pathways.

Accumulation of β-catenin in the nucleus acts as a co-activator for T cell stimulating factor (TCF)[29]. Mounting data has proved the close relation of TCF/LEF to the differentiation, development, and functional transform of various tumors[30, 31]. Furthermore, Wnt/β-catenin/TCF pathway has been shown to regulate T cell differentiation in both the thymus and peripheral lymphoid tissues[32–35]. In terms of research in RA, TCF is mainly limited to the regulation of system immune function, which to date its role in RA-FLS is still lacking.

We here used DDK1, blocker of classic Wnt signaling pathway, to eliminate the influence of the Wnt/β-catenin pathway to further testify the function of Dvl3. It turned out that in terms of the cytokine secretion, while TNF-α and IL-1β was regulated by both arm of Wnt pathway, IL-17 and IL-21 seems to be mainly determined by non-classical pathways

As an important member of small molecule GTPases, RhoA is intimately regulated by the non-canonical Wnt pathway[36] which is subsequently responsible for the activation of ROCK. Once activated, ROCK will contribute to the aggregation and polymerization of the cytoskeleton and thereby enhance the contraction of cells to drive movement[37]. Recently, a study demonstrated that a selective oral ROCK2 inhibitor could inhibit the phosphorylation of STAT3 and reduce the ability of PBMCs to produce IL-17 and IL-21[38], which was consistent to our result.

This study proposed a model in which constitutive activation of Dvl3 by synovial inflammatory exosomes in combination with inflammatory cytokines leads to activation of both arms of Wnt pathways which further stimulate the invasive properties of RA-FLS.

To clarify the specific mechanism is a problem that needs to be addressed in the current pioneer research field of inflammatory arthritis. Based on the results *in vivo* and *in vitro*, the role of exosomal Dvl3 in activating the Wnt pathway and thereby aggravating inflammatory arthritis by promoting cell proliferation, migration, and the inflammatory response in RA-FLS has been identified. Our research will provide the possibilities to develop novel strategies aimed at deletion of exosomal Dvl3 as a potential target for the treatment of RA.

Conclusion

The “semi-transformed” phenotype of RA-FLS is one of the leading causes of function defects in the chronic inflammatory lesions of Rheumatoid arthritis (RA). There have been several pathways reported in regulating this process including the Wnt pathway. In this study, we discovered the high expression level of Dvl3 in exosomes derived from RA patients. Modified exosomes harvested form RA-FLS infected with

Dvl3 over expression lentivirus possessed the ability to promote cell proliferation migration, invasion, and production of pro-inflammatory cytokines of RA-FLS. The deteriorated role of exosomal Dvl3 in CIA has been fully demonstrated in terms of cartilage destruction, apoptosis, and inflammation. The function role of Dvl3 may attribute to the activation the downstream of both classic and non-classical Wnt pathways, which provides a novel potential target and theoretical basis for future RA intervention.

Abbreviations

CIA	Collagen induced arthritis
CLL	chronic lymphocytic leukemia
CCK-8	Cell Counting Kit-8
DMEM	Dulbecco’S modified eagle medium
Dvl3	Dishevelled3
ELISA	Enzyme-linked immunosorbent assay
ESCRT	Endosomal sorting complex required for transport
FLS	Fibroblast-1ike synoviocyte
HE	Haematoxylin &Eosin
IF	Immunofluorescence
IHC	Immunohistochemistry
IL-17	Interleukin-17
IL-1β	Interleukin-1β
IL-21	Interleukin-21
JNK	c-Jun N-terminal kinase
MMP	Matrix metalloproteinas
MOI	Multiplicity of infection
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffer saline
PWT	Paw withdrawal threshold
qPCR	Real-time quantitative PCR
RA	Rheumatoid arthritis
RNA	Ribonucleic acid
ROCK	Rho kinase
SCID	Severe combined immunodeficiency
SPF	Specific Pathogen Free
STAT	Signal transducer and activator of transcription
TKA	Total knee arthroplasty
TNF-α	Tumor necrosis Factor-α

Tunel	TdT-mediated dUTP Nick-End Labeling
WB	Western Blot

Declarations

Ethics approval and consent to participate

The Institutional of Ethics Committee of the Shanghai Changhai Hospital reviewed and approved the study. All volunteers gave their written informed consent before participation.

All animal operations and handling have been approved by the Animal Care and Use Committee of Changhai Hospital.

Consent for publication

The authors give consent for publication on arthritis research & therapy.

Availability of data material

All the data material related to the research are available.

Competing interests

The authors declare there are no competing interests.

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Authors' contributions

T-WX and Z-DB designed the experiments, CN, Z-YJ and QY collected clinical specimens, CN and L-HR performed the morphological study. T-WX, CN and GJ performed the experiments. QY, F-XM and L-SW analyzed and interpreted all of the data. T-WX and QY wrote the manuscript.

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Figures

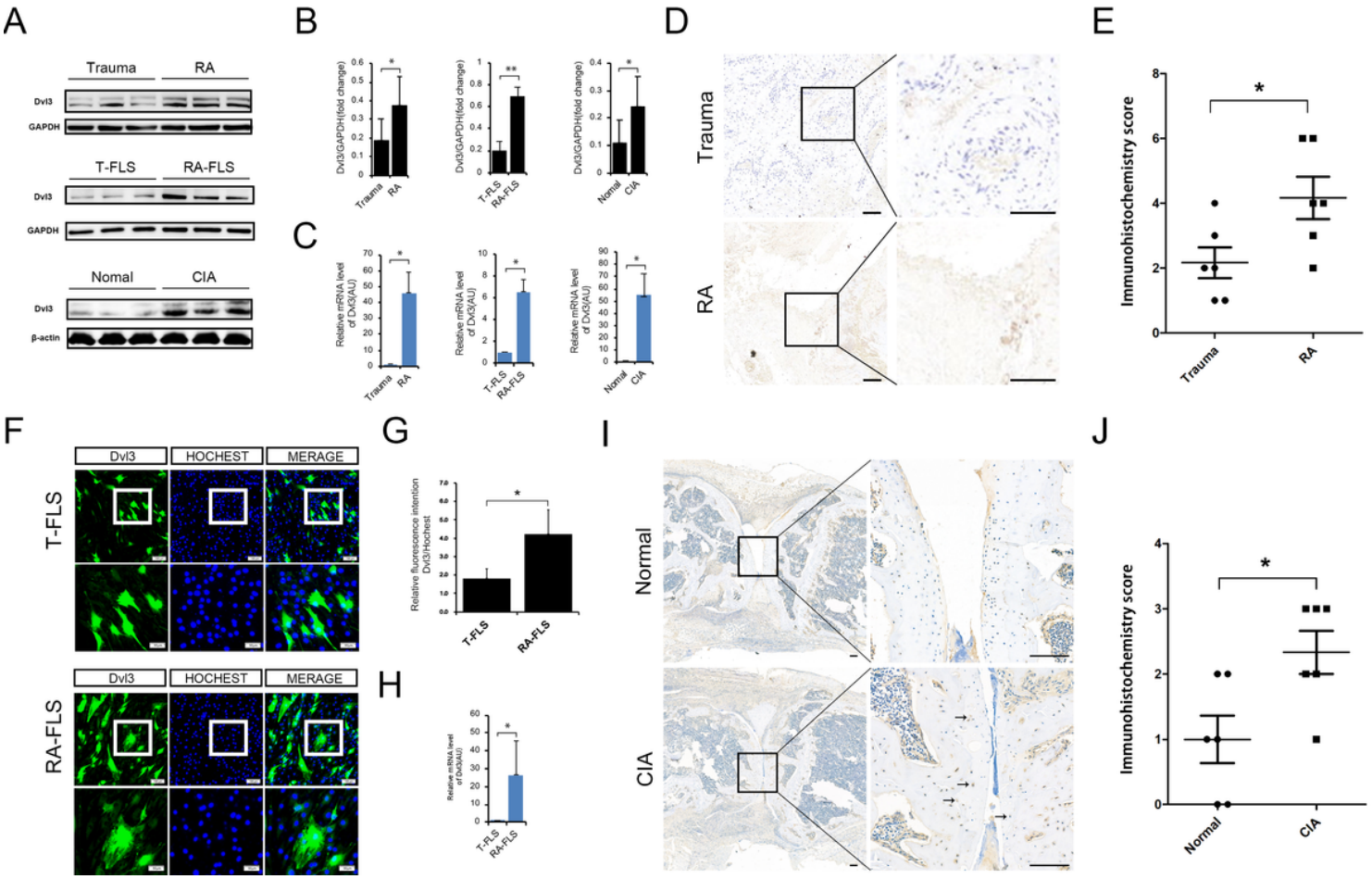

Figure 1

Dvl3 expressions validation in RA and CIA mice synovium. (A, B) The Western blot analysis tested the expression of Dvl3 in synovium or FLS harvested from RA or CIA mice. (C) The expression of Dvl3 mRNA in synovium tissue or FLS. (D) Dvl3 immunohistochemistry analysis of synovium from RA or Trauma patients; Scale bar,100μm. (E) immunohistochemical scores of Dvl3 expression. (F) Dvl3 immunofluorescence analysis of FLS from RA or Trauma patients; Scale bar, up 100μm, down 50μm (G) The quantification of relative Dvl3 fluorescence intensity at the indicated group. (H) Real-time PCR analyses of Dvl3 mRNA in exosomes derived from RA or Trauma FLS; Scale bar,100μm. (I, J) Immunofluorescence analysis of Dvl3 (arrow indicated) from CIA or normal mice synovium. Values are shown as mean ± SD (n=6). *P <0.05; **P <0.01.

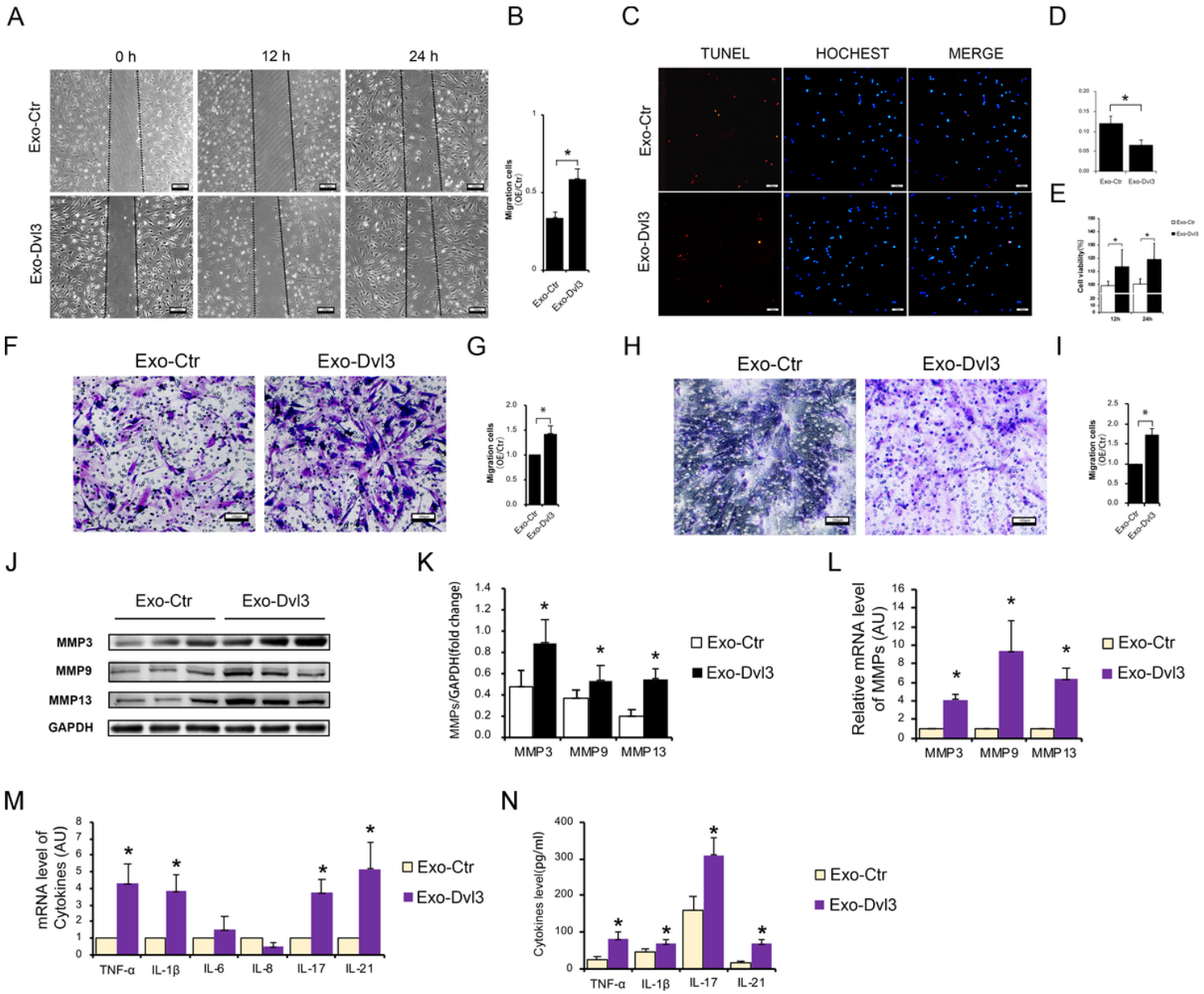

Figure 2

Exosomes derived from FLS with high Dvl3 expression enhanced the survival activity, migration ability and inflammatory responses of RA-FLS. (A) Representative images showing the Tunel+ cells (red) treated by the exosomes obtained from control or Dvl3 over-expression groups. The nuclei were stained by Hoechst (blue); Scale bar,100μm. (B) Quantification of the percentage of Tunel+ cells in indicated conditions. (C) CKK8 assay of survival activity of FLS treated by different exosomes. (D, E) The results of wound healing assay showed that RA-FLS treated by Dvl3 over-expressed exosomes exhibited higher mobility compared with control group; Scale bar=200μm. (F, G) The results of Transwell cell migration test showed that the number of migration cells in exosomes Dvl3 group was elevated; Scale bar,100μm. (H, I) Representative results of Transwell cell invasion test; Scale bar,100μm. (J) The Western blot and Real-time PCR analyses tested the expression of MMPs in RA-FLS treated by indicated exosomes. (K) Real-time PCR analyses of different cytokines expression. (L) Concentrations of TNF-α, IL-1β, IL-17 and IL-21 was examined by Elisa analyses. Values are shown as mean ± SD (n=6). *P <0.05; **P <0.01.

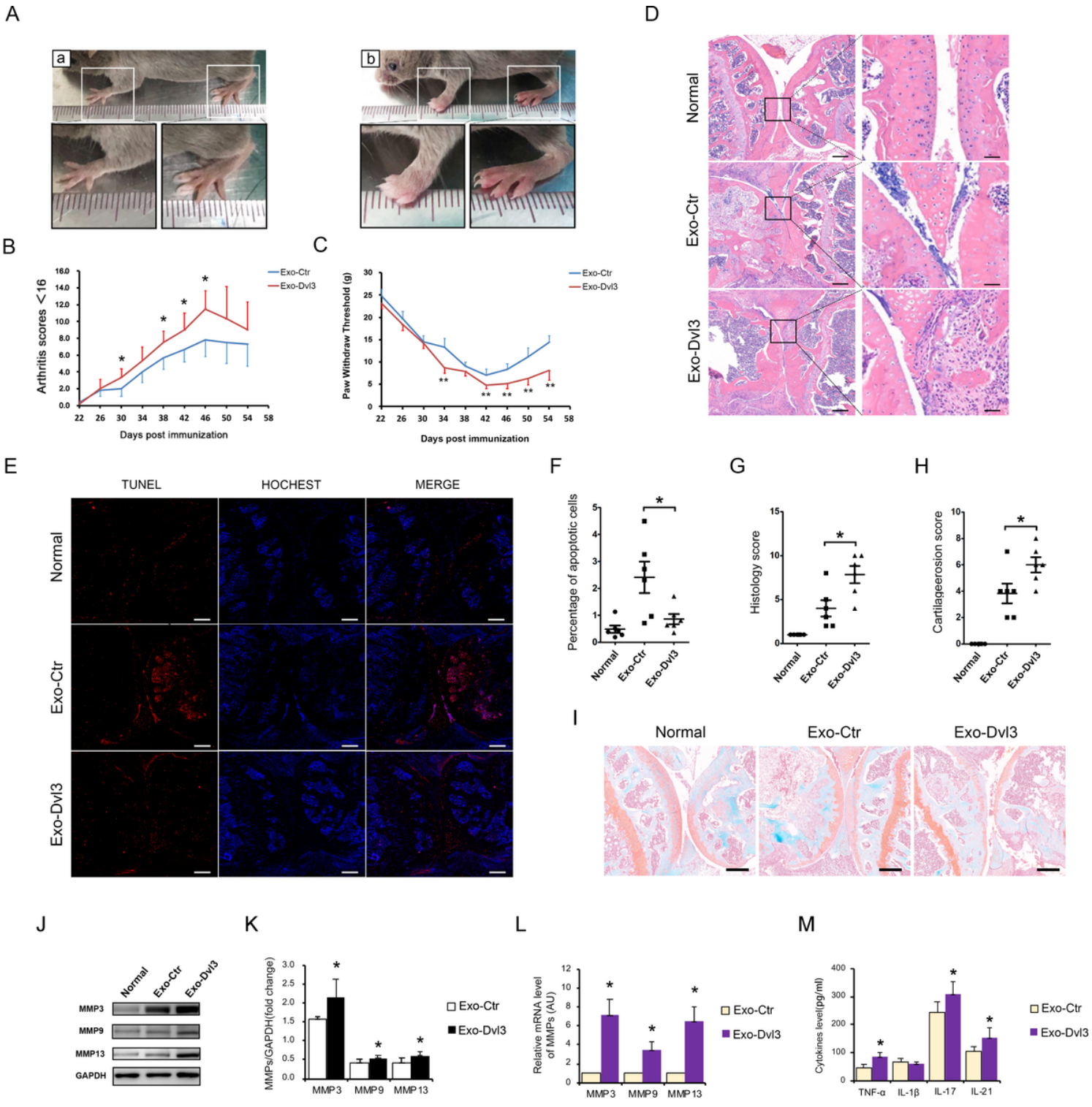

Figure 3

Exosomes derived from FLS with high Dvl3 expression aggravated CIA disease. (A) Images of forepaw and hind-paw from healthy or CIA mice showed a pronounced edematous swelling and ankylosis paw due to disease progression. (B, C) Average scores of arthritis and MWT analysis showed exosomes Dvl3 accelerated the disease severity of CIA. (D, G) The typical H&E images and histopathological scores of synovial inflammation destruction; Scale bar, right 250μm, left 50μm. (E, F) Representative images and quantification of the percentage of in suit Tunel+ cells (red) in joint of mice from indicated groups; Scale

bar, 250μm. (H) Histogram shows the cartilage destruction scores of Safranin O-Fast Green staining (I) from two groups; Scale bar, 250μm. (J-L) Western blot and Real-time PCR analyses tested the expression of MMPs in joints treated by indicated exosomes. (M) Concentrations of serum TNF-α, IL-1β, IL-17 and IL-21 was examined by Elisa analyses. Values are shown as mean ± SD (n=6). *P <0.05; **P <0.01.

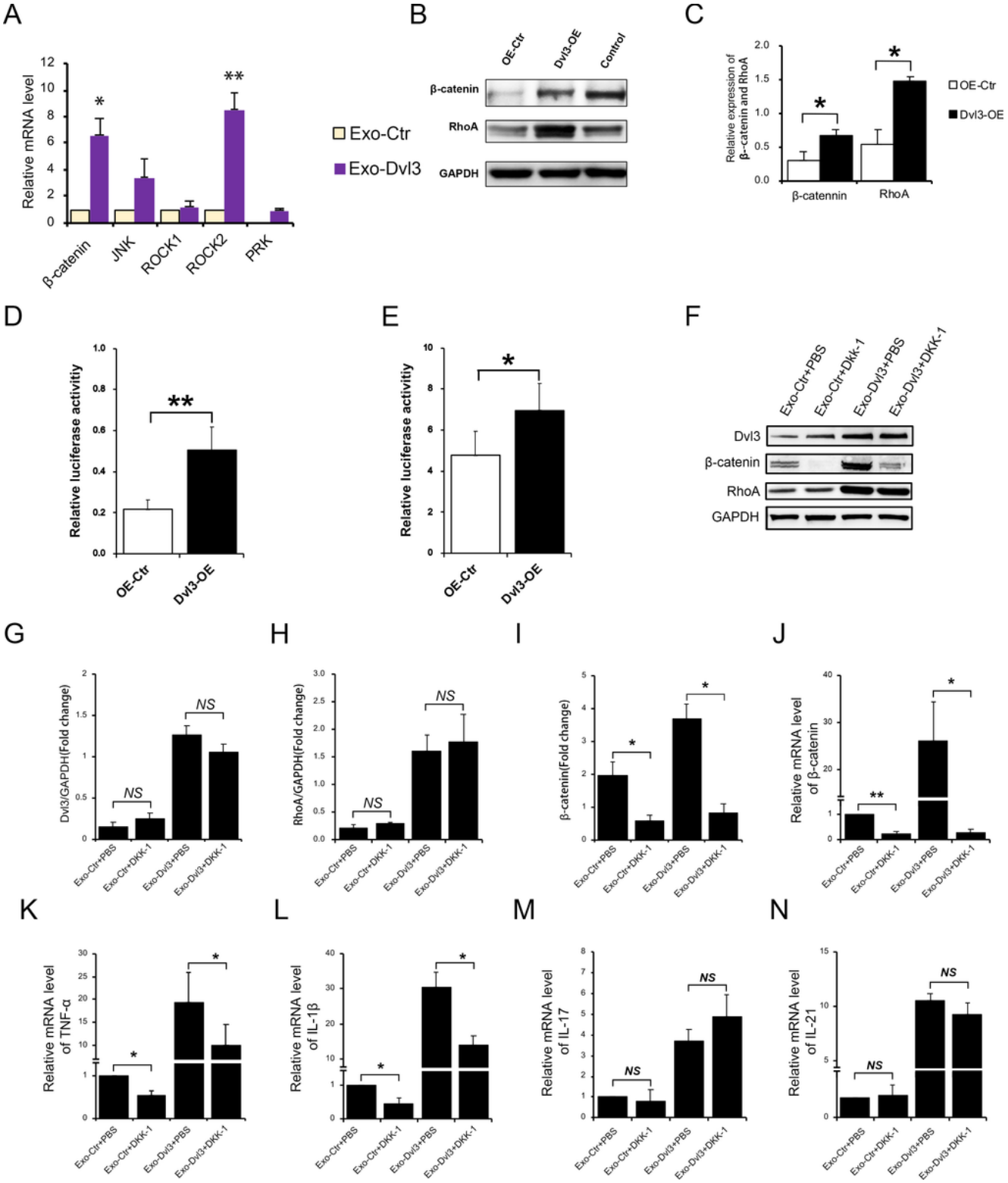

Figure 4

Over-expression of Dvl3 upregulated both canonic and non-canonic Wnt pathways. (A) Relative mRNA expression level of typical molecules of Wnt pathways in RA-FLS treated by indicated exosomes. (B, C) The protein level of β-catenin and RhoA in the RA-FLS determined by western blot. (D, E) Dual luciferase reporter gene detection results showed Dvl3 could activate both classical and non-classical Wnt signaling pathways. (F-J) The protein and mRNA level of β-catenin and RhoA in RA-FLS in indicated conditions was detected by WB and RT-PCR. (K-N) Concentrations of TNF-α, IL-1β, IL-17 and IL-21 from supernatant of RA-FLS in different conditions was examined. Values are shown as mean ± SD (n=6). *P <0.05; **P <0.01.

Supplementary Files

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