Dear Editors and Reviewers:

Thank you for your letter and for the reviewers' comments concerning our manuscript entitled "FMNL3 regulates RhoC/FAK pathway and actin assembly to promote cell invasion in colorectal carcinoma" (Manuscript NO: 39491). Those comments are all valuable and very helpful for revising and improving our paper. We have studied comments carefully and have made correction which we hope meet with approval. Revised portion are marked in red in the paper. The main corrections in the paper and the responds to the reviewer's comments are as flowing:

Reviewer #1: This is a well planned study with multiple experimental elements supporting the significant results. A reasonable conclusions were drawn from these experiments.

Response to comment: Thank you for your affirmation and appreciation.

Reviewer #2: In this manuscript, the authors clarified the underlying mechanism of formin-like 3 (FMNL3) promoting the invasion in colorectal carcinoma (CRC). That is, FMNL3 was initially activated by RhoC, followed by the activation of p-FAK and downstream of MAPK/ERK and PI3K/AKT signaling pathways, resulting in the increased expression of MMPs and VEGF. FMNL3 also accelerates the rearrangement of actin-based protrusions in a RhoC-dependent manner. These subsequently enhance the invasive capacity of CRC cells. It suggests a novel therapeutic regime for reducing cancer cell dissemination, blocking metastatic progression and prolonging life expectancy of patients with CRC. The article is comprehensive and worthy of our study. However, there is a minor problem in the article: In the result of Western Blot, the form of presentation of MMP-2 and MMP-9 is inconsistent with other bands. Please confirm it.

Response to comment: Thank you for your careful observation! I'm sorry it may be because we didn't clearly explain the figure legend, which made you misunderstand. In Figure 3-Figure5, the bands of MMP-2 and MMP-9 are not derived from the results of western blot but from the results of gelatin zymograph assays, so they display distinctly from the bands of the results of western blot. If you want to check the method of gelatin zymograph assay, please read the paragraph 9 of the materials and methods part.

Reviewer #3: In this study, the authors investigated the invasion mechanism of colorectal cancer cell via formin-like 3 protein (FMNL3)-mediated enhancing RhoC/focal adhesion kinase (FAK) pathway. They argue that this pathway shows the effect of inducing metastasis of CRC cells. This study was well arranged and presented with good quality of results. However, this reviewer raises one major and some minor concerns. [Major concern] It has been reported that the induction of nuclear signaling by cleaved FAK increases the metastasis of colorectal cancer. Therefore, only the increase of FAK expression originating from various signals including FMNL3 and RhoC cannot explain the metastasis in CRC cells clearly. Therefore, the authors will have to provide an accurate analysis of the signaling cascades that can induce the metastasis of CRC cells from FNML3 expression. [Minor concerns] * Conclusion in Abstract: There is a lack of the description what phenomenon of FMNL3 has induced metastasis by remodeling of actin-based protrusions. * Statistical analysis: In all results, statistical significance values of 'b' were presented as $p < 0.01$, which seems to require recalculation. The value inferred from the error bar is close to $p < 0.001$. * Method for Figure 1: A detailed description of the method for inducing overexpression of FMNL3 in CRC cells is required. * Figure 2: According to the Western blot results, FMNL3 was only down regulated. * It should be described what shRNA1 and shRNA2 mean when using shRNA to down-regulate FMNL3. * There are no discussion and

review of the purpose and analysis of observing the expression between FMNL3 and F-actin. * The explanation for the mechanism of metastasis by association of MMP-2 and MMP-9 expression through VEGF and/or RhoC should be discussed. * There are many typos.

Q1. It has been reported that the induction of nuclear signaling by cleaved FAK increases the metastasis of colorectal cancer. Therefore, only the increase of FAK expression originating from various signals including FMNL3 and RhoC cannot explain the metastasis in CRC cells clearly. Therefore, the authors will have to provide an accurate analysis of the signaling cascades that can induce the metastasis of CRC cells from FMNL3 expression.

Response to comment: We are very sorry for our negligence of the correlation nuclear signaling by cleaved FAK with metastasis of colorectal cancer. Considering the Reviewer's suggestion, we have consulted a large number of literatures to perfect our discussion. Please refer to the statements from line 9 to 22 of paragraph 6 of the discussion section. That is, we added some additional discussion as follows: FAK is a protein tyrosine kinase first identified at extracellular matrix and intergrin receptor cell adhesion sites and is a key regulator of cell movement[48]. Resent studies showed increased expression of p-FAK in the nuclei of cells in laryngeal cancer and four digestive cancer, including colorectal cancer[49, 50]. Nuclear FAK promotes cell proliferation and survival through enhanced P53 degradation[51], suggesting an association between p-FAK and abnormal cell proliferation. In addition, nuclear expression of p-FAK is also associated with poor prognosis in colorectal cancer[52]. In this study, we found that the phosphorylation of FAK triggered by RhoC/FMNL3 signaling induced the activation of MAPK and AKT and the subsequent upregulation of MMPs and VEGF, and contributed to the invasive potential of colorectal cancer cells. Of course, it may also be correlated with nuclear location of p-FAK triggered by RhoC/FMNL3 signaling.

Q2. Conclusion in Abstract: There is a lack of the description what phenomenon of FMNL3 has induced metastasis by remodeling of actin-based protrusions.

Response to comment: It is really true as Reviewer suggested that we should descript what phenomenon of FMNL3 has induced metastasis by remodeling of actin-based protrusions. We have re-written this part according to the Reviewer's suggestion. Please refer to paragraph 2 of the discussion section as follows: Previous study have reported that reorganization of actin cytoskeleton is responsible for enhanced cell motility that is necessary for cancer cell invasion and metastasis[4]. As the Rho-GTPase binding protein, DRF possesses conserved function in the actin cytoskeleton dynamics exerted through the formin homology 2 (FH2) domain[37]. DRF contains a NH2-terminal GBD domain, upon which binding to a Rho-GTPase, the bound NH2-terminal diaphanous inhibitory domain (DID) is dissociated from COOH-terminal diaphanous autoregulatory domain (DAD). This in turn results in the release of autoinhibition of inactive DRF and subsequently allows the FH2 domain to function as a direct regulator of actin polymerization[37]. DRFs are major actin filament nucleartors, which can bundle linear actin filaments and generate membrane protrusion such as filopodia and lamellipodia[38, 39]. Here, we found that the DRF FMNL3 could promote the elongation of filopodia and restrict the

broadening of lamellipodia. Some researchers have also reported the assembly of filopodia and lamellipodia by FMNL3[13, 22, 24] and verified the structure of the FMNL3 FH2/actin complex mediated actin nucleation and elongation[40].

Q3. Statistical analysis: In all results, statistical significance values of 'b' were presented as p <0.01, which seems to require recalculation. The value inferred from the error bar is close to p <0.001.

Response to comment: It is really true as Reviewer suggested that some statistical significance values of 'b' were presented as p <0.01, which seems to require recalculation. The value inferred from the error bar is close to p <0.001. What we thought at the time was that the range of p <0.01 contains p <0.001. Since statistically significant statistical values are usually P <0.01 and P <0.05, so we simplified the presentation to include the subset of significant statistical value of P <0.001 into P <0.01 at that time. We have revised this presentation in the revised paper according to the Reviewer's suggestion and indeed this display is more accurate. Please refer to the figures and figure legends in our revised manuscript.

Q4. Method for Figure 1: A detailed description of the method for inducing overexpression of FMNL3 in CRC cells is required.

Response to comment: Considering the Reviewer's suggestion, we have added a detailed description of the method for inducing overexpression of FMNL3 in CRC cells. Please reading the paragraph 3 of the Materials and Methods section marked in red in the revised manuscript. Here, we displayed as follows: FMNL3 gene was amplified by PCR and then inserted into the plasmid pcDNA3 (Invitrogen, Forster City, CA, USA). The primers used were as follows: forward 5'-TCCGATTCATTCTTAC-3', reverse 5'-CCGCCTCAACTCTGCTATT-3'. The PCR conditions were as follows: 95℃ for 3 min, followed by 35 cycles of amplification (94℃ for 30s, 55℃ for 40s, 72℃ for 2 min). The fragment was inserted into the pGC-FU-EGFP-3FLAG lentiviral vector. The FMNL3-overexpression vector was transfected into lentiviral packaging 293T cells. The culture supernatant containing viral particles was harvested 48h after transfection of 293T cells.

Q5. Figure 2: According to the Western blot results, FMNL3 was only down regulated.

Response to comment: It is really true as Reviewer stated that according to the Western blot results of figure 2, FMNL3 was only down regulated. Because we used two human shRNA sequences to silence FMNL3 gene, as we know, the interference efficiency of shRNA sequences is only 70-80%, but less than 100%, FMNL3 was only down regulated but not disappear in our Western blot results of figure 2.

Q6. It should be described what shRNA1 and shRNA2 mean when using shRNA to down-regulate FMNL3.

Response to comment: We are very sorry for our negligence of description of what shRNA1 and shRNA2 mean when using shRNA to down-regulate FMNL3. We have made additional remarks for that. Please read the paragraph of *Construction of pasmids and transfection* of

MATERIALS AND METHODS section marked in red in our revised manuscript.

Q7. There are no discussion and review of the purpose and analysis of observing the expression between FMNL3 and F-actin.

Response to comment: We are very sorry for our negligence of the discussion and review of the correlation of the expression between FMNL3 and F-actin. We have supplemented and written this part according to the Reviewer's suggestion. Please read the paragraph 2 of the discussion section marked in red in revised manuscript.

Q8. The explanation for the mechanism of metastasis by association of MMP-2 and MMP-9 expression through VEGF and/or RhoC should be discussed.

Response to comment: We think the paragraph 5-6 of the discussion section in revised manuscript may help to explain for the mechanism of metastasis by association of MMP-2, MMP-9 and VEGF expression through RhoC.

Q9. There are many typos.

Response to comment: We are very sorry for our incorrect writing in the first manuscript. We have tried our best to correct them and marked them in red in our revised manuscript. Thank you for your carefulness.

Special thanks to you for your good comments.

We tried our best to improve the manuscript and made some changes in the manuscript. These changes will not influence the content and framework of the paper. And here we did not list the changes but marked in red in revised paper.

We appreciate for Editors/Reviewers' warm work earnestly, and hope that the correction will meet with approval.

Once again, thank you very much for your comments and suggestions.

Best regards,

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