Pancreatic Tumor Cell Secreted CCN1/ Cyr61 Promotes Endothelial cell migration and Aberrant Neovascularization

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The complex signaling networks between cancer cells and adjacent endothelial cells make it challenging to unravel how cancer cells send extracellular messages to promote aberrant vascularization or tumor angiogenesis. Here, in vitro and in vivo models show that pancreatic cancer cell generated unique microenvironments can underlie endothelial cell migration and tumor angiogenesis. Mechanistically, we find that pancreatic cancer cell secreted CCN1/Cyr61 matricellular protein rewires the microenvironment to promote endothelial cell migration and tumor angiogenesis. This event can be overcome by Sonic Hedgehog (SHh) antibody treatment. Collectively, these studies identify a novel CCN1 signaling program in pancreatic cancer cells which activates SHh through autocrine-paracrine circuits to promote endothelial cell migration and tumor angiogenesis and suggests that CCN1 signaling of pancreatic cancer cells is vital for the regulation of tumor angiogenesis. Thus CCN1 signaling could be an ideal target for tumor vascular disruption in pancreatic cancer.

Pancreatic Ductal Adenocarcinoma (PDAC) is the fourth most common cause of cancer death in the United States, causing 37,390 deaths in 2012 alone. The median survival rate is 6 months or less, and the five-year survival rate for PDAC is only about 5%. A majority of patients present late, with locally advanced disease or with cancer already metastasized to distant organs and thus they are precluded from a resection. In a minority of patients, occasionally a curative resection is successful, however their prognosis remains poor, with the median survival rate after surgery of 11–20 months. The course of PDAC has not significantly improved even with multiple therapeutic attempts. Surgical or chemotherapeutic failure could be due to disease relapse with early metastasis, which is a complex, multistep process depending on almost mysterious tumor microenvironments and surrounding factors. Thus, there is a growing need to understand the mechanism in the progression of pancreatic cancer.

Despite conflicting views about the formation and recruitment of new blood vessels in human PDAC, decades of studies demonstrate that PDAC, like other cancers, needs new and destabilized blood vessels (tumor angiogenesis) as a prerequisite event for the growth and progression as well as dissemination of tumor cells for metastasis. Thus, targeting these blood vessels to prevent tumor growth and metastasis may provide novel strategies for PDAC therapy. Disappointingly, therapies that target angiogenesis in PDAC are not effective to all patients and show huge negative side effects, some of which may be life threatening. Thus, to achieve a new therapeutic approach, it is necessary to identify the underlying signaling cascade that is directly involved in tumor angiogenesis or aberrant blood vessels surrounding PDAC.

CCN1 (formerly known as Cyr61), a matricellular protein of CCN-family, plays a vital role in pancreatic cancer progression and metastasis. We have shown that CCN1 impacts both sonic hedgehog (SHh) and Notch pathways through integrins in PDAC cells. Both SHh and Notch signaling influence PDAC growth and contribute in the formation of tumor angiogenesis in PDAC and other cancers. During embryonic development and at the site of neovascularization, CCN1 acts as an angiogenic factor, and pro-angiogenic activities of CCN1 are mediated through integrins αvβ3 and α6β1 in human umbilical vein endothelial cells. However, the
role of CCN1 in aberrant blood vessel formation in pancreatic cancer remains unclear. Thus, the objective of this study is to evaluate whether tumor cell secreted CCN1 plays any role in aberrant blood vessel formation. We demonstrate that tumor cell secreted CCN1 promotes endothelial cell migration in recruiting aberrant blood vessel formation/tumor angiogenesis, and SHh plays a vital role in this event.

Methods

Cell Culture. Human pancreatic cancer cell lines (i.e., AsPC-1 and Panc-1) and mouse embryonic mesenchymal stem cells, C3H10T1/2, were purchased from American Type Culture Collection (ATCC, Manassas, VA). The cell lines were cultured in Dulbecco’s modified Eagle’s medium (Sigma, St. Louis, MO) and supplemented with 10% fetal bovine serum (FBS) and harvested for Western blotting to check the transfection (50 mU 100 units/ml penicillin and 100 units/ml streptomycin (Sigma) at 37°C in an incubator in the presence of 5% CO2. Human aortic smooth muscle cells (AOSMC) and human umbilical vein endothelial cells (HUVEC) were obtained from Lonza (Walkersville, MD) and maintained in smooth muscle cells basal media (SmBM) with various growth factors (SmGM-2, i.e., insulin, FGF, EGF and 2% serum) and EGM-2 bullet kit (EBM-2, the basal medium supplemented with growth factors and 5% serum) respectively. Cells were used for the experiments between four and six passages.

All experimental protocols were approved by Research and Development Committee, Kansas City VA Medical Center. Kansas City, MO 64128.

Reagents. Matrigel was purchased from BD Biosciences (San Jose, CA). Gelfoam was purchased from Pharmacia & Upjohn Company (NY, USA). CCN1/Cyr61 recombinant protein (hrCCN1) was purchased from Fisher Scientific (St. Louis, MO). Human polyclonal anti-rabbit CCN1/Cyr61 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Generation of CCN1-knockout pancreatic cancer Cells. CCN1-depleted Panc-1 [Panc-1CCN1−/−] cell line or CCN1-positive scrambled-shRNA transfected Panc-1 cell line [Panc-1Scramble/shCCN1] were generated according to our previous methods. Briefly, cloned human CCN1-shRNA or scrambled-shRNA-containing vectors (Block-iT RNAi vector, Life Technology, Grand Island, NY) were transferred into Panc-1 cells using the Neon™ transfection system. Transfected cells were treated with Zeocin™ (50 μg/ml) for stable selection. Stable cells were then cultured in regular DMEM media with 10% FBS and harvested for Western blotting to check the transfection efficiency (see Supplementary Fig.S1).

Preparation of Conditioned Media (CM) from different pancreatic cancer cells. The procedure of preparation was the same as previously described. Briefly, AsPC-1 and Panc-1 cells and genetically engineered cells (i.e., Panc-1CCN1−/− and Panc-1Scramble/shCCN1) were grown in HUVEC media for 24 hours. Media were collected and centrifuged for 10 min at 2000 rpm at 4°C followed by filtration through 0.22-μm sterile membrane (EMD Millipore, Billerica, MA) to remove the cells or cell debris. CMs were collected for endothelial cell migration and in vitro and in vivo angiogenesis assays.

Western blot analysis. The Western blot analysis was performed in different experimental cell lysates according to the method described previously. Cell lysates or CM were obtained according to our previous methods and equal amounts of protein were subjected to SDS-PAGE and Western blot. Membranes were incubated overnight at 4°C with antibodies against CCN1/Cyr61 (Cell Signaling Technologies, Danvers, MA), SHh (R&D Systems, Minneapolis, MN) and actin proteins followed by incubation with HRP-conjugated secondary antibody for 30 min at room temperature. Signals were detected with Super Signal Ultra Chemiluminescent substrate (Pierce, Rockford, IL) by using ID Image Analysis software Version 3.6 (Eastman Kodak Company, Rochester, NY).

In vitro angiogenesis assay. In vitro angiogenesis/tube formation assays were performed as described earlier. Briefly, to test the impact of CMs on in vitro angiogenesis, HUVECs (10,000 cells/well) were seeded into matrigel™ (200 μl coated chambered slides (8-well) containing endothelial cell-specific media (RM, background control) or CMs. HUVECs were incubated at 37°C for approximately 20 h, stained with Calcein AM (Trevigen, Gaithersburg, MD) to detect live cells and then the capillary-like structures were imaged using a Nikon photographic fluorescence microscope. Quantification of the number of capillary-like structures was carried out using the NIS Elements software program with modification of the protocol of Longair et al.

In vitro wound healing assays. Wound healing assays were performed with HUVECs according to our previous method. Briefly, ~25,000 HUVECs were seeded in 12-well slide chambers and grown into a monolayer culture with 100% confluency. After

Figure 1 | CCN1 protein level in pancreatic cancer cell line and different vascular cell lines. The Western blot analysis represents the expression of CCN1 in AOSMC, HUVEC, 10T1/2 and Panc-1 cells. GAPDH was used as a loading control. The bar graph represents the ratio of CCN1 and GAPDH. Data are shown as the means of three separate experiments; bars represent mean ± SEM. The figure has been cropped from original Western blots of CCN1 and β-actin, which are included in supplementary information (Figure S5).

Figure 2 | Conditioned media (CM) of different pancreatic cancer cells promotes aberrant capillary-like tube formation/tumor angiogenesis in an in vitro endothelial tube formation assay. HUVECs were seeded onto Matrigel™ in regular media (RM) or CM generated from different pancreatic cancer cells (i.e., AsPC-1, and Panc-1). After overnight, cells were stained with Calcein AM and live cells and capillary-like structures were detected by fluorescence microscope. RM is considered as a positive control. (A). Representative fluorescence images of capillary-like structures. Scale bar = 100 μm. (b). Quantification of branches using the NIS Elements software program with modification of the protocol of Longair et al. Data expressed as Mean ± SEM of three sets of experiments. For statistical analysis, a Student’s t-test was performed.
scratching through the monolayer with a pipette tip, media were replaced with RM or CMs from two genetically engineered Panc-1 cell lines. The ability to recover from the wounds (% closure) was measured using software attached to the Nikon Eclipse digital inverted photo microscope.

**In vivo Angiogenesis Assay.** The Gelfoam-implantation angiogenesis assay was performed according to our previous method. Briefly, three sets of FVB/N mice (6–8 weeks old; N = 4) were anesthetized immediately before implantation of Gelfoam (Gelfoam®, Pharmacia & Upjohn Company, NY, USA). Gelfoams (8 × 8 mm), presoaked with regular RM or CMs [Panc-1CCN1(1) or Panc-1CCN1(2)], were transplanted subcutaneously in mice. The transplanted mice were maintained for 5–6 days. The implanted Gelfoam was removed carefully after 6 days, and angiogenesis was detected and quantified using an inverted fluorescence microscope. Scale bar = 500 µm. Bar diagram of quantified capillaries is provided in supplementary figures (Fig. S4). (D). HUVECs were seeded onto Matrigel® in regular media (RM) in the presence or absence of hrCCN1 (100 ng/ml). After overnight, cells were stained with Calcein AM and live cells and capillary-like structures were detected by fluorescence microscopy. Scale bar = 500 µm.

**In vitro Cell Migration Assays.** For the detection and quantitation of migration of HUVECs in the presence of different CMs, a modified gelatin-coated Boyden chamber assay (Corning) was performed with the procedure described earlier. Briefly, ~10,000 HUVECs were seeded onto the upper chamber of the Boyden chamber with different experimental conditions as described in the figure legends. The lower chambers either contained ~90% confluent Panc-1 cells (CCN1-positive or CCN1-deleted) or CMs were added to the lower chambers. After overnight, the cells which had migrated through the membrane in response to the attractants were stained with crystal violet stain and the migration index was measured at 600-nm using VMax Microplate Reader with current version of SoftMax Pro (Molecular Devices, Sunnyvale, CA).

**Statistical Analysis.** All experiments were performed in triplicate for each of the observations. Each of the data represents the mean ± SEM from the three or more separate experiments. Statistical analysis was performed between the two groups of data by an unpaired Student’s t-test or one-way ANOVA followed by Tukey post-test to compare two groups. P-values less than 0.05 were considered statistically significant.

**Results**

CCN1 differentially expressed in different vascular cells and cancer cells. In order to determine the status of CCN1 in different cell lines involved in tumor angiogenic process, such as vascular smooth muscle cells, endothelial cells and progenitor cells of pericytes, and pancreatic cancer cells, CCN1 expression was determined in AOSMC, HUVECs, C3H10T1/2 and Panc-1 cells by Western blot analysis using CCN1-specific antibody. We found that CCN1 is constitutively expressed in AOSMC, HUVEC and Panc-1 cells (Fig. 1), while its expression was minimal or undetected in 10T1/2
pluripotent stem cells (Fig. 1). Maximum expression was identified in HUVECs as compared to AOSMC and Panc-1.

**Pancreatic cancer-cells-secreted (PCCS)-CCN1 promotes in vitro Aberrant Neovascularization.** CCN1 is a pro-angiogenic factor in normal tissues as well as in various cancers\(^{22,23,36,37}\) and plays critical role in the progression of pancreatic cancer\(^{4,15}\). In this study, we found CCN1 protein expression in both cancer cells and vascular cells (Fig. 1). Thus, these studies collectively led to the possibility that PCCS-CCN1 protein may have a tumor angiogenic function in pancreatic cancers. To test the hypothesis, first, we determined the impact of conditioned media (CM) of different pancreatic cancer cell lines (i.e. AsPC-1 and Panc-1) on the in vitro angiogenesis/capillary-like structure formation. The studies found that both regular media (RM) and CM promote in vitro angiogenesis, but CM leads to a significant increase in branching as compared to RM (Fig. 2). Moreover, the morphology of CM-induced capillary-like structures was not similar to our observations of RM-induced angiogenesis (Fig. 2), CM induces deformed capillary-like structures with unequal accumulation of endothelial cells and gaps (a possible hallmark of leakiness of blood vessels) as compared to RM, where we found few such deformed structures. (Fig. 2 and supplementary Fig. S2). Our next goal was to determine if PCCS-CCN1 plays any role in the formation of aberrant capillary-like structures. To do so, HUVECs were grown on Matrigel with the CMs collected from either Panc-1\(^{CCN1(-)}\) cells or Panc-1\(^{CCN1(+)}\) cells (Fig. 3A). As expected, in the presence of RM, HUVECs form customary capillary-like structures on the Matrigel (Fig. 3B). In contrast, when HUVECs were grown on the Matrigel in the presence of CCN1-positive CM, there was a significant increase in number of capillary-like structures as compared to Panc-1\(^{CCN1(-)}\) cells and RM, but structurally they are habitually deformed and sluggish as compared to RM [Fig. 3B(b) and Supplementary Fig. S3]. This structure is very similar to tumor blood vessels. These facets can be markedly abolished if CCN1 expression is knocked down by shRNA [Fig. 3B(c) and Supplementary Fig. S3], indicating that PCCS-CCN1 may play a critical role in tumor angiogenesis. The involvement of PCCS-CCN1 in tumor angiogenesis was further validated by growing HUVECs on matrigel with Panc-1\(^{CCN1(+)}\)-CM pre-treated with different doses of CCN1-neutralizing antibody or pre-immune serum (control) (Fig. 3C). The studies found a dose-dependent inhibitory effect of CCN1 antibody on the formation of aberrant angiogenesis promoted by PCCS-CCN1. A high dose (250 ng/ml) of CCN1-antibody exhibited a drastic effect on accumulation of endothelial cells and capillary formation (supplementary Fig. S4). Finally, in order to determine if addition of human recombinant CCN1 (hrCCN1) protein in the RM mimics the effect of tumor derived conditioned media (Fig. 3D), HUVECs were seeded on the matrigel with RM in the presence or absence of hrCCN1 (100 ng/ml) and morphological structures were determined. Unlike PCCS-CCN1, we found the same regular angiogenesis in the presence of hrCCN1 as we found in RM (Fig. 3D).

**PCCS-CCN1 promotes Aberrant Neovascularization in vivo.** To confirm the results of our in vitro angiogenesis studies, we conducted gelfoam angiogenesis assay in a mouse model. We found that, like in vitro, CM of Panc-1\(^{CCN1(-)}\) cells also resulted in enhanced aberrant angiogenesis in the mice skin as compared to RM and CCN1-negative CM (Fig. 4). In CCN1-negative CM formed a few incomplete blood vessels (Fig. 4, lower panel).

**PCCS-CCN1 promotes endothelial cell migration.** Endothelial cell migration is a hallmark of normal and pathological angiogenesis, and this process is directionally regulated by various stimuli\(^{24}\). Given the critical role of migration of endothelial cells, we sought to determine whether CCN1-positive pancreatic cancer cells contribute to the migration of endothelial cells. We assayed endothelial cell migration in the presence of Panc-1\(^{CCN1(-)}\) cells, Panc-1\(^{CCN1(+)}\) cells or regular media in an indirect co-culture system (Fig. 5A). We found that HUVEC migration was significantly less when cultured indirectly with CCN1-depleted pancreatic cancer cells as compared to CCN1-positive pancreatic cancer cells, indicating tumor cell secreted CCN1 plays a vital role in endothelial cell migration (Fig. 5A). To explore the role of CCN1 in endothelial migration in greater details, we performed in vitro wound-healing (scratch) assay. Consistent with earlier findings, the results of the scratch assay demonstrated a potential role of PCCS-CCN1 in promoting the motility of endothelial cells (Fig. 6). Additionally, the studies also found that CCN1 antibody markedly blocked the CM\(^{CCN1(+)}\)-induced motility of HUVECs, while no drastic changes were observed in the presence of CM\(^{CCN1(-)}\) + hrCCN1 protein (Fig. 6, right panel). Collectively, these results urged us to speculate that aberrant angiogenesis in pancreas could be promoted by tumor cell secreted CCN1 rather than other sources such as endothelial cells (Fig. 1). This result also indicates that PCCS-CCN1-induced tumor angiogenesis is an indirect pathway which could be generated in the tumor cells by PCCS-CCN1.

**PCCS-CCN1-induced endothelial cell migration is mediated through sonic hedgehog (SHh) signaling.** Recently, our studies...
demonstrated that CCN1 is a critical regulator of SHh in pancreatic cancer cells to exert its pathobiological functions. Moreover, SHh also regulates angiogenesis in non-classical pathways. Thus, we considered the possibility that induction of HUVEC migration by tumor-cell-secreted CCN1 is mediated through SHh. To test the hypothesis, first, we determined the status of SHh in cancer cells and vascular cells. As shown in Figure 7A, SHh protein is only expressed in Panc-1 cells and AOSMC, while it is undetected or minimally detected in HUVEC and 10T/1 cells by Western blotting. Next, we determined the endothelial cell migration in an indirect HUVEC and Panc-1 (CCN1-positive or CCN1-negative) co-culture experiment in the presence or absence of CCN1 or SHh antibodies. We found that both CCN1 and SHh antibodies significantly prevent the Panc-1 cell-secreted CCN1 induced HUVECs migration (Fig. 7B), while this effect of these two antibodies was undetected when HUVECs were co-cultured with CCN1-negative Panc-1 cells. Collectively, these studies indicate that tumor cell secreted CCN1 protein activates HUVEC migration through tumor cell generated SHh.

Figure 5 | PCCS-CCN1 promotes endothelial cell migration in an indirect co-culture assay. HUVECs were indirectly co-cultured with CCN1-positive Panc-1 [CM(CCN1(+))] cells or CCN1-depleted Panc-1 [CM(CCN1(−))] cells for 24 h. The migrated cells were stained with crystal violet stain and migration index was measured at 600 nm using VMax Microplate Reader with the current version of SoftMax Pro (Molecular Devices). Each group had nine biological replicates and the experiment was repeated twice. The left view illustrates the workflow of indirect co-culture methodology, and the right view is an endothelial cell migration assay in different experimental conditions. RM is a background control. The data represent mean ± SEM. For statistical analysis, we performed ANOVA followed by Tukey’s post-test to compare the two groups.

Figure 6 | PCCS-CCN1 promotes endothelial cell motility in in vitro wound healing assays. Representative images of scratch-wound healing exhibit the motility of HUVECs in CM of CCN1-positive Panc-1 cells with or without CCN1 antibody and CM of CCN1-depleted Panc-1 cells with or without hrCCN1 protein. Cell motility into the wound area was examined and measured by microscopy and photomicrograph was taken at 0 h and 24 h. Motility of HUVECs is indicated by red lines.

Figure 7 | SHh antibody impairs the PCCS-CCN1-induced in vitro endothelial cell migration. (A). Detection of SHh protein level in different vascular cell lines and Panc-1 cell line using Western blot analysis. β-actin is used as a loading control. The figure has been cropped from original Western blots of SHh and β-actin, which are included in supplementary information (Figure S6). (B–C). HUVECs were indirectly co-cultured with CCN1-positive Panc-1 [Panc-1CCN1(+) ] cells (B) or CCN1-negative [Panc-1CCN1(−) ] cells (C) in the presence or absence of CCN1 antibody (CCN1-ab) or SHh antibody (SHh-ab) for 24 h to test migration. The migrated cells were stained with crystal violet stain and migration index was measured at 600 nm using VMax Microplate Reader with the current version of SoftMax Pro (Molecular Devices). Each group had nine biological replicates and the experiment was repeated twice. The left view illustrates the workflow of indirect co-culture methodology, and the right view is an endothelial cell migration assay in different experimental conditions. RM is the background control. The data represent mean ± SEM. For statistical analysis, we performed ANOVA followed by Tukey’s post-test to compare the two groups.
tumor angiogenesis for therapy. Recent studies have suggested that PCCS-CCN1 blockade, which blocks tumor blood vessel formation and promotes tumor angiogenesis, can lead to therapeutic benefits in various cancers, including pancreatic cancer. Moreover, these studies have demonstrated that PCCS-CCN1 promotes tumor angiogenesis, and that the regenerative switch to tumor angiogenesis can be mediated by Sonic Hedgehog (SHh). SHh, a downstream target of PCCS-CCN1, can activate the SHh pathway in tumor endothelial cells, promoting tumor angiogenesis.

**Discussion**

Our study demonstrates that pancreatic cancer cells secrete CCN1 (PCCS-CCN1) is critical for endothelial cell migration and in vitro as well as in vivo aberrant neovascularization. The molecular mechanisms by which PCCS-CCN1 regulates endothelial migration involve modulation of SHh signaling (Fig. 8), making CCN1 an attractive therapeutic target to prevent pancreatic cancer progression.

Unlike other cancers, PDAC has been considered a non-vascular cancer. Nevertheless, multiple studies have provided ample evidence in support of a positive correlation between vascular density and PDAC progression. Moreover, studies find pancreatic cancer cells directly involved in modulation of tumor angiogenesis in vivo through paracrine-autocrine loops, and VEGF signaling has been considered a prime promoting factor. Thus, like other solid tumors, tumor angiogenesis can be a striking target for the therapy of pancreatic cancer. Despite these findings, the underlying molecular mechanism by which pancreatic cancer cells regulate tumor angiogenesis is still incompletely understood. Because CCN1 participates in pancreatic cancer progression and is known to be an angiogenic factor, the aim of this study was to uncover the role of PCCS-CCN1 in the communication of pancreatic cancer cells and endothelial cells in induction of tumor angiogenesis/aberrant neovascularization.

Tumor angiogenesis is a complex, rate-limiting and multistep process in which endothelial cell proliferation and migration followed by differentiation into aberrant capillaries are vital processes. Our combined data from different in vitro and in vivo experimental approaches displayed that PCCS-CCN1 promotes endothelial cell migration and aberrant capillary formation (Figs. 2–6). We find that tumor angiogenic response of CCN1 was strongly dependent upon the tumor microenvironment because hCCN1 was unable to promote endothelial cell migration and tumor angiogenesis when added in regular endothelial cell culture media (Fig. 3C), while tumor cell secreted CCN1 (i.e., conditioned media or co-culture system) significantly increased endothelial cell migration and promoted tumor angiogenesis. Moreover, these studies not only identify PCCS-CCN1 as a tumor angiogenic factor but also suggest that PCCS-CCN1 blockade, which blocks tumor blood vessel formation, could offer an effective strategy for disrupting tumor angiogenesis for therapy.

While we demonstrate that PCCS-CCN1 promotes tumor angiogenesis, it was not clear which molecular mechanisms link with CCN1 action. We anticipate that PCCS-CCN1 mediated tumor angiogenesis may encompass contributions of factors. Recently, we found that SHh, regulator of angiogenesis, is a downstream target of CCN1 in pancreatic cancer cells. To gain mechanistic insight into how PCCS-CCN1-directed tumor angiogenesis can be achieved, we performed indirect co-culture experiments, comparing endothelial cell migration of controls [i.e., Panc1, CCN1() and SHh-depleted (antibody-treated) conditions (Fig. 7). We found that SHh is required for CCN1-induced tumor angiogenesis. However, at this time, we do not yet understand the mechanisms by which SHh regulates tumor angiogenesis. SHh-mediated tumor angiogenic switch could be through canonical or non-canonical pathways. Thus, further studies are warranted.

In conclusion, our studies demonstrate that CCN1 of pancreatic cancer cells is vital for the regulation of tumor angiogenesis and thus could be an ideal target for tumor vascular disruption in pancreatic cancer.

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**Author contributions**

G.M. and S.M. performed research, analyzed the data and wrote the initial draft of the manuscript. I.H., S.S. and K.D. performed some of the experiments. S.K.B. and S.B. designed and supervised the project and finally reviewed the manuscript before submission to the journal.

**Additional information**

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