

***In Vivo* Effects of Conditioned Medium from Human Uterine Cervical Stem Cells in an Ovarian Cancer Xenograft Mouse Model**

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Abstract. *Background/Aim: Ovarian cancer is the most lethal of all gynecological cancers, despite advances in surgical techniques and medical treatments. During the last years, therapies based on mesenchymal stem cells and particularly their secretome (conditioned medium, CM) have emerged as promising treatments for various types of tumors. Materials and Methods: In the present study, we evaluated the in vivo antitumor effect of human uterine cervical stem cell conditioned medium (hUCESC-CM) after intraperitoneal administration in an ovarian cancer mouse model. Results: We found that intraperitoneal injection of hUCESC-CM in immunodeficient mice, injected fifty days previously with the human ovarian adenocarcinoma SKOV-3 cell line, significantly reduced abdominal tumor growth, and significantly increased overall survival, compared to control mice. Conclusion: hUCESC-CM could be an alternative approach to intraperitoneal treatment of ovarian*

cancer, either administered alone and/or with conventional chemotherapy.

Ovarian cancer is the most lethal of all gynecological cancers. This is largely attributable to the fact that approximately 75-80% of ovarian cancers are diagnosed at late stages, due to the lack of specific symptoms and of reliable means for early diagnosis. Despite advances in surgical techniques and medical treatments, median survival for advanced ovarian cancer has increased only marginally over the past two or three decades, and this is independent of geographical areas (1, 2). One feature of ovarian cancer is its limitation to the abdominal cavity for a relatively extended length of time, before spreading to distant organs, which makes it theoretically amenable to intraperitoneal therapy. Indeed, intraperitoneal chemotherapy has been widely used, mainly as an adjuvant treatment, to combat the intraperitoneal spread of ovarian cancer, or prevent recurrence of abdominal masses after radical cytoreductive surgery (3). The latest approach in this sense has been the implementation of HIPEC (hyperthermic intraperitoneal chemotherapy) protocols as an adjunct to ovarian cancer surgery. Results from two independent randomized trials on the use of this technique show a significant, but still modest increment in both overall and disease-free survival (4, 5); however, the value of HIPEC in the management of advanced ovarian cancer is still controversial (6). The main advantage of HIPEC over former protocols of intraperitoneal chemotherapy is that it is administered only once, at the end

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of the surgical debulking procedure, and with the abdominal cavity still open. However, HIPEC after radical peritoneal surgery can be carried out only in very specialized settings, mainly because of the very complicated surgery involved in the removal of all visible tumors inside the peritoneal cavity that is a prerequisite for its use. As result, the search for alternatives, or complements, to classical chemotherapeutic agents is mandatory to further improve the results.

One promising experimental approach to cancer treatment involves the use of stem cells, and particularly their secretome contained in the culture medium, also called conditioned medium (CM) (7). Our group has isolated and characterized a strain of human stromal stem cells from the uterine cervix (human Uterine Cervical Stem Cells, hUCESCs) whose secretome shows potent antitumor effects (8, 9). We have also elaborated a conceptual model, based on their site of origin, explaining their epithelium-regenerating, antitumor, anti-inflammatory, and antibiotic potential (9, 10). This theory has been partly ratified in experimental models of corneal regeneration (11, 12), acute colitis (13), and antifungal properties of hUCESC-CM against the two yeast strains most prevalent in vaginal mycoses (14).

In the present study, we tested the *in vivo* antitumor effects of hUCESC-CM after intraperitoneal administration in an ovarian cancer mouse model.

Materials and Methods

Cell cultures and hUCESC-CM. The human ovarian adenocarcinoma cell line SKOV-3 was obtained from ATCC-LGC (Barcelona, Spain). The cell line was tested and authenticated according to microscopic morphology, growth curve analysis, and mycoplasma detection in conformity with the ECACC cell line verification test recommendations. SKOV-3 were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Gibco, ThermoFisher Scientific, Waltham, MA, USA).

Culture of hUCESCs has been previously described (8). Briefly, cells were cultured in 90 mm Petri dishes at 70% confluence with 5 ml of DMEM/F12 culture medium with 10% FBS (Sigma-Aldrich), in air-CO₂ (95:5) atmosphere at 37°C for 48 h. The cells were washed three times in PBS and cultured again in 5 ml DMEM/F12 without FBS. After 48 h, the medium was centrifuged for 5 min at 300 × g to remove cellular debris, the supernatant was collected, and used either immediately or lyophilized (SP Scientific, 25L Genesis 5 Q EL-85, Gardiner, NY, USA) and stored at -80°C until used. The lyophilized hUCESC-CM was resuspended either in 5 ml (1x) or 1.25 ml (4x) of ddH₂O just before use as indicated.

MTT metabolization assays. Cell viability/proliferation experiments were carried out using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assays. SKOV-3 cells were plated at 3×10⁴ cells per well in 24-well plates. Twenty-four h later, the cells were treated with equal volumes (500 µl) of DMEM-F12 with 10% FBS (+FBS), DMEM-F12 without FBS (-FBS), SKOV-3-CM (obtained after culture for 48 h of SKOV-3 cells in DMEM without FBS), and

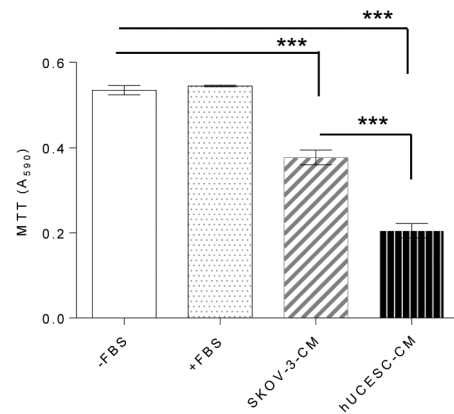


Figure 1. Treatment of SKOV-3-LUC cells with hUCESC-CM reduces proliferation/cell viability. SKOV-3 cells were cultured with incomplete medium (FBS-), complete medium (FBS+), SKOV-3-CM, and hUCESC-CM. After 48 h of culture, an MTT assay was carried out to evaluate cell viability. ****p*<0.001.

hUCESC-CM for 48h. The absorbance of samples was measured at 570 nm using a multiwell plate reader (Tecan ULTRA Evolution, Männedorf, Switzerland). Results were plotted as the mean±SEM values from three independent experiments.

Animal studies. All animal studies were approved by the University of Santiago de Compostela Ethics Committee for Animal Experiments. Age-matched female mice between 6-8 weeks old, (BALB/cAnNRj-Foxn1nu/Foxn1nu, Janvier Labs, Saint Berthevin Cedex, France) were used for xenografting studies. Twenty nude mice (10 controls and 10 treated) were injected intraperitoneally (*i.p.*) with 5×10⁶ SKOV-3 cells stably transfected with the pBabe-luciferase vector (SKOV-3-LUC cells) and selected with puromycin. After luciferin injection (150 mg/kg), tumor growth was monitored externally by luminescence using the *In Vivo* Imaging System (IVIS, Caliper Life Sciences, Alameda, CA, USA). An intensity map was obtained using the Living Image software (Caliper Life Sciences), which uses a color-based scale to represent the intensity of each pixel (from blue representing low to red representing high). Fifty days after cell injection, mice were injected *i.p.* (250 µl) with DMEM/F-12 (control) or with hUCESC-CM-4x twice a week, until the endpoint of each mouse. Two control and two treated mice were sacrificed on days 66 (one control and one treated mouse) and 77 (one control and one treated mouse), and abdominal tumors extracted, and H&E stained. Immunohistochemical analyses were also performed. The remaining 8 mice were monitored for survival analyses.

Histological and IHC analyses. Organs were removed from mice, fixed in 10% neutral buffered formalin for 24 h, embedded in paraffin and routinely sectioned. Sections of 4 µm thickness were stained with haematoxylin-eosin (H&E) and scanned at 20x using a PathScan® (Excilone, Elancourt, France). For immunohistochemistry, sections were mounted on silan-coated slides (Dako-Agilent, Glostrup, Denmark) and automatically immunostained in an Autostainer Link-48 (Dako-Agilent). As primary antibodies, we employed FLEX ready to use monoclonal antibody against Ki-67 (clone MIB1, Dako-Agilent) and caspase-3 rabbit polyclonal antibody at a dilution of 1:100 for 30 min (Cell Signalling, Technology, Danvers, MA, USA). As detection

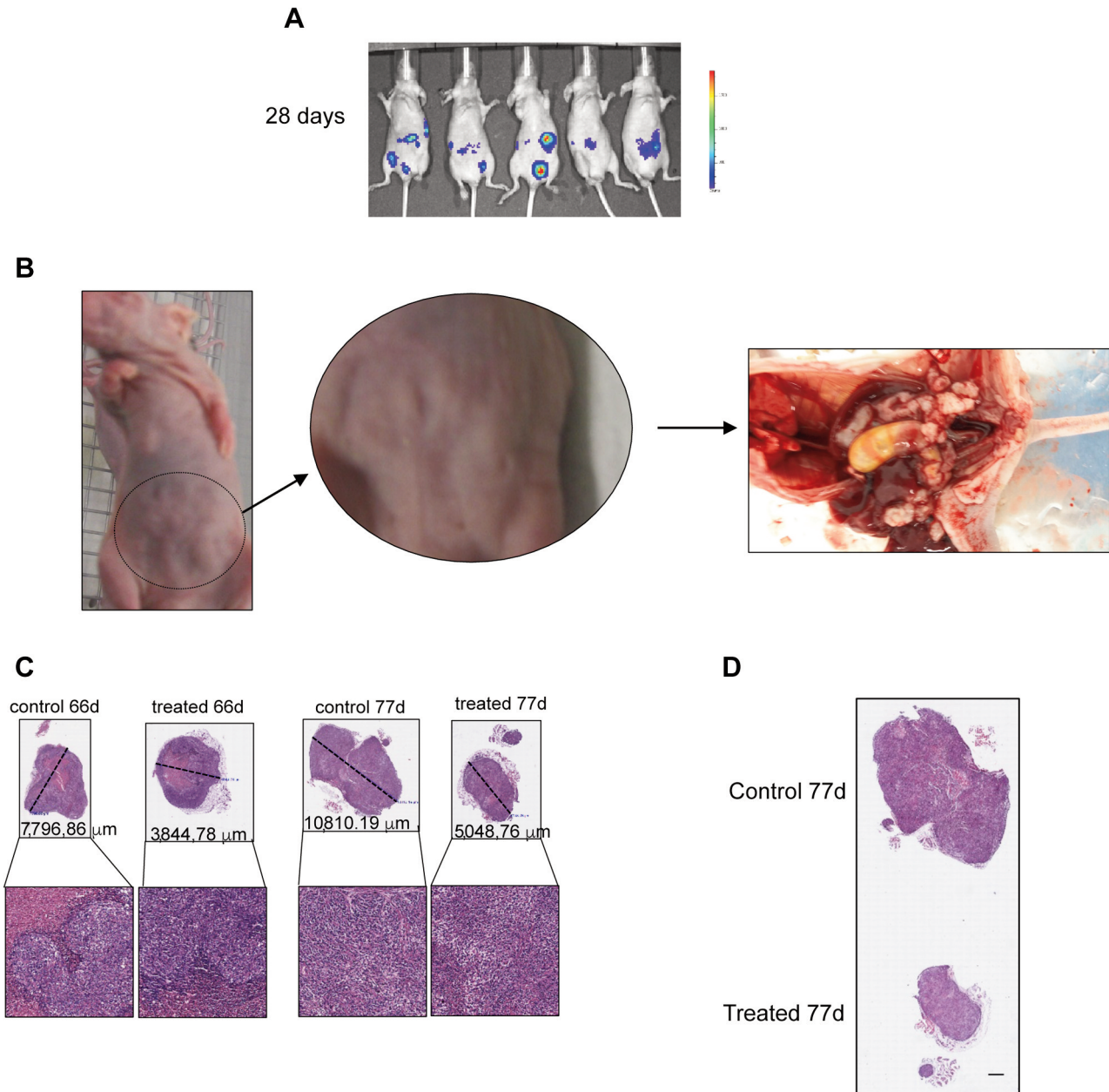


Figure 2. Treatment with hUCESC-CM reduces tumor growth in an ovarian cancer mouse model. (A) Example of five mice injected intraperitoneally with SKOV-3-LUC cells at day 28. (B) Representative image of one control mouse sacrificed on day 66, showing abdominal tumors. (C) H&E staining of peritoneal tumors in controls and treated mice (days 66 and 77 after SKOV-3-LUC cells injection). Dotted lines indicate tumor length in μm . (D) H&E staining of tumors from a control (up) and a treated (down) mice at day 77. Scale bar=1 μm .

system, we used EnVision FLEX/HRP (Dako) for 30 min and 3,3'-diaminobenzidine tetrahydrochloride (DAB) as chromogen for 10 min.

Statistical analysis. Each experiment was performed at least 3 times. Values are expressed as mean \pm SEM. Means were compared using 1-way ANOVA, with the Tukey-Kramer multiple comparison test for *post-hoc* comparisons. For overall survival analysis, the log-rank (Mantel-Cox) test was used. GraphPad Prism software was used for all calculations. The level of significance was $p < 0.05$.

Results

To explore the possible effect of hUCESC-CM on the SKOV-3 cell line, we evaluated cell proliferation/ cytotoxicity. As shown in Figure 1, a significant ($p < 0.001$) decrease in MTT metabolism was observed in SKOV-3 cells after 48 h of hUCESC-CM administration as compared to cells treated with medium with and without FBS, and with SKOV-3-CM. We

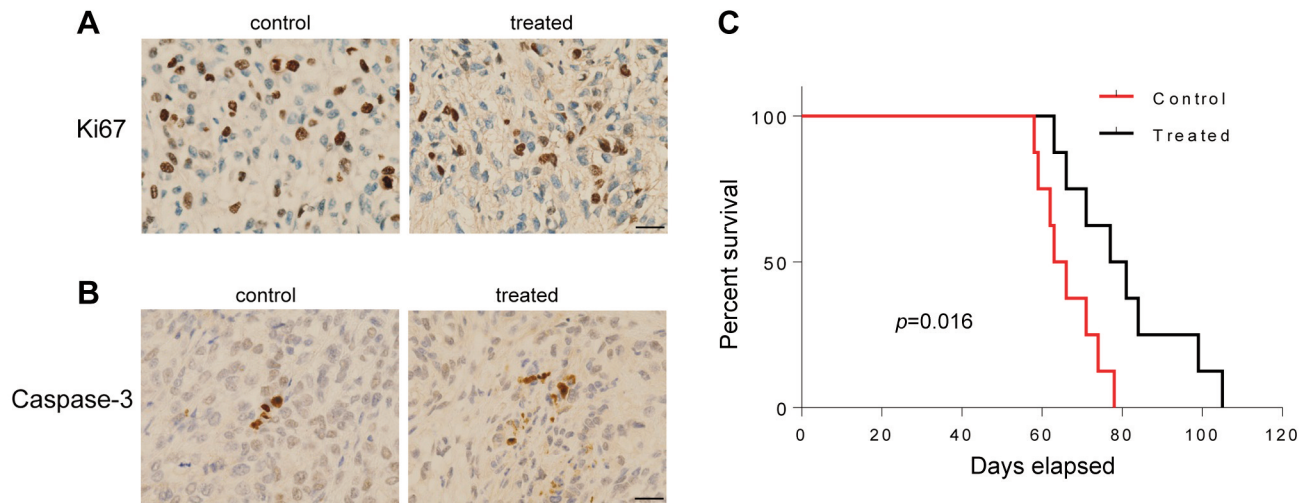


Figure 3. Treatment with hUCESC-CM increases overall survival *in vivo* in an ovarian cancer mouse model. (A) Immunohistochemistry (IHC) analysis of the Ki-67 proliferation marker in tumors of control and hUCESC-CM treated mice at day 77. Scale bar=40 μ m. (B) IHC analysis of the caspase-3 apoptosis marker in tumors of control and hUCESC-CM treated mice. Scale bar=40 μ m. (C) Kaplan-Meier plot of overall survival (OS) in CM-treated vs. control mice ($p=0.016$).

next evaluated the effect of the administration of hUCESC-CM *in vivo* in a severe immunodeficient (SCID) mouse tumor xenograft model. Tumor growth was monitored externally by luminescence using the IVIS (Figure 2A). Fifty days after *i.p.* injection of SKOV-3 cells, when peritoneal tumor masses became visible and palpable, mice were *i.p.* injected twice a week with either DMEM/F-12 (controls) or hUCESC-CM (250 μ l). On days 66 and 77 one control and one treated mouse were sacrificed, and necropsy was performed (Figure 2B). Both controls and treated mice had tumors in the abdomen, as demonstrated by H&E staining (Figure 2C). Individual abdominal tumors had about half the size in treated mice compared to controls (Figure 2C-D). IHC analyses of the ki-67 proliferation marker indicate an intense proliferative index (>25%) in both control and treated mice (Figure 3A). Similarly, caspase-3 protein expression (as an apoptosis marker) between controls and treated mice does not show significant differences (Figure 3B). Finally, to evaluate overall survival between control and treated mice, eight mice per group were followed until death. As shown in Figure 3C, Kaplan-Meier survival plots indicated that treatment with hUCESC-CM significantly increased the overall survival of mice as compared to controls ($p=0.016$).

Discussion

To the best of our knowledge, this is the first report about the *in vivo* antitumor effect of mesenchymal stem cell-CM in an ovarian cancer animal model. Our results show that intraperitoneal infusion of hUCESC-CM induced tumor

regression and significantly prolonged survival of treated mice, compared to the control group.

In a recent report, it was shown that mesenchymal stem cells from different sources (human bone marrow, adipose tissue, and umbilical cord) and their conditioned media have anti-proliferative effects *in vitro* against several human ovarian cancer cell lines (15). Our results agree with these data. Indeed, it is highly interesting to verify that *in vivo*, abdominal tumors demonstrated an obvious reduction in size by almost half in treated mice with respect to controls, although no significant differences were observed in proliferation and apoptosis, probably due to the low sample size. More importantly, hUCESC-CM treatment significantly increased overall survival compared to controls, which is not always the case after successful *in vitro* experiments.

Besides the obvious antitumor potential, the use of stem cell-CM for intraperitoneal treatment may present some additional advantages over conventional intraperitoneal chemotherapy. For example, it can be easily obtained by means of such a simple procedure as a Pap smear, a cervical biopsy, or a fragment of the cervical transformation zone at the time of surgery (8). This would eliminate any theoretical concern for tolerance. It is worth noting that despite its promising features, stem cell therapy is still in its infancy, and potential side-effects are not well known. However, CM treatment, especially as local or regional treatment, as the one applied herein, is theoretically much less likely to produce any life-threatening complications.

One conflicting and unexpected finding in this investigation was the apparent lack of any effect on either proliferation or

apoptosis in the hUCESC-CM treated tumors, in the admittedly very small number of samples tested for this purpose. This would be in apparent contradiction with our original findings, when we isolated and characterized the hUCESC line (8). In fact, after exposure to the conditioned medium from hUCESC, both inhibition of proliferation and increase of apoptosis took place in the studied breast cancer cell lines *in vitro*, especially so in the biologically more aggressive ones, markedly limiting their growth. Growth was clearly inhibited *in vivo* in the ovarian tumors of the present study, so much so, that despite its small size, the group of treated mice showed a significant increase in survival. One of the mice in this group had to be euthanized time after completion of the follow-up period and was clinically tumor-free at that point, something absolutely unusual in the face of such an aggressive tumor. Although neither Ki67 (proliferation), nor caspase-3 (apoptosis) expression, were apparently altered in the few samples tested, this does not mean, by far, that proliferation, or apoptosis, are not involved in the tumor growth restriction observed. Besides the small number of samples, another possible confounder is the technique employed (immunohistochemistry), which is purely qualitative. A larger cohort, using quantitative techniques, such RT-PCR, for caspase-3 expression measurement, and flow cytometry for proliferation assessment would possibly yield different results, more in line with those reported for our *in vitro* experiments. Moreover, alternative mechanisms of growth limitation might be involved. The decisive role played by the cross-talk between the original tumor cells and their surrounding, predominantly fibroblastic stroma (and hUCESC is a stromal stem cell line) is steadily gaining relevance, as highlighted in a recent revision (16). In this sense, the tumor microenvironment, and more broadly speaking, the immune system, plays a critical role in the progression and control of cancer through secretion of various cytokines, chemokines, and other factors. Hypothesizing that cytokines have a prominent role in mediating antitumor effects of hUCESC-CM, we used a screening approach with a human cytokine antibody array. Out of 174 cytokines analysed, 4 cytokines with known antitumor effects showed higher expression in hUCESC-CM-treated tumors as compared to controls (9). Interferon-gamma-inducible protein-10 (IP-10) which was increased more than 16-fold (2553 *vs.* 153, $p < 0.001$) in the hUCESC-CM-treated tumors compared to controls, could potentially inhibit ovarian tumor growth via suppression of tumor angiogenesis, inhibition of tumor cell proliferation and induction of apoptosis of ovarian cancer cells (17). Fms-related tyrosine kinase 3 ligand (FLT3LG) is another cytokine that demonstrated a fivefold increase (1,033 *vs.* 190, $p < 0.001$) relative to controls. FLT3LG has been reported to manifest potent antitumor activity in animal models, presumably due to its capacity to recruit dendritic cells, T cells, and Natural Killer cells *in vivo* (18). Besides, there was a more than the threefold increase (39 *vs.* 12, $p < 0.001$) in tumor necrosis factor superfamily member 14 (TNFSF14) in the hUCESC-CM-treated

tumors compared to controls. TNFSF14 has been found to induce an antitumor immune response with significant changes in the tumor microenvironment hallmarked by an increase in tumor-infiltrating lymphocytes (19). In addition, there was a 120-fold increase in Latency-associated peptide (LAP) (21376 *vs.* 178, $p < 0.001$) that activates TGF- β , which in turn functions as a potent tumor suppressor by inducing growth inhibition and apoptosis (20). Given the antitumor effects of these cytokines, hUCESC-CM might contribute to cancer suppression in our *in vivo* ovarian cancer model by mechanisms different from pure proliferation inhibition or apoptosis induction.

In summary, our study provides evidence that intraperitoneal administration of hUCESC-CM reduces peritoneal tumor volume and significantly increases overall survival in an ovarian cancer mouse model, suggesting that either hUCESC-CM alone or in combination with conventional intraperitoneal chemotherapy could be a promising therapeutic tool in ovarian cancer.

Conflicts of Interest

The Authors declare the following competing interests: Francisco J. Vizoso, Roman Perez-Fernandez, and Noemi Eiró are co-inventors of a patent ("Human uterine cervical stem cell population and uses thereof") owned by GiStem Research, of which some authors are shareholders (JS-L, SS, NE, FV, RP-F, and JS). The founding sponsors had no role in the design of this manuscript, in the collection, analyses, or interpretation of data, in the writing of the manuscript, or in the decision to publish the results.

Authors' Contributions

Conceptualization: J.S., R.P-F., F.V., M.M.; Methodology and analysis: J.S-L., S.S., N.E., L.G-C., E.A.; Writing: F.S., R.P-F., J.S. All Authors have read and agreed to the published version of the manuscript.

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