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Stem cell paracrine actions in tissue regeneration and its potential therapeutic effect in human endometrium: a retrospective study.

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Abstract:	Objective: Determining genetic and paracrine mechanisms behind endometrial regeneration in Asherman's Syndrome and Endometrial Atrophy (AS/EA) patients after autologous CD133+ bone marrow-derived stem cells (CD133+BMDSCs) transplantation. Design: Retrospective study using human endometrial biopsies and mouse models. Setting: Fundación-IVI, IIS-La Fe, Valencia, Spain. Samples: Endometrial biopsies collected before and after CD133+BMDSCs therapy, from 8 women with AS/EA (NCT02144987). And uterus from 5 mice, with only left horns receiving CD133+BMDSCs therapy. Methods: In human samples, hematoxylin and eosin (H&E) staining, RNA arrays, PCR validation and neutrophil elastase (NE) immunohistochemistry (IHQ). In mouse samples, PCR validation and protein immunoarrays. Main outcome measures: H&E microscopic evaluation, RNA expression levels, PCR and growth/angiogenic factors quantification, NE IHQ signal. Results: Treatment improved endometrial morphology and thickness for all patients. In human samples, JUN, SERPINE1 and IL4 were up-regulated while CCND1 and CXCL8, down-regulated, after treatment. The significant decrease of NE signal corroborated CXCL8 expression. Animal

	model analysis confirmed human results and revealed a higher expression of pro-angiogenic cytokines (IL18, HGF, MCP1, MIP2) in treated uterine horns. Conclusions: CD133+BMDSCs seems to activate several factors through a paracrine mechanism to help endometrium regeneration, through an immunological tolerance milieu that precedes proliferation and angiogenic processes. Insight in these processes could bring us one step closer to a non-invasive treatment for AS/EA patients. Funding: ISCIII (PI17/01039, CD15/00057); Generalitat Valenciana (PROMETEO/2018/137, ACIF/2017/118, ACIF/2015/271). Keywords: Endometrial regeneration, bone marrow-derived stem cells, paracrine mechanisms, Asherman's Syndrome, Endometrial Atrophy. Tweetable abstract: CD133+BMDSCs regenerate endometrium via an immunological tolerant milieu that heads proliferation and angiogenesis.

TITLE

Stem cell paracrine actions in tissue regeneration and its potential therapeutic effect in human endometrium: a retrospective study.

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SHORTENED RUNNING TITLE

Endometrial regeneration via CD133^{+} stem cells.

ABSTRACT

Objective: Determining genetic and paracrine mechanisms behind endometrial regeneration in Asherman's Syndrome and Endometrial Atrophy (AS/EA) patients after autologous CD133+ bone marrow-derived stem cells (CD133+BMDSCs) transplantation.

Design: Retrospective study using human endometrial biopsies and mouse models.

Setting: Fundación-IVI, IIS-La Fe, Valencia, Spain.

Samples: Endometrial biopsies collected before and after $CD133^{+}$BMDSCs therapy, from 8 women with AS/EA (NCT02144987). And uterus from 5 mice, with only left horns receiving $CD133^{+}$BMDSCs therapy.

Methods: In human samples, hematoxylin and eosin (H&E) staining, RNA arrays, PCR validation and neutrophil elastase (NE) immunohistochemistry (IHQ). In mouse samples, PCR validation and protein immunoarrays.

Main outcome measures: H&E microscopic evaluation, RNA expression levels, PCR and growth/angiogenic factors quantification, NE IHQ signal.

Results: Treatment improved endometrial morphology and thickness for all patients. In human samples, *JUN*, *SERPINE1* and *IL4* were up-regulated while *CCND1* and *CXCL8*, down-regulated, after treatment. The significant decrease of NE signal corroborated *CXCL8* expression. Animal model analysis confirmed human results and revealed a higher expression of pro-angiogenic cytokines (IL18, HGF, MCP1, MIP2) in treated uterine horns.

Conclusions: $CD133^{+}$BMDSCs seems to activate several factors through a paracrine mechanism to help endometrium regeneration, through an

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Funding: ISCIII (PI17/01039, CD15/00057); Generalitat Valenciana (PROMETEO/2018/137, ACIF/2017/118, ACIF/2015/271).

Keywords: Endometrial regeneration, bone marrow-derived stem cells, paracrine mechanisms, Asherman's Syndrome, Endometrial Atrophy.

Tweetable abstract: $CD133^{+}$BMDSCs regenerate endometrium via an immunological tolerant milieu that heads proliferation and angiogenesis.

INTRODUCTION

Stem cell therapy is a widely used technique in regenerative medicine that has provided promising results recently. Therapies using autologous stem cells can successfully treat different diseases such as limb ischemia[1], or multiple myeloma.[2] While adult/somatic stem cells are present in many tissues,[3] adult bone marrow is a well-known reservoir of mesenchymal stem cells and endothelial progenitor cells (EPCs).[4,5] CD133 is a surface antigen that defines a broad population of adult/somatic stem cells, including EPCs.[6] The regenerative properties of $CD133^+$ hematopoietic bone marrow-derived stem cells ($CD133^+$BMDSCs) have been demonstrated in many fields, most notably in ischemic heart conditions.[7]

Recent evidence supports that paracrine actions provoked by these cells play an essential role in mediating regeneration via releasing biologically active factors.[8] The main premise defining this concept was described by Baraniak and McDevitt: “a recent paradigm shift has emerged suggesting that beneficial effects of stem cells may not be restricted to cell restoration alone, but also due to their transient paracrine actions”.[9]

From all endometrial pathologies, Asherman’s Syndrome (AS) and Endometrial Atrophy (EA) are some of the most relevant for assisted reproduction. AS is characterized by intrauterine adhesions caused by curettage or uterine traumas, leading to a lack of functional endometrium.[10] Meanwhile, EA caused by poor endometrial growth resulting from several risk factors (lack of estrogens, surgical interventions or idiopathic causes). Women with AS/EA have a higher risk of impaired implantation, early miscarriage, and diminished pregnancy rate.[11] Though different treatments have been tried (exogenous estrogen, low-

dose aspirin, vaginal sildenafil citrate),[12] only stem cell therapy has demonstrated to be effective.[13–18] Moreover, BMDSCs and their paracrine effects have shown promising results in ovarian rejuvenation,[19] follicular restoration,[20] embryo culture[21,22] and chronic pelvic disease treatment[23].

In this context, our group has recently completed an innovative study showing the regenerative effects of CD133$^+$BMDSCs in human[18] and murine[15] AS/EA models. The low frequency of stem cell engraftment in our animal model appeared insufficient to explain the described significant improvement of endometrial regeneration. This observation supports the mentioned premise that the final effectors of the regenerative process are soluble factors released by the transplanted CD133$^+$BMDSCs.[24]

This report represents a continuation of these previous studies,[15,18] where we further investigate different factors and mechanisms that are induced by CD133$^+$BMDSCs and assist endometrial recovery. The identification of these transient effects could be valuable to learn the specific patterns of endometrial regeneration and to possibly create non-invasive therapies for AS/EA.

MATERIAL AND METHODS

Study participants, experimental design and histological analysis

Eight patients from our previous pilot study (ClinicalTrials.gov NCT02144987),[18] were selected for this project. A detailed description of these patients is given in S2.

Samples used for this study were human endometrial formalin-fixed and paraffin-embedded (FFPE) biopsies obtained before and three months after autologous CD133$^+$BMDSCs injection. All biopsies were taken during the

proliferative phase under hormonal replacement therapy cycles (estradiol alone before progesterone). Experimental design is detailed in Figure 1.

Hematoxylin and eosin (H&E) stain using standard protocols were performed: morphological, microanatomical and histological analysis of individual samples were carried out and compared individually in both groups (Figure 1).

RNA isolation and reverse transcription

Human endometrial tissues were cut into 5-µm sections per block and condition (before/after treatment). Samples were randomly joined into 2 pools: patients #1-#4 (pool 1) and patients #5-#8 (pool 2). These pools were treated for RNA isolation accordingly the RNeasy FFPE Handbook protocol (QIAGEN, Germany).

For reverse transcription, the First-Strand cDNA Synthesis protocol from FFPE samples (QIAGEN, Germany) was used.

Molecular analysis and gene expression arrays

Before performing the arrays, cDNA was evaluated with the housekeeping gene GAPDH (QIAGEN, Germany) by quantitative real-time PCR (qRT-PCR). Then three qRT-PCR s with RT2 Profiler PCR Arrays format C (QIAGEN, Germany) were carried out for before and after treatment (pools 1 and 2): PAHS-040ZC: Human EGF/PDGF Signaling Pathway, PAHS-041ZC: Human Growth Factors, and PAHS-072ZC: Human Angiogenic Growth Factors. These 3 arrays were selected based on previous results which suggested proregenerative and proangiogenic effects as a result of the stem cell therapy.[15,18]

Bioinformatics data analysis

Analysis of the qRT-PCR data was performed following the approach of Yuan *et al*.[25] Then, a *t*-test was calculated for each gene comparing ΔCT values between both groups. CT values of statistically significant genes were represented in a heatmap with rows and columns ordered using hierarchical clustering.

After analyzing gene expression arrays, the KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway database was used by manually annotation of the genes.[26]

Human gene array validation

To verify the results, the expression of *JUN* (jun proto-oncogene, c-Jun), *CCND1* (cyclin D1) and *CXCL8* (C-X-C motif chemokine ligand 8) was analyzed. qRT-PCR was performed using specific primers (Thermo Fisher Scientific, USA) (S3). Relative gene expression levels were determined by the ΔΔCt and normalized to GAPDH. Qiagen Data Analysis Software (https://www.qiagen.com/shop/genes-and-pathways/data-analysis-center-overview-page/) was used to calculate fold regulation (FR).

Neutrophil elastase protein expression

Immunohistochemistry for neutrophil elastase (NE) in the human samples was performed. Deparaffinized tissue sections were incubated with monoclonal mouse anti-human NE (1:100; M0752 Dako, Agilent, CA, USA); human tonsil was used as positive control. Then the Envision HRP system was used (K4065, Dako, Agilent, CA, USA).

Randomly chosen areas at X20 magnification of NE stained slides were evaluated by three blinded observers. An average of 2,400 cells were counted,

by Image-Pro Plus Software v6.3 (MediaCybernetics, MD, USA), to analyze stained cells in before/after treatment samples. Total NE expression was presented as the mean percentage of positive signals versus total cells with their corresponding standard deviation (SD).

Murine models with Asherman's Syndrome and human CD133⁺BMDSCs transplantation: validation of human results and protein immunoarrays

Uteri (n=5) from previously previous published work were used.[15] Here, both horns were mechanically damaged and the left horns were treated by intrauterine injection with human CD133⁺BMDSCs, the damaged right horns were maintained as controls (Figure 4A).

Firstly, some of the differentially expressed human genes were validated in the mouse model: *Jun*, *Serpine1* (PAI-1, plasminogen activator inhibitor-1) and *Ccnd1*. To note *IL4* (Interleukin 4) or *CXCL8* were not tested, the former cannot be detected in NOD-SCID mice due to its dynamic activity in allograft rejection via T cells[27] and the latter is not expressed in mice.[28] RNA extraction and qRT-PCR were performed as detailed before; specific primers are in S3. Secondly, cytokine profile and growth and angiogenesis factors in the uterine tissue were measured. After deparaffination and rehydration, total protein extraction was performed using Qproteome FFPE Tissue Kit (QIAGEN, Germany). Similar to the human model, two multiplex immunoarrays were done to investigate molecules involved in regeneration and angiogenic processes: Mouse Cytokine & Chemokine 26-plex ProcartaPlex Panel (Thermo Fisher Scientific, USA) and MILLIPLEX MAP Mouse Angiogenesis/Growth Factor Magnetic Bead Panel (MERCK, Germany). Quantification was carried out using a Luminex MagPix system and Luminex xPonent Software.

Statistical data analysis

Statistical analysis was performed using GraphPad Prism 7.04 software. Data are presented as mean ± SD. A paired sample *t*-test was used to analyze NE signals in before/after treatment samples and in the immunoarrays data. *P*-value<0.05 was considered as significant.

RESULTS

Endometrial reconstruction after cell therapy with CD133⁺BMDSCs

H&E staining of the human endometrium before treatment showed stromal compaction and a non-functional secretory glandular morphology in most of the samples (#1, #3, #4, #5, #8) (Figure 1, upper panel). In contrast, 3 months after treatment with CD133⁺BMDSCs, the endometrium displayed clear stromal organization and the morphology of the glands varied from inactive to secretory (Figure 1, lower panel). In all the cases, the histological pattern of the endometrial samples after treatment was improved from a functional point of view, with exceptional results in patients #1-#4, #7, and #8.

Endometrial thickness in all patients ranged between 3 and 5 mm before treatment, after treatment this broadened to a range of 5 to 12 mm. More details can be found in S2.

Gene expression arrays in samples before and after treatment

A total of 252 genes were analyzed from the three different gene arrays used. To note we are only taking those genes into account that have highly restricted significant expression patterns in both pools. Therefore, only six genes had a significantly different expression in the treatment group: *JUN* (p=0.037), *ARAF*

(*p*=0.049), and *CCND1* (*p*=0.043) from the Human EGF/PDGF Signaling Pathway array; *IL4* (p=0.041) from the Human Growth Factors array; and *CXCL8* (*p*=0.036) and *SERPINE1* (*p*=0.026) from the Human Angiogenic Growth Factors array. We discarded *ARAF* because it showed to be up-regulated in pool 1 but down-regulated in pool 2. However, the other 5 genes show to be up- or down-regulated in both pools. *JUN*, an oncogene, *SERPINE1*, an inhibitor of fibrinolysis, and *IL4*, involved in immune response, were down-regulated, but became up-regulated after treatment. On the other hand, *CCND1*, a regulator of CDk4 kinase, and *CXCL8*, a potent mediator of the inflammatory response, were down-regulated after treatment. As seen in Figure 2B, *CXCL8* was the gene with the highest FR between conditions.

Selection and validation of reference genes

Validation of the 3 genes selected from the gene array results corroborated that in human samples *JUN* was up-regulated after the treatment (FR=1.429), while *CCND1* and *CXCL8* were down-regulated (FR=-1.434 and -26.546, respectively) (Figure 2B).

Gene expression pattern analysis

The KEGG pathway database was used to characterize the differentially up-regulated gene functions.[26] Here it became apparent that *JUN*, *SERPINE1* and *IL4* could fundamentally influence cell cycle progression and angiogenesis, playing roles in anti-apoptosis, cell differentiation, proliferation and survival, cytokine production, cellular growth, and chemotaxis. Seven signal transduction pathways in wich these genes take part were identified: Wnt, MAPK, and TNF

pathways correlated to *JUN*; Wnt, HIPPO, and TGFβ pathways to *SERPINE1*; and, JAK-STAT, and PI3K-AKT to *IL4* (Figure 2C).

Neutrophil elastase protein expression before and after treatment

To demonstrate the effect of *CXCL8* downregulation, a neutrophil chemoattractant, NE immunohistochemistry was performed (Figure 3A-B). After counting all positive signals, we detected a statistically significant decrease after treatment in all patients (p=0.025) (Figure 3).

Validation of human genes results and protein expression in murine models

It was confirmed in murine samples that *Jun* and *Serpine1* genes up-regulated after the treatment (FR=1.215 and 2.231, respectively), while *Ccnd1* was down-regulated (FR=-2.921) (S1).

Multiplex immunoarrays of the uterine horns were performed (injected -treated- and non-injected -not treated- with human $CD133^{+}$BMDSCs) (Figure 4A). The expression of all proteins in the treated (n=5) and not treated (n=5) horns can be found in S4. From the 48 target proteins analyzed, four showed a statistically significantly higher expression in treated horns: IL18 (interleukin-18), HGF (hepatocyte growth factor), MCP-1 (C-C motif chemokine 2) and MIP2 (C-X-C motif chemokine 2) (Figure 4B-C). Other interesting proteins such as VEGFA (vascular endothelial growth factor A), FGF-2 (fibroblast growth factor 2), betacellulin, TNFα (tumor necrosis factor) or interleukin-10 also showed a tendency towards having a higher expression in the treated horn without being significant (S4).

DISCUSSION

Main discussion

Even though this study is based on findings and samples previously obtained by our group,[15,18] all the results showed and discussed here are completely new, reinforcing interesting and new concepts in the regenerative medicine field mainly in endometrial regeneration after stem cell therapy.

The present study elucidated for the first time some of the specific mechanisms responsible of endometrial tissue repair in patients suffering from AS/EA after specific autologous stem cell treatment. The identification of five differentially expressed genes (*JUN*, *SERPINE1*, *IL4*, *CCND1* and *CXCL8*) related with the therapeutically potential of $CD133^{+}$BMDSCs describes an immunomodulatory scenario and a subsequent dynamic regeneration. We also observed a decrease in the human NE expression influencing probably the inflammatory responses and the immune system in treated patients. After validating a number of these genes in both women and immunocompromised mice, mouse horns revealed to overexpress crucial angiogenic and reparative factors like IL18, HGF, MCP-1 and MIP2 after stem cell administration, reinforcing its regenerative potential.

AS/EA are pathological conditions strongly related to subfertility and recurrent implantation failure.[12,29] The implication of BMDSCs in endometrial tissue recovery has been widely documented in mouse models,[17,30] macaques[31] and humans[32–34] however the specific events by which this grafting may improve the restoration still remains unknown. Current research efforts include elucidating the systems implicated in tissue regeneration driven by BMDSCs.[8,9,35] Our aim is to decipher the stem cell mechanisms and paracrine signals implicated in the

recovery and regeneration of pathological endometrium after BMDSCs treatment in humans and mice.

The silencing of the immunologic milieu in treated women could be led mainly by the noteworthy down-regulation of *CXCL8* gene; described as a cytokine involved in neutrophil activation and T cell chemotactic activity[36] avoiding the production of an effective immune response.[37] Moreover, we corroborated this by the arrest of neutrophils due to the significant reduction of NE expression.[38] Interestingly, some studies correlated the decrease of *CCND1* gene expression, an oncogenic cell-cycle regulator which varies with the phase of the cell cycle in normal cells,[39] with the down-regulation of *CXCL8*.[39–41] A decline of *CCND1* indicates that cells are in S (synthesis) and G2-M (growth and mitosis) phases promoting a proper status for proliferation and functional endometrial recovery.[39,42,43]

This hypothesis is supported further by the upregulation of the *SERPINE1* gene.[44] *SERPINE1* has been described to be mainly produced by the endothelium,[45] is implicated in arterial remodeling in cardiac wound healing[46] and is required for keratinocyte migration during cutaneous injury repair.[47] In the human endometrium, the increased expression of *SERPINE1* was described throughout decidualization,[48] giving rise to vascular remodeling and morphological and functional changes in the stromal cells.[49] In our context it is likely that *SERPINE1* may be an influencer toward differentiation and neovascularization during the regeneration of the stromal compartment. The increase of *IL4* expression is correlated to higher proliferation, differentiation, and anti-apoptosis actions in several cell types including cancer cells;[36,50]

probably inferring here in the treated endometrium a cascade of regenerative events.

Beyond the events described above, affecting the endothelial and stromal compartments of the human endometrium, these effects were also accompanied by the epithelial endometrial differentiation presumably guided by *JUN*.[51–54] The moderate up-regulation of this gene in treated patients was associated with the regeneration of the epithelial endometrial compartment due to its role as an important mediator of epithelial cell development and proliferation. The central role of *JUN* in proliferation and differentiation of primary human keratinocytes was shown by the formation of an aberrant epithelium in the murine epidermis when *c-Jun* is not expressed.[55] Moreover, Salmi *et al*. described how *JUN* expression appeared to be associated with the proliferation of endometrial epithelial cells but remained relatively unchanged in the stromal compartment in human endometrium.[56]

To support our study we attempted to identify the repertoire of secreted factors in the animal model.[15] Several detected human genes were also validated in mouse uterine tissue, increasing the possibility that the events observed in the animal model could be also taking place in the human endometrium. From all the selected factors analyzed (S4), IL18, HGF, MCP-1 and MIP2 showed a higher expression pattern on treated horns when compared with controls.

IL18, commonly described as a pro-inflammatory cytokine, can also operate as an angiogenic factor,[57] suggesting its role to promote neovascularization after tissue injury. Furthermore, HGF is not only implicated in endometrial remodeling during the estrous cycle but also in cell proliferation via auto/paracrine mechanisms in the mouse endometrium and mainly in epithelial cells.[58,59] In

addition, HGF has been postulated to regulate its own activation by the upregulation of the protein product from *SERPINE1* gene.[60,61] And it has also been described to be up-regulated when *JUN* is overexpressed.[62] Additionally, MCP-1 has been widely described in tissue repair, remodelling and angiogenic processes (induction of migration and sprouting of endothelial cells and the increase of vascular permeability).[63,64] In relation to this, Butler *et al*.[65] described MCP-1 and HGF (and also VEGFA) as angiocrine factors, which are defined as factors from vascular endothelial cells that have a paracrine action. Lastly, MIP-2 has also been described to enhance cell proliferation, mainly in hepatic tissue.[66] Interestingly, the pro-angiogenic properties shared among these 4 factors correlate with the neovascularization and regenerative evidences we found on the human model.

Strengths and limitations

This study provides detailed information to explain complex mechanisms at gene and protein levels that are related to human endometrial regeneration after stem cell therapy. Findings can be generalized because the selection process is well-designed and samples are representative of the study population; moreover this was corroborated in a mouse model. Nevertheless, future studies using specific molecules identified in the murine model (cytokines, chemokines, growth and angiogenic factors) should be also tested in human endometrial tissue to assess our preliminary results as a non-invasive therapy in patients suffering AS/EA. To note that several factors identified in the murine model were related to the inflammatory response, but due to the fact we are working with NOD-SCID mice, we did not fixate our discussion/work in that direction.

Interpretation

Thanks to the elucidation of certain transient paracrine actions it is possible that these factors could be used in the future to enhance the therapeutic efficacy of stem cell approaches. In this sense, it starts to clarify the mechanisms of the regenerative process after stem cell therapy. In general, the mechanisms sustained by the transplanted stem cells were quite similar in human and murine models. Firstly, by the establishment of an immunotolerant milieu favoring regenerative events. Followed by the respective proliferation of the endothelial, stromal and epithelial compartments guided by very different and specific patterns. And all together accompanied by the global neovascularization process carried out by the well-named angiocrine factors.

CONCLUSION

In conclusion, successful human endometrial regeneration after autologous $CD133^{+}$BMDSCs therapy seems to depend on the ability of the immune system to become tolerant and receptive as well as on the capability of resident cells to promote tissue regeneration and neo-vascularization, all via paracrine actions. Taken into account the results presented here, the next steps would be the validation of these factors as truly effectors in both mouse and human AS/EA models and investigating if pregnancy and delivery rates would be improved.

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DISCLOSURE OF INTERESTS

L.dM-G., H.F., S.L-M., N.L-P., H.C., D.H. and A.F. report no conflict of interest. X.S., A.P. and I.C. have a patent to declare: STEM CELL THERAPY ON ENDOMETRIAL PATHOLOGIES (Application number: 62013121).

CONTRIBUTION TO AUTORSHIP

L.Dm-G. and H.F.: experimental studies and procedures, manuscript drafting, analysis. S.L-P. and N.L-P.: experimental studies. H.C.: analysis, manuscript drafting and critical discussion. A.F.: experimental studies and procedures. D.H.: bioinformatics procedures and analysis, manuscript drafting. X.S.: study design, A.P.: study design and critical discussion. I.C.: experimental studies and procedures, study design, analysis, manuscript drafting and critical discussion.

ETHICS APPROVAL

Samples used in this study came from 2 studies: Cervelló *et al.,* 2015 (Ethics Committee A1329228834285, University of Valencia)[15] and Santamaría *et al.,* 2016 (ClinicalTrials.gov NCT02144987).[18]

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607

FIGURE LEGENDS

Figure 1: Study design. (A) Before any treatment, an endometrial biopsy was obtained from women with Asherman's Syndrome (AS) and/or Endometrial Atrophy (EA). Histology and microanatomy were analyzed by hematoxylin and eosin (H&E) staining (pictures showed in the upper panel, 10X; scale bar 0.2 μm). (B, C) After that, human $CD133^+$ bone marrow-derived stem cells ($CD133^+$BMDSCs) were mobilized (by G-CSF, granulocyte colony-stimulating factor) and isolated from these patients by flow cytometry. (D) Isolated cells were autotransplanted in the same women and (E) another biopsy was obtained three months after the intervention (lower panel showing H&E staining after treatment, 10X; scale bar 0.2 μm). In parallel, these $CD133^+$BMDSCs were also used for an animal model represented in the right side of the diagram (Cervelló *et al.*, 2015).[15]

Figure 2: Comparison of endometrial gene expression profile before/after $CD133^+$BMDSCs therapy in patients with AS and EA. (A) Heat map showing genes with significant different expression before/after treatment conditions. *SERPINE1*, *JUN*, and *IL4* proved to be up-regulated after the treatment. Conversely, *CCND1* and *CXCL8* were down-regulated after the treatment. Fold regulation value is shown with a typical color gradation, in green for up-regulation and in red for down-regulation situations, as shown in the right side of the figure. (B) qRT-PCR array data validation of selected genes (*JUN, CCND1,* and *CXCL8*) was performed in samples before/after treatment by qRT-PCR (to note we have analyzed two pools per each condition; n=4 patients per pool). Gene expression is represented as fold regulation; **Fold Regulation < -2. (C)

633 Schematic overview of up-regulated genes, metabolic pathways in which they
634 are involved and biological processes they trigger.

635 **Figure 3. Neutrophil elastase endometrial protein expression in patients**
636 **with AS and EA before/after cell therapy with autologous CD133⁺BMDSCs.**
637 (A) Schematic overview of the relation established among *CXCL8* gene,
638 neutrophils recruitment and neutrophil elastase (NE) expression at protein level.
639 (B) Immunohistochemistry against NE of two representative histological
640 samples (at 20X magnification) before/after treatment. Positive (human tonsil)
641 and negative (absence of primary antibody) controls were used for NE
642 immunohistochemistry. (C) A graphic showing the statistically significant
643 difference in NE signal is showed, *paired samples *t*-test indicated significant
644 differences <0.05 (*p*-value = 0.025).

645 **Figure 4. Comparison of protein expression profile in treated and not**
646 **treated uterine horns (with human CD133⁺BMDSCs) in a mouse model**
647 **with damaged uterus.** (A) Diagram summarizing the methodology used in our
648 animal model, where left horn was damaged and intrauterine injection
649 performed with BMDSCs (named as treated), and right horn only with the
650 damage (not treated). (B) Proteins showing a statistically significant difference
651 in tissue expression when treated and not treated uterine horns were compared:
652 IL18, HGF, MCP-1 and MIP2; *paired samples *t*-test indicated significant
653 differences (*p*-value < 0.05). **paired samples *t*-test indicated significant
654 differences (*p*-value < 0.01). To note: the difference in expression of all proteins
655 in treated horns showed to be at least twice as much as in the not treated
656 horns. (C) Table with the main characteristics about IL18, HGF, MCP-1 and
657 MIP2.

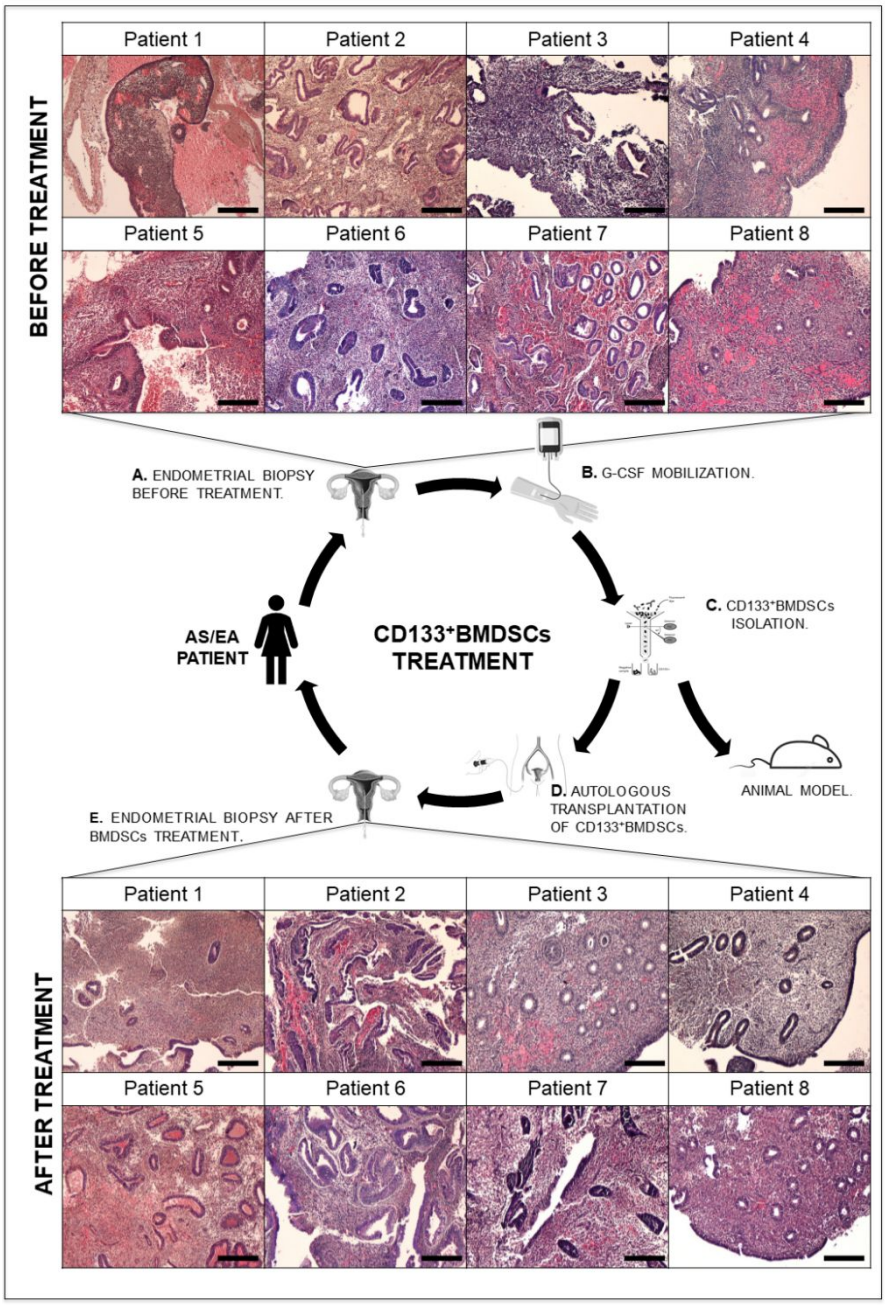
SUPPORTING INFORMATION

S1 (supplementary figure 1). PCR array data validation of selected genes in murine uterine tissue. *Jun*, *Ccnd1* and *Serpine1* were validated (as performed in human samples) in treated and not treated uterine horns by qRT-PCR (to note we have analyzed two pools per each condition, n= 4 patients per pool).

S2 (supplementary table 1). Study participants. Clinical characteristics of the 8 selected patients with Asherman's Syndrome (AS) and Endometrial Atrophy (EA). The Asherman's Syndrome Classification by 'The American Fertility Society classification of intrauterine adhesions, 1988'.[67]

S3 (supplementary table 2). Specific primers used for the validation of *JUN*, *CCND1, CXCL8, CCND1* and *GAPDH*. *GAPDH* was used as housekeeping gene. *JUN, CCND1* and *GAPDH* primers were common for both species, human (Hu) and mouse (Ms).

S4 (supplementary table 3). Multiplex immunoarrays data from animal model. All targets analyzed are shown, including detection limit, mean concentration expressed in pg/ml (with standard deviation (SD)) in treated and not treated uterine horns, and p-value. Targets in green are those showing statistically significant differences; *paired samples t-test indicated significant differences (*p*-value < 0.05); **paired samples t-test indicated significant differences (p-value < 0.01). Targets in blue are those showing an upper trend without being significants. ND: no detected. UDL: under detection limit.



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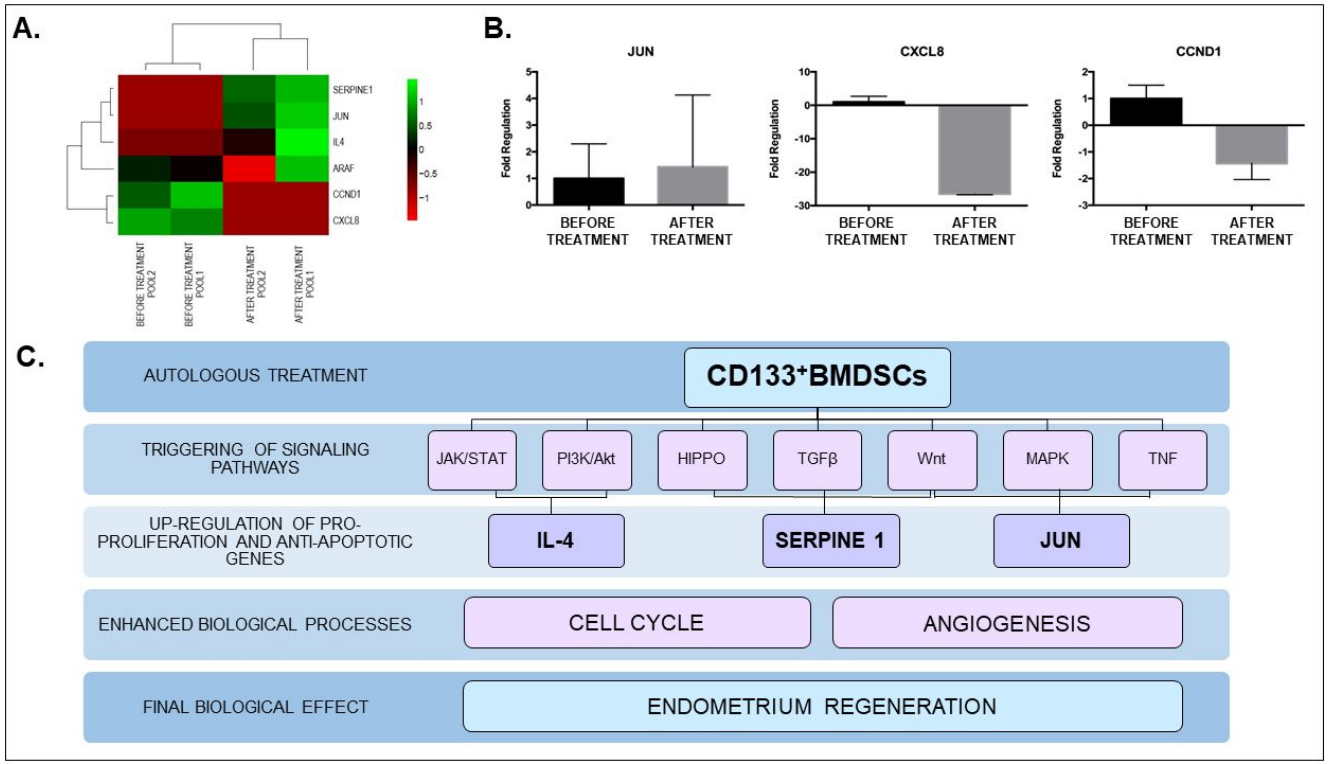

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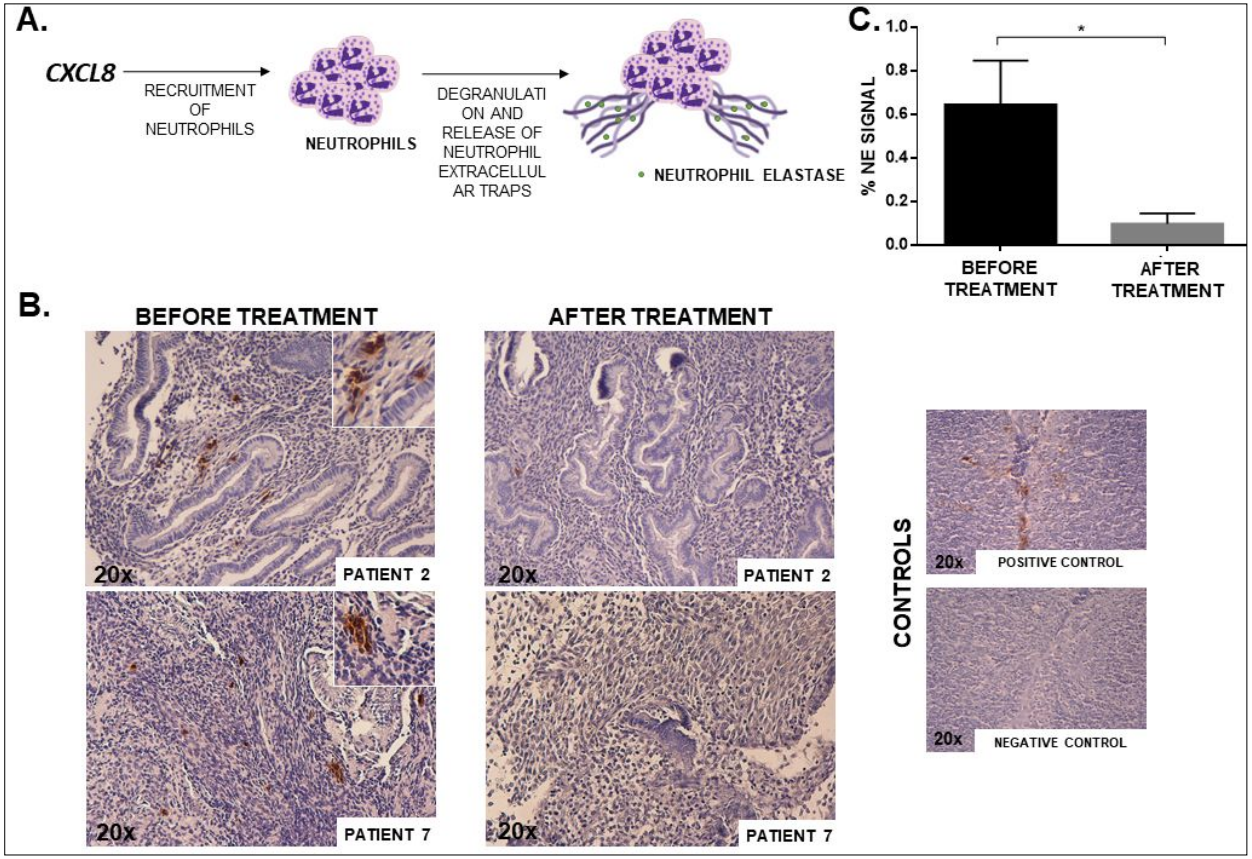
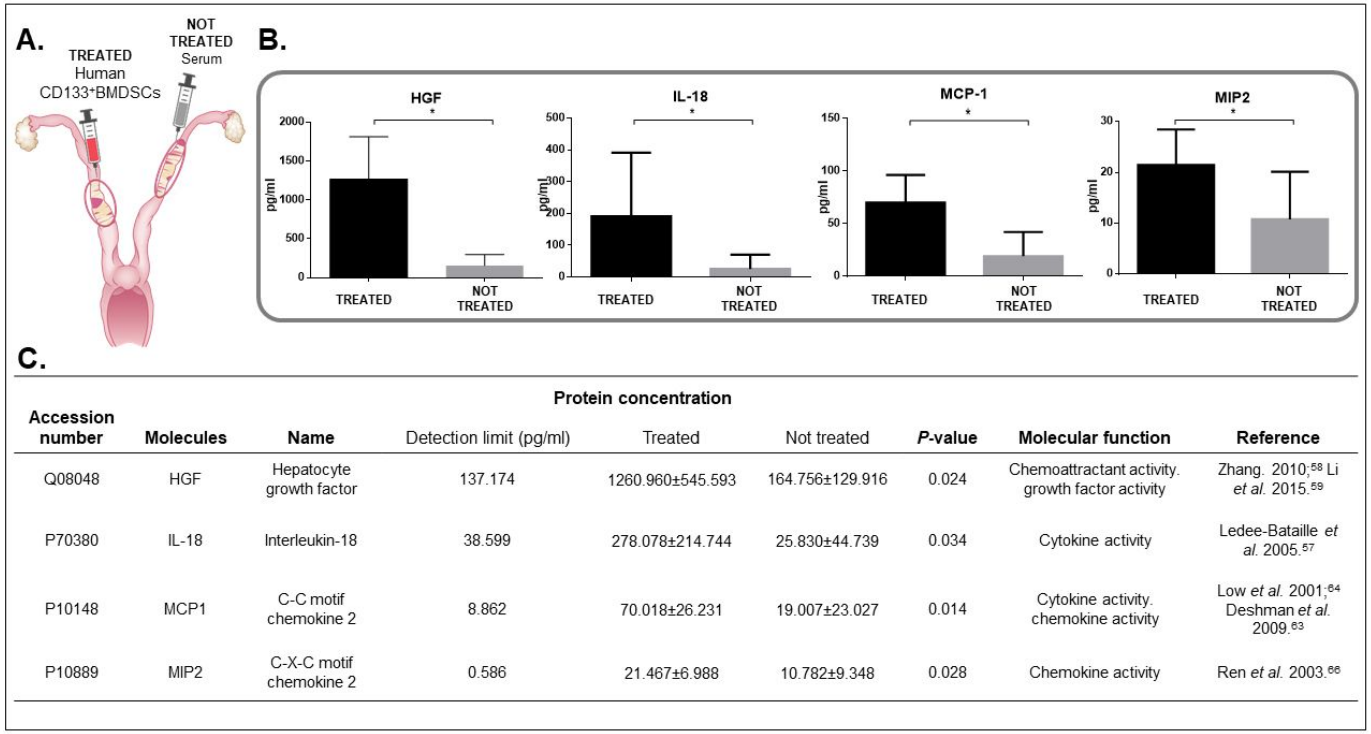


Figure 3. Neutrophil elastase endometrial protein expression in patients with AS and EA before/after cell therapy with autologous CD133⁺BMDSCs. (A) Schematic overview of the relation established among *CXCL8* gene, neutrophils recruitment and neutrophil elastase (NE) expression at protein level. (B) Immunohistochemistry against NE of two representative histological samples (at 20X magnification) before/after treatment. Positive (human tonsil) and negative (absence of primary antibody) controls were used for NE immunohistochemistry. (C) A graphic showing the statistically significant difference in NE signal is showed, *paired samples *t*-test indicated significant differences <0.05 (*p*-value = 0.025).



C.

Accession number	Molecules	Name	Protein concentration: Detection limit (pg/ml)	Protein concentration: Treated	Protein concentration: Not treated	*P*-value	Molecular function	Reference
Q08048	HGF	Hepatocyte growth factor	137.174	1260.960±545.593	164.756±129.916	0.024	Chemoattractant activity. growth factor activity	Zhang. 2010;[58] Li *et al.* 2015.[59]
P70380	IL-18	Interleukin-18	38.599	278.078±214.744	25.830±44.739	0.034	Cytokine activity	Ledee-Bataille *et al.* 2005.[57]
P10148	MCP1	C-C motif chemokine 2	8.862	70.018±26.231	19.007±23.027	0.014	Cytokine activity. chemokine activity	Low *et al.* 2001;[64] Deshman *et al.* 2009.[63]
P10889	MIP2	C-X-C motif chemokine 2	0.586	21.467±6.988	10.782±9.348	0.028	Chemokine activity	Ren *et al.* 2003.[66]

Figure 4. Comparison of protein expression profile in treated and not treated uterine horns (with human CD133⁺BMDSCs) in a mouse model with damaged uterus. (A) Diagram summarizing the methodology used in our animal model, where left horn was damaged and intrauterine injection performed with BMDSCs (named as treated), and right horn only with the damage (not treated). (B) Proteins showing a statistically significant difference in tissue expression when treated and not treated uterine horns were compared: IL18, HGF, MCP-1 and MIP2; *paired samples *t*-test indicated significant differences (p-value < 0.05). **paired samples *t*-test indicated significant differences (p-value < 0.01). To note: the difference in expression of all proteins in treated horns showed to be at least twice as much as in the not treated horns. (C) Table with the main characteristics about IL18, HGF, MCP-1 and MIP2.