Electronic supplementary material (ESM)

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Title: **Diverse impact of xeno-free conditions on biological and regenerative properties of hUC-MSCs and their extracellular vesicles**

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Supplementary materials

Table S1. List of qPCR primers (all for human sequences).

No.	Gene Name	Gene symbol	Forward (5' – 3')	Reverse (5' – 3')	Ref.
1	Nanog homeobox	NANOG	ACCTCAGCTACAAACAGGTGAAG	TTCTGCGTCACACCATTGCT	[1]
2	GATA binding protein 4	GATA4	AACGACGGCAACAACGATAAT	GTTTTTTCCCCTTTGATTTTTGATC	[2]
3	NK2 transcription factor-related, locus 5	NKX2-5	CCCCTGGATTTTGCATTCAC	CGTGCGCAAGAACAAACG	[2]
4	troponin T type 2 cardiac	TnTC	ATGAGCGGGAGAAGGAGCGGCAGAAC	TCAATGGCCAGCACCTTCCTCCTCTC	[3]
5	myosin, heavy chain 7, cardiac muscle, beta	MYHCB	CTGGAGGCCGAGCAGAAGCGCAACG	GTCCGCCCGCTCCTCTGCCTCATCC	[3]
6	myosin, light chain 7, regulatory	MYL2A	GGGCCCCATCAACTTCACCGTCTTCC	TGTAGTCGATGTTCCCCGCCAGGTCC	[3]
7	kinase insert domain receptor	FLK1	GGTTGTGTATGTCCCACCCC	GAGTGGTGCCGTACTGGTAG	[1]
8	TEK tyrosine kinase, endothelial	TIE2	TCCCGAGGTCAAGAGGTGTA	AGGGTGTGCCTCCTAAGCTA	[1]
9	interleukin 1 beta	IL-1β	AGACATCACCAAGCTTTTTTGCT	GCACGATGCACCTGTACGAT	[4]
10	interleukin 6	IL-6	TTCGGCAAATGTAGCATG	AATAGTGTCCTAACGCTCATAC	[5]
11	interleukin 8	IL-8	TTAGCACTCCTTGGCAAAACTG	CTGGCCGTGGCTCTCTTG	[4]
12	interleukin 10	IL-10	GTGGAGCAGGTGAAGAATGC	GCCACCCTGATGTCTCTCAGTT	[6]
13	interferon, gamma	IFNγ	GTTCCATTATCCGCTACATCTGAA	TCAGCTCTGCATCGTTTTGG	[4]
14	tumor necrosis factor alpha	TNFα	CCTCTGATGGCACCACCAG	TCTTCTCGAACCCCGAGTGA	[4]
15	lectin, galactoside-binding, soluble, 3	GAL-3	CCAAAGAGGGAATGATGTTGCC	TGATTGTACTGCAACAAGTGAGC	[7]
16	jagged 1	JAG-1	CGGCCTCTGAAGAACAGAAC	TCACCAAGCAACAGATCCAA	[8]
17	neurogenic locus notch homolog protein 2	NOTCH-2	TGGGCTACACTGGGAAAAAC	TAGGCACTGGGACTCTGCTT	[8]
18	neurogenic locus notch homolog protein 3	NOTCH-3	CTCATCCGAAACCGCTCTAC	TCTTCCACCATGCCCTCTAC	[8]
19	BCL2-associated X protein	BAX	TTTTGCTTCAGGGTTTCATCCAG	CGGAAAAAGACCTCTCGGGG	S.B-W
20	B-cell CLL/lymphoma 2	BCL2	GATAACGGAGGCTGGGATGC	TGACTTCACTTGTGGCCCAG	S.B-W
21	beta-2-microglobulin	β2m	AATGCGGCATCTTCAAAC	TGACTTTGTCACAGCCCAAGATA	[9]

Table S2. Phenotype of UC-MSC in various xeno-free media. Results are presented as means ± standard deviation. Values were rounded to one decimal place. Statistically significant differences at $p<0.05$ in comparison to control medium (M6) are indicated with an asterisk in bold.

	Percentage of positive cells						
Antigen/Medium	**M1**	**M2**	**M3**	**M4**	**M5**	**M6**	**M7**
CD166	99.4 ± 0.2	92 ± 10.8	94.4 ± 6.8	99.6 ± 0.5	92.2 ± 8.9	99.5 ± 0.3	95.3 ± 4.3
CD73	99.6 ± 2.2	93.4 ± 7.7	98.9 ± 1.1	99.4 ± 0.9	98.4 ± 2.2	100	99.7 ± 0.3
CD90	99.2 ± 0.1	94.9 ± 8.5	99.8 ± 0.2	99.6 ± 0.3	97 ± 4.7	99.9 ± 0.1	97.8 ± 2
CD44	99.2 ± 0.1	87.4 ± 13	99.2 ± 0.7	99.8 ± 0.2	99.3 ± 0.3	95.5 ± 8.6	96.4 ± 6.3
CD105	91.8 ± 1.2	83.6 ± 7.6	81.1 ± 14	92 ± 6.1	**65 ± 24***	94.2 ± 5.9	91.2 ± 4.1
CD29	97.8 ± 3.7	90.4 ± 11	99.5 ± 0.6	99.9 ± 0.1	97.3 ± 4	99.4 ± 1	99.8 ± 0.2
CD34	0 ± 0.3	2.6 ± 5.8	0 ± 0.1	0 ± 0.1	0.2 ± 0.2	0 ± 0.1	0 ± 0.1
CD45	1.3 ± 2.4	2 ± 4.2	2.9 ± 4.0	2.3 ± 4.3	0.9 ± 1.7	1.7 ± 2.1	1.3 ± 1.7
CD14	0.1 ± 0.4	0.1 ± 0.1	0.2 ± 0.3	0.1 ± 0.1	0.3 ± 0.4	0.2 ± 0.3	0 ± 0.1
CD16	0.1 ± 0.3	0.5 ± 1.2	0.2 ± 0.2	0.1 ± 0.1	0.2 ± 0.3	0 ± 0.1	0.3 ± 0.2
HLA-DR	0.5 ± 1.3	0.1 ± 0.1	0.6 ± 0.6	0.4 ± 1	0.2 ± 0.2	0 ± 0.1	0.1 ± 0.1

Table S3. Nanoparticle size analysis. Size distribution of UC-MSC-EVs was measured with IZON (IZON Science; Cambridge, MA, USA). Mean and mode with standard deviation for each medium type are presented. Statistically significant differences at $p<0.05$ in comparison to control medium (M6) are indicated with an asterisk in bold.

EV type	**Mean**	**Median**
M1-EVs	**163 ± 1.4***	134.5 ± 13.4
M2-EVs	180.5 ± 14.8	141 ± 7.1
M3-EVs	167.5 ± 17.7	138.5 ± 19.1
M4-EVs	170.5 ± 9.2	**127 ± 2.8***
M5-EVs	181 ± 25.5	140 ± 21.2
M6-EVs	181± 8.5	138.5 ± 3.5
M7-EVs	181.5 ± 23.3	145.5 ± 13.4

Table S4. Surface antigens detection on extracellular vesicles derived from UC-MSCs. Results are presented as means ± standard deviation. Values were rounded to one decimal place. Statistically significant differences at $p<0.05$ in comparison to control medium (M6) are indicated with an asterisk in bold.

	Percentage of positive events						
Marker/EVs	**M1-EVs**	**M2-EVs**	**M3-EVs**	**M4-EVs**	**M5-EVs**	**M6-EVs**	**M7-EVs**
RNA	**19.7 ± 1***	16.3 ± 1.2	15.1 ± 0.5	16.5 ± 0.2	**19 ± 0.5***	14.4 ± 0.7	16.5 ± 0.9
CD90	**52.6 ± 12***	45.9 ± 13	**49 ± 6.6***	36.5 ± 8.7	31.4 ± 9.5	30.9 ± 13	27.3 ± 7
CD44	**32.9 ± 12***	**22.8 ± 11***	**24 ± 5.8***	17.5 ± 8.8	21.3 ± 4.5	8.5 ± 6	12.7 ± 2.9
CD105	25.1 ± 9	28.8 ± 4.4	25.9 ± 5	25 ± 3.4	23.1 ± 4.9	23.4 ± 7.4	26 ± 13.6
MFI-CD105	82 ± 6.5	87 ± 13	73 ± 12	89 ± 17	57 ± 4	68 ± 8.1	79 ± 7
CD49e	36 ± 6.8	**63 ± 6.2***	**47 ± 0.1***	33 ± 1.1	34 ± 5.5	33 ± 3.3	31 ± 8.4
CD34	0.5 ± 0.2	1.6 ± 2.3	0.6 ± 0.3	0.5 ± 0.2	0.3 ± 0.1	0.4 ± 0.1	0.5 ± 0.2

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Supplementary figures

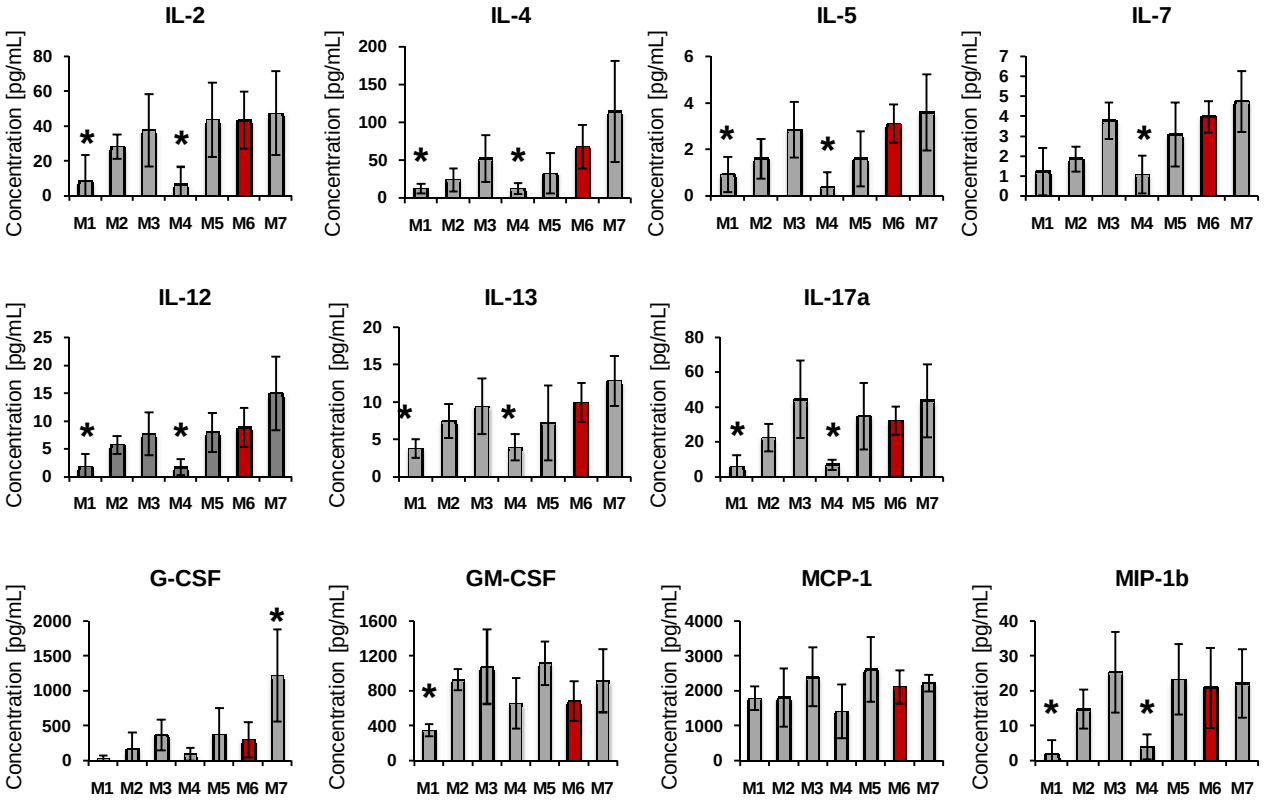

Fig. S1. Secretion of cytokines by UC-MSC cultured in xeno-free and standard media. Concentration of selected interleukines (IL-2, -4, -5, -7, -12, -13, -17a) and other cytokines, including granulocyte - colony stimulating factor (G-CSF), granulocyte macrophage – colony stimulating factor (GM-CSF), monocyte chemoatrractant protein –1 (MCP-1) and macrophage inflammatory protein – 1 beta (MIP-1b) was measured in conditioned media collected after passage 3 with the BioPlex Pro™ Human Cytokine 17-plex Assay (BioRad). Results were compared with one-way ANOVA and Dunnet post-hoc test to control conditions (M6). * $p<0.05$. Mean values ± standard deviations are shown. UC-MSCs - Umbilical Cord-derived Mesenchymal Stem Cells.

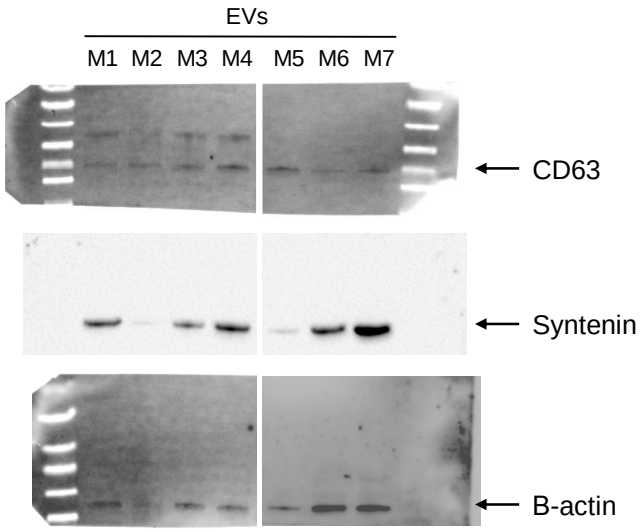

Fig. S2. Western blot analysis of proteins typical for EVs. Equal protein amounts (300 µg) were used for each EV type (M1-M7). Proteins were separated by PAGE and transferred to PVDF membranes to detect CD63, Syntenin and β-actin using specific primary and horseradish peroxidase (HRP)-conjugated secondary antibodies, as described in *Materials and Methods*. Original Western blot pictures are shown. EVs – Extracellular Vesicles derived from UC-MSCs; PAGE – polyacrylamide gel electrophoresis; PVDF – polyvinylidene fluoride.

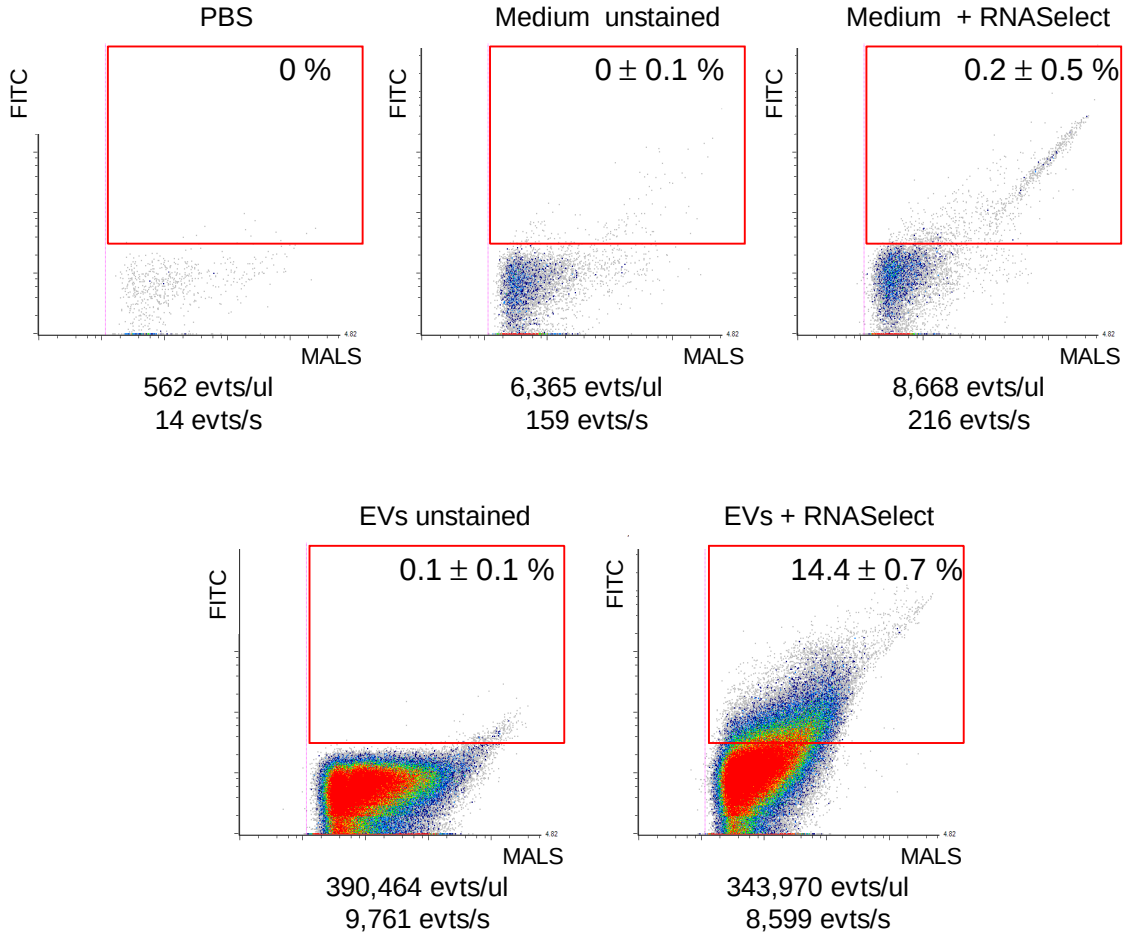

Fig. S3. Flow cytometry analysis of RNA-positive objects in the xeno-free and standard cell culture media used in the study. by A50-Micro Flow Cytometer (Apogee Flow Systems). The percentage of RNA-positive events in the samples were calculated considering concentration of the particles in the samples and validated on EVs sample stained with the SYTO® RNASelect™ dye. Representative dot plots are shown. PBS – phosphate buffered saline; EVs – Extracellular Vesicles derived from UC-MSCs; MALS - Medium Angle Light Scatter; FITC - fluorescein isothiocyanate;

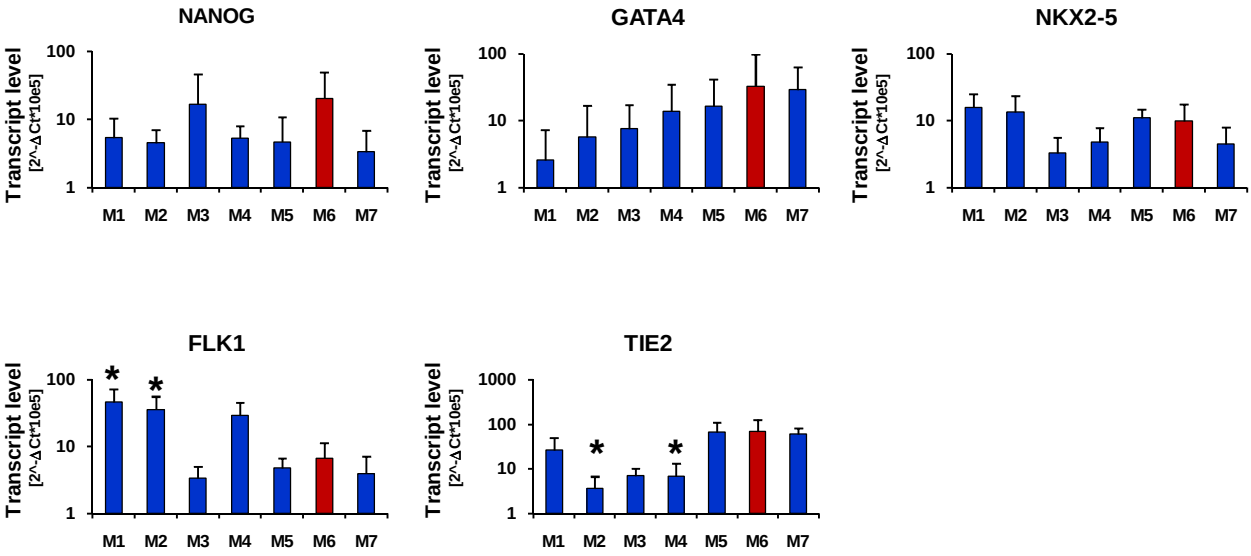

Fig. S4. Relative transcript levels in UC-MSCs cultured in xeno-free media and in standard conditions. The real time PCR method was employed to measure mRNA levels for genes from the pluripotency network (*NANOG*), regulators of cardiac development (*GATA4*, *NKX2.5*) and angiogenesis (*FLK1*, *TIE2*). Results were calculated with the ΔΔCt method using β2-microglobulin as endogenous control and compared with one-way ANOVA and Dunnet post-hoc test to control conditions (M6). * $p<0.05$. Mean values ± standard deviations are shown.

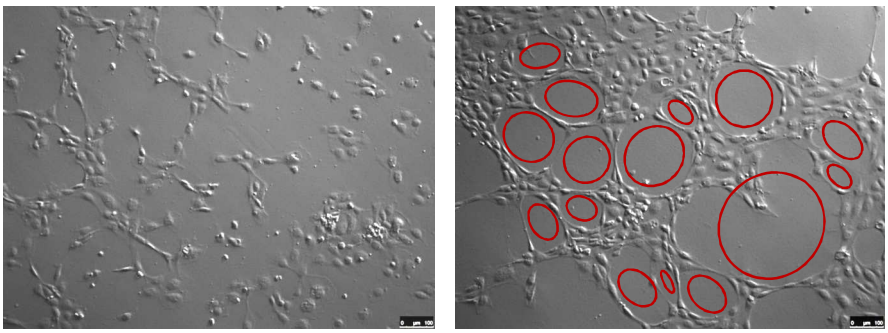

Fig. S5. Schematic representation of capillary-like tube enumeration. Capillary-like structures were formed by the human umbilical vein endothelial cells (HUVECs) treated or untreated with extracellular vesicles collected from the xeno-free cultures of umbilical cord-derived mesenchymal stem cells (UC-MSC-EVs). Representative images of endothelial cells directly after seeding on a matrigel (left) and after 6 hours of the assay (right) with capillaries indicated with red circles. Scale bar = 100 µm.

References to supplementary materials:

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