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Characteristics of MIC-1 Antlerogenic Stem Cells and Their Effect on Hair Growth in Rabbits

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Abstract. *Aim: We characterized growth factors produced by MIC-1 antlerogenic stem cells and attempted to apply those cells to stimulate hair growth in rabbits. Materials and Methods: We evaluated the gene and protein expression of growth factors by immunocytochemical and molecular biology techniques in MIC-1 cells. An animal model was used to assess the effects of xenogenous stem cells on hair growth. In the experimental group, rabbits were intradermally injected with MIC-1 stem cells, whereas the control group rabbits were given vehicle-only. After 1, 2 and 4 weeks, skin specimen were collected for histological and immunohistochemical tests. Results: MIC-1 antlerogenic stem cells express growth factors, as confirmed at the mRNA and protein levels. Histological and immunohistochemical analysis demonstrated an increase in the number of hair follicles, as well as the amount of secondary hair in the follicles, without an immune response in animals injected intradermally with MIC-1 cells, compared to animals receiving vehicle-alone. Conclusion: MIC-1 cells accelerated hair growth in rabbits due to the activation of cells responsible for the regulation of the hair growth cycle through growth factors. Additionally, the xenogenous cell implant did not induce immune response.*

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Antlerogenic stem cells participate in annual antler regeneration in the red deer (*Cervus elaphus*) (1, 2). They are intermediate cells, between embryonic and adult stem cells, which have the ability to differentiate into a number of different cell types (3-5). Rapid and simultaneous growth of various antler-forming tissues, *e.g.* velvet, cartilage, bone, nerves and blood vessels, is possible, among others, due to growth factors (6). Growth factors are conservative proteins that regulate cell growth and differentiation. Numerous tissues of the antler synthesize growth factors, *e.g.* insulin-like growth factor (IGF-1), platelet-derived growth factor (PDGF), bone morphogenetic growth factor-2 (BMP-2), epidermal growth factor (EGF), vascular growth factor (VEGF), fibroblast growth factor-2 (FGF-2) and nerve growth factor (NGF) (7-12). The scientific literature lacks reports on the investigation of growth factors and other proteins produced by cultured cells isolated from the growing antler. In our previous studies, we observed that MIC-1 cells accelerate the regeneration of damaged cartilage and bone. Defect regeneration was possible through transfer of signals stimulating protein synthesis and cell proliferation and due to the immunosuppressive and immunomodulatory activities of antlerogenic stem cells (13, 14).

Hair is skin appendages developed during the intrauterine life and cyclically regenerated throughout an organism's life. As a structural unit, the hair follicle comprises of the hair papilla, the hair bulb, outer and inner root sheaths, a fibrous capsule, the sebaceous gland and the arrector pili muscle (15). Formation and growth of a new hair depends on co-operation of numerous cells, in particular, hair follicle stem cells, hair follicle bulge area stem cells and dermal papilla cells (16). Cell interaction through growth and transcription factors results in the periodicity of hair growth. These factors include proteins of the Wingless Int (WNT), FGF, transforming growth factor-β (TGF-β), BMP, as well as sonic hedgehog homolog (Shh)

pathways (17). FGF-2 regulates the formation of the hair germ from epidermal cells (18). Keratinocyte growth factor (KGF) and IGF-1 stimulate the proliferation of the matrix cells (19, 20). VEGF is crucial for the development of blood vessels in the hair follicle (21). The BMP molecules play a vital role in the activation and stimulation of the matrix cells to differentiate into various hair-forming cells (22). However, a decrease in BMP levels along with a simultaneous increase in WNT pathway protein levels, leads to hair stem cell activation (23).

Hair growth ceases when the dermal papilla cells no longer secrete factors that stimulate hair matrix cells. The matrix cells undergo apoptosis (24). Accelerated ageing and impaired production of proteins responsible for the regulation of the hair growth cycle in the cells of the papilla under the effect of testosterone and its derivatives in androgenic alopecia leads to weakening of the hair follicles and to more rapid and excessive hair loss (25). Activation of dermal papilla and matrix cells, as well as stem cells, by growth factors can prolong the hair growth cycle and prevent hair loss. Experimentally applied stem cells can be a source of IGF-1, VEGFA, EGF and KGF.

The aim of this study was to analyze the expression of growth factors in MIC-1 antlerogenic stem cells and to define their effect on cells participating in hair growth in rabbits.

Materials and Methods

Cell lines. MIC-1 antlerogenic stem cells and 3T3 Balb mouse embryonic fibroblasts were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Lonza, Verviers, Belgium), containing 10% bovine foetal serum and 1% L-glutamine, penicillin and streptomycin solution (Sigma-Aldrich Chemie Gmbh, Munich, Germany). The cultures were grown at 37˚C in a humid atmosphere with 5% CO_2. The cells were removed from the culture flasks with TrypLe (Gibco, Life Technologies, Grand Island, NY, USA).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR). Total RNA was isolated from the MIC-1 antlerogenic stem cells with the RNeasy Mini Kit (Qiagen, Hilden, Germany). The reverse transcription reaction was performed for 1 μg RNA using the Sensiscript Reverse Transcription Kit (Qiagen). For the PCR reaction, 1,300 ng of cDNA, PCR Core Kit (Qiagen) and a pair of primers for the following genes were used: major histocompatibility complex class I (*MhcI*), major histocompatibility complex class II (*MhcII*), interleukin-10 (*Il-10*), *Fgf-2, Kgf, Ngf, Bmp-2, Tgf-β1, Igf-1*, *Igf-2*, neurotrophin-3 (*Nt-3*), and β-actin (*Actβ*). Primer sequences were designed in accordance with the gene sequences available in the GeneBank database for *Cervus elaphus* and *Bos taurus* or come from previous publications. The nucleotide sequences of growth factors for these animal species are characterized by high homology and are presented in Table I. The conditions for the reaction included preliminary denaturation for 15 min at 95˚C, proper denaturation for 35 s at 95˚C, annealing for 35 s at 58˚C for Kgf, 59˚C for Ngf, Bmp-2, Fgf-2, Il-10, Igf-1, Nt-3 and B-Actin, 60˚C for Tgf-B1, Igf-2, and extension for 1 min at 72˚C. The denaturation, annealing, as well as extension steps were repeated for 38 cycles. The PCR reaction products were analysed with 1.5% agarose gel. Ten microliters of PCR product were loaded onto the gel. As mass markers of 50-1000 bp or 100-1000 bp DNA Ladder (Fermentas Inc., MD, USA) was used. Electrophoresis was performed at 100 V in TAE×1 (Tris-acetate-EDTA) buffer. The agarose gel was visualized and photographed with Gel Doc (Bio-Rad Lab., Segrate Milano, Italy). The expression level for each gene was assessed by a semi-quantitative method with the Quantity One 1-D Analysis Software (Bio-Rad Lab.). In order to assess the amount of the amplified cDNA for each of the genes, optical density (OD) values of the band, formed after the PCR product had been loaded on agarose gel, were read. The OD of a band was calculated as the ratio of the intensity and volume of all pixels forming the band. The expression level of each gene was calculated as the ratio of the OD of a band of a given gene to the OD of a band of the control gene, β-actin.

Immunofluorescent and immunocytochemical reactions. The MIC-1 antlerogenic stem cells were seeded at 5×10^3 cells/well on a cell culture slide. A 24-h culture of MIC-1 antlerogenic stem cells was subjected to staining. The cells were washed in phosphate buffered saline (PBS) for 5 min and fixed in 4% paraformaldehyde/PBS for 15 min at room temperature. Next, the cells were washed in PBS for 2×5 min and permeabilized in 0.2% Triton-PBS for 10 min. In the reaction with an antibody conjugated with horseradish peroxidase (HRP), the endogenous peroxidase activity was blocked using 3% H_2O_2 solution. After another washing in PBS for 2×5 min, primary goat anti-cow FGF-2 basic antibody (R&D Systems, Wiesbaden-Nordenstadt, Germany) was added, at a dilution of 1:50 for the immunofluorescent reaction (IF) or at 1:500 for the immunocytochemical reaction (IC); mouse anti-human VEGF-A antibody (ReliaTech GmbH, Wolfenbuttel, Germany) at a dilution of 1:25 for the IC; mouse anti-human VEGF-C antibody (Relia Tech GmbH) at a dilution of 1:200 for the IF; mouse anti-human VEGF-D antibody (ReliaTech GmbH) at a dilution of 1:200 for the IC; mouse anti-human VEGFR-3/FLT-4 antibody (ReliaTech GmbH) at a dilution of 1:25 for the IC; mouse anti-human tubulin antibody (R&D Systems) at a dilution of 1:200 for the IF or 1:1000 for the IC. The specimens were incubated overnight at 4˚C in a humid chamber. All primary and secondary antibodies were diluted in 1% bovine serum albumin (BSA) diluted in PBS. After washing in PBS for 2×5 min, a donkey anti-goat secondary antibody, conjugated with tetramethylrhodamine isothiocyanate (TRITC) (Jackson ImmunoResearch Lab., Inc., Newmarket, Suffolk, UK), was used for the FGF-2 staining and a donkey anti-mouse secondary antibody, conjugated with fluorescein isothiocyanate-conjugated (FITC) (Jackson ImmunoResearch Lab.), was used for VEGF-C and tubulin staining. Secondary antibodies were used at a dilution of 1:50 and the specimens were incubated for 1 h at room temperature in a dark humid chamber. After the cells had been washed in PBS and deionised water, Vectashield mounting medium (Vector Lab., Biokom, Burlingame, CA, USA) was applied. In order to reveal FGF-2 expression in the immunocytochemical reaction, the cells were incubated with a biotin-labelled secondary antibody for 20 min, washed in PBS and incubated with streptavidin-HRP for 20 min (LSAB+ Dako Real Detection System; Dako, Glostrup, Denmark). In order to demonstrate the VEGF-A, VEGF-D, VEGFR-3/FLT-4 and tubulin expression in the immunocytochemical reaction, the cells were incubated with a secondary antibody conjugated with HRP for 20 min. The cells were then incubated for 5 min with 3,3'-diaminobenzidine substrate-chromogen (EnVision anti-rabbit/anti-mouse; Dako) and the cell nuclei were counterstained with haematoxylin. The specimen were dehydrated in a series of graded alcohol and Euparal mounting medium (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) was applied.

Table I. *Sequence of primers used in the Reverse Transcription Polymerase Chain Reaction (RT-PCR) reaction.*

Gene	PCR product size (bp)	Sequence of primers	References/GenBank sequence
MhcI	530	5'-GGATGAAGCATCACTCAG-3' 5'-CGCTGCTGCGCGCAGACC-3'	(26)
MhcII	207	5'-GTGTTAYTTCACCAACRGGACG-3' 5'-GTTGTGGTGGTTTAGAGCCTC-3'	(27)
Ngf	177	CAGCGTCAGCGTGTGGGTGG TGCTTCGCGTCGATGCCTCG	AF145043.1
Bmp-2	185	CAATGGACGTGTCCCCGCGT GCCCAGCTCGGGAATGAGGC	HM004075.1
Kgf	144	ACTGTTCCAGCCCCGAGCGA TCCAACGGCCACTGTCCTGA	AY923858.1
Tgf-β1	237	CGGCAGTGGCTGACCCACAG GTTGGTGTCCAGGGCTCGGC	DQ642715.1
Fgf-2	247	CGGGGTCCGCGAGAAGAGTGA CTGCCCAGTTCGTTTCAGTGCCA	AF152587.1
Il-10	252	CTCATGCTGCGGGAGCTCCG TCGCAGCCGGAGGGTCTTCA	U11767.1
Igf-1	174	ACAAGCCCACGGGGTACGG TCTTGGGCATGTCGGTGTGGC	DQ985698.1
Igf-2	400	GCTTACCGCCCCAGCGAGAC GCGGACGGTGACTCTTGGCC	EF177491.1
Nt-3	220	GCCCGAGAGTCCGCCAAGTC CGCGGTGGCTCTTGTGCTCT	U66715.1
β-Actin	219	5'-CCCAAGGCCAACCGTGAGAAGATG-3' 5'-GTCCCGGCCAGCCAAGTCCAG-3'	(6)

Western blot. After washing with cold PBS, the MIC-1 cells were lysed for 20 min in ice, with Cell Lytic MT buffer (Sigma) containing a protease inhibitor cocktail (Sigma) and 0.5 mM phenylmethyl-sulfonyl fluoride. Cell residues were removed by centrifugation at 12000 ×*g* for 10 min and the supernatant containing the total cellular proteins was stored at –80˚C for further analyses. The tests also involved the MIC-1 cell homogenate obtained through homogenization of 5×10^6 cells in aqua pro injectione (Polpharma, Stargard Gdanski, Poland). Determination of the protein concentration was performed using the bicinchoninic acid assay (Thermo Fisher Scientific Inc., IL, USA). As positive controls for the analysed antigens, a human recombinant KGF (hrKGF) (K 1757; Sigma), as well as FGF-2 (hrFGF-2) (F 3393; Sigma) were used. The cell extracts and sample buffer were mixed in a ratio of 3:1 [250 mM tris(hydroxymethyl)aminomethane (pH 6.8), 40% glycerol, 20% (v/v) β-mercaptoethanol, 100 mM dithiothreitol, 0.33 mg/ml bromophenol blue, 8% sodium dodecyl sulfate (SDS) (Sigma)] and then denatured for 10 min at 95˚C. The protein samples were loaded using 30-50 μg and were separated by electrophoresis using Laemmli's method in 4-20% polyacrylamide gel with SDS (TGX Gel; BioRad Lab.). The proteins were then electrophoretically transferred onto a nitrocellulose membrane (0.2 μm, Protran BA83; Whatman Group, Dassel, Germany) and fixed on the membrane with 0.5% glutaric aldehyde solution in PBS, in accordance with the protocol published by Karey and Sirbasku (28). Non-specific binding sites were then blocked with 3% BSA in Tris-buffered saline with 0.1% Tween 20 (TBST) buffer.

FGF-2 expression was detected with goat anti-FGF2 antibody (dilution 1:500, AF-233-NA; R&D Systems). However, for the reaction with KGF a rabbit anti-KGF antibody was used (dilution 1:4000, ab9598; Abcam, Cambridge, UK). The incubation was performed overnight at 4˚C with gentle stirring of the antibody solution, diluted in 1% BSA in TBST buffer. The membrane was washed three times with the TBST buffer and afterwards incubated for 1 h at room temperature in a solution of a donkey anti-rabbit or a donkey anti-goat antibody, conjugated with HRP (1:3000; Jackson ImmunoResearch Lab.). The detection was carried out with a chemiluminescence substrate (Immun-Star HRP Chemiluminescent Kit; BioRad Lab.) and the results were documented at different exposure times ranging from 2 s to 30 min in a Chemi-Doc XRS Molecular Imager (BioRad Lab.). As a control for the loading efficacy and transfer, we applied membrane staining for the total protein with Ponceau S.

Cell co-cultures. Fibroblasts were seeded at 1×10^5 cell/well on a 12-well plate. After 24 h the medium was replaced and inserts (Becton Dickinson, NJ, USA) with the MIC-1 cell suspension (2×10^4 cells) were added. After 72 h, the number of fibroblasts was evaluated by a colorimetric test measuring the amount of sulforhodamine B (SRB), a dye that binds to living cells (29). The cells were fixed with 50% trichloroacetic acid and later stained with 0.4% SRB solution in 1% acetic acid for 30 mins. The unbound dye was removed by washing in 1% acetic acid and the dye bound to the cell proteins was extracted with 10 mM unbuffered Tris solution. The OD was read on an Elx 800 universal microplate reader (Bio-Tek, Instruments, Inc., VT, USA), at 562 nm. The reading control included subjection of the medium alone to the above procedure. All reagents used in the test were purchased from Sigma.

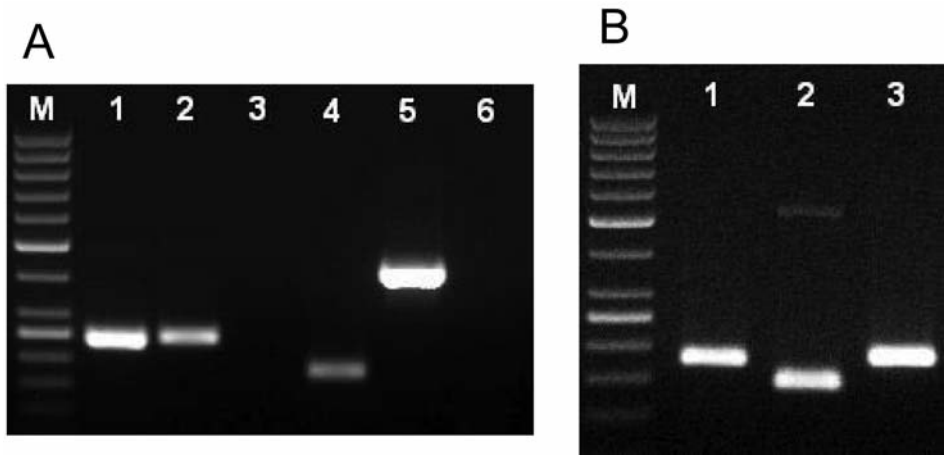

Figure 1. *Growth factor and interleukin 10 (Il-10) gene expression in MIC-1 stem cells, Reverse Transcription Polymerase Chain Reaction (RT-PCR). A. M: Mass marker, 1: transforming growth factor β1 (Tgf-β1), 2: fibroblast growth factor 2 (Fgf-2), 3: interleukin-10 (Il-10), 4: insulin-like growth factor 1 (Igf-1), 5: insulin-like growth factor 2 (Igf-2), 6: neurotrophin-3 (Nt-3). B. M: Mass marker, 1: nerve growth factor (Ngf), 2: bone morphogenetic protein 2 (Bmp-2), 3: keratinocyte growth factor (Kgf).*

In vivo studies, intradermal injection of MIC-1 line antlerogenic stem cells in rabbits. The study was performed on nine female White Californian rabbits (4.5 kg body weight) (Licensed Rabbit Breeder, Wroclaw, Poland). The experimental group included six rabbits and the control group three rabbits. All animals were purchased from a licensed rabbit breeder approved by the Veterinary Inspectorate. The protocol of the experiment was approved by the Commission of Bioethics at the Wroclaw Medical University. The animals had the skin on their left sides shaved and the sites where the intradermal injections were to be made were disinfected. Four 1-cm squares were marked for sites of injection. In the experimental group, intradermal injection of the MIC-1 stem cell at suspension of 4×10^4 cells in 0.4 ml aqua pro injectione was performed. The rabbits were given MIC-1 cells without immunosupression. The rabbits of the control group were given a random amount of aqua pro-injection. After 1, 2 and 4 weeks, specimens were collected for histological and immunohistochemical tests. On the part of the sections stained with haematoxylin and eosin (H&E stain), we evaluated the hair structure and compared the cell injection sites with the control sections, whereas on the remaining parts, we performed immunocytochemical reactions for the Ki67 proliferating cell nuclear antigen (a mouse anti-human Ki67 antibody used at a dilution of 1:200; Dako), CD34 hair follicle stem cell marker (a mouse anti-human CD34 antibody used at a dilution of 1:200; R&D Systems) and sex determining region Y-box 2 (SOX2) dermal papilla cell marker (a mouse anti-human SOX2 antibody used at a dilution of 1:500; Becton Dickinson). The presence of Ki67-, CD34- or SOX2-positive cells in hair follicles of experimental and control specimens were assessed. Intensity of cell staining indicated the level of hair cells activation.

Results

RT-PCR. For the majority of the examined growth factors, gene expression was detected at the mRNA level. The highest relative expressions were detected for *Igf-2* (0.654) and *Tgf-β1* (0.506). The expression of the remaining genes was *Fgf-2*, 0.245; *Igf-1*, 0.151; *Kgf*, 0.035; *Ngf*, 0.02; and *Bmp-2*, 0.019. We found no expression for *Nt-3* or *Il-10* in the MIC-1 antlerogenic stem cells (Figure 1 A and B).

Table II. *Optical density (OD) of fibroblasts and fibroblasts cultured with MIC-1 cells.*

OD fibroblasts	OD fibroblasts cultured with MIC-1 cells
0.603	1.26
0.613	1.382
0.622	1.439
0.649	1.36
0.635	1.232
0.682	1.18

Immunofluorescent and immunocytochemical reactions. We detected a distinct cytoplasmic expression of the FGF-2 protein and tubulin, both in the immunocytochemical and immunofluorescent reactions, for all MIC-1 antlerogenic stem cells (Figure 2 A-D). We also found that those cells demonstrated powerful cytoplasmic expression of VEGF-A and VEGF-C, and weak expression of VEGF-D proteins. The MIC-1 cells lacked expression of the VEGFR-3/FLT-4 receptor (Figure 2 E-H).

Western blot. The antibodies revealed a positive reaction, with a 17-kDa band for the human FGF-2 protein, which confirms the specificity of the antibody. For the MIC-1 cells, we obtained a 17-kDa band, thus confirming the FGF-2

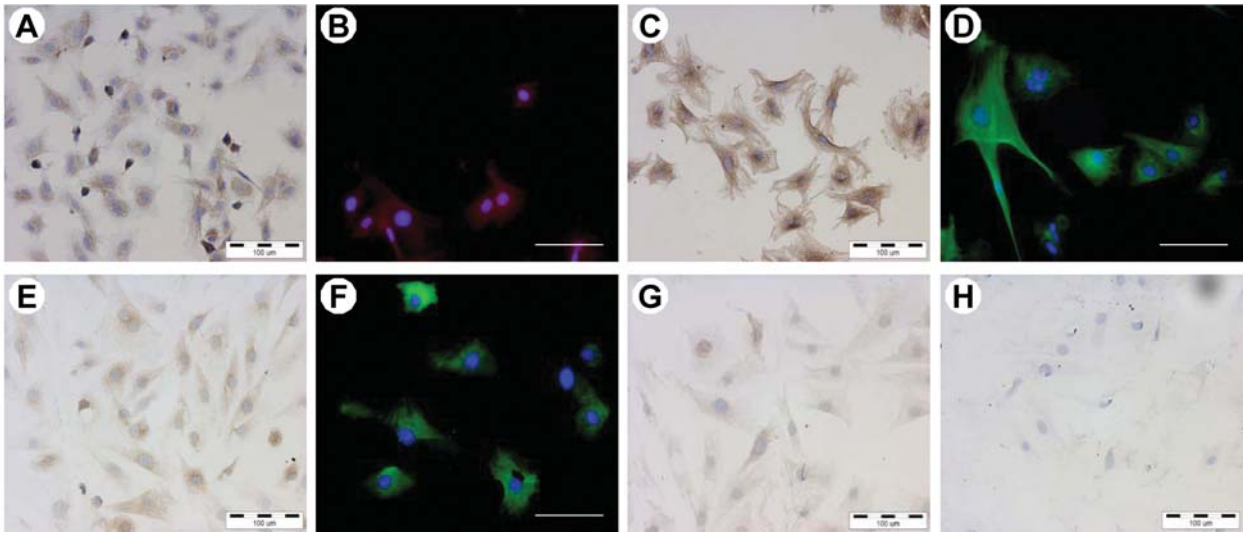

Figure 2. *Growth factor protein expression in MIC-1 stem cells. A, C, E, G, H: Immunocytochemical reaction with horseradish peroxidase-conjugated antibodies for fibroblast growth factor 2 (FGF-2) (A), tubulin (C), vascular endothelial growth factor A (VEGF-A) (E), vascular endothelial growth factor D (VEGF-D) (G) and vascular endothelial growth factor receptor 3 (VEGFR-3) (H). B, D, F: Immunofluorescent reaction with fluorescent-labelled antibodies for FGF-2 (B), tubulin (D) and VEGF-C (F).*

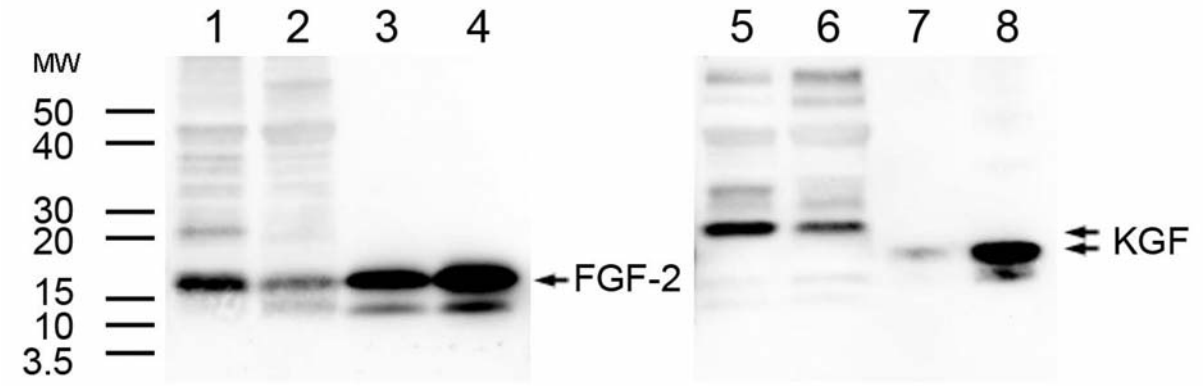

Figure 3. *Expression of fibroblast growth factor 2 (FGF2) and keratinocyte growth factor (KGF) protein in MIC-1 cells by western blot, using antibody against FGF-2 (lanes 1-4) and KGF (lanes 5-8). Lane 1: 50 μg of protein from MIC-1 cells; lane 2: 50 μg of protein from MIC-1 homogenate; lane 3: 2 ng hrFGF2; lane 4: 4 ng hrFGF2; lane 5: 50 μg protein from MIC-1 cells; lane 6: 50 μg protein from MIC-1 homogenate; lane 7: 1 ng hrKGF; lane 8: 5 ng hrKGF.*

protein expression. The expression of FGF-2 was also found in the MIC-1 cell homogenate (Figure 3). The antibodies showed a positive reaction with a band of approximately 19 kDa for the human KGF protein, which confirms the correct activity of the antibody. For the MIC-1 cells, we obtained a band of approximately 22.5 kDa, thus proving the expression of the KGF protein, whose molecular weight may differ from that of the human protein. From the reaction, we also obtained non-specific protein bands. However, they displayed a significantly weaker expression than that of KGF. The expression of KGF was also shown in the MIC-1 cell homogenate (Figure 3).

Cell co-cultures. In vitro, we observed the stimulating effect of the MIC-1 stem cells on fibroblast proliferation. After 72 h of incubation, the number of fibroblasts was increased by 100% compared with fibroblasts not exposed to stem cells (Table II).

In vivo studies, intradermal injection of MIC-1 line antlerogenic stem cells in rabbits. We studied the effect of the intradermally-injected antlerogenic stem cells on the hair growth in rabbits. Regular observation showed very rapid hair growth at the injection sites. Macroscopically, we observed an increase of hair growth in all rabbits of the experimental group

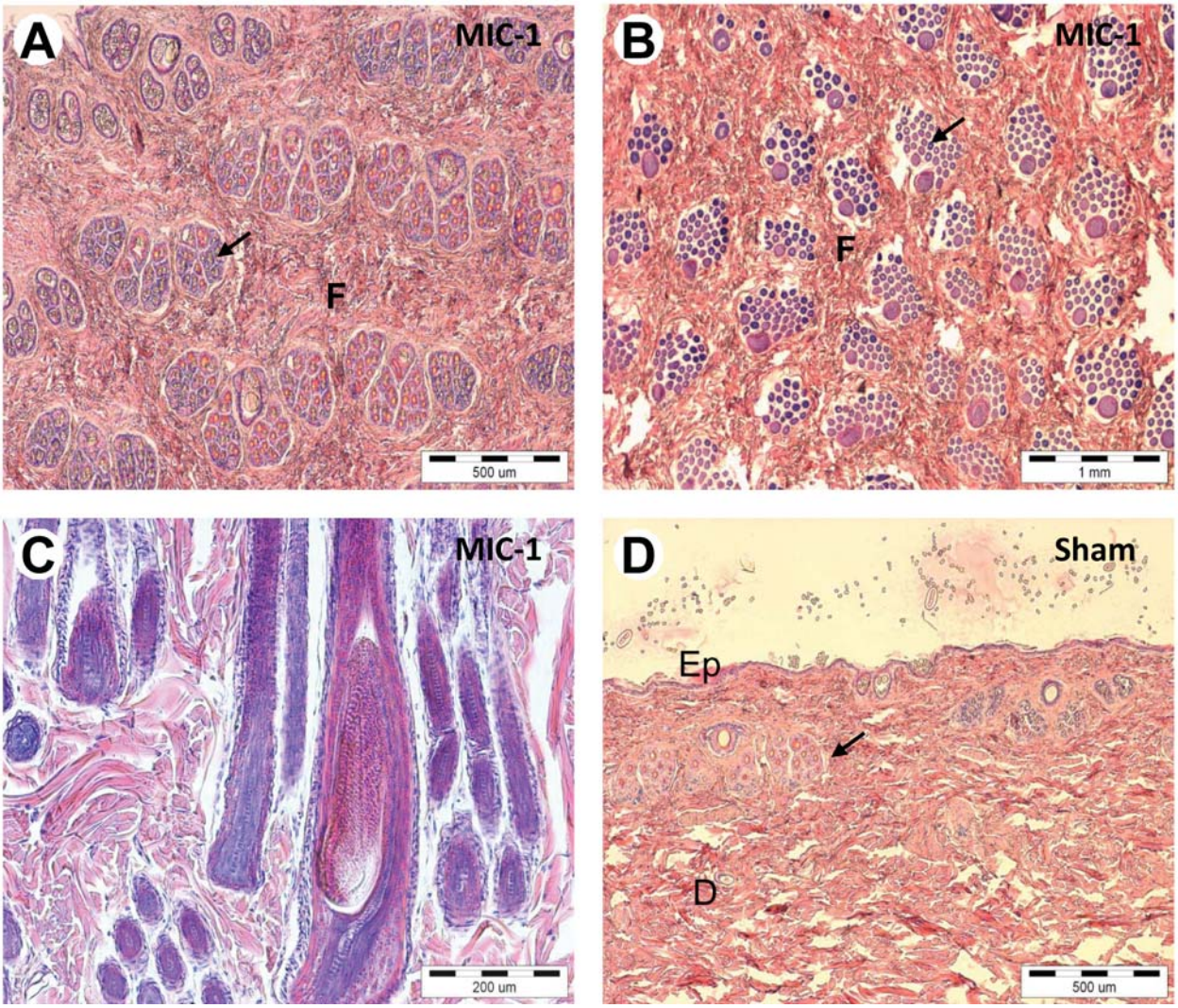

Figure 4. *The effect of MIC-1 cells on hair growth in rabbits, histological test. Experimental group, one week (A, C) and two weeks (B) after implantation of MIC-1 antlerogenic stem cells. In the skin, numerous hair follicles (↑) with secondary hairs are surrounded by thick dense collagen fibre bundles, with no lymphocyte infiltration. Control group, one week (D) after injection of vehicle-only. In the skin, the number of hair follicles remains unchanged, the follicles (↑) are surrounded by loose collagen fibre bundles. F, Collagen fibre bundles; Ep, epidermis; D, dermis. H&E staining.*

compared to the control group after one week (data not shown). Within four weeks, the hair on the shaved skin re-grew completely. We also noted a thickening of the hair. At the control sites, the hair started to re-grow at 4 to 5 weeks. Histological tests of the skin specimen performed after 1, 2 and 4 weeks of the experiment, proved increased hair growth and showed no presence of immune response after the application of cells compared with the control. In all animals, we observed an increase in the number of hair follicles, as well as the amount of secondary hair in the follicles, as early as one week after the application of the MIC-1 cells, compared with the control group (Figure 4 A, C and D). Within the hair follicle, around the main hair, we observed several to several dozens of smaller-growing secondary hairs. In the material obtained at 2 and 4 weeks after implantation, we did not observe any increase in the number of hair follicles or secondary hairs (Figure 4 B). Histological images of the examined skin layers, epidermis, dermis and subcutaneous tissue, were normal. We observed an increased number of collagen fibre bundles in the dermis, as well as in the subcutaneous tissue, when comparing the control images with the experimental ones (Figure 4A, B and D). Daily observations showed no inflammatory reaction and the injection sites showed only minor short-term reddening. At the injection sites, there was lack of implanted MIC-1 stem cells or lymphocyte infiltration both at one week and two weeks during the study (Figure 4A and B).

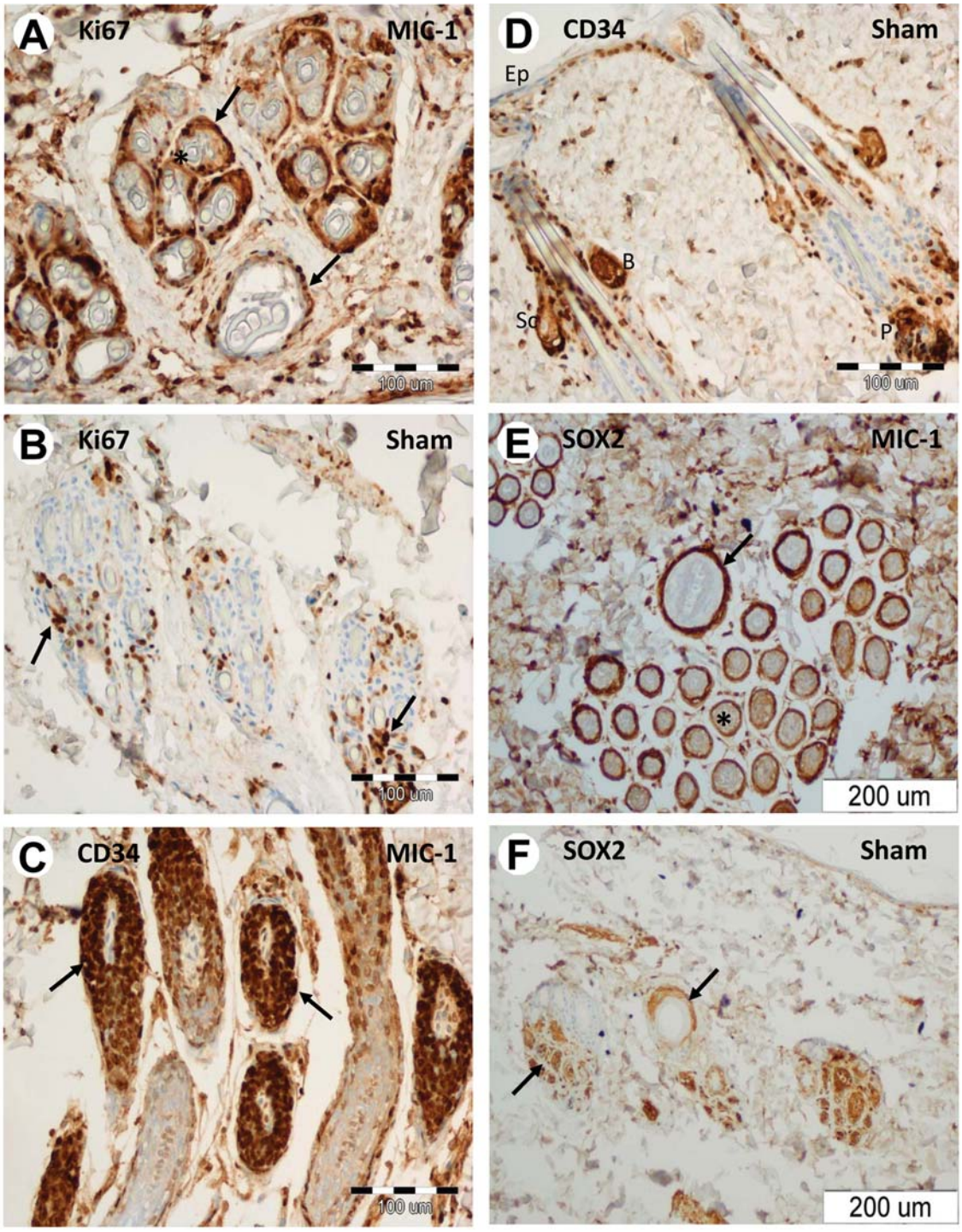

Figure 5. *One week after the injection of antlerogenic stem cells (MIC-1) or vehicle (sham). Immunohistochemical reaction was carried out for Ki67, for cluster of differentiation-34 (CD34) and sex determining region Y-box 2 (SOX2) antigen. A: Cross-section. Experimental group, high expression of Ki67 can be seen in the cells of the internal and external root sheath (↑). The Ki67 expression was stronger for the secondary hairs (*) compared to the main hair. B: Cross-section. Control group, expression of Ki67 can be seen in some of the cells of the external root sheath (↑). C: Tangential section. Experimental group, expression of CD34 in the cells of the internal and external root sheath (↑). D: Vertical section. Control group, expression of CD34 in the cells of the basal layer of the epidermis, the sebaceous gland, the bulge area and the dermal papilla. E: Cross-section. Experimental group, high expression of SOX2 in the cells of internal and external root sheath (↑). The SOX2 expression was stronger for the secondary hairs (*), compared to the main hair. F: Cross-section. Control group, expression of SOX2 in the cells of the internal and external root sheath (↑). Ep, Epidermis; Sc, sebaceous gland; B, bulge area; P, papilla.*

After one week, the immunohistochemical reactions showed higher Ki67, CD34 and SOX2 expression in the majority of the follicles in all animals from the experimental group compared to these of the control group, where the expression was limited to the single follicles. Moreover, the expression of Ki67, CD34 and SOX2 was stronger and was observed both in the secondary hairs and the main hair of the follicle. Both groups expressed Ki67, which was localized to the cells of the internal and external root sheath, the bulge area and the dermal papilla (Figure 5A and B). However, stronger reaction occurred after injection of MIC-1 antlerogenic stem cells (Figure 5A). Similarly, stronger CD34 and SOX2 expression was observed in the experimental group. Both markers were localized to the cells of the internal and external root sheath, bulge stem cells of the follicle and papilla cells (Figure 5C-F). The expression of all these markers remained unchanged and strong until the fourth week of the experiment.

Discussion

Our study, to our knowledge, is the first report, describing the expression of numerous growth factors in the MIC-1 cell line derived from deer antlers. Expression of some factors in tissues of growing red deer antlers has been repeatedly described (7, 8, 11, 12). This study, for the first time, characterizes the growth factors produced by MIC-1 antlerogenic stem cells. The highest mRNA expression was obtained for *Igf-2*. However, the expression levels of the remaining factors, namely *Tgf-β1*, *Fgf-2*, *Igf-1*, *Kgf*, *Ngf* and *Bmp-2*, underwent gradual decrease. On the other hand, expression of such growth factors as FGF-2, VEGF-A, VEGF-C and VEGF-D was shown at the protein level using the immunocytochemical method. The dividing cells exhibited the strongest cytoplasmic expression of FGF-2. Expression of FGF-2 protein was also confirmed using the western blot method. Identity of molecular weight for FGF-2 derived from deer cells with that of the human was also proven. What could be considered a novel finding is the demonstration of the expression of KGF (FGF-7) belonging to the FGF family. The expression of KGF, both at mRNA and protein levels, was lower than that of FGF-2. Similarly to FGF-2, the KGF protein detected was comparable in size to human KGF. The non-specific protein bands showing significantly weaker expression may suggest the presence of KGF isoforms. Both growth factors are mitogens and play a significant role, among others, in the regulation of hair growth (18, 19). The implanted MIC-1 cells could have accelerated the hair growth process through those factors by activating numerous cell types responsible for hair formation. Hair growth relies on good blood supply of the bulbs. The factors affecting angiogenesis during hair growth are, among others, the VEGF factors (21). There are several types of VEGF: VEGF-A, VEGF-B, VEGF-C and VEGF-D. We noted high expression of VEGF-A and VEGF-C proteins in the MIC-1 cells. However, the expression of VEGF-D was low. The lack of expression of VEGFR-3, which is a receptor for VEGF-C and VEGF-D, may indicate a paracrine effect on other cells, *i.e.* through VEGF factors, antlerogenic stem cells can regulate the blood supply of hair follicles.

In vitro, we confirmed that the antlerogenic stem cells stimulate fibroblast proliferation. Regulation could be induced through the growth factors studied, among others, FGF-2. Our observation has been confirmed in previous studies which proved that deer antler extract stimulates fibroblast and nerve stem cell growth *in vitro* (30, 31). Investigation of the mutual effect of stem cells on cells in co-cultures showed that growth factors secreted by those cells into the medium directly stimulated their proliferation (32, 33).

In the experimental group, the intradermally injected MIC-1 antlerogenic stem cells accelerated hair growth in rabbits. Rabbit fur started to re-grow as early as one week from the application of cells, unlike in the control group, in which hair growth was only observed after four weeks. During the experiment, hair growth at the site of injection was significantly more rapid compared to the control. Finally, at the injection sites, rabbit fur was thicker and hair growth visibly quicker. Hair growth in rabbits expands in waves, *i.e.* activation of one hair follicle leads to activation of adjacent ones (34). Application of stem cells and, thus, likely the expression of growth factors by these cells at the sites of injection, induced a spreading wave of hair growth activation. Activation of local stem cells responsible for hair growth took place at those sites. Histological examinations confirmed a significant increase in the number of active hair follicles and, in them, growth of several to several dozens of secondary hairs (guard hairs), which was not observed in the control group. Additionally, the immunohistochemical reactions performed confirmed that more follicles were stimulated to grow in rabbits injected with MIC-1 cells compared with the control group. In the experimental group, we noted a higher expression of Ki67, CD34 and SOX2 compared with the control group. Some stem cells at the site of the bulge were Ki67-positive, which means that they were activated as well. The expression of Ki67 in cells located below the bulge suggests that they were actively dividing progenitor cells. In the activated follicles, we observed a high expression of Ki67 in the cells of the matrix. High Ki67 expression in the majority of the secondary hairs is probably related to acceleration of hair growth. Interestingly, we observed high expression of CD34 and SOX2 in the cells of the internal and external root sheath, the bulge stem cells and dermal papilla cells. However, the previous studies indicate another typical site of CD34 expression in the bulge area and SOX2 expression only in the dermal papilla. The injection of the MIC-1 stem cells probably accelerated hair growth even though the expression of stem cell hair markers appeared in adjacent sites of typical hair follicle areas.

Among the described growth factors, FGF-2, KGF, VEGF-A and VEGF-C could be potentially significant for hair

growth. The factors which accelerated hair growth due to the activation of cells regulating the hair growth cycle were growth factors produced by the antlerogenic stem cells. This demonstrates the stimulation and transfer of signals from the applied stem cells to other cells of the hair follicle, the result of which could be immediate hair re-growth. This observation has also been confirmed in the majority of our medical experiments, in which stem cells were used in order to regenerate auricle cartilage and bone defects in rabbits (13, 14). At the sites of the analyzed tissues where the operated skin was in contact with the MIC-1 cells, we always observed more rapid hair growth (data not shown).

Numerous reports aim to explain the lack of allogenic stem cell implant rejection because of immunomodulatory activity of stem cells (35, 36). The present study investigated the effect of MIC-1 antlerogenic stem cells on the hair growth in a xenogenous animal model. The intradermal application of MIC-1 antlerogenic stem cells did not induce an immune response in the rabbits. Our previous experiments showed that after the application of the MIC-1 cells to rabbit cartilage and bone defects, no immune response occurred, while immunohistochemical reactions performed for the inflammatory T-cells, B-cells and macrophages, were negative (13, 14). The molecular analysis revealed that MIC-1 cells expressed low levels of MhcII and no expression of MhcI (37). One of the anti-inflammatory factors secreted by stem cells is TGF-β1 (38, 39). Its presence was confirmed through molecular biology tests in the MIC-1 cells. The immunosuppressive, immunomodulatory and anti-inflammatory activity of the implanted stem cells contributed to the lack of immune response and was involved in the inhibition of inflammatory cell proliferation and activity. Additionally, the proven lack of Il-10 expression, an anti-inflammatory cytokine, in the MIC-1 cells agrees with literature reports indicating its significant anti-inflammatory activity although, related to T-regulatory lymphocyte activity (40). The anti-inflammatory activity of the MIC-1 cells has also been confirmed by numerous reports on extracts and dietary supplements obtained from growing antlers which are approved as safe-to-use by the US Food and Drug Administration and show significant efficacy in rheumatoid arthritis, as well as in the inhibition of osteoporosis and acceleration of wound-healing processes (41, 42).

Final conclusion: The MIC-1 antlerogenic stem cells express numerous growth factors. Intradermal injection of the MIC-1 cells accelerated hair growth in rabbits due to the activation of cells responsible for the regulation of the hair growth cycle. The increased expression of proliferating cell and stem cell markers was mostly observed in the stem cells of the hair follicle bulge area and cells of dermal papillae. The stimulation of fur re-growth in rabbits could be induced through growth factors produced by the MIC-1 cells. Additionally, the xenogenous cell implant did not induce any immune response. The lack of immune responses may be explained by the immunomodulatory and anti-inflammatory activity of the MIC-1 cells and the decreased expression of MHCs.

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