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5-Azacytidine enhances proliferation in transplanted rat fetal epiglottis

Sandra Marinovic Kulisic[1], Gordana Juric-Lekic[2], Masa Vikic-Topic[2], Vedran Lokosek[2], Vedran Radujkovic[2], Floriana Bulic-Jakus[3], Ana Katusic[3], Maja Vlahovic[3], Ljiljana Serman[3], Nino Sincic[3]

[1]University Department of Dermatology and Venereology, Zagreb University Hospital Center and School of Medicine, University of Zagreb, Croatia, [2]Department of Histology and Embryology, School of Medicine University of Zagreb, Croatia, [3]Department of Biology, School of Medicine University of Zagreb, Croatia

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1. ABSTRACT

Fetal rat epiglottis and its developmental potential in ectopic transplants under the influence of the epigenetic drug was investigated. Epiglottises from 17-days-old rat embryos were transplanted under kidney capsules of adult rats for 14 days. 5-azacytidine (5mg/kg) was injected intraperitoneally during first three days and controls were sham treated. TEM, immunohistochemical detection and quantitative stereological analysis of the Proliferating Cell Nuclear Antigen (PCNA) expression (numerical density N_v) were performed. Typical chondroblasts with long surface processes and sparse lipid droplets were found in fetal epiglottis and chondrocytes with shorter processes, numerous lipid droplets and elastic fibers in adult. PCNA was expressed in almost all cells of the fetal epiglottis while in the adult it was expressed in minority of cells. In transplants, differentiation progressed towards the differentiation found in the adult. Application of 5-azacytidine increased the capacity for proliferation ($N_{v\ PCNA}$) in comparison to controls but no difference in differentiation was observed. Data about the developmental potential and induction of proliferation in mammalian epiglottis by epigenetic modulation is of importance for regenerative medicine.

2. INTRODUCTION

Almost two decades ago, human epiglottis was used as an autologous composite graft in eyelid reconstruction (1) and agenesis of the epiglottis was described in the adult without the history of dysphagia, stridor and respiratory infections (2).

Data about structure and development of epiglottis are scarce and mostly limited to the adult human (2,3), adult rat (4,5) and fetal and adult cat (6). Epiglottic cartilage of the adult is covered by mucosa consisting of epithelium and lamina propria with blood vessels, lymph vessels and tubuloacinar glands. At its oral surface and the majority of laryngeal surface epiglottis is covered by stratified squamous epithelium and at the bottom of the laryngeal surface with ciliated pseudostratified epithelium. Interestingly, before and at birth ciliated pseudostratified epithelium dominates over the stratified squamous epithelium in humans (7). In transplants of embryonic rat epiglottis growing under the kidney capsule epithelial differentiation was restricted to ciliated pseudostratified epithelium. Therefore it seems that the contact with air is necessary for differentiation of the stratified squamous epithelium (8).

The purpose of this study was to investigate the morphology and ultrastructure of rat embryonic epiglottal cartilage and its potential for the development of the elastic cartilage in ectopic *in vivo* transplants under the kidney capsule (9). Additionally, the epigenetic drug and DNA demethylating agent 5-azacytidine (5-azaC) known to change various developmental parameters through changes of gene expression was applied (10,11) in order to improve development of the epiglottis in transplants. Our studies are important for strategies considered in regenerative medicine (12,13).

3. MATERIALS AND METHODS

3.1. Animals

All animal experiments in this study were done with the permission of the Ethical Committee of the School of Medicine University of Zagreb.

3.2. Isolation and transplantation of epiglottises

Fisher strain rats were mated overnight and the finding of the sperm in the vaginal smear designated the day 0 of pregnancy. Females were killed in ether and 17-days-old fetuses were isolated. Epiglottises from fetuses or adult animals (three months old) were isolated under the dissecting microscope using two pairs of watchmaker's forceps and tungsten needles and rinsed in Phosphate Buffered Saline (PBS).

Fetal epiglottises were transplanted to an ectopic site in adult male Fisher rats. Rats were anaesthetized and kidney was approached through a paravertebral incision through skin and muscle. A small pocket was formed under the kidney capsule to place the explant. The wound was closed by 16 mm Michel's clumps. Transplants were grown *in vivo* for 14 days. 5-azacytidine (50mg/kg) was dissolved in PBS and applied by an i.p. injection for three consecutive days (13 animals) while sham controls (22 animals) were treated by PBS, only.

3.3. Histology

15 epiglottal explants and 35 transplants were fixed by St. Marie solution (1% acetic acid in 96% ethanol, $+4^0$C), embedded in paraffin, processed for routine histology and stained by haematoxylin-eosin, resorcin-fuxin, Verhoeff's iron haematoxylin or toluidine blue.

3.4. Immunohistochemistry

For immunohistochemical analysis mild fixation during 24 hours with St Marie's solution was used. Explants were dehydrated and embedded in paraffin at 56^0 C. Serial sections (5 μm) were put on silanized slides (S3003; DAKO, Glostrup, Denmark) and air-dried for 24 hours at room temperature. Sections were deparaffinized in xylene (2x 5 min), treated with absolute ethanol and 96% ethanol (2x 3min) and H_2O (30 seconds).

Monoclonal Mouse Anti-Proliferating Cell Nuclear Antigen (PCNA), Clone PC 10, (DAKO, M0879), was diluted to 1:100 and applied for one hour. Negative controls were treated with an unspecific antibody (No. V 1617 mouse IgG_1, DAKO). Labeled streptavidin-biotin kit (DAKO LSAB® 2 Kit, HRP) for usage on rat tissue was used according to manufacturer's instructions. Sections were briefly counterstained with hematoxylin, washed first with distilled water, then for 20 min in tap water and again for 3 min in distilled water, and covered with 50% glycerol in PBS.

3.5. Transmission electron microscopy

For analysis by transmission electron microscopy, isolated transplants were immediately transferred from culture to 4% glutaraldehyde fixative where they were kept for two hours and then postfixed in 1% OsO_4 during two hours. They were washed three times for 10 min in 0,1M phosphate buffer, dehydrated in ascending concentrations of ethanol and finally transferred to ethanol and acetone (1: 1) for 30 min and 100% acetone for 30 minutes. They were kept in Durcopan (Balzers, Lichtenstein) diluted in acetone (1: 1) for two hours, embedded in Durcopan and kept at 56^0 C for 3 days. Serial semi-thin sections (0.9μm) were cut and stained with 1% toluidine blue. Ultrathin sections (70 nm) tissue were made, mounted on copper grids and contrasted with lead citrate and uranyl acetate. A Zeiss 902A transmission microscope was used for ultrastructural analysis (Centre for Electron Microscopy, Medical Faculty University of Zagreb).

3.6. Stereology

Randomly selected paraffin blocks were used for stereological analysis of PCNA-positive cells as described previously (11). Five consecutive sections were taken in a random fashion from each series. Quantitative stereological analysis of numerical density (Nv) was performed by Nikon Alphaphot binocular light microscope (Nikon, Vienna, Austria) using Weibel's multipurpose test system with 42 points (M 42) at magnification of 400x. The area tested (A_t) was 0.0837 mm^2. For each investigated group the orientation/pilot stereological measurement was carried out in order to define the number of fields to be tested. The numerical density of PCNA-positive cells was determined by the point counting method. Numerical density (Nv) was calculated by formula: $Nv = N/A_t \times D$, where N was the number of PCNA-positive cells on tested area. The mean tangential diameter (D) was calculated by light microscopy at magnification of 400x and for 100 cells was 0.031mm. Differences in numerical densities (Nv) of PCNA-positive cells in investigated groups were analyzed by Student t-test.

4. RESULTS

4.1. Progress in development between fetal and adult rat epiglottis *in situ*

By light histology it was discovered that the 17-days–old fetal rat epiglottis consists of an immature cartilage covered by the immature epithelium (Figure 1. A, B). The proliferation marker PCNA was expressed in majority of cells forming the cartilage and epithelium (Figure 1. C, D). On the other hand, the adult epiglottis consisted of the mature elastic cartilage. One or more chondrocytes were situated in a single lacuna and between lacunae a dense dark network of elastic fibers was typically stained by Verhoeff's iron haematoxylin (Figure 1. E, F).

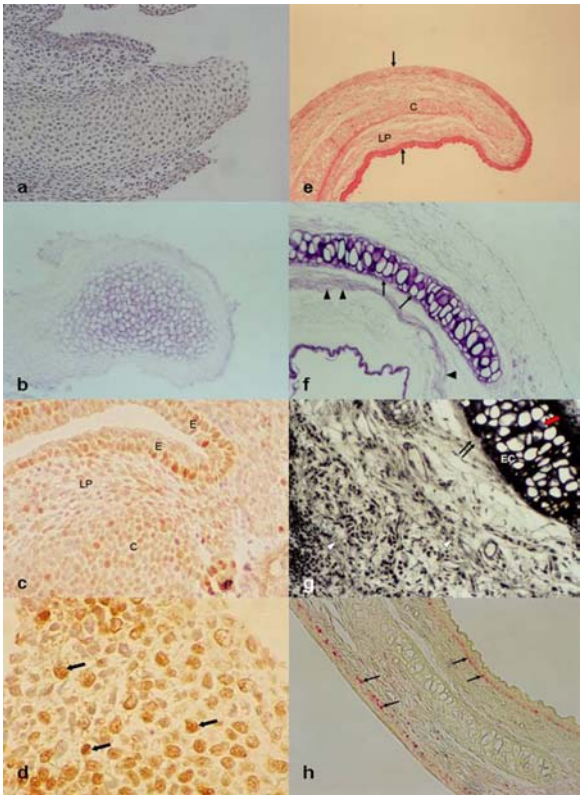

Figure 1. Comparative histology of the 17-days-old rat fetal and adult epiglottis. A) Immature fetal cartilage and epithelium. Haematoxylin-eosin, x 100. B) Immature fetal cartilage (C). Resorcin-fuxin, x 100. C) PCNA expression in the immature tissues: epithelium, connective tissue and cartilage of the fetal epiglottis. E–epithelium, LP–lamina propria, C–immature cartilage. DAB, counterstained with haematoxylin, x 200. D) PCNA expression in fetal chondroblasts (arrows). DAB, counterstained with haematoxylin, x 200. E) Adult epiglottis. C–elastic cartilage, stratified squamous epithelium (arrow), LP–lamina propria. Haematoxylin-eosin, x 40. F) Adult epiglottis composed of the elastic cartilage with typical elastic fibers in the perichondrium (arrows) and in the lamina propria (arrowheads). Resorcin-fuxin, x 100. G) Adult epiglottis. EC–elastic cartilage, chondrocytes in lacunae (arrow), elastic fibers in the perichondrium (double arrows) and in the lamina propria (arrowheads). Verhoeff's iron haematoxylin, x 100. H) PCNA expression in basal cells of the stratified squamous epithelium (arrows) of the adult epiglottis. DAB, counterstained with haematoxylin, x 100.

Expression of the cell proliferation marker PCNA was detected in only several chondrocytes and in the basal cell layer of the surface epithelium (Figure 1G, H).

TEM analysis at the ultrastuctural level revealed more precisely that 17-days –old rat epiglottal cartilage really consisted only of chondroblasts. They were scattered through future cartilage (Figure 2. A). At their surface long cytoplasmic processes typical for immature cells were present. Within the cytoplasm abundant rough cytoplasmic reticulum with numerous ribosomes, numerous Golgi apparatuses and mitochondria typical for metabolically very active immature cells were also present. Lipid droplets were present only sporadically (Figure 2. B, C, D). In the adult elastic cartilage of the rat chondrocytes typically lying in lacunae surrounded by a territorial ring were observed. The cells were either solitary or nests of several cells were found (Figure 3. A). Cytoplasmic processes at the surface of mature chondrocytes were shorter than in chondroblasts and in intercellular space elastic fibers forming wide coherent bands or smaller separated clumps were present (Figure 3. B). Numerous lipid droplets characteristic for the adult elastic cartilage of the rat epiglottis were present surrounded by densely packed parallel filaments (Figure 3. C).

4.2. Maturation of fetal epiglottis in transplants

To analyze the impact of 5-azacytidine on developmental potential of the rat fetal epiglottal cartilage 17-days old epiglottises were transplanted under the kidney capsule and animals were treated by 5-azacytidine immediately and for two more consecutive days (50μg/kg) together with sham treated controls.

Classical histological analysis by light microscopy revealed that in transplants cartilage developed further, was enveloped in perichondrium while whole epiglottis was covered by mucosa. Differentiated chondrocytes were situated within lacunae and the perichondrium surrounded the epiglottal cartilage (Figure 4). No difference between cartilage in transplants treated by 5-azaC or controls could be found upon classical histological examination. However, a higher degree of cartilage differentiation was discovered in transplants than in initial explants of 17-days-old fetal epiglottises which contained an immature cartilage without a perichondrium (Figure 2).

At the ultrastructural level again no differences were observed between 5-azaC-treated and controls. TEM analysis discovered differentiated chondrocytes with several lipid droplets in the cytoplasm (Figure 5. A) and numerous short processes on the cell surface (Figure 5 A, B; Figure 6. A, B, C). In chondrocytes rough endoplasmic reticulum was abundant and pinocytotic vesicles were present (Figure 6. D). In the intercellular space small dense granules were present. Among numerous fibers, thicker branching fibers could be distinguished from thinner fibers (Figure 6. E). At higher magnification cytoplasmic lipid droplets were seen either being connected with each other or separated from each other by thin cytoplasmic septa. The rest of the cytoplasm was characterized by cisterns of rough endoplasmic reticulum and numerous mitochondria. Thin fibers with granules and thick fibers were found within the intercellular space (Figure 6. F).

4.3. 5-azacytidine enhances the expression of the Proliferating Cell Nuclear Antigen (PCNA) in transplants

The marker of proliferating cells (PCNA) was expressed in cells of both cartilage and mucosa (epithelium and lamina propria) which differentiated in the transplants.

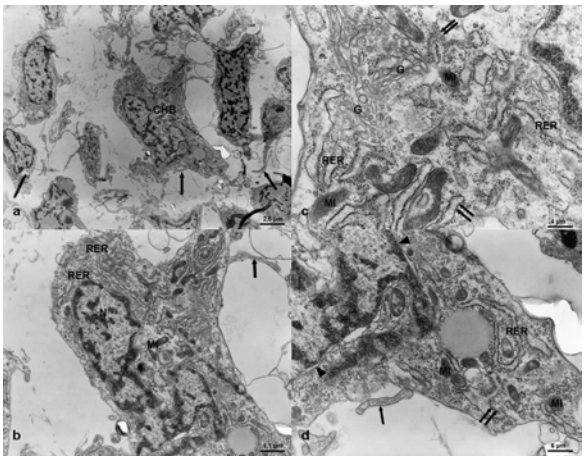

Figure 2. Ultrastructure of the 17-days-old rat fetal epiglottis. A) CHB–chondroblast, cytoplasmic processes (arrows). TEM, x 3000. B) Detail of A. A chondroblast. N–nucleus, MI–mitochondrion, RER–rough endoplasmic reticulum, cytoplasmic processes (arrows).TEM, x7000. C) A chondroblast. G–Golgi apparatus, MI– mitochondrion, RER–rough endoplasmic reticulum, ribosomes (double arrow). TEM, x 20 000. D) A chondroblast. Nuclear envelope (arrowhead), MI–mitochondrion, RER–rough endoplasmic reticulum, ribosomes (double arrow), cytoplasmic processus (arrow). TEM, x 7000.

It was present in basally situated nuclei of the pseudostratified epithelium with goblet cells and absent from nuclei situated closer to the epithelial surface. The difference was observed between its expression in sham treated controls and transplants treated with 5-azacytidine (Figure 7) only after stereological quantification. Numerical density (Nv) was significantly higher ($p<0,05$) in transplants treated for the first three days by 5-azaC (Figure 8).

5. DISCUSSION

In this study histology and ultrastructure of the 17-days-old embryonic epiglottal cartilage was described for the first time in the rat. At this stage of development, immaturity of the cartilage was represented by ultrastructural features typical for all chondroblasts regardless of the nature of their future cartilage type (14).

The Nuclear Proliferating Cell Antigen (PCNA) is a cofactor for the DNA polymerase and an endogenous marker of proliferating cells (15,16). Expression of PCNA detected by the immunohistochemistry at the single cell level has been used in developmental studies to discern between cycling cells (e.g. proliferating cells surrounding the third brain ventricle) and terminally differentiated cells (e.g. ganglionic cells) within a certain tissue type (17). Almost ubiquitous presence of the PCNA that has been found now in the 17-days-old embryonic epiglottis was also typical for immature cells that are still confined to the cycling cell tissue compartment. Keeping in mind that the transition to the G0 phase leading to terminal differentiation is characterized by the absence of the PCNA, its absence can be correlated to the transition of chondroblasts to chondrocytes. Indeed, in the adult epiglottis, where presence of typical differentiated chondrocytes was confirmed by TEM analysis, PCNA expression was restricted to a minority of cartilage cells.

During two weeks after transplantation, differentiation of embryonic cartilage progressed and epiglottal cartilage became enveloped by perichondrium. Numerous processuses necessary for metabolic exchange across a larger cell surface area with the extracellular space were present. This feature is important for nutrition of chondrocytes distant from the blood vessels contained within the perichondrium. Well developed ER found in chondrocytes was typical for mature cells secreting tissue-specific proteins. In the cat embryonic epiglottis chondrocytes appeared during the 8th and elastic fibers during the 12th week of intrauterine development (6). Multiple lipid droplets surrounded by densely packed parallel filaments found in transplants are characteristic for adult elastic chondrocytes of the rat epiglottis (5) as well as for the auricular cartilage. In the sparse intercellular space thinner fibers with granules as well as thicker, irregular, partly discontinuous, branched structures which run approximately in the middle of the intercellular space which have been found in transplanted epiglottis correspond to the ultrastructure of elastic cartilage in the rat external ear (18).

The classical ectopic *in vivo* transplantation model used here (9), confirmed its value for this particular developmental study dealing with embryonic epiglottis, because 17-days-old epiglottis survived well and its differentiation proceeded towards the mature elastic cartilage. Subcapsular kidney space has been exploited extensively before for investigation of developmental potential of a number of embryonic tissues such as the embryonic shield (19), single germ layers (20), retina (21), lacrimal gland (22), mandible (23) and lensectomized eyes (24,25). Results obtained with epiglottis are in concordance with those studies because development always progressed and tissues could exert at least a part of their full developmental potential even if pre-cultivated in poor metabolic conditions such as chemically defined media without any proteins. The reason for this lies probably in the optimal vascularization of the kidney capsule that provides an optimal microenvironment (19).

Autologous elastic auricular cartilage transplants are used for facial reconstruction in human medicine but among problems of such procedures is the difficulty in obtaining a sufficient amount of reconstructive material and a risk of cartilage absorption after grafting (12). Recently, a successful two-stage implantation method was devised were cultured auricular chondrocytes were injection-implanted at an ectopic site into the lower abdomen of the patient where

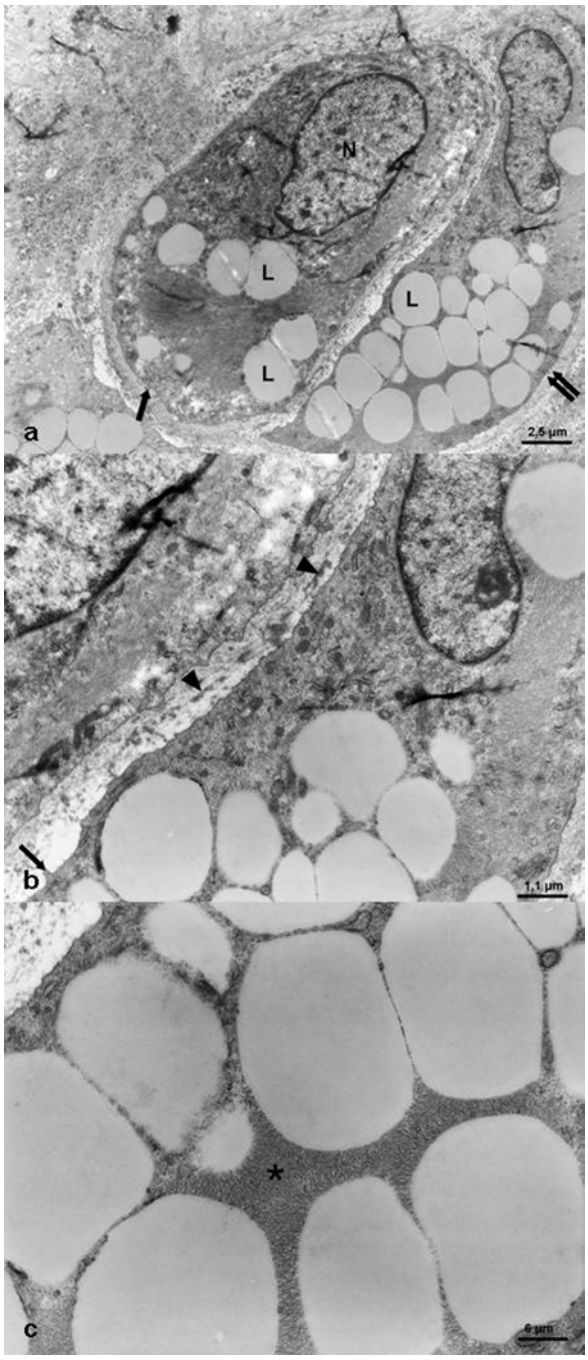

Figure 3. Ultrastructure of the adult epiglottis. A) Two chondrocytes in a lacuna of the adult epiglottal cartilage. N–nucleus, L–lipid droplets, cytoplasmic processes (arrow), lacuna (double arrow) . EM, x3000. B) Detail of A. Microvillus processes on the surface of the chondrocyte (arrow), elastic fibers in the intercellular space (arrowheads). TEM, x 7000; C) Detail of A. Lipid droplets in the cytoplasm of a chondrocyte. Between lipid droplets numerous filaments are present (asterix). TEM, x 20 000.

the cells grow into a large, newly generated cartilage with neoperichondrium in 6 months (13).

Epigenetic drug 5-azacytidine is a synthetic drug, the analogue of cytosine. It has recently been approved for the therapy of human myelodysplastic syndrome (26,27). Higher doses of 5-azaC seem to cause cytotoxic effect while lower doses can cause genome hypomethylation (10) either of which is the probable cause of its teratogenic influence discovered during pregnancy of the rat (11,17). In this investigation stereological quantification of the PCNA expression showed that 5-azaC increased the ability for proliferation of epiglottal cells similarly as was shown for the placenta in rats treated by 5-azaC during pregnancy

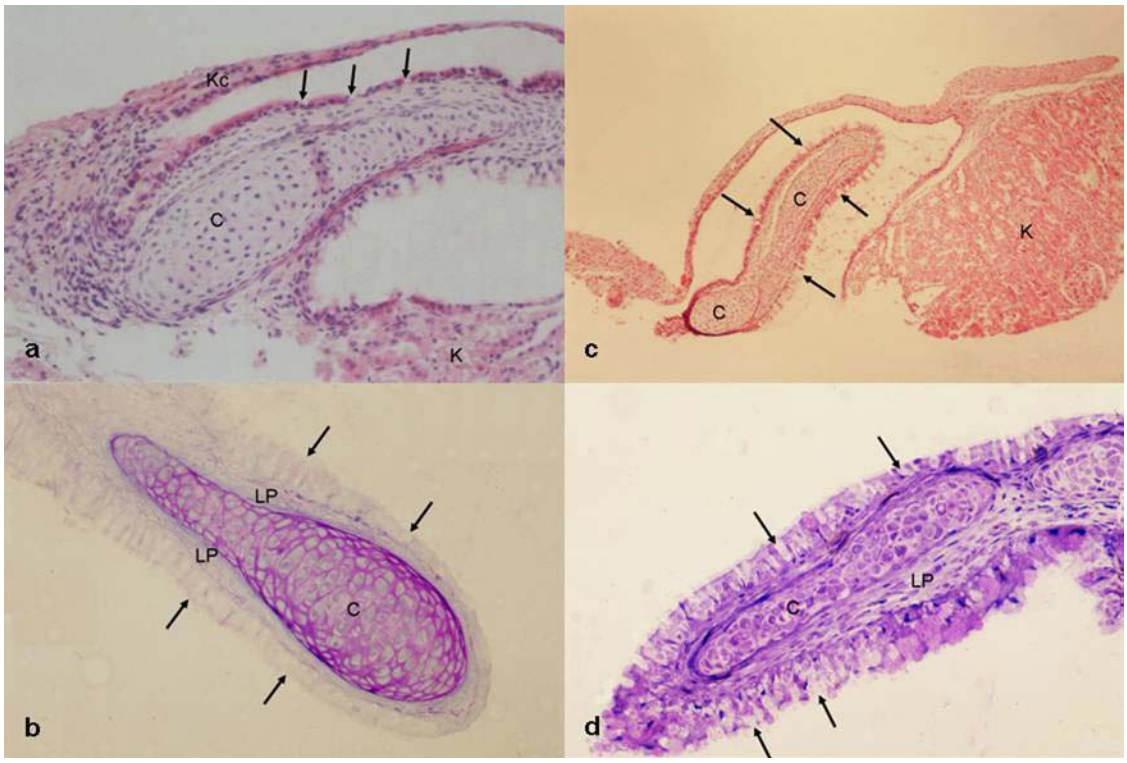

Figure 4. Histology of the 17-days-old rat fetal epiglottis in transplants. A) Control transplant without 5-azaC treatment after 14 days. C–cartilage, ciliated pseudostratified epithelium with goblet cells (arrows), K–kidney parenchyma, Kc–kidney capsule. Haematoxylin-eosin, x 100. B) Semi-thin section of a control transplant without treatment with 5-azaC after 14 days. C–cartilage, ciliated pseudostratified epithelium with goblet cells (arrows), LP–lamina propria. Toluidine blue, x 100. C) Transplant treated with 5-azacytidine after 14 days. C–cartilage, ciliated pseudostratified epithelium with goblet cells (arrows), K–kidney parenchyma, Kc–kidney capsule. Haematoxylin-eosin, x 40. D) Semi-thin section of a transplant treated with 5-azacytidine after 14 days. C–cartilage, ciliated pseudostratified epithelium with goblet cells (arrows), LP–lamina propria. Toluidine blue, x 100.

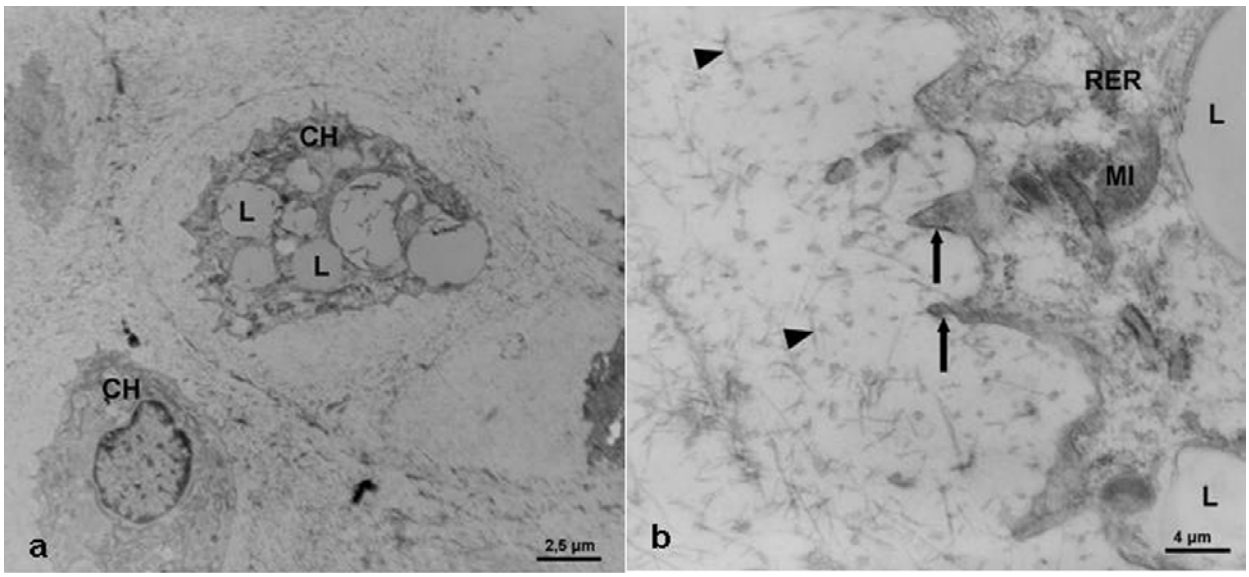

Figure 5. Ultrastructure of transplanted fetal epiglottis (sham treated control). A) Cartilage in a control transplant. CH–chondrocytes, L–lipid droplet. TEM, x 3000; B) Detail of A. L–lipid droplet, MI–mitochondrion, RER–rough endoplasmic reticulum, cytoplasmic processes (arrows), fibrils of the extracellular matrix (arrowheads). TEM, x 20 000.

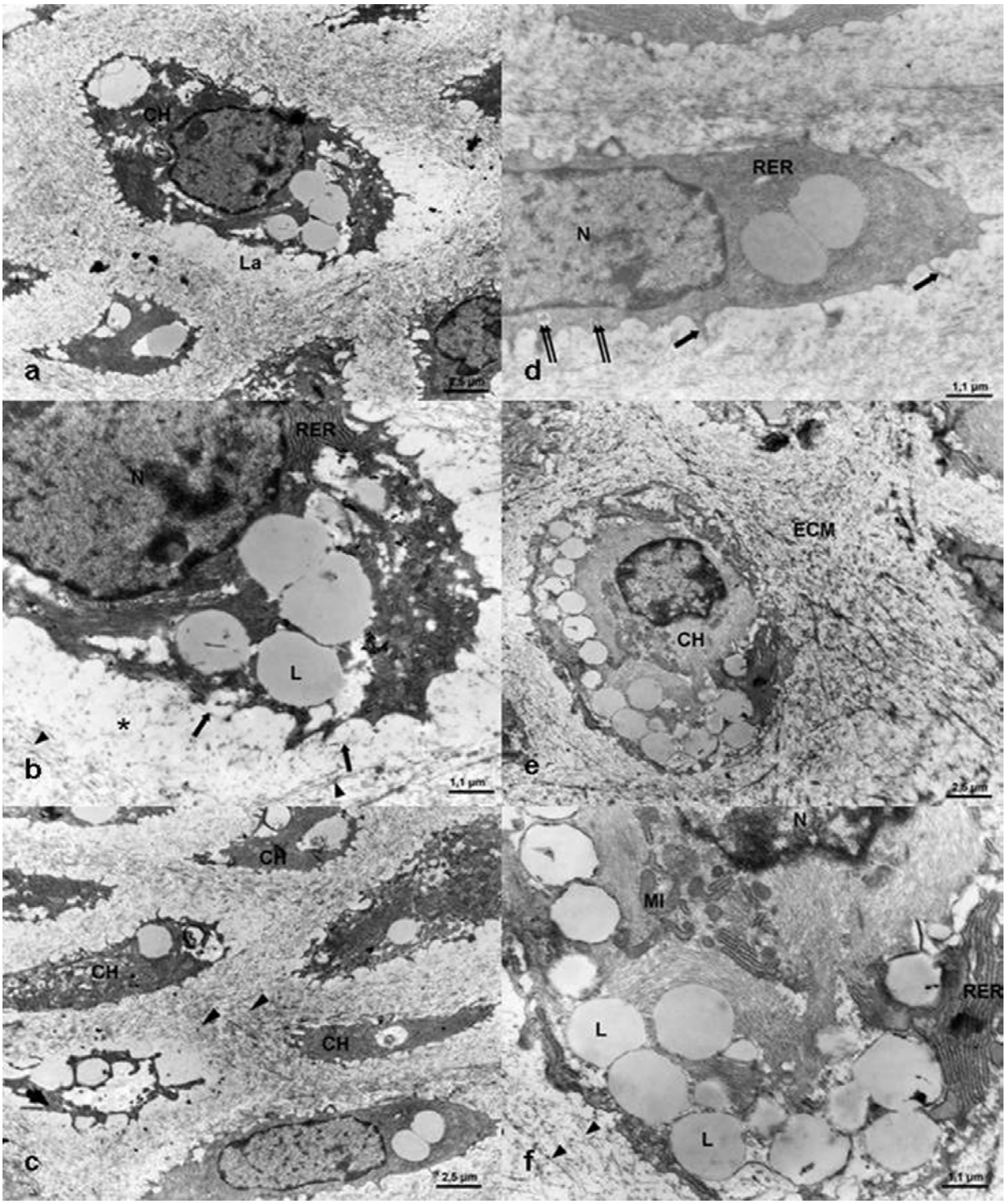

Figure 6. Ultrastructure of transplanted fetal epiglottis treated with 5-azacytidine. A) CH–chondrocytes, La–lacuna. TEM, x 3000. B) Detail of A. A chondrocyte in a lacuna. N– nucleus, RER–rough endoplasmic reticulum, L–lipid droplet, lacuna (asterix), fibrils in a lacuna (arrow), fibrils of the extracellular matrix (arrowheads). TEM, x 7000. C) CH–chondrocytes, extracellular matrix (arrowheads). D) Detail of C. N–nucleus, RER–rough endoplasmic reticulum, pinocytotic vesicles (double arrow), cytoplasmic processes of a chondrocyte (arrow). TEM, x 7000. E) CH-chondrocyte, ECM-intercellular matrix. TEM, x 3000. F) Detail of E. N–nucleus, MI–mitochondrion, RER–rough endoplasmic reticulum, cytoplasmic processus (arrows), L-lipid droplets, fibrils in the lacuna (arrowheads). TEM, x 7000.

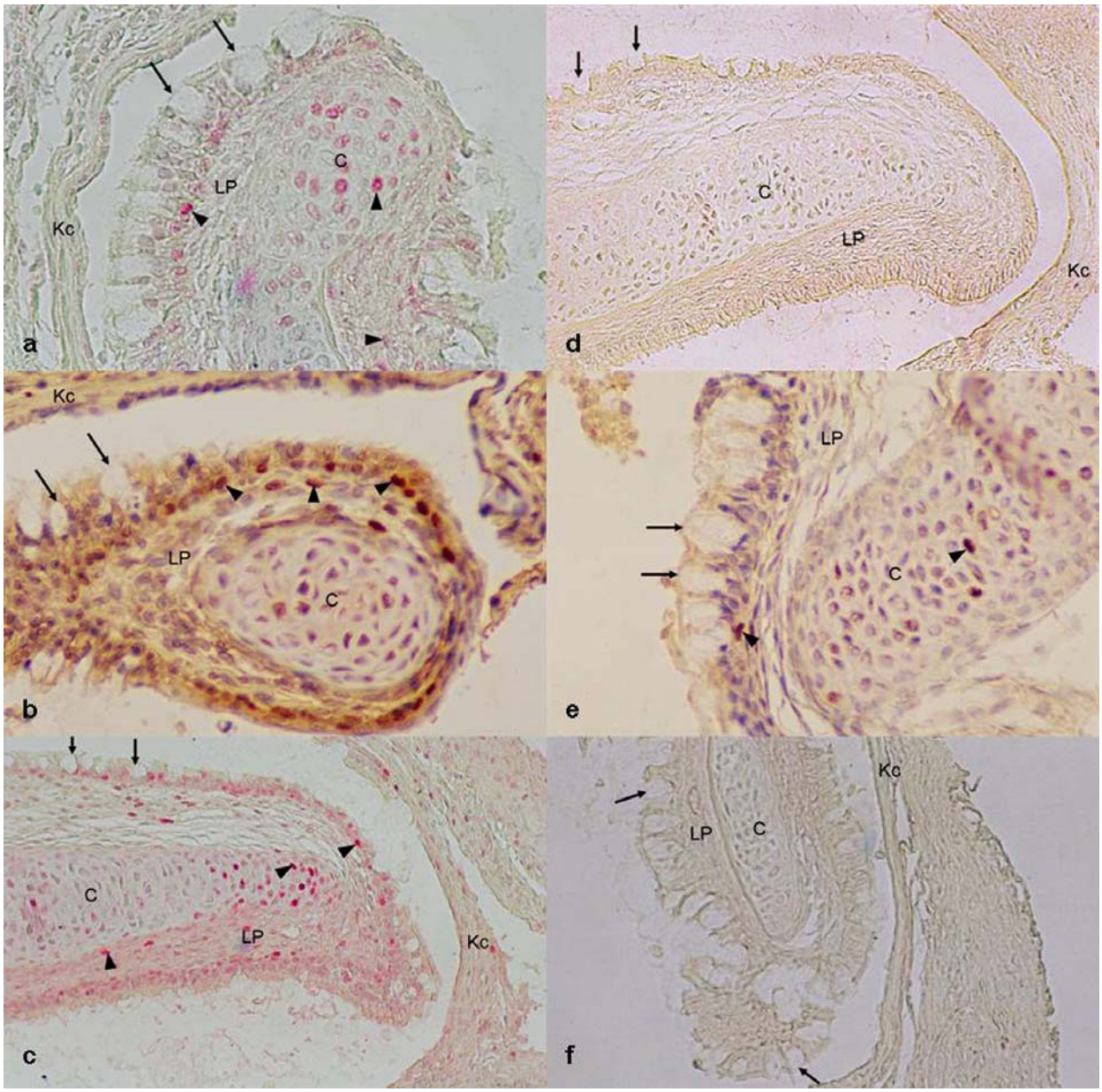

Figure 7. PCNA expression in transplants. A) PCNA expression in mucosa (epithelium and lamina propria) and cartilage of the treated transplant (arrows). C–cartilage, goblet cells in ciliated pseudostratified epithelium (arrows), LP–lamina propria, Kc–kidney capsule. Fast red, counterstained with haematoxylin, x 400. B) PCNA expression in the epithelium and cartilage of the treated transplant (arrows). C–cartilage, goblet cells in ciliated pseudostratified epithelium (arrows), LP–lamina propria, Kc–kidney capsule. DAB, counterstained with haematoxylin, x 200. C) PCNA expression in the epithelium and cartilage of the treated transplant (arrows). C–cartilage, goblet cells in ciliated pseudostratified epithelium (arrows), LP–lamina propria, Kc–kidney capsule. Fast red, counterstained with haematoxylin, x 200. D) Negative control of Figure 3. A. C–cartilage, ciliated pseudostratified epithelium with goblet cells (arrows), LP–lamina propria, Kc– kidney capsule. Fast red, counterstained with haematoxylin, x 200. E) PCNA expression in the epithelium and cartilage of the sham treated control transplant. C–cartilage, goblet cells in ciliated pseudostratified epithelium (arrows), LP–lamina propria. DAB, counterstained with haematoxylin, x 400. F) Negative control. C-cartilage, goblet cells in ciliated pseudostratified epithelium (arrows), LP–lamina propria, Kc–kidney capsule. DAB, counterstained with haematoxylin, x 200.

(11). So it seems that selective modulation of DNA methylation may have important clinical implications not only for the prevention and treatment of cancer (28) but also in increasing proliferative potential of tissues such as epiglottis that have been considered for reconstructive tissue therapy within the scope of regenerative medicine (1).

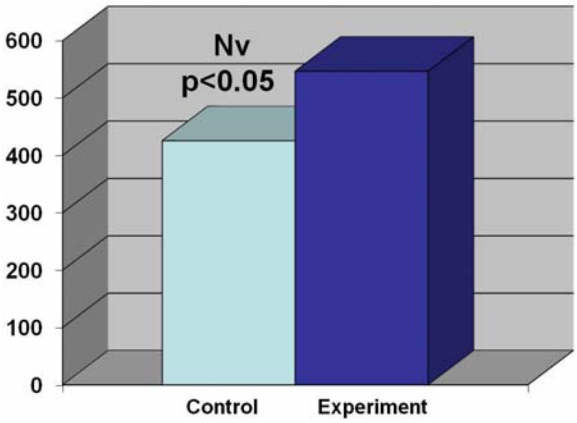

Figure 8. Numerical density of the PCNA positive nuclei. Note that Nv was significantly higher in 5-azacytidine treated transplants.

6. ACKNOWLEDGMENTS

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7. REFERENCES

1. J. L. Adams, N. R. Olson and D. B. Siders: The use of the epiglottis as an autologous composite graft in eyelid reconstruction. *Ophthal. Plast. Reconstr. Surg.* 9, 206-211 (1993)

2. K. H. Hong and Y. S. Yang: Agenesis of the epiglottis and false vocal folds with maxillary hypoplasia in an adult. *JLO* 117, 895-898 (2003)

3. M. W. Sung, J. W. Kim and K. H. Kim: Bifid epiglottis associated with Joubert's syndrome. *Ann. Otol. Rhinol. Laryngol.* 110, 194-196 (2001)

4. E. H. Nielsen and P. Bytzer: High resolution scanning electron microscopy of elastic cartilage. *J. Anat.* 129, 823-831 (1979)

5. E. H. Nielsen: The elastic cartilage in the normal rat epiglottis. *Cell Tiss. Res.* 173, 179-191 (1976)

6. M. Egerbacher, R. Krestan and P. Bock: Morphology histochemistry and differentiation of the cat´s epiglottic cartilage: a supporting organ composed of elastic cartilage, fibrous cartilage, myxoid tissue and fat tissue. *Anat. Rec.* 242, 471-482 (1995)

7. G. Juric-Lekic, V. Radujkovic, V. Kancijan, F. Bulic-Jakus, V. Lokosek, A. Katusic, M. Vlahovic and L. Serman: Differentiation of epiglottal epithelia during prenatal and postnatal human development. *Coll. Antropol*. 32, 1115-1120 (2008)

8. S. Marinovic-Kulisic, G. Juric-Lekic, F. Bulic-Jakus, V. Radujkovic, I. Parcetic, M. Vlahovic, A. Katusic, N. Sincic and Lj. Serman: Development of epithelia in the ectopic transplant of the fetal rat epiglottis. *Acta Dermatovenerol. Croat*. 16, 55-59 (2008)

9. N. J Robertson, P. J. Fairchild, H. Waldmann- Ectopic Transplantation of Tissues Under the Kidney Capsule. *In*: P. J. Fairchild, Ed. Methods in Molecular Biology, Immunological Tolerance, Methods and Protocols Part III, Volume 380, pp.347-353; Humana Press, Totowa, New Jersey, USA (2007)

10. M. Esteller: Molecular Origins of Cancer, Epigenetics in Cancer. *N. Engl. J. Med*. 358, 1148-1159 (2008)

11. Lj. Serman, M. Vlahovic, M. Sijan, F. Bulic-Jakus, A. Serman, N. Sincic, R. Matijevic, G. Juric-Lekic and A. Katusic: The impact of 5-azacytidine on placental weight, glycoprotein pattern and proliferating cell nuclear antigen expression in rat placenta. *Placenta* 28, 803-811 (2007)

12. H. Yanaga, K. Yanaga, K. Imai, M. Koga, C. Soejima and K. Ohmori: Clinical Application of Cultured Autologous Human Auricular Chondrocytes with Autologous Serum for Craniofacial or Nasal Augmentation and Repair. *Plast. Reconstr. Surg.* 117, 2019-2030 (2006)

13. H. Yanaga , K. Imai, T. Fujimoto and K. Yanaga: Generating ears from cultured autologous auricular chondrocytes by using two-stage implantation in treatment of microtia. *Plast Reconstr Surg.* 124, 817-825 (2009)

14. R.O. Greep and L. Weiss, Histology. New York: McGraw-Hill (1973)

15. E. Prosperi: The fellowship of the rings: distinct pools of proliferating cell nuclear antigen trimer at work *FASEB J.* 20, 833-837 (2006)

16. S. Maric, F. Bulic-Jakus, D. Jezek, G. Juric-Lekic, M. Kos and M. Vlahovic: Expression of the proliferating cell nuclear antigen and protein products of tumour suppressor genes in the human fetal testis . *Andrologia* 36, 24-30 (2004)

17. M. Vlahovic, F. Bulic–Jakus, G. Juric–Lekic, A. Fucic, S. Maric and D. Serman: Changes in the placenta and in the rat embryo caused by demethylating agent 5-azacytidine. *Int. J. Dev. Biol*. 43, 843-846 (1999)

18. Lj. Kostovic-Knezevic and A. Svajger: Cytoplasmic filaments associated with lipid droplets in chondrocytes of the rat auricular cartilage. *Experientia (Basel)* 31, 581-582 (1975)

19. F. Bulic-Jakus, T. Strahinic-Belovari, S. Maric, D. Jezek, G. Juric-Lekic, M. Vlahovic and D. Serman: Chemically defined protein-free *in vitro* culture of

mammalian embryo does not restrict its developmental potential for differentiation of skin appendages. *Cells Tissues Organs* 169, 134-143 (2001)

20. B. Levak-Svajger, V. Knezevic and A. Svajger: Development of separated germ layers of rodent embryos on ectopic sites: a reappraisal. *Int. J. Dev. Biol*. 35, 177-189 (1991)

21. A. Katusic, G. Juric-Lekic, N. Jovanov-Milosevic, M. Vlahovic, D. Jezek, L. Serman, N. Sincic, B. Veljanovska and F. Bulic-Jakus: Development of the fetal neural retina *in vitro* and in ectopic transplants *in vivo*. *Coll. Antropol.* 32, 201-207 (2008)

22. A. Katusic, Lj. Serman, F. Bulic-Jakus, G. Juric-Lekic, M. Vlahovic and K. Kratochwil: The expression of proliferating cell nuclear antigen and retinoblastoma protein in transplanted fetal rat lacrimal gland. *Acta Clin. Croat*. 43, 211-215 (2004)

23. F. Bulic-Jakus, G. Juric-Lekic, M. Vlahovic, A. Katusic, S. Marinovic-Kulisic, Lj. Serman and D. Jezek: Survival of rat embryonic parts after ectopic transplantation. *Acta Clin. Croat*., 44, 211-215 (2005)

24. G. Juric-Lekic, F. Bulic-Jakus, M. Vlahovic, K. G. Wiman, M. Bygdeman and G. Klein: Mammalian neural retina in different experimental systems. *Period. Biol*. 96, 67-68 (1994)

25. G. Juric-Lekic and A. Svajger: Lentoid formation in ectopic graft of 1entectomized eyes of rat foetuses. *Cell Differ. Develop*. 27, 225-232 (1989)

26. J. R. Kuykendall: 5-Azacytidine and Decitabine Monotherapies of Myelodysplastic Disorders. *Ann. Pharmacother*. 39, 1700-1708 (2005)

27. E. Kaminskas, A. T. Farrell, Y-C. Wang, R. Sridhara and R. Pazdur: FDA Drug Approval Summary: Azacitidine (5-azacytidine, Vidaza™) for Injectable Suspension. *Oncologist* 10,176–182 (2005)

28. R. Singal and G. D. Ginder: DNA Methylation. *Blood*, 93, 4059-4070 (1999)

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Send correspondence to: Gordana Juric-Lekic, Department of Histology and Embryology, School of Medicine University of Zagreb, Salata 3, 10000 Zagreb, Croatia, Tel: 00385-1-4566-790, Fax: 00385-1- 4590-251, E-mail: gjuric@mef.hr