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original research article

Epigenetic drug 5-azacytidine impairs the potential for odontogenesis but improves tooth morphogenesis in the transplanted embryonic mandible

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Running title: Influence of a DNA demethylating agent on transplanted embryonic mandible

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List of abbreviations:
5azaC – 5-azacytidine
BMP – bone morphogenic protein
FGF – fibroblast growth factor
HERS – Hertwig's epithelial root sheath
HOX – homeobox (gene)
i.p. – intra-peritoneal
MSX1 – muscle segment homeobox 1
MSX2U – muscle segment homeobox 2U
PBS – phosphate-buffered saline
PCNA – proliferating cell nuclear antigen
SHH – sonic hedgehog protein
WNT – Wingless-related integration site

Keywords: tooth; anomalies; Vidaza; embryo; grafting; epigenetics; DNA-methylation

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ABSTRACT

Background and purpose: *Epigenetic mechanisms are crucial in regulating development. The aim of the study was to investigate whether a DNA-demethylation drug 5-azacytidine (5azaC) affects odontogenesis in embryonic mandibles ectopically transplanted in vivo.*

Materials and methods: *Mandibles from 13.5- and 14.5-day-old Fischer rat embryos containing early tooth-primordia (dental laminas) were transplanted under the kidney capsule of adult males. Host animals were treated with 5azaC (5mg/kg, i.p.) for the first three days and sham-controls with PBS. After two weeks, differentiation was analysed by histology and cell proliferation by immunohistochemistry.*

Results: *In some transplants, the bell stage of incisors and molars developed. Teeth in 13.5-day-old transplants produced only dentine, and the incidence of mandibles with teeth in 5azaC-treated hosts was lower. PCNA was expressed only in odontoblasts. Several 14.5-day-old transplants developed teeth with both dentine and enamel. In 5azaC-treated hosts, Hertwig's epithelial root sheath developed, but the number of mandibles with teeth was lower than in controls ($p<0.05$). Somewhat fewer molars than incisors developed under 5azaC-treatment. Differentiation of the bone, cartilage, salivary glands, epidermis, hair, sebaceous glands, and adipose cells proceeded in all transplants, except for myotubes that were absent from older transplanted mandibles.*

Conclusions: *Embryonic mandibles retained the potential for the development of teeth at the ectopic site, but odontogenesis was more advanced in a-day-older mandibles. In older mandibles, the 5-azaC impaired potential for odontogenesis, but teeth that developed reached a higher stage of organogenesis. These results are contributing to the epigenetic explanation of the development of teeth anomalies.*

INTRODUCTION

Embryonic development depends on the coherent interaction of genetic and environmental factors. To ensure organ morphogenesis, control mechanisms, such as the embryonic induction based on the interaction of different cells, regulated cell migration, precisely regulated proliferation, and programmed cell-death (apoptosis) are activated during specific phases of development *(1, 2)*.

Tooth development is a classic example of an epithelial-mesenchymal interaction of ectodermal origin. Regulation of tooth positioning from

incisors to molars is achieved through HOX-gene expression in the mesenchyme. The development of each dental lamina to the dental bud phase is regulated by the epithelium and then by mesenchyme. The signals that control dental development are growth factors Wnt, bone morphogenetic proteins (BMP), fibroblast growth factors (FGF), and sonic hedgehog protein (SHH). Transcriptional factors MSX1 and MSX2U also take part in the process of cell differentiation and modelling of each tooth. Teeth have a signalling centre that organizes dental development, precisely the enamel knot. It is formed as a limited area in the enamel epithelial layer at the top of the dental bud. At the transition from the early cap to the bell stage, the enamel knot cells degenerate by apoptosis, which is strictly regulated by BMP4 *(3-5)*. Unlike some other mammalian species, rats have only incisors and molars. Their incisors have deep roots, three layers (pulp, dentin and enamel only on the front side of the incisors) and they grow continuously *(6)*.

The environment influences proper gene expression necessary for development through epigenetic mechanisms *(7)*, among which the DNA methylation process, one of the key epigenetic mechanisms in vertebrates *(8, 9)*. DNA methylation modulates gene expression by influencing DNA transcription and changing the response of cells to external stress *(10, 11)*. A rare study on patients with impaired odontogenesis (non-syndromic anodontia and hypodontia) showed higher global methylation level and simultaneous methylation level change of multiple developmentally important gene promoters *(12)*.

Ectopic transplantation of embryos or its parts is an optimal method for investigating the remaining developmental potential for growth and differentiation of various embryonic cell types, tissues or organs, freshly isolated or pre-cultivated *in vitro (13, 14)*. In organ-transplants, the crosstalk between organ primordia and the surrounding microenvironment necessary for organogenesis is possible also because the main interactions regulating normal development are spared. Even in the transplants of early rodent embryos that develop into experimental embryonal teratoma-tumors consisting mainly of differentiated tissues in anatomically irregular spatial relations, sometimes the development of organotypic structures, like teeth or fingers with falangae were described *(15, 16)*. Such experimental tumors can develop in different extrauterine positions, such as testis *(17)*, anterior chamber of the eye *(18)*, under the kidney capsule *(19)*, subcutaneous connective tissue *(20)* or back muscles *(21)*.

Among ectopic sites, the subcapsular space of the kidney seems to be the optimal one according to the vast experience of the Zagreb School of Mammalian Embryology and other authors, because the transplanted tissue develops between the vascularised kidney capsule and the kidney parenchyma, where the vascularisation ensures proper nutrition of transplants that are stably positioned in a subcapsular pocket. Vascularization also ensures delivery of extraneous agents applied to the host for research purposes. Cultivation under the kidney capsule was performed with embryos-proper, lentectomised eye cup, neural retina *(18, 19)*, while transplantation of tooth germs *(22)*, lachrymal gland *(23)* gonads *(24)* and epiglottis *(25)* followed.

5-azacytidine (5azaC) is a DNA-demethylating epigenetic drug used for the treatment of myelodysplastic syndrome and leukemias in humans. Changing the DNA methylation, it causes teratogenic effects during mammalian development *(26, 27)* and also influences the development of organ primordia cultivated *in vitro* or transplanted to the ectopic environment under the kidney capsule *(25, 28)*.

The aim of this study was to determine the potential for tooth organogenesis under the influence of the DNA-demethylating epigenetic drug 5azaC in the fetal rat mandible transplants containing the dental lamina as the basis for tooth development.

MATERIAL AND METHODS

All procedures on animals were conducted according to the Directive 2010/63/EU and Croatian Law on protection of experimental animals with approval from the Ethics Committee of the School of Medicine, University of Zagreb.

Animals

Females of the Fisher strain rats were mated with the males in the evening. The next morning, a finding of the sperm in the vaginal smear designated the beginning of pregnancy (0.5-day-old embryos). Animals were anesthetized with 0.8 ml/kg of ketamine (Narketan®; Vétoquinol, Bern, Switzerland) and 0.6 ml/kg of xylazine (Xylapan®; Vétoquinol, Bern, Switzerland) and uteruses were removed. From embryos recovered at 13.5 and 14.5 days of gestation, mandibles were isolated by microdissection under the dissecting microscope.

Transplantation

Mandibles were transplanted under the kidney capsule of the 3-month-old male rats of the same strain. To approach the kidney, a paravertebral incision through muscle and skin was done after the rats were anesthetized with 0.8 ml/kg of ketamine (Narketan®; Vétoquinol, Bern, Switzerland) and 0.6 ml/kg of xylazine (Xylapan®; Vétoquinol, Bern, Switzerland). A small pocket was formed after the incision of the kidney capsule, and the mandible was transplanted. Michael's clumps (16 mm) were used to close the wound.

5-azacytidine (5 mg/kg; Sigma Aldrich, St. Louis, MO, USA) was applied by the i.p. injection during three consecutive days, while controls were treated with PBS. After 14 days, host animals were sacrificed with 0.8 ml/

kg of ketamine (Narketan®; Vétoquinol, Bern, Switzerland) and 0.6 ml/kg of xylazine (Xylapan®; Vétoquinol, Bern, Switzerland). The transplants were then microsurgically isolated under a dissecting microscope, using watchmaker's forceps.

Histology and immunohistochemistry

Isolated transplants were fixed in the Saint Marie solution (+4 °C, 96 % ethanol, and 1 % acetic acid added directly before fixing), rinsed, dehydrated and embedded in paraffin. Uninterrupted 5 µm serial sections were routinely prepared for light microscopy and stained with hematoxylin and eosin or Masson's trichrome staining (*29*).

For immunohistochemical analysis, the tissue was deparaffinised and rehydrated in xylene (2 x 5 minutes), absolute and 96 % ethanol (2 x 3 minutes), and H_2O (0.5 minutes). Sections were then placed in a covered jar with buffer solution (containing Tris-HCl and distilled H_2O) and heated in a microwave oven (9 minutes at 620 W and 11 minutes at 310 W). After that, the sections were cooled in the buffer solution for 40 minutes at room temperature and then in distilled H_2O for 10 minutes.

Monoclonal mouse anti-proliferating cell nuclear antigen (PCNA) (Clone PC 10, Code No. M 0879, DAKO, Agilent, USA), 1:100 was applied for 1 hour. As a negative control, the standard negative control reagent (Code No. V 1617, DAKO, Agilent, USA; 7 ml mouse IgG1 in 0.05 M Tris buffer, pH 7.6, containing carrier protein and 15mM sodium azide) was used. DAKO Animal Research kit, peroxidase (Code no. K 3954, DAKO, Agilent, USA) accordingly to the manufacturer's instructions, was used for visualization of the PCNA signal.

Statistical analysis

Statistical analysis was performed using SPSS 20.0 software (IBM SPSS Inc. Chicago, IL, USA). All measurements were taken in triplicate. The critical significance level for the statistical tests performed was set at 0.05. Results were statistically evaluated by Fisher's exact test.

RESULTS

To analyse the impact of the ectopic environment and the epigenetic influence of 5-azacytidine (5azaC) upon

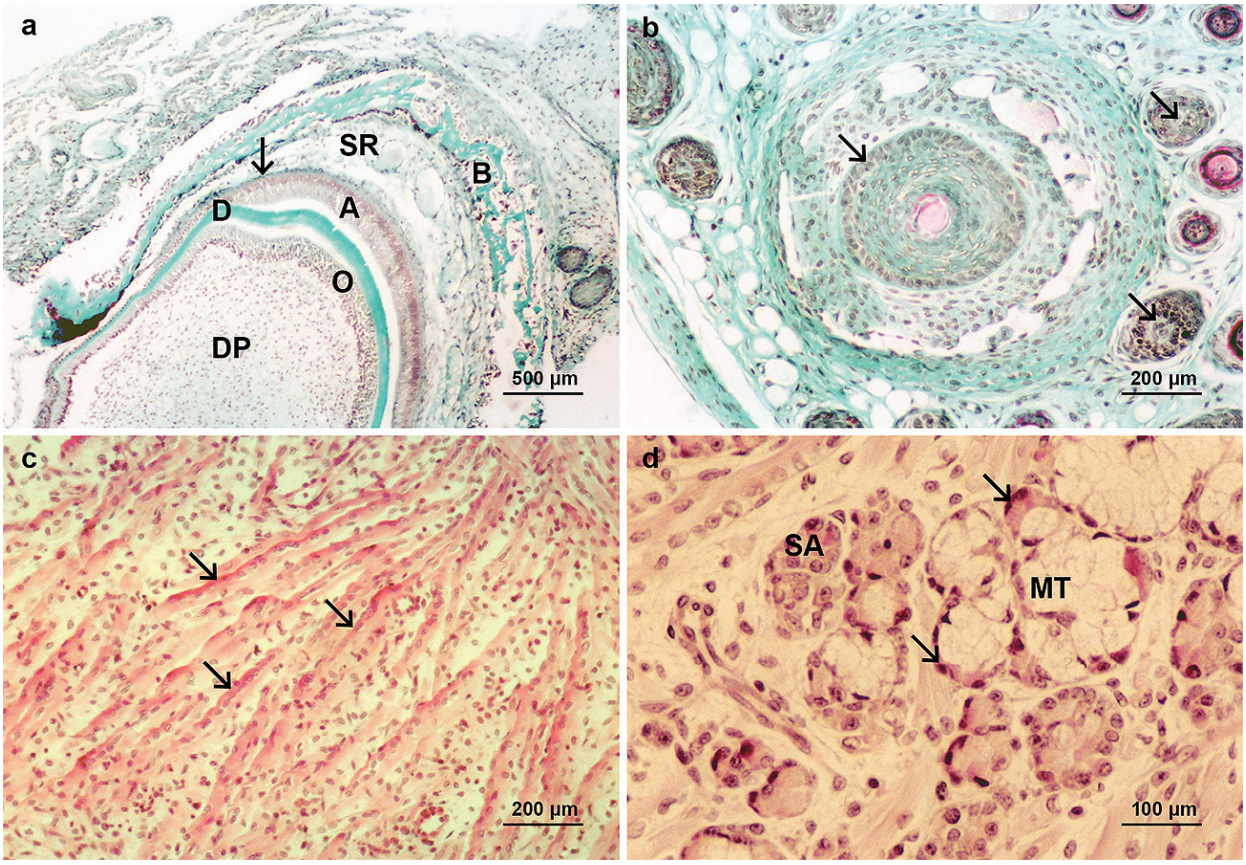

Figure 1. ***Control 13.5-day old rat embryonic mandible transplant after 14 days.*** *A- ameloblasts, D-dentin, DP-dental papilla, O-odontoblasts, SR-stellate reticulum, SA-serous acini, MT-mucous tubules, B-bone. a) incisor germ, stratum intermedium of the stellate reticulum (arrow), Masson's trichrome staining, b) well differentiated hair follicles (arrows), Masson's trichrome staining, c) myotubes (arrows), H + E, d) mixed salivary glands, serous demilunes (arrows), H + E.*

odontogenesis in embryonic mandibles, they were transplanted under the kidney capsule, and host animals were treated by PBS (controls) or by the DNA demethylating agent 5azaC. To find out whether the development was influenced stage-specifically, mandibles from two earliest consecutive stages were used.

Histological analysis of the 13.5-days old mandibular transplants

After two weeks, odontogenesis was discovered in all control transplants. Both molars and incisors (Figure 1a) differentiated to the bell stage. Odontoblasts secreted a thin layer of dentin, but ameloblasts did not secrete any enamel. Odontoblasts expressed proliferating cell nuclear antigen (PCNA) in their nuclei, while PCNA expression was absent from ameloblasts (Figure 3). Other histological structures that developed in these mandibular transplants were bone (Figure 1a), cartilage, myotubes (Figure 1c), salivary glands (Figure 1d), epidermis, hair follicles (Figure 1b), sebaceous glands and adipose cells.

All transplants, growing in animals that were treated with 5azaC, survived under the kidney capsule and were similar in size after the experimental period of 14 days. Histological analysis showed similar results as in controls. Molars and incisors differentiated to the bell stage. In the stellate reticulum, stratum intermedium was visible (Figures 2a and 2c). Again, secretion of dentine, but not of enamel, was found, and other histological structures developed such as the bone, cartilage (Figure 2d), myotubes, salivary glands (Figure 2b), epidermis, hair follicles, sebaceous glands, and adipose cells. Intranuclear expression of the PCNA was present in a few odontoblasts but was absent from ameloblasts (Figure 3).

Histological analysis of the 14.5-days old mandibular transplants.

All transplants in the control group contained molars and incisors (Table 1) that differentiated to the bell stage. In contrast to the teeth found in 13.5-days old mandibular transplants, 14.5-days old mandibular transplants produced both enamel and dentin (Figure 4), and Tome's processes were noticeable (Figure 4e). The stratum intermedium of the stellate reticulum was visible in all teeth (Figure 4). Other histological structures that developed

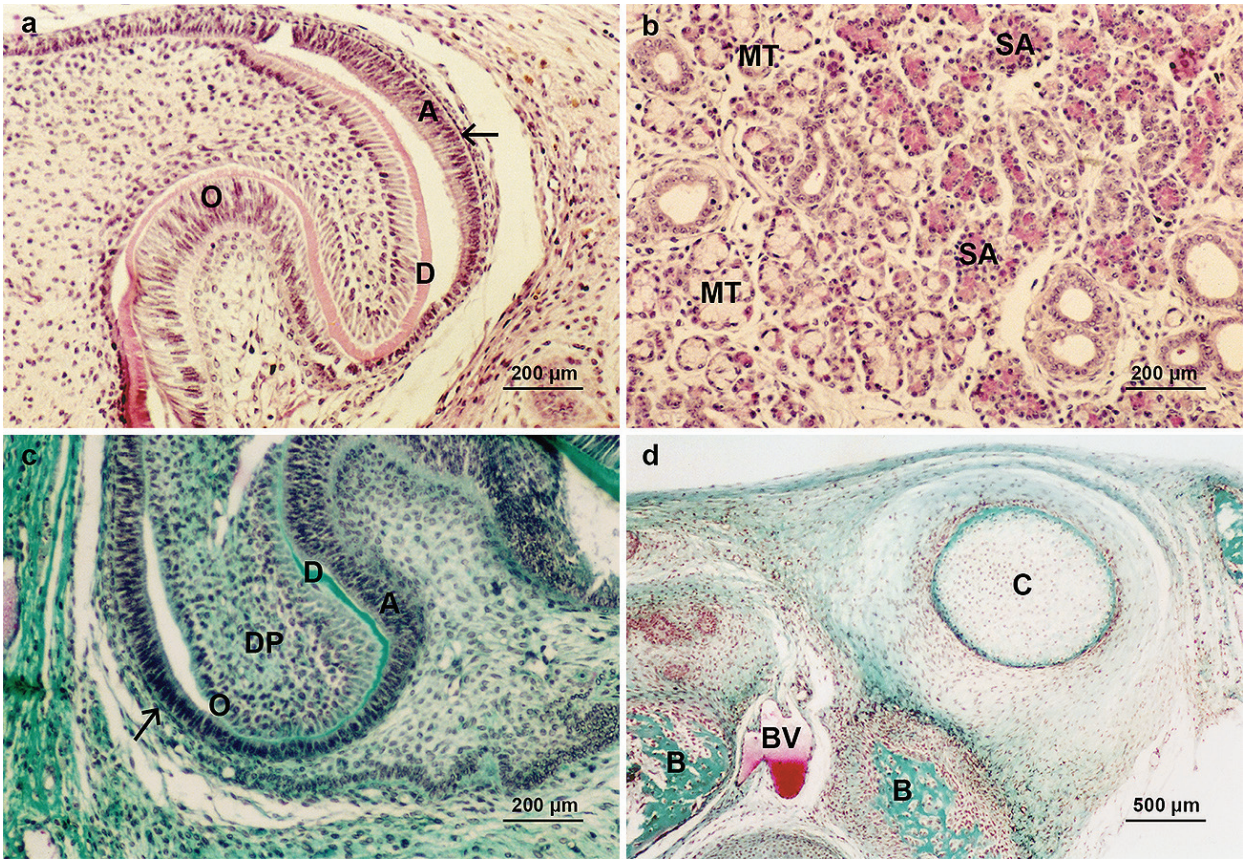

Figure 2. ***13.5-day old rat embryonic mandible transplant from adult hosts treated with 5azaC for 14 days.*** *A-ameloblasts, D-dentin, DP-dental papilla, O-odontoblasts, B-bone, BV-blood vessel, SA serous acini, MT-mucous tubules, stratum intermedium of the stellate reticulum (arrow). a) a bell stage tooth, H + E, b) mixed salivary glands, H + E, c) bell stage tooth, Masson's trichrome staining, d) embryonic mandible, Masson's trichrome staining.*

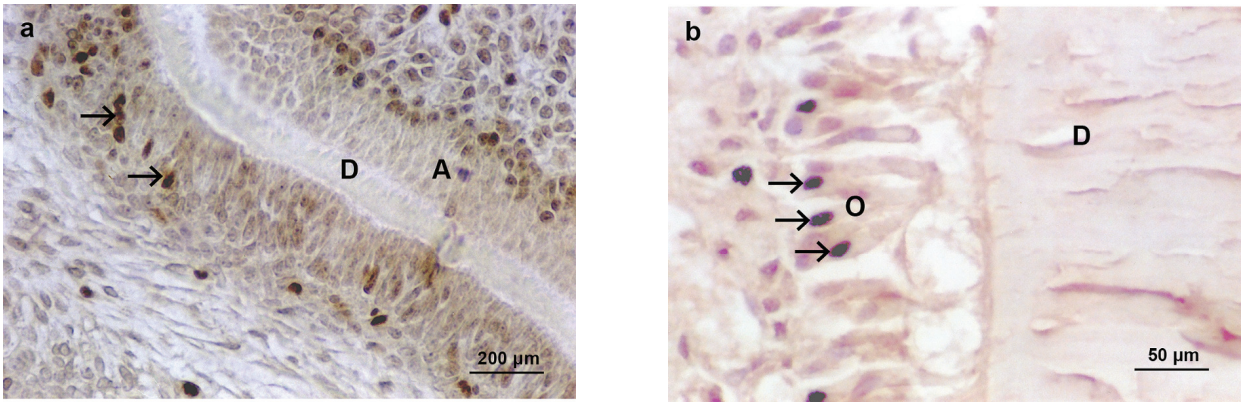

Figure 3. ***Control 13.5-day old rat embryonic mandible transplant from adult hosts after 14 days.*** *PCNA expression in odontoblasts (arrows), A-ameloblasts, O-odontoblasts, D-dentin. DAB, haematoxylin counterstain. a) treatment with 5azaC, b) control.*

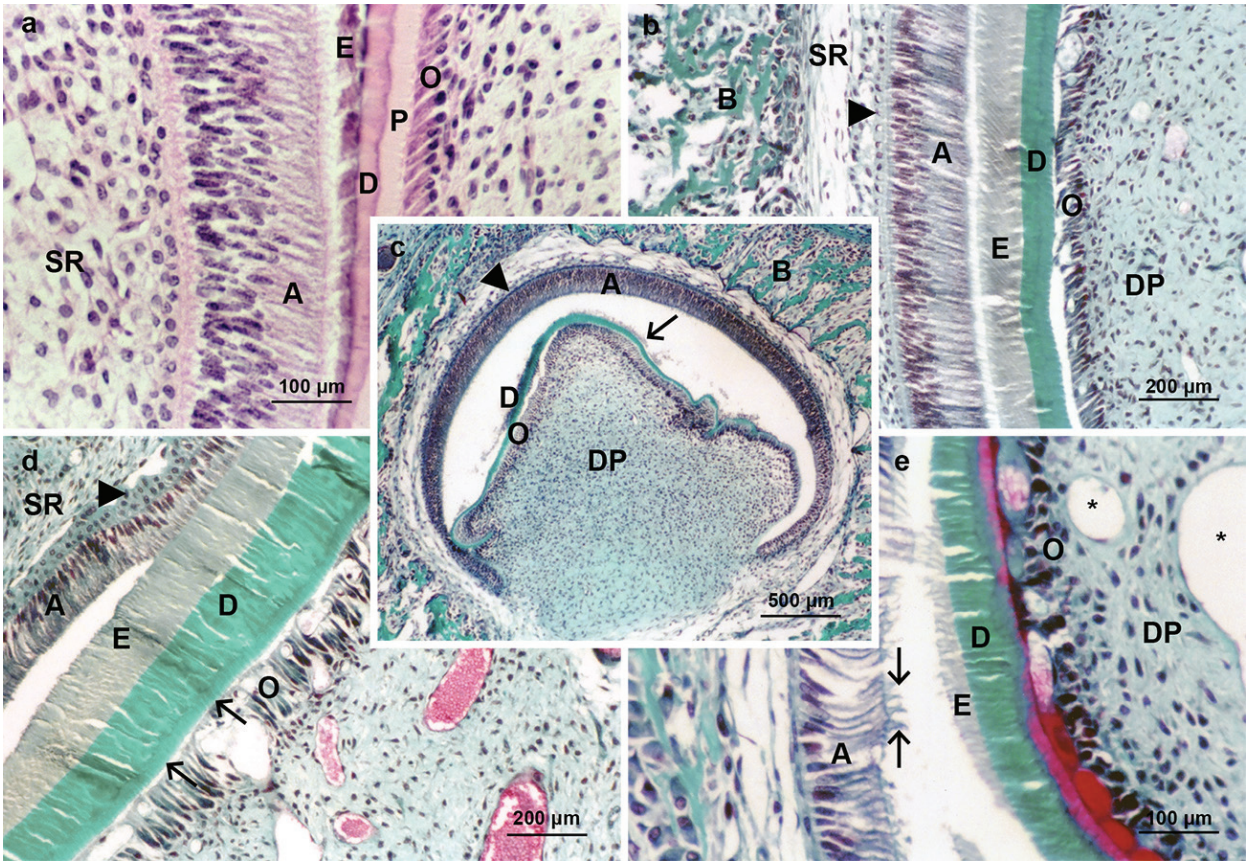

Figure 4. ***Control 14.5-day old rat embryonic mandible from adult hosts after 14 days.*** *DP-dental papilla, O-odontoblasts, A-ameloblasts, D-dentin, P-predentin, SR-stellate reticulum, B-bone, E-enamel. a) dental crown, H + E, b) dentino-enamel junction, stratum intermedium of the stellate reticulum (arrowhead) Masson's trichrome staining,* ***c)*** *tooth germ, enamel (arrow), stratum intermedium of the stellate reticulum (arrowhead), Masson's trichrome staining, d) dental crown, stratum intermedium of the stellate reticulum (arrowhead), predentin (arrows), Masson's trichrome staining,* ***e)*** *bell stage tooth, Tome's processes (arrows), blood vessels (asterisk), Masson's trichrome staining.*

in transplants were bone, cartilage, salivary glands, epidermis, hair follicles, sebaceous glands, and adipose cells.

All mandibular transplants which were treated with 5azaC survived under the kidney capsule and were similar in size after the experimental period of 14 days. Incisors and molars differentiated to the bell stage. In one of the developing teeth, only dentin was found (Figure 5a), while in all of the rest, both dentin and enamel were pro-

Table 1. *Odontogenesis in rat embryonic mandible-transplants from hosts treated with 5azaC for 3 consecutive day. Number of transplants (N) and percentage of transplants with developed teeth, molar, and incisor.*

Age	Group (N) N		Teeth		Molar		Incisor	
			%	N	%	N	%	
13.5 days	5azaC	(7)	2	28	1	14	2	28
	Control	(8)	5	71	4	50	2	25
14.5 days	5azaC	(7)	3*	42	1	14	3	32
	Control	(7)	7*	100	4	57	4	57

*p = 0.035 (Fisher's exact test)

duced (Figure 5c). The stellate reticulum clearly shows stratum intermedium (Figure 5a), but only in this group, all developing teeth showed the Hertwig's epithelial root sheath (HERS) (Figure 5d). Among other histological structures, bone (Figure 5a), cartilage (Figure 5e), salivary glands, epidermis, hair follicles (Figure 5b), sebaceous glands, and adipose cells were assessed.

Comparison of the tooth incidence between treated with 5azaC and controls

To assess the influence of 5azaC on developmental processes, we conducted a comparison of the incidence of the main histologically analysed structures between the experimental and control groups of mandibular trans-

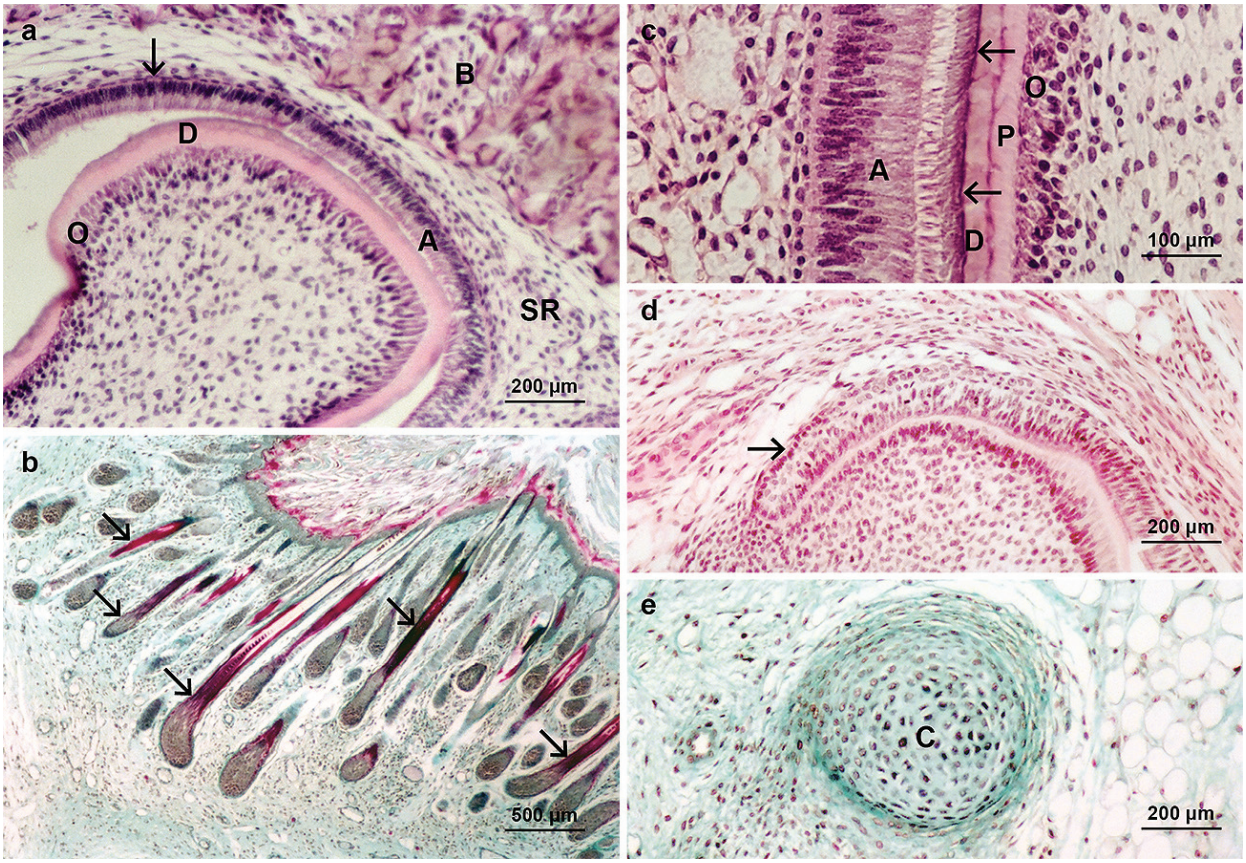

Figure 5. ***14.5-day old rat embryonic mandible from adult hosts treated with 5azaC after 14 days.*** *O-odontoblasts, D-dentin, A-ameloblasts, SR-stellate reticulum, B-bone, P-predentin, C-cartilage.* ***a)*** *bell stage tooth, stratum intermedium of the stellate reticulum (arrow), H + E, b) hair follicles (arrows), Masson's trichrome staining, c) dentino-enamel junction, enamel (arrow), H + E, d) Hertwig's epithelial root sheath (arrow), H + E, e) cartilage, Masson's trichrome staining.*

Table 2. *Differentiation of histological structures other than teeth, in 13.5- and 14.5-day-old rat embryo mandible transplants under 5azaC treatment. Number of transplants (N) and percentage of differentiated structures in transplants.*

Age	13.5 days				14.5 days			
Group (N)	5azaC (7)		Control (8)		5azaC (7)		Control (7)	
	N	%	N	%	N	%	N	%
Bone	7	100	7	87	6	85	7	100
Cartilage	5	71	6	75	3	42	4	57
Myotubes	2	28	3	37	0	0	0	0
Salivary glands	3	42	6	75	3	42	5	71
Epidermis	7	100	6	75	6	85	7	100
Hair follicles	5	71	4	50	4	57	6	85
Sebacceous glands	4	57	3	37	4	57	6	85
Adipose tissue	4	57	5	71	5	71	5	71

Note that no statistically significant difference was found.

plants. In 13.5-days old rat embryonic mandible transplants, all histological structures developed with no significant difference in the experimental and control group, although teeth were of a lower incidence in 5azaC-treated transplants (Table 1).

However, in 14.5-days old transplants, the number of mandibular transplants in which teeth developed was significantly smaller in those treated with 5azaC when compared to the controls ($p = 0{,}035$). The rest of the histological structures showed no difference in the incidence between the experimental and control group of transplants (Table 2).

DISCUSSION

Our results have shown that the DNA-demethylating drug 5-azaC changed odontogenesis in transplanted embryonic mandibles. This result is in concordance with the general importance of DNA-methylation in development *(8)* and specifically in mammalian odontogenesis *(9, 12)*. Since this DNA demethylating agent has been used in human medicine *(30)*, it is crucial to be aware of its side effects upon embryonic development.

A higher global methylation and differential methylation level of gene promoters involved in development was found in patients with anodontia and hypodontia in comparison to healthy individuals *(12)*. The results of this study have now shown that 5azaC diminished the potential for odontogenesis and enhanced differentiation of teeth in older (14.5-day-old) transplanted embryonic mandibles. Therefore, 5azaC seems to change odontogenesis in a stage-specific manner. 5azaC applied during pregnancy in the same strain of rats resulted in limb malformations of embryos treated on the 12th and 13th day of gestation, but not on the 14th day *(31)*. Therefore, individual organs and histological structures have different sensitivity to 5azaC, depending on the developmental stage. 5azaC is a DNA demethylating agent that changes spatiotemporal gene dynamics by activating gene promoters, and untimely gene expression of signalling molecules during the complicated process of odontogenesis may cause anomalies *(32, 33)*. Recently it has been shown that DNA-demethylating activity of 5azaC is also associated with enhancement of ROS/RNS in limb buds that develop into malformed limbs *(27)*. Tooth development proceeds also after birth when DNA methylation was shown to regulate odontogenesis through cell cycle inhibition *(34)*.

DNA methylation is an epigenetic mechanism that mediates between the environment and the cells and tissues of a living organism *(35)*. Some environmental factors, such as the osteogenic supplements (dexamethasone and chitosan), enhanced expression of dentin sialophosphoprotein and matrix extracellular phosphoglycoprotein for differentiation of dental pulp cells *(7)*. Although in this particular case epigenetic signatures were not investigated, epigenetic approaches to the repair of dental pulp were recently proposed *(36)*.

No impact of 5azaC on the differentiation of other histological structures, such as bone, cartilage, myotubes, salivary glands, epidermis, hair follicles, sebaceous glands, adipose cells was found in this study, so changes in epigenetic signatures seem not to play a vital role in the induction of these structures at these particular stages. Structure formation derived from mesenchyme depends upon prior interactions/cell crosstalk between the mesenchyme and maxillary or mandibular epithelia *(37)*.

Embryonic mandible transplant survival and ongoing odontogenesis showed that this model of transplantation is appropriate for studying the development of teeth. The

results confirm earlier positive findings of good survival rate and progression of differentiation in transplants of the rat embryonic epiglottis under the kidney capsule *(25, 38).*

In this study, the bell stage teeth have differentiated from the simple dental lamina structure, present in the isolated embryonic mandible *(39).* We found that the bell stage tooth germ structures were more differentiated in the 14.5-day-old mandibles than in 13.5-day-old mandibles. In 13.5-day-old mandibles proliferation marker (PCNA) was expressed only in a few odontoblasts. This suggests that most of the odontoblasts and all ameloblasts are postmitotic cells in the *in vivo* transplant, as well as is true for *in situ* odontogenesis *(40).* A postmitotic cell is a terminally differentiated one, so some odontoblasts were still undifferentiated. In 13.5 day-old transplants with only dentin production in the tooth crown, all odontoblasts seem not to have entered the terminal differentiation, thus disabling extensive enamel secretion induction in ameloblasts. Older transplants (14.5-day-old) were able to produce both dentin and enamel. Mezadri *et al. (20)* transplanted bell stage tooth germs from the newborn rats to an ectopic place, the skin pouch of the ear in an adult rat, which resulted in dentin secretion after seven days and dentin and enamel secretion after 14 days. Mezadri *et al. (20)* encompassed altogether the period of around 28-35 days from the beginning of gestation, while we encompassed 28.5-29.5 days. We can assume that the enamel production would be present even in the 13.5-day-old transplants if the *in vivo* cultivation lasted longer. Zhu *et al. (22)* showed that transplanting mice teeth already at the bell stage under the kidney capsule of adult mice leads to further development and differentiation up to 4 weeks *in vivo*, forming tooth crown, root, bone marrow, cementum-like tissue but longer transplantation period (up to 5 months) led to degeneration and forming of cyst-like lesions. Transplanting autologous tooth germs was used to substitute individual teeth as early as the seventies in the last century *(41).* To understand how the methylation alterations affect the crosstalk between the cell and surrounding ectopic microenvironment *(11)* is also important for optimization of stem-cell-based teeth engineering *(4).* In 2007 dental buds were cultivated from interacting mouse epithelial and mesenchymal cells in a three-dimensional culture system *in vitro*. Transplanting such a bioengineered dental bud to the oral cavity of an adult rat resulted in the differentiation of a functional tooth with all structural elements *(42, 43).*

CONCLUSIONS

Although odontogenesis from the earliest primordium such as the dental lamina proceeded to the bell stage of both incisors and molars in several ectopic transplants of embryonic rat mandibles, only teeth from the older 14.5-day-old embryonic mandible produced dentine and enamel in transplants, while a day younger mandible transplants exerted restriction of development by not producing enamel. DNA-demethylating drug 5azaC restricted development of teeth in some of the transplanted mandibles (significantly in older ones) and impaired incidence of molars in transplants of both stages. At the same time, 5azaC advanced morphogenesis of the tooth in older transplants in comparison to controls. Therefore, 14.5-day tooth-primordium seems to be more strictly regulated by epigenetic cues during odontogenesis. Our experimental results deepen the insight into the epigenetic causes of tooth agenesis and anomalies such as anodontia or hypodontia in mammal *(12, 44)* and may be important for tooth regeneration strategies.

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Author contributions: *VR designed the research, conducted experiments and interpreted results; MHP designed, supervised and conducted histological assessments and statistical analysis; AKB designed, supervised and conducted histological assessments and statistical analysis; MT conducted histological analysis; NS designed and conducted in vivo experiments; GJ-L conducted histological analysis and interpretation of results; DJ conducted histological analysis and interpretation of results; FB-J designed, supervised the research and interpreted the results. All authors participated in writing the article.*

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