Acta Dermatovenerol Croat 2008;16(4):183-189 MEDICAL RESEARCH

Epigenetic Deregulation through DNA Demethylation Seems Not to Interfere with the Differentiation of Epithelia from Pre-Gastrulating Rat Embryos *in vitro*

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SUMMARY One of the epigenetic mechanisms controlling differentiation during mammalian development is the process of DNA methylation. The differentiation of tissues in pre-gastrulating rat embryos cultivated *in vitro* under the influence of the demethylating agent 5-azacytidine (5azaC) was investigated. Eight-day-old Fisher rat embryos consisting of epiblast and hypoblast (primitive ectoderm and primitive endoderm) were isolated and cultivated in serum-supplemented medium by air-lifting method *in vitro*. A single dose of 5azaC (30 μM) was added to the culture medium on day 5 of cultivation. After 14 days, teratoma-like structures developed and were processed by routine histology. When compared to controls, the explants treated with 5azaC showed a statistically significant higher incidence of neuroblasts, myotubes, cartilage, and blood islands. On the other hand, the incidence of stratified squamous, columnar and glandular epithelium was not statistically different from controls. It seems that differentiation of epithelia was not sensitive to DNA demethylation caused by 5azaC like differentiation of other tissues, especially mesodermal derivatives.

KEY WORDS: epigenetics, 5-azacytidine, DNA methylation, epithelium, rat embryo, culture

INTRODUCTION

One of the epigenetic mechanisms driving the process of differentiation in the mammalian embryo is DNA methylation. It silences some regions of the genome and at the same time allows for expression of other parts of the genome without altering the DNA sequence (1). The basic mechanism is mediated by the enzyme DNA methyltrasferase (Dnmt) which attaches methyl groups to DNA and consequently modifies its activity. In other words, cytosines in CpG sequences of the DNA molecule are methylated and converted to 5-methylcytosines. The number and pattern of such methylated cytosines influence the functional state of the gene: low levels of methylation correspond to high activity, while high levels correspond to low gene activity. After DNA replication, maintenance DNA methyltransferases make sure that the methylation pattern of the parental DNA is copied to the daughter strand.

The inhibitor of DNA methylation, 5-azacytidine (5azaC), has proved to be a valuable tool to study the role played by DNA methylation in

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differentiation. It has been shown to influence differentiation in various systems (2-7). The mechanism of 5azaC action lies in its incorporation into genomic DNA, which leads to its hypomethylation (8). The molecular structure of 5azaC, with the 5th carbon atom of the cytosine ring replaced by a nitrogen atom, renders it incapable of accepting the methyl group in the enzymatic methylation reaction occurring in the newly synthesized DNA.

During development, many DNA methylation changes occur (9). Between the morula and the blastocyst stage, *de novo* methylation occurs presumably as a result of Dnmt3a and Dnmt3b expression prior to the eight-cell stage (10). *De novo* methylation is lineage-specific, so that the inner cell mass from which all adult tissues arise is highly methylated and the trophectoderm which gives rise to most extra-embryonic tissues comprising the placenta remains methylated at the same level as the morula (11). This level of methylation is consistent with that reported for hypermethylated somatic and hypomethylated extra-embryonic tissues (12). At the blastocyst stage, the bulk of the genome is hypomethylated except for the alleles of imprinted genes and some repetitive elements which remain methylated (13). After implantation, a marked *de novo* methylation is concomitant with the induction of differentiation.

Hypermethylation of genomic DNA by Dnmt3a and Dnmt3b seems to be necessary for ES cells to form teratomas in nude mice (14). Teratomas are tumors consisting of various differentiated tissues, derivatives of all three definitive germ layers (ectoderm, mesoderm and endoderm). They can also be experimentally induced by transplantation of rodent embryo-proper consisting of either two (epiblast and hypoblast) or three (embryonic ectoderm, mesoderm and endoderm) germ layers (15). Moreover, such embryos can give rise to teratoma-like structures if grown *in vitro*. They usually contain differentiated tissues such as stratified squamous, columnar and glandular epithelium, neuroblasts, myotubes, cartilage, and blood islands.

Table 1. Survival of 8-day-old embryos after two-week cultivation in serum-supplemented medium with the addition of 5-azacytidine on day 5 of culture

	No. of embryos	No. of embryos survived	Survival (%)
Controls	36	26	72.2
5azaC (30 μM)	42	32	76.2

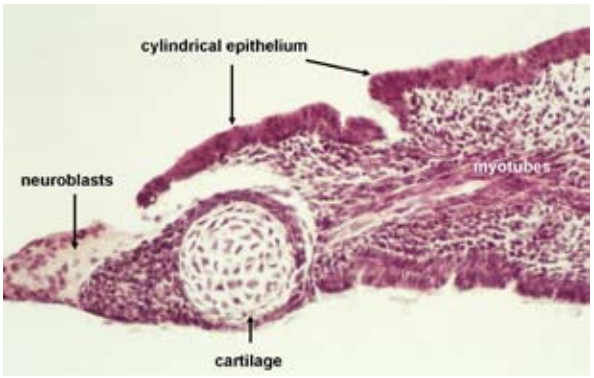

Figure 1. Control teratoma after two-week culture of 8-day-old embryo. Well differentiated cartilage, myotubes, columnar epithelium and neuroblasts. HE, x100

DNA demethylation caused by 5azaC has changed *in vitro* differentiation of experimental teratomas derived from a 9-day-old three-layered rat embryo-proper (16), but the question remains whether the response to DNA demethylation would be different in an 8-day-old two-layered rat embryo-proper considering that these embryos do not contain all three germ layers, but consist of epiblast and hypoblast only.

MATERIAL AND METHODS

Fisher rats were mated overnight and the next day vaginal plug designated the beginning of pregnancy. The rats were killed after 8 days of pregnancy and embryos were isolated from the uteri of pregnant females. Deciduomas were opened under a dissecting microscope by watchmaker's forceps and embryos extracted. They were transferred to the clean medium and Reichert's membranes were removed. The extra-embryonic part was cut off by tungsten needles and discarded.

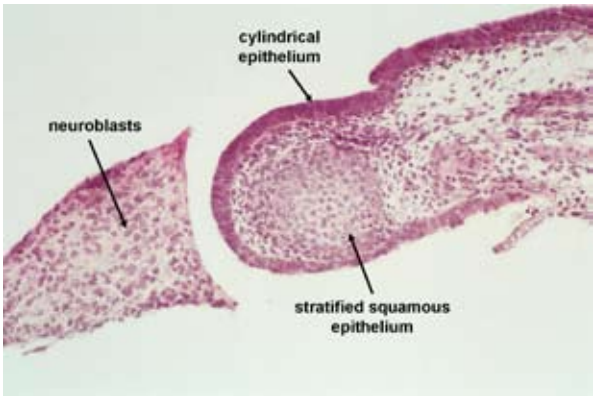

Figure 2. Control explant with differentiated stratified squamous and columnar epithelium and neuroblasts. HE, x100

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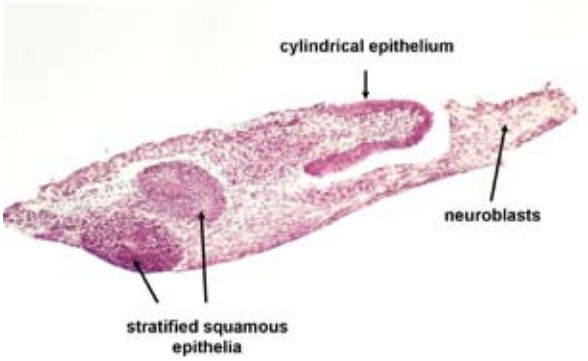

Figure 3. Eight-day-old embryo after two-week cultivation in serum-supplemented medium with 5azaC. Note stratified squamous and columnar epithelium as well as neuroblasts. HE, x40

Stainless-steel grid was covered by the lens paper and positioned in the well of the culture dish (Falcon No. 3037) filled with medium. Embryonic shields consisting of both epiblast (primitive ectoderm) and hypoblast (primitive endoderm) were transferred to the air-liquid interface by the braking pipette. Embryos were cultivated for 14 days in a humidified incubator with 5% CO_2 in the air at 37 ^{0}C. The medium used was Eagle's minimum essential medium (MEM) with Hank's balanced Salt Solution (Gibco BRL) supplemented with 50% rat serum. Rat serum was obtained from the aortal bifurcation of anesthetized male rats and immediately inactivated at 56 ^{0}C for 30 min. The medium was sterilized through a 0.22-mm Millipore filter and changed every other day during cultivation. 5azaC (Sigma A 2385-1G) was dissolved in PBS in a concentration of 30 μM and applied to the culture medium on the fifth day of cultivation. Controls were cultivated without 5azaC (Figs. 1,2,5-8).

After the period of cultivation, embryos were fixed in Zenker's solution, embedded in paraffin and processed for routine histology. Uninterrupted serial sections were stained by hematoxylin-eosin.

Figure 4. Epidermis with visible keratinization and blood island in the explant after two-week cultivation with 5azaC. HE, x200

The incidence of various differentiated tissues was established by light microscopy and compared to controls. Statistical evaluation was performed by χ^2- test and the level of statistical significance was set at $p<0.05$.

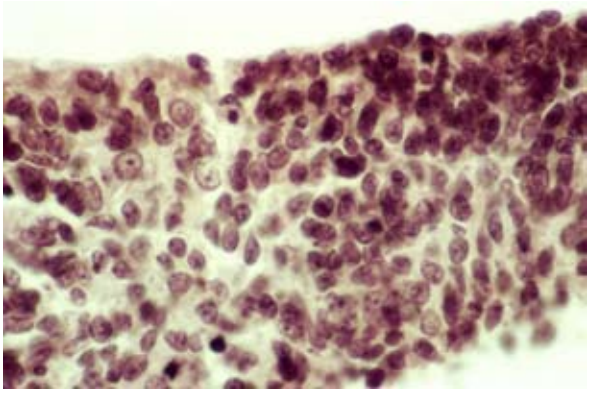

Figure 5. Clearly visible neuroblasts in control explant after two-week cultivation in serum-supplemented medium. HE, x400

RESULTS

After two-week cultivation of 8-day-old embryos in the serum-supplemented medium, approximately 70% of embryos survived in both controls and those treated with a single dose of 5azaC (Table 1). Teratoma like structures that survived the cultivation period were consisting mostly of primitive connective tissue with various differentiated tissues scattered randomly within (Figs. 1 and 2). Stratified squamous epithelia showed the highest incidence of all tissues found in explants and in only a few keratinization was observed (Fig. 4). Neural tissue was present in the form of neuroblasts (Fig. 5) and occasionally even differentiated neuroepithelium was found (Fig. 6). Columnar

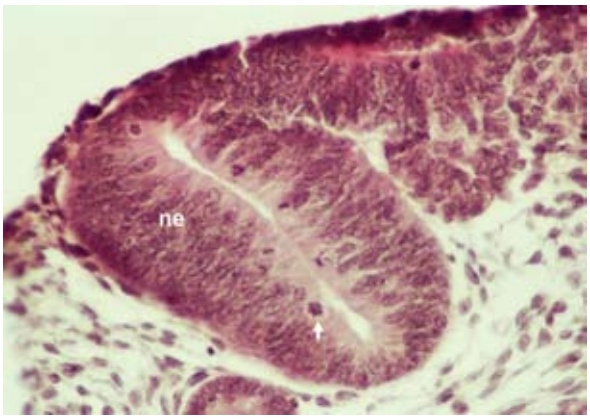

Figure 6. Impressive differentiation of neuroepithelium (ne) in control explant after two-week cultivation. Arrow points to the cell undergoing mitosis. HE, x200

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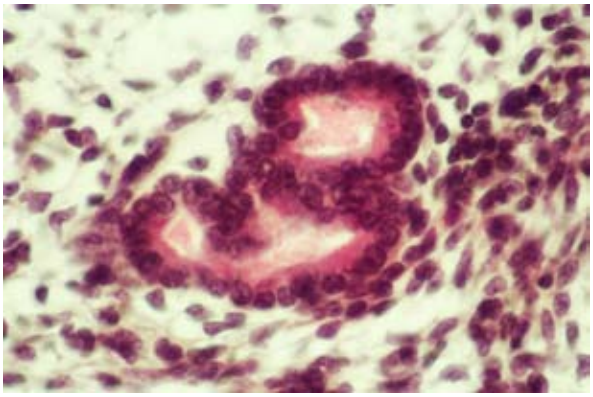

Figure 7. Glandular epithelium in control explant after two-week cultivation in serum-supplemented medium. HE, x400

and glandular epithelia (Fig. 7) were also present in a relatively high incidence, while mesodermal derivatives like myotubes, cartilage and blood islands (Figs. 8 and 9) showed lowest incidence (Table 2).

The single dose of 30 μM 5azaC added to the culture medium on the fifth day of cultivation resulted in a significantly increased incidence of neural tissue, myotubes, cartilage and blood islands. On the other hand, stratified squamous, columnar and glandular epithelia did not show statistically significant difference when compared to controls, although the columnar and glandular epithelia differentiated some less frequently, and squamous epithelia little more frequently (Table 2, Figs. 10, 11 and 12).

DISCUSSION

Cultivation of 8-day-old embryos for two weeks in serum-supplemented medium resulted in lesser survival than 9-day-old embryos cultivated in the same conditions. The survival of pregastrulating

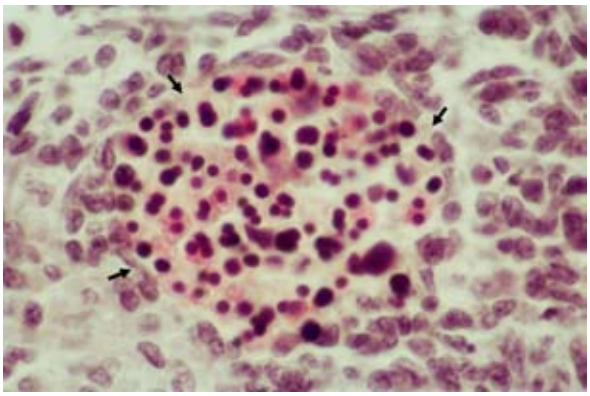

Figure 8. Arrows point to the blood island filled with blood precursor cells in control explant after two-week cultivation. HE, x400

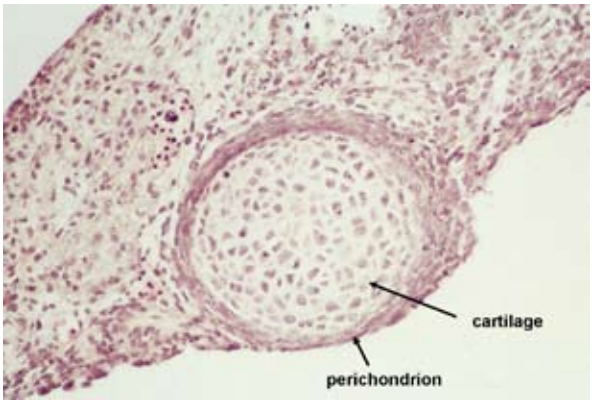

Figure 9. Cartilage with clearly visible perichondrion in 5azaC cultivated embryo after two-week cultivation. HE, x100

embryos was more like serum-free cultivation of 9-day-old gastrulating embryos (17). Although the addition of 5azaC improved differentiation of 8-day-old embryos, their survival was still poor. It is known that 5azaC is a compound with strong cytotoxic effect when used in cell cultures (18). Besides cytotoxicity, 5azaC also shows strong teratogenic effects when applied *in vivo* to pregnant rats (19).

Nevertheless, the addition of 5azaC to serum-supplemented culture medium where 8-day-old embryos were grown significantly influenced the development of those pregastrulating embryos where only two primitive germ layers (epiblast and hypoblast) are present. When compared to gastrulating embryos, which consist of three definitive germ layers, where the effect of DNA demethylation was clearly seen in the significant incidence improvement of mesodermal derivatives (16), we observed the 8-day-old rat embryos to respond in almost the same way. Although

Table 2. Frequency of various tissues in explants after two-week cultivation in serum-supplemented medium with the addition of 5-azacytidine on day 5 of culture

	Controls		5azaC		*P*
	n	%	n	%	
No. of explants	26	100	32	100	
Stratified squamous epithelium	15	58	24	75	
Neural tissue	11	42	25	78	<0.01
Myotubes	3	11.5	13	40	<0.01
Cartilage	2	8	12	33	<0.01
Blood islands	7	26.9	18	56.3	<0.02
Columnar epithelium	9	34	9	28	
Glandular epithelium	4	15.4	3	9.4	

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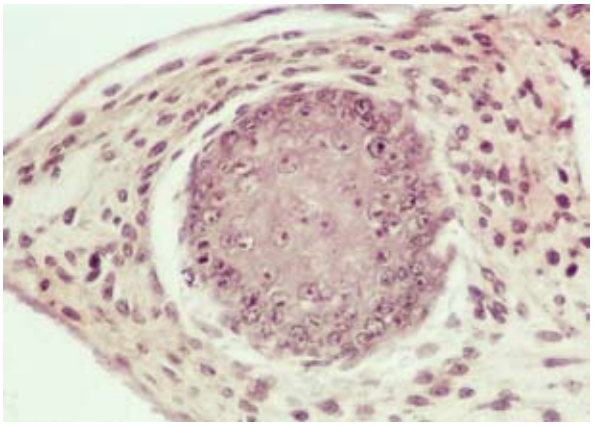

Figure 10. Stratified squamous epithelium in 5azaC cultivated embryo after two-week cultivation. HE, x200

pregastrulating embryos do not contain definitive mesoderm, it is known that the primitive ectoderm (epiblast) not yet fully differentiated at this stage of development has the potential of giving rise to all three germ layers including mesoderm (20). So, even in this early stage of development the potential of forming mesodermal derivatives is present, but it is significantly improved by 5azaC caused DNA demethylation. The processes of numerous *de novo* methylations that take place after the blastocyst stage during normal development of pregastrulating embryo are essential for tissue specific gene expression and consequently normal development (21). By disturbing this normal methylation pattern with the addition of 5azaC to the culture medium we induced the differentiation of predominantly mesodermal derivatives. Yet, the ability of 5azaC to induce differentiation is restricted to serum-supplemented embryo cultures, because if embryos are grown in a serum-free medium the muscle and the neural tissue, both sensitive to serum deprivation, are almost absent in controls and in 5azaC medium (17).

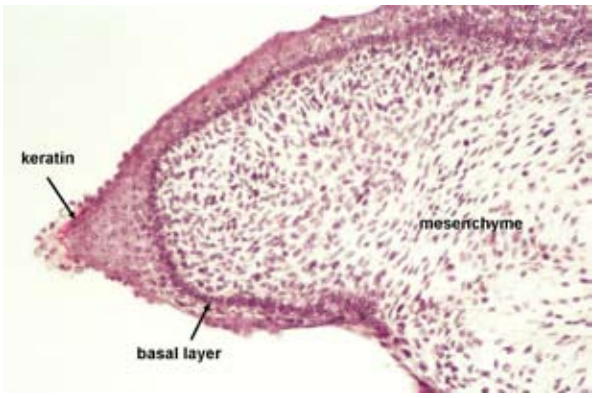

Figure 11. Keratinized stratified squamous epithelium with visible basal layer covers mesenchymal structure of the explant cultivated in 5azaC medium for two weeks. HE, x100

On the other hand, ectodermal derivatives such as squamous stratified, columnar and glandular epithelia did not respond to the addition of 5azaC followed by the process of DNA demethylation. Their differentiation was similar as in controls. It is in concordance with our previous results where 9-day-old embryos were used and the differentiation of epithelia was as good as in controls (16). Moreover, precultivation of gastrulating embryos in a simple protein-free medium and grafting *in vivo* afterwards showed that some ectodermal derivatives like skin appendages retain their developmental potential. They are still able to differentiate hair and sebaceous glands (22). It seems that even DNA demethylation caused by 5azaC did not influence epithelia in their already established developmental potential.

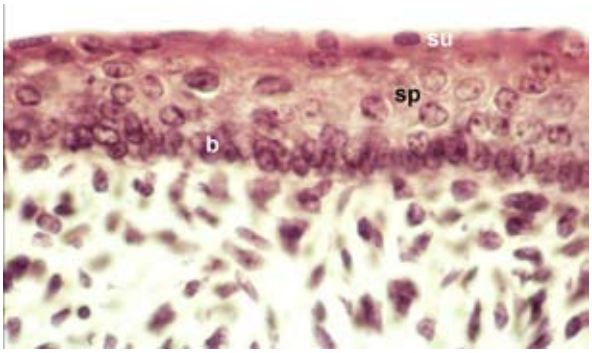

Figure 12. Stratified squamous epithelium in 5azaC cultivated embryo for two weeks. Note basal layer (b), intermediary layer – stratum spinosum (sp), and superficial layer (su). HE, x400

Although the expression of the large group of at least 43 genes called epidermal differentiation complex (EDC) is assumed to be involved in keratinocyte terminal differentiation and some of them are known to be regulated by the process of DNA methylation (23), there is evidence for a master regulatory gene of epithelial cells. When it was discovered, it turned out to be the p63 gene, a close relative to tumor-suppressor p53 gene (24). It still remained uncertain whether the primary function of p63 was in the control of differentiation of epithelia or self-renewal, or both. Surprisingly, p63 was discovered to be responsible only for maintaining the stemness of epithelia, and consequently its ability to replace the tissue, because in the p63-lacking mice normal commitment and differentiation of epithelia was established (25). Considering these new facts about gene control in

epithelial differentiation we can conclude that DNA demethylation caused by the addition of 5azaC to the developing embryos did not influence the process of epithelial differentiation.

CONCLUSION

In vitro model of studying the differentiation in pre-gastrulating embryos revealed the differentiation of mesodermal derivatives, but not the differentiation of epithelia, to be strongly influenced by changes of DNA methylation caused by the demethylating agent 5-azacytidine.

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