

REGULATION OF GROWTH AND DIFFERENTIATION IN EARLY MAMMALIAN EMBRYO BY FGF AND NGF GROWTH FACTORS IN ORGAN CULTURE *IN VITRO*

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SUMMARY – The aim was to analyze the regulation of growth and tissue differentiation in a unique *in vitro* culture model of gastrulating mammalian embryo by fibroblast growth factor (FGF) and nerve growth factor (NGF) during two weeks. They both play a crucial role during embryogenesis and the purpose of this study was to test their possible synergistic influence in the period when mammalian embryos are extremely sensitive to external factors. We cultivated 9.5-day-old rat embryos on metal grid supported lens paper, at the air-liquid interface in culture medium (Eagle's minimal essential medium (MEM) with 50% of homologous serum) with the addition of FGF, NGF and a combination of FGF/NGF in a time frame of 9 days. Other three groups of embryos were for 24 hours pretreated with 5-azacytidine (5azaC), an agent that can activate repressed genes. A parallel group of nontreated control embryos were cultivated with each experimental group. During 14 days of culture embryos grown in teratoma-like explants and growth rate were evaluated by measuring average size of explants using an eyepiece micrometer on days 5, 7, 11 and 14 after the addition of growth factors. Differences between respective groups were estimated by Student's t-test. Differentiated tissues were estimated on serial histologic sections. χ^2 -test or Fisher exact test were used to compare the proportion of tissues between respective groups. In embryo-derived teratomas NGF or FGF/NGF combination used within the 9 day time frame did not stimulate differentiation of any kind of tissues; moreover, FGF/NGF inhibited maturation of epidermis, while FGF stimulated differentiation of neural tissue, hemopoiesis and myotubes. We did not observe any kind of stimulative cooperative action of FGF and NGF in differentiation processes. So it seems that NGF hinders the stimulating effect of FGF. NGF alone impaired growth of explants, but in combination with FGF acted synergistically, thus improving the growth rate of cultivated embryos. Additional activation of genes with 5azaC had no effect on possible NGF influence on neural tissue differentiation, but resulted in improved myotube differentiation. The activation of genes with 5azaC/FGF signal and 5azaC/FGF/NGF combination improved the proportion of neural tissue and myotubes as well as hemopoiesis. Obviously, these results supported the role of FGF as neural inducer and mesoderm inducer. Anyway, FGF or NGF induced differentiation at least partially depends on the status of gene methylation.

Key words: *Embryo culture; Fibroblast growth factor; Nerve growth factor; Embryonic teratomas*

Introduction

A model of ectopic development of mammalian embryo *in vitro* devised in our laboratory¹ was developed to study

differentiation and growth of mammalian embryo during the critical period of development. The proper embryonic part of the whole gastrulating embryo cultivated *in vitro* had grown during two weeks into teratoma-like explants composed of various intermixed tissues. Therefore, this model is very successful for investigating differentiation and simultaneous growth of various tissues. During the last decade, considerable progress has been made in elucidat-

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ing the molecular basis of tissue differentiation and growth²⁻⁷. Molecular cell biology tends to look at cell function as cross talk from cell to cell in the embryo as well as in the body. Signaling molecules such as fibroblast growth factor (FGF) and nerve growth factor (NGF) take part in these processes. They are found in various parts and at various times during development of mammalian embryo^{5,8-12}. NGF is physiologically significant for survival and development of sympathetic and neural crest-derived sensory neurons¹³. Moreover, it has a wide spectrum of influence on non-neural cells^{14,15}.

FGF is a potent mitogenic agent for a wide variety of mesoderm-derived cells, including capillary and endocardial endothelial cells, myoblasts and mesothelial, glial and astroglial cells *in vitro*. FGF plays an important role in cell proliferation and differentiation in embryogenesis, tissue regeneration, CNS development, wound healing and tumor progression^{5,7,16,17}. There is some evidence that they act synergistically in neural differentiation and perhaps other tissue differentiation during embryogenesis^{5,18-20}.

We used two strategies to investigate FGF and NGF signaling in growth and differentiation of early mammalian development *in vitro*: first, we applied factors in medium for cultivation, and then treated additionally activated repressed genes with demethylating agent 5-azacytidine (5azaC) before adding growth factors.

Material and Methods

The experiments were carried out on Fischer inbred rats aged 3 to 4 months. They were kept in conventional cages, fed standard laboratory diet and water *ad libitum*. Males were used as donors of sera for culture medium supplementation.

Dissection technique used for isolation of postimplantation embryos

Fischer inbred strain rats were mated overnight. Gestation was considered to have begun early in the morning when sperm was found in vaginal smear, and it was designated as day 0.5 of pregnancy²¹. Immediately after the sacrifice of animals on gestation days, 9 embryos at the early head-fold stage (E9.5) were isolated from the uterus. The isolated uterus from pregnant females was manipulated in sterile Tyrode's saline. The decidua in which the embryo was embedded was peeled off with watchmaker's forceps. The entire egg cylinder (embryonic shield + extraembryonic part + ectoplacental cone) was isolated using a dissecting microscope. After separation of the embryo from

the uterus, the ectoplacental cone and Reichert's membrane were removed; extraembryonic part was cut at the level of the amnion and discarded. The remaining proper embryo was cultivated.

Organ culture in vitro

Cultivation of the embryonic parts of the egg cylinders was performed at the air-liquid boundary. Three embryos were put on lens paper supported by a stainless-steel grid placed in the center of an organ culture dish (Falcon No. 3037, Becton Dickinson, Oxford, UK). A certain amount of culture medium was put under the grid to keep the lens paper wet. As basal medium, Eagle's minimal essential medium (MEM) with Hank's balanced salt solution (MEM from Gibco BRL) supplemented with 50% of rat serum was used. Serum was taken from the same strain as embryos. Rat blood from aortas was immediately centrifuged and the serum was inactivated at 56 °C for 30 minutes and sterilized through a 0.22 µm Millipore filter²².

NGF or FGF (Sigma Chemical Co., St. Louis, MO, USA) or both agents together were added to basic culture medium from day 5 through 14 at a concentration of 100 ng/mL. The time of embryo exposure to NGF, FGF or FGF/NGF signals was 9 days.

In the other three experimental groups, embryos were exposed to DNA methylation inhibitor 5azaC (Sigma Chemical Co., St. Louis, MO, USA) for 24 hours before FGF, NGF or FGF/NGF treatment. 5azaC was added to culture medium at a concentration of 3 µM as a single dose from day 5 through 6, followed by the addition of FGF, NGF or FGF/NGF until the end of culture. We used this protocol because overt differentiation began on day 5 of culture²³.

Embryos were grown in an incubator in humid atmosphere containing 5% CO₂ and 95% air at 37 °C. The medium was changed every other day during the culture period. Embryos were maintained *in vitro* for 14 days.

At the same time we measured growth on days 5, 7, 11 and 14 during the culture period.

Evaluation of growth and tissue differentiation in culture in vitro

To monitor growth, dimensions of explants were measured from day 5 through day 14 of culture period, i.e. at the same time when the medium was changed, using an eyepiece micrometer. The perpendicular major and minor diameters of all explants were measured and expressed as arithmetic mean of these two values (micrometers). Thus, the growth rate of explants was evaluated by measuring av-

erage size on a particular day²³. Differences between the respective groups were estimated by Student's t-test for unlimited degrees of freedom.

After 14 days in culture, the embryos grown in teratoma-like explants were fixed in Zenker's solution, washed in tap water, and processed by routine histologic methods. Serial histologic sections (5 μ m) stained by hemalaun and eosin (HE) were made and checked for the presence of various tissues by light microscopy. We compared the proportions of differentiated tissue between the respective treated and control groups of explants. The incidence of tissues found was statistically evaluated using χ^2 -test analysis or Fisher exact test; p values of less than 0.05 were considered statistically significant.

Results

Differentiation of tissues in explants of embryos treated with FGF, NGF and FGF/NGF combination

Cultivation was performed with early head-fold stage of embryos for 14 days. We used Eagle's minimal essential medium (MEM) supplemented with 50% of serum as basic culture medium (Tables 1 and 2). The 9.5-day-old embryos at head fold stage were cultivated in the medium with serum (control, i.e. nontreated embryos) and approximately the same number of embryos were treated with NGF, FGF or both agents during 9 days of culture (Table 1).

Teratoma-like explants developed from cultivated embryos in control medium (nontreated embryos) contained tissue derivatives of all three germ layers. We found the same tissues such as epidermis, neural tissue, myotubes and smooth muscle, cartilage, etc. Epidermis was present in almost all explants (18/20), neural tissue differentiated in 65% of all cultivated explants, whereas only 30% of explants contained myotubes (Table 1). Neural cells contained typical big euchromatic nuclei with striking dark nucleoli and cells with smaller nuclei and relatively irregular nuclear envelope (glial cells).

We tried to assess the effect of FGF or NGF, or their putative synergistic action on differentiation in gastrulating embryo in the period when mammalian embryos are most sensitive to the effect of different natural substances present *in vivo*. Therefore, we treated embryos with 100 ng/mL of NGF or FGF or FGF/NGF combination during 9 days. Considering NGF, the results confirmed our previous experiments²⁴. The proportion of tissues in NGF-treated explants was approximately the same as in control nontreated explants (Table 1).

FGF treatment modified the differentiation. FGF significantly improved the rate of neural tissue differentiation ($p < 0.05$) (Fig. 1B), stimulated hemopoiesis and myotube differentiation, although the proportion of myotubes (Fig. 1A) was not significantly different from control. The same held true for combined action of FGF/NGF, although the proportion of the mentioned tissues was not

Table 1. Differentiation of various tissues in embryo-derived teratoma-like explants *in vitro* treated with FGF, NGF and FGF/NGF combination, from day 5 through 14 of culture

Treatment**	Proportion of tissues							
	Control*		FGF100		NGF 100		FGF/NGF	
No. of explants analyzed	20		13		28		14	
Differentiation:	n	%	n	%	n	%	n	%
Keratinized epidermis	18	(0.90)	8	(0.61)	26	(0.93)	7	(0.50) ^a
Immature epidermis	2	(0.10)	4	(0.30)	2	(0.07)	7	(0.50)
Neural tissue	13	(0.65)	13	(1.00) ^a	14	(0.50)	10	(0.71)
Intestinal epithelium	14	(0.70)	5	(0.38)	18	(0.64)	9	(0.64)
Gland epithelium	4	(0.20)	–	–	6	(0.21)	–	–
Cartilage	13	(0.65)	10	(0.76)	19	(0.68)	6	(0.43)
Myotubes	7	(0.35)	8	(0.61)	9	(0.32)	6	(0.43)
Smooth muscle	7	(0.35)	4	(0.30)	7	(0.25)	5	(0.35)
Heart muscle	7	(0.35)	7	(0.53)	5	(0.18)	4	(0.28)
Hemopoiesis	–	–	4	(0.30)	–	–	3	(0.21)

* Control = nontreated group grown in basic culture medium

** FGF100 = 100ng/mL FGF, NGF100 = 100 ng/mL NGF; FGF/NGF = 100 ng/mL FGF + 100 ng/mL NGF; χ^2 -test: significant difference *versus* control: a) $p < 0.05$

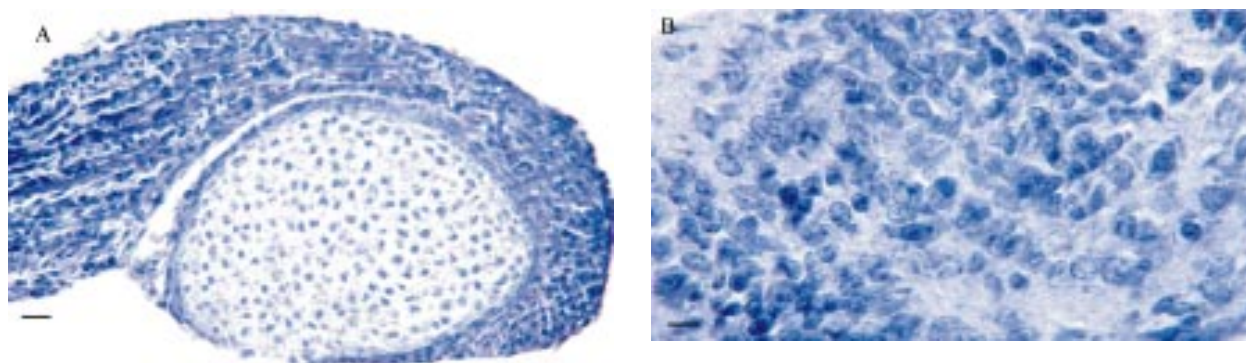


Fig. 1A. Myotubes and cartilage developed in an explant treated with FGF in concentration of 100 ng/mL from day 5 through 14 of culture. HE, scale = 50 mm. B. Detail of explant with neural tissue. HE, scale = 25 mm.

significantly different from control. Moreover, this combination prevented epidermal keratosis. The proportion of immature epidermis was significantly higher in FGF/NGF treated embryos ($p < 0.05$) (Table 1).

Differentiation of tissues in explants of embryos pre-cultivated in culture medium with 5azaC

To verify if it was possible to change the degree and pattern of tissue differentiation we treated embryos with the demethylating agent 5azaC for 24 hours before FGF or NGF or FGF/NGF treatment. 5azaC was applied from day 5 through 6 and after that embryos were exposed to particular growth factors until the end of culture period.

In explants developed from embryos treated with 5azaC/NGF differentiation of myotubes significantly improved and this was in accordance with our previous experiment²⁴. Thus, in the embryos pretreated with 5azaC, then treated with FGF on FGF/NGF, the incidence of differentiation of neural tissue, myotubes and hemopoiesis was also markedly improved (Table 2, Fig. 1).

Regulation of growth by FGF and NGF

Control explants grown in serum supplemented medium reached the maximum size in the first week of culture and then gradually decreased in the second week (Figs. 2, 3). NGF impaired growth of explants, while FGF stimulated growth, although it was not always statistically

Table 2. Differentiation of various tissues in embryo-derived teratoma-like explants in vitro pretreated with 5azaC for 24 h, followed by FGF, NGF and FGF/NGF combination, from day 6 through 14 of culture

Treatment**	Proportion of tissues in explants pretreated with 5azaC from day 5 through 6							
	Control*		FGF100		NGF 100		FGF/NGF	
No. of explants	18		14		14		14	
Differentiation:	n	%	n	%	n	%	n	%
Keratinized epidermis	17	(0.94)	9	(0.64)	13	(0.92)	10	(0.71)
Immature epidermis	1	(0.05)	5	(0.35)	1	(0.07)	4	(0.28)
Neural tissue	10	(0.55)	14	(1.00) ^a	8	(0.57)	14	(1.00) ^a
Intestinal epithelium	13	(0.72)	6	(0.42)	9	(0.64)	11	(0.78)
Gland epithelium	–		1	(0.07)	2	(0.14)	5	(0.35)
Cartilage	13	(0.72)	7	(0.50)	6	(0.42)	12	(0.85)
Myotubes	5	(0.27)	12	(0.85) ^b	11	(0.78) ^b	12	(0.85) ^b
Smooth muscle	8	(0.44)	6	(0.42)	8	(0.57)	5	(0.35)
Heart muscle	11	(0.61)	6	(0.42)	7	(0.50)	3	(0.21)
Hemopoiesis	–		4	(0.28)	–		3	(0.21)

* Control = nontreated group grown in basic culture medium

** FGF100 = 100 ng/mL FGF; NGF100 = 100 ng/mL NGF; FGF/NGF = 100 ng/mL FGF + 100 ng/mL NGF; 5azaC = 3 μ M 5-azacytidine; χ^2 -test: significant difference versus control: a) $p < 0.02$, b) $p < 0.001$

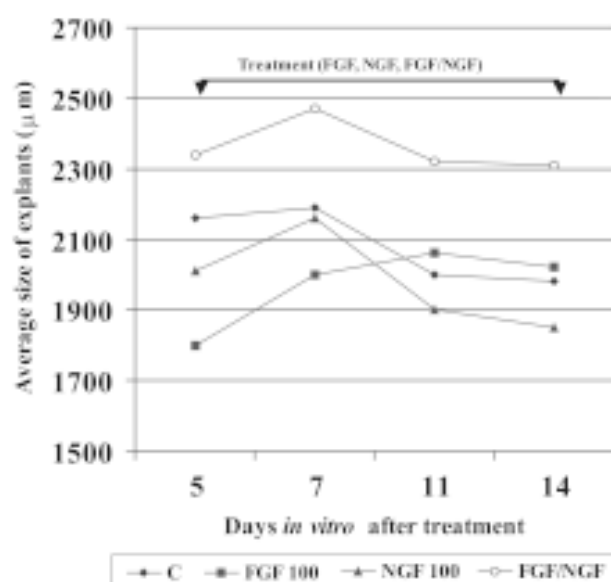


Fig. 2. Effect of FGF, NGF and FGF/NGF combination on growth of embryo-derived teratoma-like explants in organ culture *in vitro*. For the first 5 days embryos were cultivated in basic culture medium and treatments started on day 5 through 14 of culture. Control groups of explants were untreated. Each point depicts the mean of about 20 values. NGF100 = 100 ng/mL NGF; FGF 100 = 100 ng/mL FGF; FGF/NGF = 100 ng/mL FGF + 100 ng/mL NGF.

different from control groups on a particular day of measurement. During FGF treatment the average size of explants showed an increasing tendency on all measurement days. When we applied FGF and NGF together in culture media the growth of explants significantly improved in comparison to control and experimental FGF-treated or NGF-treated embryos on each measurement day (Fig. 2).

Growth rate in the groups of embryos pretreated with 5azaC was always reduced in comparison to that in non 5azaC pretreated groups of embryos and control groups. Growth curves of the groups of embryos pretreated with 5azaC on day 5 of culture followed by FGF or NGF until the end of culture gradually decreased. On the contrary, combined FGF/NGF treatment for 9 days of culture after a single dose of 5azaC improved growth (Fig. 3). In this group of embryos growth rate significantly improved in comparison to FGF- or NGF-treated embryos after 5azaC application and was similar to control growth curve, but the average size of embryos did not reach the size of those embryos which were treated without 5azaC during the experimental period (Figs. 2, 3).

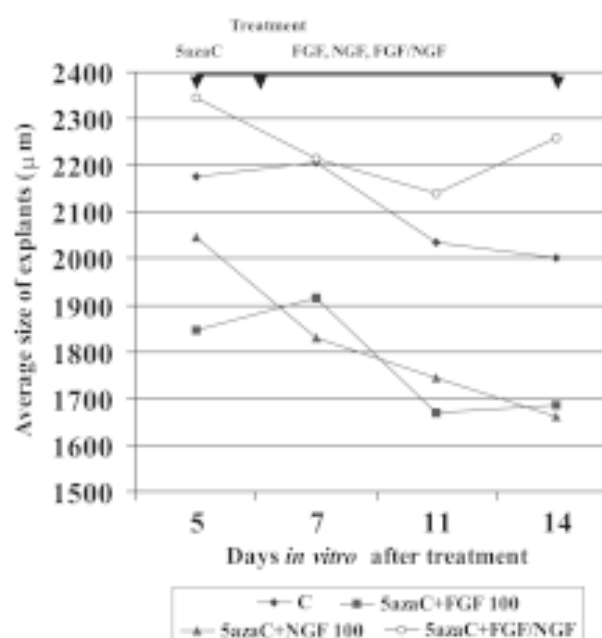


Fig. 3. Effect of FGF, NGF and FGF/NGF combination on the growth of embryo-derived teratoma-like explants in organ culture *in vitro* pretreated for 24 hours with 5azaC. For the first 5 days embryos were cultivated in basal control medium and treatments with 5azaC started on day 5 through 6, and after that with growth factors until the end of culture. Control groups of explants were untreated. NGF100 = 100 ng/mL NGF; FGF 100 = 100 ng/mL FGF; FGF/NGF = 100 ng/mL FGF + 100 ng/mL NGF; 5azaC = 3 mM 5-azacytidine.

Discussion

Embryonic parts of 9.5-day-old head-fold embryos were cultured *in vitro* for 14 days and the growth and histologic analysis of the resulting teratomas confirmed that the growth and differentiation were possible under the conditions described. Such conditions are highly desirable for precise investigation of developmental growth and differentiation-inducing agents or teratogenic factors affecting the mammalian embryo²⁵. However, for the whole 9.5-day-old embryo cultures, which are usually used in such experiments, a longer culture period is desirable²⁶ and in our experiments it lasted two weeks, although the period for the whole-embryo culture is usually limited to two days²⁶. Differentiation of the muscle tissue¹⁴, neural tissue²⁷, cartilage²⁸, etc. is often studied in cell cultures. However, there are no tissue interactions in cell cultures. Inductive interactions between embryonic cells are very important in early development, and there is no doubt that our three-

dimensional organ-culture model allows for interaction between cells in the whole embryo.

Considering growth, NGF impaired growth. In our previous experiments in serum-free and protein-free medium NGF as the only protein present, diminished growth as well⁴. Therefore, these experiments confirmed the inhibitory effect of NGF on the growth of early gastrulating embryo.

On the contrary, FGF stimulated growth although it was not always statistically different from the control group on a particular measurement day, while combined FGF/NGF treatment significantly improved growth in comparison to control and experimental FGF- or NGF-treated groups. It seems that FGF and NGF act synergistically, thus improving growth rate.

In the groups of embryos pretreated with 5azaC followed by FGF or NGF growth rate gradually reduced from day 5 till day 14 of culture. Considering 5azaC/FGF treatment, we were rather surprised with its inhibitory effect because one single dose of 5azaC in serum supplemented medium did not change growth²⁹. Therefore, we could expect similar stimulatory effect as in FGF treated embryos. The possible explanation is: all embryos were randomly chosen as control or experimental, and average masses of experimental groups of embryos were significantly lower than the control ones, at the time when the treatment started (Fig. 2), so they could not recover in 9 days. Anyway, this requires further investigation.

As the growth rate of explants pretreated with 5azaC followed by FGF/NGF combination was improved in relation to control, FGF- or NGF-5azaC-pretreated, it seems that the possible antimitotic suppressing effect of 5azaC was neutralized by combined FGF/NGF treatment for 9 days of culture (Fig. 3). In all appearance, this experiment confirmed the synergistic stimulatory action of FGF and NGF on the growth.

Although NGF could in other experimental systems induce differentiation^{5,14,27}, in our experimental system the concentration of NGF used did not affect the differentiation at all (Table 1), and simultaneously diminished growth. These results are in accordance with our previous experiments⁴.

On the contrary, FGF signal changed the differentiation of several tissues (stimulated hemopoiesis, myotubes and neural tissue). FGF, previously studied as a mesoderm inducer³⁰, has more recently been proposed as a candidate for neural induction³¹. The expression of FGF supports a possible role in neural induction and patterning³¹.

It has been found that FGF and NGF have a neural inducing activity in vertebrates at an appropriate time³²,

and other signaling factors cooperate to produce the complete pattern. Considering combined FGF/NGF treatment, the data in Table 1 show that this combination was less effective in differentiating neural tissue and myotubes than FGF alone. Moreover, combined treatment inhibited maturation of epidermis, although there was evidence for cooperative action of FGF and NGF^{5,18}.

By changing the gene methylation pattern *via* 5azaC action, more genes were activated than repressed. Therefore, additional gene activation improved differentiation of neural tissue in explants treated with FGF/NGF. This is most probably the effect of FGF signaling, because we found the same proportion of neural tissue in 5azaC/NGF treated explants in control group (Table 2). Considering myotube differentiation, we found a significantly higher proportion in all experimental series than in controls (Table 2). Results obtained with 5azaC/NGF confirm our previous experiments²⁴. In embryos treated with 5azaC/FGF and 5azaC/FGF/NGF the proportion of myotubes was the same. At the moment it is difficult to say if there was any cooperative interaction between FGF and NGF.

Conclusion

In our experimental system, NGF or FGF/NGF combination used within a time frame of 9 days did not stimulate differentiation of any kind of tissues in embryo-derived teratomas, while FGF stimulated differentiation of neural tissue, hemopoiesis and myotubes. We did not observe any kind of stimulating cooperative action of FGF and NGF in differentiation processes. Moreover, FGF/NGF combined action inhibited the maturation of epidermis. So, it seems that NGF hinders the stimulating effect of FGF. In spite of the fact that NGF alone impaired growth of explants, it seems that NGF acts synergistically with FGF, thus improving the growth rate of cultivated embryos.

Gene activation by hypomethylation action of 5azaC had no effect on the possible NGF action on neural tissue differentiation, but it resulted in improved myotube differentiation. The activation of genes with 5azaC/FGF signal resulted in changes. Improvement of the proportion of neural tissue and myotubes as well as of hemopoiesis confirmed its role as a neural inducer and mesoderm inducer. Anyway, FGF or NGF induced differentiation was at least partially dependent on the status of gene methylation.

This study offered a new approach in gaining a more precise insight into the differentiation inducing agents in early postimplantation embryos cultivated *in vitro*.

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Sažetak

REGULACIJA RASTA I DIFERENCIJACIJE EMBRIJA SISAVACA FAKTORIMA RASTA FGF I NGF U KULTURI ORGANA *IN VITRO*

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Namjera je bila istražiti regulaciju rasta i diferencijacije pomoću fibroblastnog faktora rasta (FGF) i faktora rasta živaca (NGF) u jedinstvenom *in vitro* modelu za kultiviranje embrija sisavaca u kritičnoj fazi razvoja, gastrulaciji, tijekom dva tjedna. Oba faktora imaju ključnu ulogu u embriogenezi sisavaca, a svrha je bila istražiti njihov mogući sinergistični učinak kada su embriji sisavaca najosjetljiviji na djelovanje pojedinih vanjskih čimbenika. Embrije štakora stare 9,5 dana kultivirali smo na metalnoj mrežici s lećnim papirićem, na granici plinske i tekuće faze, u temeljnom mediju za kultiviranje (Eagleovom minimalnom esencijalnom mediju (MEM) s 50% homolognog seruma) uz dodatak faktora rasta FGF, NGF i kombinacije FGF/NGF tijekom 9 dana kulture. Druge tri skupine embrija su prethodno bile tretirane 24 sata 5-azacitidinom (5azaC), agensom koji može aktivirati gene. Paralelne skupine embrija kultivirane su u temeljnom mediju kao kontrolne. Tijekom 14 dana kultiviranja embriji izrastu u teratoidne strukture, a pritom se rast procjenjuje određivanjem prosječne veličine eksplantata pomoću okularnog mikrometra u određene dane (5, 7, 11, 14) nakon dodavanja faktora rasta. Razlike u rastu između pojedinih skupina ispitane su Studentovim t-testom. Prisutnost diferenciranih tkiva u teratomima utvrđena je analizom na serijskim histološkim rezovima. Razlike u proporcijama diferenciranih tkiva između pojedinih skupina utvrđene su χ^2 -testom ili Fisherovim egzaktnim testom. U embrijskim teratomima razvijenim *in vitro* niti NGF niti FGF/NGF kombinirani tretman nisu stimulirali diferencijaciju bilo koje vrste tkiva, dapače, FGF/NGF kombinacija je inhibirala sazrijevanje epidermisa. FGF je stimulirao diferencijaciju živčanog tkiva ($p < 0,05$), miotuba i hemopoezu. Nismo mogli utvrditi sinergistični učinak FGF i NGF u procesu diferencijacije bilo koje vrste tkiva. Dapače, čini se da NGF smanjuje stimulacijski učinak FGF u procesu diferencijacije. NGF suzbija rast eksplantata, ali u kombinaciji s FGF djeluje sinergistično, značajno povećavajući rast eksplantata. NGF nije imao učinka na očekivano povećanje diferencijacije živčanog tkiva, nakon dodatne aktivacije gena pomoću 5azaC, međutim, diferencijacija miotuba je značajno povećana. Kombinirano djelovanje 5azaC/FGF i 5azaC/FGF/NGF na aktivnost gena povećalo je učestalost diferencijacije živčanog tkiva, miotuba i hemopoezu u eksplantatima. Ovaj rezultat potvrđuje djelovanje FGF kao induktora živčanog tkiva i mezoderma. U svakom slučaju diferencijacija potaknuta FGF i NGF signalima djelomice ovisi o stupnju metilacije gena.

Ključne riječi: *Kultura embrija sisavaca; Fibroblastni faktor rasta; Faktor rasta živaca, Embrijski teratomi*