Coll. Antropol. **28** (2004) 2: 681–688
UDC 57.089.3:57.017.6
Original scientific paper

Developmental Potential of Mouse Embryos without Extraembryonic Membranes in Modified Organ Culture

Nataša Stević[1], Tatjana Belovari[1], Ljiljana Kostović-Knežević[2] and Srećko Gajović[2]

[1] Department of Histology and Embryology, School of Medicine, University »J. J. Strossmayer«, Osijek, Croatia,

[2] Croatian Institute for Brain Research, School of Medicine, University of Zagreb, Zagreb, Croatia

A B S T R A C T

The long term stationary culture of postimplanatation embryos without extraembryonic membranes is a method to assess their developmental potential in vitro. The method was almost exclusively used on rat embryos, while mouse embryos were considered unsuitable due to their poor differentiation. In present study the postimplantation mouse embryos were used to verify potential of this method in mice. In addition, the course of in vitro differentiation was compared to embryo development in situ. Embryos were cultivated for maximum of 14 days and morphology and differentiation was analysed on serial semithin sections. Although anatomical relationships were lost from the beginning of the cultivation, the differentiation was only delayed, and the developmental potential after long term culture was comparable to those observed in rats. Therefore the advantages of long term cultivation could be utilized to analyse the differentiation of numerous lines of genetically modified mice with impaired postimplantation development.

Key words: *embryo, development, in vitro, in situ*

Introduction

The mammalian embryo develops in uterus, which enables plenty of nutrients and superior protection, but hinders the experimental investigation of its development. Various *in vitro* culture methods, which mimic closely the *in situ* (i.e. *in ute-*

Received for publication November 1, 2004

ro) environment, are available to study mammalian embryo development and differentiation. *In vitro* experimental systems are used for determination of gene expression and function in normal embryonic development, investigation of growth factors, teratogenic agents or effects of biologically active substances and drugs[1–10].

Embryos can be cultivated with or without extraembryonic membranes. Whole-embryo culture methods allow mouse embryos to develop outside the uterus for 2–3 days and during that period gastrulation and early organogenesis are achieved at the similar rate as *in situ*[6,7,11–13]. This model contributes substantially to understanding of developmental mechanisms and clinical problems[7,14–17]. Its major disadvantage is the relatively short period for which the embryos can be cultivated, as some tissues and organs require more time to obtain full differentiation[18]. Culture of embryos without extraembryonic membranes enables investigation of effects of short term and long term exposure to biologically active substances since embryos can be cultivated for two weeks. This model suits better for the study of early stages of development[3,19,20]. During gastrulation stage, which is critical for lineage specification and tissue patterning[21], differentiating cells are extremely sensitive to various endogenous as well as exogenous signals. This *in vitro* system enables the exploration of direct impact of substances added to the medium on embryo cells, as they are not any more protected by removed extraembryonic membranes[22]. Rat egg-cylinder during two weeks of cultivation *in vitro* develops in teratoma-like structure consisting of well-differentiated tissues, derivatives of all three germ layers[23]. Various tissues and organ development, for example, teeth development[24,25], development of retinal pigment epithelium[26,27], inner ear epithelium[28] or taste papillae development[29] can also be studied in this experimental system.

The long term studies of *in vitro* differentiation of postimplantation embryos were done mostly in rats. There are few possible reasons for that: relatively small mouse embryonic shields difficult for manipulations, observed differences in stimulation of a specific kind of tissues by different sort of serum used for cultivation *in vitro*, poor differentiation[30], and development in teratocarcinomas *in vivo*, which is not the case when rat embryos were used[31]. In order to verify potential of this method in mice, in this study postimplantation CD1 mouse embryos (Theiler's stage 11; E 7.5)[32,33] without extraembryonic parts were cultivated *in vitro* to determine sequence of appearance of various tissues and the time needed for their differentiation. Morphological development of embryos was studied by comparing appearance of various tissues and differentiation *in vitro* with that of embryos, which developed at an equal period of time *in situ*. The mouse embryo differentiation *in vitro* was comparable to those previously observed in rat, confirming that this method is suitable for analysis of developmental potential of mouse postimplantation embryos.

Materials and Methods

Three months old outbred CD1 mouse embryos, which were kept at the Croatian Institute for Brain Research, School of Medicine, University of Zagreb, were used.

The late-gastrula stage embryos (Theiler's stage 11) (E 7.5) were isolated, the Reichert's membrane was removed and extraembryonic parts cut off at the level of the amnion. Embryos were placed in disposable dishes for organ culture (Falcon No. 3037) on a lens paper supported by a stainless steel grid. The Dulbecco's Modified Eagle Medium (DMEM) (Life

Technologies, Rockville, SAD) with 20% Foetal Bovine Serum, FBS (Life Technologies, Rockville, SAD) were added to the dish in amount needed to wet the lens paper. Embryos were cultivated in an incubator in 5% CO_2 and 95% air at 37 °C for maximum of 14 days. The culture medium was changed every second day.

After 1, 2, 3, 4, 7, 10, 12 and 14 days of culture, explants were taken for histology. Explants were fixed in 1% paraformaldehyde i 1% glutaraldehyde for 2h, then postfixed in 1% OsO_4 for 1h and embedded in Durcopan (Sigma, Deisenhofen, Germany). Serial semithin sections (1mm) were cut with ultramicrotome (Reichert-Jung UltracutE), stained with 1% toluidine and analysed by light microscope.

Mouse embryos developed *in situ* were isolated on different stages of development (E8.5, E9.5, E10.5, E11.5). They were processed by routine histological methods, embedded in paraffin and serial sections (6mm) stained with hemalaun and eosin were histologically evaluated.

Results

In situ development

At E8.5 stage of development egg cylinder was cup shaped, with cranial and caudal fold. In mediosagital line neural groove was well visible. Surface ectoderm, neural groove and neural folds were made from pseudostratified neuroepithelium, notochord, optic pit, otic placode, mesenchyme tissue, somites, heart primordium with its mesocardium, liver primordium and primitive gut can be recognized. At E9.5 (Figure 1) craniocaudal folding of embryos was observed. The major divisions of the brain were apparent from the surface. The brain vesicles begun to separate: the forebrain (prosencephalon) – divided into the telencephalon and the diencephalon, the midbrain (mesencephalon) and the hindbrain (rhombencephalon). Neural tube was in process of closure cranially. Lens vesicles in concavity of optic cups, otic vesicles, notochord, dorsal root ganglion and somites were observed. Dorsal aorta and cardinal veins, primordia of lungs and liver were developed. The gut was divided into foregut, midgut and hindgut. Forelimb and hindlimb buds were formed. At E10.5 thyroid primordium, trachea and lung bud, oesophageal region of foregut and gastric dilatation of foregut, urogenital ridges, mesonephric tubule and mesonephric duct were differentiated. At E11.5 pigmentation in optic vesicles, frontal and nasal processes and Rathke's pouch appeared. In heart primordium left and right atrial and ventricular chamber of heart were separated.

In vitro development

During culture *in vitro* (for 1, 2, 3, 4, 7, 10, 12 and 14 days) embryos were developed into experimental embryonic teratomas, made of different tissues.

After explantation, the structure of embryos was disturbed and in the first two days poor differentiation of embryos

Fig. 1. Mouse embryo at E9.5 (lateral view), brain vesicles (*), heart region (→), forelimb and hindlimb buds (▶) (×15).

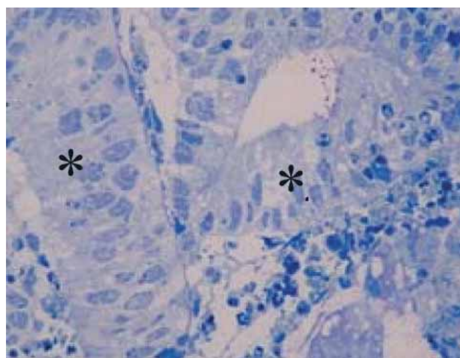

Fig. 2. Neuroepithelium (*) in explant cultivated for 3 days (×400).

was observed. After 1 day *in vitro*, explants contained few cavities, which were lined with three layers of cells like germ layers. Near the lumen there was a thin simple epithelium resembling the endoderm, then in the middle agglomeration of cells corresponding to the mesoderm and finally densly arranged cells within cuboidal epithelium resembling ectoderm. After 2 days *in vitro*, there were no recognizable anatomical structures but the mesenchyme surrounded by the epithelia. After 3 days *in vitro* neuroepithelium (Figure 2), surface cuboidal epithelium and columnar epithelium were present in the explants. The columnar epithelium formed tubular structure corresponding to the primitive gut. From day 4 till day 7 the neuroepithelium with noticeable basal lamina formed small cysts in the mesenchyme. Mesenchymal cells developed further, they were loosely arranged with cytoplasmic processes like fibroblasts. Surface epithelium became stratified. From day 10 till day 12 neuroblasts, stratified squamous epithelium and embryonic blood vessels appeared. Mesenchyme was differentiated into connective tissue, composed of cells with slender and dense nuclei and more or less extracellular matrix. After 12 days of culture striated border was observed on apical surface of simple columnar epithelium resembling epithelium of small intestine. After 14 days of culture cartilage, myotubes and pseudostratified columnar epithelium with ciliae were observed. During long term culture (14 days) derivatives of all three germ layers differentiated well: neuroblasts, stratified squamous epithelium, cartilage with perichondrium, myotubes, vessels,

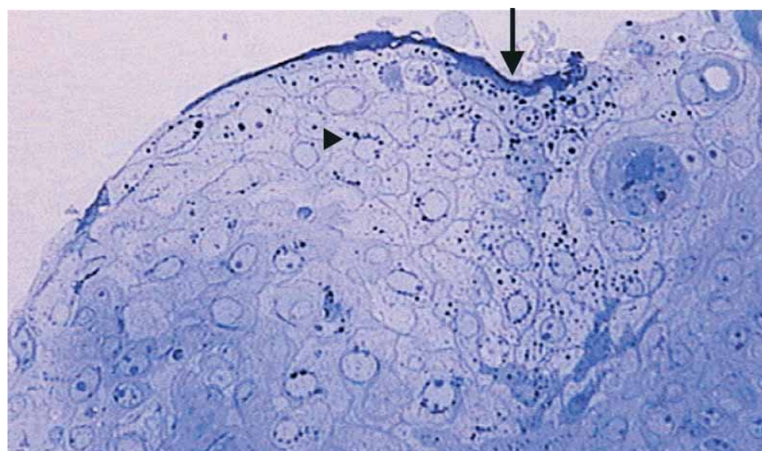

Fig. 3. Stratified squamous epithelium in explant cultivated for 14 days. Stratum corneum (→), keratohyaline granules (▶) (×400).

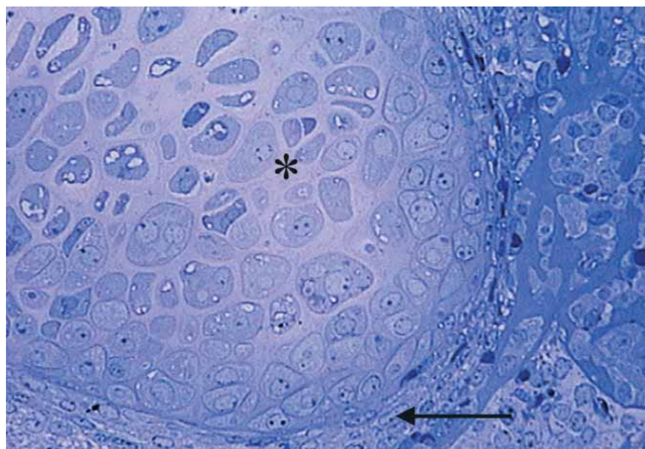

Fig. 4. Cartilage (*) with perichondrium (→) in explant cultivated for 14 days (×400).

simple columnar and pseudostratified columnar epithelium with ciliae. Nonkeratinized and keratinized stratified squamous epithelium (Figure 3) resembling the epidermis differentiated. Islands of cartilage consisting of chondocytes in lacunas scattered throughout the cartilage matrix and enclosed with the perichondrium (Figure 4). Cylindrical structures with nucleoli in line, myotubes, were rare. Although pulsations in explants were well visible during medium changing, cardiac muscle was not found on histological sections. Vessels were lined with simple squamous epithelium corresponding endothelium. Pseudostratified columnar epithelium with basal laminae and ciliae (Figure 5) resembling respiratory epithelium was abundant.

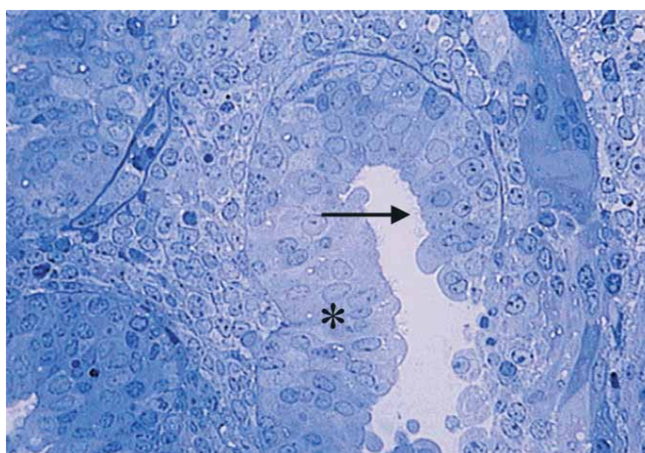

Fig. 5. Pseudostratified columnar epithelium (*) with ciliae (→) in explant cultivated for 14 days (×400).

Discussion

When compared with corresponding mouse embryos developed in situ, the *in vitro* explants, which were cultivated without extraembryonic membranes, lost from the beginning the anatomical relationships of the normal development. Nevertheless the differentiation was not impaired and, as the culture advanced, more and more complex tissues and their derivatives were observed.

Ectodermal derivatives, epidermis and neuroblasts, differentiated well and with high incidence in this experimental system, when medium was supplemented with serum[30]. After 3 and 4 days simple cuboidal epithelium, and after 7 days incompletely differentiated stratified epithelium at the surface of explants were present. The time needed for complete differentiation of nonkeratinized and keratinized stratified squamous epithelium was 12 days. This was in agreement with investigation carried out on rat embryos, in which epidermis developed after 14 days[34,35]. Neuroectoderm, as another ectodermal derivative, was differentiated in form of pseudostratified epithelium from day 3 till day 7. Comparing to *in situ*, *in vitro* development of the cysts corresponding to the neural tube was delayed for two days, compared to E8.5 embryos with closed neural tube. Neuroblasts were observed for the first time in explants after 12 days *in vitro*. For recognition of incompletely differentiated neuroblasts on earlier developmental stages (before 12 days), molecular markers, as for example nestin, should be used[36].

Mesodermal derivatives reached high degree of differentiation after 12–14 days *in vitro*. At that time connective tissue was differentiated in all explants. At the beginning of the cultivation the explants were composed exclusively from epithelial sheaths similar to the germ layers. Mesenchyme was firstly observed after 2 days in vitro and its appearance changed with the period of cultivation. As mesenchyme became morphologically differentiated, cells synthesized ground substance first and mature fibres later. Other mesodermal derivatives like vessels, myotubes and cartilage were observed from day 12 till day 14. Myotubes were spare in this study like in similar experiments with rat embryos. It was shown that myotubes need the addition of specific substances in cultivation medium for development[37]. This study confirmed that cartilage differentiated well after 14 days *in vitro*, which was the case even in cultivation in serum-free media[38]. Ossification processes *in vitro* require addition of some specific substances to the medium, for example retinoic acid, insulin-like growth factor-1, transforming growth factor-ß1, ascorbic acid or ß-glycerolphosphate[39–42]. Adipose tissue need addition of insulin, transferrin, fibroblast growth factor, rat submandibular gland extract or hydrocortisone[43,44].

In this study tube-like structures corresponding to the primitive gut lined with simple columnar epithelium were differentiated after 3 days, which was 2 days later than *in situ*. In order to get complete set of morphological features recognizable by light microscopy, for intestinal epithelium 12 days, and for respiratory 14 days *in vitro* were needed. Intestinal and respiratory epithelium in previous experiments with gastrulating rat embryos were also well differentiated[35]. Events in endoderm organogenesis are well described, but the initial formation and early specification of the endoderm is poorly understood. Several growth factors promote the survival and growth of early endoderm in culture, but did not induce further endodermal differentiation. Direct cell contacts between germ layers promote differentiation of endoderm[45], because endoderm receives instructions from adjacent germ layers and starts new gene expression[46].

For terminal differentiation of rat gastrulating embryos addition of 20–60% rat serum was required[30]. In this study 20% foetal bovine serum (FBS) was used, because in recent studies mouse embryos with extraembryonic parts, organ primordia, cell lines and embryonic stem cells were cultivated in medium containing 10–20% FBS[24,28,47–53]. However, in vitro developmental potential could substantially vary, depending on the medium used for cultivation and duration[35,54]. The amount and the quality of the serum can be one of the main causes for previously reported poor differentiation of mouse explants in *in vitro* culture[30].

Since neuroepithelium and primitive gut were observed two days later than *in situ*, it can be concluded that differentiation *in vitro* was delayed for two days in this experimental system. During *in vitro* cultivation derivatives of all three germ layers developed gradually. Despite the initial delay and *in vitro* conditions, which certainly do not match the surrounding of uterus, after 14 days all main tissue types were present and well differentiated according to morphological criteria. This developmental potential was fully comparable with those of rat embryos. All the tissues present in the rat tissues (neuroblasts, cartilage, epidermis, myotubes, gut and respiratory epithelium) were present in the mouse, and those absent (bone and adipose tissue) did not differentiate in mouse as well. Therefore we conclude that the long term modified organ culture of mouse egg-cylinder is a suitable method for analysing mouse embryos. This opens wide areas for application, from which we emphasize the possibility for prolonged in vitro analysis of embryos from numerous lines of genetically modified mice showing embryo lethality around gastrulation period.

Acknowledgements

This work was supported by the Croatian Ministry of Science and Technology (grant 108117).

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N. Stević

Department of Histology and Embryology, School of Medicine, University »J. J. Strossmayer«, J. Huttlera 4, 31000 Osijek, Croatia,

RAZVOJNA SPOSOBNOST MIŠJIH ZAMETAKA BEZ IZVANEMBRIONALNIH OVOJNICA U MODIFICIRANOJ KULTURI ORGANA

S A Ž E T A K

Dugotrajna kultura postimplantacijskih mišjih zametaka bez izvanembrionalnih ovojnica upotrijebljena je kao metoda za procjenjivanje razvojnih sposobnosti *in vitro*. Metoda je gotovo isključivo upotrijebljavana za štakorske zametke, dok su mišji zameci smatrani neprikladnima zbog njihove slabe diferencijacije. U ovom istraživanju postimplantacijski mišji zameci kultivirani su *in vitro* kako bi se odredile mogućnosti ove metode na mišjem modelu. Osim toga, tijek *in vitro* diferencijacije je uspoređen s embrionalnim razvojem *in situ*. Zameci su kultivirani najviše 14 dana, a morfologija i diferencijacija su analizirane na serijskim polutankim rezovima. Iako su anatomski odnosi izgubljeni od samog početka kultiviranja, diferencijacija je bila samo odgođena i razvojna sposobnost je nakon dugotrajne kulture bila usporediva s onom uočenom kod štakora. Stoga se prednosti dugotrajnog kultiviranja mogu primjeniti za proučavanje diferencijacije različitih linija genetski modificiranih miševa s poremećenim postimplantacijskim razvojem.