CELL TECHNOLOGIES IN BIOLOGY AND MEDICINE

Stem Cells Giving Rise to Extraembryonic Tissues

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The review is devoted to characterization of stem cells involved in the formation of extraembryonic tissues during the early development of mammalian embryos. Here we present our results of characterization of stem cells from the trophoblast and extraembryonic endoderm of voles and comparative analysis of these cells and the corresponding mouse cells and discuss possible signal pathways maintaining these cells in undifferentiated state.

Key Words: *trophoblast stem cells; alternative signal pathway; extraembryonic endodermal cells; vole; mouse*

CHARACTERISTICS OF EMBRYONIC STEM CELLS

Embryonic stem cells (SC) are indispensable model for studies of the mechanisms of maintenance of undifferentiated state *in vitro* and *in vivo* and directed differentiation of mammalian cells during the early ontogeny. In contrast to differentiated cells, they have some peculiarities allowing their use in fundamental studies and in clinical and experimental medicine. First, embryonic SC form a self-maintaining population, *i.e.* they can be maintained in an undifferentiated state after many passages in culture [60]. Second, under appropriate conditions they can differentiate into certain cell types, which is very important for replacement cell therapy [40,56,69].

There are several types of SC differing by their origin and capacity to give rise to various differentiated derivatives. Three types of SC can be obtained from pre-implantation blastocysts cultured under certain conditions. These SC are a unique model for the study of early ontogeny in mammals. They differ by the methods of isolation, culturing, contribution into chimeric embryos, and expression of specific molecular markers. Different signal pathways are realized in these cells for the maintenance of their undifferentiated state. These SC types are embryonic SC (ESC) presented by epiblast cells, extraembryonic endoderm cells (XEN-cells) derived from the hypoblast, and trophoblast SC (TSC) derived from trophoectoderm (TE, later extraembryonic ectoderm, Fig. 1). Mice are the only mammals for which all three types of SC of embryonic origin were isolated and analyzed in detail.

ESC are pluripotent cells capable of differentiating into all three germ layers and can form all types of animal cells *in vivo* [2,63,72]. Due to this pluripotency of ESC they can give rise to different organs and tissues, which gives hopes for recovery in patients with degenerative diseases, including autoimmune ones. However, there are ethical and legislative problems for application of human blastocysts. Moreover, patient-specific ESC cannot be obtained. In light of this, the search for alternative ways of obtaining human ESC

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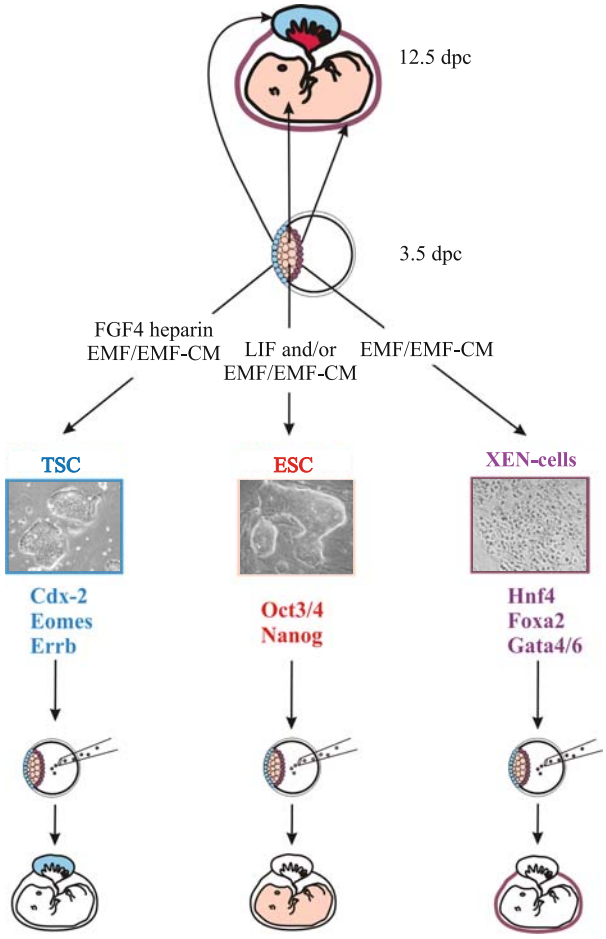

Fig. 1. Scheme of SC origination from mouse blastocyst. TSC: derivatives of polar blastocyst, form compact epithelial colonies in the presence of FGF4, heparin, and embryonic mouse fibroblasts (EMF) or conditioned media (EMF-CM). ESC are derived from epiblast; round cells form homogenous multilayer colonies in the presence of cytokine LIF and/or EMF (EMF-CM). XEN-cells originate from hypoblast, have round or stellate morphology, and grow in the presence of embryonic fibroblasts of media conditioned by embryonic fibroblasts. Here and in Fig. 2: dpc: day post-coitum.

for solving the problem of histoincompatibility is now in progress. One of them is reprogramming of somatic nuclei via their transfer into enucleated oocytes [12]. Another and more promising method of obtaining patient-specific cells consists in creation of induced pluripotent SC (IPSC) from patient's terminally differentiated somatic cells. This method is based on reprogramming of the somatic nucleus via transduction with viral vectors carrying the genes encoding transcription factors, in particular, factors maintaining pluripotency: *Oct4*, *Sox2*, *c-Myc, Klf4*, *Nanog,* and *Lin28* [45,74,79,83]. In later studies, IPSC were obtained by using adenoviral vectors not incorporating into the host genome [55,71] and carrying various chemical compounds improving the efficiency of reprogramming and/or replacing some transcription factors [43] and IPSC with lesser number of transduced factors [32,78].

TSC and XEN-cells are other important types of SC, which can be obtained from mammalian blastocysts. These cells are multipotent because they have limited development potential and differentiate only into extraembryonic cells [26,29,36,75], which allows them to be used as model objects in the studies of early development of mammalian extraembryonic tissues. Let us analyze these two types of SC.

TSC

TSC are isolated from extraembryonic ectoderm of preimplantation blastocysts. It is known that cells of extraembryonic ectoderm participate in embryo implantation into the uterine wall mucosa forming the fetal part of the placenta; disturbances in the formation or functions of this structure lead to fetal death. TSC are of particular importance because they can be used as an *in vitro* model in the studies of implantation and placentation processes and interaction between the embryonic and extraembryonic cells in early embryos.

For understanding of the importance of trophoblast cells in the early development of mammals let us review their role in implantation and formation of the placenta in mice *in vivo*.

IMPLANTATION AND FORMATION OF MOUSE PLACENTA

Implantation of the blastocyst into the uterine wall mucosa and formation and normal functioning of the placenta depend on four types of cells, which also can be derived from TSC: spongiotrophoblast cells, trophoblast giant cells, syncytiotrophoblast structure, and glycogen-producing trophoblast cells [65]. Implantation of the embryo starts on day 4.5 of embryonic development and is first provided by trophoblast giant cells [3,34]. They are formed from mural TE not contacting with the inner cell mass (ICM) after transition from the mitotic to the endoreduplication cell cycle. Trophoblast giant cells invade the maternal spiral arteries carrying blood to the site of implantation [6]. Polar TE directly contacting with ICM retains proliferative capacities and differentiates into extraembryonic ectoderm and ectoplacental cone consisting of proliferating diploid cells.

Further development of extraembryonic ectoderm cells leads to the formation of chorionic epithelium. Chorionic trophoblast cells differentiate into different types, *e.g.* into multinuclear cells of the syncytiotrophoblast, a product of cell fusion. Syncytial trophoblast invades maternal capillaries and destroys them and the contacts with endothelial cells. Maternal blood fills the forming lacunas [3,65]. The surface of syncytiotrophoblast cells directly contacts with endothelial cells of embryonic blood vessels and plays the major role in the transport of nutrients and gas exchange between the maternal and embryonic blood executed by the placenta [6].

Ectoplacental cone gives rise to a layer of spongiotrophoblast cells supporting the structure of placenta labyrinth, a precursor of glycogen-producing cells of the trophoblast. Placental labyrinth is a densely packed structure consisting of intensively branching villi of the trophoblast associated with embryonic blood vessels. Direct exchange between the maternal and embryonic blood takes place in placental labyrinth [65]. The function of glycogen-producing cells of the trophoblast is still unknown; they appear at late terms of pregnancy, first within the spongiotrophoblast layer and then penetrate into myometrium interstitium [6].

Two independent groups of researchers showed that in mice the placenta also plays a role of an independent organ of hemopoiesis on days 10.5-11 of embryonic development [22,58]. Despite 10-fold decrease in hemopoietic activity as soon as by day 15.5 of embryonic development, the placenta is believed to play an important role in the development of adult hemopoietic system.

TSC ISOLATION

During embryogenesis, all subtypes of the invasive trophoblast arise from the common precursor located in polar TE (or extraembryonic ectoderm) of the blastocyst and in the chorion of postimplantation mouse embryos [65]. The maintenance of diploid proliferating cell population of the extraembryonic ectoderm *in vivo* depends on signals from ICM and then from epiblast; in the absence of these signals the cells differentiate into giant cells or other terminally differentiated placental cell types [20,66]. The population of TSC can be obtained by *in vitro* culturing of early embryos; these cells remain in undifferentiated state for infinite time.

TSC were first isolated from preimplantation mouse blastocysts [75] and later TC-like cells of voles *Microtus rossiaemeridionalis* were obtained [23]. Despite the differences in the composition of culture medium during isolation and culturing, these cells are similar by many parameters (morphology, spectrum of differentiated derivatives, gene expression, and tumorforming capacity).

Mouse and vole TSC were isolated from preimplantation embryos (3.5 day post-coitum) and cultured using mouse embryonic fibroblast feeder or medium conditioned by fibroblasts (Fig. 2) [23,75]. This stage of embryonic development was chosen because it corresponds to the first division of equipotential blastomers of the morula leading to the appearance of ICM and TE [1], the latter being the TSC precursor. Then, polar TE directly contacting with ICM retains its proliferative capacities and differentiates into extraembryonic ectoderm; culturing of extraembryonic ectoderm yields mouse TSC on 6.5 day post-coitum [75].

In mice, the undifferentiated state of extraembryonic ectoderm cells is maintained via fibroblast growth factor FGF4 [50,51,61] affecting the trophoblast via binding with heparin followed by activation of *Fgfr2* [9,68]. Therefore, for maintaining the cells in undifferentiated state we added exogenous FGF4 and heparin to the culture medium (Fig. 2). In contrast, TS-like cells of vole were obtained in the absence of FGF4 and heparin in the culture medium [23]. In spite of this, vole TS-like cells proliferated without differentiation and expressed markers of undifferentiated TSC. A hypothesis was put forward on activation of alternative FGF-independent signal pathway [23]. Moreover, generation of rat TS-like cells in the absence of FGF4 and heparin was re-

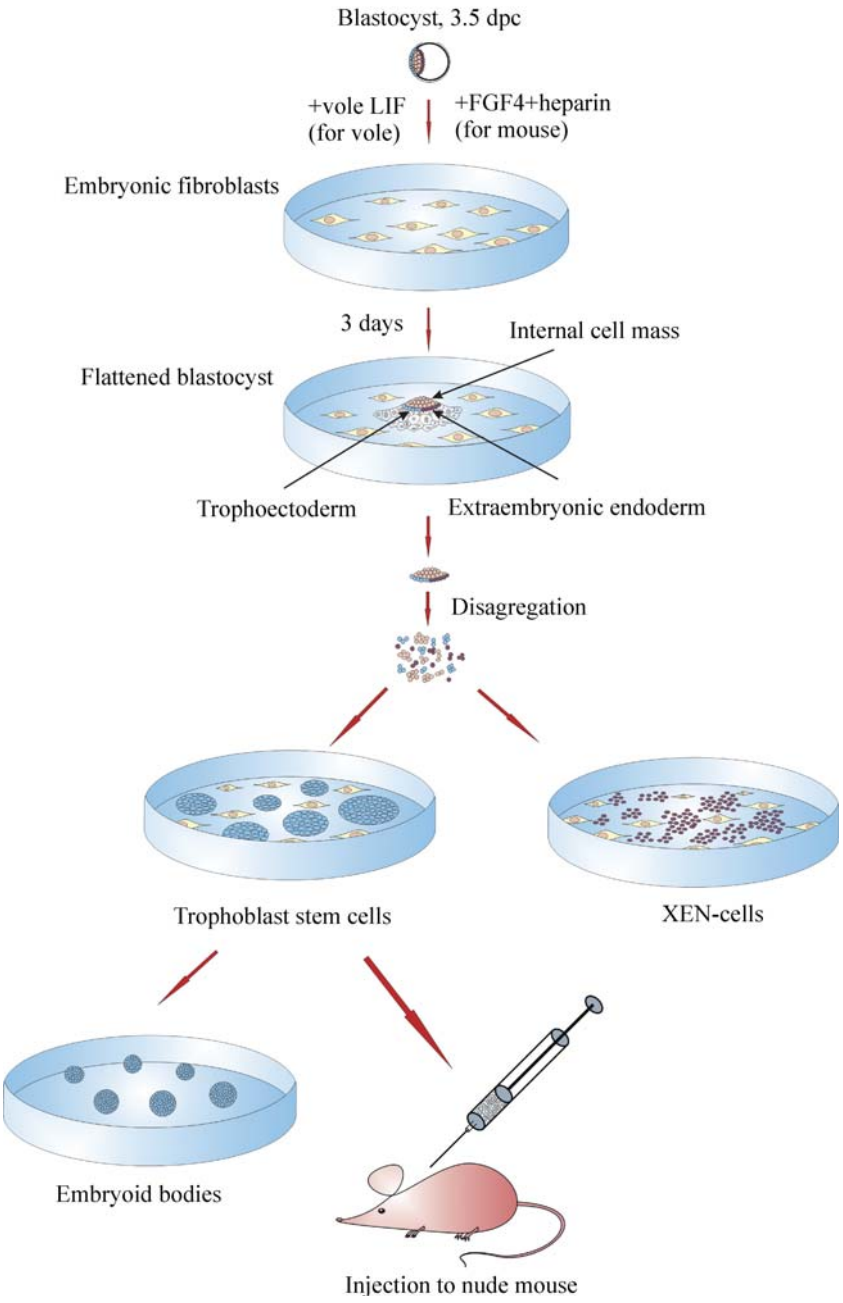

Fig. 2. Experiment for obtaining and analysis of extraembryonic SC (TS- and XEN-cells) from mouse and vole preimplantation blastocysts.

cently reported [13], which confirmed the assumption on alternative signal pathway maintaining TSC in the undifferentiated state.

TSC CHARACTERIZATION

Morphology of mouse TSC [75] is similar to that of TS-like colonies of vole cells (Fig. 3). TSC form multilayer epithelium-like colonies differing from multilayer colonies of mouse ESC [62]. The cells in these colonies are characterized by high nucleus/cytoplasm ratio and tight cell-cell contacts. Proliferating TSC of mice and voles do not express *Oct4* and *Nanog* genes maintaining undifferentiated state of pluripotent ESC (Fig. 3) [11,33,41,48,50]. Long-term proliferation of TSC is provided by genes *mEomes* and *Cdx-2* responsible for the TE-type development and typical of extraembryonic ectoderm and *Errβ* gene maintaining undifferentiated state and proliferation of trophoblast cells [75].

Multipotency of mouse TSC, *i.e.* restricted developmental potential, was demonstrated in experiments

for generation of mouse chimeras by cell injection into preimplantation blastocysts. The injected cells in chimeras were detected only in the placenta, but never participated in the formation of the embryo (Fig. 1) [17,28,75].

Another method of evaluation of the spectrum of differentiated SC descendants is subcutaneous injection of the cell suspension to immunodeficient mice followed by the analysis of developed tumors. The formation of tumor-like cysts was observed after injection of mouse TSC [31], Jeg-3 human choriocarcinoma cells [24], and vole TS-like cells [23] to immunodeficient mice (Fig. 2). These tumors represented blood-filled capsules. Histological analysis showed that the central part of the tumor induced by injection of vole TS-like cells was presented by a hematoma and a necrotic zone resulting from proliferation and differentiation of TS-like cells invading the recipient tissues and destroying blood vessels. The layer adjacent to the necrotic zone (Fig. 3, *f*) apart from the bulk of intensively differentiating cells (Fig. 3, *f, g*) contained some giant, primarily mononuclear, cells at different stages of differentiation (Fig. 3, *g*, *h*). The tumors also contained structures resembling differentiated ectoplacental cone elements and chorial plate (Fig. 3, *g*). Incubation of capsula tissues in the culture medium yields cell colonies phenotypically identical to the studied cell strains (Fig. 3, *i*), which confirms the presence of undifferentiated intensively proliferated cells in the capsule walls.

The mechanism of cyst formation is similar to the process of trophoblast invasion into the uterus during *in vivo* implantation, when giant cells expressing trophoblast giant cell markers *Plfr* and *Pl-1* penetrate into maternal spiral arteries; due to these processes, complex structure of the placenta is formed in parallel with trophoblast differentiation [6]. Thus, isolation of TSC and evaluation of their properties and contribution into implantation processes in combination with methods of targeted transduction of placenta-specific genes into the defective placenta [54] can be useful in the treatment of the pathologies of placenta organogenesis and functioning.

Some interesting properties of mouse TSC were discovered. In differentiated state these cells are characterized by enhanced adhesion and phagocytic activity similar to those of trophoblast cells *in vivo* [3,6]. Vole TS-like cells exhibit similar properties. This can be explained by vital biological role of trophoblast cells during implantation.

For more complete characterization of TSC, expression of genes typical undifferentiated and differentiated cells and signal pathways involved in the maintenance of undifferentiated status of these SC should be analyzed.

TRANSCRIPTION FACTORS AND SIGNAL PATHWAYS TYPICAL OF UNDIFFERENTIATED TSC

Genetic studies on mice allowed identification of the majority of transcription factors and genes controlling TSC proliferation. FGF4/FGFR2 signal pathway including FGF4, its receptor FGFR2 [8], and transcription factors CDX-2 and EOMES [65,75] is the main pathway for preparation and maintenance of mouse TSC in undifferentiated state and for interaction between trophoblast cells and ICM.

In vertebrates, the FGF family includes more than 20 genes, but most attention is focused on the role of *Fgf4* gene in the stimulation of trophoblast development. It is well known that FGF4 is a component of embryonic signal pathway essential for the maintenance of multipotent state of trophoblast lineage cells. The product of *Fgf4* gene is detected at the very early stage, in single-cell mouse embryo. During subsequent blastocyst development, mRNA is accumulated in ICM cells and then is detected in epiblast cells at the early preimplantation stage [61] and in 6- and 8.5-day embryos, but no peptide is present in TE. FGF4 does not directly affect the development of ICM, which is confirmed by successful isolation of ESC from blastocysts of mice with both FGF-4 alleles inactivated [80]. However, the embryos of $Fgf4^{-/-}$ mice die *in vivo*, which is most likely the result of disturbances at later stages of trophoblast development and implantation and placentation processes.

FGF4 factor acts via binding and activation of surface receptor FGFR2 expressed on trophoblast cells. In the blastocyst and at early postimplantation stages, *Fgfr2* gene is intensively expressed in diploid cells of the trophoblast and then in cells of extraembryonic ectoderm, which attests to its role as a specific receptor of TSC. These findings are confirmed by the result of the experiment, where mutant mice lacking *Fgfr2* and *Fgf4* genes died before implantation [8], which was probably caused by disturbances in trophoblast cell proliferation [61]. When analyzing the results of the experiment on reciprocal expression of *Fgf4* and *Fgfr2*, the researchers hypothesized that trophoblast can be a target tissue for FGF signals [65].

Analysis of the early development of the trophoblast led them to an assumption on the existence of genes specifically expressed in the trophoblast that are essential for its development and are the key targets for FGF signal [65]. In early embryos, TE develops in the absence of *Oct4* expression in outer cells [59]. However, the start of TE differentiation precedes *Oct4* down-regulation in the outer cells of the blastocyst, which attests to the existence of a positively acting factor stimulating TE development and serving as a target for FGF signal, *Cdx-2* gene.

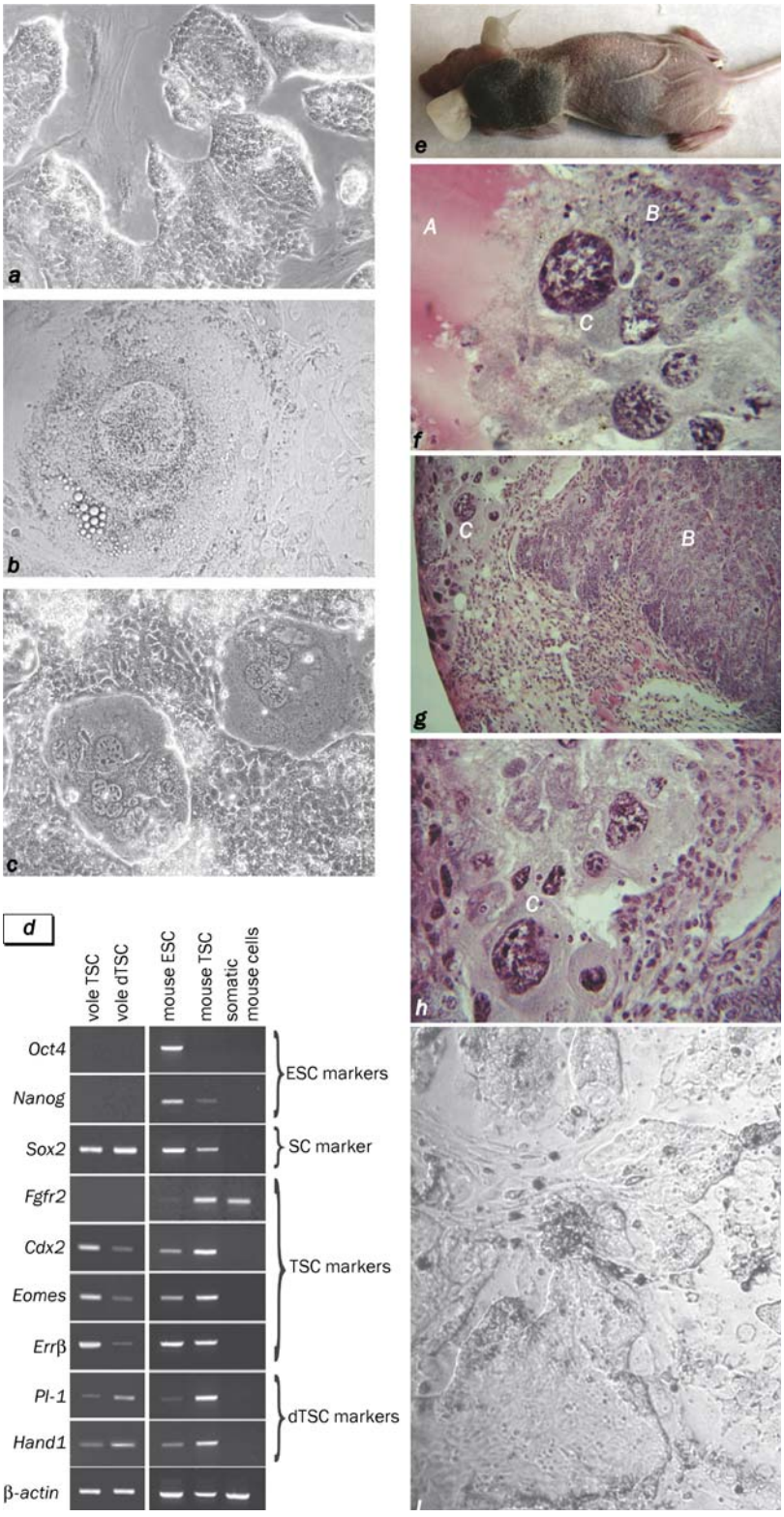

Fig. 3. Characterization of vole TS-like cells. *a*) morphology of flat monolayer colonies of vole TS-like cells: R2 cells (×60; phase contrast); *b*) giant cell with granular cytoplasm, a result of spontaneous differentiation of vole SC (×200; phase contrast); c) giant multinuclear cells formed as a result of differentiation of vole SC in passage 35 culture (×60; phase contrast); *d*) RT-PCR analysis of the expression of gene specific for embryonic and extraembryonic ectoderm in different types of mouse and vole cells and tissues; *e*) general appearance of a tumor-like cystic formation 1 week after subcutaneous injection of TS-like cells from common voles *M. rossiaemeridionalis* to a *nude* mouse; *f*) histological analysis of a fragment of the borderline cell layer on day 35 after injection of vole TS-like cells; g) a fragment of the tumor similar to chorial plate; *h*) cluster of giant cells in chorial plate-resembling tumor fragment; *A*: necrotic zone; *B*: accumulation of proliferating cells; *C*: accumulation of differentiated giant cells invading the host tissues (hematoxylin and eosin staining); *i*) colonies of TS-like cells isolated from cultured capsule walls from the tumors induced by injection of vole TS-like cells to *nude* mice (phase contrast).

Transcription factor of *Caudal-related homeobox 2* homeodomain, *Cdx-2* gene, is most actively transcribed in the outer polar blastomeres of the morula and in TE, but is absent in ICM cells [37,64]. It was hypothesized that repression of *Cdx-2* gene in the inner cells of early morula can be the first event in segregation of ICM and TE cells. It was demonstrated that excessive expression of *Cdx-2* gene in ESC leads to suppression of *Oct4* gene transcription and their differentiation into TE, though without the formation

of giant cells [52]. Culturing of these cells in the presence of FGF4, heparin, and embryonic fibroblast-conditioned medium leads to the appearance of TS-like epithelial colonies, while being injected into mouse blastocyst, they (similarly as TSC) contribute exclusively into the placenta [52].

Experiments on embryos with homozygous *Cdx-2* gene deletion showed that gene mutation led to their preimplantation death. The mutants formed normal early blastocysts, but then a block of TE determination was observed, the blastocoel collapsed, the embryos failed to hatch from the zona pellucida and, hence, were unable to implant into the uterine wall [73]. Expression of *Hand1* and *Pl-1* genes, markers of giant cells, was absent in *Cdx-2*$^{-/-}$ embryos, which confirmed disturbances in TE differentiation. It was also demonstrated that *Cdx-2* gene down-regulates expression of *Oct4* и *Nanog* genes in outer blastocysts cells [73]. Thus, *Cdx-2* gene is the earliest gene determining segregation of ICM and TE lineage cells; its expression is essential for all aspects of early TE development from proliferation of diploid cells to the development of polyploid giant cells.

Another target gene for FGF signal, *Eomes* gene, a T-box transcription factor, is also important for embryo development and TE proliferation. It is expressed in blastocyst TE and extraembryonic ectoderm of early postimplantation blastocysts [14,67] and similarly to *Cdx-2* gene can affect the TE formation. It was demonstrated for ESC cells that *Eomes* can be a target for *Cdx-2* gene [52]. Embryos homozygous by *Eomes* gene mutation form blastocysts with expression of *Oct4* gene in ICM and TE-specific gene *Cdx-2* in surface cells. Blastocyst implantation into the uterus is not followed by embryo development because of arrest of trophoblast differentiation, which leads to embryo death. This suggests that TE is formed, but does not proliferate. Thus, *Cdx-2* and *Eomes* genes are the main candidates for the role of regulators of TE differentiation.

Removal of any factor maintaining diploid TSC in undifferentiated state, FGF4 or heparin (for mice) and embryonic fibroblasts (for voles), inhibits proliferation and differentiation into trophoblast giant cells (Fig. 3, *b*) [75,77], and multinuclear syncytium-like cells (Fig. 3, *c*) [26,29]. High culturing density also induces TSC differentiation [26] accompanied by repression of extraembryonic ectoderm-specific genes, activation of placental lactogen *Pl-1*, a specific giant cell marker, and enhanced expression of *Hand1* gene, another giant cell marker belonging to basic helix-loop-helix family (bHLH) (Fig. 3, *d*) [70,75]. Despite no *Hand1* gene product was detected in extraembryonic ectoderm cells [15], it is expressed in TSC irrespective of the presence of FGF4 and fibroblast-conditioned medium [75]. This can be explained by the presence of minor amounts of differentiated derivatives in the culture of undifferentiated TSC, *e.g.* trophoblast giant cells. It was also established that overexpression of *Hand1* gene promotes differentiation of TSC into trophoblast giant cells even in the presence of FGF4 and conditioned medium. Another bHLH family gene, *Stra13*, expressed in giant cells also induces TSC differentiation towards trophoblast giant cells [29].

Genes characteristic of ectoplacental cone and spongiotrophoblast (*Tpbp/4311*, *Mash2*) are also expressed during differentiation of mouse TSC [10,15, 30,39,82]. *Mash2* gene is important for the maintenance of spongiotrophoblast layer cells; in the absence of this gene, the number of these cells decreases and more giant cells appear. At the same time, overexpression of *Mash2* gene prevents differentiation of cells into trophoblast giant cells [15]. It was also shown that this gene produced a transient stimulating effect on proliferation of mouse TSC even in the absence of exogenous FGF4 [29].

ALTERNATIVE SIGNAL PATHWAYS

FGF4/FGFR2 signal pathway is not the only pathway involved in proliferation and differentiation of SC in the early trophoblast, which is confirmed by successful isolation of TS-like cells of common voles [23] and rats [13] in the absence of FGF4 and heparin. The undifferentiated state and proliferative potential of these cells are maintained via activation of an alternative FGF-independent signal pathway. The absence of *Fgfr2* gene expression in vole TS-like cells (Fig. 3, *d*) confirms this assumption. The factors triggering and maintaining this pathway are produced by embryonic fibroblasts, because the presence of fibroblast feeder or fibroblast-conditioned medium is a necessary conditions for obtaining and proliferation of TSC in undifferentiated state in both mice [75] and voles [23].

There are data on mechanisms involved into regulation of the fate of extraembryonic ectoderm cells and TSC. These data helped to identify alternative factors essential for the maintenance of TSC proliferation [17,25]. It was demonstrated that the members of transforming growth factor-β (TGF-β) superfamily, *e.g.* NODAL are essential for the maintenance of constant *Fgf4* gene expression. Moreover, NODAL can directly affect extraembryonic ectodermal cells without activation of *Fgf4* gene by maintaining expression of *Errβ*, *Eomes*, and *Cdx-2* genes and suppressing the expression of *Mash2* gene, which prevents differentiation of TSC [25]. The important role of TGF-β superfamily was confirmed in experiments, where conditioned medium was replaced with TGF-β superfamily factors

[17], activin A and TGF-β inhibiting the expression of markers of differentiated TSC [49]. Thus, the representatives of TGF-β superfamily (activin A, TGF-β, or NODAL) can be the factors released by fibroblasts into the culture medium and triggering an alternative pathway involved in the maintenance of undifferentiated state of vole TS-like cells without cooperation with the FGF4/FGFR2 signal pathway or cooperation with other unidentified factors released by embryonic fibroblasts probably takes place. It should be noted that these factors are highly conservative, because vole TS-like cells proliferate without differentiation during culturing on both vole and mouse fibroblasts.

Errβ gene is an important component of alternative FGF-independent signal pathway. Orphan nuclear receptor ERRβ plays an important role in trophoblast development and in early placentation processes. Its expression is observed in undifferentiated TS lineage cells in early extraembryonic ectoderm and chorionic ectoderm [75]. In embryos lacking this gene, expression of *Cdx-2* and *Eomes* genes is preserved [73], but these embryos are characterized by the absence of diploid trophoblast layer, abnormal chorion development, and overproduction of trophoblast giant cells enveloping the embryo by a thick layer, which leads to its death on post-coitum day 9 [42,65,76]. In contrast to *Cdx-2* and *Eomes* genes, *Errβ* gene is not essential for the initial periods of trophoblast proliferation, but is necessary for its maintenance. Thus, *Errβ* gene is a component of a signal pathway maintaining proliferation and undifferentiated state of both trophoblast and TSC. High level of *Errβ* gene expression in vole TS-like cells supports the hypothesis on its participation in alternative FGF-independent signal pathway.

It is likely that vole LIF is also a critical factor for triggering the signal pathway participating in the generation of vole undifferentiated TS-like cells. Addition of vole LIF to the culture medium led to the appearance of TS-like cell colonies [23], while in the absence of this factor no SC were obtained from early embryos [4]. LIF belongs to the cytokine interleukins-6 (IL-6) family consisting of IL-6, IL-11, ciliary neurotrophic factor, oncostatin M, and cardiotrophin-1. The effect of LIF was mediated via heterodimer complex of surface receptors consisting of LIF receptor (LIFRβ) and glycoprotein gp130 signal transducer [21,27]. This heterodimer complex is very important for activation of transcription factor STAT3 (signal transducer and activator of transcription-3) and maintenance of undifferentiated state of ESC [47]. Vole LIF is necessary only at the initial stages of isolation of TS-like colonies, which is confirmed by the facts that stable lines proliferate for a long-time without differentiation on fibroblast feeder or in the presence of fibroblast-conditioned medium without LIF. The existence of an alternative signal pathway for maintaining the properties of TSC opens possibilities for studying signal pathways regulating the development of trophoblast. These studies are also interesting for obtaining TSC of other species, including human TSC (not obtained yet).

SC OF EXTRAEMBRYONIC ENDODERM

Another type of extraembryonic cells is XEN-cells (extraembryonic endoderm cells). These SC were obtained from cultured preimplantation blastocysts of mice [37] and common vole [5]. Morphology of XEN-cells forming colonies with minimum cell-cell contacts drastically differs from multilayer colonies of ESC with tight junctions and monolayer TSC colonies. The culture of mouse XEN-cells consists of two mutually transforming cell types [37]. Type 1 cells are round and look like spilled beads and type 2 cells have stellate shape. However, XEN-cell isolation from voles, apart from cells looking like spilled beads (Fig. 4, *a*) yielded a subpopulation of cells growing as flattened colonies looking like sheets of tightly contacting cells (Fig. 4, *b*). Culturing of XEN-cells at a high density leads to the formation of a monolayer with net-like structures. During culturing of mouse XEN-cells in the absence of medium conditioned by mouse embryonic fibroblasts on plastic not coated with gelatin for 6 days, many cells increased in size, formed vacuoles, and became intolerant to passaging, which attested to their differentiation. However, vole XEN-cells can be passaged for a long time on gelatin-coated substrate in the absence of conditioned medium [5]. Moreover, in contrast to mouse ESC and TSC, XEN-cells are FGF- and LIF-independent (Fig. 1) [53,75].

In chimeras obtained by injection of mouse *GFP/lacZ* XEN-cells into blastocysts, these cells were found only in extraembryonic endoderm forming extraembryonic organs, but not in the embryo, yolk sac mesoderm, or trophoblast (Fig. 1). In the majority of chimeras (98%), XEN-cells were found in parietal endoderm and only in 1 (2%) of 50 chimeras XEN cells participated in the formation of only visceral endoderm [36].

Analysis of mouse and vole XEN-cell cultures showed that they do not express markers of the epiblast (genes *Oct4*, *Nanog*, and *Rex1*) and trophoblast (genes *Cdx-2*, *Fgfr2*, and *Eomes*), but express genes specific for extraembryonic (parietal and visceral) endoderm (*Gata4*, *Gata6*, *Sox7*, *Sall4*, *Hnf4*, *Foxa2*, and *Afp*) (Fig 4, *c, d*) [36]. A species-specific difference of vole XEN-cells was the expression of *Errβ* gene involved in the maintenance of TSC proliferation in undifferentiated state. Marker of visceral endoderm, *Afp* gene, was detected in minor amounts in undifferentiated culture of XEN cells, but the signal increased

during differentiation, whereas expression of *Gata4* and *Sox7* genes typical of extraembryonic endoderm decreased, the levels of transcription of *Hnf4* and *Foxa2* genes, markers of visceral endoderm, remained unchanged [36]. Let us consider some transcription factors essential for the development and function of extraembryonic endoderm.

EXPRESSION OF GENES TYPICAL OF EXTRAEMBRYONIC ENDODERM

Cytokeratin-18 is a cytoskeleton protein belonging to the class intermediate filament proteins. The expression of cytokeratin-18 gene was detected in none embryonic tissues except for the endoderm [57]. Since

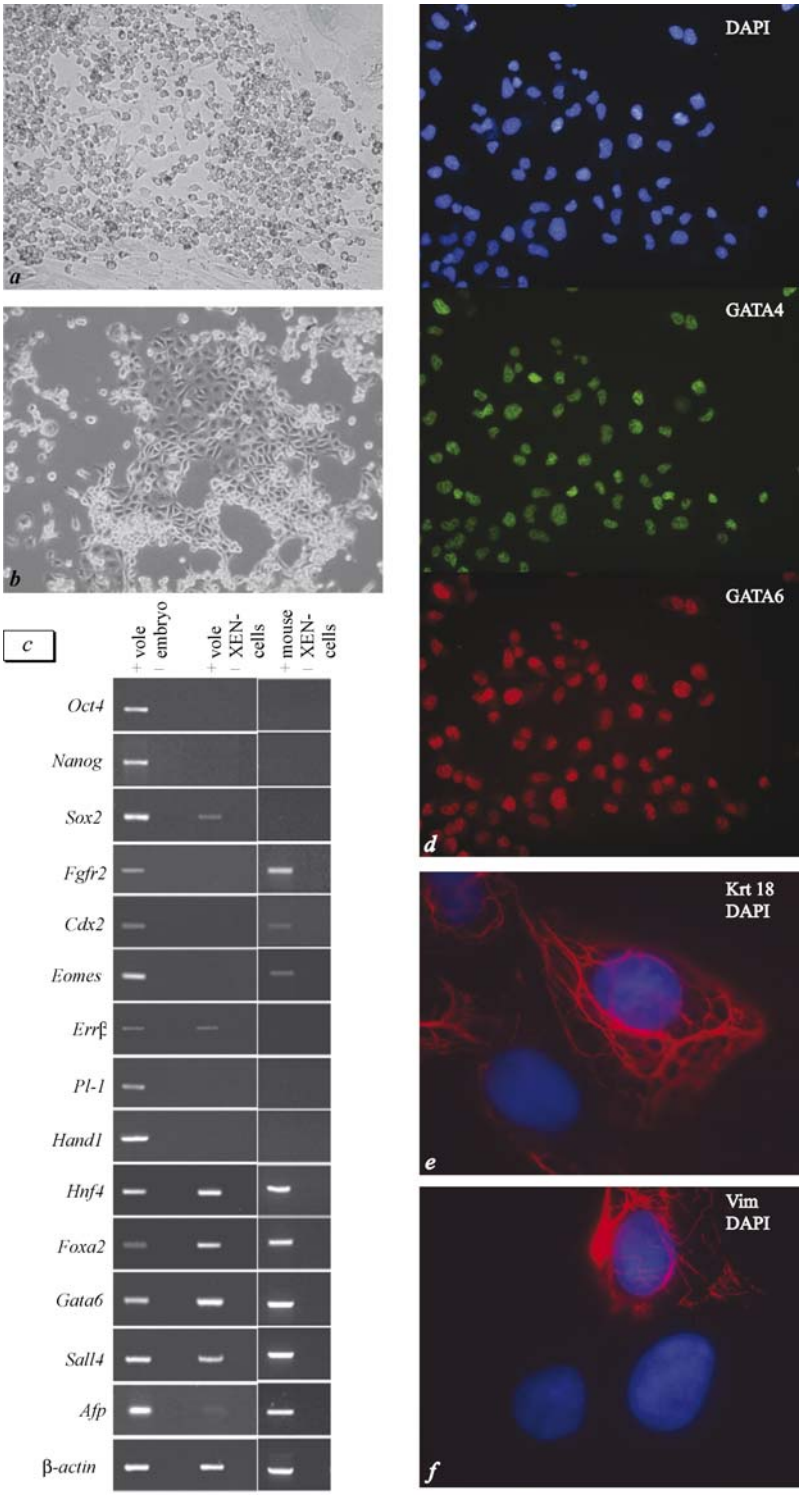

Fig. 4. Characterization of XEN-cells. *a*) morphology of spilled beads-like cell subpopulation (×60; phase contrast); *b*) morphology of the colony of vole XEN-cells with tightly contacting cells (×60; phase contrast); *c*) RT-PCR analysis of the expression of genes specific for embryonic and extraembryonic ectoderm and extraembryonic endoderm in mouse XEN-cells, vole XEN-cells, and 7.5-dpc vole embryos (control): minus denotes negative control of reverse transcription; *d*) immunocytochemical detection of transcription factors GATA4 (green) and GATA6 (red), nuclei are stained with DAPI (blue, ×60); *e*) immunocytochemical staining of XEN-cells for cytokeratin-18 (×1000); *f*) immunocytochemical staining of XEN-cells for vimentin (×1000).

the expression of this gene is specific for extraembryonic endoderm, cytokeratin-18 is intensively expressed in XEN-cells of mice [37] and vole (Fig. 4, *e*) *in vitro*.

Expression of the gene for another intermediate filament protein vimentin in extraembryonic endoderm cells was studied on *Bos taurus* embryos [44]. It was found that in the extraembryonic endoderm vimentin is expressed only in cells intended for definitive visceral yolk sac, *i.e.* belonging to the visceral endoderm. Thus, immunocytochemical analysis revealed XEN-cells belonging to the visceral endoderm, *i.e.* expressing vimentin, and to parietal endoderm, was demonstrated on vole XEN-cells (Fig. 4, *f*).

Transcription factor HNF4, a member of the steroid hormone superfamily, participates in the regulation of gastrulation [16]. In mammals, HNF4 factor binds to promoter of various genes of the visceral endoderm and liver. In developing mouse embryo, expression of this transcription factor was first detected on day 5.5 of embryonic development; at later terms, *Hnf4* mRNA was detected in the liver and gut epithelium [16].

Another transcription factor FOXA2 (HNF3β) appears in the visceral endoderm on day 6 of embryonic development. At later terms, this transcription factor is expressed in the chorda, perichordal mesoderm, and definitive gut endoderm [7]. HNF3β$^{-/-}$ embryos die on days 6.5-9 of embryonic development, they were characterized by development retardation and serious defects (*e.g.*, the absence of the chorda) disturbing the development of the neural tube in the dorsoventral direction [7]. HNF3β$^{-/-}$ embryos also demonstrate aberrant relationships between the yolk sac and the embryo [18,46]. In mutants, the visceral endodermal layer is normal, but *Afp* expression is absent in the yolk sac [18], which attests to the role of transcription factor FOXA2 in endoderm development.

Transcription factor GATA6, a member of zinc-finger transcription factor family, first appears at the blastocyst stage in some ICM and TE cells. At later terms, the product of this gene is detected in the parietal endoderm and in the mesoderm [35]. In two studies, cell lineages morphologically similar to XEN-cells were derived from stable ESC by forced expression of either GATA4 or GATA6 [19], or from mutant ESC lineage cells carrying *Nanog* gene deletion [48]. These cultures, apart from morphological similarity, express various markers of extraembryonic endoderm, which attests to the involvement of *Gata4* and *Gata6* genes in the regulation of primary endodermal cell development. Moreover, mutant embryos heterozygous by *Gata4* genes *Gata6* die on day 13.5 of embryonic development because of cardiovascular defects and primitive endoderm malformation and dysfunction [38,81]. These facts suggest that transcription factors GATA are essential for both extraembryonic tissues functioning and early embryonic development.

Thus, XEN-cells, derivatives of the extraembryonic endoderm, can be used as *in vitro* model for the study of early development of mammalian extraembryonic cell lineages.

CONCLUSION

Human TSC and XEN-cells, unlimited source of extraembryonic cells participating in the early embryonic development, are not obtained yet. *In vitro* study of extraembryonic cells from various mammalian species and signal pathways triggering the maintenance of undifferentiated state and differentiation of SC can help to elucidate general regularities of these processes and extrapolate them to humans.

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