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Pluripotency of bank vole embryonic cells depends on FGF2 and activin A signaling pathways

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ABSTRACT The objective of this study was to investigate the capability of bank vole *(Myodes glareolus)* embryonic cells to sustain their pluripotent character during *in vitro* culture, and to determine the optimal conditions for derivation of embryonic stem (ES) cells. We compared the presence of specific pluripotency (Oct4, Ssea1) and differentiation markers (Gata4 - primitive endoderm marker; Cdx2 - trophectoderm marker) in blastocysts and inner cell mass (ICM) outgrowths obtained from blastocysts of bank vole, and two mouse hybrids F1(C57Bl/6xCBA/H) and F1(C57Bl/6x129/Sv), which differ in the permissiveness of giving rise to ES cells. We found that, in contrast to mouse, the expression of pluripotency markers in the cells of bank vole ICM outgrowths is progressively downregulated and rapidly lost by the 4th day of culture. This correlates with the appearance of cells expressing *Gata4* and *Cdx2,* indicating differentiation towards primitive endoderm and derivatives of trophectoderm, respectively. We have also shown that heterologous cytokine leukaemia inhibitory factor (LIF) in conjunction with either homologous or heterologous feeder layer is unable to delay differentiation and preserve pluripotency of bank vole embryonic cells. Thus, the conditions optimised for mouse do not support the maintenance of bank vole embryonic cells in the undifferentiated state and do not allow for the isolation of the ES cells. Instead, combination of fibroblast growth factor 2 and activin A allows retention of Oct4 expression in bank vole blastocyst outgrowths during 4-day culture, indicating that signaling pathways operating in human, rather than mouse ES cells, might be involved in the process of self-renewal of bank vole embryonic cells.

KEY WORDS: *bank vole, mouse, blastocyst, pluripotency, Oct4, differentiation marker*

Introduction

During early embryogenesis mammalian embryo undergoes a series of cleavage divisions that lead to the formation of a blastocyst, composed of two distinct cell lineages: trophectoderm (TE) that surrounds the fluid-filled cavity, and the inner cell mass (ICM) composed of compact group of pluripotent cells located at one pole of the blastocyst. *Cdx2* was shown to be one of the earliest known genes responsible for TE differentiation (Beck *et al.*, 1995; Strumpf *et al.*, 2005). Shortly before implantation, the second wave of differentiation results in the establishment of two subpopulations of cells within the ICM. Cells that face the blastocyst cavity differentiate into primitive endoderm, which express *Gata4* and will contribute to extraembryonic tissue, the yolk sac (Arceci *et al.*, 1993; Morrisey *et al.*, 1996). Internal cells of ICM, referred to as epiblast, retain the pluripotency and give rise to all embryonic tissues and also to extraembryonic membranes, such as allantois and amnion.

The ICM and its successor, the epiblast, are the only cell lineages that transiently retain pluripotency. *In vivo* they differentiate into primary germ layers of the developing foetus – ectoderm, endoderm and mesoderm. Cultivation of ICM cells of blastocyst in the presence of leukaemia inhibitory factor – LIF (a member of Interleukin (Il-6) family of cytokines) (Smith *et al.*, 1988; Williams *et al.*, 1988) can result in the derivation of embryonic stem cells (ES cells) (Evans and Kaufman, 1981; Martin, 1981). ES cells maintained *in vitro* can multiply without loosing their undifferentiated character for the indefinite period of time. Similarly to ICM cells they express such pluripotency markers as *Oct4* (Scholer *et al.*, 1989; Rosner *et al.*, 1990; Palmieri *et al.*, 1994), *Nanog*

Abbreviations used in this paper: ES, embryonic stem cell; ICM, inner cell mass; Il, interleukin; LIF, leukemia inhibitory factor; TE, trophectoderm.

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(Chambers *et al.*, 2003; Mitsui *et al.*, 2003) and *Ssea1* (Solter and Knowles, 1978). Surprisingly, despite many efforts, ES cells are not readily obtained from embryos of mammals other than mouse and even not from every mouse strain. Protocols optimised for derivation and culture of mouse ES cells appear to be not suitable for the establishment of ES cells from other mammalian species. Available stable mouse ES cells lines originate predominantly from 129/Sv strain of mice and its hybrids. Except for mouse cells, only human (Thomson *et al*., 1998), some other primates (Wolf *et al*., 2004), and possibly rat (Buehr *et al.,* 2008; Lee *et al.,* 2008; Ueda *et al*., 2008) cell lines have been proven to retain their undifferentiated state and pluripotency after long-term *in vitro* culture.

The insight into molecular basis of pluripotency and conditions required to preserve this state in the early embryo is of great importance for understanding requirements for the maintenance of pluripotent cells *in vitro*. It is well known that mechanisms responsible for the preservation of the undifferentiated state and pluripotency of ES cells differ between mouse and human. In mouse ES cells, several pathways including leukaemia inhibitory factor (LIF)/signal transducer and activator of transcription 3 (STAT3) (Niwa *et al*., 1998; Matsuda *et al*., 1999), bone morphogenetic protein (BMP)/inhibitor of differentiation (ID) (Ying *et al*., 2003), phosphoinositide-3-kinase (PI3K)/AKT (Paling *et al*., 2004) and Src (Anneren *et al*., 2004) have been shown to play crucial role in self-renewal. In contrast, LIF is incapable of maintaining stem cell status in human ES (hES) cells (Daheron *et al*., 2004; Humphrey *et al*., 2004). Likewise, whereas BMP4 has been shown to block differentiation of mouse ES cells, it induces hES cells to differentiate into extraembryonic lineages (Xu *et al*., 2002). Among the factors necessary for hES cell renewal are fibroblast growth factor 2 (FGF2), transforming growth factor (TGFβ)/Activin/Nodal (Dvorak *et al*., 2005; James *et al*., 2005) and MEK/ERK signaling pathways (Li *et al*., 2007). These reports point out, that there are substantial differences in mechanisms controlling self-renewal between mouse and human ES cells. Thus, establishing the culture conditions promoting derivation of ES cell line from species other than mouse and man may allow generalizing and extending the knowledge of biology of these cells.

With this aim in mind we have attempted to derive ES line from a rodent, the bank vole (*Myodes glareolus*) whose reproduction and embryology is relatively well known and which was previously used for a number of developmental studies (Mystkowska, 1975; Ozdzenski and Mystkowska, 1976a and b). Although mouse and bank vole belong to different families (or subfamilies, according to certain taxonomic systems) they have similar timing and sequence of pre- and peri-implantation development. Unfortunately, standard procedure used for the production of mouse ES cells was inadequate for the production of vole ES cells. In order to elucidate the possible basis of the difference between mouse and bank vole, we compared the presence of specific pluripotency (Oct4, Ssea1) and differentiation (Gata4, Cdx2) markers in blastocysts and *in vitro* cultured 2-, 3-, and 4-day-old ICM outgrowths obtained from the bank voles and from two mouse hybrids, F1(C57Bl/6x129/Sv) and F1(C57Bl/6xCBA/H). We also examined the effect of increased concentration of LIF or its absence in the medium, and the presence of homologous or heterologous embryonic fibroblast feeder layers on sustaining the undifferentiated phenotype and pluripotency of mouse and bank vole embryonic cells. In order to confirm the possible involvement of self renewal mechanisms operating in human ES cells we also examined bank vole blastocyst outgrowths cultured under conditions applied for human embryo-derived ES cells i.e. in the medium supplemented with FGF2 and activin A (Vallier *et al*., 2005).

Results

Attempts to derive bank vole ES cells

We attempted to derive bank vole ES cells applying mouse ES cells derivation techniques that permitted us to isolate ES cells from F1(C57Bl/6xCBA/H) mouse hybrid (Fig. 1A). Bank vole blastocysts (n=785) or immunosurgically isolated blastocyst inner cell masses (n=234) were cultured on feeder layer of inactivated mouse embryonic fibroblasts in the ES medium supplemented with 1,000 I.U./ml of murine LIF (Nagy *et al*., 2003). Resulting outgrowths were next disaggregated either mechanically or enzymatically. Disaggregated mouse blastocyst outgrowths gave rise to primary colonies of several different morphological types of cells, including cells of stem cell-like morphology. However, cells resulted from disaggregation of outgrowths of whole bank vole blastocysts or isolated ICMs, as a rule, rapidly differentiated either into trophoblast giant cells or loosely attached primitive endoderm-like cells (Fig. 1 B,C). Isolated ICMs immediately differentiated into colonies of primitive endoderm-like cells (Fig. 1C), which, following subsequent dissociation reached the confluency during a few days (Fig. 1D).

To investigate the basis of inability to produce bank vole ES cells using standard culture conditions we turned to the study of pluripotency markers expression as a potential predictor of the

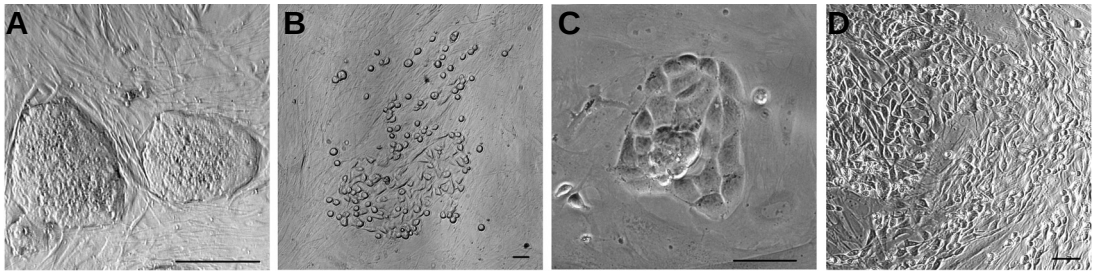

Fig. 1. Colonies of cells derived from mouse and bank vole blastocysts. (A) *ES cell line derived from blastocyst of F1(C57Bl/6xCBA/H) mouse hybrid (8th passage).* **(B)** *Colony of loosely attached primitive endoderm-like cells (4 days post-disaggregation of bank vole blastocyst outgrowth.* **(C)** *ICM of bank vole blastocyst 3 days after explantation.* **(D)** *Confluent culture of endoderm cells (6 days post-disaggregation of bank vole ICM outgrowth). Scale bar, 20μm.*

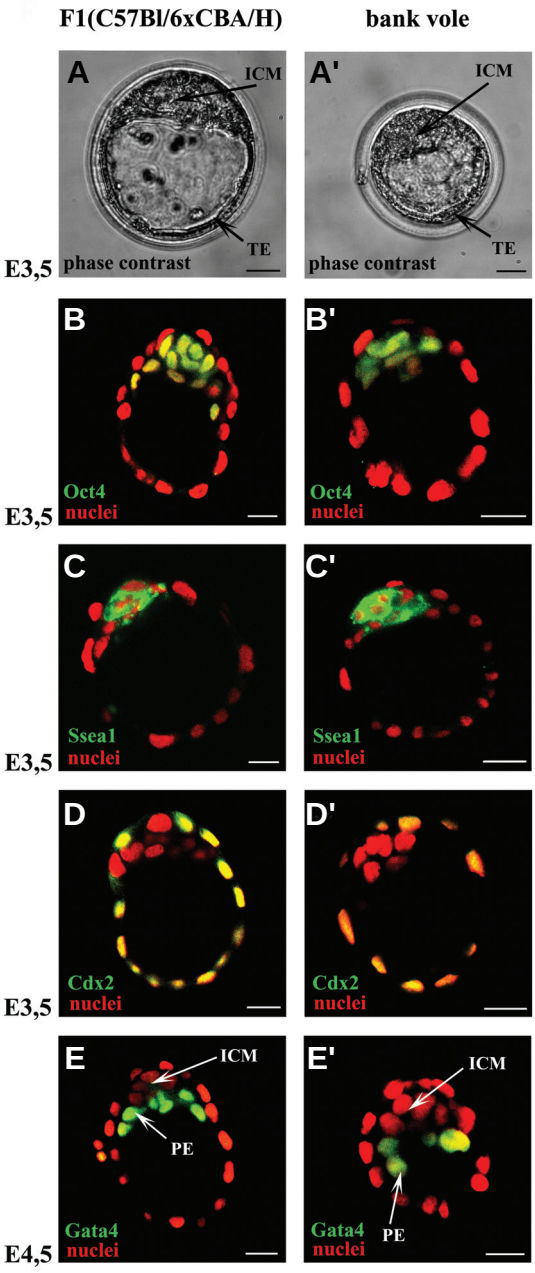

Fig. 2. Presence of lineage markers in E3.5 and E4.5 mouse (left column) and bank vole (right column) blastocysts. *Blastocysts were double-labeled with chromomycin for DNA (red) and lineage markers (green).* **(A,A')** *Phase contrast;* **(B,B')** *Oct4;* **(C,C')** *Ssea1;* **(D,D')** *Cdx2;* **(E,E')** *Gata4. Scale bar, 20 μm.*

successful derivation of ES cells. We assumed that blastocysts' outgrowths containing high number of pluripotent cells should be a better source of embryonic stem cells than the outgrowths containing few or none pluripotent cells. In order to find out whether mouse and bank vole blastocysts differ in the number of pluripotent cells, we followed differentiation processes of blastocysts of bank vole and two mouse hybrids during four days of culture. F1(C57Bl/6xCBA/H) is considered to be non-permissive for ES cell derivation, and F1(C57Bl/6x129/Sv), because of a 129/Sv parent, is considered to be the most permissive in yielding ES cell lines. We focused at the expression of *Oct4* transcription factor and Ssea1 surface antigen that are expressed in the pluripotent, undifferentiated cells, and *Cdx2* and *Gata4* that are markers for trophectoderm and primitive endoderm, respectively.

Pluripotency and differentiation markers in mouse and bank vole blastocysts

Mouse blastocysts were recovered at 3.5 day of development. From unknown reasons we were unable to recover bank vole blastocysts at day 3.5. This forced us to isolate embryos at day 3 of development, i.e. at the morula stage, and culture them *in vitro* until they reached the blastocyst stage. Bank vole blastocysts do not significantly differ in their morphology from mouse blastocysts except that they are smaller (Figs 2A and 2A'). Analysis of 46 F1(C57Bl/6xCBA/H) mouse and 39 bank vole blastocysts at 3.5 day of development revealed that in both species all ICM cells expressed Oct4 protein and cell surface antigen Ssea1 (Fig. 2 B,B' and C,C'), and trophectoderm cells expressed Cdx2 (Fig. 2 D,D'). Moreover, ICM cells of all analysed blastocysts lacked Gata4 - marker of primitive endoderm (data not shown). This clearly indicated undifferentiated character of ICM cells in both species and suggested that they could be a potential source of ES cells. At 4.5 day of development in all examined mouse and bank vole blastocysts (9 and 8, respectively) we detected the presence of a cell layer lining ICM that expressed *Gata4* that indicates differentiation of primitive endoderm (Fig. 2E and E').

Oct4, Gata4 and Cdx2 proteins in mouse and bank vole ICM outgrowths during in vitro *culture*

Blastocysts of F1(C57Bl/6xCBA/H) and F1(C57Bl/6x129/Sv) mouse hybrids isolated at 3.5 day of development, and plated onto gelatin-coated surface of culture chambers hatched from zonae pellucidae within 24 h and attached to the surface. On the 3rd day of *in vitro* culture blastocysts contained group of ICM cells positioned on the top of trophectoderm giant cells (Fig. 3A and A'). Since bank vole blastocysts were denuded from the zonae pellucidae before plating (see Material and Methods section), they attached within few hours of *in vitro* culture; consequently, they were slightly more advanced in the process of outgrowth formation than mouse blastocysts. Bank vole outgrowths were usually compact and seldom contained trophoblast giant cells (Fig. 3A").

TABLE 1

OCT4-POSITIVE CELLS IN BANK VOLE AND MOUSE ICM OUTGROWTHS DURING *IN VITRO* CULTURE IN THE PRESENCE OF 500 I.U./ML LIF

species	Number of Oct4 - positive cells	% (number) of outgrowths with Oct4-positive cells at subsequent days of culture		
		day 2	day 3	day 4
mouse F1(C57Bl/6x129/Sv)	+++ [1]	100% (18/18)	84% (21/25)	36% (5/14)
	+ [2]	0	16% (4/25)	36% (5/14)
	- [3]	0	0	28% (4/14)
mouse F1(C57Bl/6xCBA/H)	+++	95% (19/20)	42% (8/19)	35% (6/17)
	+	5% (1/20)	26% (5/19)	18% (3/17)
	-	0	32% (6/19)	47% (8/17)
bank vole	+++	56% (14/25)	29% (8/28)	0
	+	36% (9/25)	25% (7/28)	0
	-	8% (2/25)	46% (13/28)	100%(13/13)

[1] Oct4 protein in more than 7 cells per outgrowth; [2] Oct4 protein in less than 7 cells per outgrowth; [3] Lack of Oct4 protein in outgrowth

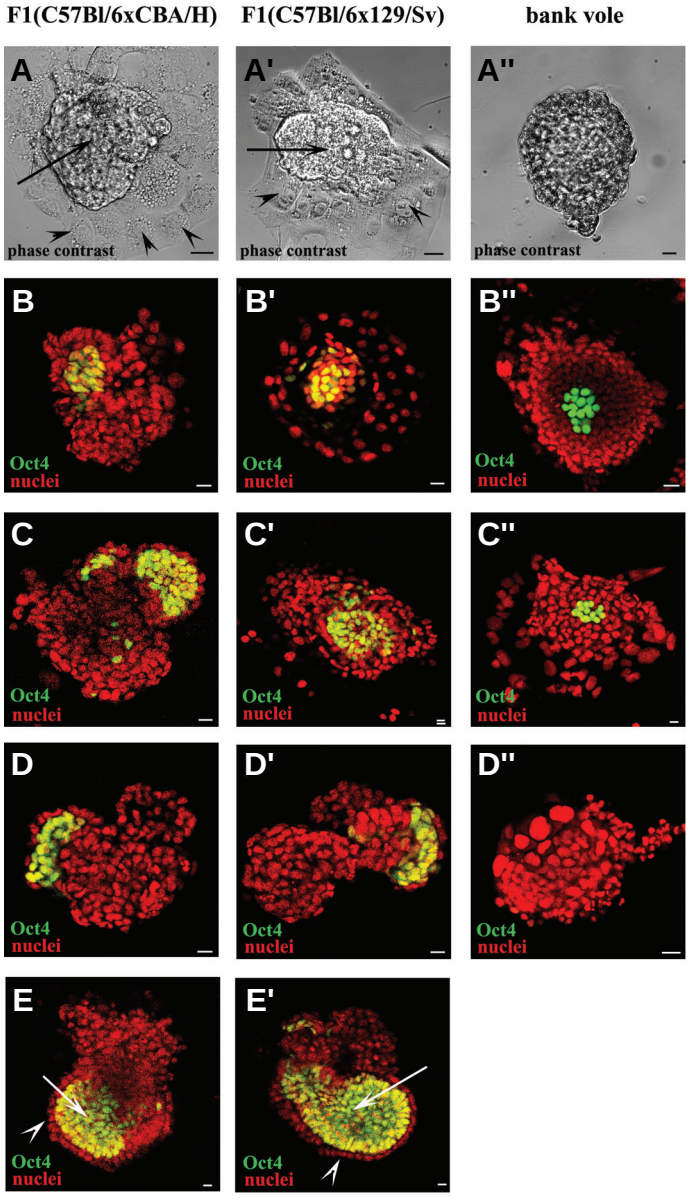

Fig. 3. Presence of Oct4 protein in mouse and bank vole blastocyst outgrowths. *Outgrowths were double-labeled with propidium iodide for DNA (red) and Oct4 (green).* **(A,A',A")** *Phase contrast of day 3 outgrowths (arrows indicate ICM outgrowths and arrowheads show trophoblast giant cells);* **(B,B',B")** *Day 2 outgrowths;* **(C,C',C")** *day 3 outgrowths;* **(D,D',D")** *day 4 outgrowths;* **(E,E')** *day 4 outgrowths - note egg cylinder-like structure with epiblast (arrow) and primitive endoderm (arrowhead). Scale bar, 20 µm.*

All F1(C57Bl/6xCBA/H) (n=20) and F1(C57Bl/6x129/Sv) (n=18) mouse outgrowths examined on the 2nd day of culture consisted of significant number of Oct4-positive, presumptive pluripotent cells (Table 1, Fig. 3B and B'), and Gata4–positive primitive endoderm cells located in the form of a ring at the periphery of the ICM outgrowth (Fig. 4A and A'). On the same day of culture only 56% (14/25) of bank vole blastocyst outgrowths contained *Oct4* expressing cells that were gathered in the middle of the outgrowths (Table 1, Fig. 2B"). In the remaining 11 outgrowths (44%) *Oct4* was either undetectable or expressed only in few cells. Moreover, in these outgrowths high number of cells expressed *Gata4* (Fig. 4A"). This indicates that the bank vole outgrowths differentiate into endodermal cells faster than the mouse outgrowths (Fig. 4 A,A',A").

On the 3rd day of culture the outgrowths of F1(C57Bl/6xCBA/H) and F1(C57Bl/6x129/Sv) mouse hybrids significantly increased in size and usually consisted of a pool of undifferentiated, Oct-4-positive cells (Table 1, Fig. 3C and C') surrounded by *Gata4* expressing endodermal cells (Fig. 4B and B'). Groups of cells expressing *Oct4* were detectable in 84% (21/25) of F1(C57Bl/6x129/Sv) outgrowths. In 16% (4/25) of outgrowths *Oct4* expression was limited to few cells. In addition, 32% (6/19) of F1(C57Bl/6xCBA/H) outgrowths lacked cells expressing *Oct4* which suggests a loss of pluripotency. On the same day of culture in 46% (13/28) of bank vole outgrowths all cells have lost pluripotency as judged by the lack of expression of Oct4 (Table 1). Thus, the number of outgrowths containing Oct4-positive cells and also the proportion of *Oct4*-expressing cells per outgrowth decreased considerably in bank vole embryos, especially in comparison with F1(C57Bl/6x129/Sv) mouse hybrids ($P<0.001$). Analysis performed on the 4th day of culture, i.e. at the time when ES cell derivation procedure requires disaggregation of ICM outgrowths, revealed that both mouse and bank vole outgrowths continued to differentiate, as evidenced by intensified expression of *Gata4* (Fig. 4C,C' and C") and *Cdx2* (Fig. 5C and C'), and considerable decrease in *Oct4* expression. Almost 30% (4/14) of F1(C57Bl/6x129/Sv) and 50% (8/17) of F1(C57Bl/6xCBA/H) outgrowths lacked cells expressing *Oct4*. Oct-4-positive outgrowths contained either patches of small number of presumptively pluripotent cells (Fig. 3D and D'), or formed structures resembling egg cylinder with large epiblast-

TABLE 2

EFFECT OF LIF CONCENTRATION ON *OCT4* EXPRESSION IN MOUSE AND BANK VOLE BLASTOCYST OUTGROWTHS OVER 4-DAY CULTURE PERIOD

species	LIF concentration (I.U./ml)	Oct4 - positive cells	% (proportion) of outgrowths with Oct4-positive cells at subsequent days of culture		
			day 2	day 3	day 4
mouse F1(C57Bl/6x129/Sv)	1,000	+++ [1]	100% (12/12)	100% (24/24)	90% (9/10)
		+ [2]	0	0	10% (1/10)
		- [3]	0	0	0
	5,000	+++	100% (13/13)	100% (17/17)	88% (15/17)
		+	0	0	6% (1/17)
		-	0	0	6% (1/17)
mouse F1(C57Bl/6xCBA/H)	1,000	+++	100% (18/18)	89% (25/28)	59% (32/54)
		+	0	7% (2/28)	19% (10/54)
		-	0	4% (1/28)	22% (12/54)
	5,000	+++	100% (15/15)	88% (21/24)	64% (9/14)
		+	0	0	14% (2/14)
		-	0	12% (3/24)	21% (3/14)
bank vole	500	+++	56% (14/25)	29% (8/28)	0
		+	36% (9/25)	25% (7/28)	0
		-	8% (2/25)	46% (13/28)	100% (13/13)
	1,000	+++	50% (8/16)	36% (4/11)	0
		+	31% (5/16)	9% (1/11)	0
		-	19% (3/16)	55% (6/11)	100% (13/13)
	5,000	+++	75% (15/20)	38% (5/13)	0
		+	20% (4/20)	38% (5/13)	0
		-	5% (1/20)	23% (3/13)	100% (17/17)
	0	+++	88% (22/25)	41% (14/34)	0
		+	8% (2/25)	24% (8/34)	0
		-	4% (1/25)	35% (12/34)	100% (18/18)

[1]Oct4 protein in more than 7 cells per outgrowth; [2]Oct4 protein in less than 7 cells per outgrowth; [3]Lack of Oct4 protein in outgrowth

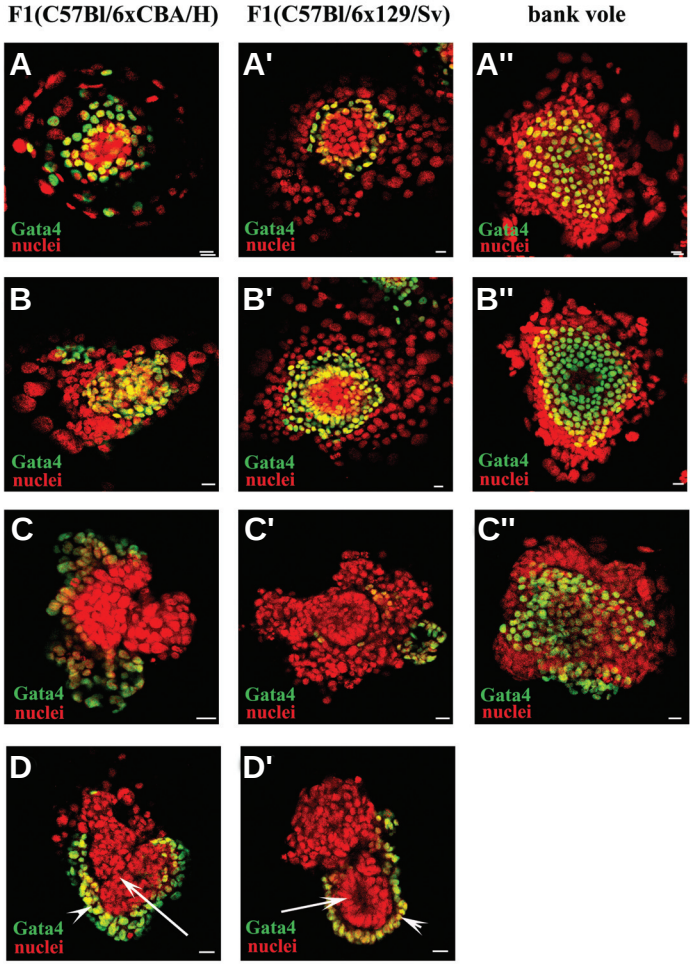

Fig. 4. Presence of Gata4 protein in mouse and bank vole ICM outgrowths. *Outgrowths were double-labeled with propidium iodide for DNA (red) and Gata4 (green).* **(A,A′,A″)** *2-day-old outgrowths;* **(B,B′,B″)** *3-day-old outgrowths;* **(C,C′,C″)** *4-day-old outgrowths;* **(D,D′)** *4-day-old outgrowths mimicking egg cylinder. Arrows point to the epiblast-like cells and arrowheads point to the primitive endoderm-like cells. Scale bar, 20 μm.*

like core (Fig. 3E and E') surrounded by primitive endoderm-like cells expressing *Gata4* (Fig. 4D and D'). On the 4th day of culture, bank vole outgrowths formed monolayers of morphologically heterogeneous cells, which did not form distinct ICM. In contrast to mouse, the bank vole outgrowths were unable to create egg cylinder-like structures. In addition, as judged by total lack of Oct4 protein, they totally lost undifferentiated character (Fig. 3D"). When compared with mouse 4-day-old cultures this difference was statistically significant (P<0.01).

Since bank vole outgrowths originated from blastocysts that - unlike mouse embryos - were subjected to overnight *in vitro* culture and removal of zona pellucida, we wanted to know whether the modified experimental procedure could be responsible for the rapid loss of undifferentiated character of bank vole ICM-derived cells. We processed 14 F1(C57Bl/6xCBA/H) mouse embryos according to the bank vole embryo protocol and cultured them for 4 days. These conditions did not accelerate differentiation processes in the mouse outgrowths. *Oct4* expression was not lowered in these outgrowths, but, surprisingly, was even slightly higher than in blastocysts processed conventionally: 70% of these outgrowths (24/34) contained Oct4-positive cells, in comparison to 53% of outgrowths treated in usual manner. These results indicate that the precocious differentiation of bank vole ICM cells was not caused by this particular experimental procedure.

Thus, we concluded that the rapid loss of cells expressing markers of the pluripotency in bank vole outgrowths is a poor prognosis for the successful derivation of embryonic stem cells. Next, we decided to check whether the modification of standard conditions applied for ES cell derivation might affect the maintenance of pluripotency markers in bank vole and mouse embryonic stem cells.

The effect of LIF on the maintenance of pluripotency of mouse and bank vole embryonic cells

The isolation and maintenance of mouse ES cells requires 500 – 1,000 I.U./ml of LIF (Nagy *et al*., 2003). However, it was previously demonstrated that derivation of ES cells from embryos of some inbred mouse strains requires higher LIF concentration (Baharvand and Matthaei, 2004). On the other hand, proliferation of ES cells of other species, such as humans and monkeys, does not involve LIF signaling pathway (Sumi *et al*., 2004; Daheron *et al*., 2004). In order to explore the possible role of LIF in retaining the pluripotency of bank vole embryonic cells, we analysed the *Oct4* expression in blastocysts of bank voles and F1(C57Bl/6xCBA/H) and F1(C57Bl/6x129/Sv) mouse hybrids cultured in ES medium supplemented with either 1,000 I.U./ml or 5,000 I.U./ml murine recombinant LIF (Table 2).

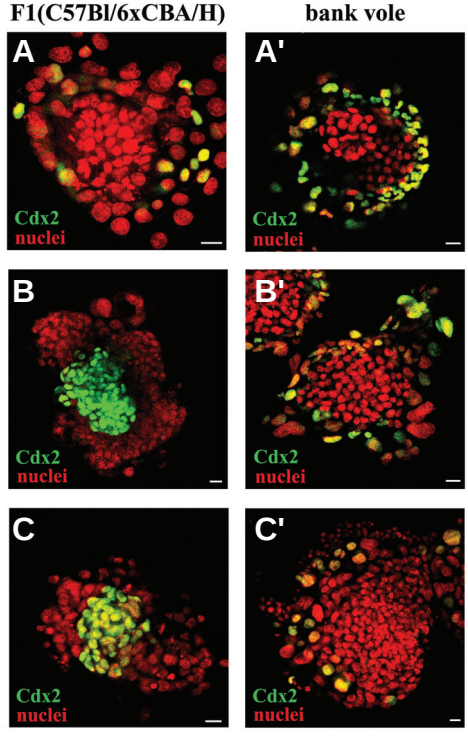

Fig. 5. Presence of Cdx2 protein in bank vole and F1(C57Bl/6xCBA/H) mouse blastocyst outgrowths. *Outgrowths were double-labeled with propidium iodide for DNA (red) and Cdx2 (green).* **(A,A′)** *2 days of culture;* **(B,B′)** *3 days of culture;* **(C,C′)** *4 days of culture. Scale bar, 20 μm.*

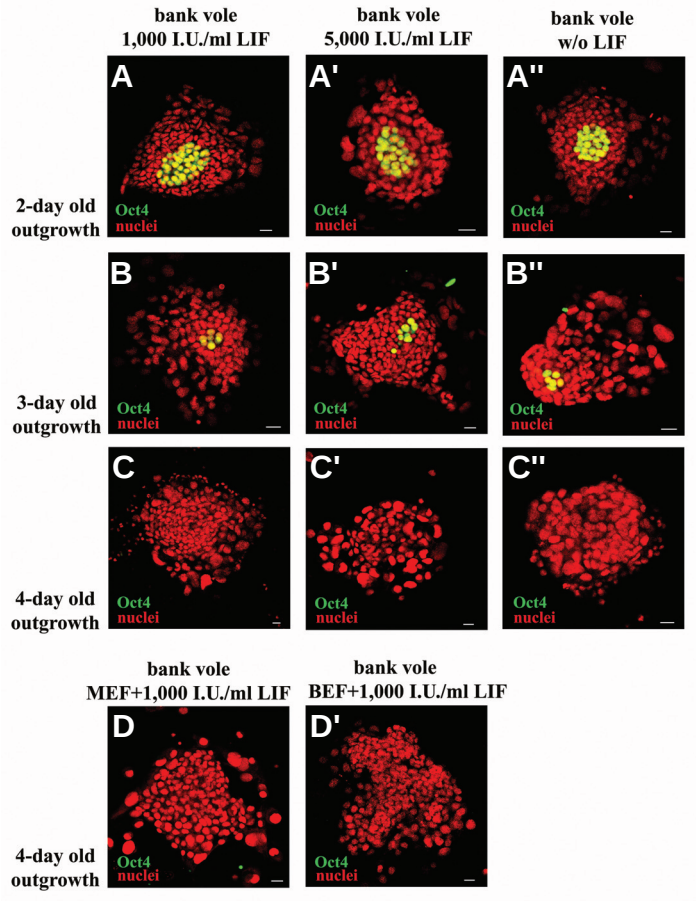

Fig. 6. Effect of LIF concentration and feeder layer on *Oct4* expression in bank vole blastocyst outgrowths. *Outgrowths were double-labeled with propidium iodide for DNA (red) and Oct4 (green).* **(A,B,C)** *Outgrowths cultured in 1,000 I.U./ml LIF;* **(A',B',C')** *outgrowths cultured in 5,000 I.U./ml LIF;* **(A",B",C")** *outgrowths cultured without LIF;* **(D')** *outgrowths cultured on the MEF feeder layer and in the 1,000 I.U./ml LIF;* **(D")** *outgrowths cultured on the BEF feeder layer and in the 1,000 I.U./ml LIF. Scale bar, 20 μm.*

The twofold increase in LIF concentration (1,000 I.U./ml) resulted in the significant increase in the number of Oct4-positive cells in F1(C57Bl/6x129/Sv) and F1(C57Bl/6xCBA/H) outgrowths analyzed on the 3rd day of culture. Moreover, this effect was still evident on the 4th day of culture (*cf* Tables 1 and 2). Concentrations of 1,000 and 5,000 I.U./ml of LIF were equally efficient in maintaining the pluripotent character of mouse embryonic cells (Table 2).

Unlike mouse outgrowths, bank vole outgrowths did not respond either to twofold or to tenfold concentration of LIF. The percentage of Oct4-positive bank vole outgrowths cultured in the presence of 1,000 I.U./ml LIF for 2 (Fig. 6A) and 3 (Fig. 6B) days respectively was not significantly higher than in cultures kept in standard medium. On the 4th day of culture, regardless of LIF concentration, none of analyzed outgrowths contained cells expressing *Oct4* (Table 2, Fig. 6C).

Because we have used commercially available murine recombinant LIF, lack of sensitivity of bank vole cells might be attributed to heterologous nature of this protein. We decided to check activity of LIF derived from *Microtus rossiaemeridionalis* (a species that is taxonomically closer to bank vole than mouse). Supplementation of ES medium with *Microtus rossiaemeridionalis* LIF did not delay the differentiation process during *in vitro* culture of bank vole blastocysts and like in cultures containing murine LIF, we could not detect cells with Oct4 protein in 4-day-old outgrowths of bank vole blastocysts (n = 15). In addition, in bank vole blastocysts cultured for 2-4 days in LIF-free medium the loss of cell pluripotency occurred at the same rate as in the presence of LIF (Table 2, Fig. 6 A",B",C"). Taken together, our data indicate that addition of heterologous LIF does not promote maintenance of the bank vole embryonic cells in the undifferentiated state.

The effect of homologous and heterologous feeder cells on the delay of differentiation of bank vole embryonic cells

It is generally accepted that the presence of feeder layer, such as inactivated embryonic fibroblasts, can support derivation of ES cells. To test the effect of feeder layer on bank vole blastocysts differentiation they were plated onto the growth-arrested mouse embryonic fibroblasts (MEFs) or bank vole embryonic fibroblasts (BEFs) in culture medium supplemented with 1,000 I.U./ml of LIF, and cultured for 4 days. None of bank vole blastocyst outgrowths that were cultured for 4 days either on MEFs (n=15) or on BEFs (n=20) contained Oct4-positive cells (Fig. 6 D,D'). Thus, regardless of whether the bank vole cultures were grown on supportive feeder layer of homologous or heterologous embryonic fibroblasts, ICM-derived cells lost the undifferentiated phenotype after the same period of culture *in vitro*. Moreover, these results did not differ from those obtained in experiments in which bank vole embryos were cultured in the presence of LIF only.

The effect of growth factors and culture conditions, which have been successfully used for human ES cells

The lack of responsiveness to LIF by bank vole embryonic cells resembles the behaviour of human ES cells, which pluripotency greatly relies on the FGF2 and activin A-dependent signaling pathway. These observations prompted us to analyze *Oct4* ex-

TABLE 3

EFFECT OF CULTURE CONDITIONS OPTIMISED FOR HUMAN ES CELL DERIVATION ON *OCT4* EXPRESSION IN MOUSE AND BANK VOLE BLASTOCYST OUTGROWTHS AFTER 4 DAYS OF CULTURE

		% (number) of outgrowths with Oct4-positive cells on the 4th day of culture	
culture conditions	Oct4 - positive cells	F1(C57Bl/6xCBA/H)	Bank vole
ES medium+LIF	+++ [1]	95% (19/20)	0
	+ [2]	5% (1/20)	0
	- [3]	0	100 % (0
ES medium	+++	35% (7/20)	65% (15/23)
LIF, FGF2, activin A	+	55% (11/20)	9% (2/23)
	-	10% (2/20)	26% (6/23)
Matrigel+ES medium	+++	67% (10/15)	57% (4/7)
LIF, FGF2, activin A	+	20% (3/15)	14% (1/7)
	-	13% (2/15)	29% (2/7)
Matrigel+BEF-CM[4]	+++	62% (8/13)	0
LIF, FGF2, activin A	+	23% (3/13)	0
	-	15% (2/13)	100% (18/18)
Matrigel+ES medium	+++	58% (7/12)	0
LIF	+	8% (1/12)	0
	-	33% (4/12)	100% (11/11)

[1]Oct4 protein in more than 7 cells per outgrowth; [2]Oct4 protein in less than 7 cells per outgrowth; [3]Lack of Oct4 protein in outgrowth; [4]ES medium conditioned by bank vole embryonic fibroblasts.

pression in bank vole blastocyst outgrowths cultured for 4 days under culture conditions commonly used for human-derived ES cells. However, since we wanted to compare the reaction of mouse (dependent on LIF) and bank vole (independent of LIF) outgrowths we decided to supplement all culture media used in the experiments described below with LIF.

First, we cultured F1(C57Bl/6xCBA/H) mouse and bank vole blastocysts in medium supplemented with 1,000 I.U./ml LIF, 20ng/ml fibroblast growth factor 2 (FGF2) (Xu *et al*., 2005; Dvorak *et al*., 2005; Levenstein *et al.,* 2006) and 20ng/ml activin A (Vallier *et al*., 2005; James *et al*., 2005; Xiao *et al*., 2006). At the 4th day of culture, 90% (18/20) of mouse outgrowths contained Oct4 positive cells (Table 3, Fig. 7A). This result did not significantly differ from the result of previous experiment in which 100% of mouse outgrowths cultured in the presence of LIF only contained cells expressing Oct4 (Table 3). Unexpectedly, under conditions described above 65% (15/23) of bank vole outgrowths contained multiple and 9% (2/23) contained single *Oct4* expressing cells (Fig. 7A').

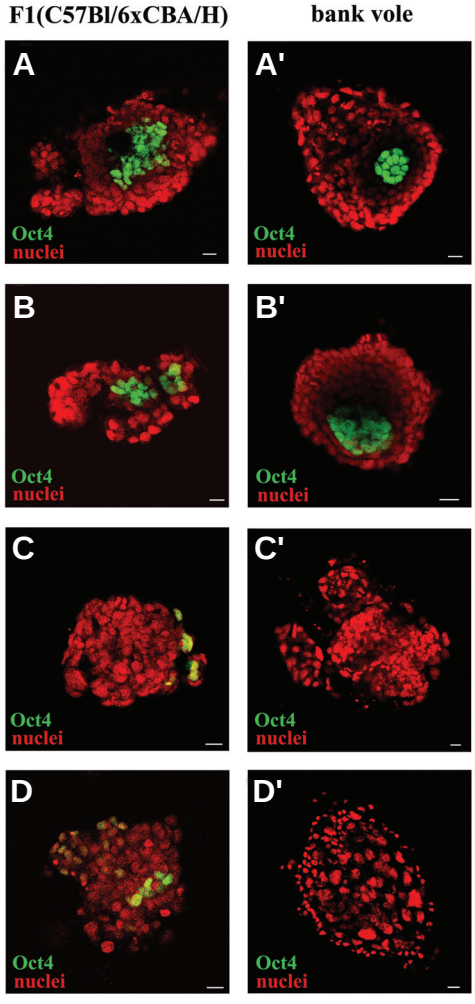

Fig. 7. Effect of culture conditions optimised for human ES cell derivation on *Oct4* expression in mouse and bank vole 4-day old outgrowths. *Outgrowths were double-labeled with propidium iodide for DNA (red) and Oct4 (green).* **(A,A')** *Outgrowths cultured in medium supplemented with LIF, FGF2 and activin A;* **(B,B')** *outgrowths cultured in medium supplemented with LIF, FGF2 and activin A on the slides covered with Matrigel;* **(C,C')** *outgrowths cultured on Matrigel, in ES medium conditioned by bank vole embryonic fibroblasts (BEF-CM) with addition of growth factors;* **(D,D')** *outgrowths cultured on Matrigel, in the presence of LIF only. Scale bar, 20 μm.*

Next, we further modified culture conditions by plating mouse and bank vole blastocysts onto slides coated with Matrigel – a complex mixture of mouse sarcoma origin, that was shown to support the hES cells culture in the absence of fibroblast feeder layer (Xu *et al*., 2001, reviewed in Rao and Zandstra, 2005). As previously, we used LIF, FGF2 and activin A- supplemented medium. At the 4th day of culture, 87% (13/15) of mouse outgrowths contained Oct4 positive cells (Table 3, Fig. 7B). Similarly, as in the presence of the growth factors only, 57% (4/7) of bank vole outgrowths contained numerous and 14% (1/7) single *Oct4* expressing cells (Fig. 7B'). Noteworthy, in contrast to the mouse outgrowths, Matrigel alone was not able to sustain the expression of *Oct4* in outgrowths of bank vole. None of 11 outgrowths remained undifferentiated, as evidenced by lack of *Oct4*-expressing cells (Table 3, Fig. 7D'). The outcome of these experiments was significantly different from the results of the previous ones, when bank vole embryos were cultured in the presence of LIF only.

Next, we further modified the conditions culturing F1(C57Bl/6xCBA/H) mouse and bank vole blastocysts in FGF2 and activin A-supplemented medium that has been also conditioned by bank vole embryonic fibroblasts (BEFs). Under such culture conditions 85% (11/13) of mouse embryos sustained *Oct4* expression on the 4th day after plating (Fig. 7C). Unexpectedly, the growth of bank vole outgrowths under such conditions was extremely poor. Twenty five blastocysts did not attach to the Matrigel at all. None of the remaining 18 blastocysts, that attached and formed the outgrowths, expressed *Oct4* (Fig. 7C'). Such a behaviour of bank vole embryos might be explained by the presence of inhibitory components, secreted by fibroblasts, that could block signaling pathway molecules responsible for sustaining the undifferentiated state of bank vole cells. This inhibitory action against activin A has been previously suggested regarding human ES cells cultured in medium conditioned by embryonic fibroblasts (Xiao *et al*., 2006).

Taken together, these observations suggest that mouse and bank vole embryonic cells differ in their requirements for maintenance of the undifferentiated state and pluripotency. Moreover, our findings strongly indicate, that FGF2- and activin A-dependent signaling pathways play a role in the process of bank vole self-renewal, similarly as it happens in human embryonic stem cells.

Discussion

The majority of presently available mouse ES cell lines are derived from 129/Sv strain and its hybrids. Derivation of ES cell lines from other strains, such as C57Bl/6, BALB/c or CBA, is very inefficient and requires modifications of primary culture conditions, for instance twofold or even tenfold increase in LIF concentration (Kawase *et al.*, 1994; Schoonjans *et al.*, 2003; Baharvand and Matthaei, 2004). Obtaining of ES cell lines from species other than mouse and man has proved even more difficult. Protocols optimised for derivation and culture of mouse ES cells were not

suitable for establishment of embryonic stem cells from hamster (Doetschman *et al*., 1988); mink (Sukoyan *et al*., 1992); dog (Hatoya *et al.*, 2006; Hayes *et al.*, 2008); cat (Yu *et al*., 2008); rabbit (Honda *et al*., 2008); horse (Saito *et al.*, 2002); pig (Notarianni *et al.*, 1990; Notarianni *et al.*, 1991; Wheeler, 1994; Li *et al.*, 2003); sheep (Notarianni *et al*., 1991); buffalo (Verma *et al*., 2007) and until recently from rat (Iannaccone *et al.*, 1994; Vassilieva *et al*., 2000; Schulze *et al.*, 2006; Demers *et al*., 2007). Bank vole seems to be yet another species supplementing the list of ES cells derivation trials.

The time of ICM disaggregation

According to Evans and Kaufman (Evans and Kaufman, 1981) the crucial factor for the success in producing ES cells is the right stage of the ICM of *in vitro* cultured blastocyst at the time of initial disaggregation, usually performed between 3rd and 5th day of culture. In our experiments the bank vole blastocyst outgrowths were disaggregated on the 3rd or 4th day of culture i.e. at the time when rapid downregulation of *Oct4* expression, accompanied by the extensive differentiation towards Gata4-positive primitive endoderm and Cdx2-positive trophectoderm derivatives, occurred. Thus, it is highly unlikely that disaggregation of such outgrowths would result in the establishment of ES cell line. Our observations were consistent with the study of Buehr *et al*. (Buehr *et al*., 2003) who showed that *Oct4* downregulation occurring in cultured rat blastocysts was the most probable reason of failure to produce rat ES cells. Moreover, Hay *et al*. (2004) showed that the siRNA-mediated decline of *Oct4* expression in ES cells causes upregulation of endoderm - (*Gata6)* and trophoblast - (*Cdx2)* - associated genes. However, it has to be noted that these results are in contrast to the findings of Niwa *et al*. (2000) who showed that the expression of *Gata4*, which is restricted to parietal and visceral endoderm, increases during differentiation of ES cells that was induced by elevation, but not suppression of *Oct4* expression level.

Nevertheless, our and other researchers' observations suggest that the key to a success in obtaining pluripotent, self-renewing cell lines may lie in setting up the conditions or treatment of embryos that allow maintaining the proper level of *Oct4* expression. Thus, we thought that by modifying the standard culture conditions used during mouse ES cell derivation we can influence and maybe sustain the *Oct4* expression in bank vole embryonic cells, and in consequence increase the chance for the successful embryonic stem cell derivation.

Feeder layer

Feeder layer provides extracellular matrix facilitating cell attachment and also secretes LIF and some undefined growth factors supporting the maintenance of stem cell phenotype, both of mouse and human ES cells. Various laboratories used different cell types as a feeder layer. Homologous embryonic fibroblasts were successfully used to produce ES, or ES-like cells in human (Richards *et al*., 2003), mink (Sukoyan *et al*., 1992), rat (Iannaccone *et al*., 1994) and buffalo (Verma *et al*., 2006). The first described procedures of propagation of ES cells utilized mouse embryonic fibroblasts as a feeder layer for human blastocysts. Later, with the goal of potential therapeutic application, mouse feeder cells were successfully replaced by many different kinds of human supportive cells such as human adult marrow stromal cells, uterine endometrium, human adult skin (Amit *et al.*, 2003; Cheng *et al.*, 2003; Richards *et al.*, 2003). Ouhibi *et al*. found that the best derivation of rat ES-like cells was achieved by culturing embryos on rat uterine epithelial cells (Ouhibi *et al*., 1995). Also mink fibroblasts were better than mouse embryonic fibroblasts for maintaining mink embryonic cells in the undifferentiated state (Sukoyan *et al*., 1992). However, embryos of other species such as cow and sheep, did not benefit from the homologous feeder layer (Piedrahita *et al*., 1990). In our hands, neither homologous nor heterologous fibroblasts, prepared from mid-gestation embryos, prolong the maintenance of undifferentiated cells in bank vole outgrowths beyond the 3rd day of culture.

LIF, FGF2 and activin A

Among defined factors that maintain proliferation, undifferentiated state and pluripotency of mouse embryonic cells is leukaemia inhibitory factor – LIF (Smith *et al.*, 1988; Williams *et al.*, 1988). Human or murine LIF was also successfully used for derivation and sustaining the undifferentiated state for the limited number of passages of mink (Sukoyan *et al*., 1992), dog (Hatoya *et al.*, 2006), horse (Saito *et al.*, 2002) and rat (Li *et al*., 2008) ES-like cells. On the other hand, it was shown previously that LIF was not beneficial for the derivation of porcine ES-like cells (Moore and Piedrahita, 1996). Similarly, our results clearly show that addition of either mouse, or vole LIF did not promote derivation of ES cells from bank voles blastocysts. It is, therefore, unlikely that bank vole embryo-derived pluripotent cell lines can be isolated under culture conditions commonly used for mouse ES cells.

It is well known that human embryonic stem cells also do not require LIF for the self-renewal and maintenance of pluripotency (Thomson *et al*., 1998). These cells utilize another mechanisms for self-renewal, i.e. fibroblast growth factor 2 (FGF2), transforming growth factor (TGFβ)/Activin/Nodal and Mek/Erk - based signaling pathways (Vallier *et al*., 2005; Xu *et al*., 2005; Dvorak *et al*., 2005; James *et al*., 2005; Levenstein *et al.,* 2006; Xiao *et al*., 2006; Li *et al*., 2007). According to Vallier *et al*. (2005) the combination of activin A or Nodal with FGF2 maintains the expression of pluripotency markers and supports the stem cell status in human ES cells even in the absence of feeder layer, feeder-conditioned medium or Matrigel. Also, activin A alone is sufficient to support long-term feeder-free growth of human ES cells, to induce the expression of other regulators, such as Oct4, Nanog, and FGF2. It also inhibits markers of differentiation (Xiao *et al*., 2006). Recent reports indicate that multiple signaling pathways, including activin A-dependent one, might be involved in keeping also rat ES-like cells in an undifferentiated state (Li *et al*., 2008). But, in rat ES cells homologous rat LIF was also important to sustain expression of *Oct4* and *Nanog* (Ueda *et al*., 2008), implicating that rat ES cells share some characteristics with both mouse and human ES cells. Importantly, also in our study, FGF2 and activin A were crucial for the maintenance of pluripotent state of cells in the bank vole outgrowths.

In conclusion, our and others' results indicate, that various species of mammals including human, mouse, bank vole and rat do differ in growth factors and the signaling pathways they

employ to sustain their undifferentiated and pluripotent character. Bank vole embryonic cells utilize mechanisms governing self-renewal operating in human, rather than in the mouse. Fibroblast growth factor 2 and activin A, that positively regulate the *Oct4* expression, appear to be of crucial importance for this process.

Materials and Methods

Animal studies were approved by Local Ethic Committee No. 1 in Warsaw, Poland according to European Union Council Directive 86/609/EEC of 24 November 1986 on the approximation of laws, regulations and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes (Close *et al.*, 1996; Close *et al.*, 1997). All animals were raised on the premises.

Embryo collection

Mouse embryos. Mice were maintained in the 14 h light/10 h dark cycle mid-pointed at 1 a.m.). Six-16-week-old F1(C57Bl/6xCBA/H) and F1(C57Bl/6x129/Sv) female mice were induced to ovulate by injection of 10 I.U. pregnant mare's serum gonadotrophin (PMSG, 'Folligon' Intervet, Netherlands) followed 46-50 h later by 10 I.U. of human chorionic gonadotrophin (hCG, 'Chorulon' Intervet, Netherlands) injection and allowed to mate with males of the same cross. The following morning, mating was confirmed by the presence of a vaginal plug. Blastocysts were collected 90-96 h after hCG injection by flushing the uterus with M2 medium (M16 medium buffered with HEPES, Sigma (Fulton and Whittingham, 1978). Being a result of a cross between F1 animals, the embryos represented, in fact, F2 generation.

Bank vole embryos. Bank voles originated from the colony reared as an outbred stock in our Animals Facility. They were maintained in a controlled photoperiod of 16 h light and 8 h dark. Eight-16-week-old females were superovulated with 10 I.U. of PMSG injection followed, 42-48 h later, by injection of 10 I.U. hCG (Mystkowska, 1975). Females were crossed with males and the mating was confirmed by the presence of vaginal plugs or spermatozoa in the vaginal smears on the next morning. Embryos were recovered approximately 72 h after hCG injection (E3.5) as morulae or after 96 h (E4.5) as blastocysts. Morulae were cultured overnight in M2 medium at 37…C, under 5% CO_2 in the air until they reached the blastocyst stage. Since bank vole embryos are unable to hatch spontaneously *in vitro*, the zona pellucida was removed from blastocysts by brief exposure to acid Tyrode's solution (pH 2.5) (Nicolson *et al*., 1975).

Derivation of bank vole ES cells

Immunosurgical isolation of ICMs (Solter and Knowles, 1975)

Blastocysts were incubated in haemolytic serum for 0.5 h (1:100; Biomed, Cracow, previously inactivated at 56°C for 0.5 h in order to inactivate complement) and subjected to guinea pig complement (1:5, 0.5h; Sigma). The dead TE cells were removed by gently pipetting the ICMs in a finely drawn pipette.

Culture conditions

Blastocysts or ICMs were placed in plastic dishes (Nunc) covered with gelatin [0.2% (w/v), Sigma] and feeder layer of inactivated primary mouse embryonic fibroblasts (see below). Medium for derivation of ES cells was composed of KnockOut Dulbecco's Modified Eagle's Medium (Knockout DMEM, Invitrogen) supplemented with 15% foetal bovine serum (FBS, Invitrogen) with addition of nonessential amino-acids (0.1mM, Invitrogen), L-glutamine (2mM, Invitrogen), β-mercaptoethanol (0.1mM, Sigma), penicillin (5000 units/ml), streptomycin (5000 μg/ml, Invitrogen) and murine leukaemia inhibitory factor (LIF; 1,000 I.U./ml, ESGRO, 10^7 units, Chemicon International). After 4-5 days of culture ICMs outgrowths were disaggregated mechanically or enzymatically into clumps of cells, by pipetting outgrowths subjected previously to 5 min. incubation in 0.25% trypsin-EDTA (Invitrogen). Clumps of cells were transferred onto fresh layer of embryonic fibroblasts and inspected daily for appearance of primary colonies.

Expression of pluripotency and differentiation markers in cultured blastocysts

Culture conditions optimised for mouse ES cells

Culture of blastocyst outgrowths on gelatin: Mouse blastocysts (zona pellucida-enclosed) and bank vole blastocysts (zona pellucida-free) were placed in gelatin-coated chambers of tissue culture slides (Lab-Tek Chamber Slides, Nunc) in 0.5ml of ES medium. The medium was composed of KnockOut Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 15% FBS, (Invitrogen), murine LIF at concentration of 500, 1,000 or 5,000 I.U./ml or *Microtus rossiaemeridionalis* LIF – a gift from Professor Suren Zakian from the Institute of Cytology and Genetics, Russian Accademy of Sciences, Novosibirsk, Russia, and remaining components (as described above). Blastocysts were cultured for 2, 3 or 4 days at 37…C, in the atmosphere of 5%CO_2 in the air (day of explantation - day '0'). During 1-2 days blastocysts outgrew on the slides. The ICMs formed clumps of cells surrounded by bigger and flatter trophectodermal cells. They were fixed and subjected to indirect immunofluorescence analysis.

Culture of blastocyst outgrowths on feeder cells: The preparation of feeder layer has been described in detail by Robertson (Robertson, 1987). Primary mouse or bank vole embryonic fibroblasts (MEF and BEF, respectively) were derived from 13.5-day-old foetuses of F1(C57Bl/6xCBA/H) hybrid mice and bank voles. The MEF and BEF cells were cultured in DMEM (with 4,500 mg/l glucose, Sigma) supplemented with 10% FBS (Biochrom) and penicillin (5,000 units/ml) - streptomycin (5,000 μg/ml, Invitrogen). Confluent layers of fibroblasts were inactivated by treatment with mitomycin C (10μg/ml, Sigma) for 2 h. Growth-arrested feeder cells were prepared one day before plating the blastocysts on the culture slides.

Culture conditions optimised for human ES cells

F1(C57Bl/6xCBA/H) mouse and bank vole blastocysts were placed in gelatin - or Matrigel - (Xu *et al*., 2005; BD Biosciences, Franklin Lakes, NJ; 1:10) - coated chambers of tissue culture slides in 0.5ml of ES medium supplemented with 1,000 I.U./ml LIF, 20ng/ml recombinant human fibroblast growth factor 2 (FGF2) (Invitrogen) and 20ng/ml recombinant human/mouse/rat activin A (R&D Systems Inc.). In one experiment medium conditioned by bank vole embryonic fibroblasts was used for culture instead of unconditioned ES medium. Conditioned medium (CM) was collected according to Xu *et al*. (2001).

Indirect immunofluorescence

3.5- or 4.5-day-old blastocysts and 2-, 3-, and 4-day old blastocyst outgrowths were fixed overnight (for Cdx2 and Ssea1 detection) or for 30 min (for Oct4 and Gata4 staining) in 4% paraformaldehyde in Ca^{2+} Mg^{2+} - free PBS at 4…C. After fixation the outgrowths were washed with Ca^{2+} $Mg2^{+}$ - free PBS and then permeabilised with Ca^{2+} Mg^{2+} - free PBS containing 1.5% BSA (bovine serum albumin; Sigma) and 0.3% Triton X-100 (Sigma) for 20 min at room temperature. To block nonspecific antibody binding, outgrowths were incubated in Ca^{2+} Mg^{2+} - free PBS with 3% BSA and 0.01% Tween 20 (Bio-Rad Laboratories) in 4…C overnight. Following the blocking step, the expression of pluripotent and differentiating cells markers was detected using mouse monoclonal antibodies against Oct4 (sc-5279, Santa Cruz Biotechnology, 1:100), Cdx2 (BG-MU392, Biogenex, 1:50) and Ssea1 (MC-480, Developmental Studies Hybridoma Bank, 1:50) and rabbit polyclonal antibody against Gata4 (sc-

9053, Santa Cruz Biotechnology, 1:100). The primary antibodies were diluted in Ca^{2+} Mg^{2+} - free PBS containing 3% BSA. Next, the outgrowths were rinsed with Ca^{2+} Mg^{2+} - free PBS three times (15 min per wash) and then incubated with FITC-conjugated goat anti-mouse IgG or donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, 1:200) for 2 h at room temperature. In order to visualize chromatin the outgrowths were covered with Vectashield Mounting Medium containing propidium iodide (Vector Laboratories) and the blastocysts were incubated in glass bottom dish (WillCo Wells B.V., The Netherlands), in droplets of chromomycin A (10 μg/ml in Ca^{2+} Mg^{2+} - free PBS, Sigma), for at least 30 min under paraffin oil, at 37ºC. Negative controls were performed using the same procedure with omitting the primary antibody step. Specimens were scanned using LSM 510 ZEISS laser scanning confocal microscope (Carl Zeiss Jena, Germany). The number of Oct4-positive nuclei was counted and determined as "+++" in case of more than 7 positive cells, "+" when less than 7 cells were stained and "-" for outgrowths devoid of *Oct4* expressing cells. Then the most representative, single optical sections were collected.

Statistical analysis

Data were analysed using χ^2 test. Differences at $P<0.05$ were considered significant.

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