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Analysis of Polarity of Bovine and Rabbit Embryos by Scanning Electron Microscopy[1]

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ABSTRACT

Cellular polarization during preimplantation development of the embryo is believed to be a crucial event in the transition of a zygote to a blastocyst stage embryo with morphologically and functionally differentiated cell types. While extensive studies have been done on polarity development in mouse embryos, little information is available in other species, particularly in cattle. The objective of this study was to examine the initiation of polarity by microvilli distribution on blastomeres of cattle and rabbit embryos using scanning electron microscopy (SEM). Bovine embryos were obtained by in vitro fertilization of in vitro-matured follicular oocytes. Rabbit embryos of various stages were collected from superovulated rabbits. Intact embryos and isolated blastomeres were examined in both species. Blastomeres from 1- to 8-cell embryos in both cattle and rabbits showed no polarity. The onset of transitional polarization of microvillous distribution occurred in some blastomeres of cattle embryos at the 9- to 15-cell stage; but typical, distinct polarity was not manifested until after the 16-cell stage with approximately 40% polar cells per embryo. In the rabbit, blastomere polarity occurred one cell cycle later, with 46% polar cells per embryo after the 32-cell stage. The difference in cell numbers at the time polarity is evident is probably related to the different cell stages for embryo compaction and blastocyst formation in the two species.

INTRODUCTION

Along with an increase in cell number in the embryo, embryonic development is characterized by progressive specialization of cells and their concomitant loss of ability to form other cell types. Mammalian embryonic cells undergo the first distinct morphological and functional differentiation at the blastocyst stage, in which two cell types arise: the inner cell mass (ICM) and the trophectoderm (TE). In the mouse, the divergent differentiation of these two cell lineages begins with the surface and cytoplasmic polarization at the 8-cell stage [1–5]. Cleavage of these 8 polarized cells results in the generation of a 16-cell embryo having an average of 9–11 outer polar cells and 5–7 inner, nonpolar cells [6–9]. The two cell types in the mouse blastocyst are thought to arise from these two populations of precursor cells [5]. The peripheral polar cells are presumed to give rise to the outer layer of the blastocyst, the TE, whereas the central nonpolar cells are believed to form the ICM [8, 10, 11]. Examination of the surface microvillous polarization of intact and separated blastomeres by scanning electron microscopy (SEM) has been reported in the mouse [3] and more recently with preliminary results in the rabbit [12]. However, surface polarization of bovine blastomere microvilli has never been reported, and bovine embryonic polarity in terms of fluorescein isothiocyanate-concanavalin A (FITC-Con A) staining has been reported only briefly [13].

The objectives of the present study were to examine systematically, by means of SEM, the characteristics of the blastomere surface microvilli and to determine the timing of polarity development of the blastomeres in both bovine and rabbit embryos in terms of the blastomere surface microvillous distribution. Our ultimate goal in this research is to examine nuclear totipotency of the morphologically differentiated polar and nonpolar cells by nuclear transfer.

MATERIALS AND METHODS

Collection of Bovine Embryos

Bovine embryos were obtained from in vitro fertilization (IVF) of in vitro-matured (IVM) follicular oocytes. Ovaries were collected at a local slaughterhouse in an insulated container and transported to the laboratory in warm Dulbecco's PBS (Gibco, Grand Island, NY) containing 0.1% polyvinyl alcohol (Sigma, St. Louis, MO) (DPBS-PVA) and antibiotics-antimycotics (Gibco). The ovaries were washed twice in warm DPBS-PVA before collection of oocytes. Follicular oocytes were collected by aspirating small antral follicles (2–7 mm in diameter) with an 18-gauge needle and a 12-ml syringe. The aspirated cumulus oocyte complexes were stored in DPBS-PVA medium filtered through a 0.2-μm-diameter Millipore filter (Bedford, MA).

The cumulus-oocyte complexes were selected, on the basis of their appearance, with at least four compact layers of cumulus cells. These oocytes were then matured and fertilized as described previously [14]. Briefly, the complexes were cultured for 22 h at 39°C (5% CO_2, 95% humidified air) in 100-μl microdroplets of Medium 199 with Earle's salts containing 25 mM Hepes and 7.5% fetal calf serum (M199 + FCS) with added ovine FSH (0.5 μg/ml), ovine LH (NIDDH; 5 μg/ml), and estradiol (1 μg/ml) [14]. The droplets were covered with medical fluid (Dow Corning Medical Fluid 360, Midland, MI). For fertilization, one or two straws of frozen semen from one Holstein bull were thawed in a 37°C water bath. The sperm were processed to induce capacitation in vitro with calcium ionophore

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A23187 or heparin [14], and sperm were coincubated with oocytes for fertilization in Brackett's defined medium [15] for 6 h. The embryos were then transferred into 100-μl droplets of M199 + FCS for further culture. The medium was changed every 48 h. The development stages of the embryos were determined by phase-contrast microscopy, and embryos were harvested at different stages.

Collection of Rabbit Embryos

Sexually mature Dutch-belted rabbits were superovulated [16]. Eight- to sixteen-cell stage embryos were collected surgically from the oviducts of superovulated does 46 h after insemination and injection with 0.5 mg of pituitary LH (PLH; Burns-Biotech, Inc., Omaha, NE). At 54 h, 17- to 32-cell stage embryos and at 63 h, 33- to 66-cell stage embryos were collected from the oviducts of the superovulated does. Embryos were flushed with DPBS-PVA at room temperature.

Removal of Accessory Coats and Isolation of Single Embryo Blastomeres

The zona pellucida of the bovine embryos, and the mucin coat plus the zona pellucida of the rabbit embryos, were removed by incubation in prewarmed acidic DPBS-PVA (pH = 2.5) for approximately 2 min at room temperature and then incubated with 0.5% pronase in DPBS-PVA at 39°C for 5–15 min [16]. The zona-free embryos in both cattle and rabbits were then incubated in Ca^{2+}-free DPBS-PVA at 39°C for 20 min. They were carefully disaggregated into individual blastomeres by pipetting with a fire-polished micropipette (50–120 μm inside diameter). All blastomeres from a single embryo were kept together in a single well of a multiple-microwell plate (Nunclon Delta SI, Nunc, Denmark).

Scanning Electron Microscopic Analysis

After incubation in Ca^{2+}-free medium, intact embryos and disaggregated blastomeres of various stages were fixed in 3% glutaraldehyde plus 0.5% paraformaldehyde in Hanks' balanced salt solution (HBSS) containing 0.1% polyvinyl alcohol (HBSS-PVA) [12]. They were then washed in three changes of HBSS-PVA and plated onto 6 × 6-mm glass coverslips coated with poly-L-lysine (M_r 4000–15 000; Sigma). The embryos on the coverslips were postfixed in 1% osmium tetroxide in HBSS-PVA for 1 h. After being rinsed with distilled water, the coverslips were incubated for 2 h in 2% tannic acid solution, rinsed, and reosmicated for 1 h in 1% osmium tetroxide in distilled water. After osmication and rinsing, the specimens were dehydrated in a series of increasing concentrations of ethanol starting with 25% ethanol. Samples were critical point dried by use of CO_2, mounted, and coated with gold. Observations were made with a Zeiss DSM-960 (Thornwood, NY) SEM at an accelerating voltage of 10 kV. A total of 95 bovine embryos and 40 rabbit embryos were used, and blastomeres isolated from these embryos were observed. Only embryos with a high proportion of blastomeres recovered (average of 96%) were evaluated for polarity development.

RESULTS

General Comments

On the basis of surface microvillous distribution of the plasma membrane, blastomeres were generally classified into three groups: nonpolar, polar, and uncertain. Nonpolar blastomeres had a uniform distribution of microvilli on the whole surface of the plasma membrane (Figs. 1a and 2a). In polarized blastomeres, one pole was completely covered, or at least half of the surface of the plasma membrane was covered, by uniform microvilli, but the remaining areas of the plasma membrane had smooth surfaces (Figs. 1b and 2b). In the case of partially polarized blastomeres (Fig. 2b), often there was a relatively higher density of microvilli at the border of the polarized area. The uncertain group includes the blastomeres without microvilli or in several cases, with a few microvilli scattered on the surface of the plasma membrane (Fig. 3a). Some blastomeres had many cytoplasmic blebbings (Fig. 3b) on the surface; these blastomeres may have been degenerating. These classifications are applicable to both cattle and rabbit blastomeres, although the characteristics of the microvilli differ among the two species. The microvilli of the rabbit blastomeres were cylinder-like structures and were much thicker than the thread-like microvilli of the bovine blastomeres (compare Figs. 1 and 2).

Embryos of both species examined in the present study were considered normal embryos on the basis of the embryonic development data in our previous publications [14, 16, 17]. The fact that more bovine blastomeres were classified as "uncertain" suggests that cattle embryos produced by IVM/IVF procedures were of lower quality.

Cattle Embryos

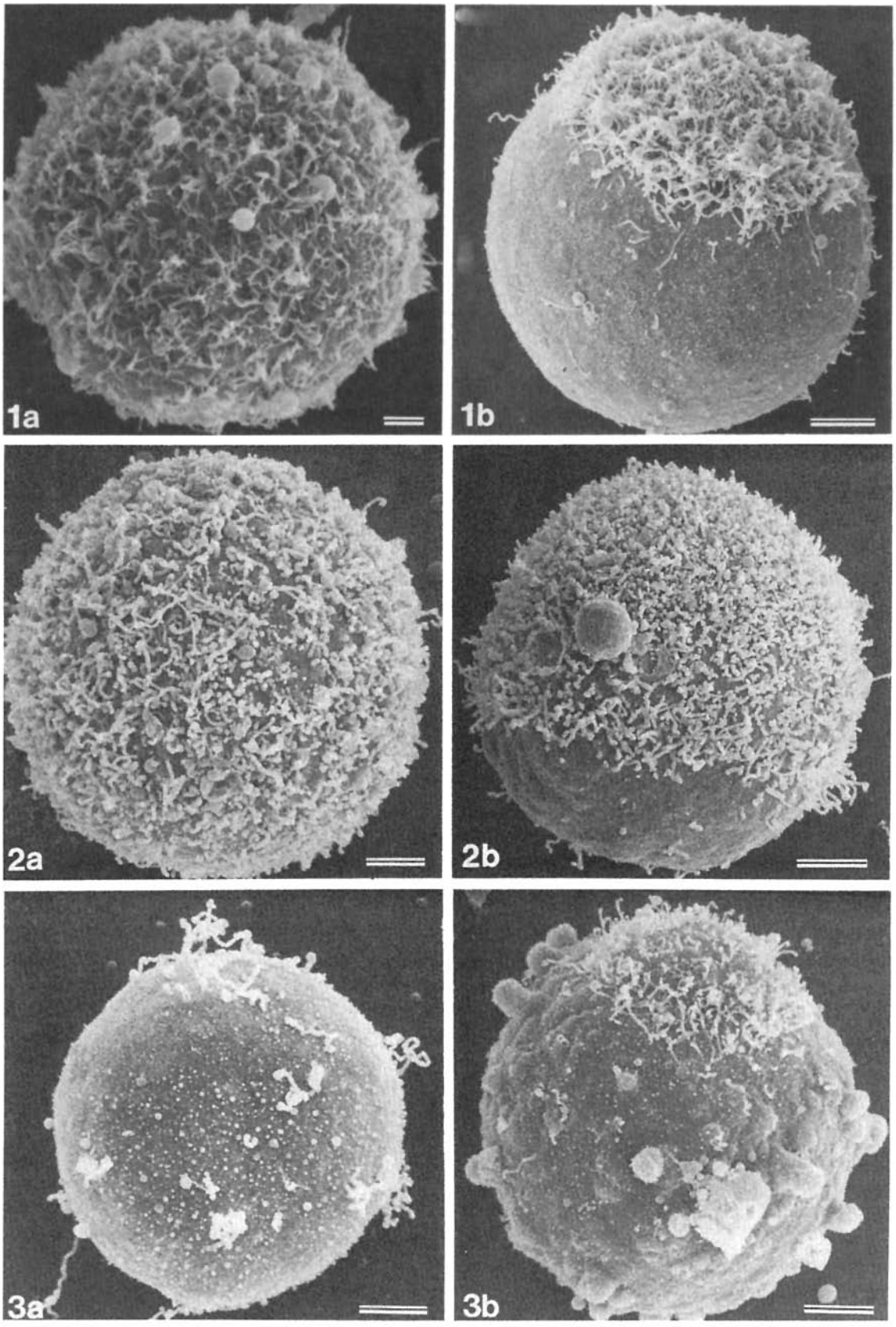
SEM observations of intact embryos were made on the free surface of approximately half of the outer blastomeres

PLATE I.

FIG. 1. a) Nonpolar blastomere from a 46-cell stage bovine embryo. The cell surface is covered with long uniform microvilli. Bar represents 2 μm. b) Polar blastomere from a 20-cell stage bovine embryo. The microvilli are concentrated at one pole of the cell. The microvilli-devoid region shows a smoother surface on the plasma membrane. Bar represents 5 μm.

FIG. 2. a) Nonpolar blastomere from a 17-cell stage rabbit embryo shows uniform microvilli on the cell surface. Bar represents 5 μm. b) Partially polarized blastomere from a 63-cell stage rabbit embryo. Bar represents 5 μm.

FIG. 3. a) Blastomere from a 34-cell stage bovine embryo classified as "uncertain" for polarity. Note that only a few microvilli are scattered on the cell surface. Bar represents 5 μm. b) Polar blastomere from an 18-cell stage bovine embryo showing some signs of degeneration (buddings and reduced microvilli) on the cell surface. Bar represents 5 μm.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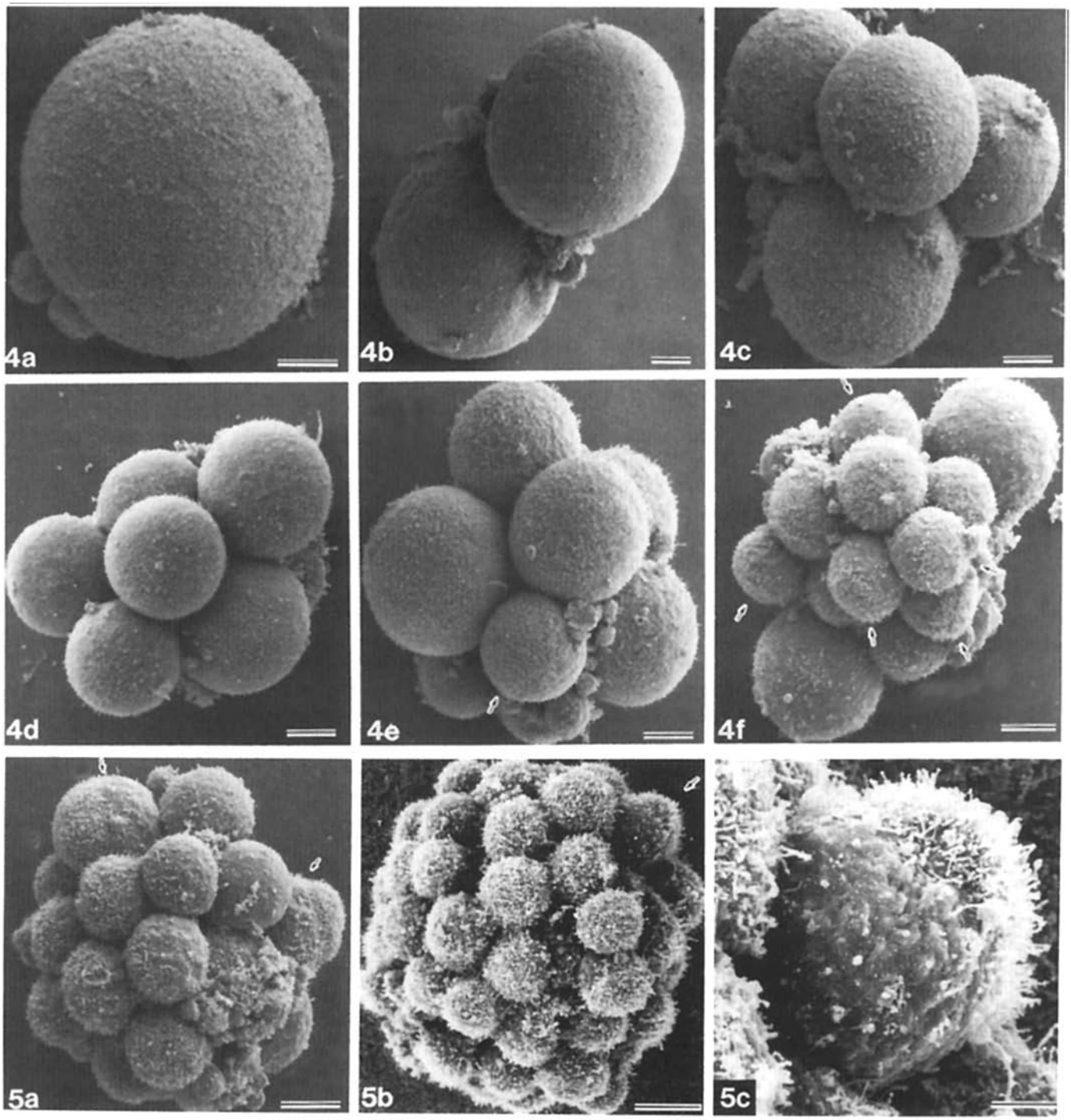
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(on the opposite side of coverslips) in situ as well as on disaggregated blastomeres. Tables 1 and 2 summarize the results on polarity of microvillous distribution of external blastomeres of intact embryos and of disaggregated blastomeres, respectively.

One-cell zygotes were characterized by uniform distribution of well-developed microvilli (Fig. 4a). The blastomeres from 2- to 8-cell embryos also had uniform distribution but with relatively short microvilli and occasionally a few long microvilli (Fig. 4, b-d). The density of the long microvilli increases at the 9–15-cell stage and becomes dominant after the 16-cell stage (compare Fig. 4, a-f).

Changes in the distribution pattern of the microvilli were first noted in the 9- to 15-cell bovine embryos, where two different cell types were observed: cells that were uniformly covered with microvilli (nonpolar) and cells having a single pole of long microvilli (polarized, Fig. 4e). Although many intact embryos (55%; Table 1) contained one or more polar blastomeres, only 15% of the isolated blastomeres showed membrane polarity at this stage (Table 2). Most other blastomeres (nonpolar) were covered with relatively dense long and short microvilli. In the polar cells at this stage, the pole region contained mostly long microvilli but the rest of the area was often covered with uniformly distributed short microvilli (not shown).

From the 16-cell stage onward, almost all (40 of 41) bovine embryos examined in this study, except for one 17-cell embryo, possessed one or more polarized blastomeres (Figs. 4f and 5a). The polarized cells constituted 40% of the total blastomeres for embryos between the 16-cell stage and the late morula stage. The ranges of variation in the proportion of polar cells among embryos are shown in Table 2. There was an increase in the proportion of uncertain blastomeres in the later-stage embryos (33–74 cells), probably due to degeneration of the cells. The SEM observations on whole embryos revealed that the poles of microvilli of the outer blastomeres were not always restricted to the free surface (facing the external milieu). In some cases, the equatorial plane of polarized cells was located on the free surface (Fig. 5a). It is possible that this was caused by partial rotation of blastomeres during processing of the embryos; blastomeres that rotated would be likely to be detached.

TABLE 1. Polarity of outer blastomeres of intact bovine embryos.*

Species	Embryo stage	No. of embryos	Embryos with polar cells (%)		
			Yes	No	Uncertain
Cattle	2–8 cells	11	0	100	0
	9–15 cells	11	55	27	18
	16–32 cells	16	94	0	6
	Late morula	5	100	0	0

*Embryos with one or more polar blastomeres were scored as yes; embryos with no visible polar blastomeres were scored as no; uncertain embryos referred to embryos with most of their visible outer blastomeres bald or covered with very few microvilli. See detailed classification in the text.

Rabbit Embryos

Cell polarity as indicated by distribution of microvilli was not detectable until after the 32-cell stage in the rabbit (Table 2). After the 32-cell stage, 17 of 18 embryos showed polarity (Table 2), with 46% of their blastomeres being polarized. The ranges of variation in the proportion of polar blastomeres among the rabbit embryos are given in Table 2. These blastomeres showed a single pole of microvilli or had a membrane surface only approximately half covered with microvilli (Fig. 2b); the nonpolar blastomeres were uniformly covered with microvilli (Fig. 2a), as observed in bovine embryos. In the rabbit embryos, the proportion of "uncertain" blastomeres was much smaller than in the bovine embryos; and in contrast to findings for the bovine embryos, this proportion remained constant throughout the stages examined after onset of polarity of those embryos.

For intact embryos, the majority of the outer blastomeres at around the 64-cell stage showed microvillous poles on the outer surface of the cells facing the milieu (Fig. 5, b and c). This observation also contrasts with findings for the bovine morula stage embryos, in which the microvillous poles were not always restricted to the free surface (Fig. 5a).

DISCUSSION

Our SEM analysis of the surface microvilli of embryo blastomeres demonstrates that the size and number of microvilli varied with species (Figs. 1 and 2) and stage of development (Fig. 4, a-f). The microvilli of the rabbit blastomeres were thicker than those of the bovine blastomeres. In addition, the initiation of polarity of microvilli on bovine blastomeres in some embryos occurred at the 9–15-cell stage. However, this initial polarity was often characterized as a

PLATE II.

FIG. 4. a) Bovine 1-cell zygote 20 h after in vitro insemination and fertilization; note the second polar body. Bar represents 20 μm. b) Bovine 2-cell embryo; microvilli are decreased in size and number compared to those in the 1-cell zygote (Fig. 4a). Bar represents 20 μm. c) Bovine 4-cell embryo; note that short and long microvilli uniformly cover the cell surface. Bar represents 20 μm. d) Bovine 8-cell stage embryo; short and long microvilli uniformly cover the cell surface. Bar represents 20 μm. e) Bovine 9–15 cell embryo. Some of the blastomeres show a single pole of microvilli (arrow), although the other blastomeres were uniformly covered with microvilli. Note the change in size of microvilli. Bar represents 20 μm. f) Bovine ≥ 16-cell embryo. Some of the blastomeres show microvillous polarization (arrows). Relatively large blastomeres located in the exterior of the cell mass show asynchronous cell division. Note that long microvilli are dominant at this stage. Bar represents 20 μm.

FIG. 5. a) Bovine morula stage (32-cell) embryo. Note equatorial plane of a polarized cell, which is dividing into the microvillous pole and the other non-microvillous pole. Bar represents 20 μm. b) Rabbit morula (approximately 64-cell) stage embryo. Note the difference in orientation of poles and the size of microvilli between the rabbit and bovine morulae (Fig. 5a). Bar represents 20 μm. c) Higher magnification of Figure 5b as indicated by the arrow. Polarization of microvilli can be observed on one pole of the cell. Bar represents 5 μm.

TABLE 2. Polarity of disaggregated blastomeres of bovine and rabbit embryos.*

Species	No. of cells per embryo	No. of embryos	Blastomeres evaluated	Blastomeres, mean %, (range)		
				Nonpolar	Polar	Uncertain
Cattle	1–8	26	69	100	0	0
	9–15	6	61	75 (44–100)	15 (0–40)	10 (0–22)
	16–32[a]	13	250	50 (31–100)	40 (0–57)	10 (0–44)
	33–74	7	322	38 (19–63)	40 (19–52)	22 (0–62)
Rabbit	8–15	10	73	100	0	0
	16–32	11	262	99 (95–100)	0	1
	33–66[a]	19	951	52 (36–100)	46 (20–64)	2 (0–8)

*Classification of polarity of blastomeres is described in the text.
[a]Only one embryo in the group had no polar blastomeres.

pole of long microvilli and a uniform distribution of short microvilli on the rest of the surface. This probably is a transitional stage of polarity development. Distinct poles of microvillous distribution in bovine blastomeres were manifested after the 16-cell stage, with approximately 40% of the blastomeres being polarized. This pattern and percentage of polar cells remained throughout the late morula stage in cattle (33–74-cell stage). The finding on the timing of embryonic blastomere polarity contrasts with observations in a recent preliminary report [13]; in that study, onset of polarity was found at the 32-cell stage by means of fluorescent microscopy after FITC-Con A staining of dissociated bovine blastomeres.

In rabbits in the present study, the onset of polarity of microvilli on embryonic blastomeres did not occur until after the 32-cell stage; this is consistent with a previous study using FITC-Con A staining of dissociated blastomeres [12]. These observations in both cattle and rabbits differ from findings in the mouse, where polarization is seen at the 8-cell stage [5,18]. However, if one considers the differences in cell numbers in relation to the events of embryo compaction and blastocoele formation among the different species, our observation on the timing of polarity development in cattle and rabbit embryos seems to be in harmony with the mouse data suggesting that polarity occurs just before compaction. In the mouse, embryo compaction occurs at the 8–16-cell stage, whereas in cattle and rabbits, embryo compaction is manifested at the 16–32-cell and 32–64-cell stages, respectively [1, 19–21]. Blastocyst formation normally occurs at the next cell cycle after firm compaction in mouse, cattle, and rabbit embryos [20, 21], i.e., the 32-, 64-, and 128-cell stages, respectively. Therefore it seems reasonable to conclude that a general pattern of timing of polarization among species is related to the onset of embryo compaction, which differs relative to embryonic cell number among the three species.

An interesting observation in the present study was that in cattle the characteristics of the surface microvilli of the 2–8-cell embryos distinguish these embryos from the zygotes and the later-stage embryos. The 2–8-cell blastomeres have a uniform distribution of short microvilli with very few long microvilli (Fig. 4, b-d). The density of the long microvilli increases at the 9–15-cell stage, but short microvilli are still obvious, particularly in polar blastomeres. The long microvilli become dominant on the blastomere surface after the 16-cell stage. In addition, the cattle embryos exhibited a transitional phase of polarity at the 9–15-cell stage, in which only a few blastomeres in some embryos showed incomplete poles of microvilli distribution. These phenomena were not observed in the rabbit embryos and have not been reported in other species.

At the 16–32-cell stage and onward, bovine embryos reached a stable proportion of polar vs. nonpolar blastomeres. However, in no stage have we found a single embryo containing only polarized blastomeres (Table 2). This is also true for rabbit embryos, as shown in this study and elsewhere [12], in contrast to mouse embryos, most of which exhibit 100% polar cells at the 8-cell stage [1, 4, 5]. These differences may be associated with the fact that polarization already has occurred in the mouse when none of the blastomeres in an 8-cell stage embryo are completely surrounded by other blastomeres. The highest proportion of polar cells found in the present study was 57% for cattle and 64% for rabbit embryos (Table 2).

We also observed a relatively high proportion of blastomeres with no surface microvilli, or only few surface microvilli, in cattle embryos (Fig. 3a). These blastomeres were classified as uncertain with respect to polarity and were interpreted as possibly degenerating, as reported in the mouse [23]. This may be partly attributable to the fact that more IVM/IVF embryos undergo degeneration than do in vivo-developed bovine embryos. Interestingly, the proportion of uncertain blastomeres increased at the 32–64-cell stage, a finding consistent with the "morula to blastocyst" block observed during culture of bovine embryos. Blastomeres devoid of microvilli have also been reported in the mouse

[23]. There were fewer such blastomeres in rabbit embryos compared to bovine embryos (Table 2), suggesting that fewer blastomeres degenerate before the blastocyst stage in the rabbit. This agrees well with our blastomere separation experiments, in which 95–100% of rabbit blastomeres were separable and viable [17] while cattle blastomeres were much more fragile and more difficult to separate without causing damage (X. Yang, unpublished observations and this study).

Because uneven numbers and sizes of blastomeres were seen during cleavage, it is likely that early cell divisions are not always synchronous, particularly in cattle as shown in the present study and reported previously [24]. In the mouse, it is believed that the inside nonpolar blastomeres are more likely to contribute to the ICM of the blastocyst than are the outside, polar cells, which more likely contribute to TE [25–27]. The totipotency of the polar, outer cells vs. the nonpolar, inner cells has been compared in the mouse by reaggregation of the different types of cells and embryo transfer [28]. Interestingly, postimplantation development of aggregates of outer, polar vs. those of inner, nonpolar cells was found to be normal and to be comparable to that of control embryos. This suggests that blastomeres of embryos that are morphologically differentiated in polarity may not have become functionally differentiated with regard to developmental potency. In contrast, a recent preliminary study with nuclear transfer of polar vs. nonpolar blastomeres showed lower developmental potential of the polar blastomeres [13]. However, it is not clear whether this variation is due to species difference or to differences in the approaches used.

In conclusion, the present research, in which microvillous distribution of embryonic blastomeres was thoroughly examined by SEM, provides strong evidence regarding the development of polarity in both cattle and rabbit embryos. There is a possible suggestion that bovine and rabbit embryos manifest their polarity on their outside blastomeres (Fig. 4, e and f and 5, a and b), but further investigation is needed to quantify the allocation of polar vs. nonpolar blastomeres. This research is significant in being the first systematic evaluation of polarity development of microvillous distribution in domestic species. The current results on the timing of polarity development and the proportion of polar vs. nonpolar cells in bovine and rabbit embryos are of considerable value for the design of future research to explore nuclear totipotency by nuclear transfer in both species [13, 21].

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