Expression of β-Galactosidase in Preimplantation Ovine and Porcine Embryos

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Abstract. **Knowledge regarding the timing of embryonic expression of the mammalian genome is of relevance for the development of preimplantation diagnostic methods for human genetic diseases. For development of preimplantation diagnosis of lysosomal storage diseases, it will be necessary to know at which embryonic stage the genes for lysosomal enzymes are expressed. In previous studies by other investigators, it has been shown that lysosomal α- and β-galactosidase and β-glucuronidase in murine embryos increase 50- to 100-fold in activity between the two-cell and late blastocyst stage. We describe here expression of lysosomal β-galactosidase in preimplantation ovine (two-cell through midblastocyst) and porcine (two-cell through late blastocyst) embryos. Expression of β-galactosidase in ovine and porcine preimplantation embryos followed a similar rate of increase as that described for murine embryos. Activity of β-galactosidase increased over 10-fold between the two- to four-cell and midblastocyst stages in ovine embryos, and 300-fold between the two-to four-cell and late blastocyst stages in porcine embryos. Activity expressed on a per cell basis was relatively constant in ovine embryos, as has been described in murine embryos, and increased approximately 5-fold on a per cell basis in porcine embryos. Activity of β-galactosidase in ovine and porcine embryos initially was >12-fold on a per cell or per embryo basis than in murine embryos evaluated. The knowledge of β-galactosidase embryonic expression may provide the basis for preimplantation diagnosis of genetic β-galactosidase deficiency in these species.** [P.S.E.B.M. 1990, Vol 194]

The time of initiation of expression of the mammalian genome is of clinical relevance to development and implementation of methods and techniques of preimplantation diagnosis of human genetic diseases (1, 2). Embryonic expression of the mammalian genome occurs as early as the four-cell stage embryo and is required for normal development (3–7). The specific genetic defect for many human inherited diseases has been documented, and this knowledge provides the basis for prenatal diagnosis and therapeutic abortion (8–11). The methods currently employed most commonly are amniocentesis and chorionic villus biopsy with the subsequent, often enzymatic, diagnosis of the specific genetic defect. The disadvantages of these methods include the risk of abortion to normal fetuses, parental stress related to abortion, and moral objections of carrier parents to abortion (9, 10, 12).

Biochemical microassays allow sensitive and direct measurement of lysosomal and cytoplasmic enzymes in single embryos (13–18). Since early gene expression has been demonstrated for both lysosomal and cytoplasmic enzymes, preimplantation diagnosis of genetic diseases, for which the specific genetic defect has been identified, using microassays is feasible. The techniques to obtain cells from early cleavage embryos for diagnostic purposes and the methods to successfully transfer intrauterinely the remainder of an at-risk embryo are well established (19–22). Successful preimplantation diagnosis in a murine model of the Lesch-Nyhan syndrome has recently been reported utilizing such tech-

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niques (18). Application of biochemical microassays to other animal models should soon result in preimplantation diagnosis of other genetic disorders including lysosomal storage diseases.

The purpose of this investigation was to determine the preimplantation embryonic expression of ovine and porcine lysosomal β-galactosidase using microfluorimetric techniques, and to evaluate the potential of these large animal species models for development of human preimplantation diagnostics.

Materials and Methods

Ovine Embryo Collections. Synchronization, superovulation, and embryo collections of 18 outbred ewes of the Suffolk, Finn, and Columbian breeds were performed during the natural breeding season as described previously (23). A progesterone pessary (Upjohn Co., Kalamazoo, MI) containing 60 mg of medroxyprogesterone acetate was placed intravaginally for 14 days for synchronization in each ewe. Pessaries were checked daily for retention. Superovulation commenced the morning of Day 13 after pessary insertion. Five milligrams of follicle-stimulating hormone (Burns-Biotech, Omaha, NB) were injected intramuscularly at 8 A.M. and 8 P.M. on Days 13, 14, and 15. The pessaries were removed the morning of Day 14. Ewes were checked for heat beginning the evening of Day 14, although none showed heat until the morning of Day 15. Those in standing heat were bred to rams. The rams used were six outbred Suffolk and Columbian rams. Each ewe was bred at least twice total, on Day 15 and the morning of Day 16. The time of the first breeding (time of first standing heat) was designated as $t = 0$.

Embryos were collected surgically under general anesthesia. Ewes were anesthetized with ketamine (2.2 mg/kg) and Xylazine (0.22 mg/kg) intravenously and placed in dorsal recumbency. After surgical preparation, a ventral midline incision was made to enter the abdominal cavity and the uterus was exteriorized. Each uterine horn was flushed separately retrograde using phosphate-buffered saline (pH 7.2) with 5% heat-inactivated fetal calf serum. Embryos were collected from five ewes at 60–62 hr postbreeding, six ewes at 84–86 hr, three at 120–122 hr, and four at 144–146 hr. Number of ovulation points in each ovary was determined and recorded.

Porcine Embryo Collections. Yorkshire/Hampshire crossbred sows and gilts were observed for heat and then hand mated to Yorkshire/Hampshire crossbred boars. Time of breeding was designated as $t = 0$. Porcine embryos were flushed with phosphate-buffered saline (pH 7.2) containing 5% heat-inactivated fetal calf serum from reproductive tracts obtained from the inseminated sows or gilts at slaughter as described previously (24). Embryos were collected at 24–30, 65–72, 89–96, 119–126, 149–156, and 173–180 hr postbreeding.

Murine Embryo Collections. Murine embryos were collected as previously described (17) and assayed in conjunction with ovine and porcine embryos as controls. Swiss-Webster female mice were placed in conjunction with Swiss-Webster male mice and observed each morning for vaginal plugs. Oviducts and uteri were dissected from female mice that had exhibited vaginal plugs and flushed with phosphate-buffered saline (pH 7.2) with 5% heat-inactivated fetal calf serum. The midpoint of the dark cycle was assumed as the approximate time of mating and was used as $t = 0$. Embryos were collected at 58–60, 66–68, 80–82, 88–90, and 102–104 hr postbreeding.

Blastomere Counts and Definitions. Direct counts of blastomeres were made in phosphate-buffered saline (pH 7.2) with 5% heat-inactivated fetal calf serum, with a phase contrast inverted microscope at ×40-100 magnification or, for embryos containing up to 12 cells, on a dissecting microscope at ×70 magnification. More advanced embryos were categorized as morulae until a blastocoel was evident. Blastocysts were categorized as early blastocyst (blastocoel just evident), midblastocyst (blastocoel expanded and occupying 50–95% of the embryo), and late blastocyst (hatched from or markedly thinned zona pellucida).

β-Galactosidase Assay. Embryos were transferred in 0.5 to 2 ml of phosphate-buffered saline with 5% heat-inactivated fetal calf serum into 5 to 10 ml of 0.14 *M* NaCl. Single embryos were then frozen at −80°C in 1.0-μl droplets of 0.14 *M* NaCl. Before assay, the droplets with embryos were lyophilized at −70°C for 2 to 4 hr. Single embryo assays for β-galactosidase were performed with modifications of the procedure described by Wudl and Paigen (25). The assay mixture was 1 m*M* 4-methylumbelliferyl-β-D-galactopyranoside (Sigma Chemical Co., St. Louis, MO) and 0.1 *M* sodium acetate (pH 4.6) with 0.1% of bovine serum albumin. Lyophilized embryos were incubated in 1.0 μl of reaction mixture for 1 hr at 37°C in 5% CO_2 under a mixture of paraffin oil/hexadecane (60:40, v:v) to prevent dessication. The reaction was stopped and pH elevated to approximately 10.6 by addition of 0.15 μl of a saturated solution of Na_2CO_3. The amount of 4-methylumbelliferone (4-MU) produced was determined by exciting the droplets at 365 nm and measuring fluorescence at 450 nm with a microscope-photomultiplier system (Nikon Diaphot-TMD inverted microscope, Nikon TMD-EF epi-fluorescence unit, and Nikon P-1 photometer) similar to the one described by Wudl and Paigen (25). The fluorescence readings were compared witho standard curves generated with 1.15-μl droplets of 0.1 *M* $NaHCO_3$ (pH 10.6) containing specific standard concentrations of 4-MU.

Preliminary assays of murine and ovine embryonic

β-galactosidase with addition of 25 m*M* glucono-δ-lactone (Sigma), an inhibitor of bovine and ovine neutral nonlysosomal β-galactosidase, indicated that the the inhibitor had no effect on production of 4-MU (data not shown). For this reason, the assays were done without glucono-δ-lactone.

Results

The numbers and stages of ovine, porcine, and murine embryos obtained are summarized in Table I. Cleavage rates were similar to those observed by other investigators (7, 17, 26). A mean of 4.6 embryos/ewe and an overall recovery rate of 42% of 199 ovulations were obtained. These values are similar to those reported by others (7, 23).

Expression of ovine, porcine, and murine embryonic β-galactosidase on a per cell and per embryo basis are shown in Table I and Figure 1. Values obtained for murine embryos closely correlate with previously published values of the strain with the Bgl-s^h (high activity) allele (17). Total embryonic β-galactosidase activity of ovine and murine embryos increased roughly 10-fold between two- and four-cell and midblastocyst stages whereas porcine embryo activity increased roughly 75-fold between similar stages. Ovine and murine embryos exhibited a plateau of β-galactosidase activity at the morulae and early blastocyst stages.

Embryonic β-galactosidase activity expressed on a per cell basis from two- to four-cell through early blastocyst stages, with estimation of the number of cells in morulae and early blastocysts at 32 (26), was relatively constant in ovine and murine embryos but in porcine embryos increased approximately 5-fold between these stages (Table I). The per cell β-galactosidase activity of ovine embryos was 12- to 24-fold greater than murine per cell activity for all comparable stages measured. The per cell activity of porcine embryos was 12- to 16-fold greater than murine per cell activity of comparable stages.

Discussion

The embryonic expression of ovine and murine preimplantation β-galactosidase increased as a function of cell number with relatively constant activity on a per cell basis after the two- to four-cell stage. The initial decrease in activity in ovine embryos on a per cell basis between two- and four-cell and six- and eight-cell stages is similar to a decrease in β-galactosidase activity reported in murine embryos between the two- and four-cell stages (17). This decrease is likely due to dilution of maternal β-galactosidase before appreciable transcription and translation of the zygotic genome. The plateau of activity seen with ovine and murine embryos is probably a result of the transition from morulae to early blastocyst in which there is a reorganization of the cellular mass and an accumulation of fluid in the blastocoel with relatively little cellular division (26). Expression of porcine embryonic β-galactosidase, however, increased also on a per cell basis, resulting in a more pronounced increase in total embryonic activity. The relative rate of increase in porcine β-galactosidase is similar to that seen with lysosomal α-galactosidase and β-glucuronidase in murine embryos (13, 14). These interspecific variabilities underline the need for caution when extrapolating from one species to a distantly related one.

Absolute activities of ovine and porcine embryonic β-galactosidase, on a per cell or per embryo basis, ranged from approximately 12- to 24-fold greater than activity of murine embryos at similar stages of development. The most significant contributing factor to this difference was a 10.5-fold greater volume of ovine and porcine embryos as compared with murine embryos (data not shown, determined by measuring diameter of embryos). Because of these large volume differences,

Table I. Summary of Embryo Collections and β-Galactosidase Activity ($\times 10^{-14}$ mol of 4-MU/hr) on a per Embryo and per Cell (Estimated)[a] Basis in Ovine, Porcine, and Murine Embryos

Stage	Ovine			Porcine			Murine		
	No. assayed	Mean ± SE activity	Activity/ cell[a]	No. assayed	Mean ± SE activity	Activity/ cell[a]	No. assayed	Mean ± SE activity	Activity/ cell[a]
2–4 cell	6	155 ± 64	51.7	19	32 ± 11	8.1	—[b]	—[b]	—[b]
6–8 cell	12	266 ± 62	34.0	5	123 ± 36	18.0	13	12.2 ± 2.6	1.5
10–14 cell	10	418 ± 42	37.0	—[b]	—[b]	—[b]	—[b]	—[b]	—[b]
16–20 cell	14	752 ± 44	45.4	2	230 ± 44	28.8	16	31 ± 4.8	1.9
Morula	22	966 ± 63	30.2	8	1326 ± 271	41.5	30	82 ± 11	2.6
Early blastocyst	12	997 ± 61	31.2	—[b]	—[b]	—[b]	14	74 ± 11	2.3
Midblastocyst	3	1489 ± 145	—[c]	8	2384 ± 197	—[c]	20	151 ± 18	—[c]
Late blastocyst	—[b]	—[b]	—[c]	7	9381 ± 1197	—[c]	16	466 ± 51	—[c]

[a] Per cell activity was estimated by dividing the mean activity at a specific stage by the cell number present at that stage.
[b] Indicates no embryos at this stage assayed.
[c] Indicates activity per cell not estimated for these stages.

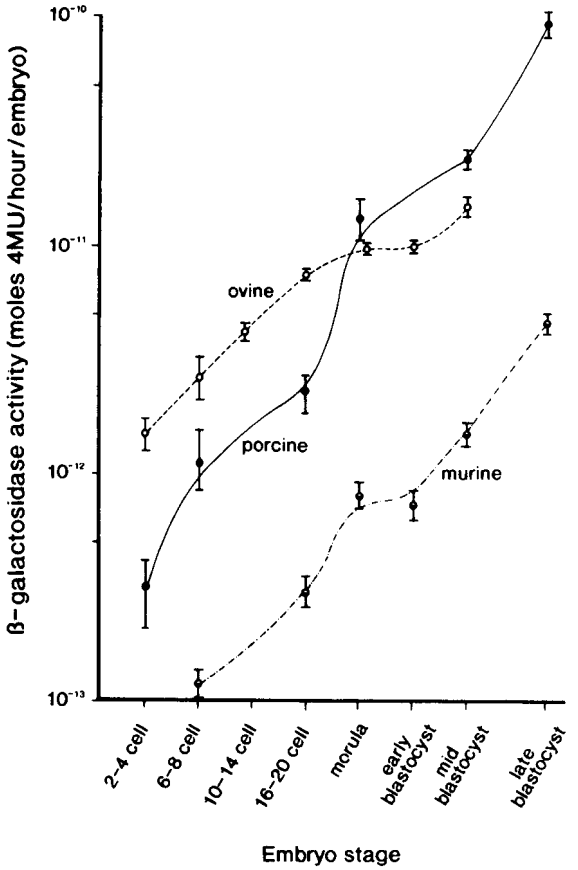

Figure 1. Embryonic expression of β-galactosidase in ovine, murine, and porcine embryos of developing stages. The Y axis is a logarithmic scale. Mean activity with standard error of the mean indicated by the vertical bars.

interspecies comparisons of preimplantation enzyme expression are best done by comparing relative rates of increase. In summary, the increases of β-galactosidase activity in ovine and murine embryos were relatively parallel, whereas porcine embryonic β-galactosidase underwent a more dramatic increase.

The increases in preimplantation enzyme expression in murine embryos have been shown to be due to embryonic expression of the zygotic genome (13, 16, 17, 27, 28). This study confirms embryonic protein synthesis in early cleavage porcine and ovine embryos, although it cannot be conclusively stated that this is due to expression of zygotic mRNA. Studies using sheep with the combined deficiency of β-galactosidase and α-neuraminidase (29–32) could be utilized to document this conclusively in the ovine species. However, the large number of carrier sheep required, the relatively poor response of sheep to superovulation, and the limitation of only surgical embryo collection currently available in sheep preclude the appropriate experiments (23).

The disadvantages of current methods of prenatal diagnosis indicate the need for earlier preimplantation diagnosis (1, 12, 18). Preimplantation diagnosis would allow either prenatal initiation of therapy, or implantation of only phenotypically normal embryos and the avoidance of the stress and risks of abortion. The individual techniques to obtain portions of at-risk embryos, tests for specific genetic defects, and successful implantation of the remainder of the embryo are currently being utilized for a combination of therapeutic *in vitro* fertilization and (late) prenatal diagnosis of genetic diseases (8, 10, 11, 19–22). These techniques should be adaptable for preimplantation diagnosis as shown by successful preimplantation diagnosis of the Lesch-Nyhan syndrome in a murine model (18). The present study confirms preimplantation embryonic expression of lysosomal β-galactosidase in phylogenetically diverse species, which probably can be extrapolated to humans (at least on a qualitative basis), and suggests further the feasibility of preimplantation diagnostic techniques for lysosomal storage diseases.

From these experiments, we conclude that expression of lysosomal β-galactosidase in early cleavage embryos increases as a function of cell number in ovine embryos, and as a function of both increase in cell number and increased activity on a per cell basis in porcine embryos. The activity on a per cell basis is relatively constant in ovine embryos and increases 5-fold in porcine embryos. This closely parallels increases seen with expression of murine embryonic lysosomal enzymes. These results are compatible with the documented early expression of the embryonic genome in sheep and pigs (4, 7). The potential for development of preimplantation diagnosis of lysosomal storage diseases, as early as the 8- to 16-cell stage, is confirmed. The ovine model with deficiencies of β-galactosidase and α-neuraminidase (29–32) would be an ideal model to test this potential.

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