The developmental capacity of blastomeres from 4- and 8-cell sheep embryos

By S. M. WILLADSEN[1]

*From the A.R.C. Institute of Animal Physiology,
Cambridge*

SUMMARY

The developmental capacity of (*a*) single blastomeres of 4-cell embryos, (*b*) pairs of blastomeres of 8-cell embryos and (*c*) single blastomeres of 8-cell embryos was studied. The blastomeres were inserted into evacuated foreign zonae pellucidae, embedded in agar, transferred to ligated oviducts of dioestrous ewes, and recovered when their total age was 5½ to 6½ days. At recovery the majority of embryos belonging to categories (*a*) and (*b*) had developed into small blastocysts, whereas most embryos of category (*c*) were vesicular forms with no clearly observable inner cell mass.

The viability of embryos which had continued their development during culture in the sheep oviduct was tested by their transfer to the uterine horns of recipient ewes which had come into oestrus on the same day as the respective embryo donors. Three lambs were produced after transfer of eight embryos of category (*a*) deriving from two parent embryos. These three lambs derived from a single 4-cell embryo. Nine lambs were produced after transfer of 16 embryos of category (*b*) deriving from four parent embryos. Four of these lambs derived from a single 8-cell embryo. When ewes which had come into oestrus one or two days after the respective embryo donors were used as recipients for 16 embryos of category (*a*) and 16 embryos of category (*b*) one and five lambs, respectively, were produced.

Finally, two lambs were produced after transfer of 31 embryos of category (*c*). These lambs derived from two different parent embryos.

The principal conclusions drawn from the study are that at least some individual blastomeres from 4- and 8-cell sheep embryos can give rise to an entire conceptus, and that the viability of embryos produced by blastomere separation depends more on the degree of reduction in cell number than on the stage of development at which separation is performed.

INTRODUCTION

The developmental potential of blastomeres isolated from cleaving mouse embryos has been extensively studied, but there are few reports of similar investigations in other mammals (see reviews by Wilson & Stern (1975) and Adamson & Gardner (1979)). In the mouse single blastomeres from 2-cell embryos have the capacity to give rise to normal conceptuses (Tarkowski, 1959) whereas single blastomeres from 4- and 8-cell embryos do not (Rossant, 1976). However, single blastomeres of either of the latter two categories can

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[1] *Author's address:* A.R.C. Institute of Animal Physiology, Animal Research Station, 307 Huntingdon Road, Cambridge CB3 0JQ, U.K.

contribute to both inner cell mass and trophectoderm derivatives when aggregated with enough blastomeres from other embryos to restore the normal cell number (Kelly, 1975). It has therefore been proposed (Rossant, 1976) that the apparent lack of totipotency of isolated blastomeres is not due to a restriction in the developmental potential of the cells, but rather that it reflects the low number of cells attained by embryos produced from isolated blastomeres at the time of blastulation. In the mouse blastulation starts approximately at the time when the embryo has undergone five cleavage divisions, and the onset of blastulation is unaffected by experimental reduction or increase in the number of blastomeres (Tarkowski, 1965). In embryos produced from single blastomeres of 4- and 8-cell embryos, therefore, insufficient cells may be in an inside position at the time of cavitation for the formation of a functional inner cell mass (Tarkowski & Wroblewska, 1967; Rossant, 1976).

If the ability of single blastomeres to develop into normal conceptuses depends primarily on the number of cells present at blastulation, then this ability may be expected to be maintained in later blastomere generations in species whose embryos blastulate after more cleavage divisions than the mouse embryo. The production of viable young from single blastomeres of 4- and 8-cell embryos in the rabbit (Moore, Adams & Rowson, 1968), in which blastulation normally occurs two cell generations later than in the mouse, lends support to this prediction. The sheep embryo normally blastulates after about six cleavage divisions (5 to 6 days after fertilization) when it contains approximately 64 cells. In experiments previously reported (Willadsen, 1979, 1980a) it was shown that sheep blastocysts containing half the normal number of cells have the capacity to develop into entire conceptuses, whether they were produced from single blastomeres from 2-cell embryos, pairs of blastomeres from 4-cell embryos or groups of four blastomeres from 8-cell embryos. In the experiments reported here the viability of sheep embryos containing one-quarter or one-eighth of the normal number of cells was investigated.

MATERIALS AND METHODS

Superovulated Welsh Mountain ewes, inseminated with Suffolk ram semen, were used as embryo donors. Welsh Mountain and Suffolk ewes were used as recipients. Proven infertile vasectomized rams were used to detect oestrus in donors and recipients. Embryos were collected on Day 2 (4-cell embryos; day of onset of oestrus: Day 0) and Day 3 (4- and 8-cell embryos). The general procedures used in sheep embryo transplantations in this laboratory have been described elsewhere (Willadsen, 1980b).

The zona pellucida was removed and the blastomeres separated mechanically. The blastomeres were then inserted into evacuated foreign zonae pellucidae and embedded in agar. The techniques used for micromanipulation and agar-embedding of embryos have been described previously (Willadsen, 1980a).

Table 1. *The development of embryos produced from single blastomeres of 4-cell embryos, pairs of blastomeres of 8-cell embryos or single blastomeres of 8-cell embryos during culture in ligated sheep oviducts*

Recipient oviducts ...	10	10	11
Composition of micromanipulated embryos	Single cells of 4-cell embryos	Pairs of cells of 8-cell embryos	Single cells of 8-cell embryos
No. embryos transferred	58 [10]	61 [10]	83 [2]
No. embryos recovered	50 (86·2%) [7]	56 (91·8%) [8]	74 (89·2%) [1]
Of these:			
No. degenerate	7 (14·0%)	0 (0.0%)	7 (9.4%)
No. retarded development	2 (4·0%)	1 (1·8%)	4 (5·4%)
No. normal rate of development	41 (82·0%) [6]	55 (98·2%) [8]	63 (85·1%) [1]

Values in [brackets] refer to no. of entire monozygotic groups.

Three categories of micromanipulated embryos were produced: (*a*) single blastomeres from 4-cell embryos, (*b*) pairs of blastomeres from 8-cell embryos and (*c*) single blastomeres from 8-cell embryos. Embryos deriving from one original 4- or 8-cell embryo were in most instances embedded together in a single agar cylinder and in any case they were embedded in such a way that they could be identified later.

The agar-embedded, micromanipulated embryos were transferred to the ligated oviducts of ewes in dioestrus and left in these temporary recipients until their total age from fertilization was 5½ to 6½ days. Upon recovery the embryos were examined as fresh specimens with the aid of a dissecting microscope. Those which appeared to have kept up the normal cleavage rate were termed 'normal'. Embryos of this category were subsequently released from the agar and transplanted to the uterine horns of ewes on Day 5, 6 or 7 of their oestrous cycle. Recipients not observed to return to oestrus were kept to full term. Representatives of 'normal', retarded and degenerate embryos recovered from the temporary recipients were fixed in acetic ethanol, 1:3, stained with lacmoid and examined by phase-contrast microscopy.

RESULTS

The results of the culture of micromanipulated embryos in ligated sheep oviducts are presented in Table 1. Of the 202 embryos transferred, 180 (89 %) were recovered, and of these 159 (88 %) had continued their development at an apparently normal rate. Among the latter were 15 of the 16 groups representing all the blastomeres of individual parent embryos. Those embryos which had developed at a normal rate had also undergone compaction and cavitation at about the normal time, a few having the general appearance of
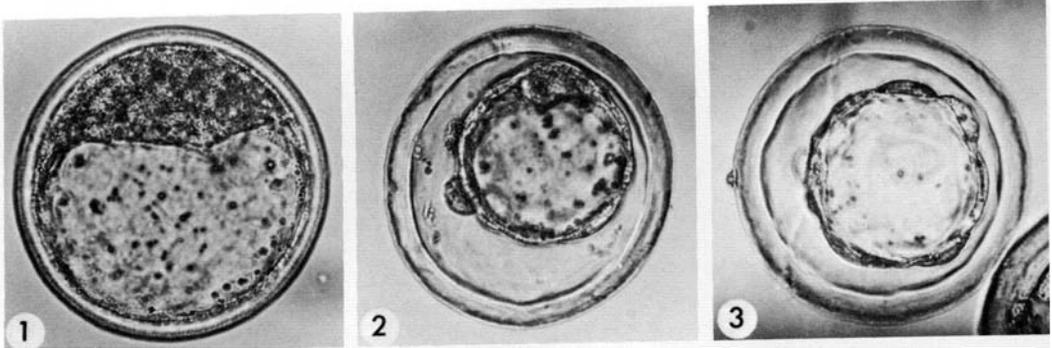
Fig. 1. Normal sheep blastocyst collected on day 7 after onset of oestrus (= Day 0).

Fig. 2. Day-7 blastocyst which developed from a single blastomere of a 4-cell sheep embryo. Note the comparatively small inner cell mass.

Fig. 3. Day-7 vesicular form which developed from a single blastomere of an 8-cell sheep embryo. Note the apparent absence of inner cells.

late morulae, while the rest were early and expanding vesicular forms. However, both the late morulae and the early vesicular forms produced in these experiments were of considerably reduced size compared to normal sheep embryos, and the expanding vesicular forms, particularly those deriving from single blastomeres of 8-cell embryos, looked much less robust than a normal expanding blastocyst. These characteristics reflected the low cell number. Thus 14 embryos, all deriving from single blastomeres of 4-cell embryos in which cavitation had just began, contained an average of 17·4 cells each (range 9–24 cells), i.e. about a quarter of the number of cells normally present at blastulation.

No morphological difference was observed between embryos developing from single blastomeres of 4-cell embryos and pairs of blastomeres of 8-cell embryos. In the majority of vesicular forms of these two categories, including all individual embryos belonging to several monozygotic groups of four of both categories, an inner cell mass could be clearly distinguished (Fig. 2). However, such inner cell masses were much less prominent and appeared to consist of disproportionately fewer cells than that of a normal embryo (Fig. 1). Vesicular forms deriving from single blastomeres of 8-cell embryos (Fig. 3) in many instances did not appear to contain an inner cell mass, although it was not possible to establish the total absence of inside cells by the microscopic examinations employed in the present study.

Two series of transplantations of groups of four monozygotic embryos were carried out to determine the viability of late morulae and vesicular forms produced from single blastomeres of 4-cell embryos or pairs of blastomeres of 8-cell embryos. In the first series ewes which had been observed in oestrus one or two days after the respective donors were used as recipients. Two monozygotic embryos were transferred to each recipient, one to each uterine

Table 2. *The viability of embryos produced from single blastomeres of 4-cell embryos or pairs of blastomeres of 8-cell embryos after transfer to definitive recipients*

Recipients (cf. text)	(A) asynchronous		(B) synchronous	
Stage of development at blastomere separation	4-cell	8-cell	4-cell	8-cell
No. embryos transferred	16 [4]	16 [4]	8 [2]	16 [4]
No. lambs born	1	5*	3†	9‡
No. recipients	8	8	4	8
No. recipients returning to oestrus Day 16 to 19	7	4	2	2
No. recipients lambing	1	3	2	5

Values in [brackets] refer to no. of groups of four monozygotic embryos.
* Two monozygotic pairs and one single lamb.
† Group of three monozygotic lambs.
‡ Group of four monozygotic lambs plus two monozygotic pairs and one single lamb.
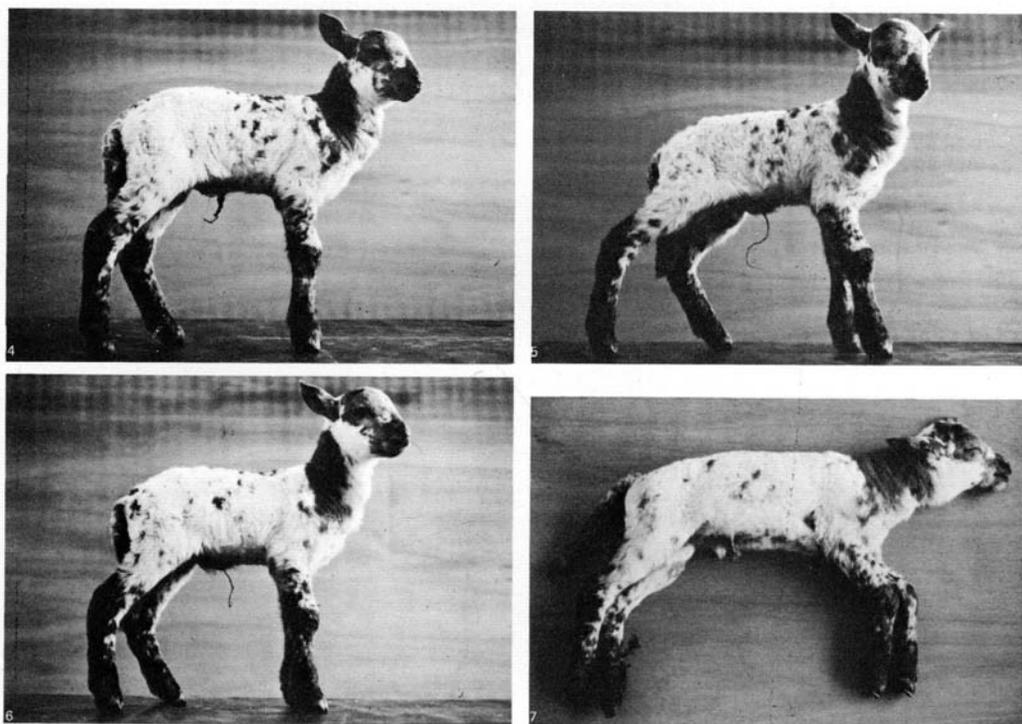
Figs. 4–7. Monozygotic quadruplet ram lambs, produced from four pairs of blastomeres of an 8-cell embryo. The two lambs in Figs. 4 and 5 were born as twins, and so were the two in Figs. 6 and 7. The dead lamb (Fig. 7) was born alive but trapped in the amnion. This lamb was killed 2 h later, having failed to breathe unaided.

horn. In this series six embryos (18·8 %) developed to term (Table 2A) but none of the original 4- and 8-cell embryos gave rise to more than two lambs.

In the second transplantation series, ewes which had been observed in oestrus on the same day as the respective donors were used as recipients. Again, two monozygotic embryos were transferred to each recipient. In this series 12 (50 %) of the embryos developed to term (Table 2B), and one group of four and one group of three monozygotic lambs were produced. The monozygotic quadruplets (Figs. 4, 5, 6 and 7) developed from the four pairs of blastomeres of an 8-cell embryo, while the monozygotic triplets derived from three single cells of a 4-cell embryo.

In a third series of transplantations the viability of vesicular forms produced from single blastomeres of 8-cell embryos was tested. Ewes which had been observed in oestrus on the same day as the respective donors were used as recipients for one group of seven, two groups of six and three groups of four monozygotic embryos. Three ewes received four embryos each, three received three embryos each, and five received two embryos each. In all instances the embryos transferred to one particular ewe were monozygotic, and all were transferred to the uterine horn ipsilateral to the ovary carrying the corpus luteum. Only two of the recipients, both of which had received four embryos, eventually lambed, producing one lamb each. This represented 6·4 % of the embryos transferred. Of the nine recipients which did not lamb, seven returned to oestrus between Day 17 and Day 20. The last two were not observed in oestrus during the remaining month or so of the breeding season.

All lambs resulting from the three series of transplantations were apparently normal.

DISCUSSION

The present experiments show that each normal blastomere of both 4- and 8-cell sheep embryos has the capacity to give rise to a vesicular form which at first glance resembles a diminutive blastocyst. However, whereas the majority of the vesicular forms developing from single blastomeres of 4-cell embryos or pairs of blastomeres of 8-cell embryos were proper blastocysts in the sense that they contained a clearly distinguishable inner cell mass, this was often not the case of vesicular forms produced from single blastomeres of 8-cell embryos. This difference was reflected in the results of the transplantations which showed that the viability of the latter category of embryos was considerably below that of embryos belonging to the former two categories.

The viability of embryos containing a quarter of the normal number of cells was enhanced when ewes which had come into oestrus on the same day as the respective donors were used as definitive recipients. This suggests that the requirement of close synchrony between the embryo and its uterine environment was accentuated by the low cell number. Although the overall embryonic survival rate in the second series of transfers was well below that obtained in

parallel experiments (Willadsen, 1980 *a*, *b*, and unpublished work) the embryonic survival rate in those recipients which did become pregnant was as high as that recorded in other experiments during the same period. It therefore seems unlikely that the relatively low overall embryonic survival rate was due to reduced developmental potential *per se*.

The low embryonic survival rate in pregnant ewes in the third series of transfers, indicates that in most instances embryos produced from single blastomeres of 8-cell embryos are unable to develop normally even in a 'synchronous' uterus and in the presence of a functional corpus luteum.

CONCLUSIONS

The principal conclusion which may be drawn from the present experiments is that at least some individual blastomeres from 4- and 8-cell sheep embryos are totipotent and can give rise to an entire conceptus. In this respect the sheep embryo clearly differs from the mouse embryo. Secondly it may be concluded that the viability of embryos produced by blastomere separation during early cleavage depends more on the degree of reduction in cell number than on the stage of development at which separation is performed. This is in agreement with observations in the mouse.

The development of a vesicular form with a clearly distinguishable inner cell mass from each blastomere of 4-cell embryos indicates that at this stage of development all blastomeres can contribute to both trophectoderm and inner cell mass, although the maximal number of lambs produced from a single 4-cell embryo in this study was only three.

The present experiments also show that each blastomere of an 8-cell embryo can develop into a vesicular form, and this may be interpreted to mean that at this stage of development all blastomeres can contribute to trophectodermal derivatives. However, they are inconclusive with respect to the ability of every individual blastomere of 8-cell embryos to contribute to inner cell mass derivatives, despite the successful development of all four pairs of blastomeres of one 8-cell embryo, since this could have been the result of chance pairing of two cells of complementary potency or of pairing of a totipotent cell with a cell of restricted potency.

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