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*TRANSPLANTATION OF LIVING NUCLEI FROM BLASTULA CELLS INTO ENUCLEATED FROGS' EGGS**

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Introduction.—The role of the nucleus in embryonic differentiation has been the subject of investigations dating back to the beginnings of experimental embryology. At first it was supposed by Roux, Weismann and others that differentiation is the result of qualitative nuclear divisions, different blastomeres thereby receiving the different kinds of nuclei which determine their subsequent differentiation. Later on this theory was disproved by numerous experiments showing that, during early cleavage at least, the distribution of the nuclei can be changed at will without altering the pattern of development. The cleavage nuclei have, therefore, been regarded as identical, and differentiation has been ascribed primarily to the well-known localizations in the egg cytoplasm.

This evidence, it should be emphasized, relates only to the early phases of development. During this time it is definitely true that the nuclei in the various blastomeres are equivalent. However, whether they remain equivalent or become differentiated as the various parts of the embryo differentiate has never been tested. The possibility that nuclei might differentiate in response to regional differences in the cytoplasm, and that such nuclear changes might have reciprocal effects on the cytoplasm during cell differentiation, was suggested by Morgan.[1] More recently Schultz[2-4] has discussed the problem more fully, indicating the known cytogenetical mechanisms that could account for nuclear differentiation, and Weisz[5] has reviewed it in relation to ciliate morphogenesis.

Obviously this problem can be solved only by the development of a method for testing directly whether nuclei of differentiating embryonic cells are or are not themselves differentiated. This sort of test could be obtained, as suggested to us several years ago by Schultz, if it were possible to transplant nuclei. Ideally, this type of experiment should be carried out by transplanting the nucleus from an irreversibly differentiated cell into an enucleated egg. The egg cytoplasm when normally nucleated is.

of course, capable of giving rise to the complete range of differentiated cell types, while in the absence of the nucleus it may cleave but fails completely to differentiate.[6-8] In other words, its differentiation, while potentially complete, is still nucleus-dependent. Therefore, if the egg nucleus could be replaced by one from a differentiated cell, the nature of the ensuing development should reveal the character of the transplanted nucleus—complete differentiation would indicate that irreversible nuclear differentiation had not occurred, while limited differentiation would indicate that it had.

In order to make such tests of nuclear differentiation it is first necessary to develop a method of transplantation that leaves both the transplanted nucleus and the recipient egg cytoplasm in undamaged condition. The only way to determine if this can be done is to work first with nuclei from undifferentiated cells which, if transplanted properly, should give rise to normal embryos. In the Amphibia the cells of choice for this purpose are those of the late blastula. They are almost as small as the differentiated cells of slightly older embryos and so present the same technical problems. At the same time they are, with the exception of the future dorsal lip cells, still undetermined and therefore their nuclei cannot be irreversibly differentiated. For these reasons we have worked out a method for transplanting nuclei from these cells into enucleated frogs' eggs. These eggs cleave and in a significant proportion of the cases develop into complete embryos.

Method.—The transplantation of nuclei is carried out in the following steps: First the recipient egg is pricked with a clean glass needle. This activates the egg and causes it to rotate so that the animal pole is uppermost and the egg nucleus can be taken out with a glass needle by Porter's[9] technique. The outer jelly coats are then removed and the egg is placed in a depression in a wax-bottomed dish in Niu-Twitty's[10] solution. A blastula or early gastrula (St. 8 to 10, Shumway[11]), placed in the same dish, is then opened up and one of the subsurface animal pole cells is dissected free from its neighbors. The cell is now drawn up into the mouth of a thin-walled glass micropipette, the lumen of which is somewhat smaller than the diameter of the cell. The pipette is held in a Leitz-Chambers holder connected via rubber pressure tubing to an ordinary 5-ml. syringe. All of the system except the tip of the needle is filled with air. The tip contains the column of solution drawn up with the cell. Provided the needle is really clean the movements of the column can be controlled accurately. Now, as the cell is drawn up into the needle it is compressed and distorted in such a way as to break the cell surface without dispersing the cell contents. The needle is then inserted into the enucleated egg and the broken cell is injected, thus liberating the nucleus within the egg. The injection can be controlled by watching the meniscus

of the fluid column within the needle, things being arranged so that the broken cell is kept near the tip of the needle while the meniscus of the column is higher up but still within the field of the microscope. Following the injection the needle is slowly withdrawn. Usually as it is withdrawn it pulls the surface coat up against the vitelline membrane so that a small canal is formed through which the egg substance may subsequently leak. This can be prevented by cutting the connection between the egg surface and the vitelline membrane with glass needles. The egg is then removed from the operating dish and placed in a small Stender dish in spring water.

Results.—When eggs (*R. pipiens*) are simply pricked with a clean glass needle they rotate, form the ephemeral "black dots" localizing the second maturation division spindle, and within a few hours show puckering of the surface or abortive and irregular cleavage furrows. There is no genuine cleavage or blastula formation. In our experiments 99% (2831 out of 2853) of the pricked eggs behaved in this way. By contrast, eggs which are pricked *and* enucleated fail to give any signs of cleavage. When observed a few hours after activation these eggs show none of the puckerings, etc., which are present in practically all of the pricked eggs at this time. Thus, 631 out of 638 enucleated eggs behaved in this way, indicating that 99% of the operations for removal of the egg nucleus were successful. Actually, the 7 eggs which did show puckers were ones in which the exovate, which forms when the egg in enucleated and which contains the egg nucleus, still retained a connection with the egg. In these the egg proper may have been enucleated, the puckers forming through the influence of the nucleus in the attached exovate.

In order to check further on the success of the operation for enucleation we always enucleated some normally inseminated eggs at the end of each experiment. From these we should obtain androgenetic haploids when the operation is successful, and normal diploids when it is not. Out of 358 operations, regarded at the time as successful, we obtained 337 embryos, all of them haploids. An additional 169 operations, regarded as not absolutely certain, gave 161 embryos of which 160 were haploids.

From these results we can say that eggs which are pricked and then enucleated will practically never retain the egg nucleus by mistake, and will never develop. When these eggs are now each injected with a diploid blastula cell nucleus more than half of them cleave, giving rise to partial and complete blastulae as summarized in table 1. Some of these blastulae were fixed. The remainder developed as shown in table 2. Of the complete blastulae the majority (74%) gastrulated normally and formed complete embryos. Approximately half of these were normal in appearance when fixed at stages ranging from stage 19 (5-mm. embryo) to the tadpole stage. The remainder formed complete neurulae but later on displayed slight to moderate abnormalities such as growth retardation, microcephaly

in some cases, larger than normal heads in others and so on. Of the 15 abnormal embryos listed in table 2, 14 were diploids and 1 was polyploid as judged from the ectodermal cell size. Among the 15 normal embryos there were 7 diploids and 8 polyploids. The only other embryos that developed far enough to give clear indications of chromosome number were 4 of the 5 abnormal neurulae listed in the table. These also were diploid (2 cases) or polyploid (2 cases). Thus, among the 35 embryos

TABLE 1

CLEAVAGE OF ENUCLEATED EGGS INJECTED WITH BLASTULA CELL NUCLEI

No. of eggs injected	197
Cleavage	
None	45
Abortive	48
Cleaved	104
Blastulae	
Partial	
$<1/2$	12
$>1/2$	29
Complete	63

NOTES: Each egg was activated, enucleated and then injected with a diploid nucleus from an animal hemisphere cell of an advanced blastula or early gastrula. Of the blastulae obtained from the injected eggs, 13 of the complete ones and 17 of the partial one were fixed during the first day of development. The remainder were allowed to develop and gave rise to embryos as shown in table 2.

TABLE 2

DEVELOPMENT OF BLASTULAE DERIVED FROM ENUCLEATED EGGS INJECTED WITH BLASTULA CELL NUCLEI

1	2	3	4		5		6		
					EFFECTIVE TOTAL			POST-GASTRULA DEVELOPMENT	
					GASTRULAE		ABN.	POST-NEURULA	
TYPE OF BLASTULA	TOTAL NO.	ARRESTED BLASTULAE	GASTRULAE NORMAL	ABN.	NORMAL	ABN.	NEU-RULAE	EMBRYOS NORMAL	SL. ABN.
Complete	50	3	37	10	30	2	2	15	15
$>1/2$	20	7	0	13	0	3	3	0	0
$<1/2$	4	4	0	0	..	..	..	..	..

NOTES: Of the total number of gastrulae obtained (col. 4) some were fixed, leaving the numbers given in column 5. These were developed into embryos as listed in column 6.

obtained there were no haploids. Had they occurred they would have been hard to account for in these experiments, since the injected nucleus was diploid. The polyploids, on the other hand, may be explained. The blastula cell nuclei are undoubtedly in various stages of mitosis when transplanted, and there should be ample opportunity for a doubling of chromosomes to occur after the nucleus has been transplanted and before the egg cytoplasm is prepared to cleave.

In order to get cytological evidence of the removal of the egg nucleus 22 of the injected eggs were fixed at stages ranging from morula to gastrula, and were sectioned and stained with the Feulgen reagent. In 21 of these eggs the exovate could be seen trapped in the inner jelly layer but separated from the egg proper. In 9 cases the egg nucleus was found in the exovate, while the egg proper consisted of cells containing normal nuclei which could only have been derived from the transplanted nucleus. In 6 additional cases Feulgen positive material was seen in the exovate, but it could not be definitely recognized as the egg nucleus. The remaining 6 cases showed no Feulgen positive material. In these the egg nucleus may have been lost from the exovate, or it could have been obscured by the pigment of the surface coat surrounding the exovate.

The evidence summarized above shows quite conclusively that living nuclei can be transplanted from blastula cells into enucleated eggs. How-

TABLE 3

DEVELOPMENT OF ENUCLEATED *Pipiens* EGGS INJECTED WITH *Catesbeiana* NUCLEI

1	2	3			4			5	
		CLEAVAGE			BLASTULAE				
					PARTIAL			ARRESTED IN	ARRESTED IN EARLY
TYPE OF NUCLEUS	NO. OF EGGS INJECTED	NONE	ABORTIVE CLEAVAGE	CLEAVED	$<1/2$	$>1/2$	COMPLETE	BLASTULA STAGE	GASTRULA STAGE
Catesbeiana (haploid)	119	27	26	66	12	31	23	66	0
Catesbeiana × *pipiens* (diploid)	46	17	7	22	1	7	14	11	11

NOTES: The nuclei used for transplantation were taken from animal hemisphere cells of hybrid blastulae 17–18 hrs. old (18°C.). The diploid hybrid nuclei were from the cross, *pipiens* ♀ × *catesbeiana* ♂. The *catesbeiana* haploid nuclei were from the androgenetic haploid hybrid, *pipiens* (♀) × *catesbeiana* ♂.

ever, in order to get an additional proof of the success of the technique we have transplanted *R. catesbeiana* nuclei into *R. pipiens* enucleated eggs. It is known from hybridization experiments[8, 12, 13] that this combination is lethal. The diploid hybrid is arrested in late blastula or early gastrula stage and dies in about 3 days, while the androgenetic haploid hybrid is always arrested in late blastula stage but dies later—at about 4 to 5 days. Thus, if the transplantation of the foreign nucleus is successful we should obtain a uniform arrest of development followed at the appropriate interval by the death of the embryo.

The experiments were done as follows: Donor blastulae were produced by inseminating *pipiens* eggs with *catesbeiana* sperm. The egg nucleus was removed from some of these, giving us androgenetic haploid hybrids. The rest of the eggs were allowed to develop as diploid hybrids. After

about 18 hours at 18° both types of hybrids had developed to stage 8 (mid to late blastula), and were usually used to provide nuclei for transplantation at this time. The hybrids are, of course, arrested shortly

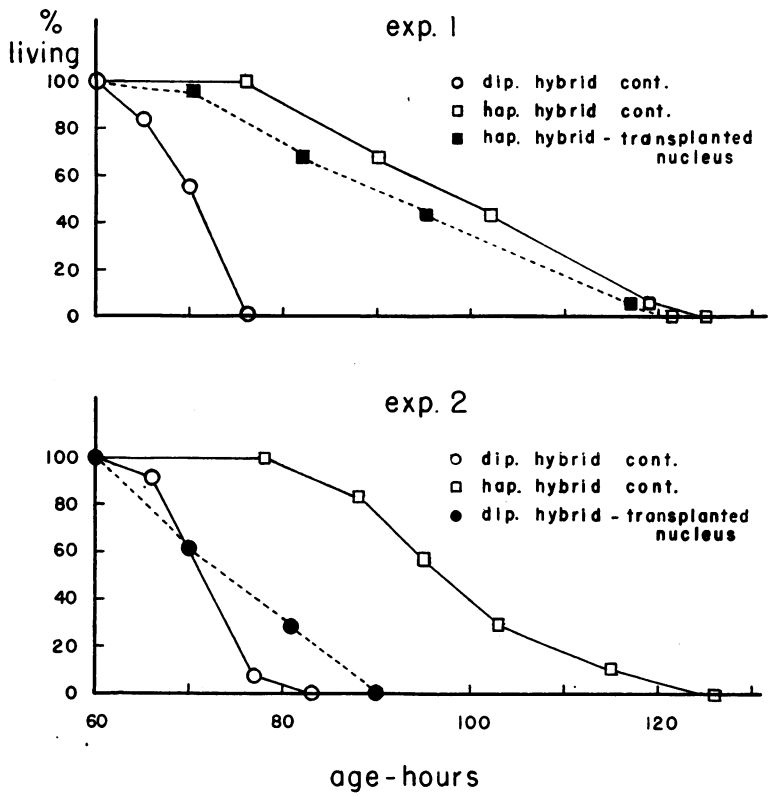

FIGURE 1

Exp. 1.—Mortality curves for (1) haploid hybrid controls, *pipiens* (♀) × *catesbeiana* ♂; (2) enucleated *pipiens* eggs injected with haploid *catesbeiana* nuclei; and (3) control diploid hybrids, *pipiens* ♀ × *catesbeiana* ♂.

Based on 55 haploid hybrid controls, 28 experimental haploid hybrids, and 147 diploid hybrid controls.

Exp. 2.—As for experiment 1, except for the experimental embryos which in this experiment are derived from enucleated *pipiens* eggs injected with diploid (*pipiens* ♀ × *catesbeiana* ♂) hybrid nuclei.

Based on 119 diploid hybrid controls, 21 experimental diploid hybrids, and 73 haploid hybrid controls.

thereafter and we have evidence, not presented here, that nuclei taken from older blastulae (stage 9) generally will not cause enucleated eggs to cleave.

The results of the transplantations of nuclei from hybrid blastulae are summarized in table 3. About half of the enucleated eggs which were injected with the *catesbeiana* haploid nucleus cleaved and developed into partial or complete blastulae, as indicated in the table. The complete blastulae, it should be emphasized, looked perfectly normal. Yet none of them showed any signs of gastrulation. They were all arrested in late blastula stage, as were the androgenetic haploid hybrid controls, and later on they all died at approximately the same time as did the controls (Fig. 1).

Enucleated eggs injected with diploid hybrid nuclei also cleaved and gave rise to blastulae, as shown in table 3. These corresponded to the diploid hybrid controls in their development. That is, they were all arrested either in late blastula or early gastrula stages, and died when their controls did. (Fig. 1.)

In order to see whether the nuclear phenomena associated with the arrest and death of the blastulae were occurring in the same way in the experimental and control blastulae we fixed several of each at ages of 42 to 72 hours. The controls consisted of 9 androgenetic haploid hybrids [*pipiens* (♀) × *catesbeiana* ♂]; the 17 experimental blastulae were all derived from enucleated *pipiens* eggs injected with haploid *catesbeiana* nuclei taken from androgenetic haploid hybrid blastulae.

A study of the arrested control blastulae revealed a variety of nuclear abnormalities. The majority of the nuclei were in an abnormal interphase condition characterized by an accumulation of chromatin around the periphery of the nucleus in the form of thick threads, leaving the central portion free of Feulgen-positive material. In addition there were arrested and abnormal metaphases, and a few anaphases which always showed bridges. In some of these figures the chromosomes were clumped. Finally, some cells contained small groups of chromosomes, or small pycnotic nuclei, presumably derived from irregular divisions that had occurred earlier.

In the majority (14 out of 17) of the experimental blastulae the nuclear abnormalities were exactly the same as those described above for the controls. In 3 cases, however, the condition was different, with the nuclei all in an abnormal interphase condition characterized by a rather diffuse Feulgen staining. We do not know yet how to account for these exceptional cases. It may be that in a larger series of cases the same type of nuclear phenomenon might appear among the controls, or occasionally there may be some effect of the transplantation on the subsequent behavior of the nuclei. In any case, in the large majority of the experimental blastulae the appearance of the nuclei is the same as it is in the controls. This fact, taken together with the fact that the extent of development and the time of death are also the same in the experimental and control blastulae

provides convincing evidence of the successful transplantation of the foreign nucleus.

Discussion.—Nuclear transplantation has been accomplished previously in amoebae,[14, 15] but to our knowledge has not been reported for other forms. The evidence summarized in this paper shows that blastula cell nuclei may now be transplanted into enucleated frogs' eggs, giving rise to nucleated embryos which differentiate normally. This means that the nuclei are not significantly damaged, and indicates that the technique of nuclear transplantation may now be used in testing nuclei from various differentiated parts of the Amphibian embryo. The advantages of being able to study the problem of nuclear differentiation with this embryo are considerable. It is one of the classical objects of experimental embryology and thereby provides us with the opportunity of correlating the properties of the nuclei, as they may be revealed by nuclear transplantation, with the known properties of the different parts of the embryo as demonstrated by the numerous transplantation and explantation experiments recorded in the literature.

The method of nuclear transplantation, described in this paper, involves transferring the cytoplasm as well as the nucleus of the blastula cell into the enucleated egg. This does not, however, bring about a significant dilution of the egg cytoplasm. The total volume of the donor blastula cell is about 2×10^{-4} cu. mm., while the average volume of the *pipiens* egg is about 3.4 cu. mm.—giving a ratio of 1:17,000. We have not calculated the volume of the blastula cell cytoplasm but it would be significantly smaller than that of the whole cell and the ratio of it to the egg cytoplasm volume is therefore probably smaller than 1:20,000. None the less, some cytoplasm is transferred along with the nucleus by this method. Refinements may make it possible to eliminate most of the cytoplasm, but it will be extremely difficult if not impossible to devise a method for obtaining nuclei which can be said to be completely free of cytoplasm. However, this is not important at present. The method as it stands should allow us to detect such irreversible changes in nuclei as may be limiting with respect to differentiation. And in the future the role of the cytoplasm may be explored by combining in transplantation nuclei and cytoplasm from different types of cells, or from the same type at different stages of differentiation.

Although the method of nuclear transplantation should be valuable principally for the study of nuclear differentiation, it may also have other uses. In particular, it can provide us with a test of whether nuclei which have been treated in various ways still retain their capacity for mitosis. This is the best if not the only test of a normal nucleus, and we hope that it may eventually be applied to the problem of developing an optimal nuclear medium—a matter which should have real importance for future studies of nuclear biochemistry.

Summary.—In this paper a method is described for transplanting nuclei from advanced blastula cells into *enucleated* eggs of *Rana pipiens*. When the nucleus is from the same species as the egg cytoplasm the egg then cleaves and can develop into a normal embryo. When the nucleus is from a different species (*R. catesbeiana*) the enucleated *pipiens* egg which receives it forms a blastula which is then arrested and subsequently dies, exactly as do the normally produced hybrids between the two species. These and other experiments prove that the blastula cell nucleus can be transplanted in undamaged condition, indicating that the technique of nuclear transplantation is now sufficiently well developed so that it may be used in studies of nuclear differentiation and possibly in other studies of nuclear function as well.

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