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STUDIES ON THE DEVELOPMENT OF PREIMPLANTATION MOUSE EMBRYOS AND BLASTOMERES FOLLOWING MICROSURGICAL MANIPULATION

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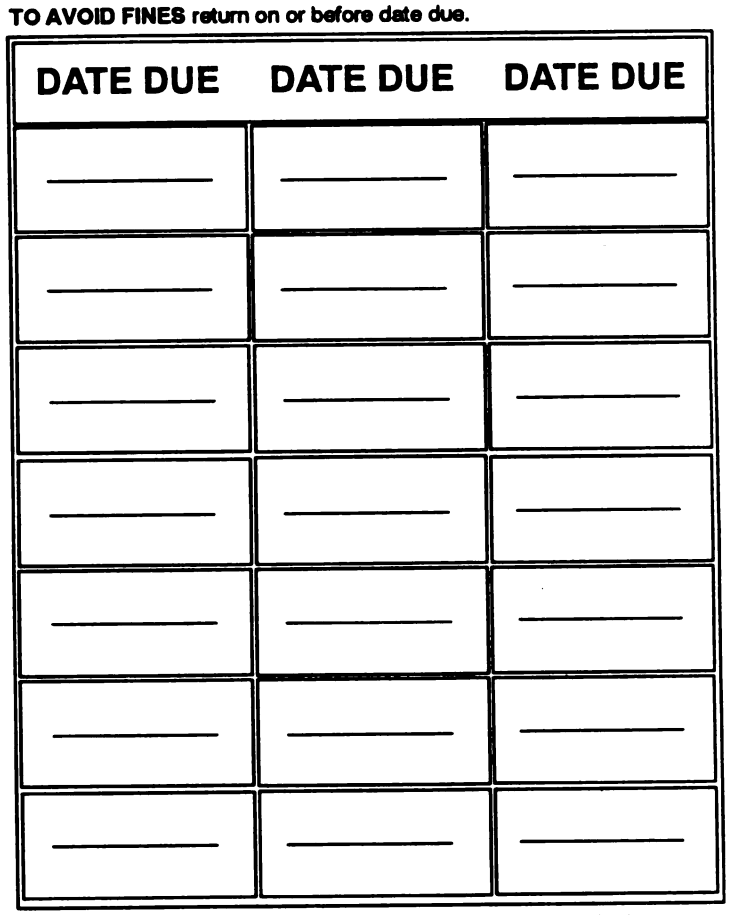

STUDIES ON THE DEVELOPMENT OF PREIMPLANTATION MOUSE EMBRYOS AND BLASTOMERES FOLLOWING MICROSURGICAL MANIPULATION

By

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ABSTRACT

STUDIES ON THE DEVELOPMENT OF PREIMPLANTATION MOUSE EMBRYOS AND BLASTOMERES FOLLOWING MICROSURGICAL MANIPULATION

By

William Edward Roudebush

Early preimplantation mouse embryos (2-8 cell stage) were evaluated for developmental potential following cell removal in two culture media with or without 20% fetal calf serum supplementation. PMSG (10 I.U.) and hCG (10 I.U.) primed female Swiss mice (3-4 weeks old) were sacrificed in the morning of day 5 for 2 cell embryos, early evening for 4 cell and on day 6 for 8 cell embryos (day 1 = day of PMSG administration). The interval between PMSG and hCG injections was 48 hours; females were placed with fertile males at the time of hCG administration. Embryos were collected, manipulated and cultured in modified TC-199 or BMOC-TALP filter sterilized media. Blastomeres were biopsied from embryos by a displacement technique. Biopsied blastomeres and embryos were evaluated for temporal development following 24 hours of incubation in a humidified atmosphere of 5% CO_2 in air, under oil at 37°C. The use of BMOC-TALP medium was found to significantly enhance the development of mouse embryos over a modified TC-199. The supplementation with 20% fetal calf serum was found to significantly enhance 2 cell embryo growth but has no overall effect. The 8 cell mouse embryo was found to be the most desirable stage for biopsy.

A maximum of three cells may be removed from the 8 cell mouse embryo without retarding development. Precompacted 8 cell embryos were more readily biopsied than the other stages, however, compacted 8 cell embryos were more likely to develop in culture. Cell determination is believed to occur at or prior to the compacted 8 cell stage in the mouse.

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Last, and certainly far from least, I once again wish to thank Mr. August Busch and family for all you do in producing that succulent product that has helped sustain me and to endure stress throughout the years.

. . . and now let the fat lady sing away . . .

Into your hands will be placed the exact results of your thoughts; you will receive that which you earn; no more no less. Whatever your present environment may be, you will fall, remain, or will rise with your thoughts, your Vision, your Ideal. You will become as small as your controlling desire; as great as your dominant aspiration....

In all human affairs there are efforts, and there are results, and the strength of the effort is the measure of the result. Chance is not. "Gifts," powers, material, intellectual, and spiritual possessions are the fruits of effort; they are thoughts completed, objects accomplished, visions realized.

The Vision that you glorify in your mind, the Ideal that you enthrone in your heart--this you will build your life by, this you will become.

- From As a Man Thinketh by James Allen (1985).

TABLE OF CONTENTS

LIST OF TABLES

TABLE

LIST OF FIGURES

FIGURE

CHAPTER I

INTRODUCTION

The ability to observe and study early embryonic growth and development *in vitro* is beneficial in our understanding of what is occurring *in vivo*. Such studies allow us to insure better health conditions and prevent inborn or induced abnormalities. This requires special techniques and methods for the collection, analysis and culture of early preimplantation mammalian embryos.

The early preimplantation embryo has been primarily studied in the mouse and have been directed towards many different and related areas. These include: the defining of culture conditions for optimal embryo growth, the timing of maternal vs. genomic control of development, biochemical analysis, developmental parameters and microsurgical manipulation techniques and applications.

Knowledge of the early embryo is incomplete from the time of nuclear fusion until complete implantation. Following fusion of the male and female pronuclei the embryo undergoes many processes and is subjected to many factors within the oviductal and uterine environments. These processes include cleavage, compaction, blastocoel formation and hatching. The initial developmental phase can be simply illustrated by following the mitotic time course of temporal development or in greater detail by defining the ever changing biochemical events that occur during development.

Current use of microsurgical manipulations include the production of embryo multiplets (identical twinning) and the assessment of developmental potentials of these embryos at various stages of development. Production of isogenic animals has been well recognized for physiological and behavioral studies and has been extensively reviewed by Rawlins (1984). Future use of this technique will lead to a better understanding of the normal development of preimplantation mammalian embryos through the use of cytogenetical or biochemical techniques.

The ability to isolate cells from an early preimplantation embryo would also be beneficial for diagnostic evaluation by cytogenetic or biochemical analysis. This was also recently suggested by Boldt (1988). It was also suggested about a need to assess chomosomal normality in embryos produced by sperm injection or zona pellucida drilling (Laws-King et al., 1987). It would be advantageous to analyze cells from transgenetically produced embryos prior to transfer, this would permit the evaluation of transfection prior to transfer.

The present investigational objectives are:

1. to develop a technique whereby blastomere(s) can be removed with minimal harm to the embryo.
2. to determine the optimal stage of development for cell removal (biopsy) which does not retard embryonic development.
3. to determine the maximal number of blastomeres that can be biopsied without retarding embryonic growth.

4. to determine the optimal culture conditions that will support the development of microsurgically manipulated embryos and biopsied blastomeres.

CHAPTER II

LITERATURE REVIEW

Interest in culturing embryos *in vitro* stemmed from the development of tissue culture techniques in the early 1900s (Biggers, 1987). It was then suggested that much could be learned, about embryogenesis and organogenesis, by studying early embryonic development *in vivo* and *in vitro*, which simulate the normal conditions as closely as possible (Mark and Long, 1912). The first reported research of culturing mammalian embryos (rabbit) was pioneered by Lewis and Gregory (1929), but it was not until the late 1940s that mouse embryos were successfully grown in culture (Hammond, 1949).

Chemically Defined Culture Media

Hammond (1949) reported that 4 and 8 cell embryos will develop in a modified saline based culture medium with 8-9% egg white supplementation. The saline medium consisted of NaCl (880 mg), KCl (30 mg), $CaCl_2$ (25 mg), $MgCl_2$ (25 mg), glucose (108 mg) and NaH_2PO_4 (10 mg) in 100 ml of water. The culture system consisted of a closed air atmospheric environment at 37°C.

The first report of successfully culturing mouse embryos in a chemically defined culture medium was by Whitten (1956a). Using a medium based on a Krebs-Ringer bicarbonate solution, Whitten (1956a) showed that the omission of calcium, magnesium, potassium

or glucose from the medium will prevent embryonic growth. It was also reported that the addition of bovine serum albumin (BSA), 0.1-0.4% by weight, will aid in the in vitro development of early embryos. Substituting Ca-lactate for Ca-chloride, Whitten (1956b) was able to grow 2 cell mouse embryos to the blastocyst stage. The following year Whitten (1957a) reported that the embryos will develop in medium gassed with 5% CO_2 in air or 5% CO_2 in nitrogen, but will degenerate when gassed with 5% CO2 in oxygen and cultured in a lactate modified Krebs-Ringer bicarbonate medium. Whitten (1957b) also reported that progesterone in low concentrations (< 2 µg/ml) had no effect on embryonic development and that high concentrations (> 4 µg/ml) decreased embryo growth and inhibited hatching of the blastocysts. The addition of estrogen was not found to reverse the toxic affect of progesterone.

The first successful transfer of cultured mouse embryos was accomplished by McLaren and Biggers (1958). They reported that 8-16 cell embryos, cultured for 2 days in a closed atmospheric chamber at 37°C, are capable of developing to term following transfer to pseudopregnant mice. The embryos were collected from superovulated mice and cultured in modified Whitten's medium (Whitten, 1956a) (Table 1) containing glucose (1 mg/ml) and BSA (1 mg/ml) and gassed with 5% CO_2 in air prior to use.

Biggers et al. (1962) co-cultured mouse zygotes (1 cell stage) with whole oviducts to the blastocyst stage using a diversified medium containing amino acids. The co-cultured embryos and

Table 1. Composition of media for mouse embryo culture (mM).

Component	Whitten (1956a)	Brinster (1963)	Whittingham (1971)	Bavister et al. (1983)
NaCl	118.46	109.23	94.59	114.0
KCl	4.74	4.78	4.78	3.2
KH_2PO_4	1.18	1.19	1.71	----
$NaH_2PO_4 \cdot H_2O$	----	----	----	0.4
$CaCl_2$	2.54	1.71	1.19	2.0
$MgCl_2 \cdot 6H_2O$	----	----	----	0.5
$MgSO_4$	1.18	1.19	1.19	----
$MgSO_4 \cdot 7H_2O$	----	----	1.19	----
Na lactate	----	10.15	23.28	10.0
Na pyruvate	----	----	0.33	0.1
$NaHCO_3$	24.88	25.07	25.07	25.0
HEPES	----	----	----	25.0
Glucose	5.55	----	5.56	5.0
BSA (mg/ml)	1.0	1.0	4.0	3.0
Phenol Red	----	----	----	10 µg/ml
Penicillin (per ml)	10.0 µg	100 U	10,000 U	100 U
Streptomycin (per ml)	10 µg	50 µg	5 µg	----

oviducts were incubated in a continuous gas flow atmosphere of 5% CO_2 in air at 37°C. Brinster (1963) cultured 2 cell embryos under light viscosity paraffin oil in a defined medium (Table 1) in a continous flow of 5% CO_2 in air bubbled through water. Biggers et al. (1965) cultured 2 cell embryos in Brinster's medium (BMOC) (Brinster, 1963) with two energy sources, lactate and pyruvate, and observed no difference in embryonic development with either energy source.

Brinster (1965a) reported that 2 cell embryos will develop optimally if the osmolarity of the cuture medium (Brinster, 1963) is adjusted to 0.276 osm and the pH is within a range of 6.42-7.21, under paraffin oil (viscosity 125/135). In a later study Brinster (1965b) reported no interactions existed between pH, osmolarity or energy used but a correlation did exist between energy source (lactate, pyruvate, oxaloacetate or phosphoenolpyruvate) and pH (7.38) on the percent of 2 cell embryo development to blastocysts in Brinster's medium (Brinster, 1963). The availability of a fixed nitrogen source on embryo growth was also studied by Brinster (1965c). He found that only the omission of cystine (0.24 x 10^{-3} mM), and BSA, will decrease blastocyst development from cultured 2 cell embryos. He suggested that the role of BSA in the culture medium could include: a nitrogen source (supplying amino acids), a chelating agent, the regulation of oxidative-reduction potential, cell surface protection and enzyme protection. Cholewa and Whitten (1970) reported that no external nitrogen source is

required for blastocyst formation from 2 cell embryos. In the same study it was observed that albumin is required for 8 cell stage growth but not for 2 cell growth. Gwatkin (1966a) reported that amino acid (arginine, cystine, histidine, leucine and threonine) supplementation is only needed for mouse blastocyst outgrowth but not for 2 cell to blastocyst stage development in BMOC (Brinster, 1963).

Whittingham and Biggers (1967) reported that a 1 cell block exists in the mouse embryo cultured in vitro. It was later reported that this 1 cell block could be overcome by the use of M16, Whittinghams medium (Whittingham, 1971) (Table 1). This medium is based on Tyrodes salt solution and contains fixed nitrogen sources.

Reports vary as to whether or not the zona pellucida is required for the in vitro culture of mouse embryos. The zona pellucida is a thick acellular layer of glycoproteinaceous material covering the outer periphery of the mammalian egg (Austin, 1982). The functions of the zona pellucida have been suggested to include mechanical protection, osmotic regulation, the prevention of polyspermy (Mintz, 1962; Pikó, 1969) and sperm binding (Saling, 1982). To study embryonic development with or without the zona pellucida requires techniques that do not harm the blastomeres while the zona pellucida is being removed. Exposure to acidic solutions (e.g. Tyrodes--pH 2.5), enzyme treatment (e.g. pronase), or mechanical disruption have all been employed to sufficiently

remove the zona pullucida from the embryo. Cholewa and Whitten (1970) reported a decrease in the number of blastocyts that developed from zona pellucida free 2 cell embryos cultured in Whitten and Biggers medium (1968) without BSA. The zona pellucida had been removed using pronase. Rottman and Lampeter (1981) reported no effect on development of zona pellucida free or zona pellucida intact day 3 embryos, morula, cultured in modified BSM II (Maurer, 1978) plus 20% fetal calf serum. Bowman and McLaren (1970) found a significant decrease in blastocyst development from zona pellucida free 8 cell embryos cultured in modified BMOC (Brinster, 1963b) in an atmosphere of 5% CO_2 in air.

There does not appear to be an ideal gas phase that will support early embryonic development. Pavlock (1967) compared continuous flow of the gases, 5% CO_2 in 95% oxygen and 5% CO_2 in air as the atmospheres for the culture of mouse 2 cell embryos in TC-199. It was found that 5% CO_2 in 95% oxygen inhibits blastocyst development (Pavlock, 1967). Quinn and Harlow (1978) reported that the optimal concentration of oxygen in the gas atmosphere should be 5% for optimal development of 2 cell embryos to blastocysts, cultured in Whittens medium as described by Hoppe and Pitts (1973). Higher concentrations of oxygen have been reported to decrease the number of cells/blastocyst following the culture of 2 cell embryos (Harlow and Quinn, 1979). The two primary gas phases used are 5% CO_2 in air and 5% CO_2, 5% O_2, 90% N_2, neither of which was superior to the other in supporting the development of

blastocysts from 2 cell embryos (Hoppe and Pitts, 1973; Hsu et al., 1974; Harlow and Quinn, 1979).

By the 1980s many investigators had studied and defined culture media to optimize embryonic development. Many factors are believed to regulate embryo growth in vitro, one of these is the addition of serum to the modified basic salt solution. A reason for this addition is that the serum may contain some unidentified growth factors (Hsu, 1980). The addition of serum, regardless of source, does not appear to be beneficial for 2 cell or 4 cell development to the 8 cell stage, but may be beneficial or required for blastocyst formation (Brinster and Thomas, 1967; Cholewa and Whitten, 1970; Saito et al., 1984). The amount of serum supplementation has varied from 5% (Pavlok, 1967) to 50% (Arny et al., 1987). Saito et al. (1984) separated human cord serum into large and small molecular weight fractions and studied the effects of the fractionated sera. The large molecular weight fraction supplemented medium was found to increase the number of blastocysts that developed from 2 cell embryos. Arny et al. (1987) reported no difference in mouse blastocyst development from 2 cell stage embryos cultured in T6 medium (Quinn et al., 1984) supplemented with 20% fetal calf serum. Shirley et al. (1985) reported no beneficial effect of human fetal cord serum or plasma (15% v/v) supplementation on blastocyst formation of 2 cell embryos cultured in Ham's F-10 medium, however 15% maternal serum or plasma supplemented Ham's F-10 medium was found to decrease blastocyst formation.

Ogawa and Marrs (1987) found reduced growth rates in embryos cultured in medium with or without protein supplementation (BSA) for 48 hours and then transferred to medium containing human cord serum. Embryos cultured in BSA medium for longer than 48 hours exhibited better growth rates than serum supplemented media. These results suggest that the nature and timing of protein supplementation may be important for the culture of preimplantation mouse embryos. Ogawa and Marrs (1987) also fractionated the human fetal cord serum and found that media supplemented with the small molecular weight component demonstrated reduced growth rates.

Embryonic Normality

Embryonic normality is difficult to measure in the preimplantation embryo. To determine normalcy a number of different analyses have been employed. These include studying the biochemical-physiological processes, cytogenetics and evaluation of temporal development of various stages of embryos based on cell size, shape and morphological evaluation.

Analysis of biochemical-physiological properties in the early embryo has been studied but only to a limited degree. Some studies may have quantified their values while others are merely qualitative.

The maintenance of the plasma membrane is regarded as an extremely important property in maintaining cell shape, permitting the transfer of metabolites and for communication between cells. A major constituent of the plasma membrane are phospholipids. Pratt

(1980) investigated the biosynthesis of phospholipids during cleavage, compaction and blastocyst formation using [methyl-^{3}H]-choline as the specific percurser. He demonstated detectable choline incorporation into lipid at the 2 cell stage and this increased 9-13 fold during the 8 cell stage. Choline kinase, required for the phosphorylation of choline, was also detectable at the 2 cell stage, increased during compaction and decreased in the blastocyst. These results demonstrated that the embryo is capable of synthesizing phospholipids and is equipped to assemble new cellular membranes (Pratt, 1980). Therefore, it was suggested that the membrane lipids could be a useful marker molecule to study the synthesis and assembly of cellular membranes and provide information on the molecular basis of changes in membrane properties which could influence the cellular responses to varying signals during early preimplantation development.

Energy needs of the early mouse embryo have been extensively studied. Pyruvate was found to be required to support the first and second cleavage divisions (Biggers et al., 1967) while glucose could only support division embryos at 4 or more cell stages (Whitten, 1956b, 1957a; Brinster, 1965b; Brinster and Thomson, 1966). Leese and Barton (1984) reported that pyruvate is the preferred energy substrate in the early developmental stages but that the glucose requirement will predominate at the blastocyst stage. However, Wales (1975) reported an apparent glucose-based metabolism in the morula. Pike and Wales (1984) suggested that the

block to glucose utilization, in early developmental stages, causes glucose to be converted to glycogen. This conversion has been suggested to be the result of deficiencies in glucose transport at the enzyme level, e.g. hexokinase or phosphofructokinase (Barbehenn et al., 1974, 1978). Edirisinghe et al. (1984a) showed that during differentiation, the outer cells of the morula acquire the ability to synthesize large amounts of glycogen. Glycogen has also been reported to be stored in trophoblastic cells by histochemical analysis (Brinster and Thomas, 1966), electron microscopy (Enders, 1971) and by autoradiographic and biochemical analyses (Edirisinghe et al., 1984a). In 8 cell embryos a measurable difference between glycogen stores in blastomeres has also been reported by these workers. This suggests that a difference in metabolism is being initiated.

Glucose utilization is by the pentose phosphate pathway and generates NADPH and ribose-5-phosphate (O'Fallon and Wright, 1986). NADPH serves as a hydrogen and electron donor in reductive biosynthesis and ribose-5-phosphate and its derivatives are constituents of ATP, coenzyme A, NAD+, FAD+, RNA and DNA. The highest activity of the pentose phosphate pathway is in the 2 cell and morula stages in mouse embryos and lowest at the blastocyst stage (O'Fallon and Wright, 1986). Wales (1986) analyzed glucose utilization by measuring CO_2 and lactate production. It was reported that glucose utilization is highest at the single cell stage and lowest at the blastocyst stage. Wales (1986) suggested that the discrepancies

found may be due in part to strain differences and not in technique. It has also been reported that the values found *in vitro* may not occur naturally *in vivo*. Edirisinghe et al. (1984b) demonstrated that the uterine environment has a marked influence on the metabolism, especially that of glycogen, in the early embryo. Embryos recovered *in vivo* have low glycogen content (Stern and Biggers, 1968) while embryos cultured *in vitro* have high levels of glycogen which increase with development to the blastocyst stage (Ozias and Stern, 1971). This problem of glycogen storage may be a contributing factor to why certain strains of embryos have poor development *in vitro*. These embryos may be unable to utilize the glucose and the resultant increase of glycogen could have a retarding effect on embryonic development. Strain differences were also found on the levels of ATP and AMP in early stage mouse embryos recovered after *in vivo* development or cultured *in vitro*. Embryos were found to have increasing levels of AMP as ATP levels decreased from *in vivo* developed embryos, the same was not found in embryos measured after development *in vitro* (Spielmann, et al., 1984). They reported that some embryonic strains would not progress beyond the 2 cell block, and their ATP levels had not decreased nor had the AMP levels increased from the 1 cell to the 2 cell stage.

Prior to blastocyst formation the embryo must enter the morula stage which is preceeded by a process termed compaction. Compaction normally occurs during the late 8 cell stage. Compaction is characterized by intracellular focal tight junctions and

gap junctions (Ducibella and Anderson, 1975a,b). Following compaction the embryo develops into a morula and then into a blastocyst. Blastocyst formation is characterized by the formation of a blastocoele cavity and the initial cell differentiation of two distinct cell populations: the inner cell mass (ICM) and the trophoectoderm. It is believed that the morula (16+ cell stage) is the initiating stage of differentiation of the blastomeres into the ICM and trophoectoderm (Ducibella, 1977; Johnson et al., 1977; Johnson, 1979). But it is not known what the initiating stage for cell determination is to program the blastomeres to develop into either the ICM or trophoectoderm. It has been postulated by Tarkowski and Wróblewska (1967) that an epigenetic mechanism exists for cellular differentiation. This mechanism is dependent upon the position of blastomeres in the embryo at the time of differentiation. This hypothesis is supported by Hillman et al. (1972), who showed that a blastomeres position in the embryo during early cleavage could influence its position in the blastocyst, i.e. exterior cells would become trophoectoderm while cells found in the interior would most likely become the ICM. Kelly (1975) also supported this hypothesis after noticing a lack of detectable morphogenetic factors up to the 8 cell stage, thus implying totipotency. Wiley (1984) preferred to call this the cell aposition hypothesis, which states that the epigenetic cue for ICM and trophoblastic differentiation arises from the sensitivity of the metabolic activity within a given cell to the percentage of the cell surface that is apposed vs. free.

Blastocyst formation is thought to be triggered by one or more possible mechanisms: (1) absolute cell number, (2) number of cytokineses, (3) number of chromosomal and/or DNA replicative cycles, or (4) nucleo-cytoplasmic ratios. Smith and McLaren (1977) have demonstrated that the blockage of cytokineses will not prevent the early embryo from developing into the blastocyst. It has also been suggested that an absolute number of cells may not be necessary for blastocyst formation based on isolated cells from early (4-8 cell stage) mouse embryos. Studies of the role of the DNA replication cycle requires the use of an inhibitor which will specifically and reversibly inhibit DNA synthesis. Cozard and Warner (1982) used an inhibitor of DNA polymerase, aphidicolin, to block DNA synthesis. Their results demonstrated that aphidicolin treated embryos were still capable of forming blastocysts, but that they failed to report on the effect of aphidicolin on DNA replication. Alexandre (1982) reported that cavitation is not dependent on a set number of completed DNA replicative cycles by continually treating 8 cell stage mouse embryos with aphidicolin. The treated embryos were prevented from cell division but were still capable of forming blastocysts. However, aphidicolin treatment of 4 to early 8 cell stage embryos does prevent blastulation. Dean and Rossant (1984) showed that treating 8 cell stage embryos for 8 hours with the DNA polymerase inhibitor will not delay cavitation and will inhibit DNA synthesis by 90%, thereby delaying cell division. Therefore, the timing of blastulation does not depend simply on the

number of DNA replicative cycles undergone since fertilization or on total cell number. It has been suggested that cell number or number of replicative DNA cycles could signal compaction which then is linked to blastocyst formation in a set temporal sequence (Johnson et al., 1979).

A final physiological event in the early mouse embryo prior to implantation is that of hatching. Hatching is believed to be dependent on prostaglandin (PG) synthesis since antagonists to PGF-2α and PGE-2 inhibit hatching in mouse embryos (Baskar et al., 1981; Biggers et al., 1978). The functional role of the antagonists is not known but may be complex, involving single or multiple sites of action. One type of antagonist, feramic acid, is known to competitively bind to the cyclo-oxygenase component of the prostaglandin synthetase system and results in the inhibition of the synthesis of all components of the arachidonic acid cascade (Cushman and Cheung, 1976).

Histamine has also been suggested to play a role in blastocyst expansion and zona pellucida hatching, by influencing prostaglandin synthesis (Biggers et al., 1978). Dey and Johnson (1980a) have reported that 8 cell to morula stage embryos produce histamine. Histidine, a precursor of histamine, may also be required for blastocyst formation since DL-α-methyl histidine (DL-α-MH) dichloride will prevent 4-8 cell stage treated embryos from developing into blastocysts (Dey et al., 1979). Histidine has also been proposed to affect early embryonic development by other roles,

growth promoting and increasing fatty acid precursors in tissues for prostaglandin synthesis since histamine increases phospholipase A2 activity (Dey et al., 1979).

Temporal development analysis for normalcy in early embryos is primarily based upon the time normal appearing embryos develop in vitro as compared to in vivo. This is a very subjective analysis and requires the observer to classify embryos based on cell size, shape and cytoplasmic characteristics.

Temporal development in the early preimplantation period has been assessed by the timing of cell cycles in different strains of mice, different ovulation procedures and by different techniques for the evaluation of each phase length. Because of the above differences it was surprising that a favorable agreement was obtained from the reports, and is summarized in Table 2. The wide range in G1 of the first cell cycle was suggested to be probably due to the lack of precision in the timing of fertilization (Smith and Johnson, 1986). The G_2 + M differences were suggested to be due to differences in strains and the increased sensitivity to stress, since lower temperatures between S phases will prolong G_2 + M. It was also reported that the third and fourth cell cycles are intermediate in position between the maternally regulated first and second cell cycles and those cells whose characteristics demonstrate a differentiated state (Smith and Johnson, 1986). Therefore, it may be possible to reason that cell determination may take place during the third and/or fourth cell cycles.

Table 2. Summary of the values for the first four cell cycle phases of mouse embryos.[a]

Cell Cycle	Length of Phase (h)		
	G_1	S	G_2 + M
First (1 cell stage)	4.5 - 12.0	4.0 - 7.0	1.0 - 8.0
Second (2 cell stage)	0.0 - 1.3	4.0 - 7.0	12.0 - 18.0
Third (4 cell stage)	1.0 - 1.5	7.0	0.5 - 5.0
Fourth (8 cell stage)	2.0	7.0	1.0 - 3.0

a From Smith and Johnson (1986).

Genetic control of development has been suggested by various researchers (McLaren and Bowman, 1973; Niwa et al., 1980; Shire and Whitten, 1980). This control is believed to be associated with the gene(s) in the major histocompatability complex (MHC) in the mouse, called the H-2 complex. This gene influences the timing of the first cleavage division and the subsequent rate of embryonic development (Goldard et al., 1982). The H-2 complex is located on the sevententh chromosome in the mouse and is involved with immunological events such as, T cell - B cell interaction and self recognition. The H-2 complex is believed to produce proteins that have pleiotropic effects (Klein et al., 1981). A difference between embryos from different strains has been found in their temporal development, separated as fast and slow embryos (McLaren and Bowan, 1973; Titenko, 1977). Slow developing embryos are associated with the $H\text{-}2_b$ haplotype (Goldard et al., 1982) and fast embryos with the $H\text{-}2_k$ haplotype (Verblanac and Warner, 1981).

Goldard and Warner (1982) reported that a Ped (preimplantation embryo development) gene influences the timing of the first cleavage division and is associated with the H-2 complex. Two reports suggest that the Ped gene products may be the the H-2 antigens expressed on the cell surface of blastocysts (Démant, 1979; Goldard et al., 1982).

Another determinate of embryonic normality is by analyzing the chromosomes by cytogenetics. Chromosomal aberrations have been identified as a primary cause of reproductive failure (Boué and

Boué, 1973) and has been suggested to be a reason for the high incidence of early embryonic loss (Roslyn et al., 1983; Rudak et al., 1984). Although there are different ways of analyzing the chromosomes in early embryos, generally, chromosomal preparations are prepared by either the air drying method of Tarkowski (1966) or by a gradual fixation using a warm-moist air drying ttechnique (Kamiguchi et al., 1976), which allows the chromosomes to spread out in the cytoplasm without rupturing the membranes. Staining with Giemsa allows for visual examination of chromosomal normality (Mizoguchi and Dukelow, 1981).

It has been suggested that a possible failure of embryonic development is the effect that superovulation regimens have on inducing chromosomal abnormalities. Hansen and Probek (1979) reported that ovulation stimulation did not affect the first meiotic cleavage in the hamster oocyte. No incidence of chromosomal abnormalities between stages of development were reported from hamsters treated with gonadotropins (Sengoku and Dukelow, 1988). Takagi and Sasaki (1976) did report on the incidence of polyploidy in mouse embryos that developed from superovulated ova fertilized in vivo.

Chromosomal analysis does have a major drawback, as does biochemical analysis, in that it requires the destruction of the total embryo and therefore prevents further studies. It would be beneficial to be able to analyze a single or group of blastomeres, for

diagnostic measures, from preimplantation embryos so as to allow the rest of the embryo to develop or be transferred to a recipient.

Effect of Cell Loss on Embryonic Development

The developmental potential of early preimplantation mammalian embryos has been evaluated using a variety of micromanipulative techniques. These include mechanical separation, by a fine needle or knife (Nicholas and Hall, 1942) or suction (O'Brien et al., 1984), cellular destruction by mechanical means (Seidel, 1952; Tarkowski, 1959,a,b) or by laser (Daniel and Takahashi, 1965) and by chemical dissociation (Nicholas and Hall, 1942; Tarkowski and Wróblewska, 1967). The use of these techniques have helped in the analysis of the regulation of development and subsequently lead to the production of monozygotic half embryos (twinning), extensively reviewed by Rawlins (1984).

Nicholas and Hall (1942) were the first to successfully culture mammalian embryos following the removal of one blastomere from a 2 cell stage rat embryo. It was not until ten years later that an embryo from another species (rabbit) was manipulated and subsequently developed not only in culture but to full term following the transfer to a suitable recipient (Seidel, 1952). The first reported live birth resulting from the manipulation of a 2 cell stage mouse embryo was by Tarkowski (1959a,b), following the technique of Seidel (1952). No technique has been reported to be the most advantagous for cell isolation from early embryos, however the use of chemical dissociation (Ca^{++}/Mg^{++} free media with 0.02% EDTA)

has been shown to help prevent membrane damage. A major disadvantage of the chemical dissassociation is that it requires the removal of the zona pellucida and aspiration of the embryo.

By removal or destruction of single blastomeres developmental potentials can be assessed for both the isolated blastomere and the host embryo. Tarkowski (1959a,b) mechanically destroyed single blastomeres of 2 cell and 4 cell mouse embryos and found no difference in the timing of blastocyst formation. The blastocysts that did form were smaller than those in the controls. This was confirmed by Mulnard (1965), where 2 cell stage mouse embryos were mechanically separated or destroyed.

Normal development following micromanipulation and transfer has been reported with low success rates (Mullen et al., 1970; Fiser and Macpherson, 1976; Gartner and Baunach, 1982) with the notable exception of Moustafa and Hahn (1978). The primary difference is not in the technique but the stage of development at the time of the manipulation procedure. Moustafa and Hahn (1978) were able to obtained high success rates by micromanipulating 8-16 cell mouse embryos into halves. Fiser and Macpherson (1976) reported that some 2 cell embryos will only produce trophoblastic vesicles following the removal of one blastomere. Single blastomeres from 4 cell or 8 cell embryos were also reported to develop primarily into trophoblastic vesicles (false blastocysts) (Rossant, 1976). Rossant (1976) suggested this pheonomena was not due to a restriction in developmental potential but the result of insufficient cell

numbers at the time of cavitation. This is not in agreement with the biochemical studies previously cited. Kelly (1977) reported that the asynchrony of cell division has a role. First division cells are more likely to develop as the ICM. This suggests that cells may already be predestined, by some unknown determinates, to progress as either the ICM or trophoectoderm, but this probably is not a strong possibility based on reaggregation studies in the mouse.

Developmental potential analysis by reaggregation studies is a useful technique. Hillman et al. (1972) reported that a single blastomere from a four cell embryo will differentiate into trophoectoderm when reaggregated with the exterior of a second 4 cell embryo. When the single blastomere is located within the interior it will most likely differentiate into the ICM. This work was supported by other workers that demonstrated the totipotency of 4 cell and early 8 cell stage embryos (Sherman, 1975; Kelly, 1975). Blastomeres that were isolated from 8 cell stage embryos did not develop in culture individually but showed dependence on others for division (Sherman, 1975). Cell fate was suggested to be based on the basis of cell position for development (Sherman, 1975).

The isolation of blastomeres from embryos usually requires the removal of the zona pellucida or the zona pellucida is lost due to the technique. Therefore, it was suggested that the low developmental rates may be due to the lack of the zona pellucida. The requirement of the zona pellucida was found only to be needed

for the in vivo development of 2 cell to 8 cell embryos (Tsundo and McLaren, 1983) and not for the in vitro development (Tsundo and McLaren, 1983; O'Brien et al., 1984). O'Brien et al. (1984) also reported that the treatment of the embryos with pronase, to remove the zona pellucida, has no detrimental effect on blastocyst formation. Pronase treatment and zona pellucida free culture, in combination, was reported to decrease blastocyst formation (O'Brien et al., 1984). It may be advantageous to develop a simple and rapid technique that will allow removal of embryonic cells without any chemical pretreatment and also will reduce the chance of embryonic injury. A possible technique may be one based on blastomere displacement. By subjecting the embryos to displacement with a micropipete, this would allow loosely associated blastomeres to be isolated with less stress. The technique would have a micropipete, pulled to patent point, enter the zona pellucida and cause the blastomere(s) to become displaced. Further penetration of the micropipete into the zona pellucida would further displace the blastomere(s) and allow a single or group of blastomeres to vacate the zona pellucida. Cell isolation or biopsy would allow for the acquisition of embryonic cells, for diagnostic analysis or developmental potential analysis, while maintaining normal embryonic growth and development.

CHAPTER III

MATERIALS AND METHODS

Production and Collection of Embryos

Three to four week old female Swiss mice were used for the production of embryos. Animals were maintained on a 12L:12D photocycle. Each animal received 10 I.U. of pregnant mare serum gonadotropin (PMSG) (Serotropin,® Teizo, Tokyo, Japan) i.p. at 1500 hours on Day 1 and injected 48 hours later at 1500 hours on Day 3 with 10 I.U. human chorionic gonadotropin (hCG) (Sigma Chemical Co., St. Louis, MO). The females were mated with males of proven fertility on Day 3 following the administration of hCG.

Different stages of embryonic development were obtained by sacrificing the females by cervical dislocation at various post mating intervals. Two cell embryos were recovered on the morning of Day 5, 4 cell stage in the early evening of Day 5 and 8 cell stage embryos were recovered in the morning of Day 6.

Embryos were collected by oviductal fragmentation. The reproductive tracts were excised from the females and the oviducts were dissected away from the uterine horns and ovaries. Isolated oviducts were placed into welled glass dishes containing 1.0 ml of filtered sterilized (0.22 μm) (Millex-GS, Millipore, Bedford, MA) culture medium. Microforceps were used to tear, fragment and remove the oviducts. This technique allows the embryos to settle on the bottom of the dish. Embryo recovery was carried out at room

temperature, but the medium was maintained at 37°C by warming on a slide stage warmer. Embryos were sorted according to stage of development, under a Bausch & Lomb stereomicroscope (70X magnification), by aspiration into a 5 µl micropipettor (SMI, Emeryville, CA). The embryos were transferred to a second welled dish containing fresh filtered sterilized medium and maintained at 37°C.

Microsurgical Manipulation of Embryos

Embryonic manipulations were carried out in a manipulation chamber, which is an inverted staining glass cover dish (No. 08-813A, Fisher Scientific, Pittsburgh, PA), coated with Sigmacoat® (Sigma) to prevent adherence of isolated blastomeres to the glass dish. The manipulation chamber rests within the micromanipulation area (Figure 1): consisting of a Bausch & Lomb stereomicroscope, bordered on two sides by Emerson Model B micromanipulators (J.H. Emerson Co., Cambridge, MA). Embryos were microsurgically manipulated with a 5 µl glass Yankee Micropet (Clay Adams, Parsippany, NJ) pulled to a patent point. Pulled micropipetes were bent at two right angles, opposed and in the same plane over an open flame of a microburner. The microburner was constructed from a pulled and bent 9 in. Pasteur pipete.

The technique for blastomere isolation was by displacement (Figure 2):

a. the micropipete was gently lowered upon and through the zona pellucida

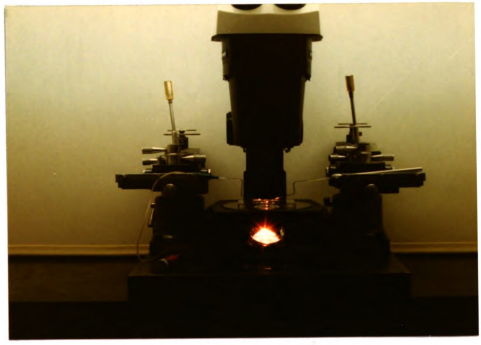


Figure 1. Manipulation area

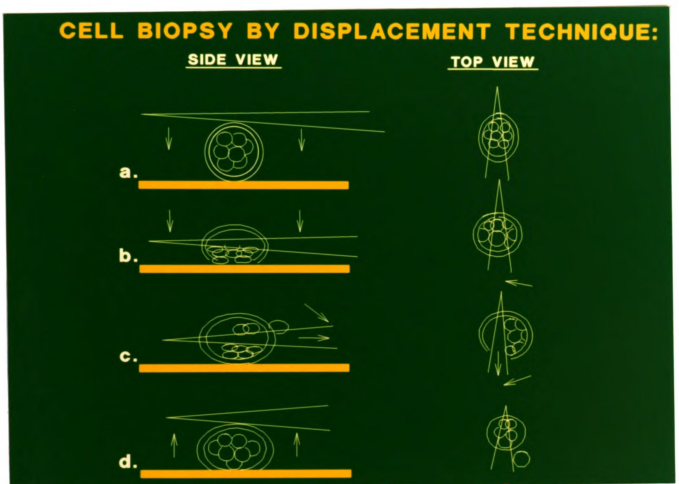


Figure 2. Cell biopsy by displacement technique

b. as the micropipete is lowered onto the embryo the blastomeres will be gently depressed and displaced within the zona pellucida

c. slight withdrawal and change in angulation permits further displacement of the blastomeres and permits the desired number of cells to vacate the zona pellucida

d. when the desired number of blastomeres have been removed, the micropipete is raised allowing the zona pellucida to close and results in the biopsy of cells from the embryo with minimal injury

Isolated, or biopsied, blastomeres and manipulated embryos were then transferred directly to microdrops (0.1 ml) of culture medium under light mineral oil (Sigma) in 35 mm Tissue Culture Dishes (Corning Glass Works, Corning, NY). All embryos, control and manipulated, were incubated for 24 hours at 37°C in a humidified atmosphere of 5% CO_2 in air, then evaluated for further development.

Assessment of advanced cell stages (16+) was aided by the use of Hoechst 33258 (10.0 µg/ml) (Sigma) and fluorescent microscopy. Hoechst 33258 is a vital nuclear stain that binds to adenine-thymidine base pairs on the chromosomes. Fluorescent microscopy was carried out on a Nikon-Diaphot inverted microscope (Nikon, Garden City, NY) equipped with an epi-fluorescence filter combination UV-1A. The filter combination consisted of a 400 dichroic mirror, 365/10 excitation filter and a 400 barrier filter. The main wavelength for ultraviolet (UV) excitation was 365 nm.

Embryo Culture

Mouse embryos were cultured at 37°C in a humidified atmosphere of 5% CO_2 in air for 24 hours of incubation. Embryonic development was assessed for 2-, 4- and 8 cell stage, microsurgically manipulated embryos and for biopsied blastomeres. Two culture media were evaluated, with or without fetal calf serum (20%) (Gibco, Grand Island, NY) supplementation. The culture media tested were a modified TC-199 (HEPES) (Gibco) and Embryo Culture Medium (ECM) based on a modified BMOC (Brinster, 1963b) and TALP (Tyrodes salt solution, albumin, lactate and pyruvate) (Bavister et al., 1983).

TC-199 was modified by the addition of sodium pyruvate (Sigma), bovine serum albumin (Sigma) and penicillin-streptomycin (Sigma) (Table 3). The medium was sterilized by filtration through a 0.22 μm Millex-GS filter prior to use. Embryo Culture Medium (ECM) was developed based on prior work in the mouse (Brinster, 1963b) and the hamster (Bavister et al., 1983) in order to produce a medium which would support the development of both mouse and hamster embryos *in vitro*. The components of ECM, Table 2, were added to sterile water to a final volume of 100 ml. The use of amino acids has been shown to be required for the *in vitro* development of hamster embryos (Bavister et al., 1983), but is not required for mouse embryos. The amino acid solution was still used in the culture media for mouse work, when it was observed to help decrease the rapid rise in pH while the medium was in an open air environment. The amino acids were added to final volume of 100 ml

Table 3. Composition of culture media used.

Embryo Culture Medium (ECM)

Component	Mean Weight	mM	Grams
NaCl	58.4424	114.00	0.6662
KCl	74.5510	4.78	0.0356
$CaCl_2 \cdot 2HOH$	147.0207	1.71	0.0251
KH_2PO_4	136.0853	1.19	0.0162
$MgSO_4 \cdot 7HOH$	246.4700	1.19	0.0293
Phenol Red	----	----	0.0020
Glucose	180.1589	5.56	0.1002
HEPES	238.3000	25.00	0.5958
$NaHCO_3$	84.0071	25.00	0.2100
Na pyruvate	110.0	1.17	0.0188
Na lactate (60% syrup)	112.1	21.58	0.2419 (0.37 ml)
BSA	----	----	0.3000
Penicillin-Streptomycin (10,000 U/ml - 10 mg/ml)		----	1.0 ml
Amino Acid Solution	----	----	6.25 ml

Modified TC-199

TC-199	100.0 ml
NA pyruvate	0.0188 g
Bovine Serum Albumin	0.3000 g
Penicillin-Streptomycin	1.0 ml
Amino Acid Solution	6.25 ml

Amino Acid Solution

Amino Acid	Grams/100.0 ml
L - Isoleucine	0.0520
L - glutamine	0.2936
L - methionine	0.0150
L - phenylalanine	0.0330

of sterile water and stored in 6.25 ml/10 ml Vacutainer (Becton-Dickinson, Rutherford, NY) at -5°C.

The addition of fetal calf serum (Gibco) was used to determine if serum would enhance embryonic development. Heat inactivated serum was stored in 8.0 ml/10.0 ml Vacutainer at -5°C until needed, then thawed by placing in a warm water bath.

In all experiments, the same culture medium was used for embryo recovery, microsurgical manipulation and embryo culture. Media was prepared fresh daily and was discarded after each trial. Osmolarity was measured by freeze point depression using a Micro-osmometer (Precision Systems, Inc., Natick, MA). Osmolarity and pH was maintained between 250-310 mOSM and 7.30-7.40, respectively.

Statistical Analysis

All data were analyzed by two-way contingency Chi-squared test according to Gill (1978).

CHAPTER IV

RESULTS

A total of 909 embryos were recovered from three to four week old female Swiss mice. Embryos were used to evaluate two culture media for optimal *in vitro* growth and to develop the cell biopsy by displacement technique (Table 4). A total of 729 mouse embryos (2-8 cell stage) served as controls or were used to evaluate the media. The cell biopsy technique was performed on 180 embryos, of which only 12 (6.7%) were not successfully manipulated and resulted in induced cell death. Cell death was defined as a blastomere that was destroyed within the zona pellucida and was incapable of being removed. Only two of the induced cellular death embryos, compacted 8 cell stage, developed in culture.

The Effect of Media and Serum Supplementation

The effect of two culture media and fetal calf serum supplementation (20%) on their ability to support mouse embryo development *in vitro* was examined. Table 5 compares the development to at least one further developmental stage of 2-, 4- and 8 cell mouse embryos in Embryo Culture Medium (ECM) and a modified TC-199, with or without 20% fetal calf serum supplementation.

ECM (68.8%) was found to enhance embryonic growth over the modified TC-199 medium (50.7%) ($P < 0.001$). There was no overall beneficial effect of adding fetal calf serum to ECM or TC-199.

Table 4. Mouse embryo (2-8 cell stage) recovery.

Total embryos recovered	909
Control & Media Test	729
Total number of embryos biopsied	180
(no. developed)	(67)
(no. failed biopsy)	(12)

Table 5. The effect of culture medium and fetal calf serum supplementation on embryonic growth *in vitro*.

Cell Stage	Medium: Embryo Culture Medium (ECM) +20% FCS	Medium: Embryo Culture Medium (ECM) -20% FCS	Medium: TC-199 +20% FCS	Medium: TC-199 -20% FCS	Total
2	24/29 (82.8%)[a]	65/105 (61.9%)[a]	11/33 (33.3%)	29/78 (37.2%)	129/245 (52.7%)
4	11/21 (52.4%)	18/26 (69.2%)	11/28 (39.3%)	9/31 (29.0%)	49/106 (46.2%)[b]
8	29/42 (69.0%)	74/98 (75.5%)	112/186 (60.2%)	35/52 (67.3%)	250/378 (66.1%)[b]
Total	64/92 (69.6%)	157/229 (68.6%)	134/247 (54.3%)[d]	73/161 (45.3%)[d]	----
TOTAL	221/321 (68.8%)[c]		207/408 (50.7%)[c]		428/729 (58.7%)

a significantly different ($P > 0.05$)
b significantly different ($P > 0.001$)
c significantly different ($P > 0.001$)
d not significantly different ($P < 0.1$)

There was enhanced development with serum supplementation at the 2 cell stage ($P < 0.05$). The supplementation of FCS was not found to enhance overall embryonic development, however, it did approach significance ($P < 0.1$).

The Effect of Microsurgical Manipulation on Embryo Growth

A preliminary study was conducted to determine the optimal stage for the microsurgical manipulation of preimplantation mouse embryos. The effects of microsurgical manipulation and media on embryonic development *in vitro* are shown in Table 6. ECM (43.1%) was found to enhance the development of manipulated mouse embryos over TC-199 (30.3%). There was no difference between the manipulation of 4 cell (38.1%) and 8 cell (39.5%) mouse embryos. Based on these results it was decided to limit studies on the effect of cell biopsy by displacement technique to the 8 cell embryo since they were more readily available and developed better in culture over the 4 cell embryo, 46.2% vs. 66.1% ($P < 0.001$), respectively (Table 5).

The Effect of Cell Loss on Embryonic Growth

A total of 120, 8 cell, Swiss mouse embryos were subjected to the cell biopsy by displacement technique (Table 7). Figure 3b illustrates an example of an 8 cell embryo immediately following the technique and after culture (Figure 4a). Control embryos are illustrated in Figures 3a and 4b.

Table 6. Development of embryos subject to micromanipulation in two media.

Cell Stage	Medium		Total
	ECM	TC-199	
2	4/12 (33.3%)	1/6 (16.7%)	5/18 (27.8%)
4	6/13 (46.2%)	2/8 (25.0%)	8/21 (38.1%)
8	34/77 (44.2%)	17/52 (32.7%)	51/129 (39.5%)
Total	44/102 (43.1%)	20/66 (30.3%)	64/168 (38.1%)

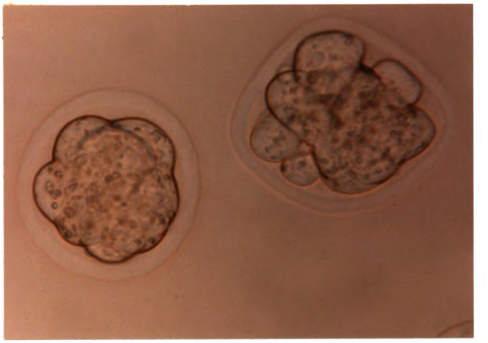
No significant difference found.

Table 7. The effect of cell loss and media on 8 cell embryo growth.

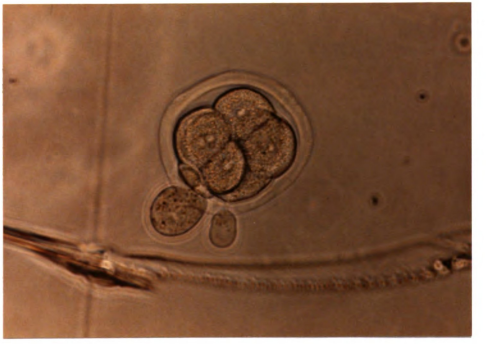
Cell Loss	Medium		
	ECM	TC-199	Total
0 (control)	103/140 (73.6%)	147/238 (61.8%)	250/378 (66.1%)
- 1	14/24 (58.3%)	6/16 (37.5%)	20/40 (50.0%)[b]
- 2	2/14 (14.3%)	4/12 (25.0%)	6/26 (23.1%)[ab]
- 3	7/16 (43.8%)	1/4 (25.0%)	8/20 (40.0%)[b]
- 4	9/25 (36.0%)	3/18 (16.7%)	12/43 (27.9%)[a]
- 5	1/15 (6.7%)	2/10 (20.0%)	3/25 (12.0%)[a]
- 6	1/13 (7.7%)	1/10 (10.0%)	2/23 (8.7%)a
- 7	0/13 (0.0%)	0/12 (0.0%)	0/25 (0.0%)[a]
Total	34/120 (28.3%)	17/82 (20.7%)	51/202 (25.2%)[a]

a significantly different from control ($P < 0.001$)

b combined data, not significantly different from control ($P > 0.05$)

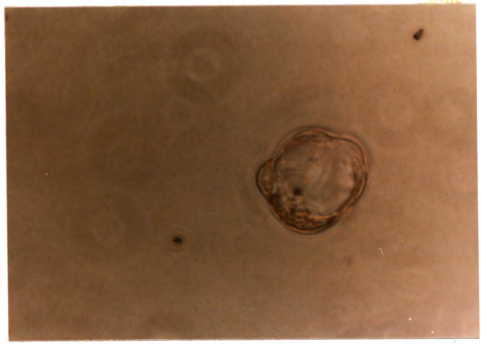


a. 8 cell stage embryos

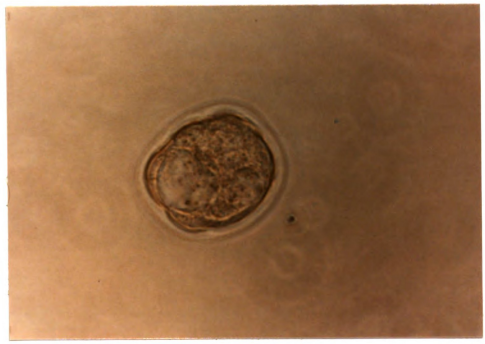


b. 2 cells biopsied from an 8 cell embryo

Figure 3. Preimplantation mouse embryos prior to culture



a. blastocyst from biopsied 8 cell embryo



b. blastocyst from control 8 cell embryo

Figure 4. Preimplantation mouse embryos following culture

The biopsy of 1 or 3 cells did not decrease the developmental potential of 8 cell embryos from the controls. Removal of 4 or more cells was found to prevent development in biopsied embryos ($P < 0.001$). It was observed that precompacted 8 cell embryos were more readily biopsied than compacted 8 cell embryos. However, it was found that biopsied compacted embryos would develop in culture over precompacted stage embryos ($P < 0.001$) (Table 8).

Cell Differentiation Observation

During the assessment following the culture of biopsied and control 8 cell mouse embryos, it was observed that precompacted embryos would be delayed in blastocyst formation but not for similarily treated compacted embryos. If a precompacted 8 cell embryo has 3 cells removed then it will behave as if it had just reached the 5 cell stage, whereas in the compacted 8 cell embryo the remaining 5 cells would develop into a blastocyst or a trophoblastic vesicle.

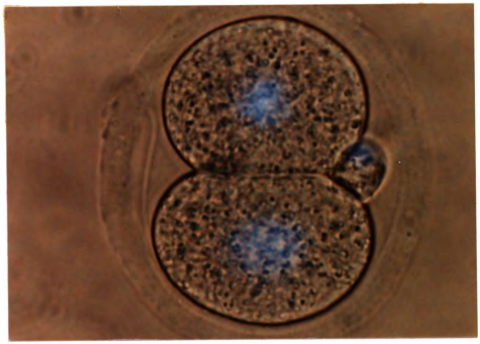
Analysis of Development Using Hoechst 33258

The use of Hoechst 33258 (10.0 μg/ml) greatly aided developmental stage analysis, by cell number quantification in all embryos (Figures 5 and 6). This confirms previous work demonstrating the effectiveness of nuclear fluorochromes for the visualization of embryonic nuclei (Ebert et al., 1985; Crister and First, 1986).

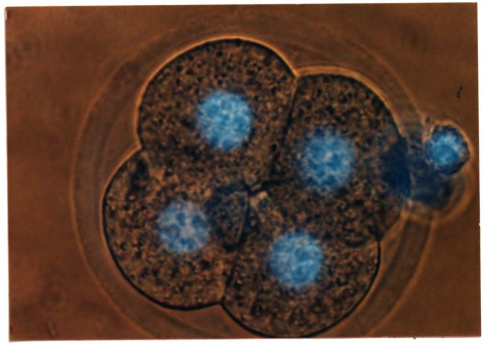
Table 8. Percent of developing embryos progressing to the morula and blastocyst stage.

Group	Cell Development Stage	
	Morula	Blastocyst
Control		
4	6/49 (12.2%)	0/49 (0.0%)
8 precompacted	134/169 (79.3%)	28/169 (16.6%)[a]
8 compacted	64/81 (79.0%)	29/81 (35.8%)[a]
Biopsied		
4	1/8 (12.5%)	0/8 (0.0%)
8 precompacted	26/36 (72.2%)	5/36 (13.9%)[b]
8 compacted	15/15 (100%)	14/15 (93.3%)[b]

a,b similar scripts are significantly different ($P < 0.001$)



a. 2 cell stained embryo



b. 4 cell stained embryo

Figure 5. Hoechst 33258 stained mouse embryos

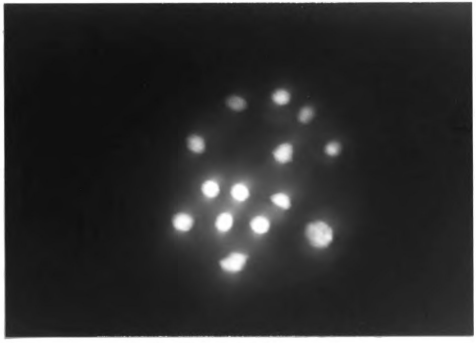


Figure 6. Fluorescent nuclei from manipulated 8 cell embryo after *in vitro* culture

CHAPTER V

DISCUSSION

The objective of this study was to develop a procedure for the removal of blastomeric cells from preimplantation mouse embryos and analyze the biopsied embryos developmental potential in vitro. Developmental potentials of biopsied mouse embryos were lower than expected for two cell embryos (27.8%). Tsundo and McLaren (1983) reported 65% development of divided 2 cell embryos. The development of divided 4-8 cell embryos (39.4%) was similar to previous previous reports (30-40%) (Tsundo and McLaren, 1983; Nagashima et al., 1984). The results suggest that the precompacted 8 cell embryo is the optimal stage to obtain cells by the biopsy technique. The maximal number of cells that can be biopsied from an 8 cell embryo without significantly disrupting temporal development is three.

It was observed that 4 cell and early precompacted 8 cell embryos maintain totipotency, which is in agreement with Kelly (1975). Compacted 8 cell embryos appear to have lost total potency suggesting that the blastomeric cells may have begun differentiation to the ICM and trophoectoderm. However, it is still not known what causes the cell differentiation. Suggestions include cell position (Fleming, 1987), cytoplasmic determinants (Tarkowski and Wróblewska, 1967) or cellular interactions (Dyce et al., 1987). The observation that some divided compacted 8 cell embryos

developed into false blastocysts or trophoblastic vesicles confirms the report of Rossant (1976). However, Rossant (1976) believed that this was not due to restriction in developmental potential but the result of insufficient cell numbers at the time of blastocoele formation. While the present study confirms that there is no apparent loss of developmental potential, one can not totally agree with the theory of insufficient cell numbers. A possible explanation is that there are insufficient numbers of ICM determined cells. That is to say that during the biopsy technique the cells that were removed might have been destined to become the ICM. Therefore, the results may suggest that sufficient cell numbers are required for compaction to occur and blastocyst formation would subsequently follow (Johnson et al., 1979). Boldt (1988) suggested that the manipulated embryo must have at least 50% of its cells to form the inner cell mass. It was observed in the present study that true blastocysts will form if 3 or fewer blastomeres are biopsied from the 8 cell embryo.

The reason for the inability of single or pairs of isolated blastomeres from 8 cell embryos to develop in vitro is unclear. The lack of cell-cell communication by cellular interactions is a possibility, however Kelly (1975) suggested that this restriction may only be due to their small size and not their intrinsic ability. Further studies are required to determine if development can be enhanced if cells are co-cultured with other whole embryos.

Overall embryonic development was supported in both Embryo Culture Medium (ECM) (68.8%) and TC-199 (50.7%), but ECM was clearly superior. The results were less than anticipated since other workers have reported greater development (75-95%) (Whitten and Biggers, 1968; Hsu et al., 1974; Arny et al., 1987). One possible reason may be strain differences rather than culture conditions. Additional studies are required to determine the optimal culture medium for each mouse strain. Supplementation of serum was not beneficial on an overall basis, but did enhance two cell development for embryos cultured in ECM, this confirms the previous work of Arny et al. (1987). It also was found that serum retarded 4 cell embryo development. Thus serum supplementation may be stage specific, beneficial at certain stages and harmful at others. This is supported by previous studies showing that serum supplementation is deleterious to mouse oocytes fertilized in vitro and cultured to the 4 cell stage (Hans and Kiessling, 1988). However, Saito et al. (1984) reported no effect of serum addition on 2 or 4 cell mouse embryos but increased 8 cell growth. The only major difference between the studies is that Saito used human cord serum and a different strain of mouse (BALB C/6 x C_3H). The differing results may not be due to the serum source since Bates et al. (1985) reported no significant difference in mouse embryos grown in media supplemented with human cord serum or fetal calf serum. The overall effect of serum supplementation for embryos cultured in TC-199 may be explained by that what ever factors present in the serum which

stimulate embryonic development does not work as an additive thus stimulating growth but rather as a replacement (mimic) for whatever may be missing in the medium that is present in other media, e.g. ECM.

Therefore, mouse embryos can be biopsied at the 8 cell stage and cultured with optimal conditions to the blastocyst stage. A maximum of three blastomeres can be removed without retarding development. There are differentiational effects of serum supplementation on mouse embryo development in vitro. Addition of serum to media is beneficial for 2 cell embryos but is not for 4 and 8 cell embryos. Serum supplementation retards the development of 4 cell mouse embryos to the 8 cell stage.

CHAPTER VI

SUMMARY AND CONCLUSIONS

The primary objective of this study was to develop a protocol for the removal of blastomeric cells from preimplantation mouse embryos. It is believed that these biopsied cells could be used for diagnostic analysis to aid in the prediction of embryonic normality. Two culture media and serum supplementation was used to determine suitable culture conditions to support embryonic development in vitro.

The following conclusions have resulted from this study:

1. The use of Embryo Culture Medium (ECM) (a modified BMOC-TALP medium) was found to significantly enhance the development of mouse embryos (2-8 cell stage) over a modified (TC-199) medium.

2. The supplementation with 20% fetal calf serum was found to enhance 2 cell embryo development but reduces 4 cell embryo growth. No overall effect of fetal calf serum supplementation was found.

3. A total of 168 (93.3%) mouse embryos were successfully biopsied, of which 38.1% developed in culture.

4. The 8 cell embryo was found to be the most desirable stage for biopsy.

5. A maximum of three cells may be removed from the 8 cell mouse embryo without retarding development.

6. Precompacted 8 cell embryos were more readliy biopsied than the other cell stages including the compacted stage.

7. Compacted 8 cell embryos were more likely to develop in culture than precompacted embryos.

8. Cell determination is believed to occur at or prior to the compacted 8 cell stage in the mouse.

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APPENDIX A

MANIPULATION OF HAMSTER EMBRYOS

Preliminary work on microsurgical manipulation of mammalian embryos was carried out on hamster embryos. This work was based on previous studies by Rawlins (1984).

Embryos (2-8 cell stage) were recovered from superovulated female hamsters (6-8 weeks) as described by Rawlins (1984). Embryos were recovered in Ca++/Mg++ free PBS (Gibco) and cultured in TALP + 20% FCS (Bavister et al., 1983) at 37°C, under oil in an humidified atmosphere of 5% CO_2 in air.

A total of 215 hamster embryos were recovered. Of these embryos 156 served as controls and 59 were microsurgically manipulated. Only one hamster embryo at the 6 cell stage, developed *in vitro* after having embryonic cells removed. This embryo had two blastomeres removed and the remaining 4 cells developed to the 8 cell stage. Control embryonic development was as follows:

2 cell (n=54) 0.0% development (2 cell block)

4 cell (n=50) 32.0% developemnt

8 cell (n=52) 13.5% development.

Difficulties were found in not only developing the technique for cell biopsy but also in the *in vitro* culture of the control hamster embryos. It was not known if cells removed from an early embryo would develop or be blocked at the two cell stage.

Therefore, studies were discontinued in the hamster and initiated in the mouse.

APPENDIX B

PUBLICATIONS BY THE AUTHOR

1. Effects of estradiol 17-beta and/or zeranol upon the seminal vesicle epithelium and stroma of young Angus bulls. W.E. Roudebush. Master's Thesis. The Ohio State University. 1985.

2. In vitro fertilization normality of Squirrel monkey (Saimiri sciureus) embryos. W.R. Dukelow, D.L. Pierce, W.E. Roudebush, J.K. Graham, and T. Asakawa. Proc. Fourth World Congress on In Vitro Fertilization, 1985, Melbourne, Australia.

3. In vitro fertilization in nonhuman primates. W.R. Dukelow, W.E. Roudebush, D.L. Pierce, J.K. Graham, and K. Sengoku. XIth. Congress of the International Primatological Society, 1986, Gottingen, FRG. Primate Report (14) July, 1986.

4. In vitro fertilization in nonhuman primates. W.R. Dukelow, D.L. Pierce, W.E. Roudebush, S.J. Jarosz, and K. Sengoku. J. Med. Prim. (in press, 1987).

5. Blastomere isolation and culture of preimplantation embryos. W.E. Roudebush, D.L. Pierce, K. Sengoku, and W.R. Dukelow. The American Fertility Society Annual Meeting. Reno, Nevada. Sept. 1987.

6. Induced cell loss: effects on the potential for embryos to develop in vitro. W.E. Roudebush, S.D. Kholkute, D.L. Pierce, and W.R. Dukelow. Biol. Reprod. 38 (suppl 1): 189. 1988.

7. Cell differentiation as determined by micromanipulation of mouse embryos. N.C. Cosby, W.E. Roudebush, L. Ye, and W.R. Dukelow. Biol. Reprod. 38 (suppl 1): 128. 1988.

8. Induction of follicular growth in the squirrel monkey with clomiphene citrate (CC). D.Pierce, Ye Lian, W.E. Roudebush, and W.R. Dukelow. Amer. J. Primat 14:437. 1988.

9. Timed-mating and gestation. W.R. Dukelow, S.D. Kholkute, Ye Lian, N.C. Cosby, W.E. Roudebush, and S. Bruggemann. Inter. J. Primat. 8:558. 1988.

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American Society of Animal Science

American Society of Primatologists

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