final report

Project Code: MU.021

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The Production of Elite Breezing Stock by Embryo Multiplication

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Nuclear Transfer (using embryonic cells)

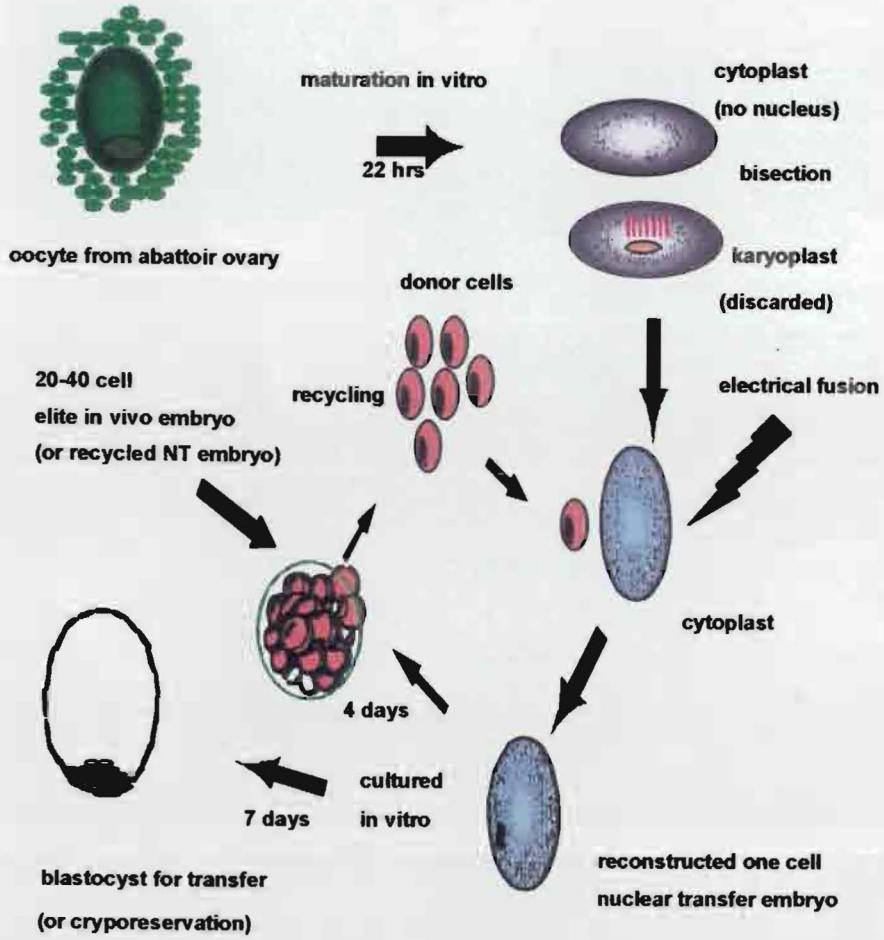

TABLE OF CONTENTS

Executive Summary

The aim of the project was to produce large numbers of low cost, genetically identical, elite cattle embryos for transfer into recipient cows to generate genetically identical, elite offspring. The embryos were to be produced by novel, simplified nuclear transfer procedures using embryonic cells as the source of donor nuclei.

Considerable progress was made towards meeting the project goals.

Using simplified nuclear transfer and cryopreservation techniques developed during the project, it was demonstrated that over 70 transferable (morula and blastocyst stage), recycled nuclear transfer embryos could be generated from one original embryo during three rounds of recycling.

Nine cloned calves derived from embryonic cells were born during the project including two sets of genetically identical bull calves. There are a further 25 diagnosed cloned pregnancies generated late in the project awaiting calving data.

The production of nuclear transfer embryos and pregnancies using somatic cells such as fetal fibroblasts as the source of donor nuclei was added as an objective because of rapid advances being made in this area overseas.

Eighty-seven somatic cell nuclear transfer morula and blastocysts for transfer were generated in the last six months of the project using microinjection techniques. These embryos were transferred, either fresh or after cryopreservation, into recipient cows, and generated three pregnancies.

Vitrification techniques for the cryopreservation of early stage nuclear transfer embryos, developed and successfully applied during the project, have the potential to overcome the logistical problems associated with multiple rounds of nuclear transfer. These novel vitrification methods will also allow the cryopreservation and direct transfer of in vitro produced embryos produced by cloning, which is a prerequisite for widespread adoption of the technology.

The absence of abnormalities, or grossly oversized calves in the cloned offspring born so far in the project is an advance on overseas experience. However, the birth of more calves will be required before it can be assumed that the nuclear transfer and culture techniques developed during this project do not lead to abnormal offspring.

Approximately 50% of nuclear transfer pregnancies positively diagnosed by ultrasound around 35 days of gestation were lost later in gestation. Losses of this magnitude are not observed in in vitro produced embryos. Whether the problems causing such losses are placental or fetal in origin, it is likely that they stem from inadequate reprogramming of the donor nucleus following its transfer into a recipient cytoplast.

A considerable amount of scientific data in relation to cloning has been generated during this project. This includes information on recycling, donor and recipient cell cycle stages, cryopreservation and reprogramming of both embryonic and somatic cells in nuclear transfer.

The Project saw the successful transfer of technology from Monash University to Genetics Australia. This has included establishing an efficient production process at Genetics Australia for the maturation of bovine oocytes and for the production of in vitro embryos.

Systems were also developed for the management of recipients prior to and after embryo transfer and for managing calving and calves after delivery, which maximize outcomes from embryo transfer.

Unravelling the mechanisms involved in nuclear reprogramming may hold the key to the production of nuclear transfer embryos which result in ongoing pregnancy rates acceptable for widespread agricultural applications of the technology. There are, however, applications of the technology in which lower pregnancy rates will give commercially viable outcomes. These applications are high value added products such as genetically enhanced animals, elite beef bulls, or elite dairy cows.

Project MU035 which follows MU021 aims to answer fundamental scientific questions about nuclear reprogramming and its effect on embryo viability and pregnancy rates. It will evaluate outcomes from present efficiencies with embryo recycling combined with vitrification techniques, and will continue to develop the efficiencies possible. The project will continue to develop and evaluate somatic cell nuclear transfer as a source of donor cells, including testing embryo viability and pregnancy results following recycling embryos generated from somatic cells. It will evaluate outcomes possible by combining these techniques with transgenic enhancement.

Objective 1:

To maximize the number of viable embryos produced from the disaggregation of bovine preimplantation embryos by optimizing each step in the nuclear transfer procedure. The research will initially concentrate on synchronizing the donor embryonic cells with the recipient cytoplasm and on activation of the reprogramming events which result in normal embryo development to the blastocyst stage for transfer to recipients.

Quantifiable objective:

The nuclear transfer techniques described will be considered successful when two or more morphologically normal morula and blastocyst stage nuclear transfer embryos can be produced from donor and recycled embryo. This is equal to an embryo multiplication factor of 2 for each generation of nuclear transfer.

This objective has been addressed in the following milestone:

- " Development of optimal conditions for the production of genetically identical nuclear transplant (NT) embryos" (Year 1, Milestone 1)

Completion of objective:

The development rate to the morula and blastocyst stage of nuclear transfer embryos after one round of cloning routinely reaches 20 to 30%. As a result of the techniques developed it is possible to produce 3 to 6 (in vitro derived) or 4 to 12 (in vivo derived) transferable embryos per one original donor embryo in the first round of nuclear transfer, thereby achieving the objective.

Summary of progress

- **Oocyte and cytoplast activation has been optimized.**
- **A novel enucleation method, which is easy to apply to large-scale experimentation and production, has been developed**
- **Zona-free cell fusion methods which can be combined with the novel enucleation technique have also been developed.**
- **A bi-phasic *in vitro* culture system for zona-free embryos and clones was developed and adopted by the Project.**
- **The above methods have been used to produce nuclear transfer embryos derived from *in vivo* and *in vitro* derived fresh and cryopreserved embryos at developmental rates comparable with non-NT *in vitro* produced embryos (15-35% transferable embryos from reconstituted nuclear transfer embryos)**
- **The advantage of using donor blastomeres with a chemically synchronized cell cycle for nuclear transfer has been demonstrated, however this method is not yet conveniently applicable to routine production.**
- **Various other studies related to optimization of nuclear transfer procedures have been conducted and the methods applied for routine production when appropriate.**

(i) Oocyte and cytoplast activation

Initial oocyte activation studies found that Ca^{2+}-Ionophore treatment combined with 6-DMAP incubation to be the most efficient method of oocyte activation, when compared to treatments consisting of Ca^{2+}-Ionophore alone, 6-DMAP alone or as a negative control, or with neither Ca^{2+}-Ionophore or 6-DMAP treatments. This applied to both the parthenogenetic development and the development of nuclear transfer embryos derived from centrifugation-enucleated and activated cytoplasts. Once the oocyte enucleation

method based on bisection was adopted, the activation of cytoplasts was modified to consist of activation treatment after, rather than before, oocyte enucleation. Various factors such as exposure to fluorescence microscopy and kinetics of activation were also addressed.

As a result of these studies, a routine activation method of bisection-enucleated cytoplasts consisting of 10 min incubation in 38 mM Ca^{2+}-Ionophore in a protein-free HEPES buffered handling medium, followed by 4 h incubation in 2mM 6-DMAP in $NaHCO_3$-buffered and bovine serum albumin (BSA) containing medium, has been adopted.

(ii) Novel enucleation method

At the beginning of this project a novel oocyte enucleation method based on centrifugal separation of karyoplasts and cytoplasts was used for nuclear transfer studies. Although this method offered clear advantages over the micromanipulation based enucleation procedures, the post-transfer viability of such cytoplasts was found to be very low. Thus a new method for enucleation was developed, which still fulfilled the expectations of easy adaptation to large scale application of the technology.

This new method relies on the removal of the zona pellucida of mature oocytes, followed by bisection of the oocyte. One half containing the nucleus (the karyoplast) is discarded and the other half containing only cytoplasm (the cytoplast) is used as the "recipient" for a "donor" nucleus in nuclear transfer. Initially, the oocytes were stained with chromatin specific fluorochrome (Hoechst 33342), bisected, and both halves then evaluated by fluorescence microscopy to separate karyoplasts from cytoplasts. However an additional step of phytohemagglutinin (PHA) incubation prior to zona removal was introduced to the protocol. PHA acts by binding the first polar body to the oocyte, facilitating identification of the part of the oocyte containing chromatin for its proximity to the polar body. This in turn facilitates the bisection of oocyte into uneven halves.

As it is widely recognized that the loss of cytoplasmic volume may affect the subsequent development of nuclear transfer embryos produced with such cytoplasts, extensive experiments were carried out to elucidate this point with the new enucleation method. Nuclear transfer embryos were produced using one or two cytoplasts per reconstituted embryo, employing one or two fusion steps. Subsequent development rates and cell numbers of resulting embryos were assessed.

As a result of these studies, the routine method of using two bisection-enucleated cytoplasts for each reconstituted embryo was adopted.

(iii) Novel approach to cell fusions

When using either centrifugation or bisection enucleated cytoplasts, the subsequent fusion of donor blastomere to the recipient cytoplast had to be adapted to accommodate the absence of the zona pellucida. Such methods were developed early on in the project, and relied on the use of strong alignment pulses and careful manual manipulation and handling of cells. As mentioned above, the zona-free approach also facilitates the fusion of two or more cytoplasts together, something which is not feasible with zona-intact cytoplasts.

(iv) Culture of zona-free embryos and clones

The production of zona-free nuclear transfer embryos required some modifications into the existing embryo culture systems. Firstly, culture of more than one embryo in a drop results in spontaneous aggregation of zona-free embryos into giant embryo aggregations. Secondly, at the early cleavage stages the tendency of blastomeres to attach to the bottom of the culture dish interfered with establishment of the close cell to cell contacts required for proper development. A substantial amount of work was therefore done on a zona free embryo culture system.

To prevent embryo aggregation, embryos have been routinely cultured singly in drops of between 2 to 30 µl. In order to achieve the beneficial effects of culturing embryos in groups, several different approaches

were tested. The first approach was to culture up to four embryos in one specially shaped drop ("tetra-drop"), the shape of the drop physically preventing embryos coalescing. However, experiments performed with normal in vitro produced zona free embryos failed to show improved development in these drops. The second approach was to culture embryos in special hybridoma-dishes, whose culture surface consists of hundreds of tiny wells separated by walls. Zona free embryos were cultured in these wells singly or in groups, in one drop. The results of these experiments did not reveal differences in embryos cultured singly or in groups. Thirdly, zona free embryos were embedded in small agar columns singly or in groups and subsequently cultured in drops. Again, no significant differences could be observed in development between single and grouped embryos.

The overall conclusions from these studies are that any extra handling of embryos required for grouping might be counter-productive in regard to possible benefits achieved by group culture. In addition, the beneficial effect of group culture may require such an intimate contact between embryos that even the slightest physical separation would be inhibitory for manifestation of such effects.

To further facilitate the culture of zona free embryos, embryos have been cultured in high concentration of BSA. A macromolecule such as BSA prevents blastomere attachment to the bottom of the culture dish. Initial concentration of 32 mg/ml (as opposed to 8 mg/ml used for culture of zona intact embryos) has been subsequently dropped to 20 mg/ml because of the possibility that too much BSA might interfere with the action of some cell surface molecules.

These experiments, together with general improvements made into bovine in vitro embryo culture by Dr. Gardner et al. at Monash University, have led to the currently used routine culture system of nuclear transfer embryos. Embryos are cultured singly in 20 µl drops of modified SOF with 20 mg/ml BSA and non-essential amino acids for the first three days, after which they are transferred singly into 30 µl drops of modified SOF with 20 mg/ml BSA and all 20 amino acids for further three days. If embryos are not utilized at Day 6, they are transferred once more into fresh 30 µl drops of the latter medium.

Nuclear transfer embryos of same genetic origin have also been deliberately aggregated together to form aggregates of 2 to 4 clones. This has been done in order to improve the cell numbers of resulting embryos and consequently, their developmental viability. Improved pregnancy rates were observed with embryo aggregates as compared with individual embryos. However, in the long term this approach can be counterproductive as it reduces the actual number of clones, and hence further studies have been aimed at improving the developmental competence of single clones. Given the lowered cell numbers seen with recycled (multigenerational) nuclear transfer embryos (see table 1), such aggregation techniques may again be adopted for such embryos until their developmental competence can be improved.

(v) Cell cycle in nuclear transfer

It has been unequivocally established that the cell cycle compatibility between donor nucleus and recipient cytoplasm is important for nuclear transfer success. Hence throughout the project we have mainly used pre-activated cytoplasts ("universal recipients") as recipients of mainly DNA-synthesis phase (S-phase) donor embryonic blastomeres. However, in one set of experiments the development of nuclear transfer embryos derived from fusions of G1-phase arrested blastomeres with non-activated cytoplasts was compared with above mentioned nuclear transfer control embryos.

Blastomeres which were chemically arrested into G1-phase of the cell cycle yielded higher development rates than control embryos, indicating possible beneficial effect of that particular cell cycle compatibility. Although the finding itself was interesting from the scientific point of view, extra chemical treatments and donor embryo manipulations required for this approach made it less appealing for the routine nuclear transfer procedures. However, further more detailed studies on this field have been planned for the future.

(vi) Other studies related to optimization of nuclear transfer procedures

Various other approaches have been addressed throughout the project in order to improve efficiencies of

the nuclear transfer procedures. Among these have been studies on kinetics of oocyte fusion in relation to activation-fusions performed 4 or 10 h after Ca^{2+}-Ionophore activation of cytoplasts, or fusions performed after Ca^{2+}-Ionophore activation but prior to 6-DMAP incubation.

An important undertaking in the project was the commissioning the design and manufacture of a new model of electrofusion machine to a local electrical company. The machine was custom-made to meet the specific requirements identified in this project. Two such machines are now in routine use after careful testing and have proved superior to previously used commercially available models.

A spin-off from the Project is that the commercial collaborator, Genetics Australia, now produces these machines for sale (The Genaust Electrofusion machine - see attachment).

Objective 2:

To develop techniques for multiplying viable embryos for transfer to recipients, by recycling the cells of nuclear transfer embryos for further generations. If the recycled nuclear transfer embryos give acceptable ongoing pregnancy rates after transfer, this will increase the potential numbers of offspring exponentially.

Quantifiable objective:

The success of the project requires that at least four generations of nuclear transfer embryos be produced. This requires the production of four or more morphologically normal embryos from the first generation of recycling and eight or more in the second generation of recycling and increasing numbers of embryos from additional generations of nuclear transfer recycling.

This objective has been addressed in the following specific milestones:

- "Transfer and establish pregnancies from recycled embryos" (Year 2, Milestone 1)
- "Establishment of optimal conditions for the production of recycled embryos" (Year 2, Milestone 2)
- "Establish that the development of fetuses and calves from NT and recycled embryos is normal" (Year 3, Milestone 1)
- "Establishment of optimum systems for cryopreservation of recycled NT embryos (comparison of slow freezing with vitrification methods combined with "direct transfer thawing")" (Year 3, Milestone 2)
- "Modification of the recycled embryo and its environment to approximate normal in vivo development (Year 3, Milestone 3)
- "Pilot scale production and transfer of recycled bovine embryos" (Year 3, Milestone 7)

Completion of objective:

Over 70 morula and blastocysts have been produced from three rounds of nuclear transfer from one original donor embryo, and a number of NT embryos have been produced from rounds four to seven. The objective has therefore been achieved.

The development rate of second and third generation nuclear transfer embryos to morula and blastocysts is similar to development rates of the first generation embryos. Approximately 30% of the clones can be used as donors for subsequent rounds of recycling. Thus the number of clones that can be theoretically produced from one original donor embryo in the first, second and third generation nuclear transfer is 20, 120 and 720, respectively, leading to approx. 180 morula and blastocysts from the third generation. However, due to practical limitations in personnel and material, the real figure will be lower. With currently used methods it is possible for two people to perform up to 120 cell fusions in one day, leading in three (or two) generations to approximately 30 morula and blastocysts.

Application of cryopreservation into the procedures has allowed up to seven rounds of cloning. Although vitrification of donor embryos leads to some losses of donor material, overall it leads to more efficient

utilization of material. Therefore in three cloning generations, the third round of nuclear transfer can be done on three different days. At 120 fusions per day this finally resulted in over 70 morula and blastocysts.

Summary of progress

- **A maximum number of three generations of cloning can be performed when using fresh (non-cryopreserved) material**
- **Fusion, cleavage and development rates to 8-10 cell stage do not differ between three generations of fresh nuclear transfer**
- **Embryo sexing can be successfully applied to embryo recycling using at least two different approaches**
- **Confirmation of enucleation has been shown to be more important in recycling than in the normal first generation nuclear transfer**
- **Application of novel vitrification method for cryopreserving recycled donor embryos facilitates an indefinite number of cloning generations, as well as providing extremely efficient utilization of donor material.**

(i) Embryonic recycling

Embryonic recycling, i.e. the use of nuclear transfer embryos as donors for the subsequent rounds of nuclear transfer, has been adopted into our embryonic nuclear transfer procedures. Up to three generations of nuclear transfer can be done consecutively, however, after that the inability to get ovaries and oocytes during the weekend prevents the production of further generations. In the majority of the studies the developmental ability of consecutive generations has not been addressed, as all clones from a particular round have been used as donors rather than left for *in vitro* culture. However, the fusion, cleavage and development rates up to day 3 after fusion don't appear to be influenced by recycling. Although none of the experiments have been directed exclusively towards the production of maximum number of embryos from one donor, more than 70 morula and blastocysts have been produced from one original donor embryo in three rounds of recycling. In another experiment, more than 300 one-cell stage reconstituted nuclear transfer embryos were produced from one original donor embryo.

(ii) Sexing combined with embryonic recycling

Ways to incorporate embryo sexing into embryo recycling were studied and two possible approaches were tested. In the first, few blastomeres were removed from the donor embryos before cell fusion, sex analyzed within few hours by PCR-methods and only embryos of the desired sex were selected for cloning. In the second, several embryos were cloned as usual, and blastomeres from resulting embryo clones after few days in culture were used for sex analysis by PCR. After the results were known, only embryonic clones of the desired sex were used for additional rounds of nuclear transfer. With this approach, the use of developmentally retarded blastomeres and actively dividing blastomeres for use in sexing was compared, showing no difference in results obtained. Both methods worked well and the use of non-viable blastomeres can be therefore be included into any nuclear transfer recycling program, avoiding wastage of valuable genetic material.

(iii) Importance of efficient enucleation in recycling

The enucleation efficiency achieved with our methods routinely exceeds 95%. In order to evaluate the impact of the approximately 5% non-enucleated cytoplasts in nuclear transfer outcomes, experiments were conducted to find out the developmental potential of non-enucleated cytoplasts used for nuclear transfer. The studies revealed that only 7% of such possible tetraploid embryos (containing chromatin both from the donor as well as recipient cell) developed to morula or blastocysts. In the first generation of nuclear transfer this figure is too small for serious consequences. However, studies also revealed close to normal development rates up to day 4, to the stage when clones would be used as donors in recycling. This represents a more serious problem, as it may lead to the selection of tetraploid embryo for subsequent recycling. Thus confirmation of successful enucleation (by staining with Hoescht 33342) has been added as

an additional step to the recycling procedures.

(iv) Embryonic recycling and cryopreservation

Early experiences in recycling confirmed the need to have a cryopreservation system available for storage of valuable recycled donor embryo material. Firstly, using only fresh material, a maximum of three rounds of nuclear transfer can be performed for logistical reasons, including closure of abattoirs on weekends. Secondly, as the number of clones increases exponentially after each recycling round, the number of clones produced even in only two or three generations is too big to be handled efficiently, leading to unnecessary waste of the donor genetic material. Thirdly, use of fresh material alone leaves the production system extremely vulnerable for any unforeseen breakdowns in the system. Thus extensive work has been performed to apply a novel vitrification method, recently developed in Denmark, to donor embryos. After optimization of the procedures, this vitrification system has been successfully applied into our recycling procedures. It is now possible to perform three rounds of nuclear transfer, cryopreserve resulting round 3 donor clones, warm them later and perform another three rounds, again cryopreserving the resulting round 6 clones and so on.

Objective 3:

To determine that the development of the nuclear transfer (NT) embryos produced by these techniques is normal, and that they give rise to normal calves.

Quantifiable objective:

Identification and control of abnormalities so that all offspring produced are normal.

This objective addressed the following milestones:

- " Determining patterns of normal development in the bovine for a) fetal size as determined by ultrasonography; b)growth factor expression." (Year 1, Milestone 2).
- " Expression profile of TEC antigens on in vivo and in vitro embryos." (Year 1, Milestone 3).
- " Determining growth factor effects on metabolism and their expression in recycled embryos. " (Year 2, Milestone 3).
- " Expression profiles of TEC antigens on NT embryos." (Year 2, Milestone 4).
- " Establish that the development of fetuses and calves from NT and recycled embryos is normal." (Year 3, Milestone 1).
- " Construction of cDNA libraries for bovine oocytes and a range of developmental embryonic stages." (Year 3, Milestone 4).
- " Determination of embryo viability marker." (Year 3, Milestone 6).

Completion of objective

The nuclear transfer embryos produced during the project showed good development rates to the blastocyst stage (20% for NT embryos produced from embryonic cells). Cell numbers of first round embryonic cell NT embryos were only slightly lower than normal IVP control embryos, however multigenerational NT embryos had significantly reduced cell numbers (see table 1). Nuclear reprogramming, to the extent that it can be measured by TEC antigen expression, occurred normally in second and third round embryonic cell NT embryos, but not in first round NT embryos. Ultrasonography of pregnancies produced, failed to show abnormalities in those pregnancies which carried on to term. The nine nuclear transfer calves born during this project did not show significant abnormalities, with average birth-weights around those expected from (non-NT) in vitro produced embryos.

Summary of progress

- **Nine nuclear transfer calves (derived from embryonic cells) born since the beginning of the**

Two sets of genetically identical cloned bull calves generated by novel embryonic cell nuclear transfer techniques developed during the Project MU021


191
192

DAIRY RESEARCH AND
DEVELOPMENT CORPORATION

22 February 1999

Dr Len Stephens
General Manager
Livestock Production Innovation
Meat & Livestock Australia Ltd
Locked Bag 991
NORTH SYDNEY NSW 2059

Dear Len

MU021 ***Production of elite breeding stock by embryo multiplication***

We received the report for the above project in November 1998.

The report was reviewed and cited as being "written in a clear logical manner and provides an easy way to understand the technology". It was also noted that "the achievements have been very significant and have been in large part the work of some very talented young staff'.

DRDC is pleased to accept the report and Monash University has been notified.

Should you have any queries regarding the next phase of the project, please contact Tom Davison.

Yours sincerely

Alex Russell
Research Liaison Officer

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Project, all had birth-weights within the normal range (see table 1).

- Serial ultrasonographic examination of a number of nuclear transfer pregnancies failed to reveal any detectable abnormalities, including increased fetal size. (see table 2)
- Approximately half of the pregnancies positively diagnosed by ultrasound after 35 days of gestation, were lost, mostly between 40 and 90 days (see table 3).
- It was demonstrated that certain cell surface antigens, called TEC antigens, are stage specific in their expression during bovine preimplantation development in normal (non-NT) bovine IVP embryos. This knowledge was then used to examine nuclear transfer embryos. It was demonstrated that second and third round nuclear transfer embryos showed normal TEC antigen expression, but that most first round NT embryos showed abnormal TEC expression. This may indicate incomplete reprogramming in first round NT embryos which is then corrected during subsequent exposure to cytoplasmic reprogramming factors (see table 4).
- A novel technique allowing the expression of certain genes over time to be detected on very small amounts of material (differential display polymerase chain reaction - DDPCR) has been applied to cattle embryos during this Project. This has enabled certain positive bovine genomic fragments to be isolated, cloned and sequenced. This in turn may lead to the identification of genes coding for various factors necessary for normal bovine embryonic development which could facilitate improved in vitro embryo culture..
- Since the beginning of this project there has been a considerable amount of work published from other groups on bovine growth factors, especially on the genomically imprinted growth factor gene IGF II and also on bovine cDNA libraries. This, together with the lack of significant abnormalities observed in nuclear transfer calves born to date using the novel techniques developed during this project, lead to a Project management decision to discontinue work on the milestones 3 and 4 in year 3.

(i) Table 1: Average cell numbers of blastocysts produced by nuclear transfer compared with those from normal (non-NT) in vitro produced controls.

	Controls (Non-NT, IVP embryos)	First round NT embryos	Multigenerational NT embryos
Average cell number per embryo (number of embryos tested)	100.5 (84)	89.1 (15)	55.8 (11)

(ii) Monitoring nuclear transfer pregnancies and births

Table 2: Fetal ultrasound data and birth-weights* of nuclear transfer calves born since 1.7.95

Calf number	Weeks of gestation for ultrasound	Fetal size greater than, equal to or less than that expected for gestation length	Birth-weight
151	11	less than	52 kg
190	6 9	equal to equal to	43 kg
191	5 8	less than less than	41 kg
192	5 8	less than less than	53 kg
193	7 10	less than greater than	45 kg
199	9	less than	35 kg
270	not done		45 kg

353	not done		34 kg
354	not done		36 kg
		Average birth-weight =	42.7 kg

***Seven of the nine calves were "induced" to give birth up to one week prior to the "due" date, and the observed birth-weights would therefore be slightly lower than those for full-term calves. All calves were born alive, and none died neonatally. The genetically identical twin bull calves 352 and 354 were born naturally to the one mother, without induction. Bull calves 191 and 192 were also genetically identical, but were born to different mothers after induction.**

(iii) Pregnancy and calving information

Table 3: Pregnancy and Calving Data on Embryonic Cell Nuclear Transfer Embryos (including cryopreserved NT embryos) Transferred for Project MU021 (1st July '95 to 30th June '98)

	Number of Embryos Implanted	Number of Recipients Used	Number of Recips Pregnant (%)	Number of of Calves Born (% preg recips calved)	% calves from embryos
Round 1 fresh	186	67	25 (37%)	awaiting some calving data	
Multigenerational fresh	49	20	3 (15%)		
Total fresh	**235**	**87**	**28 (31%)**	**15 (53%) (estimate)**	**6% (estim.)**
Round 1 cryopreserved	8	3	0	awaiting some calving data	
Multigenerational cryopres.	55	21	6 (29%)		
Total cryopreserved	63	24	6 (25%)	2 (33%) (estimate)	2% (estim.)
Total fresh & cryopres.	***298***	***111***	***34 (31%)***	***17 (50%) (estimate)***	***6% (estim)***

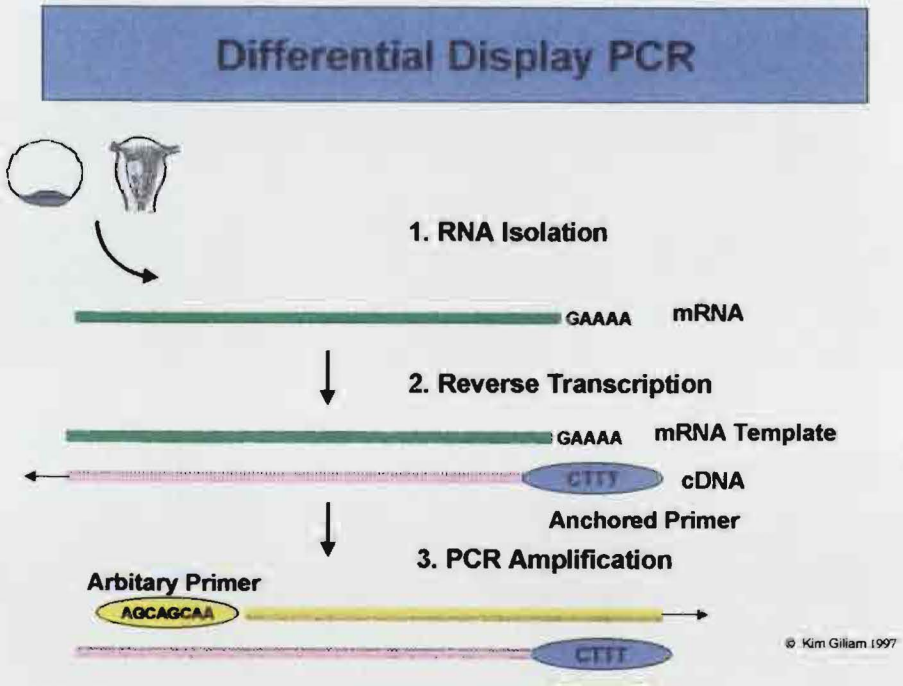
On the basis of the ultrasound data, albeit on a limited number of fetuses, it was decided not to utilize further resources on this aspect unless and until abnormal birth-weights are shown to be a problem with the nuclear transfer calves produced by the methods used in this project.

The high loss of nuclear transfer pregnancy observed in this project is consistent with that reported by other groups doing significant numbers of transfers of nuclear transfer embryos.

A number of these NT calves will be taken through to puberty to assess subsequent fertility.

(iv) Identification of developmentally regulated genes in bovine embryos which code factors of potential importance in normal bovine embryonic development

The development of the novel technique of differential display polymerase chain reaction (DDPCR) by Liang and Pardee in 1992 has enabled the more simplified detection of differential gene expression. In the past gene expression studies utilized gene libraries which required in excess of 10,000 embryos and specific PCR reactions that were linked with hybridization reactions. The amount of starting material (bovine embryos) is very limiting, therefore, the use of DDPCR was developed for use in our system. Through our ongoing research into this area we have successfully modified the technique of DDPCR so that it is suitable for small numbers of bovine embryos. Having optimized the DDPCR technique, our next aim was to elucidate the genes that are expressed during vital stages of bovine embryo development. We compared various embryonic stages with each other to determine what stimulatory factors (growth factors,


Differential Display PCR
1. RNA Isolation
GAAAA
mRNA
2. Reverse Transcription
GAAAA
mRNA Template
CTTT
cDNA
Anchored Primer
3. PCR Amplification
Arbitary Primer
AGCAGCAA
CTTT
© Kim Gilliam 1997

cytokines, transcription factors etc.) are involved in these crucial stages of development.

The technique involved collecting 1500 in vitro produced day 7 embryos and 10 in vivo produced day 15 embryos. Briefly, mRNA was extracted and reverse transcribed to produce cDNA. The resulting cDNA was amplified with an anchored primer (poly T tail) and an arbitrary primer that anneals to a matching sequence on the cDNA (fig 1). The PCR products were then separated on a polyacrylamide sequencing gel and the differences between two or more embryonic stages visualized and compared on an X-ray film (fig 2). Day 7 and day 15 embryos were compared in order to identify the genes important at these stages of development and to begin to elucidate the controlling mechanisms involved in bovine embryo development. A total of 55 bands were found to be differentially expressed between day 7 and day 15 embryos. These gene fragments were isolated and used as probes to hybridize cDNA isolated from bovine embryos at day 7 and day 15 to confirm their presence and/or absence at the specific bovine embryonic developmental stages of interest. A new technique known as 'Virtual Northerns' was modified and optimization for use in our system. This new technique is based on the principal of whole genome PCR in which the mRNA is amplified into cDNA and results in a ten-fold reduction of the amount of embryos required for reliable analysis. Positive fragments have been isolated and are now being cloned and sequenced. These fragments may lead to the identification of the genes coding for various factors necessary for normal bovine embryonic development. Such findings may then be used to provide the necessary ingredients/additives for future culture media to improve in vitro bovine embryo culture.

(v) Expression profiles of the cell surface TEC antigens on fertilized and nuclear transfer embryos.

TEC-1, -2, -3 and -4 are cell surface antigens located on a glycoprotein backbone. Through this research it was shown that the TEC antigens are stage-specific in their pattern of immunohistochemical expression during bovine preimplantation development (Table 1). In oocytes and IVF embryos TEC-1 and TEC-2 are expressed on the oocyte and blastocyst stage, with all intermittent stages negative. TEC-3 antigen expression occurs on morula and blastocyst stage embryos. TEC-4 is expressed from the oocyte to the 8-cell embryo, with all subsequent stages negative. In vivo matured oocytes and in vivo fertilized and cultured blastocysts were stained for TEC antigen expression and were found to have the same expression pattern as the in vitro fertilized embryos. The expression pattern of the TEC antigens was also examined on bovine parthenotes activated using calcium ionophore and 6-DMAP. The expression pattern was very different to that observed in the fertilized embryos. There was no TEC-1, -2 or -3 antigen expression on the parthenotes, including blastocyst stage embryos which are positive for these TEC antigens in fertilized embryos. Parthenotes displayed TEC-4 presentation throughout the embryonic stages examined, from the 2-cell to the blastocyst.

We examined the expression of these antigens on bovine embryos that had undergone serial nuclear transfer, as summarized on table 4. Round one nuclear transfer embryos showed a different pattern of TEC-2 and TEC-4 antigen expression to the pattern observed for fertilized embryos. TEC-1 and TEC-3 expression was the same as the IVF embryos. Two round one nuclear transfer blastocysts stained negative for TEC-2 expression and one blastocyst stained TEC-4 positive. These embryos showed the same TEC antigen expression as the parthenogenetic embryos. This suggests that these embryos have not been adequately reprogrammed. An alternative explanation is that the embryos were parthenotes, however as there was confirmation of enucleation this explanation appears unlikely. This difference was not seen in second and third round nuclear transfer embryos which displayed the same TEC antigen expression as the fertilized embryos. Therefore the second and third round nuclear transfer embryos showed "normal" TEC expression whereas a few of the first round nuclear transfer embryos showed altered TEC antigen expression compared to fertilized embryos.

Table 4: TEC antigen expression profiles of IVF and NT embryos

	Antibody	UF	F	2'	4-8'	9-16'	M	Bl
IVF	TEC-01 +	-	-	-	-	-	+	
1st Gen NT								3+
IVF	TEC-02 +	-	-	-	-	-	+	
1st Gen NT								2+/2-
2nd Gen NT							1-	4+
3rd Gen NT								2+
IVF	TEC-03 -	-	-	-	-	+	+	
1st Gen NT							2+	5+
2nd Gen NT							3+	4+
3rd Gen NT							1+	3+
IVF	TEC-04 +	+	+	+	-	-	-	
1st Gen NT					4+			1-/1+
2nd Gen NT							1-	3-
3rd Gen NT								2-

1st Gen NT = Round one nuclear transfer embryos
2nd Gen NT = Round two nuclear transfer embryos
3rd Gen NT = Round three nuclear transfer embryos
Oocytes and IVF embryos have more than 20 samples in each group
\+ = Fluorescence above background, considered positive
\- = Fluorescence ≤ background, considered negative

UF	=	Unfertilized	4-8'	=	4-8-cell	M	=	Morula
F	=	Fertilized	9-16'	=	9-16-cell	Bl	=	Blastocyst
2'	=	2-cell						

(vi) Determination of embryo viability marker

The aim of this milestone was to determine an effective metabolic marker of embryo viability for the bovine embryo in order to increase pregnancy rates through embryo selection. It was hypothesized that energy metabolism would reflect embryo viability following transfer. This hypothesis was based on previous research in our laboratory, which showed that the measurement of the metabolism of glucose to lactate in morphologically identical blastocysts could be used to successfully differentiate between viable and non-viable embryos in the mouse. At the commencement of DRDC MU021, our laboratory was in the process of trialing this metabolic viability marker in the sheep. On the basis of metabolic activity, 2 separate groups of morphologically identical blastocysts were transferred back to the uteri of sheep: one group hypothesized to be viable embryos and the second group hypothesized to be non-viable embryos. The experiment determined that, unlike the mouse, the metabolism of glucose was not a good marker of viability of sheep blastocysts (pregnancy rates were approximately 73% in both groups of embryos from singleton transfers).

Given the results of this experiment in sheep and the large financial investment for embryo transfers in the cow, the determination of a metabolic marker of embryo viability was not pursued.

Alternatively, an experiment was designed to determine the potential of pre-pubertal cows as oocyte donors, **using the uptake of 2 energy substrates (glucose and pyruvate) as markers of developmental**

competence and blastocyst viability. There is considerable interest in the use of pre-pubertal animals as oocyte donors as they offer the potential to increase the number of oocytes retrieved per ovary and to increase the rate of genetic gain in populations of cows with elite traits. While oocytes from pre-pubertal animals can be matured and fertilized in vitro, development to blastocyst is often poor. In the present study, oocytes were retrieved from the ovaries of stimulated pre-pubertal cows (5-7 months) and from adult cows of unknown age. The 2 groups of oocytes were fertilized in vitro and cultured for 6 days to the blastocyst stage. At successive stages of development from the 1-cell stage thought to the blastocyst, the rates of glucose and pyruvate uptake were measured non-invasively by microfluorescence, for individual embryos.

Fertilization was equivalent in embryos from pre-pubertal and adult cows (70.4% versus 70.8%, respectively; $P > 0.05$). Development to the blastocyst stage however, was significantly lower in embryos from pre-pubertal animals (9.8% versus 33.7%; $P \leq 0.05$). Despite poor development, blastocysts from the pre-pubertal group of embryos had equivalent total cell numbers to blastocysts from the adult group of embryos (85.9 versus 88.7, respectively; $P > 0.05$). The rate of glucose uptake was significantly lower in embryos from pre-pubertal cows than adult cows at the 2- to 4-cell stages (1.5 versus 3.0 pmoles/embryo/hour, respectively; $P \leq 0.05$), but was not significantly different at the blastocyst stage (20.8 pmoles/embryo/hour versus 17.6 pmoles/embryo/hour, respectively; $P > 0.05$). The pattern of pyruvate uptake by the early cleavage stage embryos (zygote to 8- to 16-cell stage) was different in pre-pubertal and adult cows. Pyruvate uptake by pre-pubertal embryos was significantly different to that of the adult cow at the zygote (2.7 versus 4.6 pmoles/embryo/hour, respectively; $P \leq 0.05$) and the 2- to 4-cell stages (4.9 versus 3.6 pmoles/embryo/hour, respectively; $P \leq 0.05$). Pyruvate uptake by embryos from the 2 groups was not significantly different at the blastocyst stage (pre-pubertal: 11.3 versus adult: 12.6 pmoles/embryo/hour; $P > 0.05$).

Observed perturbations in the uptake of nutrients by embryos from pre-pubertal cows during development of the early cleavage stages were most likely due to the presence of a high proportion of developmentally incompetent embryos. Perturbations in the uptake of both glucose and pyruvate could result in a reduction in energy production, a change in the redox state of the embryos and a reduced ability to counteract oxidative stress. Interestingly, the viability of blastocysts from pre-pubertal cows was not compromised as indicated by nutrient uptake and total cell number.

In conclusion, blastocyst development from pre-pubertal material is low compared with that from adult cows. However the viability of blastocysts from pre-pubertal material (as measured by nutrient uptake and total cell number) is not different to that from adult cows.

Objective 4:

To demonstrate that nuclear transfer and recycled embryos can be cryopreserved successfully and retain their developmental competence when transferred to recipients.

Quantifiable objective:

The cryopreserved nuclear transfer embryos will be required to achieve pregnancy rates of at least 60% that of non-frozen nuclear transfer embryos.

The following milestones addressed this objective:

- "Developing protocols for cryopreserving and then directly transferring embryos produced by improved in vitro techniques" (Year 2, milestone 8).
- "Establishment of optimum conditions for cryopreservation of recycled nuclear transfer (NT) embryos" (Year 3, milestone 2).
- "Modification to cryopreservation procedures to include antioxidants" (Year 3, milestone 5).

Completion of objective:

This objective was achieved with the development of novel cryopreservation techniques (vitrification) allowing the direct transfer, following warming, of cryopreserved first round and recycled nuclear transfer embryos which gave pregnancy rates exceeding 60% of those achieved with such non-frozen embryos.

Summary of progress

- **In collaboration with Danish scientists, novel vitrification techniques were applied to first and subsequent round nuclear transfer embryos produced in our laboratories. Following the warming and transfer of two or more such embryos per recipient, pregnancy rates (per recipient) were achieved which were close to 80% of those achieved with such NT embryos transferred fresh (non-crypreserved). However ongoing pregnancy rates were low (see below).**
- **The application of these novel vitrification techniques to normal (non-NT) IVP embryos gave conception rates per embryo transferred of 50% using a "direct" transfer method (without the use of a microscope) following warming.**
- **The use of antioxidants prior to cryopreservation of non-NT embryos was examined and the results indicated that further work in this area is warranted.**

(i) Vitrification

In collaboration with Dr Gabor Vajta from the Embryo Technology Center, Danish Institute of Agricultural Sciences, novel cryopreservation techniques, based on vitrification (very rapid cooling with high concentrations of cryoprotectant) were trialed in cattle using non-nuclear transfer IVP embryos. A summary of the vitrification technique used is given below. On the basis of these successful studies the techniques were then applied to nuclear transfer embryos. During the Project, an overall recipient pregnancy rate of 32% was achieved (28 pregnancies from 87 recipients), following the transfer of 2 or more fresh (non-cryopreserved) **embryonic cell nuclear transfer (ECNT)** embryos into each recipient. The transfer of 2 or more cryopreserved (vitrified)/warmed nuclear transfer embryos per recipient gave a pregnancy rate of 25% (6/24) which represents 78% of that achieved with fresh NT embryos (see table 3).

For logistical reasons, the fresh ECNT embryos mostly came from non-recycled nuclear transfer embryos, while the cryopreservation results were primarily from recycled embryos. There was a greater percentage of abortions lost from the cryopreserved embryos, after positive pregnancy diagnosis from 35 days onwards (see table 5). However it is not clear whether this increased loss was due to cryopreservation or recycling, as only a small number of non-cryopreserved recycled nuclear transfer embryos have been transferred. This question is being addressed in the current project (MU035). However it is interesting to note that with non-nuclear transfer IVP embryos which were vitrified, then warmed and transferred during experiments to determine the most effective cryopreservation method for IVP embryos, there has been minimal loss of pregnancies from day 35 onwards (see table 5).

Table 5. Pregnancy rates following in-straw dilution and ex-straw dilution of vitrified* embryos

Dilution Method	No. embryos vitrified	No. embryos transferred (2/recip)	No. recips pregnant (over 5 months) (%)	No. fetuses (%)
In-straw (IS)	22	22	7 (64)	11 (50)
Ex-straw (ES)	20	20	4 (40)	7 (35)

***Vitrification technique**: embryos were OPS (Open Pulled Straw) vitrified in 2 steps: (i) 10% ethylene glycol (EG) and 10% dimethylsulfoxide (DMSO) in holding media (HM - TCM199 and 20% FCS) for 1.5

min at 39°C. (ii) 20% EG and 20% DMSO in HM for 25 s at 39°C. Two embryos were loaded per straw.

In collaboration with Dr Jill Shaw and Mr Mulyoto Pangestu of the IRD, the use of antioxidants was examined. Briefly, the results demonstrated that injection of the antioxidant α tocopherol into early embryos provided some protection against chilling injury. During the current project (MU035) it is planned to further test the use of antioxidants with the vitrification system described.

Objective 5:

To ensure adequate and consistent pregnancy rates for non-frozen (cryopreserved) and cryopreserved nuclear transfer and recycled embryos produced by these techniques.

Quantifiable objective :

The success and adoption of this research will require a consistent pregnancy rate of 25% or more for all fresh and frozen nuclear transfer embryos.

This objective addressed the following milestones:

This objective addressed the fundamental principles of the Project which were aimed at developing commercially successful embryonic cell cloning technology. Therefore all milestones were considered by this objective.

Completion of objective:

The initial pregnancy rates (diagnosed by ultrasound at 35 days or later) of recipients receiving two or more fresh or cryopreserved (vitrified) embryos over the total project was 31% and over the last 6 months to 30/6/98 was 37% (see table 6). This meets the milestone for pregnancy rates.

Summary of progress:

- **The large scale production of transferable (blastocyst) stage embryonic cell derived cloned embryos, at a relatively low cost of $15 per embryo has been achieved.**
- **The ongoing pregnancy rates to term have been approximately half of the initial pregnancy rate during this project and are still below commercially viable levels.**

(i) Embryo costs and initial and ongoing pregnancy rates

The recycling and cryopreservation techniques developed during this project have enabled the large scale production of blastocyst stage, embryonic cell nuclear transfer embryos from one original embryo, at relatively low production costs. Taking account of all costs, including labor, consumables, depreciation on capitol etc, the present Project costs of producing significant numbers of such embryos is $15 per embryo.

The overall ability of such embryos to produce pregnancies which go to term is sill relatively low and requires considerable further development. The high loss of approximately half of those pregnancies from 35 days onwards seen in this project is similar to that reported from other significant groups working in cattle cloning (David Wells, Ruakura, New Zealand; Will Eyestone, PPL, U.S.; Yves Hayman, INRA, France; pers comm).

Table 6: Recipient pregnancy rates for fresh and cryopreserved nuclear transfer embryos for whole of Project and for the last 6 months.

	Number of Embryos Implanted	Number of Recipients Used	Number of Recipients Pregnant (%)	Number of of Calves Born (% preg recips calved)
Total fresh & cryopres. 1.1.98 to 30.6.98	**56**	**30**	**11 (37%)**	Awaiting calving data
Total fresh & cryopres. 1.7.95 to 30.6.98	***298***	***111***	***34 (31%)***	***17 (50%) (estimate based on 9 calves born, and on ongoing pregnancy rates)***

(ii) Recipient synchrony and stage of transfer for cloned embryos

With the nuclear transfer procedures and embryo culture techniques developed during his project, the embryo development rate is relatively rapid and approximates that seen in vivo. Transferable stage embryos (morula and blastocyst) are reached at approximately six days after the fusion of blastomere and cytoplast. Consequently it was decided to transfer embryos into recipients whose ovulation time approximated the fusion event and to culture embryos for six days instead of seven. With in vitro produced embryos, including those produced by nuclear transfer, it is usual to transfer them into recipients whose ovulation time is one day later than fertilization or fusion to allow for the normally slower development of IVP embryos compared with in vivo embryos. It has also been a standard (world-wide) practice to transfer embryos that have been cultured for seven days rather than six.

Encouraging results were achieved with the first group of NT embryos so transferred, with 71% (5/7) recipients diagnosed pregnant by ultrasound at 35 days, and 43% (3/7) of these ongoing over 5 months gestation.

With present pregnancy rates per embryo transferred, generally 3 or 4 embryos are transferred per recipient, to help contain recipient costs which are high for any type of embryo transfer in cattle. As the ongoing pregnancy rate per embryo increases with improvements in the technology, the number of embryos transferred per recipient will need to be reduced because of pregnancy losses due to uterine overcrowding which can occur will multiple fetuses. This is already likely to have reduced ongoing pregnancy rates in some instances, and is likewise recognized as a potential problem in similar projects elsewhere (David Wells, Agresearch, Ruakura, New Zealand, pers comm.).

ADDITIONAL OBJECTIVES

In addition to the five objectives discussed above which were specified at the beginning of the Project, the following objectives were included.

Objective 6:

To further optimize bovine embryo culture systems to improve the quality and quantity of bovine embryos

produced in vitro.

The inclusion of this objective in the project recognized that the separate DRDC Project funding the work in Dr David Gardner's Embryo Physiology Laboratory at the Institute of Reproduction and Development would fit appropriately within Project MU021.

Quantifiable objectives:

A significant, measured improvement in the overall quality and/or quantity of in vitro produced (IVP) bovine morula and blastocysts

This objective was undertaken by Embryo Physiology Laboratory at the Institute of Reproduction and Development, Monash University, under the direction of Dr David Gardner and concentrated on non-nuclear transfer IVP embryos. Positive findings were then adopted by the Monash University groups undertaking bovine nuclear transfer.

The objective has been addressed in the following milestones:

- "Formulation of New Culture Media Based on the Embryo's Changing Requirements" (Year 2, Milestone 6)
- "Development of More Suitable Oocyte Maturation Conditions" (Year 1, Milestone 5)
- "Determination of Carbohydrate and Amino Acid Requirements of the Developing Embryo" (Year 1, Milestone 6)
- "Determination of Effectiveness of Protein Source" (Year 2, Milestone 5)
- "Determination of Embryo Viability Marker" (Year 3, Milestone 7)

Completion of Objectives:

The objective was met by the development of in vitro embryo production culture systems which significantly improved overall IVP morula and blastocyst quality and quantity. This end was partly achieved by determining embryo requirements during culture thus allowing the system to be made more "robust" by reducing the variability brought about by such things as variations in oocyte quality.

Summary of progress

- **The development of a "bi-phasic" culture system which significantly improved bovine embryo development to the morula and blastocyst stage. In this system embryos are cultured for the first 72 hours in media containing SOF (synthetic oviduct fluid), Eagle's non-essential amino acids plus glutamine and a small amount of glucose. They are then changed into media containing Eagle's essential and non-essential amino acids, glutamine, vitamins and significantly more glucose for the remainder of the culture period.**
- **Culture of embryos in groups after the 8 to 16 cell stage has a positive effect on the rate of blastocyst development, on the numbers cells in the inner cell mass and on total cell numbers.**
- **More suitable bovine oocyte maturation conditions were developed by optimizing FSH and LH concentrations in the maturation media. Further information was gained by determining patterns of energy metabolism during bovine oocyte maturation.**
- **It has not been possible to date to develop an embryonic viability marker in the bovine despite earlier work from Dr Gardner's laboratory showing a positive correlation in murine embryos.**

(i) Formulation of New Culture Media Based on the Embryo's Changing Requirements

Results from the previous experiments, including those reported below, have given a better understanding of the requirements of the bovine embryo during culture. The embryo's requirements for carbohydrates, amino acids, vitamins and embryotrophic factors were determined. This has lead to the formulation of a two-step culture system which supports the changing requirements of the bovine embryo during development. To date it remains difficult to significantly increase the number of embryos developing to the blastocyst stage. However a better understanding of the embryo's requirements during culture has (1) improved the quality (viability) of those embryos that due develop to the blastocyst stage and (2) decreased the immense variation that we see with each culture (due partly to differences in the quality of ovaries from the abattoir). Recommendations for culturing normal IVP bovine embryos are as follows:

Step 1 Culture from the zygote to the 8- to 16-cell stage (first 72 h culture):

- Embryos should be cultured in SOF (8 mg/ml BSA) with either (1) non essential amino acids and glutamine (with 4 mM NaCl added to adjust osmolarity) or (2) in SOF (8 mg/ml BSA) with non essential amino acids, glutamine and the essential amino acids at ¼ the concentration (with 1 mM NaCl added to adjust osmolarity).
- The addition of vitamins is not necessary during this time.
- Embryos can be cultured singly during this time if necessary.
- Glucose should be included in culture media (no higher than 1.5 mM).
- The non essential amino acids should be made up in the laboratory and not purchased pre-made. Testing of the contents of the pre-bottled supply revealed that there was some variation in concentration of individual amino acids which lead to variation in total cell numbers.

Step 2 Culture from the 8- to 16-cell stage to the blastocyst (second 72 h culture):

- Embryos should be cultured in SOF (8 mg/ml BSA) with non essential and essential amino acids and glutamine. The essential amino acids should be at ¼ the concentration (with 1 mM NaCl added to adjust osmolarity).
- Glucose must be included in the culture media.
- MEM vitamins should be added to the culture media (media must be kept in the dark as some vitamins are light sensitive).
- Embryos should be grown in small groups (4 per 30 µl) as embryotrophic factors are needed during this period.

(ii) Development of More Suitable Oocyte Maturation Conditions

Several studies addressed this milestone.

(a) Energy metabolism of bovine oocytes

The aim of the present study was (1) to determine the pattern of energy metabolism during maturation of the bovine oocyte (2) to compare the utilization of energy substrates during maturation of oocytes from pre-pubertal and adult cows, to determine whether poor development of pre-pubertal embryos is due to metabolic perturbations in the oocyte. The metabolism of glucose and glutamine increased significantly during oocyte maturation and are thus important nutrients for the maturing oocyte. Pyruvate oxidation was highest at 12 h maturation. Peak metabolism of all energy substrates was significantly lower in oocytes from pre-pubertal cows. This however, was found to be a function of oocyte size. Oocytes from pre-pubertal cows were significantly smaller than oocytes from adult cows at all 0, 12 and 24 h maturation. This has serious implications for the developmental potential of the oocyte/embryo as oocyte size has been correlated with subsequent ability to develop to the blastocyst stage. Irrespective of oocyte size, there was a

delay in the increase in glucose metabolism of oocytes from pre-pubertal cows. This would have an effect on energy production, the generation of NADPH for reductive biosyntheses and the formation of ribose moieties for nucleotides. While maturation to metaphase II was equivalent in the two groups after 24 h maturation, there was a delay in germinal vesicle breakdown in oocytes from pre-pubertal cows. Thus oocytes from adult cows most likely reached metaphase II earlier than oocytes from pre-pubertal cows. Oocytes reaching metaphase II at an earlier time have been shown to be more developmentally competent. Thus the poor development to blastocyst observed in pre-pubertal embryos is evident in the oocyte.

(b) The effect of different concentrations and ratios of gonadotrophins on the in vitro maturation of bovine oocytes

The gonadotrophins, luteinizing hormone (LH) and follicle stimulating hormone (FSH), have been shown to be major components of oocyte in vitro maturation systems and are known to improve in vitro oocyte maturation and subsequent embryo development. Even with the addition of these hormones to maturation media, only 30-40% of in vitro matured oocytes survive to the blastocyst stage of embryonic development following in vitro fertilization and culture. It is likely that incomplete nuclear and cytoplasmic maturation account for a significant part of the 60-70% of oocytes that fail to reach the blastocyst stage.

This project examined the effect of different LH and FSH concentrations on oocyte maturation.

The results indicated that high concentrations (100 x greater than concentrations currently used) of LH in oocyte maturation media adversely affected further development, by causing premature maturation of oocytes. Also, high concentrations of FSH when combined with normal LH levels reduced subsequent embryonic growth.

In summary, varying concentrations and ratios of LH and FSH influenced the maturation of bovine oocytes in vitro, and optimal levels were determined.

(iii) Determination of Carbohydrate and Amino Acid Requirements of the Developing Embryo

Previous studies determining the requirements of the bovine embryo have used a variety of culture media making it difficult to draw conclusions. In addition, previous studies have not taken into account the dramatic changes in the embryo's physiology with the switch in genetic regulation from maternal to embryonic at the 8- to 16-cell stage. In the present study, the requirements of the bovine embryo during culture were determined using a 2-step culture system (step 1: zygote to 8- to 16-cell stage; and step 2: 8- to 16-cell stage to blastocyst). Further, the majority of studies have only assessed the effect of various media components on blastocyst development and not on known markers of viability and developmental competence such as total cell number, differentiation of cells into the inner cell mass and the trophectoderm and metabolic activity.

(1) Amino acid requirements of the bovine embryo

In the first series of experiments, it was determined that amino acids have both a temporal and differential effect during culture of the bovine embryo. When added to SOF during the first 72 h culture, Eagle's non essential amino acids and glutamine significantly increased development to the 8- to 16-cell stage (day 4 pi) (SOF: 35.4 % versus SOF/Negln: 62.7%; $P \leq 0.05$) and subsequent blastocyst (day 7 pi) development (SOF: 20.9% versus SOF/Negln: 41.5%; $P \leq 0.05$). Glutamine alone or glutamine in combination with Eagle's essential amino acids was unable to stimulate development to the 8- to 16-cell stage. This is in contrast to the mouse embryo where glutamine is as effective as the non essential amino acids in stimulating development of the early cleavage stage embryo. In a further contrast, the addition of the essential amino acids to the non essential amino acids and glutamine during the first 72 h culture did not negate the stimulatory effects of the non essential amino acids. It was concluded however, that the essential amino acids should be eliminated or reduced during the first 72 h culture as there was a significant inverse correlation between the concentration of essential amino acids during the first 72 h culture and development to the 8-16 cell stage (r = -0.999; $P \leq 0.01$) and subsequent blastocyst development (r = -0.992; $P \leq 0.05$). There was no effect on blastocyst cell number. The presence of glutamine was found to be

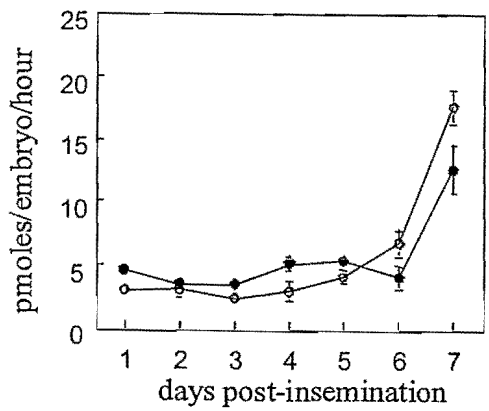
necessary during the first 72 culture and it was shown to partially function as an osmolyte. Replacing glutamine with the known organic osmolyte betaine maintained subsequent blastocyst development.

Beyond the 8- to 16-cell stage, the bovine embryo had a requirement for all of the amino acids (non essential, essential and glutamine). Culture with all 20 amino significantly increased blastocyst development (46.3%), total blastocyst cell number (99.0), the number of cells in the inner cell mass (31.2) and trophectoderm (67.8) and the proportion of cells allocated to the inner cell mass (0.32), compared to culture with other combinations of amino acids. The size of the inner cell mass (which becomes the fetus) has been positively correlated with viability following transfer in the mouse. In the mouse, the essential amino acids were found to increase the size of the inner cell mass, however our result show that the non-essential amino acids and glutamine stimulate differentiation of cells to the inner cell mass in the bovine blastocyst. While the essential amino acids are required during the second 72 h culture, our results suggest that the concentration should be reduced to one quarter of that used in mouse embryo culture. There was a significant inverse correlation between concentration and development to the blastocyst stage ($r = -0.995$; $P \leq 0.05$). The concentration of essential amino acids did not affect blastocyst cell number.

While it is known that amino acids improve embryo development, there has been little effort to investigate the mechanisms behind the actions of amino acids. In the present study, different amino acid groups had differing effects on glucose metabolism (activity of the Embden-Meyerhof pathway), during the first 72 h culture. There was a significant inverse correlation between glucose metabolism and development to the 8- to 16-cell stage, following culture with different groups of amino acids. Thus it is likely that the non essential amino acids and glutamine stimulated development of the early cleavage stages by reducing glucose metabolism (a high glucose metabolism has been shown to be inhibitory to development of the mouse embryo). A further correlation was found between intracellular pH of the 8- to 16-cell embryo and development to the 8- to 16-cell stage, following culture with different groups of amino acids. The non-essential amino acids and glutamine reduced the intracellular pH of the embryo. No correlation was found between development to the 8- to 16-cell stage and the oxidation of pyruvate or glutamine, following culture with different groups of amino acids. Amino acids have been shown to breakdown in culture media to form ammonium which is detrimental to embryo development and viability. The observed differences in development of embryos during the first 72 h culture with different groups of amino acids were not due to ammonium concentration ($P > 0.05$). During the second 72 h, culture with all 20 amino acids was beneficial as it significantly increased glycolytic activity in the embryo (compared to culture with individual amino acid groups). A high glycolytic activity is very important for energy production in the blastocyst.

(2) Carbohydrate requirements of the bovine embryo

This is the first study to non-invasively measure the uptake of the carbohydrates, by individual embryos, in the media in which embryos were cultured in (SOFaa). The graph below shows the uptake of glucose (open circles) and pyruvate (closed circles) from the zygote (day 1 pi) through to the blastocyst (day 7 pi).



The uptake of both glucose and pyruvate are fairly low and constant during development of the early cleavage stage embryo. Pyruvate uptake remains low until the blastocyst stage, while glucose uptake starts to increase from the 8- to 16-cell stage (day 4 pi). Several laboratories have decided to remove glucose from culture media for the early cleavage stage embryo, with some laboratories leaving glucose out of culture media for the entire duration. During the early cleavage stages, glucose is most likely required (albeit at low levels) for its metabolism via the phosphate pathway for the production of ribose moieties for nucleotide synthesis and NADPH for reductive biosyntheses. Low levels of pyruvate uptake are most likely important to the early cleavage stages for the maintenance of a redox potential and its use as an antioxidant. Beyond the 8- to 16-cell stage, pyruvate and glucose are needed for the production of the large quantities of ATP required by the blastocyst. The dramatic change in the uptake of carbohydrates beyond the 8- to 16-cell stage illustrates the changing requirements of the embryo after activation of the embryonic genome and thus the need to consider a 2-step culture system for determining requirements of the embryo during culture from the zygote to the blastocyst.

(3) Vitamin requirements of the bovine embryo

Vitamins are important for energy production as they are cofactors in many biochemical reactions. When added to culture media for the bovine embryo, vitamins (MEM vitamins which are mainly B-group) do not affect development. As a result, there has been very little research into the requirements of the bovine embryo for vitamins. Embryo morphology alone however, is not necessarily an indicator of viability. It has recently been shown that the absence of vitamins from culture media used to briefly hold flushed in vivo developed blastocysts, resulted in the reduction of viability following transfer (4).

The first series of experiments were carried out to determine whether vitamins had any effect on development, cell number or hatching when added at different times during culture. When added during the first 72 h culture, MEM vitamins did not effect development to the 8- to 16-cell stage. In the mouse, it has previously been shown that vitamins are inhibitory to the early cleavage stages. When added during the second 72 h culture, MEM vitamins had no effect on blastocyst development to day 7 or day 8 pi, total cell number or hatching (vitamins increase hatching of the rabbit blastocyst). They did however, significantly increase the diameter of blastocysts day 8 pi (no vitamins: 179.8 μm versus with vitamins: 205.7 μm; $P \leq 0.05$). Previous research in our group has shown that both MEM vitamins and serum are required for continued blastocyst development beyond day 7-8 pi. Further experiments were therefore carried out to determine whether there was a synergistic effect between serum and vitamins in blastocyst expansion. Culture with serum increased blastocyst diameter (day 8 pi) compared with BSA, however the addition of vitamins to media containing serum had no further affect on expansion of the blastocoel.

The blastocyst requires large amounts of energy for the formation and expansion of the blastocoel. A further series of experiments were performed to investigate the effect of MEM vitamins on energy metabolism in the blastocyst. Vitamins had no effect on glucose metabolism but they did affect oxidative metabolism. Pyruvate oxidation was significantly decreased while glutamine oxidation significantly increased in the presence of vitamins. Previous research has shown that glutamine oxidation increases greatly with blastocyst expansion. MEM vitamins are most likely stimulating blastocyst expansion by increasing the utilization of glutamine.

The effect of several individual MEM vitamins on blastocyst development and metabolism was also determined. The addition of nicotinamide, riboflavin, biotin or folic acid had no effect on blastocyst development or total cell number, but they did affect energy metabolism. Nicotinamide, riboflavin and folic acid all increased the oxidation of both glutamine and pyruvate in the expanded blastocyst. This indicates that these vitamins are having a significant, positive effect on total energy production in the blastocyst. Interestingly, a deficiency in folic acid during early fetal development has been implicated in neural tube defects. We a presently determining the effects of other individual vitamins (myoinositol and pantothenate) and the combination of folic acids, riboflavin and nicotinamide.

(4) The requirements of the embryo for embryotrophic factors
During the production of embryos in vitro, especially those produced by nuclear transfer, embryos may be grown as individuals and not in groups. It was recently shown that culture of the mouse embryo in small groups rather than singletons, led to an increased total cell number in the blastocyst and a larger inner cell mass. The size of the inner cell mass has been positively correlated with viability. A series of experiments were thus carried out to determine the effect of culturing the bovine embryo in small groups versus single embryos. Culture of the early cleavage stages in groups of 4 per 30 µl (zygote to 8- to 16-cell) had no effect on embryo development when compared to culture of single embryos. Beyond the 8- to 16-cell stage however, culture of embryos in small groups resulted in an increase in blastocyst development, total cell number and the number of cells in the inner cell mass. The results indicate that embryotrophic factors are important during culture of the bovine embryo beyond the 8- to 16-cell stage, and thus embryos should be cultured in groups from the 8- to 16-cell stage to the blastocyst.

(iv) Determination of Effectiveness of Protein Source

There are a large variety of protein sources and macromolecules presently used in the culture of bovine embryos (different types of sera and BSA's, PVA, PVP and hyaluronate). This study investigated the effect of different macromolecules on blastocyst development, in an attempt to eliminate variability and culture induced artefacts that occur when embryos are cultured in media containing BSA or serum.

Experiment 1: Culture with the macromolecules PVA or hyaluronate was compared to culture with BSA, serum or no protein. Embryos developed to the blastocyst stage in the absence of any protein or macromolecule, however development was lower than culture with serum or BSA. Both PVA and hyaluronate were found to be unsuitable as a substitute for BSA or serum, due to their inability to remain at a stable pH in SOF (development to blastocyst was less than 15%). Development to blastocyst was equivalent in BSA and serum. Due to the larger number of unknown components in serum, the darker colour of embryos grown in media containing serum and the fact that culture with serum caused mitochondrial damage in sheep embryos, it is recommended that BSA is used in the culture of bovine embryos.

Experiment 2: Our laboratory currently uses a partially lipid-stripped BSA for culture of bovine embryos. This experiment was designed to test whether the lipid content of serum affects embryo development. Embryos were cultured with 4 different types of human serum (serum from an individual on a high fat diet: charcoal stripped and non-stripped; and serum from the same individual on a low fat diet: charcoal stripped and non-stripped). The lipid content of serum had no effect on blastocyst development. While the lipid content did not affect the number of cells in the inner cell mass of the blastocyst, it did appear to reduce the total cell number. These findings require further investigation.

(v) Determination of Embryo Viability Marker

-see Objective 3

Objective 7:

To assess the ability of somatic cells to be successfully reprogrammed following fusion with, or microinjection into, enucleated oocytes. Successful reprogramming would be demonstrated by the development of blastocysts from such reconstructed ***somatic cell nuclear transfer (SCNT)*** *embryos, and then the establishment of normal ongoing pregnancies following their transfer into suitably prepared recipients.*

This objective was added during the project because of reports from other groups working in this area of pregnancies and live-births from somatic cells used in nuclear transfer. Because of the late addition of this objective, it was unlikely that live-births would be produced within the time-frame of the project.

Quantifiable objective:

Establishment of blastocysts and one or more ongoing pregnancies from somatic cell nuclear transfer embryos.

Milestones

- **The development to blastocysts of at least 10% of reconstructed somatic cell nuclear transfer embryos.**
- **The production of at least one ongoing pregnancy by 30.6.98.**

Completion of objective

The objective was achieved with the production of a relatively high overall rate of blastocyst development (14%) from reconstructed somatic cell nuclear transfer (SCNT) embryos using fetal fibroblasts as the source of donor nuclei, and the establishment an of ongoing pregnancy.

Summary of progress

- **Fetal fibroblasts electrofused with enucleated oocytes resulted in reconstructed SCNT embryos which cleaved, but which did not develop to the blastocyst stage.**
- **Microinjection of fetal fibroblasts into enucleated oocytes resulted in a high rate of development to blastocysts, which was 14% by the end of the Project, and at the time of writing this report was averaging over 20%.**
- **Transfer of 87 such fresh and cryopreserved morula and blastocyst stage SCNT embryos into 33 recipients, resulted in 2 pregnancies (6%), one of which is ongoing (over 5 months) at the time of writing.**
- **The passage of fetal fibroblasts for between four and nine passages produced similar blastocyst development rates.**
- **The embryos produced by the transfer of nuclei from fetal fibroblasts into cytoplasts by microinjection showed the same pattern of cell surface antigen expression of TEC-3 and TEC-4 stage specific epitopes during development as fertilized oocytes.**
- **The present studies indicate that nuclear transfer by microinjection of isolated somatic cell nuclei is an efficient method for producing embryos that are capable of developing to blastocysts in vitro. The choice of cell type and cell cycle manipulation are likely to influence success rates of nuclear injection. In addition, more sensitive indicators of nuclear reprogramming are necessary to improve the efficiency of nuclear transfer.**

(i) Somatic cell nuclear transfer by electrofusion

The fusion rates of whole cytoplasts with fibroblasts (25/108 – 23%) was significantly lower ($P<0.05$) than half cytoplasts (40/108 – 37%) and quarter cytoplasts (50/116 – 43%). In the experiments using a single whole, half and quarter cytoplast for fusion with fibroblasts, 43% (91/212), 26% (9/35) and 16% (6/38) cleaved respectively, but only one expanded blastocyst was produced. With double whole, half and quarter cytoplasts fused with fibroblasts, 54% (113/211), 6% (2/36) and 6% (2/35) cleaved respectively, and no blastocysts were produced. The division of cytoplasts appeared to reduce their developmental capacity severely. For the parthenogenetically activated controls, 84% cleaved (216/258) and 20% (52/258) developed to expanded blastocysts.

When blastomeres of day 4 embryos derived from double cytoplast fusion with fibroblasts were

disaggregated and re-fused with cytoplasts, 70% (23/33) cleaved in culture but none developed to blastocysts. For the parthenogenetically activated controls, 91% (30/33) cleaved and 12% (4/33) developed to blastocysts.

(ii) Somatic Cell Nuclear Transfer by Microinjection

The cleavage and development of embryos after reconstitution by subzonal electrofusion of fibroblasts into cytoplasts and the microinjection of isolated nuclei into the cytoplasts, is shown in Table I. Significantly more reconstituted embryos cleaved to 4-cells or more in culture and developed to blastocysts after isolated nuclear injection, and 14% of activated cytoplasts developed to expanded blastocysts. However, this was significantly lower than the cleavage rate of parthenogenetically activated oocytes and the development of these activated oocytes to blastocysts (24%).

Table I **Development of Embryos after Somatic Cell Nuclear Transfer**

	Number of activated cytoplasts	Number of embryos $\geq$ 4 cells	Number of expanded blastocysts
Subzonal – electrofusion	64	18 (28%)[a]	1 (2%)[d]
Nuclear injection	173	109 (63%)[b]	24 (14%)[e]
Parthenogenetic activation (control)	346	275 (79%)[c]	84 (24%)[f]

a vs b $\chi_1^2 = 21.5$; $P<0.001$, b vs c $\chi_1^2 = 15.4$; $P<0.001$
d vs e $\chi_1^2 = 6.25$; $P<0.025$, e vs f $\chi_1^2 = 6.95$; $P<0.01$

(iii) Fetal fibroblast passage number

There was no apparent relationship between passage number of the fetal fibroblasts and the developmental competence of embryos formed by nuclear injection (Table II).

Table II: **Fibroblast Passage Number and Success of Nuclear Injection**

Fibroblast passage number	Number of injected cytoplasts	Number of embryos $\leq$ 4-cells	Number of expanded blastocysts
4	5	5 (100%)	1 (20%)
5	56	47 (84%)	12 (21%)
6	11	6 (55%)	0
7	5	3 (60%)	0
8	34	30 (88%)	4 (17%)*
9	5	4 (80%)	1 (20%)

*23/30 embryos cultured for 7 days

(iv) Expression of TEC-3 and TEC-4 Epitopes on Nuclear Transfer Embryos Formed by Microinjection

The presentation patterns of TEC-3 and TEC-4 epitopes on cytoplasts, normally fertilized embryos, nuclear transfer embryos formed by electrofusion of embryonic blastomeres and cytoplasts and those recycled as second and third generation embryonic cell nuclear transfer embryos (Peura et al., 1998a, b) are shown in Table III. Fibroblasts do not express TEC-3 or TEC-4 epitopes and no 70kDa glycoprotein band was identified by Western blot analysis when the TEC-03 and TEC-04 antibodies were used as probes.

Embryos that developed after fibroblast nuclear injection showed the normal pattern of stage-specific TEC-3 and TEC-4 presentation (Table III).

From both sets of experiments there was little encouragement that somatic cell nuclear transfer could be efficiently achieved by electrofusion using the technique very successfully used by Peura et al. (1998a, b) for embryonic blastomeres.

Table III **Cell surface TEC epitope presentation after embryonic and somatic cell nuclear transfer**

Cell and nuclear transfer type	TEC-3 epitope (+ present - absent)	TEC-4 epitope (+ present - absent)
Oocyte – cytoplast	-	+
Embryos: 1-8 cells	-	+
9-32 cells	-	-
morula	+	-
blastocysts	+	-
Embryonic cell nuclear transfer		
- 1st generation	Normal[a]	Normal (± 50% at blastocyst stage)
- 2nd generation	Normal	Normal
- 3rd generation	Normal	Normal
Fibroblasts	-	-
Somatic cell nuclear transfer		
- 1st generation	Normal	Normal

[a] Normal – same distribution as bovine embryos formed after fertilization

(iv) Table 7: Pregnancy data from the transfer of SCNT embryos

	Number of Embryos Implanted	Number of Recipients Used	Number of Recips Pregnant (%)	Number of of Calves Born (% preg recips calved)	% of embryos resulting in calves
From 13.3.98 to 9.9.98 (still awaiting calving data)					
Total fresh	**24**	**9**	**1**		
Total cryopreserved	**63**	**24**	**1**		
Total fresh & cryopres. 13.3.98 to 9.9.98	**87**	**33**	**2 (6%)**	**1 (3%) (estim.)**	**1% (estim.)**

Objective 8:

(a) To transfer the technologies developed at Monash University to Genetics Australia in preparation for widespread commercial application.
(b) To begin the development of production systems, based on these technologies, which allow the efficiencies necessary for cost effective product production.
(c) To develop systems for embryo cryopreservation and transfer which allow the sale and distribution of the product (cryopreserved cloned embryos) to fit in with existing systems for the sale of semen.
(d) To develop recipient and calf management systems which maximize outcomes from cloned embryos.

Completion of objective:

This objective has been achieved with the establishment of a fully functioning in vitro embryo production laboratory at Genetics Australia's facility at Bacchus Marsh which employs three full time and one part-time staff. Systems have been developed (and patented) which greatly increase the efficiencies of oocyte recovery from abattoir ovaries. Embryo cryopreservation and transfer methods, combined with recipient management (including calving) and calf management systems which maximize outcomes from cloned embryos, have been developed and are now in routine use.

Summary of progress

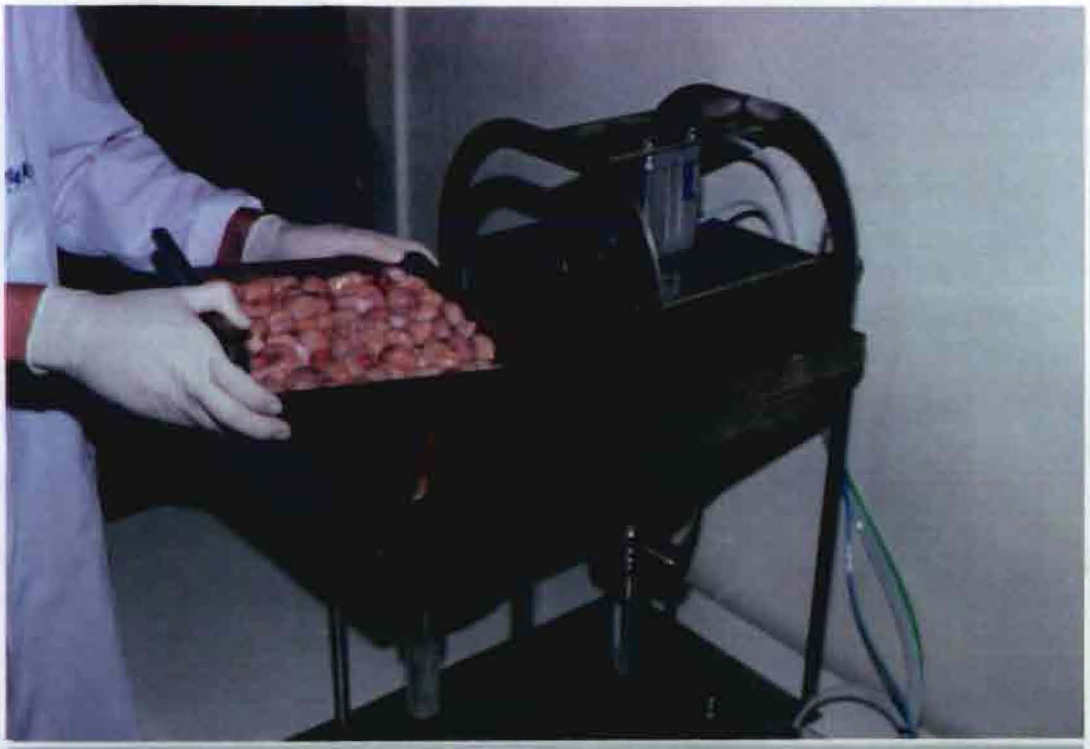
- **The embryo production (IVP) laboratory at Genetics Australia produces 3,000 matured oocytes weekly from abattoir material, for use in research at Monash University, in product development at Genetics Australia and for sale to other organizations requiring such oocytes. To increase efficiencies of oocyte production, an automated oocyte recovery machine has been developed at the Genetics Australia which has the potential to more than treble current efficiencies.**
- **The laboratory also has the capacity to fertilize and culture matured oocytes to the blastocyst stage for transfer or cryopreservation, at development rates which are world standard (35 to 50% blastocyst development from matured oocytes).**
- **Systems for cryopreservation (vitrification) have been developed which allow the direct transfer (without the use of a microscope) of cryopreserved in vitro produced embryos with excellent results (see Objective 4 (i)).**
- **Recipient, calving and calf management systems have been refined from the Genetics Australia Eureka program (see below) and are used to optimize outcomes after the transfer of cloned embryos produced during the project.**

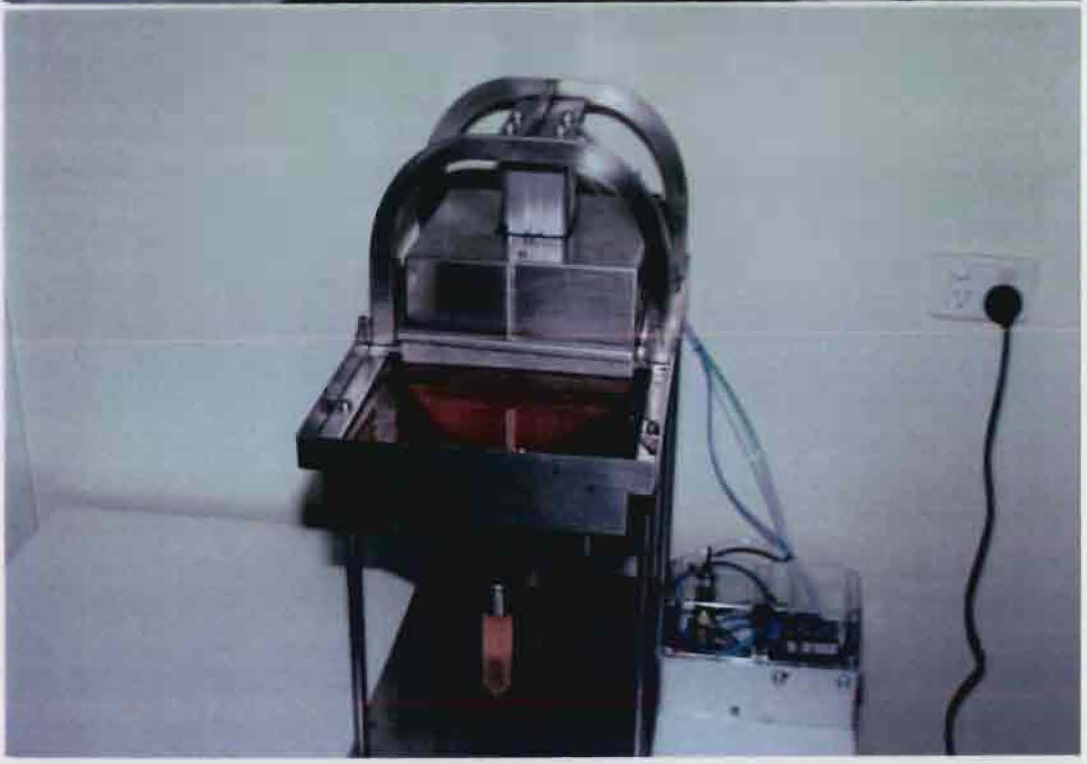
(i) IVP Laboratory

This laboratory was established at a set up cost of over $250,000 (building and equipment) during the Project, and has an annual operating budget of over $200,000. The laboratory presently produces matured oocytes for Monash University, Genetic Australia and other Institutes such as VIAS (which purchase matured oocytes from the Genetics Australia laboratory) and also produces IVP embryos for commercial purposes. The three full time staff have had training at Monash University in nuclear transfer procedures, and now have a electrofusion machine and other equipment necessary for the commercial production of cloned embryos which is due to begin early in 1999.

The automated machine for extracting oocytes from abattoir derived ovaries was designed by, and built under contract for, Genetics Australia which has taken out a patent on the machine. The current machine (fifth prototype) currently extracts a greater number of oocytes per ovary as manual methods and has at least a three fold labour saving (see table 8). Additional refinements to the current model will produce further efficiencies in this critical area of production.

Automated oocyte recovery machine
designed and patented by Genetics Australia





Genetics Australia's in vitro oocyte and embryo production laboratory and cattle handling facilities at Bacchus Marsh, Victoria

Figure

Table 8: Comparison of automated and manual oocyte recovery

	No. of "usable" oocytes per ovary obtained from "Ovary Machine"* (12 replicates)	No. of "usable" oocytes per ovary obtained by aspiration** (12 replicates)
	459 oocytes from 120 oocytes	**2835 oocytes from 1040 ovaries**
Average oocytes/ovary	**3.8**	**2.7**

* This includes results from a number of early prototypes of the machine which were suboptimal.
** This includes results from ovaries of young cows and from ovaries from drought conditions, both of which are known to reduce the number of "usable" oocytes per ovary.

(ii) Recipient management

The Genetics Australia "Eureka " program has imported over 700 high cost, elite dairy embryos, implanted them into optimally managed recipients, calved them down and reared the resulting calves. This has allowed the development of systems which produce world class outcomes from such embryos, with over 60% of transferred embryos currently resulting in live offspring with minimal neonatal and post-calving losses.

This recipient herd has been used for the transfer of all research embryos produced by the Project, including those produced by nuclear transfer. The optimal recipient and calf management techniques developed by Genetics Australia ensure the best possible outcomes from such embryos, and provide systems which will be commercially applicable.

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