TRANSGENIC LIVESTOCK: STUDIES IN IMPROVED EFFICIENCY OF PRODUCTION AND GENE REGULATION

by

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ABSTRACT

This thesis reports on studies aimed at increasing the efficiency of livestock transgenesis programs.

A murine model was used to investigate the effects of altering the DNA structure on the frequency of integration following microinjection. Exposing embryos to ultraviolet light prior to microinjection increased the frequency of integration of all transgenes investigated. The data suggest that pretreatment of DNA with intercalating agents such as Hoechst stain or a specific DNA restriction enzyme also increased the integration frequency to a degree that varied dependent on the transgene. Cleavage rates of treated embryos tended to be delayed indicating that this may be the mechanism underlying increased gene integration observed rather than DNA structural perturbation *per se*.

In studies with the porcine, several protocols were evaluated to determine a convenient source of one cell pronuclear embryos for transgenesis. It was shown that pregnant (day 21-80) postpubertal gilts treated with Cloprostenol for oestrous synchronization and successfully superovulated with gonadotrophins provided an efficient source of suitable embryos. An *in vitro* culture system for porcine embryos was established using simple medium. The system was validated by the production of transgenic and nontransgenic offspring from embryos cultured *in vitro* for five to six days. The culture system allowed long distance transport of porcine embryos and provided a basis for the development of an adaptable porcine cryopreservation procedure.

Several breeding lines of transgenic pigs with variants of a metallothionein promoter/porcine growth hormone construct were established. Preliminary studies on the action of enhanced pGH production were carried out on nontransgenic pigs treated with recombinant porcine growth hormone. Two lines of transgenic pigs demonstrated that levels of growth hormone could be regulated above normal endogenous levels. Regulation of the transgene was achieved by additions of zinc to the diet

resulting in increased growth and performance. Each transgenic line was examined to determine if the transgene effected animal growth, reproduction and could be inherited by its offspring.

Results provided from this thesis establish the feasibility of creating transgenic livestock with enhanced production characteristics using a regulated transgene. The efficiency of production of transgenic animals has been improved by (1) providing a convenient source of viable embryos and (2) establishing an *in vitro* culture system that did not compromise viability. Gene regulation in porcine growth hormone transgenic pigs was demonstrated and could provide significant benefits for commercial use. Overall the experiments provided an improved basis for understanding the application of animal biotechnology to the pig.

DECLARATION

I hereby declare that this thesis contains no material which has been submitted for the award of any other degree or diploma in any university. To the best of my knowledge and belief, no material described herein has been previously published or written by another person, except where due reference is made in the text.

If accepted for the award of a Ph.D. degree I consent to this thesis being available for loan and photocopying.

A.J. French

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PUBLICATIONS

French, A.J. (1987). Transgenic Pigs. *Pig Industry News* **26**:5

French, A.J. (1987). Transgenic pigs have 'enormous potential'. *Pork J.* **4**:14-15.

French, A.J. (1990). Transgenic and recombinant porcine growth hormone treated pigs: their potential to biotechnology in agriculture *Tech. Report SA Aust. Soc. Anim. Prod.* **2**:17-25.

French, A.J., Du, Z.T. and Seamark, R.F. (1990). Ultra Violet Light: Increased the integration frequency of microinjected DNA. *Proc. Aust. Soc. Reprod. Biol.* **22**:54 (abstr.).

French, A.J., Zviedrans, P., Ashman, R.J., Heap, P.A., and Seamark, R.F. (1990). Comparison of prepubertal and postpubertal young sows as a source of one cell embryos for microinjection. *Theriogenology* **35**:202 (abstr.).

French, A.J., Cecil, A. and Seamark, R.F. (1991). Viability of frozen hatched blastocysts cultured from one-cell zygotes. *Proc. Aust. Soc. Reprod. Biol.* **23**:119 (abstr.).

Nagashima, H. and French, A.J. (1991) Cryopreservation of porcine embryos (submitted).

French, A.J. and Seamark, R.F. (1991) Postpubertal young sows as a source of one cell embryos for microinjection (submitted).

ABBREVIATIONS

Chemicals

BSA	bovine serum albumin
CO_2	carbon dioxide
DNA	deoxyribonucleic acid
$HC0_3$	bicarbonate
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
N_2	nitrogen
O_2	oxygen
PBS	Phosphate Buffered Saline
Tris	2-amino-2-hydroxymethyl-propane-1-3-diol

Hormones

FSH	follicle stimulating hormone
GH (s)	growth hormone (s) (porcine, bovine, human)
HCG	human chorionic gonadotrophin
IGF-I	insulin-like growth factor-I
IGFBP's	insulin-like growth factor binding proteins
LH	luteinizing hormone
PMSG	pregnant mare serum gonadotrophin

Symbols and Units

Ci	curie
cm	centimetre (s)
g	gram (s)
h	hour (s)
i.u.	international unit (s)
l	litre (s)
M	molar
µg	microgram (s)
mg	milligram (s)
min.	minutes (s)
µl	microlitre(s)
ml	millilitre (s)
µm	micrometre (s)
mm	millimetre (s)
mOsm	milliosmolar
ng	nanogram
nMol	nanomole (s)
pl	picolitre
xg	unit of gravitational field
°C	degrees centigrade

Media

BMOC	Brinster's medium for ovum culture
Cl	corpora lutea (corpus luteum)
HTF	human tubal fluid medium
MEM	minimal essential medium
PBS	phosphate buffered saline
Whitten's	Whitten's media

Others

Bl	blastocyst
DIC	differential interference contrast
DNA	deoxyribonucleic acid
EB	early blastocyst
et al.	et alia (and others)
FCS	fetal calf serum
HB	hatched blastocyst
HIFCS	heat inactivated fetal calf serum
HIHS	heat inactivated human serum
HS	human serum
i.m.	intramuscular
i.v.	intravenous
IVF	*in vitro* fertilization
IVM	*in vitro* maturation
mRNA	messenger RNA
PCR	polymerase chain reaction
RNA	ribonucleic acid
UV	ultraviolet light

CHAPTER I

LITERATURE REVIEW

1.1 INTRODUCTION

The advent of transgenic animals has established new horizons of experimental possibilities for the study of mammalian development. The full impact of this revolutionary technology has yet to be felt, but it is already clearly evident that no areas of biological investigation will remain unaffected. The effect on agriculture will be equally revolutionary. The galvanic studies of Palmiter *et al.*, in 1982 demonstrated the remarkable enhancement of growth following incorporation of functional growth hormone genes into the mouse genome and adaptation of this procedure to livestock breeds became a priority for many groups world wide. Whilst the global experience has been encouraging it has also shown that there are fundamental biological constraints which need to be addressed before transgenesis can be regarded as being generally available to the livestock breeder. In particularly it has shown the need to increase the efficiency of gene integration and achieve control of integrated foreign DNA expression. This thesis reports on the Adelaide program of establishing commercial lines of transgenic pigs with transgenes designed to achieve controlled growth. Comparisons are made between the performance characteristics of the transgenic pigs produced and targeted objectives modelled using recombinantly pGH treated pigs. The limitations associated with availability, (collection micromanipulation and propagation) of embryos required for the production of transgenic pigs are highlighted and procedures developed to minimize them. Finally, this thesis examines mechanism associated with increasing the efficiency of gene integration following DNA microinjection in the mouse.

1.2 Literature Review

This literature review is presented in four parts. The first part discusses techniques associated with DNA microinjection and the establishment of transgenic animals. The second part examines factors that perturb DNA structure and the possible use of alteration in gene structure to enhance the frequency of transgene integration following microinjection. The third part investigates the limitations presently associated with the collection and transfer of embryos for microinjection. Finally, it reports on the subsequent performance of transgenic and recombinant GH treated animals compared to controlled sex matched littermates in relation to enhanced growth, reproduction and heritability.

2.0 TRANSGENESIS

2.1 Early Embryo development

The alteration of genomes with insertion of extrachromosomal DNA and preserving the inheritance in subsequent germlines requires the use of early stage embryos or totipotent cell lines as early mammalian embryogenesis is known to proceed by successive, dichotomous formation of cell lineages (Gardner *et al.*, 1975) resulting in homogeneous populations of cells with specific features and development pathways (Monray and Rosaki, 1979). The process of embryo development is generally classified into three general categories, (1) growth, an increase in size (2) differentiation, the acquisition of phenotypic variation amongst cells of identical genotypes and (3) morphogenesis, attainment of correct spatial and temporal relationships between phenotypically distinct cells. The blue print for all this, resides within the single cell of a newly fertilized zygote (Johnson, 1977). Early preimplantation development which includes fertilization by sperm, extrusion of the first and second polar body, pronuclei formation, cleavage, and differentiation occurs within the oviducts and uterus of the female reproductive tract (for recent comprehensive review see, 'The Mammalian

Preimplantation Embryo: Regulation of Growth and Differentiation *in Vitro*' (Ed. Bavister, 1987)).

2.2 The Potential for Manipulating Animal Embryos

Whilst the laboratory manipulation of mammalian eggs started more than 90 years ago (Heape, 1981) only in recent years have techniques been developed which allow mammalian zygotes to be subjected to experimental manipulation (Market, 1984).

2.3 Transgenic Animals

The term transgenesis was coined by Gordon and Ruddle, (1983) to describe a technical process enabling transfer of inheritable functional genes between organisms irrespective of species barriers. Animals whose germ lines have been modified to include foreign DNA introduced by this means are said to be transgenic (Gordon *et al.*, 1980). The foreign DNA is often referred to as the transgene (see review, Palmiter and Brinster, 1986). Mammalian cells were first shown to incorporate foreign DNA into their genome using cell culture techniques. Initially, adenovirus DNA fragments were mixed with calcium phosphate and the resulting combination added to culture cells which were subsequently shown to be transformed by the adenovirus DNA-encoded functions (Graham and Van der Eb, 1973; Graham *et al.*, 1975). Though a rare event, the cells could be detected by imposing selection procedures that demanded foreign gene expression for cell survival (Wigler *et al.*, 1977; Maitland and McDougall, 1977; Bachetti and Graham, 1977). This approach made it possible to insert foreign genes in teratocarcinoma cells (Pellicer *et al.*, 1980), by cell fusion (Illmensee *et al.*, 1978), to select and to select the surviving cells for use in the production of chimeric mouse (Tarkowski, 1961; Mintz, 1962; Papaioannou *et al.*, 1975; Dewey *et al.*, 1977).

The demonstration that mammalian cells in culture could be made to incorporate foreign DNA and be used to create germline chimeras, stimulated

efforts to improve and develop methodologies for the transfer of exogenous DNA to mammalian genomes. Most somatic cell techniques commonly used by molecular biologist such as the calcium phosphate technique, were precluded from embryo studies due to the low and variable integration frequencies achieved and the tendency of the transgenes to become associated in large "transgenomes," which remained unintegrated for many cell divisions (see review, Scangos and Ruddle, 1981). Consequently it was only following the development of micromanipulation equipment (Diacumakos, 1973) that made possible the direct injection of DNA into cells to achieve an integration frequency of between 5-20% (Graessmann *et al.*, 1979; Capecchi, 1980). The potential was further enhanced by the demonstration in cell culture that high transformation rates could be achieved by the direct microinjection of DNA into cells (Anderson, 1980; Graessmann *et al.*, 1979; Capecchi, 1980).

2.3.1 Direct Microinjection

The successful incorporation of foreign DNA in mouse embryos by direct microinjection was first shown by Jaenisch, (1976) who showed integration of the SV-40 virus within the mouse genome following microinjection into the cytoplasm.

It was several years later when the first report describing transgenic mice stemming from microinjection of non viral DNA into the pronucleus emerged (Gordon *et al.*, 1980) and this was followed quickly by several reports of similar success in stably integrating foreign DNA into the genome of the mouse (Brinster *et al.*, 1981; Constantini *et al.*, 1981; Wagner, E. *et al.*, 1981; Wagner T. *et al.*, 1981). Moreover, there was evidence that at least some of the foreign genes could be expressed (Brinster *et al.*, 1981; Wagner, E. *et al.*, 1981; Wagner T. *et al.*, 1981) and that integrated transgenes were incorporated into the germline (Constantini *et al.*, 1981; Gordon *et al.*, 1981; McKnight *et al.*, 1983; Stewart *et al.*, 1984; Palmiter *et al.*, 1982,1983).

Furthermore offspring of transgenic founder mice continued to express the foreign genes (Palmiter *et al.*, 1982).

Transgenesis through microinjection is now routine in many laboratories world wide and has provide numerous mouse lines that express a variety of integrated genes. The technique has been particularly important in studies of factors which control the expression of both endogenous and hybrid genes in which regulatory (promoter) elements of one gene have been fused to the structural elements of another gene. Palmiter and Brinster, provided a comprehensive review of both types of transgenes in 1986, however the field is progressing rapidly and continuing review is warranted (see recent review, Seamark and Wells, 1991).

The technique of DNA microinjection is conceptually straight forward, although demanding special equipment and technical skill. The basic technique involves impaling the pronucleus with a glass injection needle (1.3 mm I.D.) and then forcing buffer containing DNA into the pronucleus. Swelling of the pronucleus in response to the pressure is an essential sign of successful penetration. The volume of injection fluid injected is generally about 2 picolitres, which is equivalent to the volume of the pronucleus, and the content of the DNA is adjusted so that each injection involves transfer of between 400 and 1000 copies of the transgene. The appropriate methodology, limitations and results of recent studies have been reviewed by several authors (Brinster and Palmiter, 1982; Church *et al.*, 1985; Evans *et al.*, 1985; First, 1990; Massey, 1990; Palmiter and Brinster, 1986; Pursel *et al.*, 1987, 1989; Rexroad and Pursel, 1988, 1990; Seamark, 1989; Wagner, 1985).

Microinjection remains the most preferred technique for introducing genes into the germline of both laboratory and livestock species. Studies with livestock have resulted in the production of transgenic pigs (Hammer *et al.*, 1985; Brem et al. 1985; Pinkert *et al.*, 1987; Ebert *et al.*, 1988; Pursel *et al.*, 1987,1988; Vize *et al.*, 1988), sheep (Hammer *et al.*, 1985; Nancarrow *et al.*, 1987b; Pinkert *et al.*, 1987; Pursel *et al.*, 1987; Simons *et al.*, 1988), goats

(Fabicant *et al.*, 1987), cows (Biery *et al.*, 1988) and fish (Cloud, 1990). Expression of transgenes has been reported in the pig (Hammer *et al.*, 1985; Ebert *et al.*, 1988; Pursel *et al.*, 1987; Vize *et al.*, 1988), sheep (Pinkert et al. 1987; Pursel *et al.*, 1987; Simons *et al.*, 1988), cow (Biery *et al.*, 1988) and fish (Cloud, 1990).

The rapid and successful application of the microinjection techniques has only been possible due to concomitant advances in animal breeding technologies which include:-

(1) Techniques for the culture of animal preimplantation embryos *in vitro* (Whitten, 1956; Wright and Bondioli, 1981) and the demonstration that normal young could be born after transfer of the cultured embryos into surrogate recipients (McLaren and Michie, 1956; McLaren and Biggers, 1958; Wright and Bondioli, 1981).

(2) Techniques for oestrous synchronization and superovulation in animals (Gates, 1969; Gates and Runner, 1957; Edwards *et al.*, 1959; Paterson, 1982).

(3) Development of micromanipulation and microsurgical techniques for mouse and their suitability for extension into domestic embryos (Tarkowski, 1961; Mintz, 1962; Gardner, 1968).

2.4 Gene Integration

When linear DNA molecules are microinjected into the pronuclei between 0.2% to 0.5% of the embryos injected result in mice born with one or more copies of the integrated transgene. While low, this success rate has been sufficient for the technique to be usefully employed for studies involving the control of gene function and early mammalian development (Brinster *et al.*, 1985) but has constrained, for which reasons will be discussed, as a severe restriction for application to livestock species. A major constraint in trying to devise ways of improving the efficiency of gene transfer processes is that we know little about the mechanism by which integration of foreign DNA occurs. Mammalian cells are known to possess a repertoire of DNA

processing enzymes which could be involved in the cutting of chromosomal DNA and integration of foreign fragments of genetic information.

Generally, integration of transgenes following microinjection usually occurs prior to DNA replication, about 70% of the resulting animals carrying the transgene in all of their cells, including the sperm cells (Palmiter *et al.*, 1985b). In about 30% of the mice, integration occurs after one or more rounds of replication, and a mosiac results. Mostly such mosiac mice show the same degree of mosaicism in somatic and germ cells, but in some the germline is deficient (Wilkie *et al.*, 1986).

The salient feature is that when one or more copies of the transgenes integrates, it usually occurs at a single chromosomal site with multiple copies of the transgene arranged in a tandem, head to tail array, although other arrangements have been documented (Gordon *et al.*, 1981, 1989; Hammer *et al.*, 1984; Palmiter *et al.*, 1983). The chromosomal site of integration is probably determined randomly in that there is no evidence for integration of foreign DNA on specific chromosomes and there are reports of integration in autosomes (Krumlauf and Stewart, 1985a, 1985b; Lacy *et al.*, 1983) and X (Lo, 1986) or Y chromosomes (Wagner *et al.*, 1985). In general, there is individuality with respect to chromosomal sites and integration within different transgenic mice (Lacy *et al.*, 1983).

To explain the predominance of single integration sites, Brinster *et al.*, (1986) proposed that the rate limiting step to integration is due to randomly generated chromosomal breaks. Features supporting this model are:

- that the ends of injected DNA molecules initiate integration at these breakpoints (this accounts for a 5 fold increase in integration frequency with linear versus circular DNA).
- that homologous recombination may occur among injected molecules (to explain the prevalence of tandem arrays), but not between injected DNA and chromosome (Possibly due to differences in chromatin structure).
- another alternative explanation is that tandem arrays occur before integration.

Although this model provides explanations for findings such as homologous recombination among transgenes as seen when functional MT-hGH transgenes were generated following injection of two different MT-hGH genes with non over-lapping deletions (Palmiter *et al.*, 1985). However it does not explain the generation of concatamers and reduplication of restriction fragments not found in the original injected sequence (Gordon, 1989). Nevertheless it should be noted in the cited example, that no concatamer has ever been found in transgenic mice that does not contain at least one complete copy of the original injected DNA fragment and no concatamers have been found which are made entirely of modified elements. Consequently, no modification occurs prior to concatamerization and homologous recombination must play some role in integration. Differences have also been noted in the integration pattern between placenta and fetuses (Burki and Ullrich, 1982; Wagner, E. *et al.*, 1986).

Further experimental observations are needed to resolve these and many other questions concerning integration. More detailed information is likely come from cloning junction fragments between the transgenes and chromosomal DNA particularly in regard to events that occur before integration (Palmiter *et al.*, 1985). Although only a few such junctions have been characterized so far (See Chapter 2.4.2) it is clear that deletions, duplications and translocations of chromosomal sequences can occur at the site of integration and some junctions contain short novel DNA sequences not found in either of the injected DNA or in the vicinity of the integration site (Cory, 1986).

2.4.1 Factors Affecting the Efficiency of Gene Integration by DNA Microinjection of Zygotes.

Several of the parameters that effect the efficiency of introducing foreign DNA into mice have already been defined (Brinster *et al.*, 1985). Factors identified as critical for successful gene transfer into the germ line of mice are:-

-(1) The concentration of the DNA with integration efficiency improving as the DNA concentration in the injection fluid is increased to about 1 ng/ml (equivalent to several hundred copies of DNA injected into the pronucleus).

-(2) The form of the DNA with linear molecules integrating about five times more efficiently than circular ones. Data presently available indicate the overall efficiency for linear molecules is 26%. with linear DNA molecules with either similar or dissimilar staggered ends integrated with essentially the same efficiency. These may be superior to linear molecules with blunt ends, although this has yet to be proved. Single stranded ends do not improve integration frequency to those generated by common restriction enzymes.

-(3) The site of injection of DNA. Usually the male pronucleus of one cell zygotes is injected, although injection into the smaller, female pronucleus has proven to be as efficient with respect to integration frequency. By contrast the efficiency of integration following cytoplasmic injection is very low.

-(4) The vehicle used for DNA transfer or ideal buffer for microinjection of DNA should contain low concentrations of $MgCl_2$ or EDTA as both are toxic at high concentrations.

-(5) The size of the transgene. This usually range is between (0.7-50 kb) and does not seem to be an important parameter in integration.

-(6) The amount of DNA injected. Injection of large amounts of DNA (up to 50,000 gene copies) resulted in considerable modification of the DNA with the formation of supercoiled and concatenated structures. This is presumably due to the package of exonucleases and ligases present in the nucleus of the mouse zygote. Additional no injected DNA is replicated extra chromosomal, nevertheless despite this modification, gene integration occurs at a reasonable efficiency (Brinster *et al.*, 1985).

2.4.2 Insertional Mutagenesis

As previously noted the available evidence suggests that the site at which the foreign DNA inserts into the host chromosome is probably random.

Although there is probably a tendency for the foreign DNA to be integrated preferentially into open chromatin. Occasionally the foreign DNA would be expected to disrupt the function of an endogenous gene or possibly activate a gene by enhancer/promoter insertion. Judging for the rate of producing recessive or dominant mutations, the frequency of occurence is less than 7% (Brinster *et al.*, 1986). This is probably an underestimate because it would not include those mutations causing subtle phenotypic effects or embryonic loss. The frequency of insertional mutagenesis appears to be higher with microinjection techniques than with retroviral insertion, this is probably related to the method of microinjection, reflecting the damage caused by microinjection and the fact that large regions of chromosomal DNA may be deleted or duplicated during integration of microinjected DNA (Schnieke *et al.*, 1983; Jaenisch *et al.*, 1985, Lohler *et al.*, 1984; Mark *et al.*, 1985).

2.4.3 Mechanisms for Increasing Gene Integration following DNA Microinjection

Studies in this thesis explore the possiblity of increasing the integration frequency by altering the DNA structure prior to microinjection. Chromosomal structure is known to be perturbed by commonly available treatments such as ultraviolet light and restriction endonucleases which are expected to cause chromosomal breaks and Hoechst 33342 stain which is expected to cause relaxation by intercalating with DNA.

2.4.3.1 Ultraviolet Light

Both eukaryotes and prokaryotes respond to exposure to DNA damaging agents by inducing a multitude of repair and recombination functions, termed SOS or DIN (damage inducible functions). These inducible functions are responsible for mechanism that enhance repair which presumably are mechanism for enhancing cell survival (Dubbs *et al.*, 1974; Hall *et al.*, 1980; Sarsin *et al.*, 1980). Ultra Violet light not only damages DNA, but also blocks DNA synthesis and induces repair pathways (Hall and

Mount, 1981). Postel, (1985) reported that ultra violet enhanced the frequency of stable plasmid transformation in several different cell lines. The improved frequency was due more to increases in the competent (transformable) cell population rather than changes in integration frequency between treated and untreated cells. This was further supported by showing the more resistant a cell type was to transformation the more it responded to the enhancing effects of DNA damaging treatment. In general, evidence regarding a molecular basis for UV enhancement of mammalian systems is unclear. Overall however, the oncogenicity of UV light and its role in DNA damage may play a role in the unscheduled integration of extrachromosomal DNA (Postel, 1985).

2.4.3.1.1 Mechanism of Ultraviolet light induced damage

DNA efficiently absorbs light in the range of 240 to 300 nm (Haseltine, 1983). In doing so the bases acquire excited energy states and the photochemical reactions that ensue have been studied in detail (Wang, 1976). The principal products in DNA at biologically significant doses are cyclobutane pyrimidine dimers formed between adjacent pyrimidines. The pyrimidines-pyrimidine (6.4) lesions are formed in DNA at sites at which cytosine, and much less frequency a thymine is located 3' to a pyrimidine base, and is the precursor to the UV-induced photoproducts (Brash *et al.*, 1982). Although all these products form in DNA to varying degrees, the principal biological effects of UV radiations-lethality and mutagenesis have been attributed to cyclobutane dimers for several reasons. Firstly cyclobutane pyrimidine are the most prevalent lesions induced by UV light (Haseltine, 1983). Secondly mutants defective for repair of cyclobutane dimers show increased sensibility to the lethal and mutagenic effects of UV light (Bianchi *et al.*, 1985). Finally, photoreversal of cyclobutane dimers by visible light reverses the lethal and most of the mutagenic consequences of UV light induced DNA damages (Haseltine, 1983).

There is a linear relationship between base damage incidence and mutation incidence (Brash *et al.*, 1982). However the dose response curve for the inhibition of DNA synthesis by UV light, is a multiphasic curve determined under conditions of controlled growth and uniform temperature (Cleaves, 1983).

2.4.3.1.2 Repair Mechanism

Biochemical studies in several organism reveal that removal of cyclobutane pyrimidine dimers, occurs via enzyme-mediated excision, followed by resynthesis of DNA in the damaged area (Haseltine, 1983) and is supported by numerous genetic, biochemical and enzymatic studies (Brash *et al.*, 1982). The postulated excision-repair involves an incision 5' to the dimer by a UV specific endonuclease, initiation of DNA polymerization at the 3' hydroxyl terminus of the incision site, displacement of the dimer-containing strand via elongation of the nascent DNA and excision of the dimer-containing nucleotide followed by resealing of the break by DNA ligase (Haseltine, 1983).

The chemical DNA sequencing reaction of Maxam and Gilbert (1977) have elucidated possible mechanism for the action of purified UV-specific endonucleases and further experiments have indicated they are not simple endonucleases, but have two additional distinct activities.

-(1) an N-glycosylase activity that cleaves the N-glcosyl bond between the 5' pyrimidine of a dimer and the corresponding sugar.

-(2) an adenosine phosphate (AP) endonuclease activity that cleaves a phosphodiester bond 3' to a newly created AP site (Haseltine *et al.*, 1980; Gordon *et al.*, 1980; Demple *et al.*, 1980; Nakabeppu *et al.*, 1981; Radany *et al.*, 1980; Seawell *et al.*, 1980). This results in a 3' phosphosugar remaining at one of the terminus, which is not a substrate for DNA polymerase (Gordon *et al.*, 1981) and requires removal of the dimer, resynthesis and ligation by enzymes for which not all are identified (Haseltine, 1983). Lindahl, (1982) characterized a low molecular weight and highly specific family of N-

glycosylase enzymes which recognize a variety of specific single base modifications, this is likely to be UV-specific endonucleases, for which cyclobutane pyrimidines dimers are the only known substrate. The repair (cyclobutane dimers and other bulky addicts) is controlled by multiple genetic loci and is followed by repair synthesis in which a short segment of DNA is inserted into the genome.

2.4.3.1.3 The (6-4) Photoproduct

A minor product of UV-induced damage, occurring at a frequency of about 10% when compared to cyclopyrimidine dimers, is a compound called Thy (6-4) Pyo or (6-4) photoproducts (Franklin *et al.*, 1982). The Thy(6-4)Pyo lesion is formed by reaction of the 5,6 bond of a 5' thymidine with the exocyclic group of the 3' pyrimidine (amino or keto of cytosine and thymine respectively) the end product of this type of reaction is the formation of stable bonds at the 6 position of the 5' pyrimidine and at the 3' pyrimidine. The lesions can form between all four combinations of dipyrimidine sequences upon irradiation of dinucleotides (Franklin *et al.*, 1982).

It seems unlikely that (6-4) photoproducts are responsible in a significant way for the biological effects of UV light, because at low UV dose (1-100 Jm^2) there are 10 times more cyclobutane dimes than (6-4) photoproducts, however there is strong evidence that the (6-4) photoproducts may be mutagenic (Haseltine, 1983).

2.4.3.2 Hoechst 33342 Stain

An alternative way of perturbing DNA structure is to use intercalating agents. Hoechst stain is a bis-benzimadole dye, which is non toxic specific vital intercalating agent for DNA (Lydon *et al.*, 1980; Richards *et al.*, 1985). Hoechst 33342 fluoresces strongly (brilliant blue) with native DNA or chromatin by readily entering living cells and complexing with DNA (Pursel *et al.*, 1985). Observation requires a fluorescence microscope as opposed to

phase contrast or bright-field microscopy used for other procedures (Pursel *et al.*, 1985).

Fluorescence is limited only to structures containing DNA, more specifically, the fluorescence is limited to chromatin and is most intense at A-T rich centromeres (Hilwig and Gropp, 1972).

The relaxation induced in the DNA structure as the Hoechst-DNA complex is formed may preferential predisposed increased integration following DNA microinjection when compared to an unrelaxed site.

2.4.3.3 Restriction Enzymes

A more precise way of creating breaks in DNA is through the use of specific endonucleases. In 1970, Smith and Wilcox at Johns Hopkins Medical School isolated the first restriction enzyme that cut DNA at a specific recognition site from *Hemophilus Influenzae*. Over the next few years the increasing availability of these bacterial restriction enzymes (or restriction endonucleases) from many distinct bacterium provided molecular biologist with a very flexible, simple and useful technique for partial sequence analysis of DNA. The enzymes recognize specific short oligonucleotides from 4 to 8 residues long in DNA and cleave the DNA at all such sites (Sambrook *et al.*, 1989). Several hundred restriction enzymes with specific recognition sites are now available (Roberts, 1983). The hosts bacteria's DNA is protected from cleavage because the recognition sites are modified, usually by methylation at one or more of the bases in the site, making the site no longer a substrate for cleavage. With a 6 b.p. recognition sequence the enzyme will cut, on average, every 4,000 b.p. Since there is approximately $5x10^9$ base pairs/cell a specific restriction enzyme should cut if all DNA was free of proteins approximately 10^6 times/cell. Since only 1-10% of DNA is thought to be freely accessible to the enzyme, cutting will be much less frequent within the nuclei and may increase sites for DNA integration.

2.5.4 Further Prospects for Increasing Gene Integration

Further improvements in the efficiency of introducing genes into the germline of animals are likely to encompass technologies which allow the (1) development of simple means to introduce single copies of genes, (2) development of protection of transgenes from neighbouring chromatin, (3) discovery of techniques for homologous recombination and (4) development of methods for inactivation of gene expression (control of gene expression).

3.0 POTENTIAL APPLICATION OF TRANSGENIC ANIMALS.

The application of recombinant DNA technology has been reviewed by Ward *et al.*, 1984; Wagner *et al.*, 1984; Church *et al.*, 1985; Wagner, 1985; Kräulich, 1986; Petters, 1986; McConnell, 1986; Wagner and Jochle, 1986; Land and Wilmut, 1987; Smith *et al.*, 1987; Renard and Babinet, 1987; Rexroad and Pursel, 1988; Murray *et al.*, 1988; First, 1990; Massey, 1990; Rexroad and Pursel, 1988; Rexroad *et al.*, 1990 and Seamark 1989,1991.

In general, there are three categories of genes that are potentially useful to animal production. These include (1) Transgenes which influence reproduction and lactational performance which are selected from large groups of interacting genes. (2) Transgenes that regulate or influence immune responses and disease resistance (interferon and major histocompatibility complex). (3) Transgenes comprising of single genes or a small number of genes with a major effect (i.e wool growth (Ward, 1982), growth hormone production (Woychik *et al.*, 1982), litter size (Piper and Bindon, 1985; Jonmundson and Adalsteinson, 1985; Hanrahan and Owens, 1985) and milk quality (Lathe *et al.*, 1986; Simons *et al.*, 1987; Wilmut *et al.*, 1990).

The technology of recombinant DNA and transgenesis is not restricted to selection of genes from within a species and can include novel metabolic pathways from any source. Of particular interest are projects aimed at giving sheep the capacity to synthesize cysteine the essential amino acid rather

than rely on microbial production (Ward, 1982). Bawden *et al.*, (1987) produced a transgene comprising a serine transacetylase and o-acetylserine sulphydrase from microrganisms.

3.1 Transgenic Pigs

The potential of gene transfer to be used as a means of enhancing growth and feed conversion efficiency was most dramatically demonstrated by the "supermouse" (Palmiter *et al.*, 1982). Crucial to the sucess was the avoidance of normal GH regulatory mechanisms by using the mouse metallothionein (MT) promoter gene and the structural gene for rat GH.

A similar approach is used in all accounts of transgenic pigs. So far all transgenic pigs harbouring transciptionally active genes have been produced by the DNA microinjection technique (Brinster and Palmiter, 1986; Pinkert *et al.*, 1987). Primarily transgenes used have involved enhancing growth performance using genes such as growth hormone, human growth hormone releasing factor and insulin like growth factor-1 (Wieghart *et al.*, 1990). The initial focus on the cloning of the GH gene (Woychik *et al.*, 1982) for pratically reasons was because of the dramatic effect of exogenous pituitary derived GH on enhanced liveweight gain, milk production and feed conversion efficiency (Evans and Simpson, 1931; Machlin, 1972; Peel *et al.*, 1981,1982). To date, eleven different regulatory sequences fusion genes have been transferred into pigs (see review, Pursel *et al.*, 1990a). The transgenes have been constructed without introns from either cloned RNA libraries or with introns from genomic DNA libraries. A few constructs have been developed from the combination of both genomic and cDNA so some introns can be included (see review, Pursel *et al.*, 1990b). While research has been mainly focuscd on growth hormone related transgenes, a few notable exceptions include the mouse MX gene for disease resistance, the whey acidic protein gene for mammary gland expression and the sheep β^c globin expression gene for investigations into juvenile expression (see review, Pursel *et al.*, 1990a).

3.1.1 Genes Constructs

As previously noted, the form of DNA does not seem to make any difference as long as its linear (see Chapter 1.6.1). In attempts to gain tissue specificty and control over expression of the transgene in pigs a variety of promoter sequences have been used, these include mouse albumin, mouse and human metallothionein I and IIA respectively, moloney murine leukemia virus, bovine prolactin and rat phosphoenolpyruvate carboxykinase (see review, Pursel *et al.*, 1990a). The most commonly used metallothionein (MT) promoter sequence allows the trangenes to be expressed in virtually all cells although at markedly different levels, furthermore it is responsive to a multitude of inducers (glucocorticoids, heavy metals, inflammatory signals and interferon). Recently, other promoters such as the rat phosphoenolpyruvate carboxykinase (PEPCK) gene have been used. This sequence is specific to the liver and expression is determined on the ratio of carbohydrates to protein (McCrane *et al.*, 1988).

3.1.2 Integration

The frequency of transgenes integration in pigs approaches that reflected for mice. In a review, by Pursel *et al.*, (1990b) between 0.31-1.73% of microinjected zygotes were transgenic at birth. In contrast though, higher efficiency (10%) have been achieved in mice (Vize, 1988; Pursel *et al.*, 1990b) and a possible reason for the variation between species is unknown. Wieghart *et al.*, (1990) speculated that differences in integration frequencies between animals could be due to variations in microinjection technique, animal management, zygote care and transfer to recipients and much of the observations are difficult to evaluate due to relatively small sample size. Pinkert *et al.*, (1989) showed egg survivability was enhanced when using mature gilts and sows.

As in rodents the number of integrated copies can vary from 1 to 500 copies in predominantly tandem head to tail arrays but occasionally head to

head arrays (see review, Pursel *et al.*, 1990). Previously it was thought that no correlation existed between number of copies and serum concentration of hGH (Pursel *et al.*, 1987), however, Miller *et al.*, (1989) recently reported that a positive correlation ($P<0.05$) existed in transgenic pigs between 30-180 days of age when comparing the number of gene copies per cell and the concentration of bGH. The genomic site of integration undoubtedly plays a crucial role in the transgene ability to express and this could affect the correlation between number of copies per cell and the level of expression (Rexroad and Pursel, 1988). Further research on the DNA around the site of transgene integration which may explain some of the anomalies observed (Gordon, 1989).

3.1.3 Expression

Between 17 and 100% of transgenic animals express the transgene. Large variations in amounts of transgene product are observed which is probable due in part to the type of promoter selected (see review, Pursel *et al.*, 1990). Expression of the transgene gene occurs in a variety of tissues. McCrane *et al.*, (1988) using the PEPCK/bGH transgene reported specific expression in the liver and kidneys. Wieghart *et al.*, (1990) using the same construct showed tissue specificity in transgenic pigs but aberrant expression patterns were observed and may reflect the high integration rates. Wieghart *et al.*, (1990) concluded further investigation into ways to enhance efficiency of expression was needed.

3.1.4 Transgene control

Regulation of expression has been achieved using two different constructs. Polge *et al.*, (1989) using a prolactin promoter produced episodic levels of GH following induction with thyrotrophin releasing hormone or the dopamine antagonist, sulphride. Using a MT promoter, transgenic pigs fed Zinc between 1000 and 3000 ppm resulted in the doubling of bGH in serum (see review, Pursel *et al.*, 1990a). However, the level of response was

significantly less than observed in transgenic mice (Palmiter *et al.*, 1983; Hammer *et al.*, 1985). The variation may be explained by investigating the general activity at the locus of integration and the characteristics (i.e. methylation) of enhancer sequences in genes located either side of the fusion gene (Pursel *et al.*, 1990a).

3.1.5 Inheritance

Transgenes are inherited as a Mendelian autosomal dominant trait (see review, Pursel *et al.*, 1990a). Once the fusion genes is stably integrated into pig genome the progeny usually function in the same manner as the founder (Hammer *et al.*, 1985).

3.2 Performance of transgenic livestock

The production of transgenic animals with expressing transgenes of biologically active growth hormones have shown increases in IGF-I, insulin, feed conversion ratio and decreases in subcutaneous fat (Pursel *et al.*, 1990a). Ebert *et al.*, (1988) and Vize *et al.*, (1988) have reported superior growth rates with MThGH and MTpGH transgenic pigs respectively. However transgenesis does not necessarily guarantee the production of superior animals. Pursel *et al.*, (1990a, 1990b) reported growth was enhanced in some lines but not in others. Initial populations of Mt-bGH/hGH transgenic pigs did not grow even though hormone was in biologically active form (Pursel *et al.*, 1987). However further generations feed a diet containing 18% protein grew 16% better than nontransgenic littermates (Pursel *et al.*, 1989a, 1989b). This work was based on pigs treated with recombinant porcine growth hormone where maximal growth rate was not possible without adequate protein, in particular lysine additions to the diet (Goodband *et al.*, 1988; Newcomb *et al.*, 1988). This phenomenon is reported in other transgenic animals (Nancarrow *et al.*, 1988).

3.2.1 Health Status

The phenotypic side-effects observed in pigs harbouring constructs combining metallothionein, phosphoenolpyruvate carboxykinase or Moloney murine leukaemia virus promoters and growth hormonal structural genes is attributed to the chronic overexpression of growth hormone. High concentrations of GH have been found to be associated with a variety of health problems such as lethargy, lameness, gastric ulcers, susceptibility to stress, parakeratosis, *osteochondritis dissecans* (MLV-rGH) while non transgenic littermates and non expressing MT-hGH and bGH show no pathological signs (see review, Pursel *et al.*, 1990a). Transgenic pigs with low levels of expression using prolactin/bGH transgene showed no associated health problems (Polge *et al.*, 1989). The reproductive capacity is reduced with anovulatory transgenic gilts and lack of libido in boars, however the semen is fertile when used for A.I. (see review, Pursel *et al.*, 1990a, 1990b).

Growth hormone secretion is normally episodic and not continuous as observed in transgenic pigs and this continous exposure could predispose the associated health problems. Robinson and Clarke, (1989) showed continuous infusion of growth hormone into rats produce no enhanced growth which could underlie the finding that transgenic pigs with high expression show no growth.

Surprisingly, the commercial situation indicates that between 10-30% of market weight pigs have gastric ulcers (O'Brien, 1986) and 90% have osteochrondrosis lesions (Reiland *et al.*, 1978; Carlson *et al.*, 1988) a primary cause of arthritis. However the frequency is increased in transgenic pigs.

3.3 Alternative Techniques for the Transfer of Genes.

Although pronuclear injection is anticipated to remain the principal route for the production of transgenic animals for the next five to ten years (Murray *et al.*, 1988) The technique has two major limitations, firstly, the integration frequency is low and little is known of the processes likely to

improve this rate (Palmiter and Brinster, 1986). The second limitation is the tendency for transgenes to integrate in tandem arrays, with up to several hundred copies being incorporated.

3.3.1 Retroviral Vectors

Both intact (Jaenisch, 1976; Jaenisch *et al.*, 1985; Jaenisch and Mintz, 1974; Robertson *et al.*, 1986) and genetically engineered retroviruses (Jahner *et al.*, 1985; Van der Putten *et al.*, 1985) infect the cells of mouse embryos resulting in the production of transgenics. Genes can be transferred into embryos either by injecting infectious virus into the blastocoele cavity or by bathing blastomeres on cultured cells shedding viral particles. Integration of gene sequences were highly efficient and genes were incorporated as single units rather than as tandem arrays (Stewart *et al.*, 1982). Retroviral vectors recently have been used to produce transgenic porcine and ovine fetuses (Petters *et al.*, 1989; Hettle *et al.*, 1989).

3.3.2 Sperm Mediated DNA Transfer

Recently presented data indicated that it might be possible to by-pass the microinjection procedure and simply absorb the transgene on sperm prior to IVF procedures (Lavitrano *et al.*, 1989). The frequency of integration is comparable to that of microinjection and if proven reliable in practice, would make transgenesis widely available. However, attempts at reproducing this observation by other laboratories have been disappointing (Barinaga, 1989; Brinster *et al.*, 1989).

3.3.3 Embryonic Stem Cells

The advent of mouse embryonic stem cell lines (Evans and Kaufman, 1981; Martin, 1981) and in particular the subsequent demonstration of their functional totipotency (Bradley *et al.*, 1984) offered enhanced new propects for the production of transgenic animals. Foreign DNA can be transferred into pluripotent cells by calcium phosphate precipitation (Wagner and Mintz,

1982) or by retroviral vectors (Evans *et al.*, 1985; Stewart *et al.*, 1985; Kuehn *et al.*, 1987). Chimeras are produced by combining a blastocyst and genetically engineered pluripotent cells, if however the embryonic stem cells contribute to the germ line, progeny are produced that contain the genetically engineered genome of the stem cell. The techniques have been established for the mouse (Robertson *et al.*, 1986,1987) and hamster (Doetschmann *et al.*, 1988) however, the challenge for developing embryonic stem cell lines for livestock and other laboratory species remains of interest and determining the specialized conditions required to avoid differentiation and loss of totipotency remains to be addressed (Seamark, 1989). Albeit, Notarianni *et al.*, (1990) has recently reported on the isolation and preliminary characterization of cell lines from pig and ovine embryos.

3.3.4 Liposome Carriers

Liposomes containing encapsulated DNA have been used to transform L-cells in culture (Schaefer-Ridder *et al.*, 1982). Germline transformation was unsuccessful with liposomes, but gene transfer into somatic cells of animals has occurred (Nicolau *et al.*, 1983; Soriano *et al.*, 1983).

3.3.5 Autonomous Replicating Vectors

Bacterial plasmid based vectors, containing the origin of replication and large T antigen of poloma virus, produced 100% transformation of mice following pronuclear injection. Transmission of the vector DNA to subsequent generations resulted in 80-100% of the progeny, but because of rearrangement no expression was recorded. Future gene transfer studies may be benefited by the use of autonomously replicating viruses, including the bovine papilloma virus (Gilbert and Cohen, 1987; Lacey *et al.*, 1986; Braam-Markson *et al.*, 1985).

3.4 Limitations to the Production of Transgenic Animals.

3.4.1 Identification of Transgenes

Land and Wilmut (1987) drew attention to the fact that so far biologist have been able to identify single genes with favourable effects for transgenesis. Future transgenic programs may need to consider selection of a range of regulatory sequences that will permit full control (Pursel *et al.*, 1990a, 1990b; Wieghart *et al.*, 1990)

3.4.2 Supply of Zygotes

The production of domestic animals is restricted in part by the cost associated with the procurement of a large viable source of embryos. This is further hampered by the low and variable response of donor animals to gonadotrophins. Additionally the technology of embryo transfer has been developed around the collection of embryos at much later stages of development which has resulted in very little information being available on the optimal timing for pronuclear injection. There are a few reports pertinent to the collection of embryos for DNA microinjection these include the loss of cumulus cells and timing of first division within 4 to 6 hours respectively some 19-24 hours after ovulation (Dziuk, 1965; Thompson and Wale, 1987) and the male pronucleus forms approximately 3 to 9 hours after ovulation (Dziuk, 1965).

3.4.2.1 Future Prospects for Zygote Availability

Recently the technology of *In Vitro* Maturation (IVM) provides the possibility of producing a large supply of low cost embryos. This technology involves collection of ooyctes by aspiration from 2-6 mm. follicles obtained from abattoir supplied ovaries. Certain *in vitro* conditions are then used to mature and fertilize the immature zygotes (Moor *et al.*, 1990; Gordon and Lu, 1990). The ability of IVM embryos to initiate normal pregnancy rates (60-70%) and acceptable twinning rates (50%) have been reported (Gordon,

1991). This technology may provide a cheaper alternative source for the supply of zygotes for microinjection, however further research is required before they become an acceptable source for microinjection (Gordon, 1991).

3.4.3 Visualization of Pronuclei

The cytoplasm of zygotes from farm animals is considerable denser and more opaque when compared to those of the mouse (Wagner *et al.*, 1984) which increases the difficulty of visualizing the pronuclei. Centrifugation (e.g 15,000 x g for three minutes) stratifies the cytoplasm of the zygote and results in pronuclei becoming discernible in the equatorial segment of the zygote. This method of visualization has been sucessfully applied to the pig (Wall *et al.*, 1985) and cow (Lohse *et al.*, 1985; Hammer *et al.*, 1986) with between 80-90% of pronuclei becoming visible. Centrifugation does not impair subsequent development of the zygotes *in vivo* (Wall *et al.*, 1985; Wall and Hawk, 1988). Other procedures used to increase visibility of pronuclei include staining with Hoechst (Minhas *et al.*, 1984; Pursel *et al.*, 1985) and treatment with cytochalasin-β neither of which influenced the viability of the zygote.

3.4.4 Viability after Microinjection

Microinjection significantly reduced the viability of zygotes of domestic animals (Hammer *et al.*, 1986). Rexroad and Wall (1987) showed only 18% of microinjected embryos developed to blastocyst. Walton *et al.*, (1987) showed that zygote viability was affected by pipette size, taper of bore and the time of injection relative to the first cell division. Pursel *et al.*, (1988) showed only 21.4 and 13.5% of 2 and 1-cell microinjected embryos survived *in vivo* to the blastocyst stage.

3.4.5 Culture of Microinjected Zygotes

The inability to asses zygote viability after microinjection contributes to increasing the cost of producing transgenic livestock (Walker, 1989).

Studies on the culture of porcine zygotes have been reviewed in Chapter 2.5. Hammer *et al.*, (1986) cultured microinjected zygotes *in vitro* for 12 hours and found 10 and 23% of sheep and pig zygotes respectively developed into blastocyst in recipient ewes. While 26% of uninjected sheep zygotes and 52% of centrifuged uninjected pig zygotes survived to blastocyst, indicating that optimum conditions for culture were not met. Intermediate hosts have been used to reduce the cost and/or assess the viability of microinjected zygotes (Biery *et al.*, 1988; Rexroad and Pursel, 1988). The development of a reliable *in vitro* culture system for microinjected zygotes is of a high priority (Kane, 1987; Murray *et al.*, 1988).

4.0 BIOLOGICAL FACTORS INFLUENCING THE PRODUCTION OF TRANSGENIC PIGS

4.1 Oestrous Synchronization

Techniques used for the synchronization of oestrus in pigs have been extensively reviewed by Paterson, 1982; Webel and Day, 1982; Polge, 1982. Briefly they include (1) treatment with crude and purified pituitary preparations, GnRH, PMSG alone or in combination with HCG have stimulated oestrous synchronization in prepubertal, lactating sows, weaned and anoestrous sows, (2) utilizing the post weaning oestrus in mature sows, (3) induction of luteolysis by prostaglandins in animals with an extended luteal phase either as result of pregnancy or treatment with oestrogen, (4) establishing an artificial luteal phase in animals by feeding an orally activated progestional agent for 17 days, (5) timed gonadotrophin treatment on day 17 with PMSG and day 20 with HCG during a monitored oestrous cycle. The following protocols have been examined but are not used because of their tendency to induce cystic follicles, decrease litter size, lack precise synchronization, decrease fertility or result in teratogenic activity. Treatment with Methallibure a non-steroidal compound, progesterone and synthetic progestagens other than altrenogest, inducing accessory corpora lutea with

gonadotrophins and shortening the life span of corpora lutea with oestrogens.

4.2 Superovulation

In 1935 Casida and then later Du Mesnil du Buisson (1954), Dziuk and Gehlback (1966), Baker and Coggins (1968) induced ovulation in prepubertal gilts with multiple injections of PMSG and purified pituitary preparation. PMSG is a glycoprotein consisting of two dissimilar sub-units which resemble pituitary follicle stimulating hormone (FSH) and luteinizing hormone (LH) (see review, Papkoff, 1981). Pituitary gonadotrophin preparation, PMSG, HCG and hypothalamic releasing hormones have been used singularly and in combination to induce follicular growth or ovulation in prepubertal gilts during luteal and follicle phase of oestrous cycle, anoestrous sows and gilts, lactating or early weaned sows following suppression of the oestrous cycle. (see reviews, Paterson, 1982; Esbenshade *et al.*, 1990). Typically, doses of 500-2,000 i.u. PMSG followed 48-96 hours later with 500 i.u. of HCG induce a follicle ovulation and pregnancy.

4.3 Precise timing of ovulation.

Ovulation timing can be precisely controlled by injecting HCG or GnRH 48-96hrs after PMSG (Dziuk and Baker, 1962). Though attempts to induce follicular stimulation, superovulation or oestrus with GnRH have been varied (Baker and Downey, 1973, Guthrie, 1977; Webel, 1978). Increases in ovulation and follicular development have been observed in the prepubertal gilts but were not accompanied by increases in embryonic survival or litter size (Archibong *et al.*, 1987) unless additional progestagens or gonadotrophin were given during pregnancy (Shaw *et al.*, 1971).

4.4 Embryo Collection and Transfer

Collection of porcine embryos from the reproductive tracts of the pigs was first established in the 1960 (Hancock and Hovell, 1962; Dziuk *et al.*, 1964; Vincent *et al.*, 1964). Repeated number of collections per animal inevitably leads to a build up of scar tissue/adhesions, though 3-4 operations performed have been successful (see review, Polge, 1982). Attempts to overcome this problem have include application of dextran or saline solutions with or without heparin (Cameron *et al.*, 1989).

4.4.1 Application of Embryo Collection and Transfer

The numerous research applications of embryo transfer technology have been reviewed by Polge, (1982). Research concerning early embryo development, embryo survival, implantation, maintenance of pregnancy, embryo and cellular manipulation are largely dependent on reliable collection, culture and subsequent transfer methodologies developed for embryos. Attempts at non surgical transfer in the pig via the cervix are conflicting with reported success by some (Sims and First, 1987) while others are not (Polge and Day, 1968). Commercially, the adaptation has been slower than in other domestic species due to higher fecundity and reproductive rates in the pig. Nevertheless, because of the inability to freeze porcine embryos, several studies (Baker and Dziuk, 1970; James *et al.*, 1980, 1983; Niemann *et al.*, 1989) have demonstrated disease control and transfer of porcine embryos between countries. Experimentally, embryo transfer is an integral part of new technologies involving embryo manipulation and genetic engineering.

4.5 Embryo Transfer

The surgical procedure for recipients is similar to the one used in donors. Transfers are made to the uterus or oviduct depending on the stage of embryo development. Dziuk *et al.*, (1964) showed that it was only

necessary to transfer to one side of the reproductive tract since migration and embryo spacing occurs throughout the whole reproductive tract. Pregnancy rates of between 45 and 86% have been achieved from embryos collected from donors 2-5 days after onset of oestrus (Cameron *et al.*, 1989; Dziuk *et al.*, 1964; Vincent *et al.*, 1964; Niemann *et al.*, 1989).

4.5.1 Synchrony of Donors and Recipients

Pregnancy rate and embryonic survival are reduced if the stage of the reproductive cycle of the recipient is more than about one day forward of phase with that of the donor (see review Polge 1982). Polge (1982) showed a pregnancy rate up to 86% when embryos were transferred to a recipient either synchronous or one to two days behind the onset of oestrus in the donor. This confirms observations in other species where deviation in favour of transferring advanced embryos into 'younger' uteri is more beneficial than the reverse situation (Polge, 1982). Broermann *et al.*, (1990) showed no difference between survival rates or developmental morphology of recovered embryos when transferred to the oviduct or uterus on day 4. However day 6 embryos transferred to the oviduct showed a reduction in embryo survival. Embryonic survival was not reduced when 2-cell embryos collected from the oviduct were transplanted to the uterus of recipients at the same stage of the reproductive cycle (see review, Polge, 1982). Optimally at least 18 embryos are required per transfer (Cameron *et al.*, 1989) and at least four embryos are necessary for consistent maintenance of luteal function during early pregnancy (Polge *et al.*, 1966,1967).

4.6 Early Embryonic Development and Mortality

Early embryonic mortality accounts for approximately 30% of embryo loss in the pig during the first 25 days of gestation (Hanly, 1961). Most prenatal mortality in swine occurs before attachment of the embryos in the uterus on day 19 of gestation (Perry and Rowlands, 1962) and is evident by day 15 and increases after day 30 (Archibong *et al.*, 1987). During the pre-

attachment period the embryo must rely on histroph production by the uterus for survival (Bazer *et al.*, 1982; Bazer and Roberts, 1983). Ovarian steroids play a supporting role in regulating uterine secretions prior to embryo attachment (Maurai *et al.*, 1981; Geisert *et al.*, 1982a, 1982b).

Major factors in limiting production in swine herds is the reduced reproductive performance of pubertal gilts. Ovulations are fewer when compared with their multioestrus counterparts (Robertson *et al.*, 1951; Andersson and Einersson, 1980; Knott *et al.*, 1984; Archibong *et al.*, 1987) and, if mated, sustain greater embryonic losses (Warnick *et al.*, 1951, MacPherson *et al.*, 1977). This is not reflected in fertilization rate which do not differ significantly from prepubertal and postpubertal gilts (98 vs 100% Archibong *et al.*, 1987; 100 vs 93% Robertson *et al.*, 1951; 100 vs 96% Perry and Rowlands, 1962). Dziuk, (1969) and Rampacek *et al.*, (1975) showed that the uterus of the gilt can accommodate approximately twice as many fetuses as are normally present. Measurements of progesterone and oestrogen levels in the gilts indicated the possible reason for the discrepancy where oestrogen levels increased significantly only after day 30 while progesterone levels showed no difference between the same period when comparing first and third oestrous gilts (Tillson *et al.*, 1970; Guthrie *et al.*, 1972; Magness and Ford, 1983; Archibong *et al.*, 1987). Increased embryo losses may be attributed to inadequacies in the concentration of progesterone to stimulate the gravid uterus or to provide an initial priming concentration of progesterone prior to prepubertal oestrus (Esbenshade *et al.*, 1982). Moore (1985) demonstrated progesterone primed oestrus in ovarectomized recipients and showed increases in embryo survival after transfer. The ratios of progesterone to estrogen between first and third oestrous gilts were significantly different from the first 30 days of pregnancy (Archibong *et al.*, 1987). Therefore deviation of the ratio may decrease embryo viability as uterine secretion activity may be out of synchrony with stage of development in the embryo. Porcine embryos increase oestrogen concentration after day 11 and 12 (Perry *et al.*, 1976; Bazer *et al.*, 1982) which promotes migration

and spacing of embryos throughout the uterus (Pope *et al.*, 1981) and modulate the effect of uterine secretions (Geisert *et al.*, 1982 a,b). Therefore decreases of oestrogen after day 11 could effect embryo spacing and histroph production.

4.7 Culture and Preservation of Embryos.

Experimentation involving the collection of embryos for genetic manipulation requires a capcity to store embryos for both short and long term periods.

4.7.1 Short Term Storage

Embryo Transfer programs require media for short term storage. These simple media are also used extensively for the flushing of embryos from the reproductive tracts. Routinely, phosphate buffered saline medium (Whittingham, 1971) is used as it does not effect the viability of embryos collected up to seven days post oestrus (Polge, 1982). However, embryo viability from early stages up to the hatched blastocyst decreases with extended culture in phosphate buffered saline (Davis, 1985).

4.7.2 Long Term Storage

Long term preservation of embryos requires specialised media to support normal development *in vitro*. Wright and Bondioli (1981) published a comprehensive review on the variety and success of media used in the culture of pig embryos. All authors reported on the difficulty of culturing 1 and 2 cell embryos past the four cell stage. However, others studies have consistently demonstrated that 4-cell embryos can be cultured to the blastocyst stage in a simple salt solution containing bovine serum albumin (BSA) (Davis, 1985).

4.7.2.1 Energy Source

Davis and Day (1978) found that embryos cultured in medium without lactate and pyruvate formed blastocyst more readily than when they were added. Pyruvate alone inhibited development when BSA was used in conjunction with the inorganic salt solution.

4.7.2.2 Atmospheric Gases.

Wright (1977) used a wide variety of culture media and gaseous atmospheres under incubation conditions described by Brinster (1963). Optimal porcine blastocyst formation occurred in Whitten's medium with 1.5% BSA under a reduced oxygen atmosphere of 5% CO_2, 5% O_2, 90% N_2.

4.7.3 Co-Culture of Porcine Embryos

Complete and defined media may not contain the essential criteria needed to reproduce the *in vivo* tubal or uterine environment (Davis and Day, 1978; Davis, 1985; Bavister, 1987, 1988). Several species including outbred strains of mice (Whittingham and Biggers, 1967), hamster (Whittingham and Bavister, 1974), cow (Thibault, 1966), pig (Polge, 1982; Davis, 1985) and sheep (Gandolfi and Moor, 1987) have demonstrated developmental blocks in early stage embryos cultured *in vitro* on complete and defined media. The oviductal changes its secretory processes, fluid volume, and concentration of ions and organic constituents concomitant with the secretory patterns of ovarian steroids (Hammer, 1973; Mastroianni *et al.*, 1969; Sjöberg *et al.*, 1986). The synchrony between the hormonally regulated and embryo stimulated maternal environment and developmental age of the embryo is important for successful completion of pregnancy (Chang, 1950; Pope *et al.*, 1982). Biggers *et al.*, (1961), Gwatkin and Biggers (1963) and Whittingham, (1968) reported enhanced development of early stage embryos cultured in the presence of another cell type to the blastocyst stage. Subsequently the development of embryos of domestic species as examined in the presence of a

variety of cell types including oviduct epithelium (Eyestone *et al.*, 1987; Gandolfi and Moor, 1987; Rexroad and Powell, 1988a, 1988b; Sakkas *et al.*, 1988; Eyestone and First, 1989), trophoblastic vesicles (Heyman *et al.*, 1987; Clarkson and Nancarrow, 1988; Rexroad and Powell, 1988a), fibroblasts (Kuzan and Wright, 1981, 1982a; Gandolfi and Moor, 1987), endometrial cells (Allen and Wright, 1984; Voekle *et al.*, 1985; Rexroad and Powell, 1988b) and kidney cell monolayers (Rexroad and Powell, 1988b). Whittingham *et al.*, (1968) demonstrated that mouse oviducts explanted at metaoestrus will support development of more zygotes into blastocysts than their diestrous counterparts. Currently, data indicates that co-culture with foetal fibroblast or oviductal monolayer appears to contribute to an increase in embryonic development in ovine, porcine and bovine preimplantation embryos (Rexroad and Powell, 1988; White *et al.*, 1989). Kuzan and Wright, (1981) using porcine fibroblast on monolayers, showed that cell contact was not vital for development. Gandolfi and Moor (1987) indicated the importance of the support by both the type of feeder layer as well as the developmental state of the embryo. Oviductal feeder layers and fibroblast maintain equal embryo cleavage for the first three days. Thereafter, oviductal feeder layers maintain cleavage of embryos while viability of embryos cultured in the presence of fibroblast declined; 33% of embryos co-cultured in the presence of oviductal cell developed to the expanded blastocyst and 80% of these further survived *in vivo* compared with 5% and 46% co-cultured in fibroblast.

Porcine embryos have developed from the one and two cell stage to blastocysts (Kuzan and Wright, 1981; Allen and Wright, 1984; Wright and O'Fallon, 1987; White *et al.*, 1989). Blastocyst formation of around 70% have been observed with porcine embryos cultured in porcine oviductal epithelial cells (POEC) and combined POEC and porcine foetal fibroblast monolayer (PEF), compared with 30% and 16% with PEF on its own and DMEM respectively (White *et al.*, 1989). Uncertainties have remained over the beneficial effects of secretory proteins and the use of conditioned media. Archibong *et al.*, (1989) indicated the addition of oviductal fluid media to

culture media improved *in vitro* development of porcine embryos from 1-cell to blastocyst (No. of Blastocysts 64-83%). Enhanced development in co-culture with oviductal cells has also been reported with embryos of sheep (Gandolfi and Moor, 1987), cow (Kuzan and Wright, 1982a,; Camous *et al.*, 1984; Heyman and Menezo, 1987; Eyestone and First, 1989) and goat (Sakkas *et al.*, 1988).

5.0 GROWTH HORMONE AND REGULATION OF GROWTH

The growth of vertebrates as defined by the accretion of body weight which results from component tissue hypertrophy is regulated by a complex process involving the interplay of a number of circulating peptide hormones. The cascade of hormones involves growth hormone releasing factor (GRF); the anterior pituitary peptide, growth hormone (GH) and the liver and peripheral tissue proteins, insulin-like growth factor (IGF-I) or somatomedin-C. Ultimately, this interplay of hormones is influenced by the prevailing nutritional status of the animal as well as the changing responsiveness of tissues to these hormones.

Growth hormone is synthesized by the somatotroph cells of the anterior pituitary (Daughaday, 1981). Production and secretion of GH is regulated primarily by two polypeptides: one stimulatory (growth hormone releasing factor, GRF; Rivier *et al.*, 1982) and one inhibitory (somatostatin; Brazeau *et al.*, 1973) which are released from the hypothalamus via the hypothalamic-hypophysial portal blood system in response to neurotransmitters (see review, Frohman and Jansson, 1986). Endogenous GH is released from the pituitary in pulses in spikes that are brief in duration, low in amplitude and variable in frequency before transport to a wide variety of target tissues via the circulatory system (Schalch and Reichlin, 1966; Takahashi *et al.*, 1981; Owens *et al.*, 1991). Chapman *et al.*, (1991) estimated the half life of endogenous growth hormone in rats was approximately 3.3 minutes. The rapid disappearance suggest that the end of spontaneous GH bursts is marked by sudden cessation of GH release and

may provide an indication of the rapid rate of change in the levels of the underlying hypothalamic hormones which control GH release. In the pig GH changes with age with a 50% increase occurring between 10 and 20 kgs liveweight (Owens *et al.*, 1991). Amongst its actions include the stimulation of longitudinal bone growth (Cheek and Hill, 1974) and of metabolic actions on the utilization of protein, lipids and carbohydrates (Kostyo and Nutting, 1974; Goodman and Schwartz, 1974; Altszuler, 1974).

The growth of animals is influenced by a variety of other hormones some of which are inhibitory while others act synergistically (see reviews, Daughaday *et al.*, 1975; Muir, 1985).

5.1 Structure and Action of Growth Hormone

The growth of animals is under a complex of genetic and hormonal controls, included in this cascade is neuro peptides, somatostatin and growth releasing factor (GRF) which inhibit or stimulate the somatotrophs cells in the anterior pituitary which synthesize growth hormone (GH). Growth hormone binds to receptors in the liver where it stimulates the production of insulin-like growth factors (IGF, somatomedin C) which are thought to stimulate cellular proliferation.

Growth hormone belongs to a family of related protein hormones which include prolactin and placental lactogen (see review, Wallis, 1981). Its structure comprise a single polypeptide chain of approximately 190 amino acids with two inter chain disulphide bridges (Niall *et al.*, 1973). It synthesis and manufacture from the precursor state is described by Sussman *et al.*, (1976); Seeburg *et al.*, (1977).

Growth hormone has been purified and its amino acid sequence for a number of species, including human, bovine, ovine, porcine and equine (see review, Dayhoff, 1972). In addition, the primary structures of growth hormone from rat (Seeburg *et al.*, 1977), mouse (Linzer and Talamantes, 1985), chicken (Souza *et al.*, 1984) and salmon (Sekine *et al.*, 1985) have been deduced from the nucleotide sequences of cDNA clones.

Multiple forms of GH resulting from post-translational modifications have been detected and the many variants and activities have been reviewed by Lewis, (1984).

The importance of the pituitary and its regulation of growth was first demonstrated by Evans and Long (1921) where giants rats were produced from intrapertioneal injections of anterior pituitary extracts. This was later confirmed when the inhibited growth rate of hypophysectomy rats was reversed with treatment of crude preparations of GH (Smith, 1927). Failure of animals to produce physiological responses to different species GH indicated the concept of species specificity (see review, Friesen, 1980) this concept was later confirmed with GH receptor studies (Carr and Friesen, 1976; Lesniak *et al.*, 1977). It is general accepted that GH regulates metabolism by influencing protein, carbohydrate and lipid metabolism (Kostyo and Nutting, 1974; Altszuler, 1974; Goodman and Schwartz, 1974). In tissue culture, GH has enhanced protein synthesis by directly effecting amino acid transport, ribosome numbers, mRNA and enzymatic apparatus involved in protein synthesis (Kostyo and Isaksson, 1977; Daughaday, 1981).

Purified human GH isolated in 1956 (Li and Papkoff, 1956; Raben, 1956) was first used successful to treat hypopituitary dwarfism (Raben, 1958) and its importance was later corroborated by serum levels of patients with growth disorders; acromegaly and dwarfism were found to be due to elevated and deficient GH secretion respectively (see review, Franchimont and Burger, 1975).

In recent years, with the advent of recombinant DNA technology, GH's have become more available in a recombinant form from many species. The recombinantly derived hormones were shown to be equally efficient with respect to growth stimulation when compared to pituitary derived hormone (Olson *et al.*, 1981; Hintz *et al.*, 1982; Kaplan *et al.*, 1986).

The effects of GH on cell and somatic growth have been studied extensively (see review, Cheek and Hill, 1974). The growth is dependant on increases in cell numbers and or size of the individual cells.

Despite the considerable body of information gained on the physiological effects of GH in animals (see review, Smith *et al.*, 1955) the mechanism of the hormone stimulation of growth is still not well understood.

Early observations of GH inability to stimulate anabolic processes in cartilage from hypophysectomized rats, however serum from untreated hypophysectomized rats restores the anabolic processes, led to formation of the somatomedin hypothesis (Salmon and Daughaday, 1957) where GH stimulates somatomedins (intermediate hormones) from the liver (Daughaday *et al.*, 1972). Several somatomedins have been purified and one of the family, Somatomedin-C or insulin-like growth factor-1 (Klapper *et al.*, 1983) is thought at least in part, to mediate the stimulatory effects of GH on postnatal somatic growth and cellular proliferation (Herington *et al.*, 1983).

The correlation between IGF-I and GH was demonstrated by measuring IGF-I levels in the plasma of patients with growth hormone disorders; acromegalics high IGF-I and GH deficient, low IGF-I (Bala and Bhaumick, 1979). Administration of GH to pituitary dwarfs resulted in an increase in circulating IGF-I as well as hypophysectomized animals (Copeland *et al.*, 1980; Kaufmann *et al.*, 1978). IGF-I and IGF-II increase between 10 and 35 kgs correlate with IGF-BP activity with IGF-BP's controlling the IGF-I and IGF-II levels in blood by mediating their rate of clearance from the circulating (Owens *et al.*, 1991). The normal secreted endogenous GH exerts no significant immediate control over plasma IGF-I and IGF-II (Owens *et al.*, 1991).

The growth promoting effects of IGF-I on cultured cells *in vitro* has been reviewed by Froesch *et al.*, (1985). The infusion of pure IGF-I in hypohysectomized rats caused dose dependant stimulation of body weight, tibial epiphysial width and thymine incorporation into costal cartilage (Schoenle *et al.*, 1982) and then later recombinantly derived IGF-I demonstrated growth promoting properties in normal rats (Hizuka *et al.*, 1986).

Despite the evidence there a a number of reports which dispute the somatomedin hypothesis. A summary of these findings is giving below:-

(1) GH at site of epiphysial growth plate of the tibia stimulated local bone growth (Isaksson *et al.*, 1982; Russel and Spencer, 1985; Isgaard *et al.*, 1986), (2) specific GH binding sites on rabbit chrondocytes and epiphysial growth plates (Eden *et al.*, 1983), (3) production of IGF-I in a number of different cell and tissue types (Clemmons *et al.*, 1981; D'Ercole *et al.*, 1984) and (4) that GH-treatment *in vitro* generates IGF-I sensitive predispose 3T3 cells (Zezulak and Green, 1986) which was later confirmed by Lindahl et al. (1987) using hypophysectomized rats showed GH promoted formation of chondrocyte colonies and makes chrondocytes susceptible to IGF-I.

5.2 Organization and Regulation of Growth Hormone Genes

The identification and cloning of GH gene from different species using cDNA sequences reveals a high degree of homology and structural similarities. All genes encode for the pre-hormone of approximately 216 amino acids which contained the signal peptide and mature GH protein of 188-191 amino acids. Characterization of the genomic genes isolated from rat (Barta *et al.*, 1981; Page *et al.*, 1981), human (DeNoto *et al.*, 1981) and cow (Woychik *et al.*, 1982) contain four introns of similar length and position. Sequence analysis of genomic clones of human GH gene has shown the existence of at least five GH related genes (Seeburg, 1982; Barsh *et al.*, 1983) Three of genes are for human placental lactogens and the remaining two are for growth hormone, one of which is the normal 190 amino acid sequence (Niall, 1971) and the other a variant gene (hGH-V) (Seeburg, 1982). The two growth hormones differ at multiple sites and there is no evidence for *in vivo* expression of the variant (Seeburg, 1982) though it can be expressed in an SV-40 vector system (Pavlakis et al. 1981). In the case of the rat (Page *et al.*, 1981) and cow (Woychik *et al.*, 1982) growth hormone genes contain only one gene per haploid genome.

Expression is regulated in a tissue specific manner and is limited to the somatotrophs of the pituitary (Daughaday, 1981). Transcription is stimulated by GRF (Barinaga *et al.*, 1983) and a number of factors including thyroid hormone (Spindler *et al.*, 1982) and synthetic glucocorticoid-dexamethasone (Yu *et al.*, 1977; Spindler *et al.*, 1982).

5.3 Alteration of Growth by Exogenous Growth Hormone Treatment

The endocrine system plays an important part in regulating animal growth and in the particular the partitioning of nutrients between muscle and adipose tissue (Etherton, 1982; Etherton and Kensinger, 1984). Nutrient partitioning is defined on the basis of the tissues most crucial for the survival of the species (Steele and Elsasser, 1989). The priority of a tissue positively correlates with the metabolic rate of the tissue (Steele and Elsasser, 1989). The physiological state can be influenced by genetic and environmental factors for example, parturition in the cow indicates priority is shifted towards the utilization of nutrients for increased production of milk (Peel *et al.*, 1981,1982). The effects of exogenous growth hormone on animal growth have been known 60 years. Pituitary derived dwarfs were treated with human GH (Raben, 1958) and the procedure is now applied routinely in treating growth hormone deficiency in man (see review, Frasier, 1983). Due to the scarcity of the purified GH, studies of the effect of exogenous GH on farm animals were carried out only recently. Machlin (1972) reported that daily injections of porcine growth hormone significantly improved growth performance in young pigs by increasing daily weight gain and feed efficiency with out effecting palatibility (Evock *et al.*, 1988). This effect has also been observed sheep (Johnson *et al.*, 1985), cows (Bauman *et al.*, 1985) and fish (scc review, Cloud, 1990).

Studies from the pig indicate that treatment with either pituitary derived porcine growth hormone (ppGH) and recombinantly derived porcine (rpGH) results in increased growth rates, feed conversion efficiency, free fatty acids, glucose, insulin and decreased adipose tissue and blood urea nitrogen

with no adverse effects (Chung *et al.*, 1984; Etherton *et al.*, 1986, 1987; Boyd *et al.*, 1986; McLaren *et al.*, 1987). There is also additional body water as a result of increased protein and decreased adipose tissue (Campbell *et al.*, 1989) which results in more ash per kilogram (Campbell *et al.*, 1988). Recombinantly derived GH is equipotent to ppGH in there growth stimulating capacity with rpGH mimicking the binding to pig liver membranes (Evock *et al.*, 1988), additionally no antibodies to rpGH have been found (Etherton *et al.*, 1987).

A dose relationship exists in administration of GH and IGF-I response in a linear fashion (Evock *et al.*, 1988; Boyd *et al.*, 1986). Large dose of GH decrease pig motility as a result of osteochrondrosis. Therefore there is a need to determine the maximal doses of GH that regulate its growth promoting effects (Etherton *et al.*, 1987; Daughaday, 1983; Froesch, 1983). Growing pigs responsiveness to pGH Administration increase with liveweight (Chung *et al.*, 1984; Etherton *et al.*, 1986) and the response is modified by factors such as sex, genotype, pGH dosage and source (ppGh, rpGH) and protein amino acid intake (Campbell *et al.*, 1988a). Additionally the stimulating effects of pGH on growth rate are sustained following cessation of hormone treatment with a larger response occurring in females than males (Campbell *et al.*, 1988b). Changes in growth by pGH are mediated at least in part via stimulation of muscle protein deposition and the possibility exists that the magnitude of the pGH-response depends on the pigs intrinsic capacity for protein growth. As indicated by Campbell *et al.*, (1988a) and Etherton *et al.*, (1982) where barrows and gilts responded more to GH treatment than did intact males.

Analysis of lung, thyroid, adrenal, liver, kidney, spleen, myocardium, skeletal muscle and intestine tissues after chronic treatment with GH at a maximal efficient dose indicated no deleterious affects upon animals (Machlin, 1972). Further analysis indicated the pituitary function and rate was not affected but pituitary growth hormone rate content decreased (μg/gland and concentration/gland). The size of the somatotroph did not

differ but there was significantly fewer of them after GH treatment (Machlin, 1972).

5.3.1 Physiological Response to GH Treatment

GH treatment induced hyperglycaemia with increases of glucose and insulin occurring in feed and fasted states as a result of an increase in hepatic glucose output and a concurrent impairment in glucose clearance (Gopinath and Etherton, 1989a, 1989b).

5.3.2 Energy Intake and GH Interaction

Investigation into raising the levels of energy intake indicate that increase in growth rate and protein and fat accretion occur with no effect on feed conversion ratio (FCR). Administration of GH results in the same increases including enhanced FCR with changes occurring faster in higher energy intake pigs. This indicates effects of pGH on growth performance and energy and protein metabolism were largely independent, but additive to the effects of energy intake (Campbell *et al.*, 1988a).

5.3.3 Mineral Status in GH Treatment

Children with increased concentrations of GH exhibited significantly lower Cu and Zn status when compared to normal children. Likewise children with low GH showed significantly higher levels of plasma Cu and Zn (Henken, 1976; Aihara *et al.*, 1985). However, when diet was adequate to meet the demands for increased growth (Kraft *et al.*, 1986; Wolfrom *et al.*, 1986) Zn, Cu and Fe levels were similar or only marginally different in GH treated and control pigs (Caperna *et al.*, 1989). Serum Fe levels were lower in GH treated pigs and serum Cu was more of a function of increase feed restriction and not of pGH treatment. In contrast Zn status may influence growth and plasma levels of GH and somatomedins (Cossak, 1984; Oner *et al.*, 1984; Bolze *et al.*, 1987) as in observed in the sheep (Braithwaite, 1975). Davis *et al.*, (1987) showed IGF-I alters the interaction between transferrin,

its receptor and its subsequent internalization into cultured cells therefore the *in vivo* Fe utilization and mobilization may be altered by GH treatment. The effects of mineral status after GH treatment in pigs does conflict with human studies and a reason for the difference is unknown (Henken, 1976; Aihara *et al.*, 1985).

5.3.4 GH Treatment and IGF Mediation

IGF-I is the major mediator of the somatogenic actions of GH (Froesch *et al.*, 1983; Sillence and Etherton, 1987). IGF-I and IGF Binding proteins increases in plasma of growing pigs administered with GH (Etherton, 1987; Owens, 1989). The maximum effective dose of GH induces a relatively small increase in IGF-I which neither provides a sensitive marker nor represents a rate limiting step to the anabolic effects achievable in pigs treated chronically with pGH (Sillence and Etherton, 1987). The independent actions of GH on protein and lipid metabolism is mediated via IGF-I the release and (or) synthesis of which appears to be inhibited by dietary protein (amino acid) deficiency in particular lysine (Campbell *et al.*, 1989). Glucocorticoids hormones are potential counter regulation of GH effects and increase after injection of GH for 2 hours and then return to normal (Sillence and Etherton, 1987; Hall and Sara, 1983; Froesch *et al.*, 1985; Unterman and Phillips, 1985).

Conflicting reports on the actions of IGF-I in its mediating role (see Review Sara and Hall, 1990). In a study using lambs injected with bGH there was a significant bGH/nutrition interaction for IGF-I but there was no such interaction for body weight/components or specific GH binding to the liver (Bass *et al.*, 1991). This indicates that an increase in IGF-I does not necessarily result in increases in growth or carcass composition.

5.3.5 Reproductive Performance and GH Interaction

Growth hormone may act with other peptide growth factors present in uterine lumen fluid through autocrine or paracrine mechanism, to mediate

uterine and conceptus growth and differentiation (Letcher *et al.*, 1989). Bryan *et al.*, (1988) showed GH treatment impaired ovarian development of prepubertal gilts and predisposed a lower incidence of oestrus. The lower incidence of puberty in GH treated pigs may be explained by an *in vitro* study where there is a reduced response of granulosa cells to gonadotrophins (Letcher *et al.*, 1989). Machlin, (1972) showed uterine oedema with chronic injections of GH but determinations were based on a very small sample size. Chronic injections of GH in rats delays puberty (Groesbeck *et al.*, 1987) though dose used were 1000 times higher on a body weight comparison. In the pig, Kverages *et al.*, (1986) showed GH treatment in late pregnancy did not effect the number of young born alive or dead or their subsequent survival to weaning. Instead GH treatment in the lactating sow enhanced the survival of young pigs due to increases in the concentrations of blood glucose, body lipids and glycogen. The improved traits were influenced by the quality of the diet. In the cow, GH treatment increased milk production as a result of nutrient partitioning (Peel *et al.*, 1981,1983; Bauman *et al.*, 1982) but this was not observed in the pig (Kverages *et al.*, 1986).

6.0 SUMMARY

The period following fertilization when sperm chromatin loses protamine regulated structure and acquires a maternally determined histone structure provides, fortuitously, an opportunity when foreign genes can be microinjected into the genome and remain transcriptionally active. Whilst there is much still to be discovered about the factors which set the low efficiency of present gene transfer procedures and the genetic and other determinants that result in most genes integrating at a single random site within the genome, the technology is now being widely exploited for developing novel animal breeds for research and commercial interest.

Techniques are available which allow access to the single cell zygotes required for microinjection in most of the livestock species of agricultural importance, but there is still room for considerable improvement. A primary

constraint is the availability of donor and recipient animals to provide suitable embryonic material. For transgenic pig programs, the young gilt is the most readily available animal resource for this purpose. However, compared with the sow, the overall fertility status of the gilt is compromised and in order to utilize this resource, special protocols need to be developed to achieve the required efficiency and flexibility in the collection of viable zygotes for microinjection.

Most transgenic livestock programs have so far been focussed on transgenes incorporating growth hormone sequences, with the ambition of producing breeding stock with inheritable improved production characteristics these sequences would confer. Initial results have been disappointing due to health and other complications related to uncontrolable transgene expression, and the development of regulatory sequences which permit full control of transgene expression is now recognised as an essential primary step to realizing the full potential of gene transfer in animals.

The production of transgenic pigs (or other livestock) is an extensive undertaking, requiring a multidisciplinary approach, large animal resources and back up capital. The ultimate success of such enterprises will be largely dependent on achieving the highest level of efficiency and quality control in all aspects of the program. This thesis aims to address some of the problems challenging the animal biologist, participating in a transgenic pig program targeted at developing breeding lines of transgenic pigs for commercial use.

CHAPTER II

GENERAL METHODS

2 Animal Maintenance and Embryo Collection

2.1 *Suis domesticus* (Pig)

Large-White, Landrace and Large-White/Landrace cross sows and boars were provided by Northfield Pig Research Unit, Department of Agriculture, S.A., Commercial Pig Company, Bendigo, Vic. and Metro Farms Piggery, Wasleys, S.A, respectively. All animals were maintained under commercial housing and feeding conditions. Rations were formulated appropriate to age (see Appendix I). Animals used in all trials were vaccinated against parvovirus (Porcine Parvac, Commonwealth Serum Laboratories, Australia). Antibody titres were measured in blood samples by VetLab, Dept. of Agriculture.

2.2 Hormonal Stimulation and Mating

Donor and Recipient pigs were selected from a variety of programmes, specific details of which are given in the appropriate chapters. Superovulation and synchronization in general were induced by an intramuscular injection of PMSG (Pregnecol, Heriot AgVet Pty. Ltd., Australia) in a dose range of between 500 i.u. and 1500 i.u. Recipients received 500 i.u. of PMSG to induce synchronization whilst avoiding superovulation. The time of ovulation was controlled by an intramuscular injection of 500 i.u. HCG (Chorulon, Intervet, Holland) 44 to 72 hours after PMSG. The donor sows displaying standing oestrus were mated naturally or by artificial insemination on the evening of the next day. Recipients sows were checked for standing oestrus in the presence of boars but were not mated.

2.3 Embryo Collection and Culture

Anaesthesia was induced in donors sows by an intravenous infusion via a peripheral ear vein of 5% (v/v) solution of sodium thiopentone (Pentothal, Abbott Australia Pty. Ltd., Sydney, Australia) at a rate of one gram of anaesthetic per 90 kg body weight. Animals were maintained under anaesthesia with Halothane (Fluothane, ICI, Australia) administered through a rubber face mask. Reproductive tracts were then exteriorized by mid-ventral laparotomy and the preimplantation embryos were flushed from the appropriate region of the reproductive tract using Dulbecco's phosphate buffered saline supplemented with 1% (v/v) Heat Inactivated Fetal Calf Serum (HIFCS) maintained at 39^{o}C. Collected embryos were washed twice in HEPES-MEM and incubated for a period of no more than 5 hours. Following anaesthesia animals were treated with an intramuscular antibiotic injection (Terramycin L/A, Pfizer Agricare Pty. Ltd., Australia).

2.4 Culture Media

2.4.1 Composition of HEPES-MEM medium

HEPES-MEM medium used for transport and microinjection was prepared from powdered Minimal Essential Medium (Eagle, with Earle's salts, with L-glutamine, GIBCO, N.Y., USA) dissolved in freshly purified water. Additional supplements included 21mM HEPES, 4mM sodium bicarbonate, BSA (5mg/ml) and penicillin (100 i.u./ml). The pH and osmolarity of the prepared medium was adjusted to 7.4 and 280 mOsm before filter sterilisation (Millipore, 0.22 μm).

2.4.2 Long Term Embryo Culture

Porcine embryos requiring longer term culture were transferred to culture medium, the composition of which will be given in the appropriate chapter.

2.6 Culture System

All embryos were cultured in microdrops of media (10ml) overlaid with approximately 2.5 ml of light paraffin oil (BDH) in 35mm petri dishes. These dishes were placed inside a humidified chamber (95%) with a constant flow of prewarmed, humidified special gas mixture (5% O_2, 5% CO_2, 90% N_2). This chamber was placed inside an incubator at a temperature between 37°C (murine) and 39°C (porcine) with 95% humidity and a gas atmosphere of 5% CO_2 in air.

2.6.1 Serum

2.6.1.1 Supply

Human serum was collected from laboratory staff. Fetal Calf serum was obtained from Commonwealth Serum Laboratories (Australia). Blood was refrigerated overnight and serum separated by centrifugation at 2500xg for 20 minutes followed by heat inactivation at 56°C for 15 minutes. Aliquots of HIHS and HIFCS were stored at -20°C until required.

2.7 Embryo Transfer

Recipients underwent the same surgical procedures as donors except no antibiotic cover was given unless complications arose after surgery. Embryos were transferred surgically as described in chapter 2.3 to the appropriate region of the reproductive tract using a 1 ml syringe attached to a tom cat catheter ($3^1/_2$ Fr., $4^1/_2$ in., Sovereign, Sherwood Medical, USA) filled with a minimal amount of HEPES-MEM.

2.8 Assessment of Pregnancy rate

Recipients were checked for pregnancy by non return to oestrus (Dyck, 1982) between days 19-22 and by oestrone sulphate levels at concentrations above 1.9 nM in a blood sample collected by ear vein puncture on days 22 to 29 (Stone *et al.*, 1986; Robertson and King, 1974; Horne *et al.*, 1983). Pregnancy was again confirmed at day 60 and in the last week of pregnancy by Doppler or real time ultrasound (Fraser and Robertson, 1967; Too *et al.*, 1974).

2.9 Nuclei Staining

Nuclei identification counting was determined using the DNA specific dye Hoechst 33342 (Sigma, USA). The procedure for use on embryos was basically as described by Pursel et al (1985).

Dye solution was prepared to a concentration of 1 mg/ml in freshly purified water and stored away from direct light at room temperature. Dye maintained activity for up to six months as such. Embryos or material to be stained were placed a solution of the same media or PBS with dye stock added to a final concentration of 5 µg/µl. Embryos were incubated in this solution for 5 minutes before transfer to fresh medium for a 30 seconds followed by a further treatment of a 5 minute wash in fresh medium and mounted under a coverslip with or without paraffin waxed edges depending on the degree of cell flattening desired. Fluorescence microscopy was performed with minimum exposure as the dye faded noticeably within several minutes of UV exposure.

2.10 Construction of Transgenes

Human metallothionein IIA promoter/porcine growth hormone fusion genes ($pHMPG_{.03}$, $pHMPG_{.03A}$, $pHMPG_{.04}$, $pHMPG_{.05}$, $pHMPG_{.07}$,

$pHMPG_{.08}$)(Vize *et al.*, 1988; pers comm J.R.E. Wells; pers. comm. A. Robins) and various other transgenes pUC-19, SB-2 (pers. comm. C.S. Bawden), Gc-3a (pers. comm. G. Cam), Bp-2 (Powell, B.C. and Rogers G.E., 1990), Bp-Ms (pers. comm. B.C. Powell, Adelaide University Centre for Gene Technology) and Bp-ML(pers comm. B.C. Powell, Adelaide University Centre for Gene Technology) were provided by Dept. of Biochemistry, University of Adelaide. The organization of the most used transgene pHMPG is shown in its plasmid form in Figure 2.1 (Vize *et al.*, 1988).

2.10.1 Basal Level Deletion Derivatives ($pHM_{\Delta}PG_{*}$)

The molecular structure of the hMT-IIA promoter and localization of specific controlling elements have been described by Karin *et al.*, (1984). To obtain a derivative of the element which could induced by a predetermined concentration of a heavy metal, such as zinc, several constructs were derived by removing selected nucleotides of the basal level sequence. This process was achieved by oligonucleotide directed mutagensis and checked by nucleotide sequencing (Vize *et al.*, 1987).

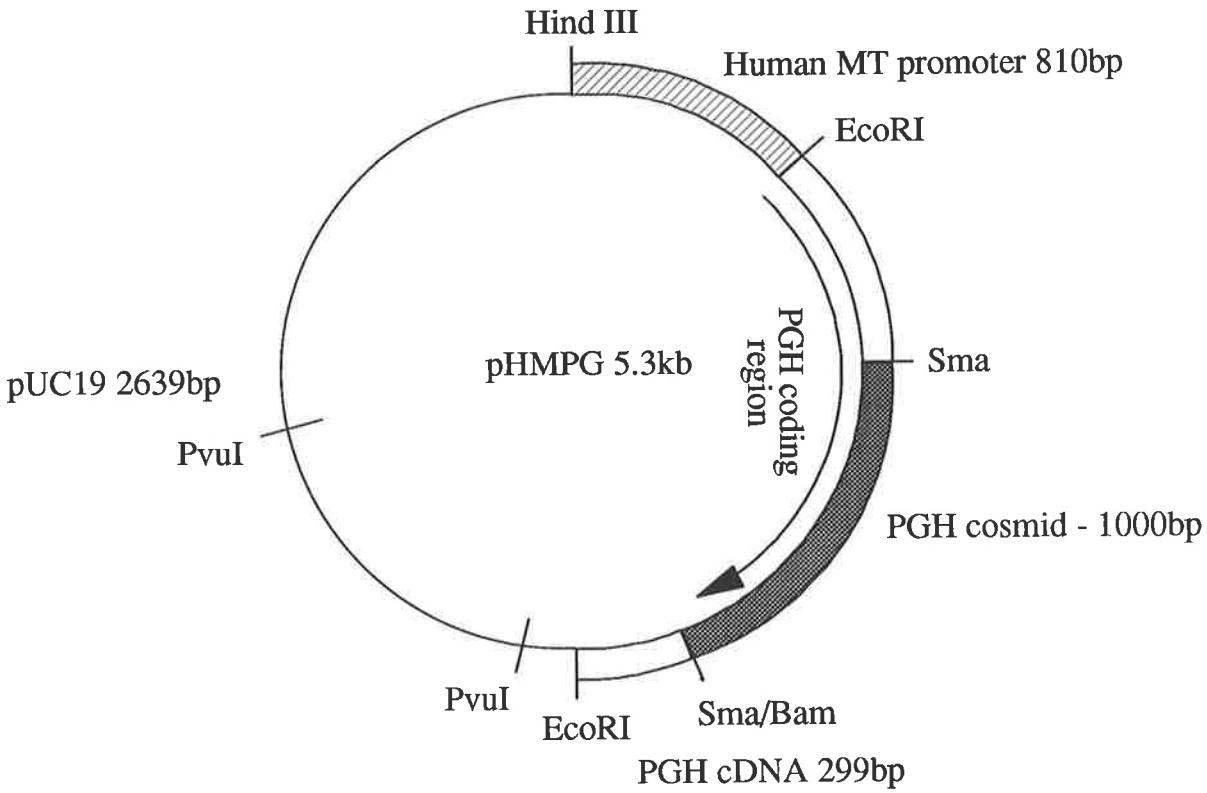
2.11 Micromanipulation

2.11.1 Methodology of Microinjection

Procedures and equipment for microinjection of mouse and pig zygotes have previously been described by Michalska (1988). Brief details including modifications have been provided below.

FIGURE 2.1 THE RESTRICTION MAP AND ORGANIZATION OF THE PORCINE GROWTH HORMONE EXPRESSION VECTOR pHMPG (VIZE *ET AL.*, 1988).

The transgene constructed combined the human MT-IIA promoter sequences, porcine GH coding sequences from the cDNA and the 3' processing signals of the genomic clone. The 2.7 kb Hind III/Pvu I fragment was used for production of transgenic animals.


Hind III
Human MT promoter 810bp
EcoRI
PGH coding region
Sma
pHMPG 5.3kb
pUC19 2639bp
PvuI
PGH cosmid - 1000bp
PvuI
EcoRI
Sma/Bam
PGH cDNA 299bp

2.11.2 Micromanipulation System

2.11.2.1 Microscope

Two three-dimension movement micromanipulators (NT-8, Narishige Scientific Pty. Ltd., Japan) were attached to an inverted microscope (Diaphot, Nikon, Japan) fitted with DIC optics. The objective turret was fitted with a LWD 20x, LWD 40x, and 10x DIC objectives and a 4x bright field objective. Microphotographs was taken with a 35 mm automatic camera (Nikon) attached directly to a 35 mm camera bayonet fitting on the microscope, using 50% of the total transmitted light. Photographs taken used 100 and 400 asa colour slide and print film.

2.11.2.2 Microinjection and Holding Pipettes

Microinjection of DNA was controlled by connecting a Leitz instrument holder containing the microinjection pipette to a high pressure nitrogen cylinder via a 3-way valve gas flow switch (Michalska, 1988) using thick walled plastic tubing (Masterflex Tubing 6409-16, Norton Performance Plastics, U.S.A.). To control meniscus movement in the holding pipette, a gas tight (threaded plunger) 0.5 ml Hamilton syringe (Alltech Pty. Ltd., Australia) was connected to a Leitz instrument holder containing the holding pipette by teflon tubing. The entire holding pipette system was filled with silicon oil (200 fluid/20 centistokes, Dow Corning, U.S.A.).

2.11.2.3 Manipulation Pipettes

Microinjection and Holding Pipettes were manufactured with glass capillary tubing directly from company packaging (Clark Electromedical, U.K.). Capillary tubing of a 150 mm in length was pulled on a pipette puller (P-77B, Brown-Flaming, U.S.A.) to produce 2 pipettes with gradually tapered tips.

Holding pipettes were pulled from thick walled tubing (GC100-15) and cut on a microforge (Defonbrune, France) to an O.D. of 150 to 180 μm before being polished with the microforge filament to an I.D. of 80 to 100 μm. Microinjection pipettes were manufactured from thin walled tubing (GC-100TF-15) to produce a closed tip of 0.1 μm O.D. DNA was loaded into the pipette from the blunt end using capillary action. Opening of microinjection pipettes to an O.D. of approximately 1 μm occurred by gently brushing the tip of the holding pipette. This procedure was conducted inside the microinjection chamber and observed through DIC optics on the microscope.

Using the microflame or microforge all pipettes were bent 100° twice at 6 mm and 14 mm intervals from the tip to facilitate entry into the manipulation chamber. Glass jars were used to store and protect pipettes until required.

2.12 Transgene Preparation

The human metallothionein/porcine growth hormone genes were prepared and purified as described in Chapter 2.10. The DNA samples were diluted in PBS (Flow Laboratories, North Ryde, New South Wales) to a concentration of 5 to 10 ng/μl, frozen (-20°C) in aliquots. Prior to microinjection aliquots of DNA were centrifuged for 3 min. at 13,500xg, at 4°C to remove particulate matter.

2.13 DNA Microinjection into Murine and Porcine Zygotes

Zygotes were washed twice in culture medium and then placed directly into a microinjection chamber identical to that described by Michalska (1988). Pronuclear one-cell zygotes were manipulated into the best focal plane under 400x Normaski optics using the holding pipette. The tip of the injection pipette was then brought into focus with one of the

pronuclei, using the fine control the injection pipette was inserted into the pronucleus. The gas pressure tap (Chapter 2.11.2.2) was then used to inject 2-4 picolitres of DNA solution into the pronucleus. The success of manipulation was confirmed by a rapid expansion of the pronucleus and the subsequent survival of the plasma membrane. Zygotes were then transferred immediately into synchronised recipients or cultured in HTF (murine) or MEM, BMOC-2 or Whitten's (porcine)(see Chapter 7) for assessment or transfer at a later stage.

2.13.1 Visualisation of Porcine Zygotes.

The pronuclei of porcine zygotes are obscured by dense cytoplasmic inclusions. To enable adequate visualization of pronuclei prior to microinjection, cytoplasm of the zygote was stratified using centrifugation (Wall *et al.*, 1985). Zygotes were transferred to sterile Epindorf tubes in HEPES-MEM and centrifuged at 13,500xg for 5-6 min. at 25°C.

2.14 Identification of Transgenic Animals

2.14.1 Isolation of DNA

Tail tissue samples were taken from mice at approximately four weeks of age and pigs at birth. Samples were stored in liquid nitrogen until required. Total nucleic acids were extracted by phenol-chloroform and ethanol precipitation. Concentrations of DNA were determined by measuring the UV light absorption at 260 nm.

2.14.2 Polymerase Chain Reaction (PCR)

Incorporation of the pHMPG transgenes in both mice and pigs was assessed by the polymerase chain reaction techniques (Mullis and Faloona, 1987) in tissue taken from tails at weaning. DNA samples (1 µl) were added to a reaction mixture (RM) contaning 36 µl H_2O; 3 µl $MgCl_2$ (25 mM); 5 µl

Amplification Buffer (500 mM KCl, 100 mM Tris Cl, pH 8.3 and 0.1% gelatin); 2.5 µl Oligo's (Primer 1 1 µl, Primer 2 1.5 µl); 2 µl 4 dNTP's mix; 0.5 µl *Taq* DNA polymerase enzyme. Each sample was overlaid with light mineral oil and run for 90 cycles in a Perkin Elmer Cetus thermal sequencer (3 hours). For cycle sequence see Appendix II. Appropriate positive (Human DNA + RM) and negative (RM) controls were included in each assay. Following amplification samples were electrophoresed on a 4% Agarose gel (1.5-2 hrs, 120 mA) and stained in ethidium bromide. Photographs using ultraviolet light were used to detect the presence of a band corressponding to the transgene. All porcine samples were analysed by the Department of Biochemistry, University of Adelaide.

2.14.3 DNA-DNA Hybridization

Incorporation of the constructs pUC-19, SB-2, Gc-3a, Bp-2, Bp-Ms and Bp-ML was by DNA-DNA hybridisation (slot and dot-bot analyses). DNA samples collected plus appropraite negative and postive controls were hybridised to approriate ^{32}P labelled probes to detect the posssiblity of transgene incorporation. Except for pUC-19, all other DNA samples were analysed by the Department of Biochemistry, University of Adelaide.

2.14.4 Southern Blot Analysis

DNA (3 µg) from transgenic pigs, along with human DNA postive and pig DNA negative controls were digested with Bam HI or Eco RI restriction enzymes. Digested samples were electrophoresed on agarose gels and then transferred onto Zetaprobe membranes (Bio Rad). The membranes were then hybridized to the nick translated hMT-IIA promoter Hind III/Ava I insert, washed at high stringency and autoradiographed.

2.15 Assay of Serum Hormones

2.15.1 Blood Sampling

Blood from new born piglets was collected either by optic sinus puncture or from the tail after tissue removal, samples from 30-120 day old pigs was collected by venopuncture or ear vein puncture. Samples were centrifuged at 2,500xg for 20 minutes, at 4°C. Serum was stored frozen (-20°C) until assayed.

2.15.2 Growth Hormone Immunoassay

The concentrations of growth hormone (pGH) in blood serum were measured by radioimmunoassay (RIA). The assay employed a rabbit antiserum to pig pituitary GH (UCB-Bioproducts, Brussels, Belgium), porcine growth hormone as a standard and [^{125}I]-iodo-pGH (80 Ci/g, pGh-I-1, AFP-6400, USDA) as radioligand. The assay was performed in 0.01M sodium phosphate containing 0.01% BSA at pH 7.5. Replicate polystyrene tubes containing 35,000 cpm [^{125}I]-pGH, rabbit anti-pGH antiserum (UCB) at dilution of 1:20,000 and either variable amounts of pituitary pGH standard or 50 µl serum (or diluted serum) were incubated in a final volume of 0.3 ml at room temperature for 16 to 20 hr. Cellulose-coated donkey anti-rabbit immunoglobulins (Dar-SacCell, Welcome), 50 µl, were added, mixed and allowed to stand for 30 min. Distilled water (0.5ml) was then added and the tubes centrifuged at 3,000xg for 10 min. The supernatant was aspirated and the radioactivity in the pellet was measured in a LKB Walllac 1272 Gamma counter. The assay sensitivity was 0.7±0.3 ng pGH/ml of assay incubate (mean±sd, n=4) or 0.2 ng per tube. The limit of detection for 50 µl of tested serum was 4.2 ng/ml

2.15.3 Oestrone Sulphate Immunoassay

The concentration of Oestrone Sulphate in blood serum from pigs was measured by radioimmunoassay. The assay employs a goat antiserum raised against oestrone-3-CEA, oestrone-3-sulphate, potassium salt, (Sigma, USA) as a standard and oestrone sulphate, ammonium salt, [6,7-^{3}H(N)]-, (60 Ci/mmol, Dupont, USA) as the radioligand.

The assay was performed in 0.15M phosphate buffered saline with 0.1% azide and 0.1% BSA, at pH 7.4. Replicate tubes containing 2,500 cpm ^{3}H-oestrone sulphate, goat anti-oestrone sulphate, antiserum at a dilution of 1:40,000 and either variable amounts of estrone-3-sulphate or 20 µl of serum (or diluted serum) were incubated in a final volume of 0.8 ml for 15 min at 37°C and for at least 1 hr at 4°C. Charcoal (v/v, 0.9%), 100 µl was added, mixed and left to further incubate at 4°C for 12 min Tubes were centrifuged at 4,000xg for 15 min. The supernatant was decanted into scintillation vials and radioactivity was measured in a Beckman 3801 beta scintillation counter. The assay sensitivity at 68% binding was 0.451±0.2 pmoles/ml (mean±sd, n=6). The cross-reactivity of antiserum is listed below:-

Oestrone Sulphate	100%
Oestrone*	>100%
Estradiol	7.5%
Estriol	1.5%
Progesterone	0.09%
17-OH Progesterone	<0.01%
Androstenedione	0.75%
Testosterone	0.75%
DHEA-SO_4	1.5%

*Despite high cross-reactivity with oestrone, levels of estrone in plasma of sows during the phase of pregnancy investigated (22-30 days) are 0.44% of the oestrone sulphate concentrations. This estrone cross-reactivity

would therefore account for <1.5% of the derived estrone sulphate levels in serum.

2.15.4 IGF-I Immunoassay

The concentrations of Insulin-like Growth Factor-1 (IGF-I) in blood plasma from pigs was measured by radioimmunoassay (Owens, P.C. *et al.*, 1990) following ethanol extraction. All assays of IGF-I were kindly performed by Dr. Owens and his staff. Details of the protocol are given in Appendix III.

Chapter III

INVESTIGATIONS INTO INCREASING THE FREQUENCY OF INTEGRATION FOLLOWING DNA MICROINJECTION

3.1 Introduction

Increasing the frequency of DNA integration would greatly enhance the possibilities of producing transgenic offspring. Currently the overall efficiency of producing transgenic animals by pronuclear injection is estimated to be between 0.0-2.0% for injected zygotes (Rexroad and Pursel, 1988). The mechanism whereby transgenes integrate into chromosomal DNA after microinjection is unknown. One salient feature indicates that when more than one copy of the transgene integrates, they are usually all integrated in the same chromosomal site in a tandem, head to tail array, although other arrangements have been documented (Gordon *et al.*, 1981; Hammer *et al.*, 1984; Palmiter *et al.*, 1983). On the basis of this finding, Brinster *et al.*, (1985) proposed that the limiting step to integration was breakage of chromosomal DNA that allowed insertion of the linear microinjected molecule with the tandem arrays forming by homologous recombination. The proposal is supported by homologous recombination amongst injected molecules derived from experiments following injection of two different MT-hGH genes with non over-lapping deletions which resulted in an intact MT-hGH genes producing a functional hGH in some transgenic mice (Palmiter *et al.*, 1985). Although plausible, evidence for the Brinster's hypothesis remains scant and the hypothesis does not explain all characteristics of the integrated elements (Gordon, 1989).

Once the foreign DNA is integrated, it is stably transmitted for many generations with no evidence of rearrangement (Palmiter *et al.*, 1985).

Several parameters effecting the efficiency of introducing foreign DNA have already been identified and are critical for successful gene transfer into the germline (Brinster *et al.*, 1985). However as indicated studies in this area are heavily constrained by the limited amount of information available on the mechanism by which exogenous DNA gains access to the embryo genome. This chapter explores the possibility of enhancing gene transfer efficiency by perturbing chromosomal structure so as to enhance integration frequency. Through these studies it was also hoped that more information

on mechanisms of gene integration would emerge. Three approaches were used:-

3.1.1 Intercalating Agent (Hoechst Stain (33342)

Hoechst stain is a bis-benzimidazole dye, that is a non toxic specific vital intercalating agent for DNA (Richards *et al.*, 1985). Hoechst stain readily enters cells and strongly fluoresce as a result of the interaction between native DNA and chromatin (Pursel *et al.*, 1985). Experiments were conducted using a non toxic intercalating agent to test the ability of a transgene to be integrated at a relaxed DNA site (ie relaxed sites might be expected to be more susceptible to integration compared with unrelaxed DNA) following DNA microinjection.

To demonstrate the effect of Hoechst stain on DNA structure the sensitivity of treated DNA to Deoxyribonuclease I (DNAase I) digestion was examined. DNAase I preferentially selects relaxed chromatin over tightly complexed DNA. The intercalating effect of Hoechst stain is expected to relax DNA structure by inserting between the DNA bases, thus making sites available for DNAase I to attack, resulting in increased fragments of DNA. The intercalating effect of Hoechst stain was compared to the known toxic intercalating agent ethidium bromide.

3.1.2 Restriction Enzyme (Hind III)

Restriction enzymes recognize specific short oligonucleotides in DNA and cleave the DNA at all such sites (Sambrook *et al.*, 1989). Hind III recognizes the sequence AAGCTT and was used in initial studies to isolate the transgene from its expression vector (ie. there is a base pair recognition sequence for Hind III at the 5' end of the transgene, see Chapter 2.10). The 6 b.p. recognition sequence of Hind III will cut, on average, every 4,000 b.p., since there is $5x10^9$ b.p/cell (Boulikas, 1990) the restriction enzyme should cut approximately 10^6 times/cell if all DNA was free of proteins. Since only 1-10% of DNA may be freely accessible to the enzyme, cutting will be much

less frequent within nuclei. Microinjection of Hind III with the transgene is expected to increase the number of chromosomal breaks which might contribute to an increased integration frequency.

3.1.3 Ultraviolet light (254 nm)

Both Eukaryotes and Prokaryotes respond to exposure to DNA Damaging agents such as ultraviolet light by inducing a multitude of repair and recombination functions which are directly responsible for enhancing repair (Dubbs *et al.*, 1974; Hall *et al.*, 1980; Sarasin *et al.*, 1980). Short-wavelength UV radiation (254 nm) is one of the best studied DNA-damaging agents and inducing damage just prior to microinjection might contribute to increased integration at the induced break points. However it should be noted not only does UV-light cause damage but it can block DNA synthesis and disrupt other cellular mechanisms (Rattan, 1989; Hall and Mount, 1981).

3.2 Materials and Methods

3.2.1 Animal Maintenance and Embryo Collection

3.2.1.1 Laboratory Mice

Hybrid Strain CBA x C57BL/6 Specific Pathogen Free Laboratory Mice were obtained from the Adelaide University Central Animal House, S.A. They were housed at 20°C, in artificial light for a period of 12 hours every day. Commercial prepared food (Mouse Cubes, Milling Industries Ltd., S.A.) and water were available *ad libitum*.

3.2.1.2 Hormonal Stimulation and Mating

Immature (3-4 weeks old) female F1 hybrid CBA x C57Bl/6 were superovulated by intraperitoneal injection of 10 i.u. PMSG (Folligon, Intervet, Holland) followed 48 hours later by 10 i.u. HCG (Chorulon, Intervet, Holland). Immediately following the HCG injection females were placed with one male. All matings were initiated using sexually mature

males, isolated for a period of two weeks prior to the first mating and for at least two days before any actual mating. On the following morning, females were checked for vaginal plugs. Detection of a copulatory plug was considered day $^1/_2$ of pregnancy. Recipient animals were prepared by placing mature females (10-15 weeks) with vasectomized males.

Females undergoing natural oestrous cycling were monitored for the presence of a copulatory plug. The copulatory plugs in donors and recipients animals provided a marker for maintaining synchronization of embryo transfer between the donor embryos and the surrogates reproductive tract.

3.2.1.3. Embryo Collection and Culture

Zygotes were extracted from oviducts using HEPES-HTF and 300 i.u. Hyaluronidase/ml (Sigma, USA) at 20 to 24 hours post HCG. Following collection zygotes were transferred and cultured in 10 µl microdrops of HTF at 37°C until required. All micromanipulations were carried out in HEPES-HTF.

3.2.2 Culture Media

3.2.2.1 Human Tubal Fluid Medium

TABLE 3.1 COMPOSITION OF HTF MEDIUM (QUINN *ET AL.*, 1985, MODIFIED).

Component	Concentration (mM)
NaCl	101.6
KCl	4.69
$MgSO_4.7H_2O$	0.20
KH_2PO_4	0.37
$NaHCO_3$	25.00
$CaCl_2.2H_20$	2.04
Na. lactate	21.40
Na. pyruvate	0.33
glucose	2.78
penicillin	100 U/ml
phenol red	0.001% (w/v)

3.2.2.2 HEPES-HTF Medium

This medium was identical to HTF medium except that sodium bicarbonate was reduced to 4 mM and 21 mM HEPES was added to enhance buffering capacity during micromanipulations on an open stage (Quinn *et al.*, 1984).

3.2.3 Media Supplements

HTF and HEPES-HTF media's were supplemented with 5mg/ml of BSA (Sigma, USA) and filter sterilized (Millipore 22μm).

3.2.4 Embryo Transfer

Anaesthesia was induced and maintained with a single (15ml per one gram of bodyweight) intraperitoneal injection of Avertin (Michalska, 1988). A small surgical incision was made through the dorsal surface and the ovaries were exteriorised. One and two-cell embryos were inserted into the infundibulum located under the bursa. Transfer of blastocyst occurred by exteriorising the uterus so that a small puncture could be made into the lumen.

3.2.5 Assessment of Implantation Rate

When each litter was weaned, the recipients were sacrificed, their reproductive tracts dissected and uteri checked for the number of implantation scars (Soares, 1972).

3.2.6 Hoechst Stain (33342)

3.2.6.1 Establishment of L_D-50 Dose

Groups (n=25) of one cell mouse embryos in HEPES-HTF were incubated for 30 min. in various concentrations of Hoechst stain and then cultured to the blastocyst stage in HCO_3-HTF to establish a dosage causing

50% mortality (L_D-50). Control embryos received the same incubation but without Hoechst stain.

3.2.6.2 Digestion of Hoechst Treated DNA with DNAase I

Genomic DNA samples were incubated with various concentrations of Hoechst 33342 stain and ethidium bromide (toxic intercalating agent). All treated samples and appropriate controls were digested with DNAase I before separation by gel electrophoresis.

3.2.6.3 Comparison of Integration Frequency

Comparisons were made between the number of transgenes incorporation into embryos microinjected following incubation with Hoechst stain at the L_D-50 dose and the appropriate controls.

3.2.7 Restriction Enzymes

3.2.7.1 Establishment of L_D-50 Dose

Groups (n=25) of one cell mouse embryos in Hepes-HTF microdrops were divided into (1) DNA construct (Injection controls), (2) Hind III, (3) DNA construct + Hind III and (4) No microinjection (Controls). Groups 1 to 3 were microinjected with or without DNA or various concentrations of restriction enzyme. All groups were then cultured to the blastocyst stage in HCO_3-HTF to establish a concentration causing 50% mortality.

3.2.7.2 Comparison of Integration Frequency

Comparisons were made between the number of transgene incorporation into embryos microinjected with DNA construct and DNA construct plus Hind III restriction enzyme.

3.2.8 Ultraviolet Light

3.2.8.1 Establishment of L_D-50 Dose

Groups (n=25) of one cell mouse embryos in HEPES-HTF microdrops were exposed to ultraviolet light (254 nm, UV-C, Model No. VL-42, Vilder Lourmat) for various periods and intensities and then cultured to the blastocyst stage in HCO_3-HTF to establish a dosage causing 50% mortality (L_D-50). Control embryos received the same treatment without UV light.

3.2.8.2 Comparisons of Integration Frequency

Comparisons were made between the rate of transgene incorporation into embryos microinjected following treatment with no ultraviolet light (Control) or the ultraviolet light at the L_D-50 dose (treated).

3.2.8.3 Measurement of Ultraviolet light

The output of the lamp was measured at 265 mW/cm^2. Therefore the standardise L_D-50 dose of UV light at 5 sec. was calculated (W=J/s) to be equivalent to 1325 mJ/cm^2 or 0.1325 mJ/m^2.

3.2.8.4 Embryo Cleavage

Based on preliminary observations an experiment was designed to test the effect of ultraviolet light on the rate of cleavage for the first cell division and then all subsequent divisions to the hatched blastocyst stage.

Groups (n=30) of one cell embryos were treated with the L_D-50 dose of ultraviolet light and compared with normal embryos on the rate of cleavage from 27 hrs until 142.5 hrs post HCG.

3.2.9 Detection of Transgenics and Southern Analysis

In order to determine if integration occured irrespective of transgene a variety of contructs were used. Incorporation of the pUC-19, $pHMPG_{.04}$, $pHMPGH_{.07}$, Cys-M, Gc-3a, SB-2, Bp-ML and Bp-Ms constructs were determined by DNA-DNA hybridization and Southern blot (Southern, 1975)(Chapter 2.14.4). These constructs were made available as part of microinjection service offered by the Dept. of Obstetrics and Gynaecology. Incorporation of the $pHMPG_{.03A}$, $pHMPG_{.05}$, $pHMPG_{.08}$ constructs was assessed by polymerase chain reaction techniques (see Chapter 2.14.2 and Plate (1)).

3.3 Statistical Analysis

Comparison of Integration frequency in treated and control microinjected mice were analyzed by one-way analysis of variance (SAS Institute Inc., 1988). The log transformed regression lines of cleavage rates for control and UV-light treated embryos were compared using analysis of covariance (Snedecor and Cochran, 1989) and Student's *t* test (Zar, 1974). The proportion of control and UV-light treated embryos were compared using ANOVA (SAS Institute Inc., 1988).

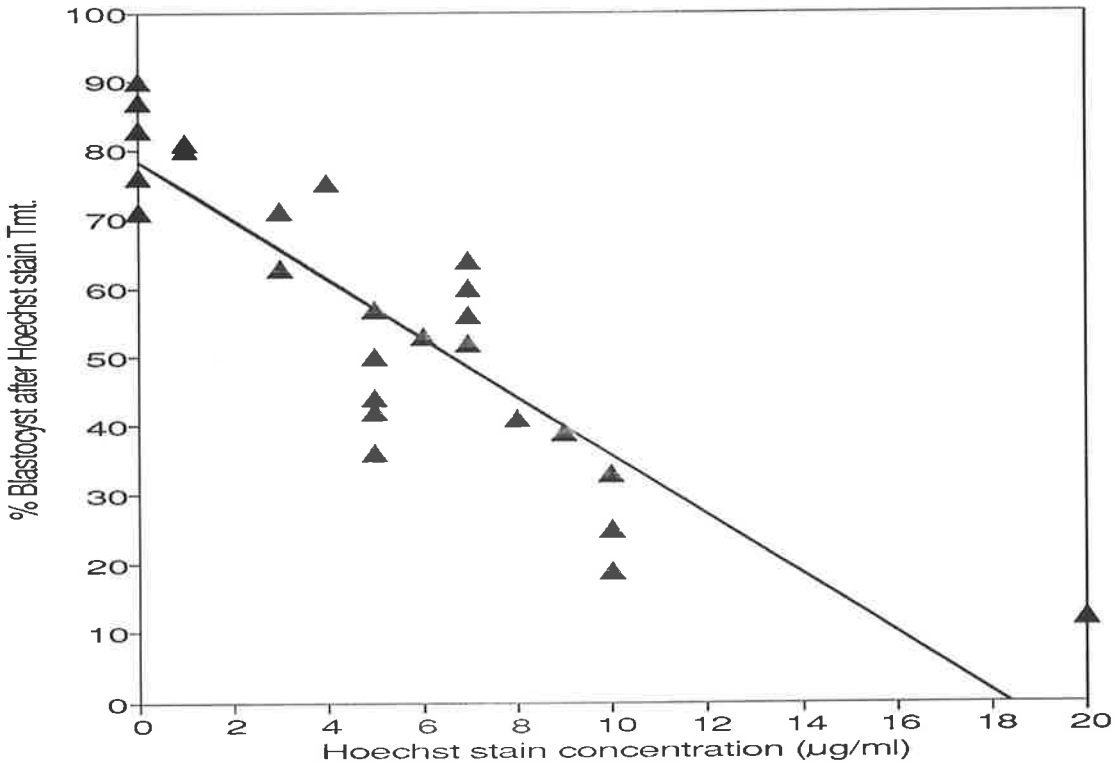
3.4 Results

3.4.1 Hoechst Stain (33342)

3.4.1.1 Establishment of L_D-50 Dose

A total number (n=810) of one cell embryos were incubated for 30 minutes in various concentrations of Hoechst Stain and cultured to the blastocyst stage to determine by linear regression the L_D-50 dose of 5µg/ml of Hoechst stain 33342. Figure 3.1 shows the effect of various concentrations of Hoechst stain 33342 on embryo development to the blastocyst stage.

FIGURE 3.1 VIABILITY OF ZYGOTES CULTURED TO BLASTOCYSTS FOLLOWING INCUBATION WITH HOECHST STAIN 33342.



3.4.1.2 Digestion of Hoechst 33342 and Ethidium Bromide Treated DNA with DNAase I

The effect of ethidium bromide and Hoechst 33342 on the DNAase I digestion pattern of total mouse genomic DNA can be seen in Plate (1). The results indicate that Hoechst 33342 does effect DNA sensitivity although not as effectively as ethidium bromide. Based on the concentration ranges and the incubation time tested, Hoechst stain is approximately $^1/_{10}$ as effective as ethidium bromide, nevertheless DNA structure is altered by its intercalating effect.

3.4.1.3 Comparison of Integration Frequency

DNA samples taken from mice near birth or at weaning and assessed for integration of the transgene by DNA-DNA hybridization indicate that treatment with Hoechst stain 33342 did not significantly increase the

integration frequency ($p>0.05$, ANOVA) of microinjected DNA (see Table 3.2 and Plate (1)). However, while not significant there appeared to be a trend for an increase in integration frequency with certain transgenes, though further investigation would be needed to confirm this (eg SB-2 control 1/11 vs treated 1/5 compared to Cys.M control 4/10 vs treated 3/9).

TABLE 3.2 NO. OF TRANSGENIC OFFSPRING FOR CONTROL (-) AND HOECHST STAIN (33342) INTERCALATING AGENT (+) TREATMENT GROUPS USING DNA-DNA HYBRIDIZATION.

Gene construct	Tmt.	Embryo Transferred*		Total No. Offspring		Transgenic No. (% live born)	
		-	+	-	+	-	+
pUC-19	-	168		23		7 (30)	
	+		89		12		5 (41)
$pHMPG_{.04}$	-	210		22		7 (22)	
	+		76		10		4 (40)
Cys.M	-	105		10		4 (40)	
	+		84		9		3 (33)
$pHMPG_{.07}$	-	420		87		0	
	+		250		46		1 (2.2)
SB-2	-	74		11		1 (9.1)	
	+		58		5		1 (20)
Gc-3a	-	84		16		0	
	+		52		8		1 (13)
Bp-2	-	130		16		0	
	+		126		21		2 (10)
Bp-Ms	-	84		3		0	
	+		62		3		0

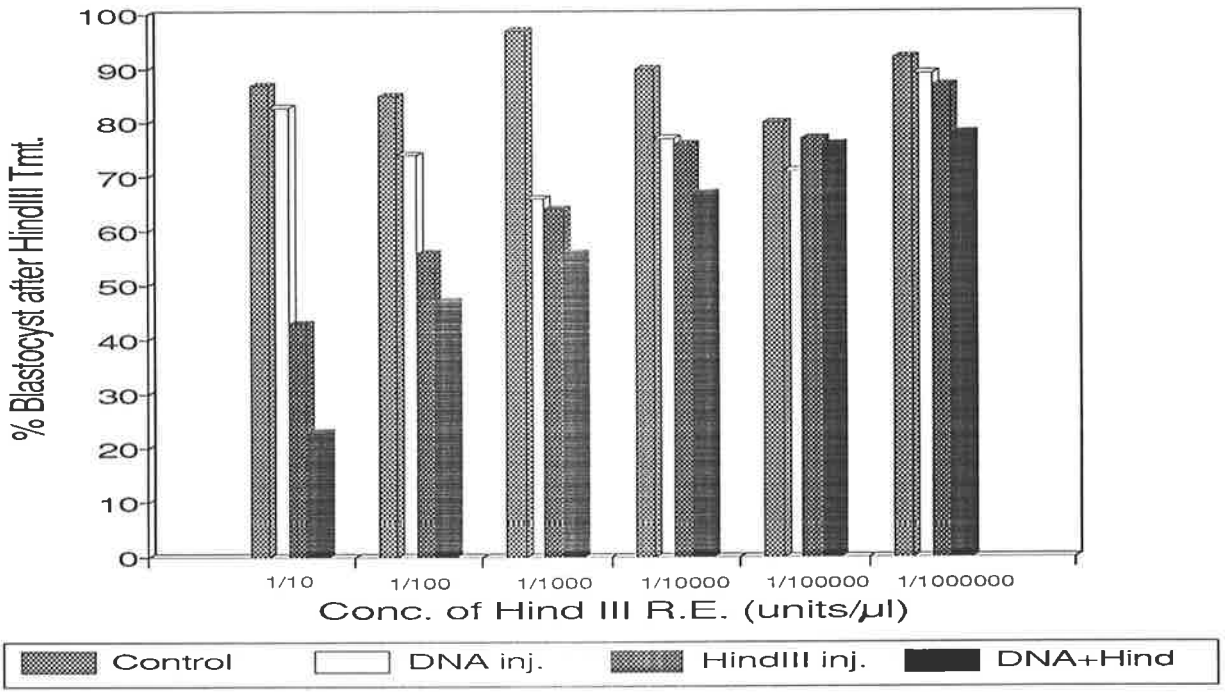
*(average transfer no. per mouse 35 ± 5)

3.4.2 Restriction Enzyme

3.4.2.1 Establishment of L_D-50 Dose

A total number (n=1665) of one cell embryos were microinjected with a 1:1 cocktail of DNA (10 ng/µl) and various concentrations of ($^1/_{10}$ to $^1/_{10^6}$ units/µl) Hind III restriction enzyme cultured to the blastocyst stage to determine an L_D-50 dose of $^1/_{1000}$ units/µl of Hind III restriction enzyme (see Figure 3.2). The reason for the large decrease in viablity between embryos injected with DNA and those injected with DNA+Hind III, particularly at high concentrations was not known, but was confirmed with several repititions of the procedures (see Figure 3.2).

FIGURE 3.2 VIABILITY OF ZYGOTES CULTURED TO THE BLASTOCYST STAGE FOLLOWING MICROINJECTION WITH (1) DNA, (2) DNA + HIND III AND (3) HIND III.



3.4.2.2 Comparison of Integration Frequency

DNA samples taken from mice near birth or at weaning and assessed for integration of the transgene by PCR indicate that treatment with the restriction enzyme Hind III at the L_D-50 dose did not significantly ($p>0.05$) influence the rate of integration (see Table 3.3). However, the results were similar to that observed using Hoechst stain where there apeared to be an increase in integration that varied with the type of transgene used, though larger number would be required to validate this ($pHMPG_{.05}$ Ctrl (36%) and Tmt (41%) compared to $pHMPG_{.08}$ Ctrl (23) and Tmt (21%)).

TABLE 3.3 NO. OF TRANSGENIC OFFSPRING FOR CONTROL (-) AND RESTRICTION ENZYME (HIND III) TREATMENT (+) GROUPS DETECTED BY PCR.

Gene construct No.	Tmt.	Embryo Transferred*		Total No. Offspring		Transgenic (% live born)	
		-	+	-	+	-	+
$pHMPG_{.05}$	-	91		14		5 (36)	
	+		81		12		5 (41)
$pHMPG_{.03A}$	-	290		52		10 (20)	
	+		158		25		8 (32)
$pHMPG_{.08}$	-	148		26		6 (23)	
	+		135		19		4 (21)

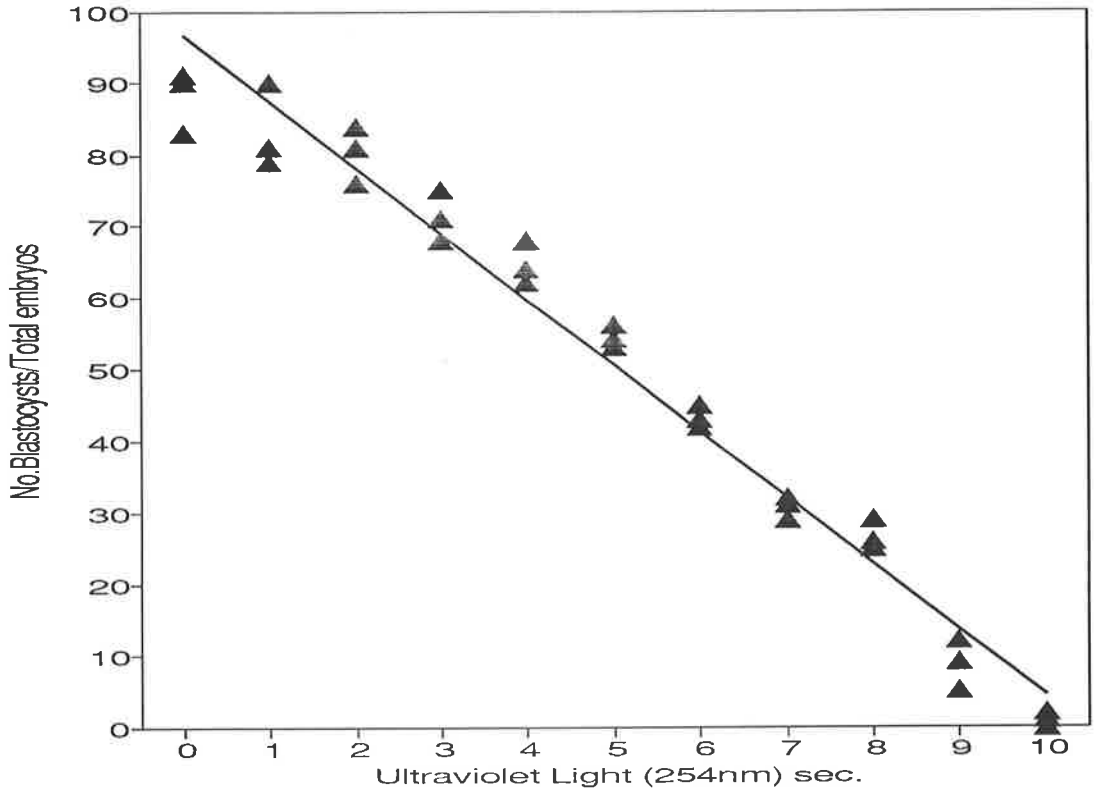
*(average transfer no. per mouse 35±5)

3.4.3 Ultraviolet Light

3.4.3.1 Establishment of L_D-50 Dose

A total number (n=990) of one cell embryos were treated with ultraviolet light (254nm) at various time intervals and cultured to the blastocyst stage to determine by linear regression an L_D-50 dose of 5 sec. (see Figure 3.3).

FIGURE 3.3 VIABILITY OF ZYGOTES CULTURED TO THE BLASTOCYST STAGE FOLLOWING TREATMENT WITH ULTRAVIOLET LIGHT.



3.4.3.2 Comparison of Integration Frequency

DNA samples taken from mice near birth or at weaning and assessed for integration of the transgene by DNA-DNA hybridization indicate that treatment with ultraviolet light can significantly increase the integration frequency ($p=0.011$, ANOVA) of microinjected DNA (see Table 3.4 and Plate (1)). The increase in integration occurred irrespective of construct providing the construct could be integrated and the results were not compounded by transfer of one to two cells to the oviduct or blastocyst to the uterus.

TABLE 3.4 NO. OF TRANSGENIC OFFSPRING FOR CONTROL (-) AND UV LIGHT TREATMENT (+) GROUPS USING DNA-DNA HYBRIDIZATION.

Gene construct No.	Tmt.	Embryo Transferred*		Total No. Offspring		Transgenic (% live born)	
		-	+	-	+	-	+
pUC-19	-	120		19		4 (21)	
	+		60		8		4 (50)
$pHMPG_{.04}$	-	183		28		9 (32)	
	+		80		12		7 (58)
SB-2	-	132		9		1 (11)	
	+		178		11		5 (45)
Bp-ML	-	115		15		1 (7)	
	+		118		18		4 (22)
Bp-MS	-	60		3		0	
	+		52		9		0
$pHMPG_{.07}$	-	276		46		0	
	+		147		21		2 (0.1)

*(average transfer no. per mouse 35±5)

3.4.3.3 Embryo Cleavage

Figure 3.4 compares the embryo cleavage rate from one-cells to two cells after UV-light treatment at the L_D-50 dose. Ultra Violet-light (254 nm) significantly ($p<0.05$) delayed the time taken to develop to the two cell stage when compared to controls. This delay in cleavage did not continue after the two cell stage of development as proportions of control and treated embryos at the 4 to 8-cell, morula, blastocyst and hatched-blastocyst were not significantly different (Table 3.5).

FIGURE 3.4 RATE OF FORMATION OF 2-CELL EMBRYOS AFTER ULTRAVIOLET LIGHT TREATMENT AT THE L_D-50 DOSE.

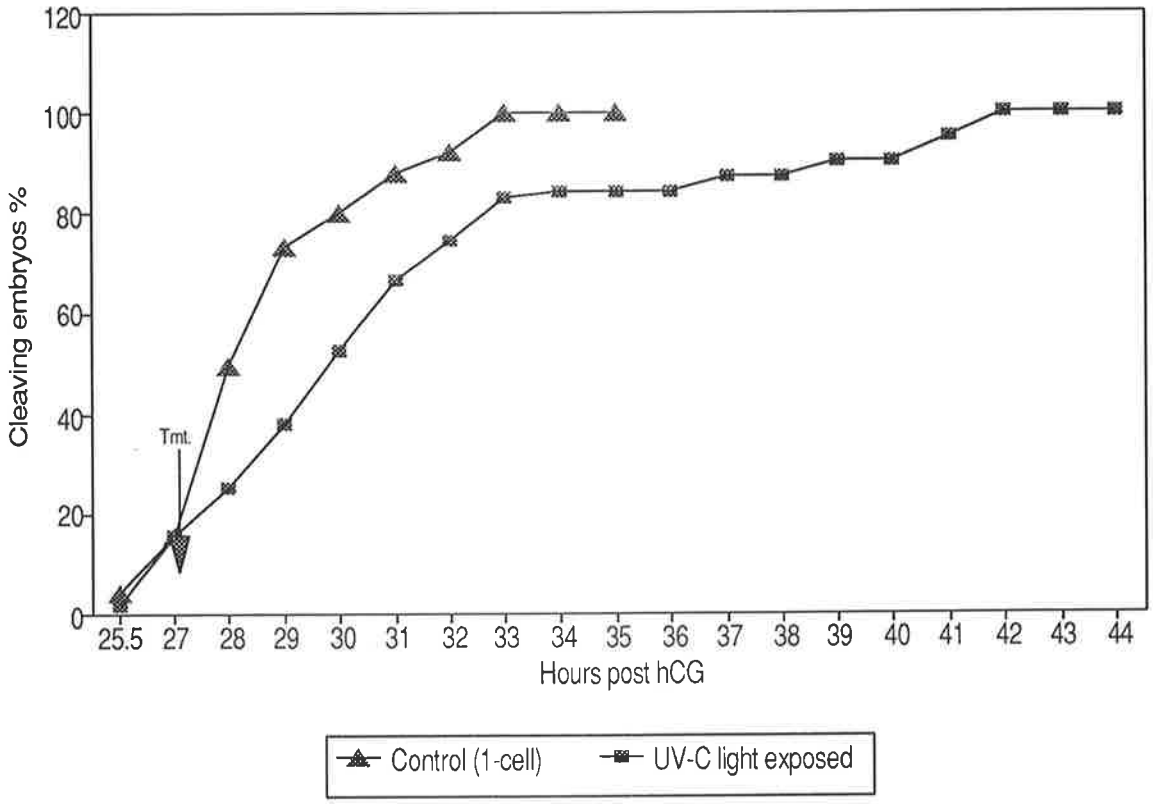


TABLE 3.5 EMBRYOS DEVELOPING AFTER ULTRAVIOLET LIGHT TREATMENT AT THE ZYGOTE STAGE WITH THE L_D-50 DOSE.

Time (hrs) post-Tmt. Bl.	Tmt.	Embryos cleaving from the 2-cell stage (%) 2-cell	4/8-cell	Morula (16/32)	Bl.	H-
70.5	UV-L	16	84			
	Ctrl	8	92			
94.5	UV-L		87	3		
	Ctrl		88	7.8		
118.5	UV-L			71	16	
	Ctrl			84	11	
142.5	UV-L					87
	Ctrl					94

3.4.4 Implantation Rates of Treated and Control Embryos

Morphologically viable embryos were transferred at either the two cell stage or blastocyst stage of development to the oviduct or uterus respectively. Non viable cells were not transferred. There was no difference between the number of offspring obtained from either uterine or oviduct transfers ($p>0.05$). Pregnancy rates varied depending on the constructs but did not differ significantly between microinjected controls and treated embryos. The ratio of implantation scars to number of fetuses in pregnant animals did not differ significantly ($p>0.05$) from treated microinjected zygotes when compared to microinjected controls (Hoechst stain, 0.40$\pm$ S.E. 0.07; Restriction enzyme, 0.43$\pm$S.E. 0.05; UV-light treated, 0.41$\pm$S.E. 0.06; Controls, 0.5$\pm$S.E. 0.03).

3.4.5 Southern Analysis

In order to establish if the various treatments were causing unusual integration patterns, a random sample of 90 pHMPG transgenic DNA samples were selected for Southern Analysis. The transgenic mice samples selected were from microinjected control (n=30) and treated groups (3 x n=20). Table 3.6 a,b,c show the results of copy number and gene arrangement as determined by DNA-DNA hybridization and Southern Analysis (see Plate (1)). The number of transgenes were analysed by Slot-Blot DNA-DNA hybridization and ranged from 0.5 to 140 copies for all treatment and control groups. No significant differences ($p>0.05$) were evident between the number of transgenes integrating from any of the treatment groups when compared to the controls.

The integration patterns, analysed by digesting DNA with Bam HI (the Bam HI recognition sequence is towards the 3' prime end of the transgene, see Figure 2.1) and separation of the fragments using agarose gel electrophoresis resulted in a variety of patterns which could be interpreted as indications of one integration to multiple integrations. There was no treatment which preferentially caused a certain type of integration pattern.

TABLE 3.6A SOUTHERN ANALYSIS OF CONTROL AND TREATED TRANSGENIC ANIMALS

Hind III Restriction Enzyme			
Construct		**Integration (Copies/cell)**	**Arrangement**
Controls			
$pHMPG_{.05}$	1	5	1 HT
	2	1	1
	3	15	1 HT
$pHMPG_{.03A}$	1	90	M R
	2	<1	1
	3	10	M HH
	4	1	1
$pHMPG_{.08}$	1	10	1 HT
	2	120	1 HT
	3	20	1 HT
Treated			
$pHMPG_{.05}$	1	25	1 HH
	2	<1	1
	3	20	M R
	4	5	1 HT
	5	15	1 HH
	6	30	1 HT
	7	15	1 HT
$pHMPG_{.03A}$	1	1	1
	2	1	1
	3	10	1 HT
	4	5	1 R
	5	<1	1
	6	10	1 HH
$pHMPG_{.08}$	1	1	1
	2	130	1 HT
	3	50	M R
	4	15	1 HH
	5	1	1
	6	5	1 HT
	7	25	M R

1 or M	One or multiple sites of integration
HH	Head to head tandem array
HT	Head to tail tandem array
R	Rearranged

TABLE 3.6B SOUTHERN ANALYSIS OF CONTROL AND TREATED TRANGENIC ANIMALS

Hoechst Stain (33342)			
Construct		**Integration (Copies/cell)**	**Arrangement**
Controls			
$pHMPG_{.04}$	1	10	1 HT
	2	5	1 HT
	3	<1	1
	4	20	1 HH
	5	110	M R
	6	10	1 HT
$pHMPG_{.07}$	1	15	1 HT
	2	1	1
	3	1	1
	4	1	1
Treated			
$pHMPG_{.04}$	1	15	M HT
	2	5	1 HH
	3	10	M R
	4	60	1 HT
	5	1	1 R
	6	5	1 HT
	7	35	1 R
	8	70	M HH
	9	1	1 R
$pHMPG_{.07}$	1	10	1 HT
	2	1	1
	3	5	1 R
	4	15	1 HT
	5	45	1 HT
	6	1	1
	7	25	1 HT
	8	1	1
	9	5	M R
	10	1	1
	11	1	1 R

1 or M	One or multiple sites of integration
HH	Head to head tandem array
HT	Head to tail tandem array
R	Rearranged

TABLE 3.6C SOUTHERN ANALYSIS OF CONTROL AND TREATED TRANGENIC ANIMALS

Ultra violet light (254 nm)			
Construct		**Integration (Copies/cell)**	**Arrangement**
Controls			
$pHMPG_{.04}$	1	1	1
	2	10	1 HH
	3	15	1 R
	4	250	M R
	5	1	1 R
	6	10	1 HT
	7	1	1
$pHMPG_{.07}$	1	1	1
	2	30	M R
	3	51	1 HH
	4	140	1 HT
Treated			
$pHMPG_{.04}$	1	1	1 R
	2	1	1
	3	20	1 HT
	4	5	1 HT
	5	15	1 HH
	6	30	1 HT
	7	15	1 HT
	8	100	M R
	9	1	1
	10	5	1 HT
$pHMPG_{.07}$	1	5	1 HT
	2	15	M HT
	3	45	1 HH
	4	1	1
	5	130	1 HT
	6	15	M HT
	7	25	1 HT
	8	1	1
	9	5	1 R
	10	1	1

1 or M	One or multiple sites of integration
HH	Head to head tandem array
HT	Head to tail tandem array
R	Rearranged

PLATE 1

Photograph 1

Digestion of total genomic DNA with DNAase I following treatment with ethidium bromide and Hoechst 33342 stain

Legend from left to right to identify samples is shown below

Track 1	Uncut Control DNA (total genomic)
Track 2	DNA incubated with 5 μg/ml of Ethidium Bromide
Track 3	DNA incubated with 2 μg/ml of Ethidium Bromide
Track 4	DNA incubated with 1 μg/ml of Ethidium Bromide
Track 5	DNA incubated with 0.1 μg/ml of Ethidium Bromide
Track 6	Uncut Control DNA
Track 7	DNA incubated with 10 μg/ml of Hoechst 33342 stain
Track 8	DNA incubated with 5 μg/ml of Hoechst 33342 stain
Track 9	DNA incubated with 2 μg/ml of Hoechst 33342 stain
Track 10	DNA incubated with 1 mg/ml of Hoechst 33342 stain

Photograph 2

Murine DNA samples isolated from tissue were examined by P.C.R. analysis to determine integration of transgene.

Legend from left to right to identify samples is shown below

Track 1	7-6 Ultraviolet light	
Track 2	2-1 Hoechst stain	positive
Track 3	4-7 Control	
Track 4	2-3 Hoechst stain	
Track 5	1-7 Ultraviolet light	
Track 6	3-3 Restriction enzyme	positive
Track 7	5-1 Control	
Track 8	5-2 Control	
Track 9	2-6 Hoechst stain	positive
Track 10	2-4 Hoechst stain	
Track 11	6-2 Ultraviolet light	
Track 12	positive control human	
Track 13	negative control mouse	
Track 14	markers	

Photograph 3

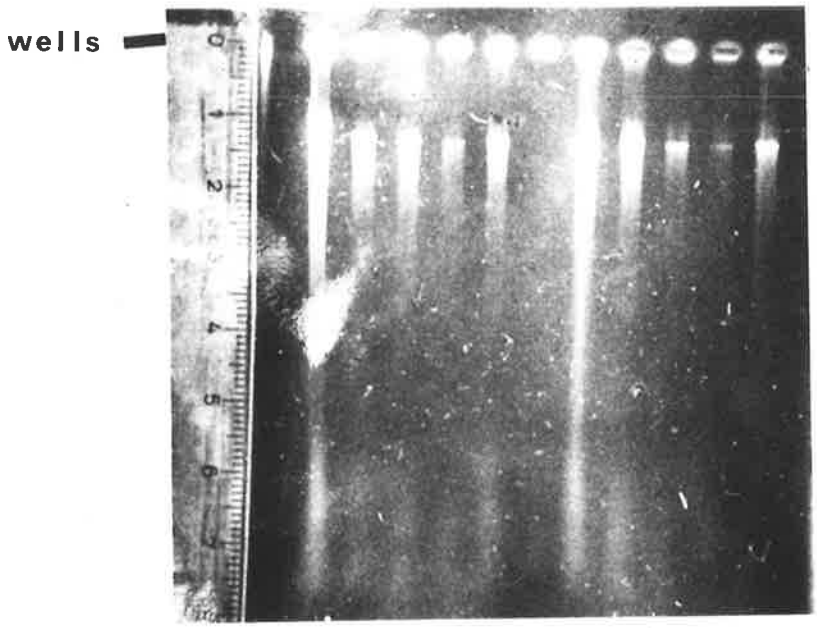
Southern blotting analysis of transgenic mice

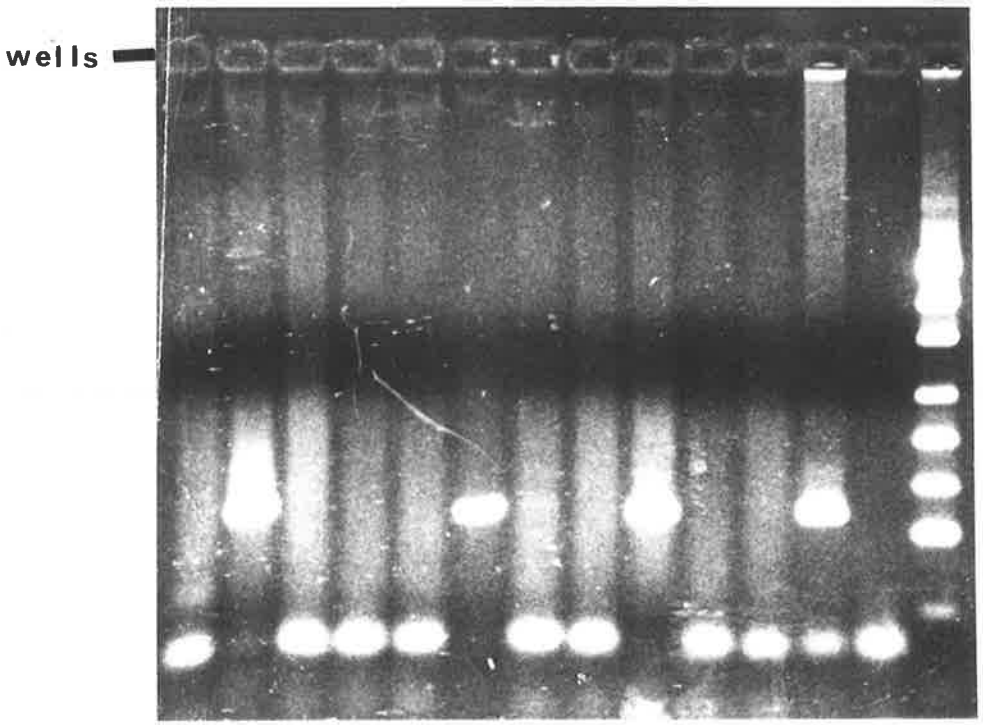
EcoRI digested (+) and undigested (-) DNA samples from transgenic mice and human (Hum.) positive and mouse (Mur.) negative controls.

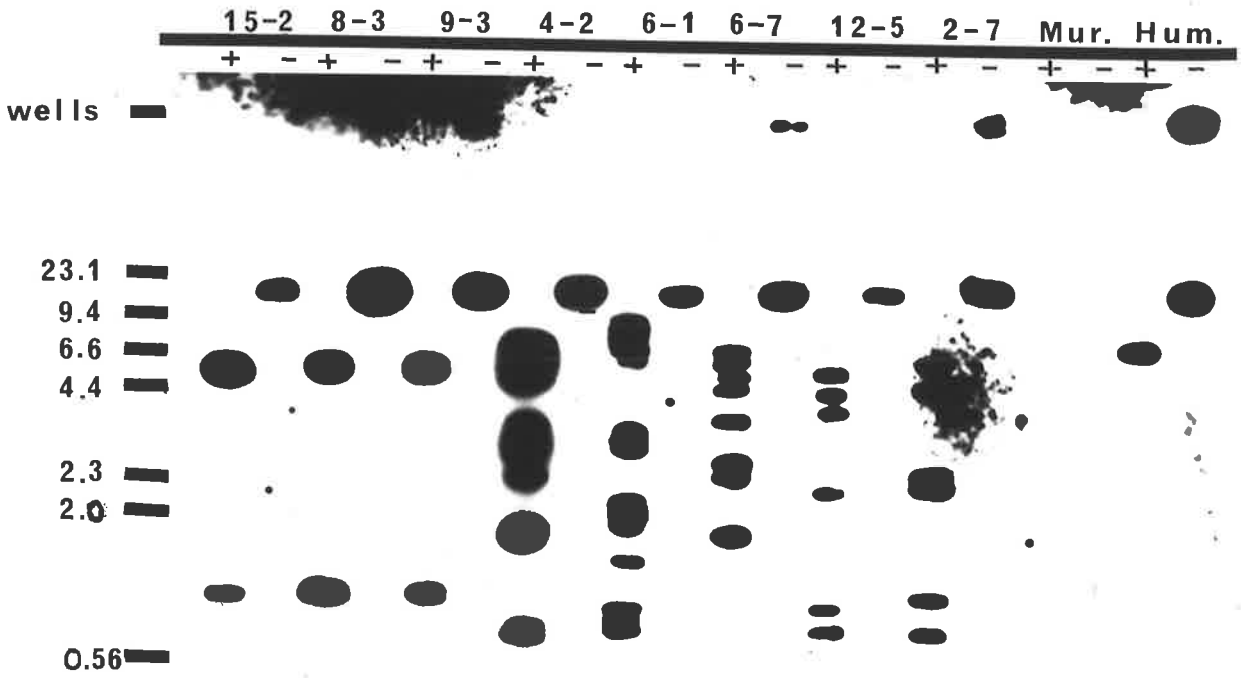
Legend from left to right to identify samples is shown below

Track 1	15-2	1 HT (R.E.)	Track 6	6-7	1 R (C)
Track 2	8-3	M HT (UV)	Track 7	12-5	1 HH (R.E.)
Track 3	9-3	M HT (C)	Track 8	2-7	M HH (H)
Track 4	4-2	M R. (H)	Track 9	Murine (Mur.) negative	
Track 5	6-1	M R. (UV)	Track 10	Human (Hum.) positive	

1 or M	single or multiple copies	(UV)	ultraviolet
HH	head to head arrangement	(H)	hoechst stain
HT	head to tail arrangement	(C)	control
R	rearranged arrangement	(R.E.)	restriction enzyme


wells


wells


15-2
8-3
9-3
4-2
6-1
6-7
12-5
2-7
Mur.
Hum.
wells
23.1
9.4
6.6
4.4
2.3
2.0
0.56

Further analysis for integration pattern using an EcoRI digest of the DNA produced banding patterns corresponding to head to tail arrangements, head to head arrangements as well as a number of rearranged sequences. Similarly there was no significant difference ($p>0.05$) between any one type of arrangement with respect to treatment group.

3.5 Discussion

The need to increase the efficiency of producing transgenic animals, with enhanced gene integration and controlled transgene expression has been clearly identified (Gordon, 1989; Pursel *et al.*, 1990; Weighart *et al.*, 1990). This study identifies several possible mechanisms whereby this may be achieved following pronuclear injection in the mouse. Brinster *et al.*, (1985) identified the form and concentration of the transgene and site of injection for the DNA as critical factors in determining efficiencies in the mouse. Optimal conditions for integration entailed injection of a few hundred linear molecules into the male pronucleus of a zygotes. On the basis of these findings Brinster *et al.*, (1985) proposed the rate limiting step to integration was breakage of chromosomal DNA. Enhanced integration should follow if breaks or areas of relaxation in chromosomal DNA are initiated. Hoechst stain relaxes DNA structure, which subsequently could increase the sites available for integration for microinjected DNA. Both the restriction enzyme and UV-light are likely to induce conditions for increased integration by causing chromosomal breaks. Using the L_D-50 dose would overcome limitations expected to occur as a result of other enzymes repairing Hind III ends or UV-induced breaks or removing Hoechst-DNA complexes. However ultraviolet light is known to damage DNA. Postel, (1985) reported that ultraviolet light enhanced the frequency of stable plasmid transformation in several different cell types. However he found that, there was no difference in integration frequency between treated and untreated cells, instead enhancement was due to an increase in the competent (transformable) cell population. Using and established L_D-50 dose,

Ultraviolet light (254 nm) increased the frequency of integration regardless of transgenes used, while the data on the use of restriction enzymes and Hoechst stain appeared to show an increase in integration that was dependant on the transgene used. It is of importance to note that the succcss of these procedures was dependant on the transgene being integrated under normal microinjection procedures.

Southern analysis of the transgenic DNA indicated that a variety of integration patterns were recorded that were similar to those documented by other research workers (Gordon *et al.*, 1981; Hammer *et al.*, 1984; Palmiter *et al.*, 1983). However, no significant differences were found with respect to integration site or arrangement when using any of the treatments. Surprisingly, there was no increase in multiple site integration for treatment with ultraviolet light and restriction enzymes. These observations do not support the proposal of Brinster *et al.*, (1985) where the rate limiting step for integration was breakage of the chromosomal DNA that allowed insertion of the linear transgene. The fact that increases in integration when using Hoechst stain and restriction enzymes with certain transgenes warrants further investigations.

The overall impression gained from these observations is that chromosomal breaks may not directly influence the number of genes integrated. Where increased integration occurred the cleavage rate of the first cell cycle was delayed indicating disruptions of the mechanisms controlling embryo cleavage. This delay was restricted to the first cell cycle and subsequent development occured at the normal rate. Recent insights into control of the mitotic phase of development is centred on the evolutionary conserved protein kinase $p34^{cdc2}$ which precisely controls the orderly cell division of embryogenesis (see reviews, Nurse, 1990; O'Farrell *et al.*, 1989). The protein kinase is itself controlled by a number of regulatory proteins (O'Farrell *et al.*, 1989) geared to an appropriately timed pulse that triggers mitotic events. Activation of the kinase involves phosphorylation via a histone H1 kinase or mitosis promoting factor (MPF) which interact with

cyclins to control the major M-phase events, including chromosome condensation, cytoskeletal reorganization, nuclear envelope breakdown and cell shape changes (Nurse, 1990). Additionally, the Poly (ADP -ribosylation) complex is likely to significant role as it controls dramatic post-translational modifications of histones and several other proteins including the enzyme poly (ADP-ribose) polymerase (Boulikas, 1990). Poly (ADP-ribose) polymerase activity increases following damage of DNA (Boulikas, 1986). Poly (ADP-ribose) polymerase displays a strong preference for free ends of DNA and remains inactive unless stimulated by single or double stranded breaks on the DNA, once activated the enzyme modifies nucleosomal histones in the vicinity of the DNA cuts (Boulikas, 1990). Farzaneh, F. *et al.*, (1988) showed Poly (ADP-ribosylation) complex is required for the integration of donor DNA into the host genome and suggest its involvement in eukaryotic DNA recombination events. Therefore, induced DNA breaks cause nuclear modification where ADP-ribose moieties from NAD^+ are enzymatically transferred to proteins, whereby they deplete the cell of NAD(P) and ATP and slow the cell development rate. Speculatively then, the rate limiting step of integration may not be solely dependant on chromosomal breaks but include, at least impart repair mechanism which delay the signals for activation of the protein kinase and subsequent mitotic events. The increase in cell cycle time resulting from Ultraviolet exposure may preferentially allow increased integration after DNA microinjection, this however was not tested.

In summary, agents which effect DNA structure can increase the frequency of creating transgenic animals, Ultraviolet light significantly increased integration regardless of the transgene used while Hoechst stain and restriction enzymes only appeared to effect integration with certain transgenes. Interestingly the integration site and transgene arrangement did not vary significantly with any of the treatments and was similar to those previously reported for untreated microinjected zygotes. The observations are interpreted as indicating that chromosomal breaks are not the sole

determinant of rate of integration, but that repair mechanism associated with embryo development and length of cell cycle may be more important.

CHAPTER IV

OESTROUS SYNCHRONIZATION AND SUPEROVULATION PROCEDURES SUITED TO TRANSGENESIS PROGRAMS IN THE PIG

4.1 Introduction

Studies concerning embryo manipulation and gene injection require the procurement of a large source of viable specific stage porcine zygotes. Various protocols have been developed to obtain synchronization of oestrus in pigs. These include (1), treatment of prepubertal gilts with PMSG to induce a timed puberty (see review, Paterson, 1982; Pinkert *et al.*, 1989), (2), utilizing the post weaning oestrus in mature sows (Longenecker and Day, 1968), (3), induction of luteolysis by prostaglandins (cloprostenol) in animals with an extended luteal phase (Guthrie and Polge, 1978), (4), establishing an artificial luteal phase in animals through feeding a safe, orally activated progestational agent (Altrenogest) for approximately 17 days (Polge, 1982; Martinat-Botté *et al.*, 1990) (5), timed gonadotrophin treatment (day 17 (PMSG), day 20 (HCG)) during monitored oestrous cycles (see review Paterson, 1982). Other procedures based on the use of progesterone and synthetic progestational compounds have been largely abandoned because of the association with cystic follicles, lack of synchronization and reduced fertility at the next cycle (see review, Webel and Day, 1982). Methallibure, an inhibitor of pituitary gonadotrophic function, which was also used, in conjunction with gonadotrophins (Polge *et al.*, 1968) is prohibited because of teratogenic activity (see review, Webel and Day, 1982). In the present study an alternative procedure was developed on the basis of administering cloprostenol to synchronize oestrus in the readily available young gilt and this procedure was used in all subsequent experiments unless specified in the text.

As the pig corpora luteum is refractory to the luteolytic action of Prostaglandin (PG) $F_{2\alpha}$ except on days 11-12 of the oestrous cycle (Gleeson, 1974; Hallford *et al.*, 1975; Guthrie and Polge, 1976), little use has been made of this approach which is widely employed in ruminant livestock species. Several techniques have been devised to extend the luteal sensitive phase, including treatment with oestrogen (Guthrie, 1975; Kraeling and

Rampacek, 1975) and gonadotrophins to induce accessory Cl (Guthrie and Polge, 1976) and treatment during pregnancy (Guthrie and Polge, 1978). Pregnancy has the advantage that the period of sensitivity can be extended up to 45 days allowing animals to be accumulated for treatment.

To enhance the ovulation rate numerous workers have reported on the use of gonadotrophins as a means to stimulate follicle development in gilts (see review, Paterson, 1982) and identified a positive correlation between the number of stimulated follicles and dose of gonadotrophin (Baker & Coggins, 1966; Phillippo, 1968; see review, Paterson, 1982). The ova produced by this means have been shown to be capable of fertilization and yield viable offspring (Dziuk and Polge, 1965). Typically, doses of 500-2,000 i.u. PMSG, followed by 48 to 96 hours later with 500 i.u. of HCG are utilized and result in induced ovulation in up to 100% of gilts over a range of ages and weights.

The aims of this study were to examine the efficiency of a procedure based on treatment of pregnant gilts with synthetic prostaglandin $F_{2\alpha}$, Cloprostenol together with timed gonadotrophins treatment as a means of obtaining pronuclear porcine zygotes required for DNA microinjection.

4.2 Materials and Methods

Experiments were conducted at the Northfield Pig Research Unit, Department of Agriculture using Large White postpubertal gilts.

4.2.1 Method of Oestrous Synchronization and Superovulation

Postpubertal donors (231 $\pm$ 29 days) were mated on the first detected oestrus and confirmed pregnant by non return to oestrus (days 18-22) and estrone sulphate (days 23-36). Cessation of luteal function and the subsequent abortion was induced by two prostaglandin (PG) intramuscular injections (1.0, 0.5 mg Cloprostenol, Estrumate, Coopers Animal Health Australia Ltd.) 24 hours apart and treated with PMSG and HCG before being mated (Day 0) either naturally or by artificial insemination.

4.2.2 Experimental Procedures

4.2.2.1 Prostaglandin Synchronization

Cloprostenol treated postpubertal gilts at either 25-40 (n=30), 41-70 (n=19) or 70-105 (n=15) days of pregnancy were superovulated with PMSG (i.m.), simultaneously with the second PG treatment and ovulation was synchronized 72 hours later with 500 i.u. HCG (i.m.). Animals were observed on day 6 (post PG) for return to oestrus.

4.2.2.2 Gonadotrophin Superovulation

The effect of varying the PMSG dosages (500 i.u., n=15; 750 i.u., n=17; 1000 i.u., n=34; 1250 i.u., n=16 and 1500 i.u., n=12) on follicular development after treatment with Cloprostenol was examined. Gonadotrophin HCG was administered 72 hours after PMSG. Animals displaying standing oestrus were mated 32 hours post HCG. The optimal timing of HCG administration to synchronize ovulation was examined in a second study. In this study, all gilts received PG, PMSG and 500 i.u. of HCG (i.m.) at 66 (Treatment 1, n=31), 72 (Treatment 2, n=36) or 78 (Treatment 3, n=34) hours after PMSG injection.

4.2.2.3 Observations

A midline laparotomy (Chapter 2.3) was performed 15-20 hours post mating. Ovaries were exteriorised and the number of corpora lutea recorded. Day 1 zygotes were collected surgically, centrifuged (3,000xg, 6 min) and morphologically assessed under differential interference contrast optics for the presence of pronuclei. Zygotes without visible pronuclei were stained in Hoechst 33342 (see Chapter 2.9).

4.3 Statistical Analysis

Return to oestrus was defined as standing heat for a period of at least 24 hours in the presence of a boar. The superovulation and

synchronization effects of gonadotrophins PMSG and HCG on embryo morphology after synchronizing oestrus with cloprostenol were analysed using ANOVA (SAS Institute Inc., 1988).

4.4 Results

4.4.1 Oestrous Synchronization using Cloprostenol

Cloprostenol proved equally efficient at all stages of pregnancy in gilts in causing return to oestrus in the presence of a boar (See Table 4.1).

Table 4.1 Number of postpubertal gilts showing return to oestrus after treatment with Cloprostenol at various stages of pregnancy.

Stage of Pregnancy (days)	Total No. (N)	Detectable oestrus (5 days post PG) (%)
25-40	30	24 (80)[a]
40-70	19	16 (84)[a]
70-105	15	13 (86)[a]

[a]Values do not significantly differ (χ^2; $p>0.05$).

4.4.2 Gonadotrophic Superovulation with PMSG after Oestrous Synchronization with Cloprostenol

The effect of PMSG on follicle type is summarised in Table 4.2. The dose of PMSG has a significant effect on the mean number of Cl, unovulated and cystic follicles. The effect of PMSG dose on follicle type can be seen in Plate (2). A significant difference ($p<0.05$) between mean Cl numbers were observed from PMSG doses of 1500 i.u. (40.1) 1250 i.u.. (34.3) and 1000 i.u. (27.3) when compared with 750 i.u. (14.8) and 500 i.u. (14.1).

The mean number of cystic follicles tended to decrease with decreasing amounts of PMSG. The comparison of two groups of PMSG

doses, (1) 1250 i.u., 1000 i.u. and 750 i.u. and (2) 100 i.u., 750 i.u. and 500 i.u. were not significantly different ($p>0.05$) between mean numbers of cystic follicles, however there was a significant difference ($p<0.05$) between the mean number of cystic follicles resulting from treatment with 1250 i.u. and 500 i.u. of PMSG.

TABLE 4.2 RESPONSE OF PMSG ON NUMBER AND TYPE OF FOLLICLES AFTER OESTROUS SYNCHRONIZATION WITH CLOPROSTENOL.

Dose of PMSG (i.u.)	Donors (N)	Total No. of Follicle types. C. luteum (mean)	Unovulated (mean)	Cystic (mean)
500	15	211 (14.0)^{c}	19 (1.27)^{b}	3 (0.20)^{b}
750	17	252 (14.8)^{c}	29 (1.76)^{b}	5 (0.88)^{b}
1000	34	930 (27.4)^{b}	39 (1.15)^{b}	48 (1.41)^{b}
1250	16	549 (34.3)^{a}	37 (2.33)^{b}	32 (2.0)^{b}
1500	12	481 (40.0)^{a}	71 (5.92)^{a}	87 (7.25)^{a}

^{abc}Values differ significantly (ANOVA; $p<0.05$)

4.4.3 The Effect of HCG Timing on the Morphology of Embryos after Oestrous Synchronization and Superovulation with Cloprostenol and PMSG

Table 4.3 is summary of results on the morphology of embryos using HCG after Oestrous Synchronization and Superovulation with Cloprostenol and PMSG.

There was a significant ($p<0.05$) effect of PMSG on the mean number of fertilized, unfertilized and retarded embryos but not of timing of HCG or the interaction of PMSG and HCG ($p>0.05$). However, the proportion of 2-cell embryos was significantly ($p<0.05$) influenced by the combined effect of PMSG and timing of HCG.

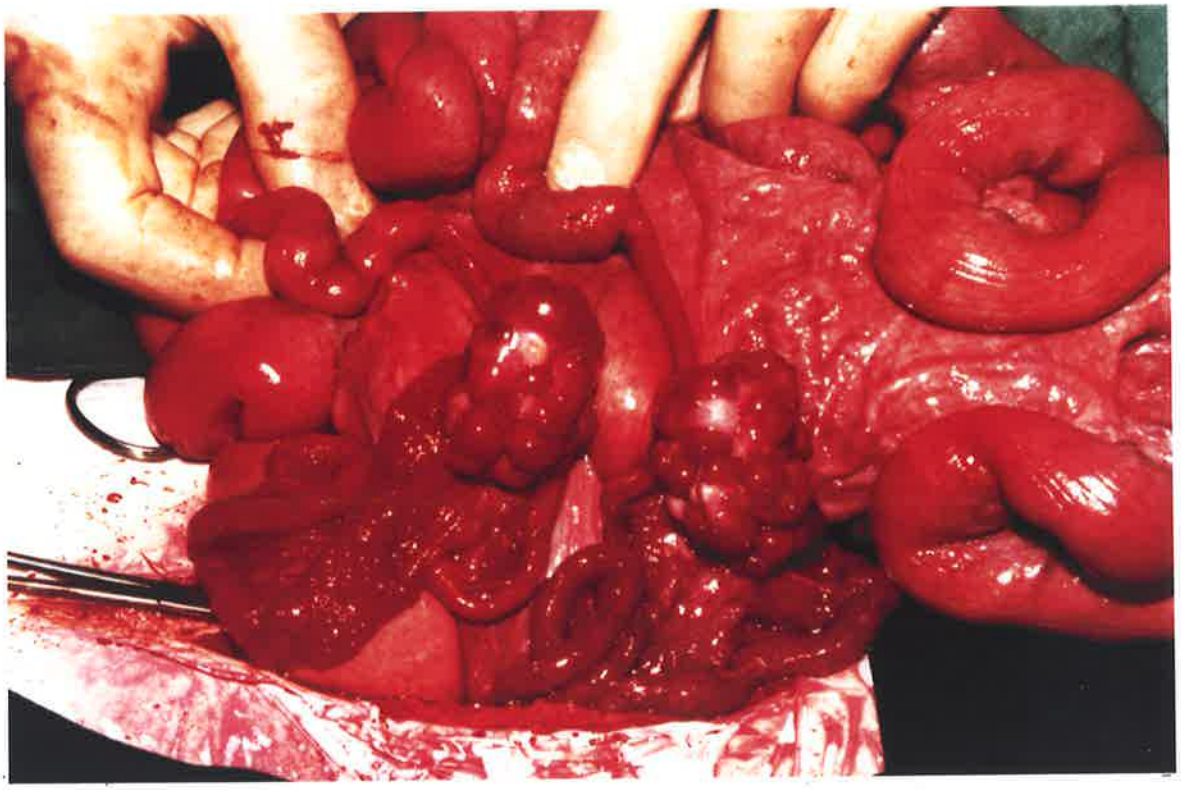
PLATE 2

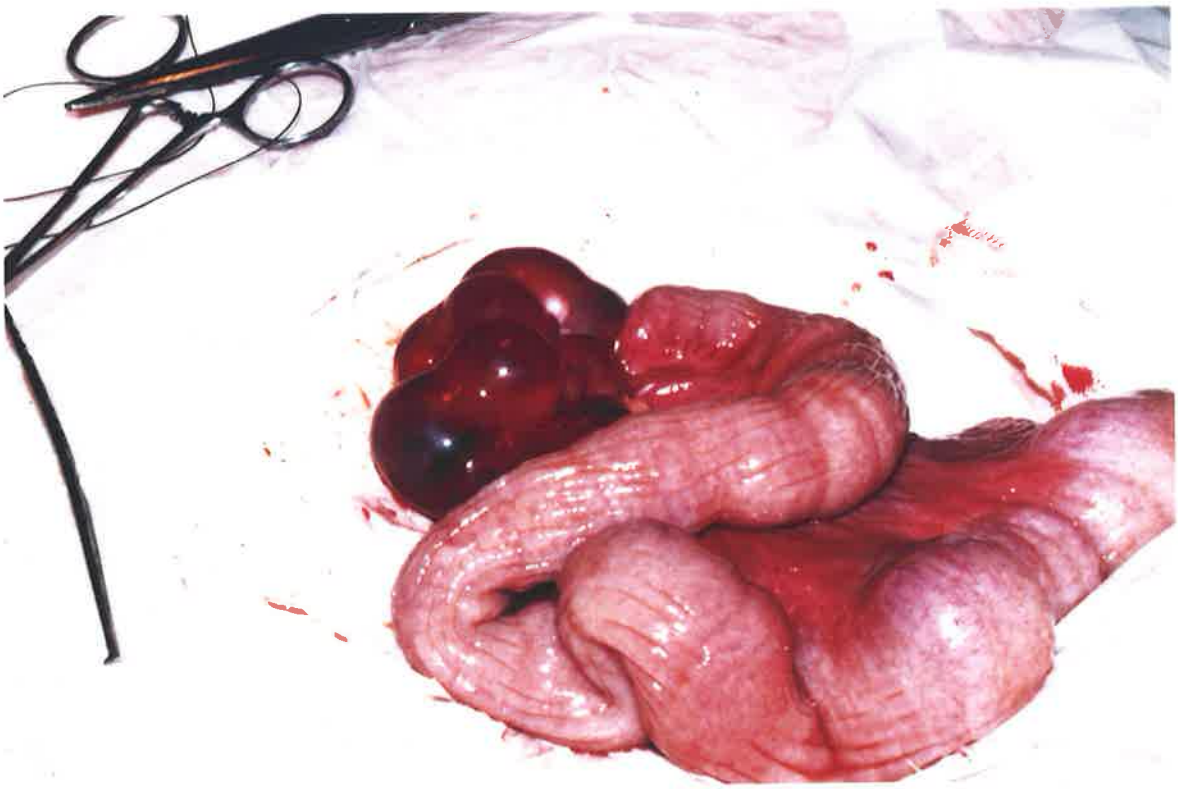
Photograph 1.

Superovulation response in gilts treated with 1000 i.u. of PMSG and cloprostenol

Photograph 2

Cystic follicles after superovulation in gilts with 1500 i.u. of PMSG and Cloprostenol





The highest yield of fertilized embryos was dependent on the dose of PMSG and was not influenced by the interaction of HCG timing as shown with 1000 i.u. of PMSG (mean, 9.4) and 1500 i.u. dose (mean, 9.0). Reducing the dosage to 500 i.u. significantly ($p<0.05$) reduced the yield of fertilized embryos (mean, 5.8). Significantly ($p<0.05$) more unfertilized embryos (mean, 11.75) were obtained from 1500 i.u. of PMSG than 1000 i.u. (mean, 2.39) or 500 i.u. (mean, 0.5). Also the 1500 i.u. dose of PMSG resulted in a significantly ($p<0.05$) higher proportion of retarded embryos (mean, 2.2) compared to 1000 i.u. (mean, 1.03) and 500 i.u. (mean, 0.45) dosages. The yield of 2-cell embryos was similar for all PMSG dose (500 i.u., mean 0.55; 1000 i.u., mean 0.5; 1500 i.u., mean 0.33).

Further analysis combining groups for the different PMSG doses indicated that the timing of HCG had no significant ($p>0.05$) effect on the proportion of embryos that were fertilized (mean values, Tmt 1, 6.97 vs Tmt 2, 8.9 vs Tmt 3, 8.38) or were not fertilized (mean values, Tmt 1, 6.9 vs Tmt 2, 5.80 vs Tmt 3, 4.59). However it did influence the yield of 2-cell embryos (Tmt 3 (mean, 1.95) > Tmt 1 (mean, 0.55) > Tmt 2 (mean, 0.23)) and may have influenced the proportion of retarded embryos (Tmt 1 (mean, 1.74) > Tmt 2, (mean, 0.93) ($p<0.05$)). No significant ($p>0.05$) differences were seen between Tmt 1 (mean, 1.74) and Tmt 3 (mean, 1.19) as well as Tmt 2 (mean, 0.93) and Tmt 3 (mean, 1.19).

TABLE 4.3 THE EFFECT OF HCG TIMING IN RELATION TO PMSG AFTER OESTROUS SYNCHRONIZATION WITH CLOPROSTENOL ON EMBRYO MORPHOLOGY AND QUALITY.

PMSG	HCG Tmt.	Donors	Embryos Collected	Retarded.	Fertilized (Average number of embryos per donor)	Unfertilized.	2-cell.
500	Tmt. 1	9	78	7 (9)	48 (62)	21 (27)	1 (2)
	Tmt. 2	12	105	4 (4)	74 (72)	18 (18)	4 (4)
	Tmt. 3	10	89	3 (3)	67 (75)	8 (8)	11 (13)
1000	Tmt. 1	10	154	14 (9)	83 (54)	53 (34)	4 (3)
	Tmt. 2	15	231	14 (6)	150 (65)	62 (27)	5 (2)
	Tmt. 3	11	163	9 (5)	109 (67)	36 (22)	9 (65)
1500	Tmt. 1	12	268	33 (12)	86 (32)	137 (51)	12 (4)
	Tmt. 2	13	306	19 (6)	136 (44)	151 (50)	0
	Tmt. 3	11	230	26 (11)	101 (44)	103 (45)	0

Tmt. 1 = HCG (500 iu) injection at 66h post PMSG
Tmt. 2 = HCG (500 iu) injection at 72h post PMSG
Tmt. 3 = HCG (500 iu) injection at 78h post PMSG

4.5 Discussion

The protocol for gilts developed provides a convenient and reliable means of timing ovulation for the collection of pronuclear zygotes which compares favourable with protocols reported by other workers (Dziuk and Baker, 1962; Dziuk *et al.*, 1964; Hunter, 1964,1966; Buttle and Hancock, 1967). Following treatment with Cloprostenol pregnant animals returned to oestrus at frequencies between 80% and 86%. There was no significant difference between the stage of pregnancy and the efficiency achieved. However for ethical reasons, the acceptable compromise for prostaglandin treatment for oestrous synchronization is probably best restricted to days 25-40 of pregnancy.

The frequency of animals displaying oestrus is comparable with other research workers using the same and different techniques. Guthrie and Polge, (1978) showed 87% of gilts exhibited a synchronized oestrus 4-7 days after the first PG injection and Pressing *et al.*, (1987) showed between 91% of midgestation sows (60 to 90 days) returned to oestrus approximately 6 days after treatment with cloprostenol and continued with a normal oestrous cycle. Regumate-treated gilts displayed oestrus up to an observed frequency of 100%, 4 to 7 days after removal of the prostagen from the diet (Busch *et al.*, 1988; Davis *et al.*, 1985; Davis, 1986; Day, 1984; Johnston, 1988; Kraeling *et al.*, 1981; Martinat-Botté *et al.*, 1985; Mauleon *et al.*, 1979; Pursel, 1981; Stevenson and Davis, 1982; Varley, 1983; Webel and Day, 1982; Webel *et al.*, 1980). Using the now prohibitive agent for oestrous synchronization, Methallibure and gonadotrophins 93-95% of gilts displayed oestrus between days 4 and 5 (Christenson *et al.*, 1973). Oestrous synchronization using post-weaning sows and gonadotrophins PMSG and HCG resulted in 69 to 100% of animals displaying oestrus (Day *et al.*, 1965,1967; Christenson and Teague, 1975; Longenecker and Day, 1968). The highest incidence of observed oestrus following treatment of prepubertal gilts with gonadotrophins (75%) was reported by Dziuk and Dhindsa, (1969), although it should be noted that a wide variation with respect to the

percentage of gilts that ovulated and displayed oestrus has been reported (see review, Paterson, 1982). Subsequently the procedure requires careful observation to detect the onset of oestrus and is therefore not suitable for collection of zygotes for DNA microinjection.

With alternative procedures such as Regumate only 45% of gilts display oestrus on day 5 with the majority of oestrus occurring on days 6 and 7 (Martinat-Botté *et al.*, 1990; Pursel *et al.*, 1981). Whilst PMSG and HCG can be used to offset some of the limiting effects such as follicle maturation, ovulation and corpus luteum formation (Guthrie and Bolt, 1983,1985), it fails to offset the mechanisms by which oestrogen triggers the preovulatory LH surge and oestrus. Induction of superovulation in Regumate treated-animals by means of PMSG and HCG is highly variable (Polge, 1981). Guthrie *et al.*, (1988) showed that substitution of purified FSH for PMSG in Regumate treated-gilts increased the number of follicles 3-6 mm in diameter, but failed to induce changes in plasma concentrations of LH, oestrogen or progesterone or influence the number of follicles 1-2 mm. or 7-8 mm. in diameter.

The inability to detect the silent or overt oestrus in animals treated with PMSG and HCG on days 17 and 20 respectively may have directly influenced the observed lower frequency of animals displaying oestrus. Post weaning provides a reliable oestrous synchronization method but the large size of the sow introduces further complications to surgical procedures involved in the collection of early stage embryos.

A positive correlation exists between the PMSG dosages and the response in terms of follicle numbers which are stimulated to develop (see review, Esbenshade *et al.*, 1990). This is evident in numerous studies concerned with the induction of superovulation using prepubertal, regumate treated, prostaglandin treated, post weaning and oestrous managed donors (Davis *et al.*, 1986; Day and Longenecker, 1968; Day *et al.*, 1965, 1967; Dziuk and Baker, 1960; Christenson *et al.*, 1973; Guthrie and Polge, 1978; Karalus *et al.*, 1990; Polge *et al.*, 1968; Pope *et al.*, 1968, 1972; Pressing *et*

al., 1987; Srikandakumar and Downey, 1989; see review, Paterson, 1982; see review, Polge, 1982; see review, Webel and Day, 1982; Webel *et al.*, 1970; Welp and Holtz, 1988).

Increasing the dosage of PMSG results in an increase in the number of unovulated and cystic follicles but detailed comparison with previous studies (see review, Paterson, 1982) is constrained by interactions related to genetics, breeds, age, weight and environment. Whilst a cheap and convenient source of gonadotrophin, PMSG has acted as a consistent source of frustration to workers in this field due to the highly variable responses seen when using superovulatory dosages. A variety of genetic and environmental factors undoubtedly contribute to this but related contributions are difficult to determine as much of the data procured is more often circumstantial than experimental (Bindon and Piper, 1982). Saumande *et al.*, (1978) showed a large variation in the antral follicle population of sheep that responded to PMSG.

Batches of PMSG vary in their content of FSH and LH like activities (Wollen, 1985) and the variation in intrinsic LH activity of PMSG may contribute to the number of cystic and unovulatory follicles (Bindon and Piper, 1982). In response to the LH like content of PMSG, follicles are exposed to this gonadotrophin at a much earlier stage of their development than normal (Cahill, 1982) and their precocious and possible inappropriate response could interfere with normal endocrine regulator mechanisms.

In this study the dosages of PMSG had a significant effect on the yield of fertilized, unfertilized and retarded embryos. The high proportion of unfertilized embryos observed with higher doses of PMSG could possible be due to an abnormal endocrine environment induced by PMSG. This response could influence sperm transport and embryo quality by producing an asynchrony between ova and sperm at the time of fertilization (Dziuk and Polge, 1965). Including HCG to time ovulation influenced the proportion of 2-cells which is undesirable because of the possibility of chimera production when using gene injection. The increased prevalence of 2-cell embryos with

Tmt 3 (78 hours post PMSG) indicates interaction with the natural endogenous surge of LH. In order to control ovulation it would be desirable to inject exogenous LH or induce an endogenous LH surge via GnRH just prior to any normal endogenous surge which probably (not tested) occurs between 66 and 72 hours post PMSG and is dependant on dose, age of animal and reproductive status.

In summary cloprostenol is shown to be a useful means of synchronizing oestrus at any stage of pregnancy in the gilt. No detrimental effect on subsequent oestrous cycles or fertility were evident and animals can be used as both donors and recipients. Combined with gonadotrophins to induce a controlled superovulation in the gilt it provides a useful means of obtaining zygotes. The maximum number of fertilized zygotes and minimal number of retarded, unfertilized and 2-cell embryos were obtained 15-20 hours post HCG with the procedure devised in which pregnant sows (25-40 days) were treated with 1000 i.u. of PMSG followed 72 hours later by 500 i.u. of HCG.

CHAPTER V

COMPARISON OF EMBRYO DONORS AS A SOURCE OF ZYGOTES FOR DNA MICROINJECTION

5.1 Introduction

Maximising the efficiency of producing transgenic animals requires fundamental embryological research to obtain a reliable source of viable ova. The ova produced must be capable of maintaining viability in supportive media from the time of collection until return to recipients. Successful superovulation procedures are dependent upon specific timing of the oestrous cycle in the gilt or sow. The most readily available pig is the prepubertal gilt. The yield of zygotes from prepubertal gilts when subjected to superovulation procedures is similar to those from mature gilts. However the reproductive performance following mating at the induced oestrus is poor (Pinkert *et al.*, 1989) and embryos obtained from prepubertal donors fail to develop to term when transferred to mature cyclic recipients sows.

Numerous workers have shown that when prepubertal gilts displaying induced oestrus are mated there is a great variation in the farrowing rate and litter size (Paterson, 1982), though acceptable rates have been obtained 67% (Dziuk and Dhindsa, 1969) and 80% (Holtz *et al.*, 1977). The failure of pregnancy in prepubertal gilts induced to ovulate with PMSG usually occurs around day 25 of gestation and is attributed to the premature regression of the corpora lutea (Shaw *et al.*, 1971; Ellicott *et al.*, 1973). The failure of prepubertal gilts to maintain cyclic activity is attributed to many factors, including an inability to maintain progesterone secretion (Shaw *et al.*, 1971; Segal and Baker, 1973; Rampacek *et al.*, 1976b; Kineman *et al.*, 1987a ,1987b), an imbalance in the luteotropic-luteolytic complex (Rampacek *et al.*, 1979), variations in numbers of LH and HCG receptors (Estienne *et al.*, 1988) and ovary shape (Grasso *et al.*, 1988), age/weight dependency (Karalus *et al.*, 1990), increased sensitivity to prostaglandin $F_{2\alpha}$ and decreased sensitivity to gonadotrophins (Puglisi *et al.*, 1978, 1979). However, the cyclic activity of prepubertal gilts can be restored and maintained by administration of steroids or gonadotrophins (Shaw *et al.*, 1971; Ellicott *et al.*, 1973; Segal and Baker, 1973; Rampacek *et al.*,

1979). Only limited information is available on zygote viability, independent of the influences of the female reproductive tract. Evidence suggests that zygotes obtained from some superovulation procedures do not possess the same capacity for *in vivo* and *in vitro* development.

The aims of this study were (1) to compare morphologically zygotes collected from prepubertal, postpubertal, mature and abattoir donors, (2) to examine the viability of microinjected zygotes after transfer to synchronised recipients.

5.2 Materials and Methods

Experiments were conducted at Northfield Pig Research Unit and Metro Farms Piggery.

5.2.1 Method of Superovulation

Postpubertal donors were treated with Cloprostenol and injected with PMSG (1000 i.u.) and HCG (500 i.u., 72 hours post PMSG) to increase and control ovulation (see Chapter 4.2.2, for details of treatment). Animals in oestrus were mated naturally or subjected to artificial insemination 32 hours after HCG. Prepubertal donors were treated with PMSG, HCG and mated as per postpubertal donors (see Chapter 4.2.2, for details of treatment). Mature donors were selected from a group of multiparous, culled for age females. Oestrous cycles were synchronised by using the time of weaning. Donors were treated with PMSG (1000 i.u.) on the day following weaning, followed 44 hours later by HCG (500 i.u.) and mated as per postpubertal gilts 32 hours after HCG. Abattoir donors were monitored for oestrus and treated with PMSG (1000 i.u., Day 15), HCG (500 i.u., Day 18) and mated 32 hours after HCG (Polge, 1981).

5.2.2 Surgical Collection

Zygotes were collected by mid-ventral laparotomy approximately 45-50 hours after HCG treatment. Details of the relevant protocols for zygote collection and surgical procedures can be found in Chapter 2.3. Mated abattoir donors were slaughtered by electrical stunning and exsanguination, 46-52 hours after HCG treatment. Reproductive tracts were removed within 30 minutes of death and zygotes were collected in a similar manner to surgical operations within the next 60 minutes. After morphological assessment, embryos from several donors were pooled to eliminate effects of individual donors.

5.2.3 Zygote Assessment

Zygotes collected were recovered from flushings, washed twice and stored in HEPES-MEM until required. Morphological examination of zygotes was achieved by centrifugation and assessment under DIC optics. Zygotes were microinjected with variants of the human metallothionien constructs (see Chapter 2.13, for details). Zygotes without visible pronuclei were rechecked for pronuclei 4 hours later before culturing overnight. The following morning zygotes were checked for cleavage and stained in Hoechst 33342 stain (see Chapter 2.9, for details).

5.2.4 Zygote Transfer

Microinjected zygotes were surgically transferred to synchronised recipients either immediately or after 24 hours of culture. Recipients were synchronised (see Chapter 2.2, for details of treatments), prepared surgically (see Chapter 2.6, for details) and monitored for pregnancy (see Chapter 2.7, for details). Due to farrowing times and the risk associated with farrowing it was necessary in unusual cases to implement a delay in farrowing. Problem recipients showing imminent signs of farrowing during the night were injected with Clenbuterol (5ml, i.m., Planipart, Boehringer

Ingelheim Pty. Ltd., Australia) to cause a relaxation of the uterus for approximately 10 hours, after which normal farrowing occurred. If induction was required, Oxytocin (1.5ml, i.m., Heriot AgVet Pty. Ltd., Australia) was given in conjunction with a β-Adrenergic blocker (Suacron, Carazolol, 4ml. i.m.) on the day preceeding Clenbuterol injection (pers. comm. M. Spicer, Dept. of Agriculture, Bendigo, Australia). Savaging by recipients was controlled by treatment with Azaperone (Stresnil, Jansen Pharmceutica, Belgium).

5.3 Statistical Analysis

Comparison were made on the proportion of viable embryos obtained from four different donor sources for the purpose of microinjection. Further comparison were made on each embryos ability to survive after transfer. Observations were analysed using the chi-square statistics (SAS Institute Inc., 1988).

5.4 Results

5.4.1 Detection of Oestrus

There was no significant difference between the number of animals displaying oestrus between days 4 and 6 after synchronization with either PMSG or prostaglandin in each of the four donor groups (87% prepubertal, 92% postpubertal, 85% mature and 82% abattoir).

5.4.2 Comparison of Embryos Collected from Different Donor Procedures.

Table 5.1 summarises the data concerning embryos obtained from prepubertal, postpubertal, mature and abattoir donors.

The average number of embryos collected was highest in prepubertal (18.6) and postpubertal (17.7) donors compared with mature (8.2) and abattoir (7.4) donors following superovulation with gonadotrophins.

Differences reflecting the fertilization rate were evident between the yield of viable embryos derived from prepubertal and postpubertal animals. However, the rate of fertilization was comparable in mature (69%), postpubertal (65%) and abattoir (57%) donors. The average fertilization rate of 64% for animals having attained puberty was significantly different when compared to prepubertal donors (41%) ($p<0.05$). The difference between fertilization rate was inversely proportional to the percentage of embryos unfertilized; prepubertal (56%), post pubertal (28%), mature (25%) and abattoir (38%). There was no significant difference between the number of retarded embryos; prepubertal 3%, postpubertal 6%, mature 6% and abattoir 5%.

5.4.3 *In Vivo* Viability of Microinjected Embryos from Different Donor Groups.

The number of animals confirmed pregnant and average litter size for each of the donor groups are shown in Table 5.2. The pregnancy rate was significantly different between recipients receiving microinjected embryos from different donor groups. The highest pregnancy rate of 100% was observed in postpubertal donors compared with 60% in prepubertal, 33% in mature and 14% in abattoir.

The embryo survival rate for all embryos transferred was highest (29%) for embryos obtained from postpubertal gilts compared to 5% for prepubertal, 4% for mature and 3% for abattoir. It is of interest to note that the embryo survival rate was similar in prepubertal (9%), mature (9%) and abattoir (11%) when the pregnancy status of the recipient was known.

TABLE 5.1 VIABILITY OF ZYGOTES COLLECTED FROM PREPUBERTAL, POSTPUBERTAL, MATURE AND ABATTOIR DONORS.

Donor Group	Donors (n)	Embryo Collected (Average±S.E.)	Fertilized (%)	Retarded (%)	Unfertilized (%)
pre-pubertal	28	520 (18.6±10.2)	214 (41)	17 (3)	289 (56)
post-pubertal	41	726 (17.7±9.8)	473 (65)	47 (7)	206 (28)
mature	21	164 (7.8±6.4)	116 (69)	6 (6)	42 (25)
abattoir	19	149 (7.8± 6.2)	85 (57)	8 (5)	56 (38)

TABLE 5.2 VIABILITY OF MICROINJECTED ONE-CELL EMBRYOS AFTER TRANSFER.

Source of Zygotes	No. of Transfers (sows,n)	Preg. Testing +ve (%)	Avg. Litter Size (n)
pre-pubertal	5	3 (60)	2 (3 live:3 dead)
post-pubertal	9	9 (100)	6 (47 live:5 dead
mature	6	2 (33)	3 (5 live:1 dead)
abattoir	7	1 (14)	3 (3 live:)

(A total of 26±5 embryos were transferred to synchronised postpubertal recipients)

5.5 Discussion

Comparison were made between prepubertal and postpubertal sows as a source of embryos for microinjection. Puberty was defined as the time at which ovulation and oestrus occur in association with normal luteal function and is usually observed around 200 days of age (Duncan and Lodge, 1960).

Using the synchronization treatment described the overall frequency of animals returning to oestrus from each of the treatments (86%) compares favourable with the highest incidence of return reported by other groups of (69%, Guthrie, 1977, 160 days; 75%, Dziuk and Dhindsa, 1969, >250 days and 94%, Hunter, 1966, 1st or 2nd parity).

The mean yield of embryos was higher for prepubertal gilts than for postpubertal gilts but the differences were not significant and these observations are similar to previous reports (Baker and Coggins, 1966; Schlieper and Holtz, 1986). The cause of the low numbers of embryos collected from mature (8.2) and abattoir (7.4) donors is unknown. It may relate to the stage at which the embryos were collected and the technical problem stemming from the large oviduct size in the mature sows which tends to expand dramatically during flushing. Embryos could therefore be more easily missed in the initial flushing of the oviduct. Embryos collected at later stages do not appear to have the same problems because they are collected from the uterus with a different internal structure and in much larger volumes of flushing medium.

Fertilization rates in the prepubertal gilts (41%) are comparable with those reported by Baker and Coggins, (1966, 1968) (43%) but lower than those observed by Holtz and Schlieper (1991); Schlieper and Holtz (1986), (63% and 67.5% respectively). Fertilization rates (avg. 64%) in animals having attained puberty were not significantly different from previous reports. However fertilization rates were not as high as those observed from Cameron *et al.*, (1989) of (91%) and Hunter, (1966) of 84%. Possibly the

variation in fertilization rates we obtained for both prepubertal and postpubertal donors when compared with other workers could be related to the stage at which embryos were collected. Generally the other groups collected embryos at and above the 4-cell stage of development although it is of interest to note that all reported a significant proportion of the embryos collected (up, to 18.6%) at the 2-cell stage. The objective in this study was to collect zygotes for DNA microinjection approximately 12 hours after expected ovulation (40 to 41 post HCG) and donors were mated once, 30 hours post HCG to minimise variations in cleavage divisions. It is speculated that if embryos were left longer in the reproductive tracts and animals were mated twice as seen in a normal commercial situation then fertilization rates would increase and this would account for the variation seen in cleavage divisions with other workers. Inducing ovulation with HCG may narrow the window of ovulation but the overall gain may be offset due to the timing and nature of the single mating.

The proportion (5%) of retarded or degenerative embryos was not significantly different between each of the four donor groups (prepubertal 3%, postpubertal 6%, mature 6% and abattoir 5%). These observations are comparable with that of Schlieper and Holtz, (1986) who reported 4.6%.

The pregnancy rate achieved for prepubertal (60%) and postpubertal (100%) donors is comparable to that observed by other groups. The lower pregnancy rate observed for mature sows (33%) may have been influenced by the environmental conditions as the trial was carried out during a hot spell when for an unprecedented 2 weeks the daily temperature remained between 35° and 40°C (March 1989). The lower pregnancy rate in embryos collected from the abattoir (14%) suggests that embryo survival played a critical role and is discussed in the next paragraph.

The embryo survival rate for all embryos transferred was highest (29%) for embryos obtained from postpubertal gilts and compares with other research workers (Holtz and Schlieper, 1991 (28%); Schlieper and Holtz,

1986 (42.4) and Cameron *et al.*, 1989 (37.6%). This is a satisfactory result considering microinjection decreases embryo viablity (Walton *et al.*, 1987). Microinjection procedures would have undoubtedly influenced the low embryo survival for prepubertal embryos of (5%) and this plus the harsh environmental conditions resulted in the lower survival observed in embryos obtained from mature donors (4%). In hindsight the poor embryo survival of abattoir derived embryos could have been prevented. Wollenberg *et al.*, (1989) showed a reduced embryo survival rate for 4,8-cell embryos collected from the abattoir in relation to the time spent in the tracts. Evidence from this study suggests that 1-cell embryos are more sensitive to post mortem changes occurring in the oviduct environment than embryos collected from the uterine environment at a later stage embryos.

Researchers have previously focused on the hypothalamic, pituitary, ovarian, age/weight and uterine components associated with the failure of some embryo donors to maintain pregnancy (Rampacek *et al.*, 1976, 1985; Flowers *et al.*, 1989; Puglisi *et al.*, 1979; Lutz *et al.*, 1984; Karalus *et al.*, 1990; Estienne *et al.*, 1988; Grasso *et al.*, 1988; Dufour *et al.*, 1988; Kineman *et al.*, 1987a, 1987b; Pinkert *et al.*, 1988). Pinkert *et al.*, (1989) using an *in vitro* model compared the viability of embryos from pre- and postpubertal sows and showed that exposure of eggs to the prepubertal reproductive tract was detrimental to survival (for the reasons shown above); however, the short exposure time was not the sole influence on egg viability. This suggests and is supported by this study that zygotes from prepubertal gilts do not possess the equivalent development potential of zygotes from mature gilts either *in vivo* or *in vitro*. These studies would support the study of Gandolfi and Moor (1987) in sheep, who suggested that *in vitro* development does not necessary mirror *in vivo* capacity.

The results of this study indicate that using the procedure described, the more readily available postpubertal gilt can be successfully

synchronised and superovulated to provide a convenient and efficient source of embryos for transgenesis.

CHAPTER VI

PRONUCLEAR DNA INJECTION OF PORCINE ZYGOTES

6.1 Introduction

The production of mice carrying foreign DNA integrated in the genome (Gordon *et al.*, 1980) by pronuclear microinjection established the possibility of inserting recombinant derived genes into the mammalian genome. Animals with cloned genes integrated within their genome are called transgenic (Gordon *et al.*, 1980). The subsequent demonstration by Palmiter *et al.*, (1982) that it was possible to achieve control of the transgene by including a regulatory sequence or promoter that determined the tissue in which the gene would be expressed and the factors which would determine its expression was the first important step in the appreciation of this new technology to livestock (Palmiter *et al.*, 1983; see reviews, Brinster and Palmiter, 1986; Palmiter and Brinster, 1986; Gordon, 1989). The initial dramatic demonstration of the potential of transgenesis used a transgene comprising the rat (rGH) or human growth hormone (hGH) structural gene fused with a zinc sensitive promoter from the mouse metallothionein gene (Palmiter *et al.*, 1982). The resulting 'supermice' showed superior growth due to the secretion of hGH and rGH from the liver, the normal site of expression of the metallothionein gene without endogenous regulation. Whilst these experiments can rightly be claimed to have heralded a new era for the animal breeder, initial experience in studies aimed at extending the technology to livestock species proved disappointing although various research groups have now produced transgenic pigs, sheep and cattle (reviewed by Pinkert, 1987; Pinkert *et al.*, 1989b; Pursel *et al.*, 1989, 1990a). There are also several reports of transgenic animals with enhanced production characteristics (see review, Pursel *et al.*, 1990b).

Several reproductive characteristics of the pig (lack of seasonality, multiparity and the relatively short generation interval) make it the livestock species of choice for research into genetic engineering. The most common of the procedures used for gene transfer remains microinjection of the foreign transgene into one of the pronuclei of a recently fertilised zygote. In the pig, the efficiency of integration with various constructs and laboratories is

between 0.31 to 1.73% of the microinjected ova developing into transgenic pigs (see review, Pursel *et al.*, 1990b) and of the transgenic animals born between 17% and 100% express the transgene (see review, Pursel *et al.*, 1990a).

The aim of this study was to extend the work of Vize *et al.*, (1988) through developing a more efficient process for the production of transgenic pigs with a metallothionein promoter/porcine growth hormone transgene. The studies were carried out using herds of pigs with different genetic constituents and maintained under dramatically different conditions.

6.2 Materials and Methods

Animal holding facilities and procedures at two piggeries, Northfield Pig Research Unit a small closed SPF research facility and Metro Farms Piggery a 27,000 head commercial unit with extremely limited research. Surgical facilities were approved by the Institutional Biosafety Committee (IBC) following inspection by a representative of the Australian Genetic Manipulation Advisory Council.

6.2.1 Zygote Supply

Zygotes were obtained following treatment of prepubertal, postpubertal gilts with or without Cloprostenol, PMSG and HCG; details on the relevant protocols are outline in Chapter 4.2.2. and 5.2.1.

6.2.2 Microinjection Procedures

Procedures and equipment for microinjection follow the protocols set out in Chapter 2.11. Microinjection occurs into pronuclei which are visible only after centrifugation (see Plate (3)).

PLATE 3

Photograph 1

Centrifugation of porcine zygotes stratifies the cytoplasm allowing visualization of pronuclei

(Optics: DIC 400x)

Photograph 2

Pronuclear microinjection of porcine zygotes

(Left injection pipette 1-2 μm)
(Right holding pipette 90 μm)

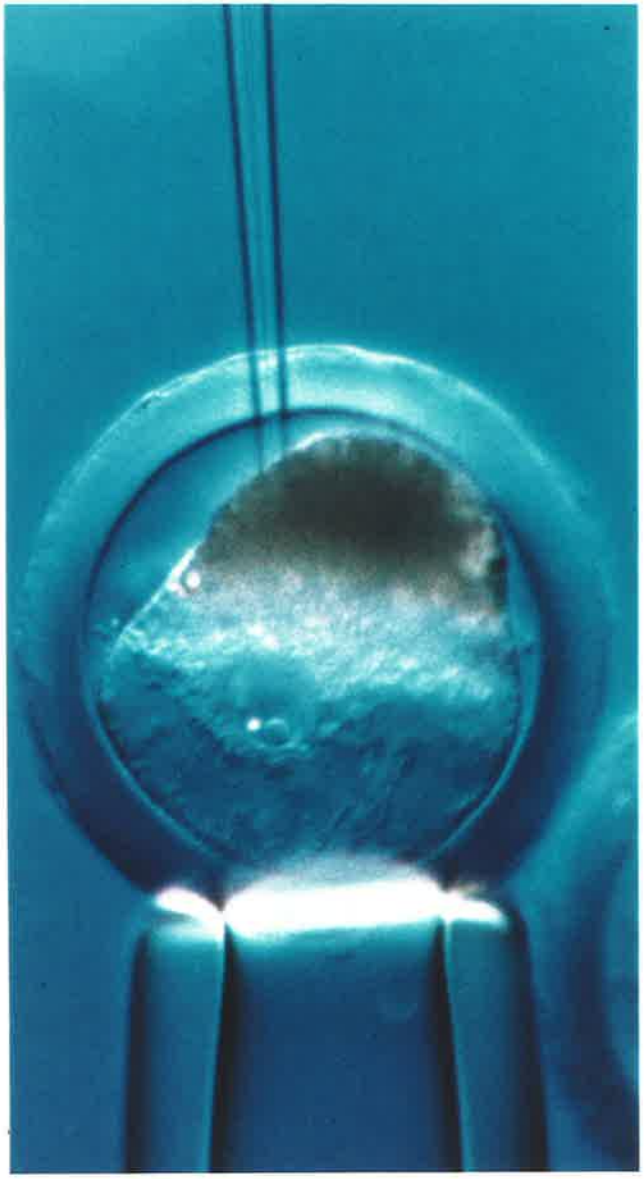
(Optics: DIC 400x)

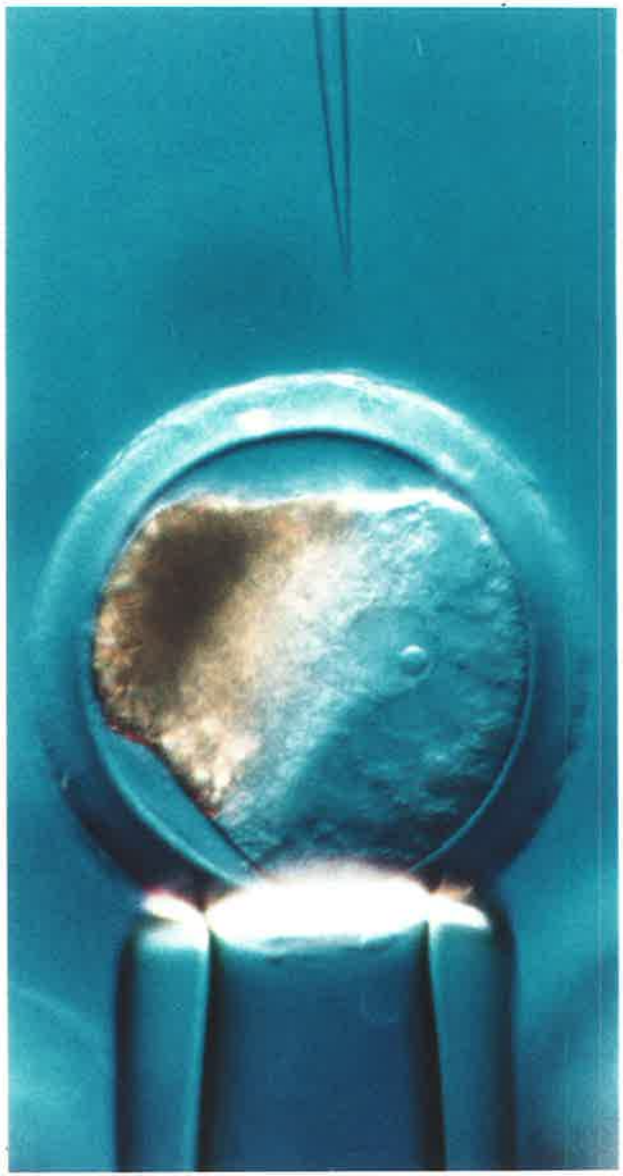
Photograph 3

Porcine zygote following pronuclei injection

Larger pronuclei has been microinjected with 1 to 2 picolitres of DNA

(Optics: DIC 400x)





6.2.2.1 Gene Constructs

Porcine zygotes were microinjected with variants of the human metallothionein IIA promoter/porcine growth hormone fusion gene; see Chapter 2.10 for details. Transgenes were loaded into microinjection pipettes using protocols outlined in Chapter 2.12.

6.2.3 Viability of Microinjected Zygotes *In Vitro* and *In Vivo*

Following microinjection with the porcine growth hormone transgene, zygotes were randomly allocated to either *in vitro* culture (see Chapter 7) or returned to recipients within 8 hours (see Chapter 5, for details of transfers). After up to six days of *in vitro* culture, embryos were transferred to uteri of synchronised recipients.

6.2.4 Preparation and Monitoring of Recipients

Postpubertal recipients were synchronized following treatment with Cloprostenol, PMSG and HCG; details on the relevant protocols are outlined in Chapter 2.2. Surgical procedures followed protocols outlined in Chapter 2.6. Following embryo transfer, recipients were checked for pregnancy by non-return to oestrus and ultrasound, details of which are outlined in Chapter 2.7.

6.2.5 Assessment of DNA Incorporation

Transgenic animals were identified from tissue samples taken at birth (see Chapter 2.14.1, for details) by either DNA-DNA hybridization (see Chapter 2.14.3, for details) or PCR techniques (see Chapter 2.14.2, for details).

6.2.6 Assessment of Expression Status in Transgenic Pigs

Serum porcine growth hormone levels and Insulin-Like Growth Factor-1 levels were assayed by radioimmunoassay (see Chapter 2.15.2; 2.15.3 for details respectively).

6.3 Statistical Analysis

Observations were analysed using chi-square statistics (SAS Institute Inc, 1988).

6.4 Results

Table 6.1 provides a summary of data which indicates the efficiency of procedures used to create transgenic pigs at the two units. Zygotes were injected with a human metallothionein promoter/porcine growth hormone a growth related fusion gene similar to those used by other groups. Variants of this construct involved deleting the elements associated with controlling the sensitivity of the metallothionein promoter in an effort to regulate the expression of porcine growth hormone with specific concentrations of zinc added as sulphate to the diet. The transgene constructs $pHMPG_{.03}$, $pHMPG_{.03A}$ and $pHMPG_{.05}$ (see Chapter 2.10, for details) were developed by the Department of Biochemistry, University of Adelaide. All constructs were proven in mice created in the Department of Obstetrics and Gynaecology University of Adelaide, to have acceptable integration and expression characteristics (see Chapter 3.4). The integration frequency of the three constructs used in transgenic pigs were not significantly different.

6.4.1 Superovulation and Embryo Collection

The average number of corpora lutea (C.l.) in donors varied from 19 per donor in trial 1 at Northfield to 30 in trial 2 for the reasons discussed in Chapters 4 and 5.

An increase in efficiency of zygote collection as assessed by the technique described by Perry and Rowland, (1962) from 52% at Northfield in trial 1 to 63% at Northfield in trial 2 and 64% at Wasleys probably reflected improved operator experience. The average number of zygotes collected, 10 trial 1, 19 trial 2 at Northfield and 11 at Wasleys reflected the numbers of C.l. present.

The percentage of embryos injected in the two trials at Northfield was comparable (58% trial 1 vs 64% trial 2). The lower result experienced at Wasleys of 40% could have been affected by three weeks of extreme temperature (35°C-40°C, March 1989) which decreased both embryo quality and pregnancy rates.

The mean zygote survival rate indicates that only 13% Northfield trial 1, 11% Northfield trial 2 and 8% of Wasleys transferred microinjected zygotes were represented by offspring at birth.

6.4.2 Integration Frequency

Transgenic detected by PCR and DNA-DNA hybridization indicated that the integration frequency was 2.59% for Northfield trial 1, 1.09% for Northfield trial 2 and 1.10% for Wasleys when expressed as the percentage of injected zygotes that resulted in transgenic pigs. When represented as the number of transgenic animals as a % of offspring born, 20% resulted from Northfield trial 1, 10% from Northfield trial 2 and 13% from Wasleys.

6.4.3 Transgene Expression

Detecting transgene expression involved sampling at various ages and measurement of elevated porcine growth hormone levels and insulin-like growth factor-1 levels (Biological activity) by radioimmunoassay. The frequency of expression for integrated gene was 40% for Northfield trial 1, 33% for Northfield trial 2 and 33% for Wasleys.

TABLE 6.1 EFFICIENCY OF PRODUCING TRANSGENIC PIGS - PERSPECTIVE

	Donors (n)	C.l. (Avg ±S.E.)	Zygotes (Avg/Don. ±S.E.) (% Rec.)	Injected (%)	Recipients	Transfer (n) (%)	Preg. Test (%) Piglets (Avg)	Transgenic (n) (%) Expressing (n) (%)
Northfield								
Trial 1	36	684 (19±9.6)	355 (10±7.4) (52%)	205 (58%)	10	193 (94%)	7 (70%) 25 (3.57)	5 (20%) 2 (40%)
Trial 2	69	2075 (30±12.5)	1307 (19±12.8) (63%)	840 (64%)	20	823 (98%)	18 (90%) 90 (5)	9 (10%) 3 (33%)
Wasleys	129	2161 (17±9.4)	1385 (11±8.4) (64%)	554 (40%)	29	543 (98%)	8 (28%) 3 ** (11%) 7 *** (25%) 46 (5.75)	6 (13%) 2 (33%)

* Average number of embryos transferred in both groups 22±6
** 3 Pregnant recipients aborted
*** 7 Recipients returned - possible reason 36°C for 3 weeks

6.5 Discussion

6.5.1 Superovulation and Embryo Collection

The increases in average numbers of corpora lutea per sow in the Northfield trials could be attributed to an increased understanding of superovulation procedures however, genetic differences were also important as when the trial was shifted to the Wasleys site, the average number of C.l. (17) decreased. The Northfield Piggery is a closed specific pathogen free, 70 sow unit and elite breeding stock is selected from a small (10%) pool. The piggery is run on a batch farrowing system where mated animals are selected on the basis of inbreeding coefficients (<12) to maximise heterogeneity. Wasleys on the other hand, is a commercial operation of some 2,700 breeding sows utilising three genetic pools an elite (PIC) stud, Wasleys stud and terminal sire line with selection on the basis of production characteristics. Animals were selected from the terminal sire lines after initial selection by Wasleys.

6.5.2 *In Vivo* Viability of Transferred Microinjected Zygotes

Pregnancy rates were significantly higher than reported by Vize *et al.*, (1988) who utilised abattoir derived zygotes, overnight culture and transfer to mature recipients. The increase in pregnancy rate could be attributed to the fact that zygotes were microinjected and transferred at the same site of collection. The lower pregnancy rate experienced at Wasleys was not significantly different from the Northfield Trials if you took into consideration that 11% of animals aborted and 25% of animals returned during 3 weeks of very hot weather. These new protocols may have overcome problems initially experienced by Vize *et al.*,(1988) who reported that no pregnancies survived to term when an average of 13 injected zygotes were transferred per recipient; therefore, they subsequently transferred 30 injected zygotes per recipient. In this study the numbers of embryos transferred varied from 16 to 28 and resulted in the birth of offspring. The

number of stillborn and mummified piglets born was not significantly different from the frequency of their occurrence in the piggery used.

6.5.3 Embryo Survival Rate

Overall the average 10.7% mean of embryo survival rate was slightly higher than 8.3% reported by Pursel *et al.*, (1990a) though comparison is limited because of different experimental conditions. However, it is of interest and relevant for future transgensis studies aimed at improving the efficiency that embryos derived from postpubertal donors have a much higher embryo survival rate (29%) with the anticipated result of more offspring resulting from injected embryos. Pursel *et al.*, (1990) reported a significant increase in pregnancy rate when using recipients with a one day delay in their oestrous cycles when compared with donors. The pregnancy rate observed in this study using recipients in oestrus on the same day was comparable with oestrous delayed recipients (Pursel *et al.*, 1990). Comparison may be difficult because of differing conditions.

6.5.4 Gene Integration

The overall integration frequency of 1.59% is similar to those reported by Brem *et al.*, (1985,1988), Ebert *et al.*, (1988), Vize *et al.*, (1988), Pursel *et al.*, (1990a), Polge *et al.*, (1989) and for other fusion genes (see review Pursel *et al.*, 1990b).

6.5.5 Expression of Integrated Transgenes

The overall percentage of expression was 35.3% for the pHMPG construct. Pursel *et al.*, (1990a) indicated that expression of integrated transgenes was dependant on the construct and type of animal used.

In conclusion, the robust protocols devised for the creation of transgenic pigs within the two units with a growth hormone fusion gene were as efficient, with respect to embryo survival, integration and expression at frequencies as those obtained by other research groups. Further

assessment of the transgenic pigs produced in these experiments is provided in Chapter 9.

CHAPTER VII

COMPARISON OF MEDIA FOR THE CULTURE OF PORCINE ZYGOTES

7.1 Introduction

Research into embryo manipulation and embryogenesis would be facilitated dramatically by the availability of *in vitro* culture systems that could support normal development without jeopardizing viability. In particular, procedures associated with the production of transgenic animals need to access zygote viability as microinjection of DNA is known to damage plasma membranes (Walton *et al.*, 1987) with only 4 to 25% of pig zygotes surviving after this procedure (Pursel *et al.*, 1989).

A variety of defined media have been examined for the culture of pig embryos; see review Wright and Bondioli (1981). Most are unable to support development *in vitro* of one- and two- cell embryos beyond one or two cleavage divisions (Davis, 1985). In contrast, embryos collected at the 4-cell stage and cultured *in vitro* cleave and form blastocysts at rates approximating those expected during normal *in vivo* development (Davis, 1985). It should be noted however that an embryos ability to cleave *in vitro* may not reflect its ability to survive after transfer to a recipient (Polge, 1982).

Development of porcine zygotes can be enhanced when cultured in the presence of porcine oviductal fluid (OVF) (Archibong *et al.*, 1989) or oviductal epithelial cells (White et. al., 1989). White *et al.*, (1989) reported 67 % blastocyst formation from 2-cells cultured in a combined porcine oviductal epithelial cells and fetal fibroblast monolayer co-culture system. Archibong *et al.*, (1989) reported 100% blastocyst formation from 1-cell cultured for 48 hours in OVF and then transferred to Kreb's Ringer carbonate medium. These authors concluded that oviduct cells or their secretory products improved *in vitro* development of porcine embryos from 1-cell to blastocyst when used in conjunction with a culture medium.

The lack of freezing methodologies have handicapped the genetic improvement of pigs offered through traditional embryo transfer practices. Several studies have reported difficulties in freezing porcine embryos (Polge, 1982). It is speculated that the high lipid concentration of the pig embryo,

particularly of the embryonic membranes adversely affects sensitivity of the embryo to cooling (Polge, 1977). This has led to the development of specialised techniques for the transport of porcine embryos over long distances. Several researchers have reported that, in appropriate media it is possible to transport porcine embryos over long distances without significant loses in viability (Baker and Dziuk, 1969). James *et al.*, (1980) and Niemann *et al.*, (1989) demonstrated that 26 to 28% of day 5-6 embryos transferred to recipients within 20-30 hours after collection developed into liveborn.

The initial aims of this study were twofold, namely to develop a simple culture medium that would support the development of normal and microinjected porcine zygotes to the blastocyst stage and in the absence of cryopreservation protocols for pig embryos (Polge, 1977) develop methodology to establish protocols for the successful long distance shipment of early stage embryos.

Recently however, Kashiwazaki *et al.*, (1991) have demonstrated that porcine embryos can be successfully cryopreserved at the hatched blastocyst stage with viable offspring produced. Therefore the studies were extended to examine whether *in vitro* derived blastocysts could be cryopreserved.

7.2 Materials and Methods

7.2.1 Supply of Zygotes

Zygotes were obtained from Metro Farms Piggery, Wasleys and Northfield Pig Research Unit. Mature sows, postpubertal donors and recipients were synchronized and gonadotrophin treated as per Chapter 4. Embryos were collected by midline laparotomy (see Chapter 2.3) and stored under conditions described in Chapter 2.3 until required.

7.2.2 Culture Media and Conditions

Three different culture media were used to compare the growth of porcine, (1) MEM plus 10% HIHS (Michalska, 1988) (2) BMOC-2 plus 10% HIHS (Brinster, 1963) (3) Whitten's medium plus 1.5% BSA (Whitten, 1971). Details on the composition of the various media can be seen in Table 7.1. Media were prepared either one or two days before embryo collection and then placed in the incubator for gas equilibrium (see Chapter 2.8, for details).

Groups of porcine zygotes (n=25) were randomly assigned to various culture media and either microinjected (see Chapter 2.11, for details) or not (controls). All embryos were assessed on their ability to divide to the hatched blastocyst stage (see Chapter 4.2, for details of assessment). Embryos reaching blastocyst stage were either transferred to recipients (see Chapter 2.6, for details) or stained in Hoechst 33342 (see Chapter 2.9, for details) to determine the number of nuclei.

TABLE 7.1 CONSTITUENTS OF SIMPLE MEDIA USED IN THE CULTURE OF PORCINE ZYGOTES.

Constituents	Whitten's medium g/litre	BMOC-2 g/litre	MEM g/liter
Inorganic salts			
$CaCl_2$		0.189	0.264
KCl	0.356	0.356	0.4
KH_2PO_4	0.162	0.162	
$MgSO_4.7H_2O$	0.294	0.294	0.200
$NaCl_2$	4.00	6.975	6.80
$NaHCO_3$	2.106	2.106	2.106
Other components			
Glucose	1.00		1.00
Na pyruvate	0.036	0.028	
Ca lactate. $5H_2O$	0.527		
Na lactate	2.416	2.264	

7.2.3 Long Distance Embryo Transfer

Long distance embryo transfer was conducted between Metro Farms, Wasleys, Sth. Aust. and Commercial Pig Company (C.P.C.), Bendigo, Vic. The distance separating the two piggeries was approximately 820 km. Embryos were maintained *in vitro* conditions for at least 30 hours.

7.2.3.1 Supply of Zygotes and Embryos

Wasleys and C.P.C. zygotes were obtained surgically (see Chapter 2.3) from superovulated postpubertal donors (see Chapter 4,. for details). Four-cell embryos were obtained from superovulated Wasleys abattoir donors (see Chapter 5.2.2) and surgically from superovulated C.P.C. mature and postpubertal donors (see Chapters 2.3 and 4, for details).

7.2.3.2 Storage and Transfer of Zygotes and Embryos

Following collection, embryos were washed in gas equilibrated MEM (see Chapters 2.8 and 7.2.2) and stored sealed in gas tight containers. Normal air in the containers was replaced with special gas mixture (see Chapter 2.8, for details). Temperature was regulated by maintaining the containers in close contact with human skin (37.4°C) for the period of the *in vitro* culture. Embryos and zygotes were transported between piggeries by domestic airlines and commercial vehicles. Following 30 hours of extended culture, embryos were removed to fresh HEPES-MEM, morphologically assessed and surgically transferred (see Chapter 2.6, for details) to either oviducts for 2-cells or uterus for 8 to 16 cells. The number of embryos transferred was based on the relationship between the number of embryos transferred and the numbers of piglets born (Dziuk, 1987; Cameron *et al.*, 1989) .

7.2.3.3 Recipients

Synchronized recipients (see Chapter 4, for details of synchronization) were monitored for pregnancy (see Chapter 2.7, for details) and offspring were recorded at birth.

7.2.4 Porcine Embryo Freezing

Groups of porcine zygotes (n=25) obtained from superovulated postpubertal donors (see Chapter 4, for details) were cultured in microdrops at 39°C of Whitten's medium plus 1.5% BSA in conditions described in Chapter 2.8 to assess the development to the hatched blastocyst stage.

Hatching blastocysts were transferred to straws and equilibrated in 1.5 M glycerol diluted in PBS plus 16% HIFCS for approximately 30 minutes. Then cooled from room temperature to -6.8°C at 1°C/min and held at this temperature 10 minutes, then seeded, and cooled at 0.3°C/min to -38°C using a programmable freezer (Cryologic Pty Ltd. Victoria, Australia) and plunged into liquid nitrogen. Embryos were maintained in liquid

nitrogen for a week then thawed at 35°C in a water bath, followed by the stepwise removal of glycerol using two solutions with decreasing concentrations of glycerol and sucrose (0.75 M glycerol, 0.3M sucrose, O.3M glycerol, 0.15 M sucrose). After washing, recovered embryos were cultured for a further 24 hours in Whitten's medium with 1.5% BSA to morphologically assess their viability.

7.3 Statistical analysis

The percentage of zygotes that developed into the blastocyst were analysed using the chi-square test. The observations were summarized into zygotes that developed to (1) 2-cell, (2) morula, (3) blastocyst and (4) hatched blastocyst. The viability of embryos transferred after *in vitro* culture was summarised by the number of offspring produced. The chi-squared statistics (SAS Institute Inc., 1988) was used in the analysis of data.

7.4 Results

7.4.1 Comparison of Culture Media

Zygotes collected from superovulated donors were randomly allocated to *in vitro* culture in one of three culture media either before or after microinjection. The development of control and microinjected zygotes to the hatched blastocyst stage is shown in Table 7.2.

Microinjection significantly ($p<0.05$) decreased the number of zygotes developing to the hatched blastocyst stage, regardless of the type of culture media used. The 46% loss of microinjected zygotes when compared with controls was initially detected in the failure of zygotes to divide to the two-cell stage.

Whitten's medium significantly ($p<0.05$) increased the percentage of control and microinjected zygotes that developed to the hatched blastocyst stage (77% control and 40% microinjected; BMOC-2 28% and 16%; MEM 5% and 3%).

Developmental stages of embryos cultured from the zygote stage are shown in Plate 4.

TABLE 7.2 DEVELOPMENT OF PORCINE ZYGOTES CULTURED IN VARIOUS MEDIA.

Tmt (n)	Total No. (%)	2-cell (%)	Morula	Bl. Day 5 (%)	H/B. Day 6 (%)
Control					
Whitten's	95	87 (92)	85 (91)	82 (87)	73 (77)
BMOC-2	147	124 (84)	102 (69)	66 (45)	41 (28)
MEM	220	158 (72)	104 (47)	26 (12)	10 (5)
Microinjected					
Whitten's	40	25 (63)	21(53)	19 (48)	16 (40)
BMOC-2	150	90 (60)	61 (41)	49 (33)	24 (16)
MEM	130	46 (35)	21 (16)	10 (8)	4 (3)

7.4.2 Viability of *In Vitro* Cultured Zygotes

Results for *in vivo* viability are summarised in Table 7.3. There were no significant differences ($p>0.05$) between the pregnancy rate (avg 80%) for groups of control or microinjected embryos transferred, irrespective of the culture medium used. In comparison, there were significant differences between the total numbers of embryos that developed into offspring after transfer with 56% of zygotes cultured in Whitten's to the hatched blastocyst stage resulting in offspring compared with 36% of the embryos for BMOC-2 transferred at the blastocyst stage and 29% of embryos for MEM transferred at the 4,8-cell stage. The transferred embryo survival rate was also significantly different when the pregnancy status of the recipient was known with 56% of embryos cultured in Whitten's for a recipient that was later confirmed pregnant resulting in offspring compared with a 45% in BMOC-2 and 36% in MEM.

The choice of media also affected significantly ($p<0.05$) the viability of cultured microinjected zygotes with only 35% of embryos transferred after

culture in BMOC-2 medium to the blastocyst stage developing into live offspring compared with 14% for embryos cultured in MEM. Unfortunately the availability of animals allowed comparison of only two media and the viability of microinjected embryos cultured in Whitten's medium needs to be confirmed.

TABLE 7.3 VIABILITY OF CULTURED EMBRYOS AFTER TRANSFER TO SYNCHRONIZED RECIPIENTS

Media	Embryo Stage (n)	Transfers* (Preg)	Live born (Avg)
Control			
MEM	4,8-cell (236)	15 (12)	69 (5.7)
BMOC-2	Bl. (120)	8 (6)	43 (7.17)
Whitten's	H/B. (84)	6 (6)	47 (7.83)
Microinjected			
MEM	4,8-cell (185)	8 (5)	25 (5)
BMOC-2	Bl. (220)	16 (13)	77 (5.92)

* An average of 16±2 control and 24±4 microinjected embryos respectively were transferred per synchronised recipient.

7.4.3 Long Distance Embryo Transfer

Results for the long distance transfer of embryos are summarised in Table 7.4. There were no significant differences between pregnancy rates (avg 43%) of embryos derived surgically (1, or 4,8-cell) or from the abattoir at the 4,8-cell stage. However, the proportion of embryos that developed into live offspring was significantly higher for 4,8-cell embryos obtained either surgically or from abattoir donors than those collected surgically at the 1-cell stage (47% vs 14%). When pregnancy was confirmed there were no significant differences between the embryo survival rate with 31% of 4,8-cell embryos transferred to a recipient that was later confirmed pregnant resulting in offspring compared with 22% for 1-cell embryos. The overall percentage of embryos resulting in live born piglets was 14% for 1 to 2-cell transfers and 26% for 4,8-cell to 8,16-cell surgically and abattoir transfers.

PLATE 4

Photograph 1	**Photograph 2**
Zygotes collected from superovulated prepubertal gilts (Optics: DIC 100x)	Two-cell embryos after 18 hours of *in vitro* culture in Whitten's medium plus BSA (Optics: DIC 200x)

Photograph 3	**Photograph 4**
Four-cell embryos after 42 hours of *in vitro* culture in Whitten's medium plus BSA (Optics: DIC 200x)	Eight-cell embryos after 60 hours of *in vitro* culture in Whitten's medium plus BSA (Optics: DIC 200x)

Photograph 5	**Photograph 6**
Blastocysts after 120 hours of *in vitro* culture in Whitten's medium plus BSA (Optics: DIC 200x)	A single hatched blastocyst derived from a zygote cultured in Whitten's medium plus BSA for 136 hours. (Stained in Hoechst 33342) (Optics:UV Fluorescent 400x)

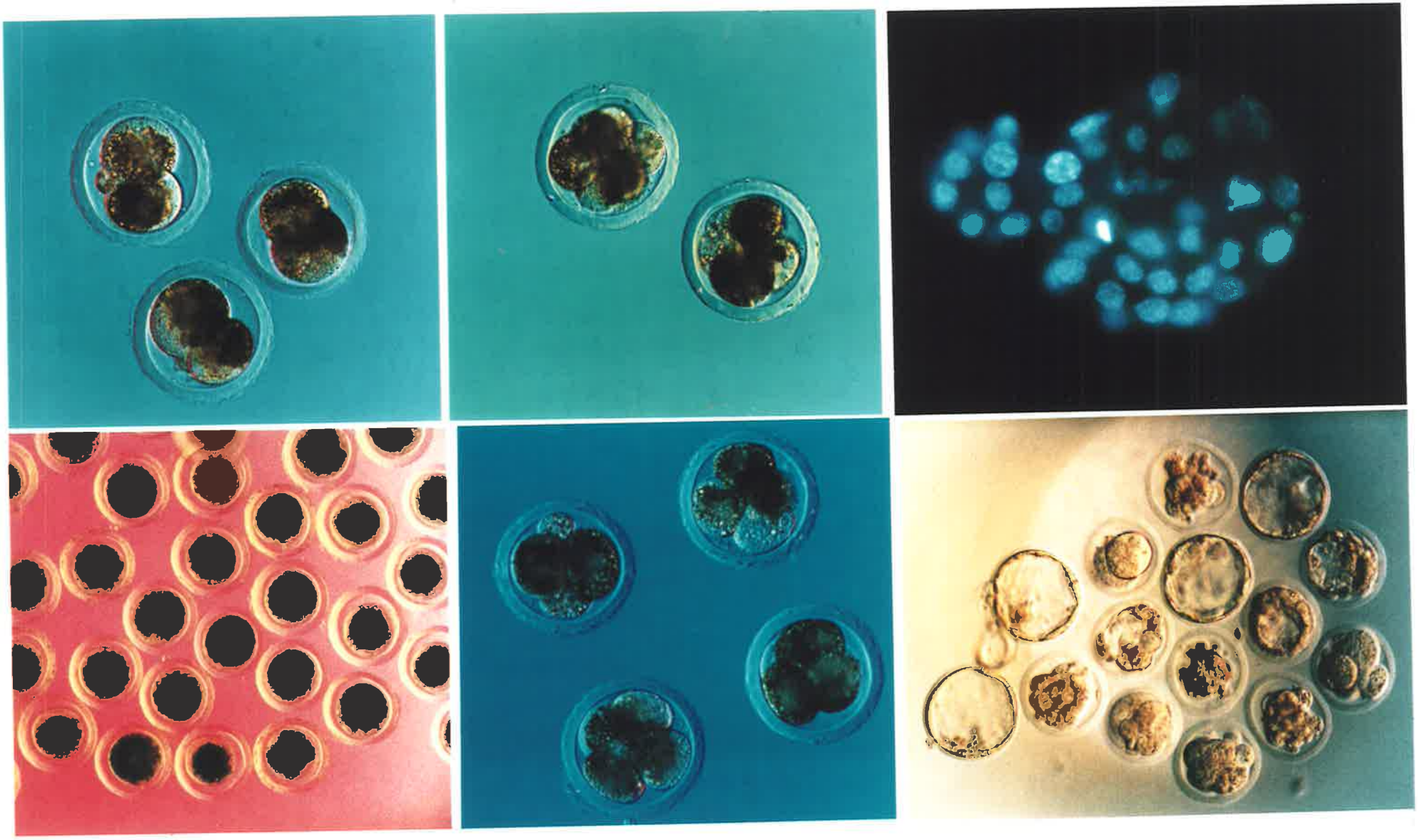


PLATE 5

Photograph 1

Porcine blastocysts (Day 5) derived from zygotes cultured in Whitten's medium for 142 hours.

(Optics: DIC 200x)

Photograph 2

Hatching porcine blastocyst (Day 6) derived from zygotes cultured in Whitten's medium.

Subjected to cryopreservation procedures.

(Optics: DIC 400x)

Photograph 3

Hatching porcine blastocyst (Day 6) derived from zygotes cultured in Whitten's medium.

Subjected to cryopreservation procedures.

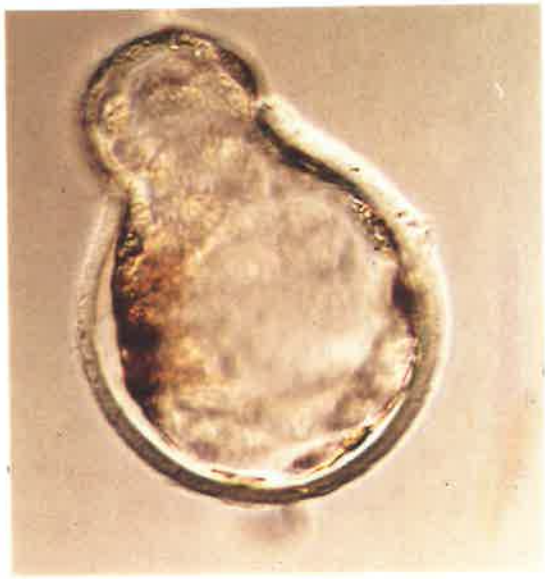
(Optics: DIC 400x)

Photograph 4

Hatching porcine blastocysts after cryopreservation at -196°C.

Cultured in Whitten's medium 3 hours after thawing.

(Optics: DIC 100x)



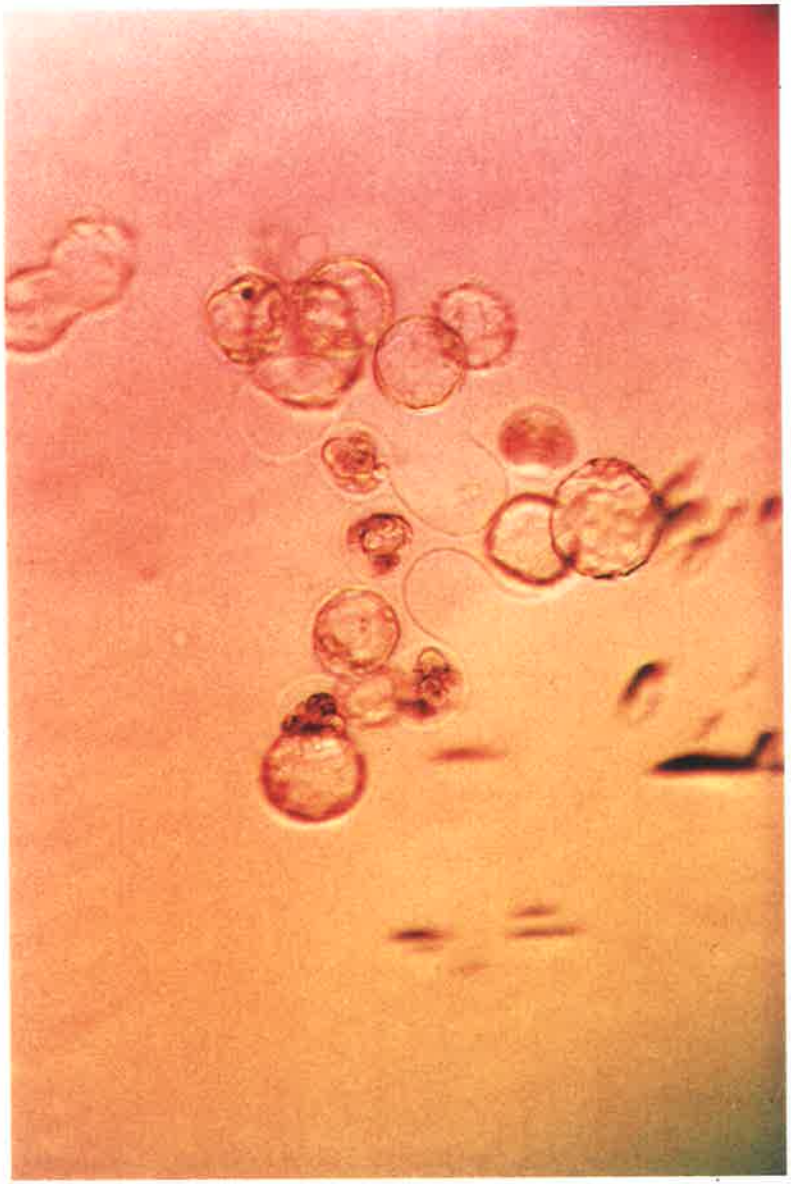


TABLE 7.4 VIABILITY OF LONG DISTANCE EMBRYO TRANSFER

Embryos Collected (n)	Transferred* (%)	Recipients (Pregnant)	Offspring (Avg)
1-cell	**2-cell**		
(134)	101 (75)	7 (4)	14 (3)
4 to 8-cell	**8 to 16 cell**		
Abattoir			
(230)	182 (79)	14 (10)	47 (4.7)
Surgical			
(306)	254 (83)	16 (12)	63 (5.25)

* An average of 16±2 embryos were transferred to each recipient.

7.4.4 Porcine Embryo Freezing

Of a total of 61 hatching blastocysts subjected to freezing following culture from the zygote stage, 35 (57%) recovered without evidence of gross morphological damage and continued their development to the hatched blastocyst stage during the 24 hour culture period. The viability of blastocyst before and after freezing is presented in Plate 5.

7.5 DISCUSSION

7.5.1 Comparison of Culture Media

The results of this study indicate that a modified Whitten's medium is the superior medium for the culture of porcine zygotes to the hatched blastocyst stage. Using this medium 87% of zygotes developed into blastocyst, 73% developed into hatched blastocyst and 56% resulted in offspring from zygotes cultured for a six-day period. Two other groups have comparable results, Archibong *et al.*, (1989) who cultured 1-cell embryos for 48 hours in oviductal fluid and then Kreb's Ringer carbonate medium with 100% of the zygotes developing to the blastocyst stage and Beckman and

Day (1991) who cultured one and two-cells embryos to the morula and blastocyst stage in modified Whitten's medium with 97% efficiency. However, in each of these cases the viability of the embryos was not assessed. Successful cleavage of most domestic livestock embryos requires additions of BSA or sera to the medium (see reviews by Kane, 1978; Brackett, 1981). Kane, (1985) presented evidence that commercial BSA preparations contain an embryonic growth factor and it is of interest that Menino and Wright, (1982) were first to report the successful culture of zygotes to the blastocyst stage using Whitten's medium containing 1.5% BSA.

Viability of zygotes cultured to the hatched blastocyst stage in BMOC-2 (28%) and MEM (5%) was severely comprised when compared with Whitten's medium. The enhanced development in this study of zygotes cultured in BMOC-2 (28 vs 18%) compared with previous reports (Wright and Bondioli, 1981) could be attributed to the addition of HIHS to the medium. Stone *et al.*, (1984) reported the development of 4 to 8 cell porcine embryos was enhanced in media containing HIHS when compared with other protein sources. Overall, it is evident that these two simple media may be inadequate for supporting activation of the embryonic genome which normally occurs at the time when the conceptus enters the uterus and media may need to be devised which closely mimic the environment of the uterine lumen (Bavister, 1988). Alternatively, the loss of viability may reflect the lack of exposure to critical oviductal factors during normal *in vivo* development which influence development past the 16-cell stage (Gandolfi and Moor, 1987).

Microinjection of porcine zygotes and subsequent culture of zygotes to the blastocyst stage in the various media caused an overall loss of 46% in viability. This reduction in viability was detected in the failure of zygotes to divide to the two-cell stage most probable reflecting damage done to plasma membranes by the microinjection procedure (Walton *et al.*, 1987). Whilst no experiments were performed to examine this possibility it would seem

unlikely that the frequency of lethal mutations caused by transgene integration is a significant cause of loss in the preimplantation embryos. Of the microinjected embryos cultured in modified Whitten's medium, 48% developed to the blastocyst stage and 40% developed to the hatched blastocyst stage. These results were significantly higher than microinjected embryos cultured in BMOC-2 (33 and 16%) and MEM (8 and 3%). The survival rates of microinjected embryos in modified Whitten's medium is superior to that reported by Pursel *et al.*, (1988) who used *in vivo* egg boxes with only 21.4% and 13.5% of 2-cell and 1-cell microinjected embryos developing to term. Comparisons with other studies are limited due to differences between experimental conditions.

The viability of cultured embryos was assessed by transfer to synchronised recipients. The overall pregnancy rate of 80% for transfer of control and microinjected embryos cultured up to 6 days was comparable with data derived from embryo transfer programs performed for commercial purposes using day 4 to 6 embryos (Davis, 1985; Cameron *et al.*, 1989). However, the survival rate for embryos cultured in Whitten's medium (56%) was higher than that previously reported by other workers of 10% for early 1 and 2-cell porcine embryos, cultured for 48 hours (Pope and Day, 1977; Davis and Day, 1978) and 36-50% for embryos obtained at a later stage (Davis, 1985; Cameron *et al.*, 1989). The embryo survival rate of 35% for BMOC-2 and 14% for MEM microinjected embryos transferred to recipients was higher than reported by other workers, though comparison is difficult because of the different experimental conditions.

It is concluded that of the simple media tested, Whitten's proved superior for maintaining for up to 6 days both control and microinjected zygotes without loss of viability. Additionally, the establishment and maintenance of cell lines for co-culture which are expensive and laborious in effort are not required.

7.5.2 Long Distance Embryo Transfer

The potential impact of embryo transfer technology to pig herd development has been heavily constrained due to the lack of suitable cryopreservation procedures. The outcome of this experiment demonstrated with the birth of live offspring that 1-cell and 4,8-cells can successfully be transferred between two piggeries. The transfer involves more than 30 hours of *in vitro* storage and transport over a distance of approximately 830 km without any specialised equipment for the storage of embryos.

The pregnancy rates after transfer (43% Avg) for both 2-cell and 8,16-cell were comparable with 46% by Niemann *et al.*, (1990) who transferred day 4-5 embryos using a modified thermos filled with steel beads connected to a battery (Minitüb, Landshut, F.R.G.). However, only embryo survival transferred at the 8,16 cell stage of 26% was comparable with embryos transferred at the morula or blastocyst stage (28%) (Neimann *et al.*, 1990). Viability of zygotes collected surgically was reduced indicating early stage embryos have increased susceptibility to extended *in vitro* culture. However, as pregnancy rates were similar to later stage embryos, survival of embryos after transfer may be dependant on uterine components (Dziuk, 1987) and secretions (Roberts *et al.*, 1984; Roberts and Bazer, 1988) rather than embryo stage.

No significant difference in viability was observed between 4,8-cell embryos collected either surgically or post mortem from the abattoir allowing future collections of embryos to be determined by the value of the animals being used. This is contrary to work reported by Wollenberg *et al.*, (1989) and Schlieper and Holtz, (1986) where the viability of embryos were seriously impaired by any delay involved in the removal of embryos from the reproductive tract. In this study tracts were not removed until 30 minutes after slaughter and flushing usually took at least another 60 minutes. Schlieper and Holtz, (1986) observed no significance difference between

embryo survival between surgical and abattoir collection only when reproductive tracts were removed immediately after slaughter.

Further improvements in efficiency may be anticipated as the trial was completed using MEM a medium which has subsequently been proved to be inferior for the culture of porcine embryos. Future experiments may improve the efficiency of transfer by using Whitten's medium but experiments will be required to validate this.

The established protocol was successful in producing offspring from 30 hours of extended *in vitro* culture using human body heat. Pregnancy rates and embryonic survival in embryos collected either surgically or by abattoir methods at the 4,8-cell stage were comparable with those reported by other workers. Further work involving the selection of media for transport may increase the percentage of embryos surviving to term.

7.5.3 Porcine Embryo Freezing

With the development of techniques that allow the cryopreservation of specific stage embryos, experiments were conducted using an *in vitro* culture model. It is concluded that embryos can be collected at the one-cell stage, cultured to the hatched blastocyst stage *in vitro* and subjected to cryopreservation procedures with similar results to embryos collected on days 5,6 or 7 (Kashiwazaki *et al.*, 1991). The *in vitro* culture model described has the advantage that it will allow pretreatment of embryos to enhance freezing capacity. Studies concerning the viability of frozen hatched blastocysts *in vivo* were underway, however, results were not available at the time of writing.

CHAPTER VIII

VIABILITY OF TRANSGENIC PIGS: MODEL STUDIES USING RECOMBINANT PORCINE GROWTH HORMONE ADMINISTRATION

8.1 Introduction

There is now compelling data supporting the initial claims, first made over 2 decades ago that production characteristics of growing pigs can be dramatically enhanced following pGH administration (Machlin, 1972; Boyd *et al.*, 1986; Campbell *et al.*, 1988a, 1988b, 1988c; Chung *et al.*, 1985; Etherton *et al.*, 1986; Evock *et al.*, 1988; McLaren *et al.*, 1987; Walton *et al.*, 1987). The improvements recorded include increased growth rate, feed conversion efficiency and improved carcass composition resulting from an increase in protein accretion and decreases in carcass lipid (Campbell *et al.*, 1988a, 1988b).

With the advent of transgenesis immediate attempts were made to incorporate GH genes into pigs, while successful the results varied. Initial reports on the performance of transgenic pigs incorporating heterologous GH genes indicated that although the transgenes expressed and produced high levels of GH, the pigs failed to thrive and suffered from health problems including lethargy, lameness, gastric ulcers, susceptibility to stress, parakeratosis and *osteochrondritis dissecans* (see review, Pursel *et al.*, 1990)

However a report from Adelaide (Vize *et al.*, 1988) showed that transgenic pigs created using homologous GH sequences were viable and could show the expected improved production characteristics resulting from pGH administration. The question then became why were there differences between groups.

One obvious difference is in the nature of the transgenes used by the two groups, the Beltsville groups used heterologous GH gene constructed from genomic DNA which resulted in plasma levels of up to 1000 ng/ml of h/bGH. Whereas the Adelaide group used a homologous gene constructed from cDNA with controlled albeit incomplete expression of 20-30 ng/ml of homologous GH.

This indicates that the differences between the potency of the heterologous GH or levels of expression of GH was detrimental to the viability of transgenic animals.

The aim of this present study was to establish animal models to further explore the physiological consequences of sustained exposure to pGH production and to determine whether prolonged exposure due to enhanced production during the growth period is detrimental to animal health. Initial experiments were aimed at establishing a model using recombinant pGH (Bresatec Pty. Ltd.). Daily injections of GH at a safe predetermined level demonstrated no detrimental effects, however investigations into a slow release formulation to more closely mimic the transgenic pigs may target the desired gene expression required for viable transgenic animals.

8.2 Materials and Methods

8.2.1 Animal Availability

Twenty Large-White/Landrace cross 30 kg pigs were obtained from Metro Farms and transported to Davies Field Laboratory, Dept. of Animal Science, Waite Agricultural Research Institute. Animals were maintained individually at 21°C in artificial light for a period of 12 hours every day. Commercial available grower feed (see Appendix I) was feed twice daily at 3x maintenance requirement.

8.2.2 Average Daily Gain (ADG) and Feed Conversion Efficiency (FCR)

Pigs were weighed and FCR determined weekly. Feed requirements were adjusted on this weekly basis so animals always received 3x maintenance diet at two restrictive feedings.

8.2.3 Recombinant pGH Administration

Following the weekly adjustment of daily feed allowance the quantity of pGH to be injected was adjusted accordingly to maintain 90 μg/kg/day. The required amount of rpGH (Bresatec) for one weeks treatment was weighed out weekly and solubilized in filter sterilized sodium borate buffer, pH 9.4, before storage in a sealed sterile container at 4°C until required.

Injection (i.m.) of rpGH or sodium borate buffer occurred between 0800 and 0830 hours into the extensor muscle of the neck. The timing of injection corresponded to the first feeding time for easy of injection and in an effort to minimise stress to the animal.

The rpGH dose of 90 $mg.kg^{-1}.day^{-1}$ was based on optimized levels obtained from initial trials completed by Bresatec (see Figure 8.1). Animals at 30 kgs were allocated to a rpGH dose of 0 (males, females, n=3) or 90 $\mu g.kg^{-1}.day^{-1}$ (males, females, n=3) for 60 days.

8.2.4 Intravenous, Subcutaneous and Slow Release Pellet Administration of rpGH

The remaining eight males were used for clearance studies and investigations into the use of administering rpGH via a slow release pellet. After catheterization animals were allowed to recover from surgical procedures for 12 hours. Following recovery, 2 animals each were injected with 360 $\mu g.kg^{-1}.day^{-1}$ either intravenously via the catheter or subcutaneously. Intravenous catheters (see Chapter 8.2.4) were flushed immediately after administration of rpGH with heparinized saline. Two control animals were injected with an equivalent volume of heparinized saline. The palmitic acid slow release rpGH pellet (200 mg of rpGH) was inserted subcutaneously near the extensor muscle under general anaesthesia in one pig. Blood sampling (5 ml.) commenced immediately after administration of either rpGH or buffer and continued for up to 254 hours.

8.2.5 Carcass Analysis

At 90 kg body weight animals treated with 90 µg/kg/day of rpGH were removed from the trial and slaughtered locally before being sent to Divison of Food and Meat Technology, CSIRO, Queensland for carcass analysis. At slaughter the area surrounding rpGH pellet was cut from the animal for further examination.

Dose-Response Relationship for rpGH Treated Pigs (30-80 kgs.)

Figure 8.1a Feed Conversion Ratio (Source: Bresatec)

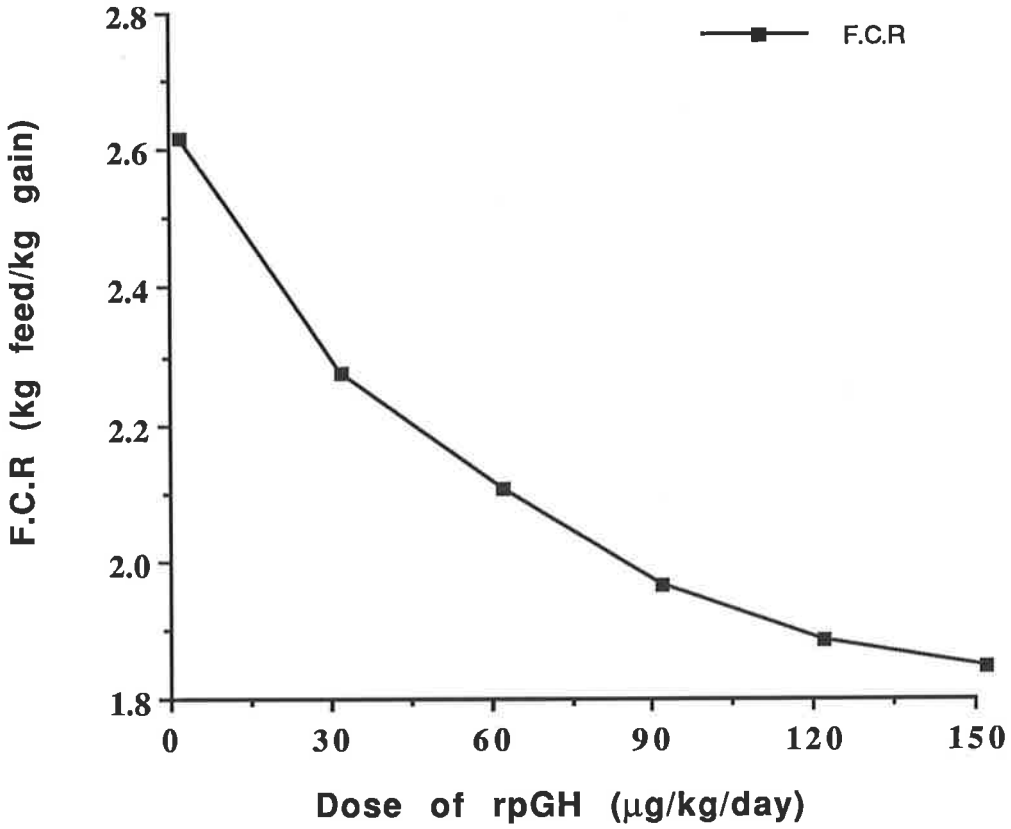
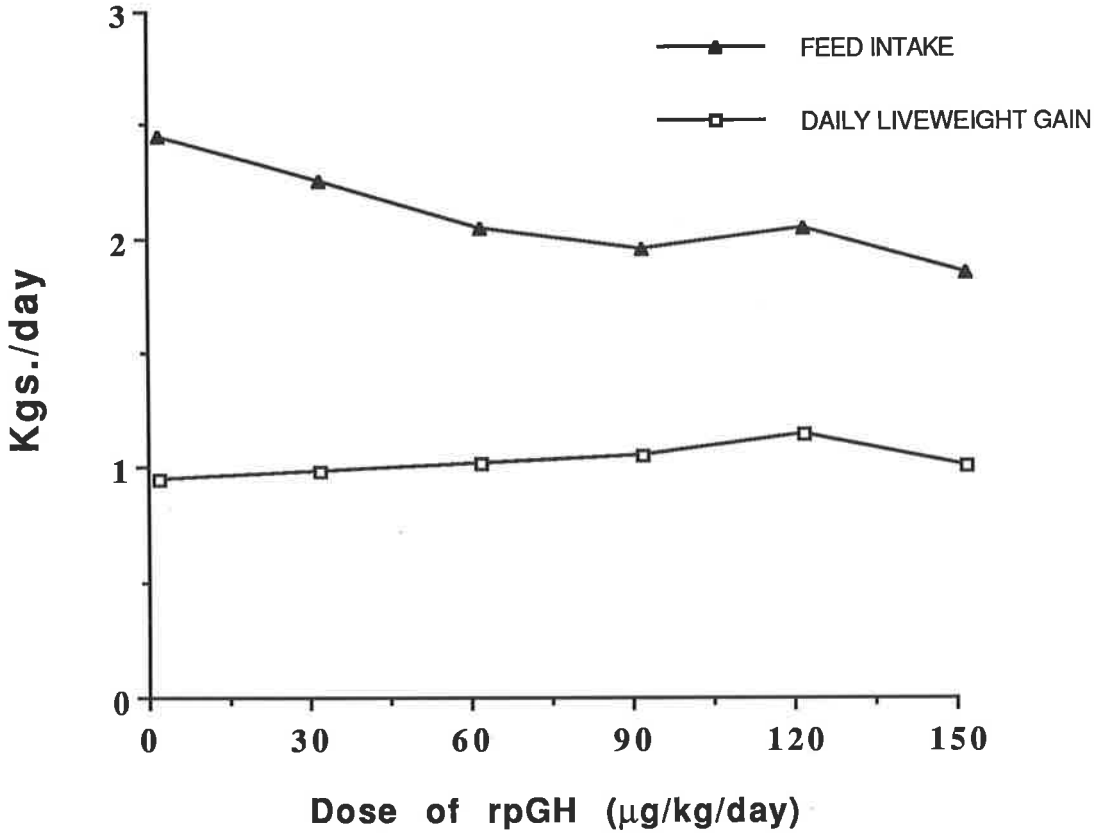


Figure 8.1b Feed Intake and Live Weight Gain



8.2.6 Catheterization

In order to facilitate the regular sampling of blood for assays, animals were catheterized using the following procedure. Following induction and maintenance of general anaesthesia (see Chapter 2.3) a peripheral ear vein was exteriorised and polyethylene tube (SP 70) ((I.D. 1.0 mm, O.D. 1.5 mm) Dural Plastics and Engineering, Dural, NSW Australia) fitted with a 19 gauge needle was inserted into the vein to a length of 20 cm. The end of the catheter was attached to the neck of animal using a belt made from adhesive tape. Catheters were cleaned daily with 250 i.u. of heparinized saline (multiparin, Fisons Pharmaceuticals, Australia) and potency was maintained up to a period of one month.

8.2.7 Measurement of Bioassay Components

8.2.7.1 Blood Sampling

Blood sampling commenced prior to the injections of intravenous or subcutaneous rpGH (360 $\mu g.kg^{-1}.day^{-1}$) or insertion of slow release pellet containing 200 mg of rpGH (equivalent to 4 weeks treatment at 90 $\mu g.kg^{-1}.day^{-1}$ to establish basal levels and continued thereafter at increasing intervals starting at 10 minutes and completing 264 hours later. Serum was collected and processed as per Chapter 2.15.1.

8.2.7.2 Growth Hormone and IGF-I Assays

The concentrations of rpGH were measured by RIA (see Chapter 2.15.2) and IGF-I by RIA following ethanol extraction (see Chapter 2.15.4).

8.2.7.3 Multiple Blood Analysis

The concentrations of various metabolites in serum were determined in samples taken from treated and control animals over a period of 60 days. All biochemical analyses were carried out by Vetlab, Dept. of Agriculture, Sth Aust. All samples were assayed on a Roche™ Cobas Mira (a random access analyzer) at 37°C using methods as recommended by the manufacturer of the kit for each specific biochemical assay.

Albumin (g/l)	Trace Albumin, Bromcresol Green method, Cat. No. 36025.
Bile Acids (μMol/l)	Nycomed, Enzabile Kit, Cat. No. 231540, Nycomed AS Pharma, Norway.
Calcium (mMol/l)	Trace Calcium, cresolphthalein method, Cat. No. 29025.
Chloride (mMol/l)	Direct Electrode method - Cobas Mira.
Cholesterol (mMol/l)	Trace Cholesterol Colorimetric Endpoint assay, Cat. No. 13012, Enzymatic single vial assay.
Creatine (mMol/l)	Roche Bilirubin (Malloy and Evelyn, modified) Kit No. 0717533.
Globulins (g/l)	Total protein less albumin
Glucose (mMol/l)	Trace Glucose Hexokinase Reagent, Cat. No. 15015, End point assay.
Lactate Dehydrogenase	Trace LDH-L Reagent (Tris Buffer) Cat. No. 20012.
Osmolarity (mMol/l)	Calculated 1.86 (Na + K) + Glucose + Urea.
Phosphate (mMol/l)	Trace Inorganic Phosphate reagent, Cat. No. 30025, Direct Phosphomolybdate method without reduction
Potassium (mMol/l)	Direct Electrode method - Cobas Mira.
Sodium (mMol/l)	Direct Electrode method - Cobas Mira.

Total Bilirubin (μMol/l)	Roche creatinine-kinetic picrate method without deproteinization, Kit No. 0711152.
Total Protein (g/l)	Trace Total protein Kit, Cat. No. 34025, Biuret Reagent.
Urea (mMol/l)	Blood urea nitrogen, trace, Cat. No. 12012

Manufacturer of Kits

1: Trace Scientific Pty. Ltd., P.O. Box 310, Clayton, Victoria, AUSTRALIA.

2: Roche Products Pty. Ltd., AUSTRALIA.

8.3 Statistical Analysis

Observations were analyzed using either χhi-square or grouped *t*-test (SAS Institute Inc., 1988).

8.4 Results

8.4.1 ADG and FCR in Growth Hormone Treated Pigs

Administration of 90 μg/kg/day of rpGH over a period of 60 days (30-90 kgs) increased ADG (11%) and FCR (25%) for all treated animals when compared with controls (see Figures 8.2 a,b) and confirmed initial results regarding the potency of the rpGH. Increases in ADG and FCR were detected in as little as four weeks. At the end of the 4 week period ADG had increased only 6% while FCR had increased to 25% (see Figures 8.3 a,b). The administration of rpGH decreased variation in the overall growth rate as a result of females being more responsive to GH than males (see Figures 8.4 a,b).

8.4.2 Clearance of Porcine Growth Hormone

Serum pGH concentrations in control animals were found to be relatively constant over the 254 hour period ranging from 8.0 ng/ml to 36 ng/ml (see Figures 8.5a, 8.6a, 8.7a). Serum pGH levels were elevated in animals injected with 360 μg/kg/day of rpGH either intravenously or

subcutaneously. Intravenous injection of rpGH elevated serum pGH 15 minutes after injection, returning to within range of baseline levels, 2 hours after injection (see Figure 8.5a). Subcutaneous injections of rpGH elevated serum pGH 30 minutes after injection, returning to within range of the baseline, 24 hours after injection (see Figure 8.6a). A subcutaneously inserted slow release rpGH pellet increased growth hormone levels approximately 4 hours after insertion, returning to within range of the baseline after 5 hours (see Figure 8.7a).

8.4.3 IGF-I Response after Treatment with rpGH

Serum IGF-I levels in control pigs remained relatively constant over a 254 hour period ranging between 100 and 260 ng/ml (see Figures 8.5b, 8.6b, 8.7b). Serum IGF-I concentrations were gradually elevated following treatment with rpGH. Injection intravenously or subcutaneously of 360 μg/kg/day of rpGH gradually increased IGF-I 40 min. and 3 hours after injection, returning to within base line ranges 5 and 20 hours after injection respectively (see Figures 8.5b, 8.6b). The Subcutaneous inserted implant increased IGF-I from 4 hours after injection and returned to within base line ranges at approximately 42 hours (see Figure 8.7b).

The integrated response of IGF-I was 2.7 times greater following subcutaneous administration (0.743 units) of GH compared to intravenous (0.275 units).

8.4.4 Metabolite Profiles

Table 8.1 summarises data on selected physiological profiles in serum from rpGH treated pigs versus saline injected pigs. Variation amongst individual pigs was more significant than variation made between animals grouped for treatment with rpGH or saline. No significant differences ($p>0.05$) were observed in any of the parameters examined for differences between rpGH and control pigs over a variety of ages.

8.4.5 Slow Release Pellet of rpGH.

Analysis of serum samples indicated that the palmitic acid pellet of rpGH released rpGH over a period of 2 hours, starting approximately 5 hours after insertion (see Figure 8.7a). The packaging procedure did not affect biological activity as IGF-I concentrations increased significantly after pellet insertion. Examination of the pellet at the time of slaughter found no traces of the pellet only small amounts of adhesive tissue which was probably associated with the surgery at the time of insertion.

8.5 Discussion

The observation that rpGH enhanced growth performance of pigs supports previous findings (Machlin, 1972; Boyd *et al.*, 1986; Campbell *et al.*, 1988a, 1988b; Chung *et al.*, 1985; Etherton *et al.*, 1986; Evock *et al.*, 1988; McLaren *et al.*, 1987; Walton *et al.*, 1987). The results confirmed the sex related magnitudes of response to rpGH administration in males in females (Campbell *et al.*, 1988a; Etherton *et al.*, 1982). The results show FCR is a more sensitive indicator of rpGH effects than ADG.

The comprehensive analysis of physiological profiles from serum indicated that adequate dietary components were supplied and met the extra demands of rpGH administration. The rpGH at the dose selected did not cause any detrimental effects for the parameters tested in serum. Results of calcium levels confirm the observation of Caperna *et al.*, (1989) where no significant interaction with GH was observed. Several reports have indicated an increase in glucose and insulin with GH treatment in fed and fasted states (Chung *et al.*, 1985; Etherton *et al.*, 1986, 1987; Evock *et al.*, 1988; Gopinath and Etherton, 1988a, 1988b). Since our measurement was made on a single sample at several different ages, more frequent sampling over 24 hours might be required to demonstrate an increase in glucose concentration.

The results show that the slow release pellet did not perform as anticipated. Alternative methods that are not constrained by constant

monitoring or loading volumes in osmotic pumps to model the continuous GH pulse as seen in GH transgenic pigs are required. Further research on slow release formulations are under commercial development to determine a suitable mechanism for administration of rpGH that will be commercially acceptable.

Combining the time of injection with feeding reduced stress to an unnoticeable level. The use of an ear vein catheter provides a useful method for the short term catheterization of pigs. It has the advantage that is easily used and animals can be released to abattoirs without devaluation as in other cases (Wangsness *et al.*, 1977).

The pattern of IGF-I response for subcutaneous injections of rpGH was similar to that observed by other workers following intramuscular injections of GH in pigs (Chung, 1985; Sillence and Etherton, 1987). Both modes of GH administration resulted in a dramatic elevation of serum GH within 10 minutes (see Figures 8.5a, 8.6a). The highest level of serum GH seen with intravenous injection of rpGH was twice that following subcutaneous injection. However, serum GH following intravenous injection returned to basal levels within 2 hours, whereas serum GH following subcutaneous injection remained elevated for at least 6 hours, indicating a continuous slow release from the injection site. Serum IGF-I levels reached a similar maximum of 800-900 ng/ml following both modes of administration, but at significantly different times of 1.5 hrs for intravenous and 6 hrs for subcutaneous administration. The elevated levels of IGF-I were sustained for longer following subcutaneous compared to intravenous administration. Consequently the integrated response of IGF-I as assessed by area under the graph is 2.7 times greater for subcutaneous than for intravenous administration. This presumably reflects the longer duration of elevated GH following subcutaneous administration. What remains puzzling is the difference between time lag in serum IGF response to GH between the two modes of administration, particularly as both obtained the highest serum GH peak at the same time.

In the classical hypothesis advanced by Daughaday, IGF synthesis in response to and regulation by GH occurs in liver in post-natal life. More recently, both GH receptors and IGF-I production have been found to occur in other tissues, particularly muscle (Clemmons *et al.*, 1991). In the present study the difference between IGF-I response to mode of GH administration may reflect differences between the exposure of liver and peripheral tissues to GH (Jorgensen *et al.*, 1990). The brief exposure following intravenous injection may result in largely hepatic clearance of the administered GH with relatively little exposure to muscle, with its fewer GH receptors (Clemmons *et al.*, 1991; Jorgensen *et al.*, 1990). Subsequently the rapid transient increase in serum IGF-I following intravenous injection may be largely due to hepatic synthesis and release. In comparison, the sustained serum GH following subcutaneous administration may result in a prolonged exposure of muscle to GH, due to perhaps non-vascular (lymphatic) distribution from the injection site. The failure to detect earlier elevated peaks in serum IGF-I due to hepatic output following subcutaneous administration, may reflect down-regulation of GH receptors in liver following sustained levels of serum GH. Finally, venous blood levels of GH may not accurately reflect GH levels in blood directly perfusing liver and muscle which may differ with the different modes of injection.

More recently IGF binding proteins have been recognized as having a major role in modulation of the activities of the IGF's (Coleman and Etherton, 1991; Baxter, 1991; Zapf *et al.*, 1989). Presently 6 IGFBP's have been identified which influence IGF's anabolic and mitogenic activities in post-natal life by regulating the transport of IGF across the endothelial cell barrier to target cells and IGF availability to IGF receptors on those cells (Baxter, 1991). The modulation can be one of enhancement or inhibition depending on the phosphorylation or glycosylation state of the IGFBP's, which influences the affinity of IGF for binding proteins or their association with cell surfaces respectively (Guyda, 1991). The major correlate, if not determinant, of IGF levels in blood in humans and pigs is the predominant

IGFBP-3 (Walton and Etherton, 1989; Blum and Ranke, 1991; Owens *et al.*, 1991a). The different patterns of GH elevation following each mode of administration may result in induction of different IGFBP's, particularly if liver and peripheral tissues are experiencing different concentrations of GH. For example, induction of smaller IGFBP's (which readily transfer IGF's out of the blood) as opposed to synthesis of IGFBP-3 (which in a tertiary complex with IGF and acid labile subunit is found in the blood) could partly explain differences observed in serum IGF-I patterns (Guyda, 1991). More simply if the synthesis of IGF-I in response to intravenous GH exceeds the binding capacity of IGFBP'S then IGF-I would be subsequently cleared rapidly from the blood in its free form. Additionally, IGFBP's could contribute to the discrepancy by interfering with the IGF-I RIA (Owens *et al.*, 1991a). All samples were acid-ethanol extracted to remove IGFBP's. Whereas this procedure is effective in removing IGFBP-3, it does not remove the smaller IGFBP's. Its relevance remains uncertain, as evidence for pigs of this age indicate IGF-I levels obtained following acid ethanol extraction correlate well with those following acid gel chromatography (Owens *et al.*, 1990,1991b).

Further research is required following unequivocal separation of IGF's and IGFBP's prior to assay or direct analysis of IGFBP's response to GH in these experiments would be desirable to explain the variation in IGF-I concentration with different modes of administration.

Further analysis with only limited data indicates a correlation ($R^2 = 0.944$) exists between the amount of growth hormone and the time to the maximal IGF-I peak (see Figure 8.8). Speculatively, this indicates the higher the level of growth hormone the more rapid the IGF-I response. Further experimentation is still required to understand the complex nature of IGF-I response to growth hormone.

The results of this chapter indicate that the Bresatec rpGH is equal in performance to other exogenous GH treatments reported in use world wide. Daily injections (90 μg/kg/day) confirmed the efficacy of the rpGH

provided in enhancing production characteristics without compromising the health of the animal. Dramatic gains in production parameters are achieved with only modest increments in circulating homologous GH and IGF-I and much of the complications in the past have been due to the markedly enhanced effects of potent heterologous hormones in transgenic pigs. An anticipated trial using a slow release formulation of pGH to closely mimic the transgene situation was unsuccessful due to technical reasons. The production of pigs with functional pGH transgenes may obviate the need for such model experiments. However evidence presented indicates parameters for targeted levels of GH transgene expression in transgenic pigs so as to obtain enhance production characteristics without causing detrimental effects.

Figure 8.2a Mean Growth for rpGH Treated Pigs

The figure compares the mean growth (kgs) of six rpGH treated (squares) and control (closed diamonds) pigs over a period of 10 weeks.

(rpGH = 90 μg/kg/day rpGH)

Figure 8.2b Mean F.C.R. for rpGH Treated Pigs

The figure compares the mean feed conversion ratio (kg gain/kg feed) for six rpGH treated (squares) and control pigs (closed diamonds) over a period of 9 weeks.

The figures compare the increased production characteristics of pigs administered with 90 μg/kg/day of rpGH.

Figure 8.2a Mean Growth Rate for rpGH Treated Pigs

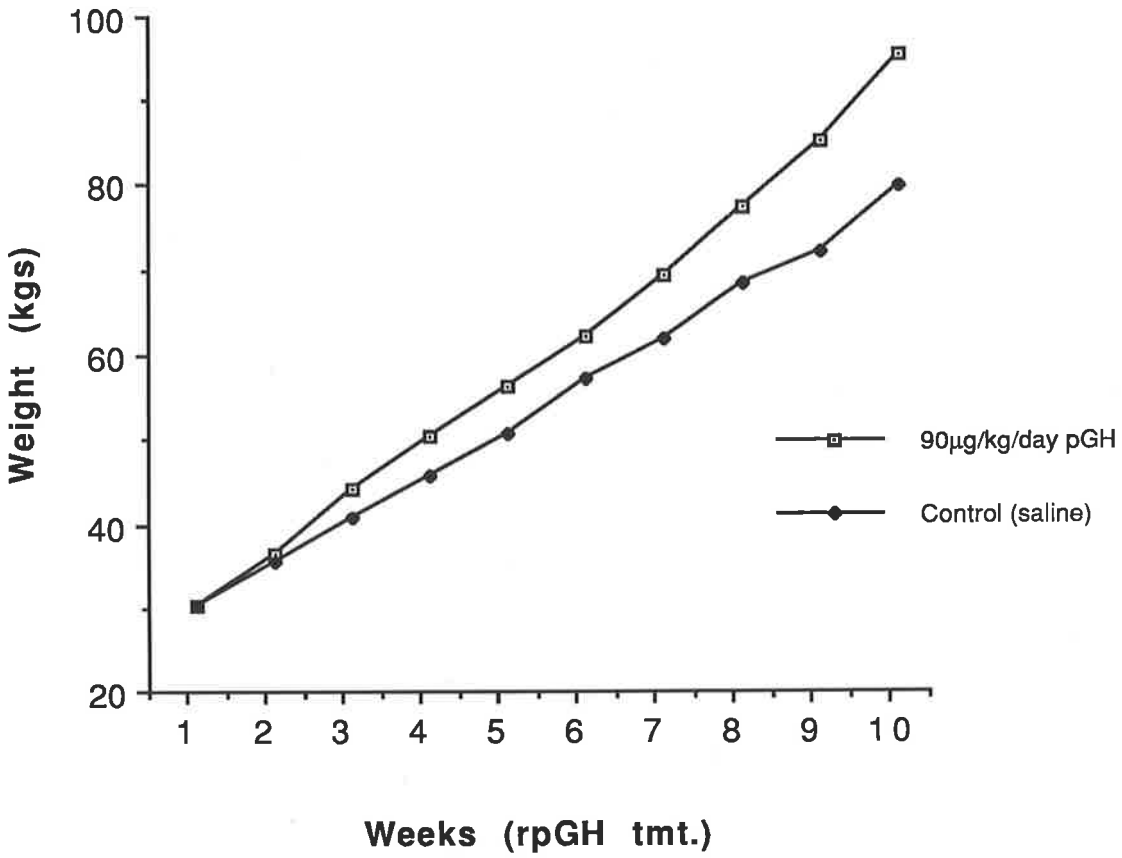


Figure 8.2b Mean F.C.R. for rpGH Treated Pigs

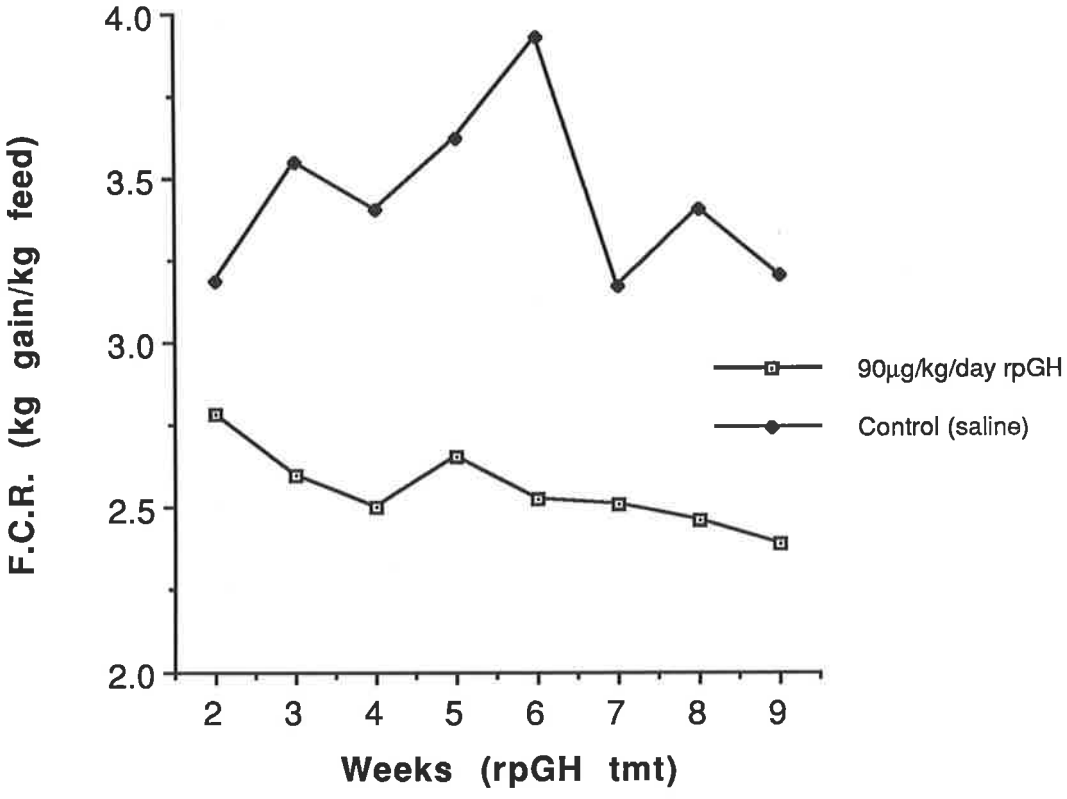


Figure 8.3a GR of Intact Male Pigs Treated with rpGH

The figure compares the weight of four pigs over a period of 5 weeks.

Symbols represent different treatments :-
90 μg/kg/day rpGH i.m. (squares)
rpGH (sub. cut.) slow release pellet (closed diamonds).
(pellet inserted week 0)
control (i.m. saline) (closed squares and open diamonds).

Figure 8.3b F.C.R. of Intact Male Pigs Treated with rpGH

The figure compares the individual F.C.R. of four pigs over a period of 4 weeks.

Symbols represent different treatments :-
90 μg/kg/day rpGH i.m. (squares)
rpGH (sub. cut.) slow release pellet (closed diamonds).
(pellet inserted week 0)
control (i.m. saline) (closed squares and open diamonds).

The figures demonstrate the effectiveness of administering 90 μg/kg/day of rpGH using a slow release pellet.

Figure 8.3a GR of Intact Male Pigs Treated with rpGH

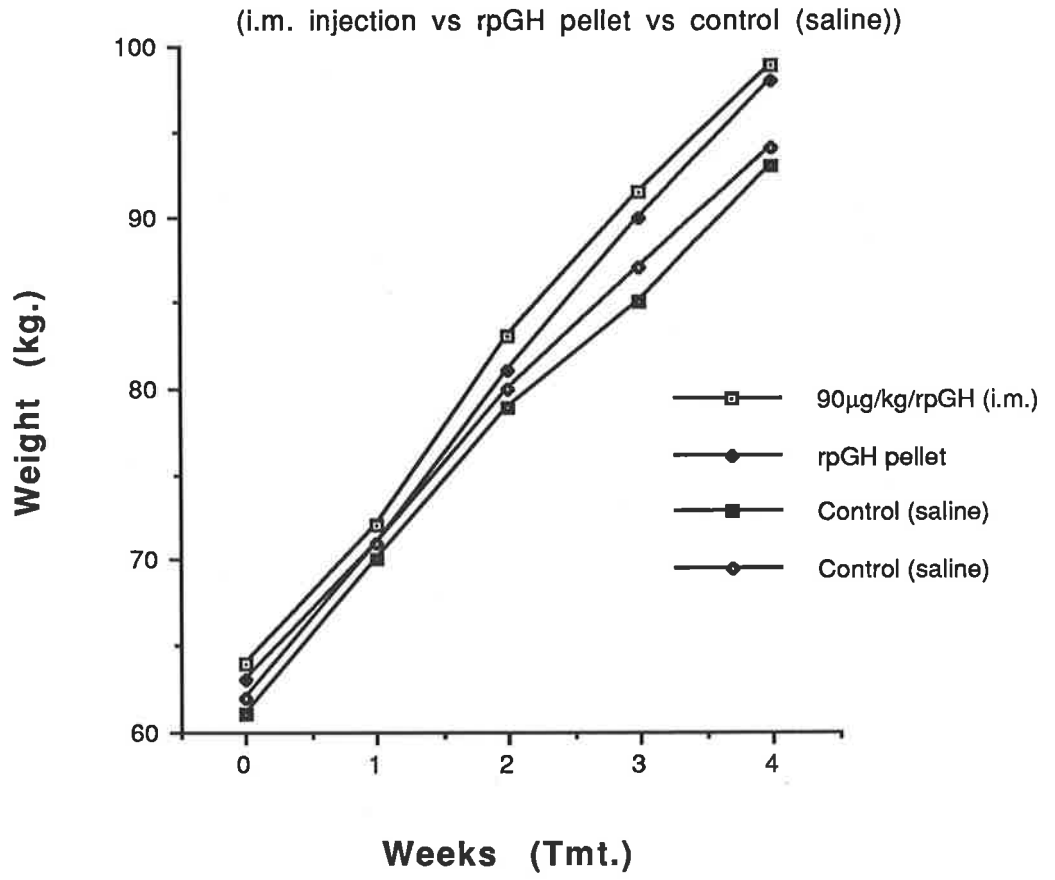


Figure 8.3b F.C.R of Intact Male Pigs Treated with rpGH

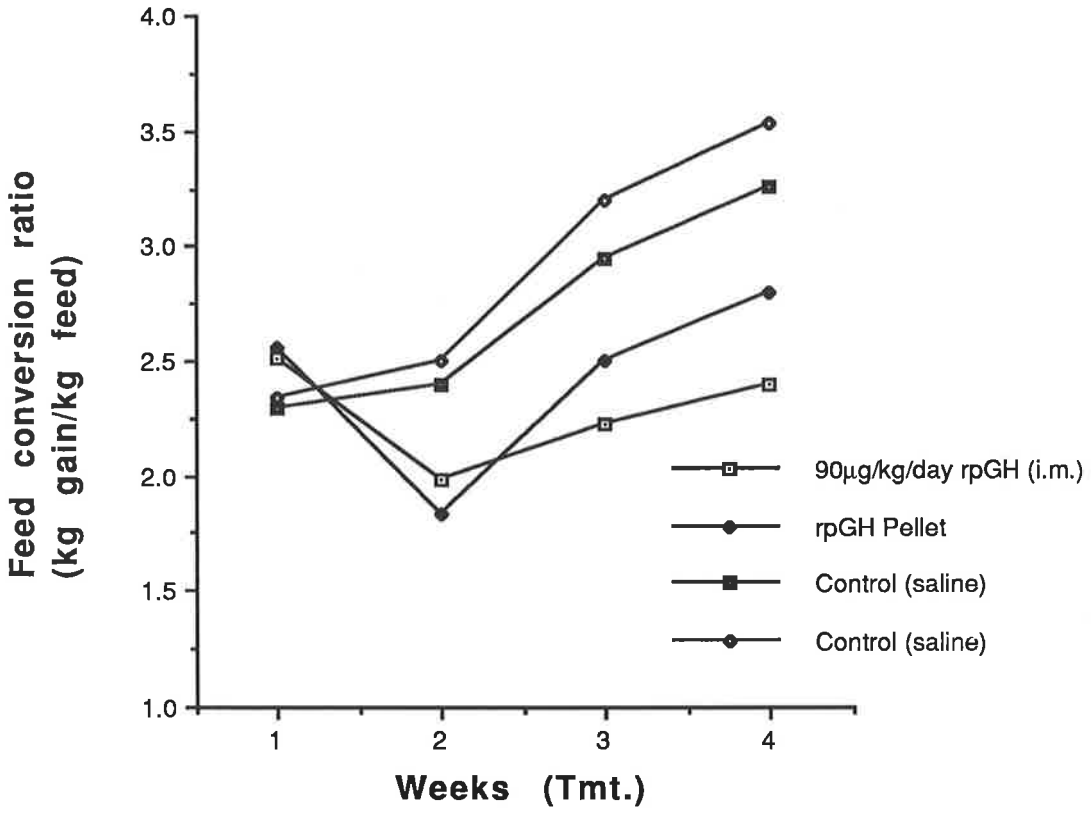


Figure 8.4a Growth Rate of rpGH Treated Pigs

The figure shows the weight of six pigs treated with rpGH (90 μg/kg/day, i.m.) over a period of 10 weeks.

(Individual pigs are represented by different symbols)

Figure 8.4b Growth Rate of Control Pigs

The figure shows the growth rate of six pigs treated (control, i.m.) with saline over a period of 10 weeks.

(Individual pigs are represented by different symbols)

Figure 8.4a Growth Rate of rpGH Treated Pigs

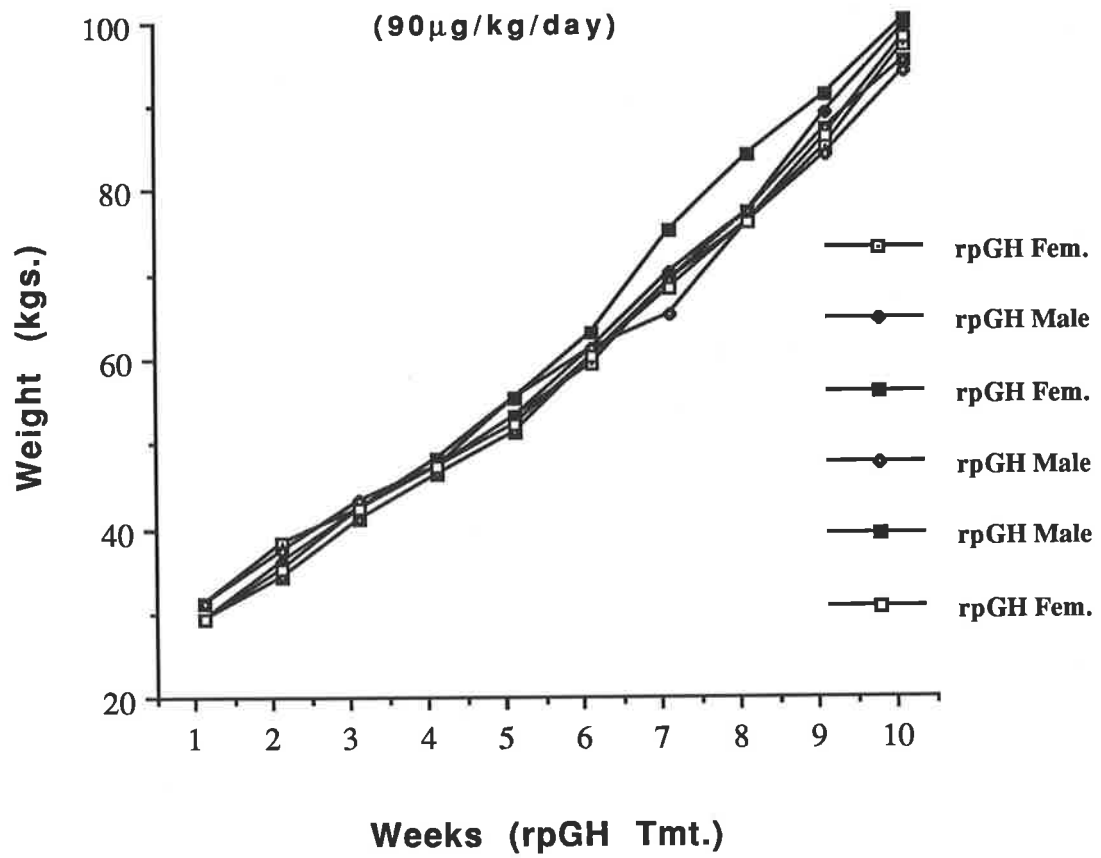


Figure 8.4b Growth Rate of Control Pigs

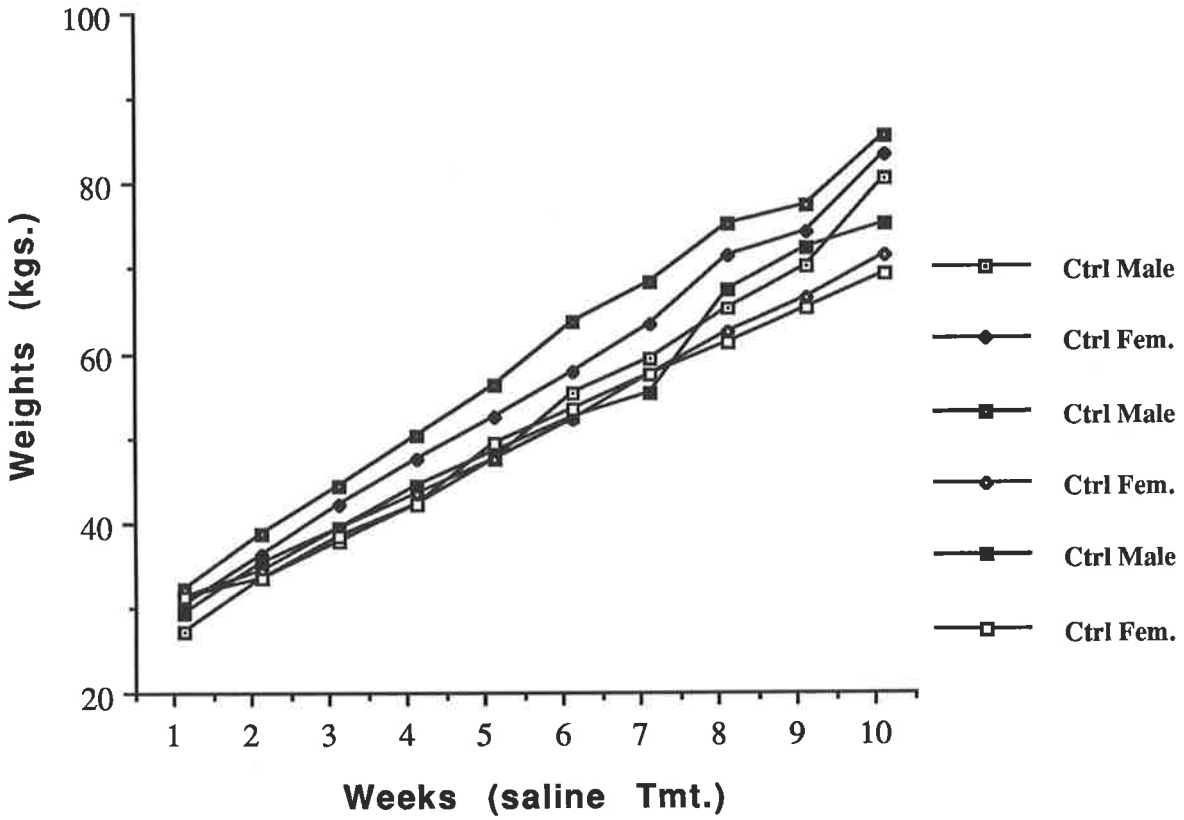


Figure 8.5a Clearance of I.V. Injected rpGH

The figure shows the mean clearance of rpGH (360 μg/kg/day, i.v.) in three pigs compared with three control (saline, i.v.) pigs over 6 hours.

Figure 8.5b IGF-I Response after I.V. rpGH

The figure shows the mean IGF-I response in three pigs treated with 360 μg/kg/day of rpGH (i.v.) compared with three control (saline, i.v.) pigs over 6 hours.

Figure 8.5a Clearance of I.V. Injected rpGH

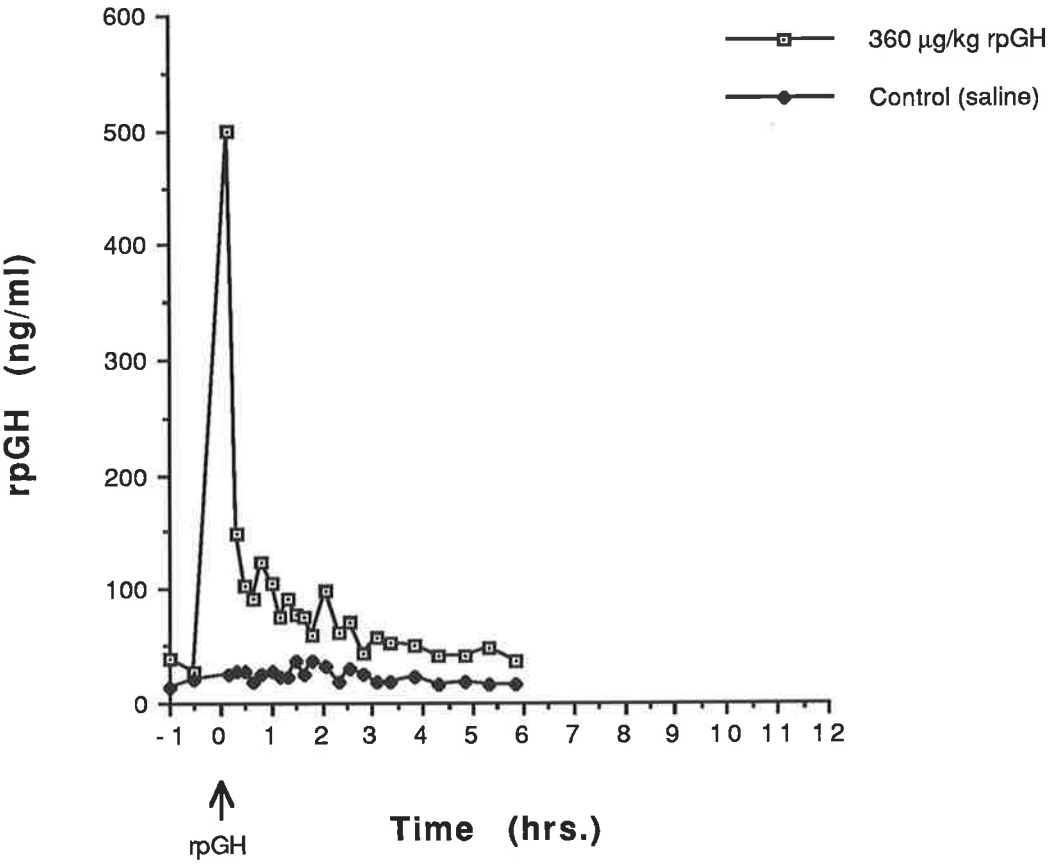


Figure 8.5b IGF-I Response after I.V. rpGH

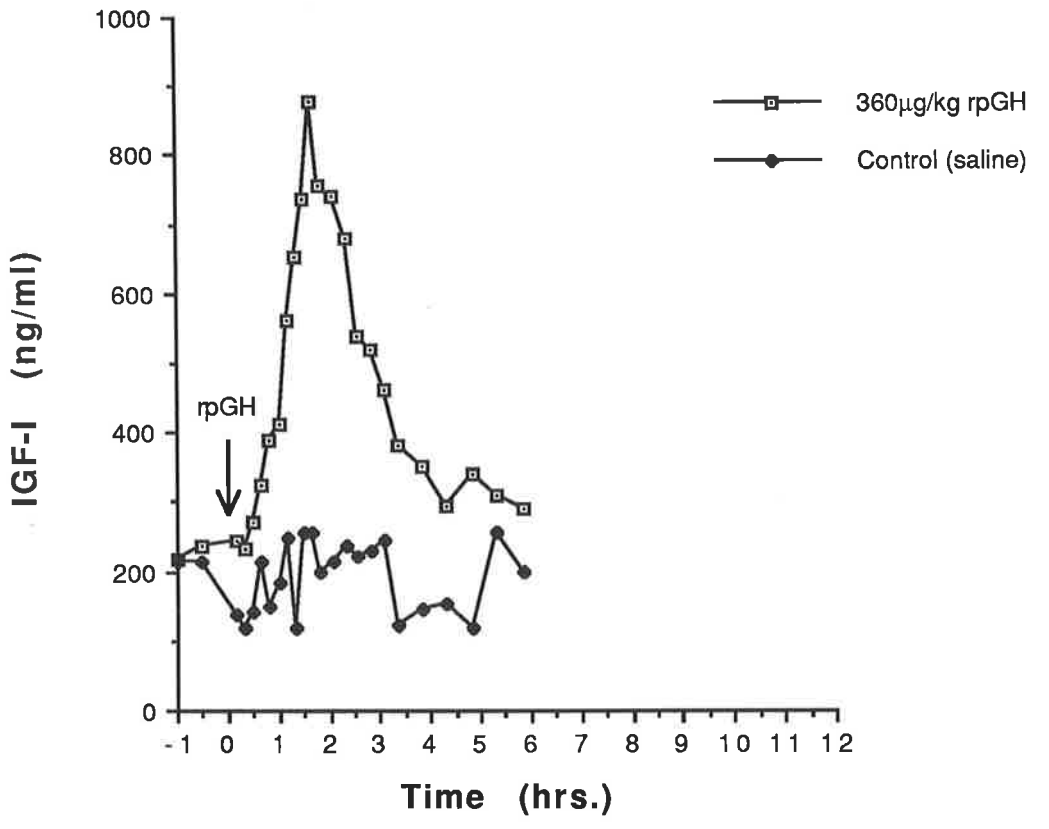


Figure 8.6a Clearance of Sub. Cut. Injected rpGH

The figure shows the mean clearance of rpGH (360 μg/kg/day, sub. cut.) in three pigs compared with three control pigs (saline. sub. cut.) over 34 hours.

Figure 8.6b IGF-I Response after Sub. Cut. Injection of rpGH

The figure shows the mean response of IGF-I after rpGH (360 μg/kg/day. sub. cut.) in three pigs compared with three control pigs (saline, sub. cut.) over 34 hours.

Figure 8.6a Clearance of Sub. Cut. Injected rpGH

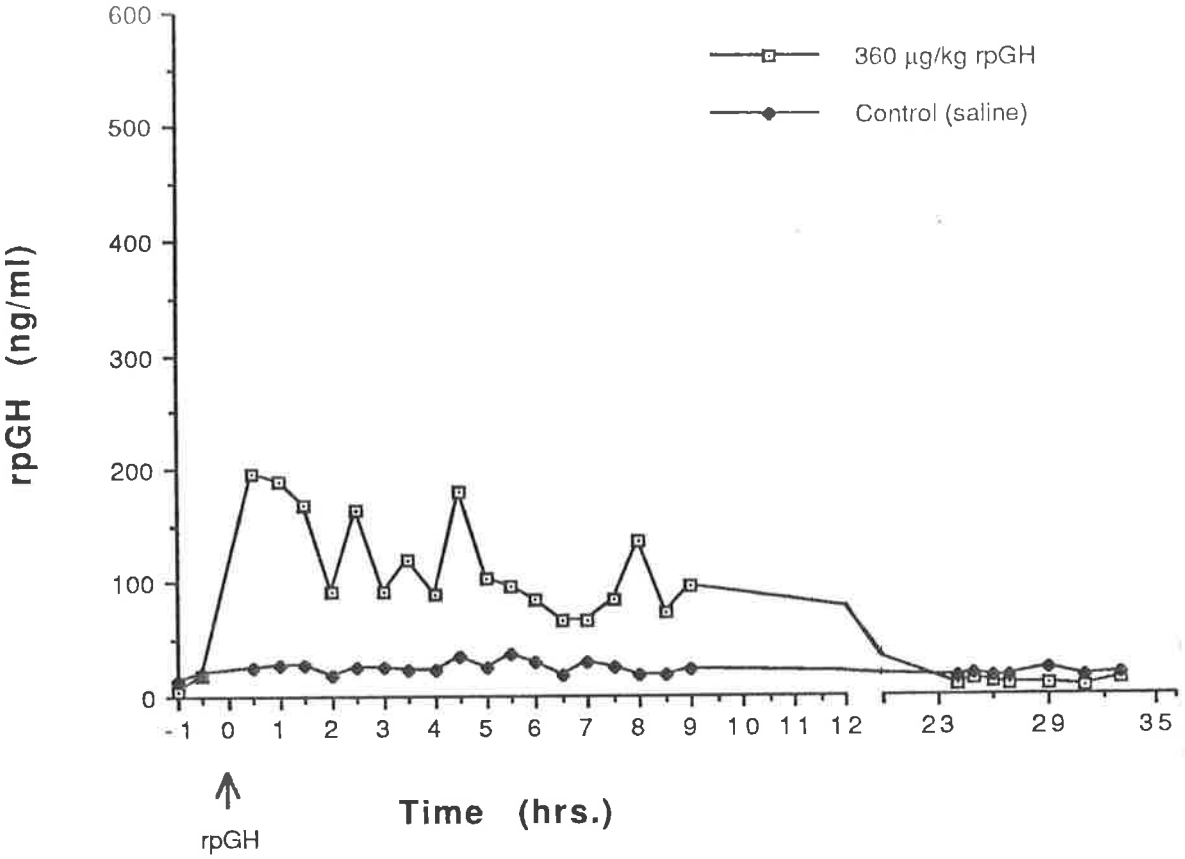


Figure 8.6b IGF-I Response after Sub. Cut. Injection of rpGH

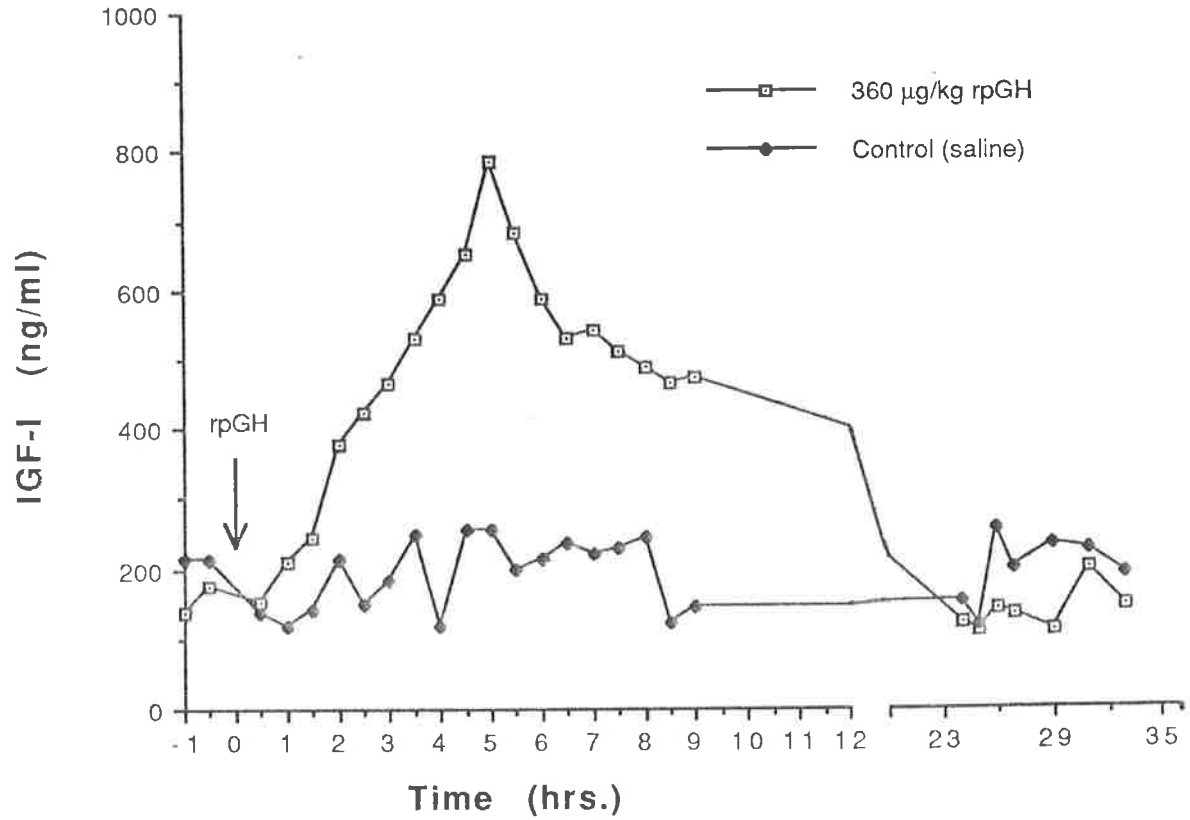


Figure 8.7a Clearance of rpGH from Sub. Cut. rpGH implant

The figure shows the clearance of rpGH (slow release pellet, 90 μg/kg/day, sub. cut.) in a pig compared with a control pig (saline. sub. cut.) over 35 hours.

Figure 8.7b IGF-I Response after Sub. Cut. rpGH implant

The figure shows the response of IGF-I after rpGH (slow release pellet, 90 μg/kg/day. sub. cut.) in a pig compared with a control pig (saline, sub. cut.) over 240 hours.

Figure 8.7a Clearance of rpGH from Sub. Cut. rpGH implant

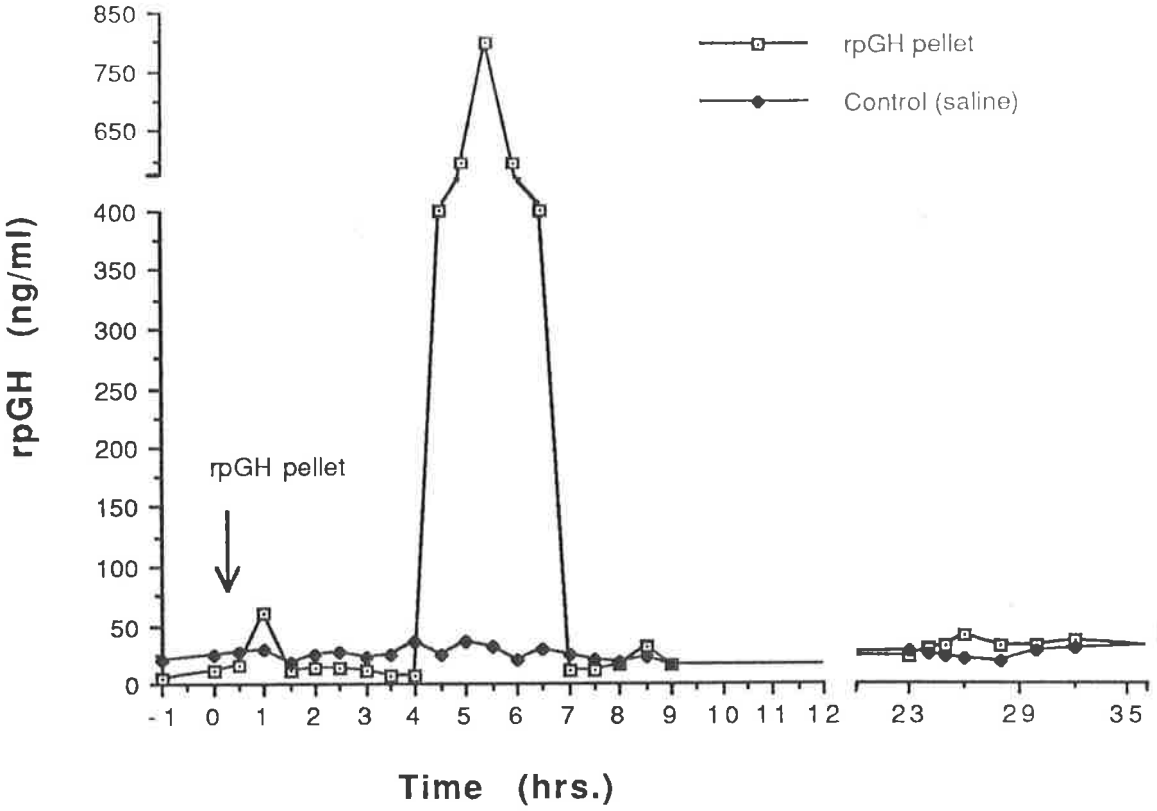


Figure 8.7b IGF-I Response after Sub. Cut. rpGH Implant

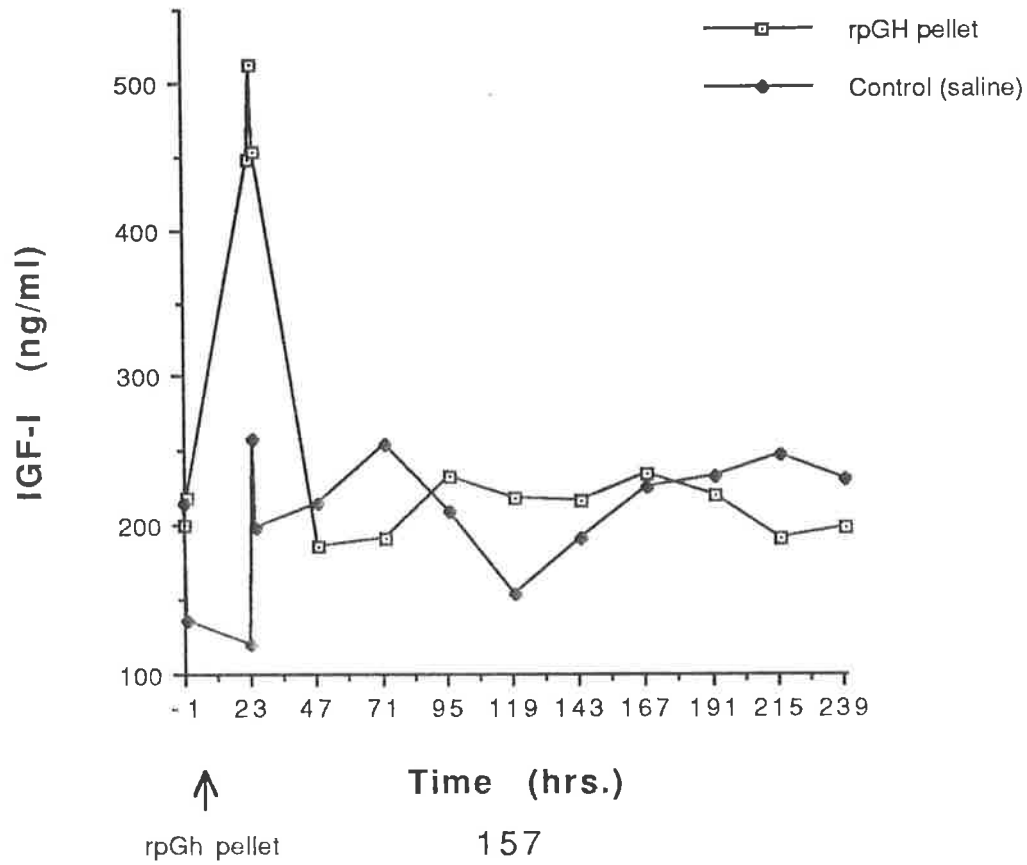


TABLE 8.1 METABOLIC PARAMETERS OF RPGH TREATED PIGS

	Status	Age (Days)	Albumin	Bile Acids	Calcium	Cholest.	Chloride	Creat.	Globulin	Glucose	Potassium	LDH	Sodium	Osmolarity	Phos.	T. Prot.	T. Bili.	Urea
-100	Ctrl M	108	40 +	6	2.05	2 -	101	0.09 -	30	3.5	6.8 +	494	139	280 -	2.54	70	0.1	5.4
-200	Ctrl F	108	41 +	9	2.44	2.2 -	101	0.07 -	31	4.4	6.4	490	139	280 -	2.54	72	0.1	5.2
-300	Ctrl M	108	33	7	2	2 -	100	0.07 -	30	3.9	6.1	504	138	277 -	2.23	63 -	0.1	5.1
-400	Ctrl F	108	39 +	14	2.16	1.5 -	102	0.09 -	23 -	3.3	6.6	544	138	278 -	2.52	62 -	0.1	5.5
1 1/11	rpGH M	120	38	5	2.25	3.4	92 -	0.1	35	4.5	6.3	425 -	134 -	270 -	2.49	73	0.1	4.4
1 5/11	rpGH M	125	39 +	9	2.15	2.9	92 -	0.09 -	35	4.1	6.5	365 -	135	274 -	2.62	75	0.1	6.6
1 Ser.	rpGH M	152	42 +	10	2.84	3	99	0.11	37	10.6 +	11.8 +	508	142	304 +	2.7	79	0.1	7.6
2 1/11	rpGH F	120	32	7	2.51	2.3 -	93 -	0.09 -	31	0.3 -	7.2 +	605 +	136	271 -	2.72	63 -	0.1	3.9
2 5/11	rpGH F	125	35	9	1.78 -	2.7 -	90 -	0.17	31	5	11.4 +	10989 +	129 -	270 -	3.7	66	0.1	4.1
2 Ser.	rpGH F	152	34	16	2.8	2.7 -	94	0.13	34	8 +	15.5 +	367 -	133 -	288	3.29	67	0.1	3.8
3 1/11	rpGH M	120	33	5	2.76	2.6 -	95	0.04 -	27	0.5 -	5.8	185 -	135	266 -	2.92	60 -	0.1	3.8
3 5/11	rpGh M	125	36	6	2.5	2.9	95	0.1	29	4.9	6.9 +	554	136	275 -	2.82	65	0.1	4
3 Ser.	rpGH M	152	37	6	3.07 +	2.2 -	96	0.14	30	0.4 -	12.9 +	515	139	288	3.36	66	0.1	3.4
4 1/11	rpGH F	120	33	7	2.66	2.7 -	94	0.11	34	0.3 -	7.2 +	250 -	135	269 -	2.79	67	0.1	4
4 5/11	rpGH F	125	37	7	2.67	2.8	93 -	0.11	32	5	7.7 +	657 +	134 -	272 -	2.53	68	0.1	3.5
4 Ser.	rpGH F	152	45 +	9	3.26 +	3.4	114 +	0.12	39	9.8 +	18.2 +	582	158 +	344 +	3.08	84	0.1	6.8
5 1/11	Ctrl M	120	34	14	2.53	2.5 -	92 -	0.1	30	5.7	6.7	99 -	132 -	270 -	2.99	63 -	0.1	5.9
5 Ser.	Ctrl M	152	34	19	2.57	2.3 -	97	0.11	29	10.3 +	14.3 +	577	133 -	288	2.57	62 -	0.1	3.7
6 1/11	Ctrl F	120	36	9	2.58	2.5 -	93 -	0.09 -	30	0.4 -	6.7	61 -	135	272 -	2.49	67	0.1	8.2
6 5/11	Ctrl F	125	36	15	2.41	2.8	92 -	0.1	33	4.6	6.9 +	401 -	134 -	274 -	2.25	69	0.1	7.1
6 Ser.	Ctrl F	152	43 +	8	3.53 +	2.7 -	99	0.11	37	5	12.4 +	622 +	148	309 +	3.57	80	0.1	5.2
7 1/11	Ctrl M	120	37	14	2.76	1.7 -	93 -	0.1	25	0.5 -	5.8	39 -	135	266 -	2.68	62 -	0.1	3.6
7 5/11	Ctrl M	125	38	3	2.56	2.9	94	0.09 -	38	1.7 -	7.3 +	272 -	138	277 -	2.76	76	0.1	5.4
7 5/11 B	Ctrl M	125	38	4	2.57	3	95	0.11	38	4.5	7.4 +	254 -	139	282	2.72	76	0.1	5.2
7-1	Ctrl M	110	36	11	2.53	2 -	99	0.09 -	26	0.3 -	4.7	231 -	142	277 -	2.83	63 -	0.1	3.8
7 Ser.	Ctrl M	152	44 +	8	2.85	2.7 -	97	0.14	30	8.5 +	11.3 +	527	141	296	3.16	74	0.1	3.9
8	Ctrl F	110	23	6	1.89 -	2.2 -	101	0.1	27	1.5 -	7.4 +	138 -	131 -	262 -	2.02	50 -	1	3.2 -
8 1/11	Ctrl F	120	37	5	2.75	2.9	94	0.12	35	3.9	6.9 +	292 -	135	273 -	3.06	72	0.1	4.8
8 5/11	Ctrl F	125	39 +	7	2.65	2.5 -	94	0.1	28	4.7	6.9 +	200 -	136	276 -	2.27	67	0.1	5.6
8 Ser.	Ctrl F	152	35	6	3.31 +	2.8	100	0.12	32	12.4 +	12.4 +	72 -	138	297	3.19	67	0.1	4.5

Reference:

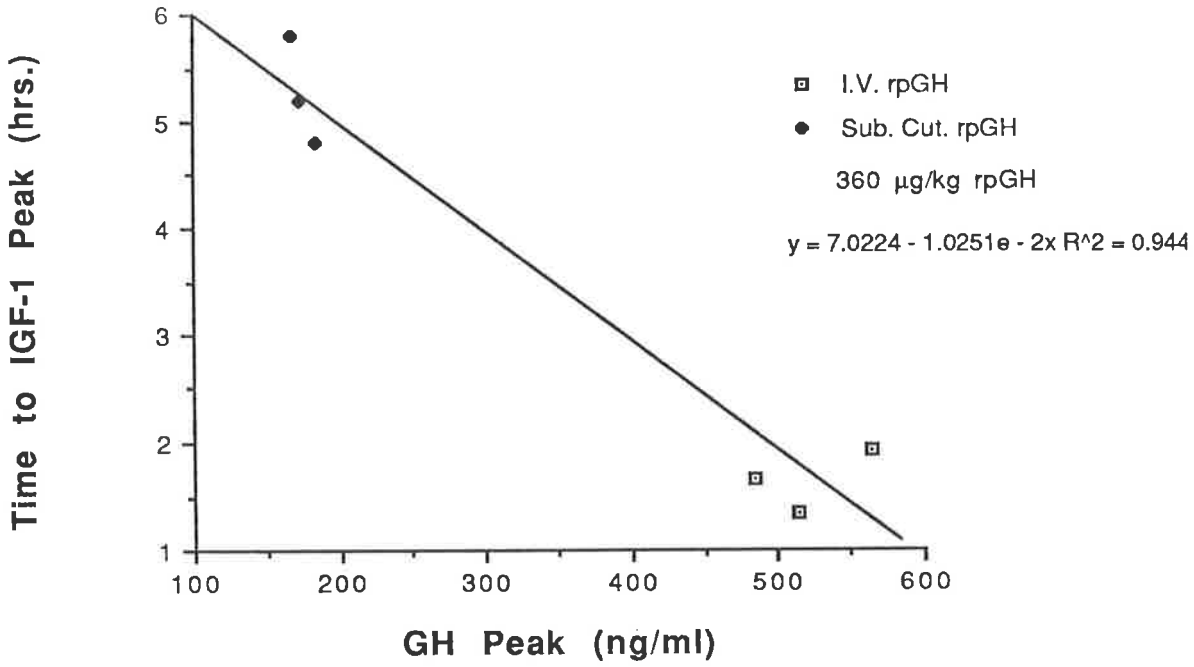
M/F male or female
Ctrl Control
rpGH recombinant porcine growth hormone
+/- outside of normal range

Figure 8.8 IGF-I Response for I.V. and Sub. Cut. rpGH (IGF-I Peak Time vs GH Peak)

The figure shows the mean time to the IGF-I Peak compared with the GH peak in pigs (n=3) treated with rpGH (360 μg/kg) either sub. cutaneously or intravenously.

(sub. cut., rpGH = closed diamonds)
(i.v., rpGH = open squares)

Figure 8.8 IGF-1 Response for I.V. and Sub. Cut. rpGH
(IGF-1 Peak Time vs GH Peak)



CHAPTER IX

ASSESSMENT OF PGH TRANSGENIC PIGS

9.1 Introduction

Following the production of transgenic mice harbouring a multifarious of transgenes (see reviews, Brinster and Palmiter, 1986; Gordon, 1989; Palmiter and Brinster, 1986) several laboratories have reported the successful insertion of transgenes into a variety of livestock species including pigs, sheep and cattle (see review, Pinkert, 1987; Pinkert *et al.*, 1989; Pursel *et al.*, 1989,1991).

In the pig several methods of introduction transgenes have been evaluated including using viral vectors and embryonic stem cells, however direct microinjection of the transgene into the zygote is the only approach has so far resulted in the production of transgenic offspring (see review, Pursel *et al.*, 1990).

A major concern arising from the initial transgenic experiments with pigs is that many of the transgenic animals produced have shown undesirable side effects associated with growth, health and reproduction status a consequence of uncontrolled transgene expression.

This study is based on transgenic pigs produced using a transgene comprised of a modified human metallothionien promoter attached to a homologous growth hormone. Model experiments in a murine model demonstrated the construct performed to produce modestly elevated levels of circulating growth hormone without basal or runaway expression whilst maintaining zinc inducibility. This study aimed at assessing transgenic pigs created using this construct for their potential for commercial use. Production parameters studied included growth efficiency, reproductive capacity, heritability of the gene and its expression and responsiveness to dietary zinc.

9.2 Materials and Methods

9.2.1 Animal Availability

All animals assessed as transgenic together with transgenic sex matched littermates used as controls were produced from protocols established for the production of transgenic animals (see Chapter 5) or from selected breeding of transgenic animals.

9.2.2 Heritability of Transgene

The heritability of the MT-pGH construct was assessed by breeding selected male and female transgenic animals from both Northfield and Wasleys piggeries. Blood and tissue samples were taken from offspring at birth for assessment of transgene status using DNA-DNA hybridization protocols and measurement of GH and IGF-I plasma levels using validated immunoassays (see Chapters 2.14.1, 2.15.1, 2.15.2, 2.15.4).

9.2.3 Production Characteristics

Due to constraints set by the University Biohazard Committee, the available experimental facilities that could be utilised for the project were extremely limited precluding extensive investigations. All pigs were checked daily to ascertain health status and weighed weekly to determine average daily gain (ADG). Accurate measurement of feed conversion efficiency (FCR) could not be obtained. Backfat (P2) measurements were made at 90 kg market weight and designated animals were slaughtered on site for tissue, carcass analysis and production disease status.

9.2.4 Control of Transgene Expression

In order to establish if the metallothionien promoter of the integrated transgene regulated the expression of pGH, grower diets (see Appendix I) were formulated to include 1000 ppm of zinc as sulphate plus 100 ppm copper as sulphate. Allowances were made to account for 100 ppm of zinc

and 50 ppm of copper in a non supplemented diet and an environmental component of between 40-80 ppm of heavy metals.

In supplementation trials animals were fed *ad libitum*, zinc supplemented grower ration from 40 to 80 kgs, then a finisher ration (see Appendix I) with no zinc supplementation.

Three blood samples were taken every 6 hours over an 18 hour period during zinc supplemented and non zinc supplemented diets for analysis (see Chapter 2.15.1) of zinc by atomic absorption spectrophotometry (VetLab) and GH and IGF-I by RIA assays (see Chapters 2.15.2, 2.15.4).

Further multiple blood analysis of metabolic parameters were analysed by VetLab (see Chapter 8.2.7.3) to determine changes in metabolic profiles.

9.2.5 Health Status

The health status of animals was continually monitored for any signs similar to those identified as causing problems in the USA transgenic stock. At slaughter animals were also subjected to extensive post mortems with special focus on abnormal pathology of articular surfaces or the digestive systems for arthritis and gastric ulcers respectively.

9.3 Statistical Analysis

Statistical analysis between transgenic and non transgenic littermates were analysed by linear regression and ANOVA (SAS Institute Inc., 1988).

9.4 Results

9.4.1 Northfield Transgenic Program

1987 Gene Construct pHMPG$_{0.01}$

Microinjection protocols using the heavy metal sensitive construct of Vize *et al.*, (1988) yielded 5 transgenic animals from 25 liveborn (see Chapter 6). Two transgenic animals died as a result of savaging by the recipient gilt

(see Table 9.1). Sow F5 was subsequently mated to three different non transgenic boars to create 3 litters (see Table 9.2).

TABLE 9.1 NORTHFIELD TRANSGENIC OFFSPRING: TRIAL 1

I.D.	GH (ng/ml)
Sow F1 died at 133 days old, gastric ulcer	272
Boar F7 died (1week)	240
Sow F5	45

TABLE 9.2 SUMMARY OF DATA ON LITTERS RESULTING FROM MATING OF TRANSGENIC SOW F5

Litter 1	*Litter 2*	*Litter 3*
24/10/88 5/9 transgenic	14/4/89 7/10 transgenic	10/9/89 0/9 transgenic

Litter 1

I.D.	Sex	T'genic	ADG (40-90 kg)	GH (birth) (ng/ml)
F44	M	T	0.81	28
F45	M		0.75	29
F46	M	T	0.86	90
F47	M		0.75	30
F48	M	T	0.80	40
F49	F		ND	23
F50	M	T	0.76	27
F51	F		ND	38
F52	F		ND	7

Litter 2

I.D.	Sex	T'genic	ADG	P2	Birth	GH (ng/ml) Zn-	Zn+	Zn-
F53	F	T	0.53	11	33	16.8	12.5	9.9
F54	M	T	0.58	11.5	41	29.8	31.8	13.5
F55	F	T	0.54	12	60	16.6	6.4	3.9
F56	F	T	0.55	14	49	11.9	16.7	7.7
F57	F	T	0.53	12	38	15.3	24.5	7.4
F58	M		0.64	14	25	12.0	19.8	8.4
F59	M		0.60	13	39	16.1	14.8	4.5
F60	F	T	0.52	14.5	53	11.3	12.4	5.4
F61	F	T	DAB		119			
F62	M		0.60	13.5	7	19.0	11.6	5.1

Litter 3 Nine live offspring, no transgenics detected (data not presented).

1988 Gene Construct pHMPG$_{0.05}$

This construct showed evidence of regulation by zinc in both tissue and murine model studies.

The program in pigs resulted in 9 transgenic animals from 43 live offspring. In one recipient 6 transgenic and all non transgenic offspring died during farrowing from problems relating to the adverse health of the recipient at parturition (see Table 9.3). Transgenic Boar F10 was successfully mated to a non transgenic sow before it died at 203 days old and a litter was born on 3/8/88 of which 9 of the 12 proved to be transgenic.

TABLE 9.3 NORTHFIELD TRANSGENIC OFFSPRING: TRIAL 2

	GH (ng/ml)
Boar F20 failed to thrive	197 mg/ml
Boar F40 chimera not breed,	110 mg/ml
Boar F10	103 mg/ml

The progeny identified as, F63-75 were reared on a commercial diet (see Appendix I) and tested at 68 days of age for Zn responsiveness by feeding them for 1 week on a Zn supplemented diet (see Chapter 9.2.4). Data from both sites for transgenic status, sex, ADG between 40-90 kgs., P2 and plasma GH and IGF-I levels both before, during and after zinc supplementation are summarised for all transgenic and non transgenic littermates in the following tables (Tables 9.4, 9.5 and 9.6). Plasma samples were taken day -1 and day +7 of Zn supplementation (see Table 9.4) for GH analysis (see Chapter 2.15.2).

TABLE 9.4 SUMMARY OF PROGENY OF BOAR F10: ZINC INDUCIBLE TRANSGENE EXPRESSION

I.D.	Sex	T'genic	ADG	P2	GH (ng/ml) Zn-	Zn+	Zn-
F63	F	Died at birth					
F64	F	T	0.5	17	11.2	14.5	17
F65	F	T	0.36	7	49.5	31.2	43.1
F66	M	T	0.62	12	14.2	15.8	12.4
F67	F	T	0.57	9	22.3	15.8	9.6
F68	F	T	0.49	13	19.0	60.0	12.4
F69	M		0.58	11	39.0	18.4	8.6
F71	M	T	Died (Day 7)				
F72	M	T	Culled prolapse (Day 112)				
F73	M	T	0.46	10	10.9	19.6	10.0
F74	F	T	0.51	11	11.7	12.7	6.6
F75	F		0.55	13	10.9	10.6	8.7

Sows F64 and F68 were retained and mated to Transgenic Boar F54 to create potentially double transgenic (ie pHMPG$_{.05}$ and $_{.01}$) The resulting litters produced F05-F12 (2/8 Transgenic) and F93-F04 (6/11 transgenic) respectively (see Table 9.5).

All Wasleys animals were reared from 40-80 kgs on a Zn supplemented diet. Blood samples were taken one day prior to, during and 3 weeks following termination of Zn supplementation over a 48 hour period (see Chapter 9.2.4).

TABLE 9.5 SUMMARY OF NORTHFIELD TRANSGENIC OFFSPRING: ZINC INDUCED TRANSGENE EXPRESSION

Litter F64

					GH & IGF-I (ng/ml)					
					Zn-		Zn+		Zn-	
I.D.	Sex	T'genic	ADG	P2	GH	IGF	GH	IGF	GH	IGF
F05	F		0.61	12	23.4	85	28.1	186	26.4	112
F06	F		ND							
F07	F		0.61	9.5	14.9	127	15.6	154	13.4	148
F08	F		0.67	12	18.3	131	19.1	192	20.4	187
F09	F		0.69	12	17.4	87	20.5	113	24.6	107
F10	M	T	0.84	11.5	16.1	218	33.2	553	17.4	465
F11	F	T	0.64	12	19.5	136	31.4	174	21.3	158
F12	M		0.74	13	14.0	236	21.6	215	18.9	194

Litter F68

					GH & IGF-I (ng/ml)					
					Zn-		Zn+		Zn-	
I.D.	Sex	T'genic	ADG	P2	GH	IGF	GH	IGF	GH	IGF
F93	F		0.66	10.5	18.0	53	14.5	89	16.4	64
F94	M	T	0.82	13	34.2	302	14.4	328	12.3	309
F95	M		0.78	10.5	32.5	112	14.8	124	16.9	104
F96	F	T	0.93	10	27.6	191	43.9	469	24.0	185
F97	M	T	0.93	12	24.6	346	24.8	448	23.5	394
F98	M	T	0.73	13.5	23.9	120	24.4	117	22.5	109
F99	F	T	0.82	12.5	18.9	94	28.9	220	8.6	67
F01	M	T	0.61	10.5	23.6	288	23.1	515	18.8	128
F02	F		0.67	13	16.3	71	19.6	109	18.4	85
F03	M		0.74	12	22.0	113	8.3	197	12.3	142
F04	M		0.78	13	15.3	247	19.1	303	16.4	212

9.4.2 Wasleys Transgenic Program

1989 *Foundation stock created with* $pHMPG_{0.05}$

Wasleys transgenic program produced 6/46 transgenic pigs with 3 expressing animals, Boars EM 1027, EM 1025 and Sow EM 1045. Boar EM 1025 died of natural causes on 26/7/89 without mating. Boar EM 1027 and Sow EM 1045 were grown without Zn supplement and then mated to non transgenic stock with the aim of creating 5-6 litters that could be evaluated

for performance under commercial (Wasleys) conditions on a Zn supplemented diet.

Five litters were created and all litters were reared post weaning on commercial diets supplemented with Zn (see Chapter 9.2.4) until animals achieved targeted market weight of 90 kgs (see Table 9.6). Animals were then returned to a commercial diet without supplement for 3 weeks prior to slaughter, carcass appraisal and tissue analysis. Plasma samples were taken over a 24 hour period at weaning, 60 kg body weight and just prior to sacrifice for GH and IGF-I RIA analysis (see Chapter 9.2.4).

Post mortem assessment confirmed that the health of Wasleys animals had been severely compromised by maintaining animals as an isolated group as required by the Biohazard Committee. The number of animals failed to produce enough body heat to maintain acceptable environmental temperature and as a consequence all Wasleys animals suffered ill health as exhibited lung legions consistent with varying levels of pneumonia and evidence of peritonitis (*streptococcus suis*) (Dr. B. Lloyd B.V.M.S.).

TABLE 9.6 SUMMARY OF WASLEYS BOAR 1027 TRANSGENIC PROGENY: ZINC INDUCIBLE TRANSGENE EXPRESSION

					GH IGF-I (ng/ml)					
					Zn-		Zn+		Zn-	
I.D.	Sex	T'genic	ADG	P2	GH	IGF	GH	IGF	GH	IGF
Litter 1										
2013	F		0.67	11.5	15.9	104	16.5	129	16.2	113
2014	M		0.87	11	10.5	210	16.1	288	12.4	219
2015	M		Died at birth							
2016	M		0.87	10	10.9	122	11.8	146	13.6	139
2017	M		0.91	14	10.8	181	15.3	169	9.5	124
2018	M		0.89	13	11.8	144	8.5	132	8.4	112
2019	M	T	0.84	9.5	24.9	152	39.4	201	11.0	104
2031	F	T	1.10	7.8	13.9	114	14.6	146	11.4	120
2034	F		0.70	12.5	11.8	114	19.2	136	13.5	119

TABLE 9.6 'CONTINUED'

I.D.	Sex	T'genic	ADG	P2	GH IGF-I (ng/ml) Zn- GH	Zn- IGF	Zn+ GH	Zn+ IGF	Zn- GH	Zn- IGF
Litter 2										
2035	F		0.55	12	19.9	121	18.1	139	18.9	118
2035	M		Hernia (54 kg)							
2037	F		0.59	10	15.3	128	15.8	102	15.4	132
2038	F		0.56	14	14.3	102	13.3	63	12.9	87
2039	F		Hernia (66 kg)							
2041	M	T	1.04	8.2	15.1	245	34.3	301	15.9	114
2045	M		Meningitis (13.5 kg)							
Litter 3										
2046	M		0.74	13.5	13.5	108	9.7	114	8.4	97
2047	M		Meningitis (6.0 kg)							
2048	M		Hernia (54 kg)							
2049	F	T	1.06	7.5	12.0	164	41.3	177	13.4	118
Litter 4										
2048	F		0.89	12.5	4.0	160	8.5	177	12.3	159
2048	M		Failed to thrive							
2051	F	T	Failed to thrive							
2053	F	T	0.98	11.3	14.8	128	39.8	159	16.1	148
2054	M	T	0.98	11.5	16.7	356	15.9	273	18.2	271
2057	M	T	0.69	10.5	12.5	124	13.4	164	14.6	136
2058	F		0.79	9.5	17.0	90	12.8	77	13.7	84
2061	M		Dead at birth							
2063	F	T	0.71	12.5	10.7	143	12.6	213	9.8	115
2064	M		0.92	10.6	4.1	105	9.4	125	12.3	114
Litter 5										
2065	M	T	1.06	9.3	11.3	185	27.1	285	18.5	108
2067	F		Failed to thrive							
2068	F		0.84	13.5	21.1	164	14.3	155	17.7	147
2069	M		0.64	11.5	8.9	86	12.5	76	16.5	75
2071	M		Dead at birth							
2073	F		Lameness destroyed (36 kg)							
2074	M		Failed to thrive (17 kg)							
2075	M		Injury destroyed (35 kg)							
2076	M		Failed to thrive (36 kg)							

*ADG Average daily weight (kg) gain (40-90kg).

9.4.3 Average Daily Gain and Index of Carcass Quality (P2)

Table 9.7 summarises the average daily gain and P2 values for Northfield and Wasleys transgenic pigs when compared with the littermate

controls. Overall Northfield transgenic males average daily gain ranged from (0.58-0.93 kg.day^{-1}) when compared to non transgenic males (0.58-0.78 kg.day^{-1}) while the transgenic females ranged from (0.36-0.93 kg.day^{-1}) when compared to non transgenic females (0.55-0.69 kg.day^{-1}). Several Northfield transgenic animals (ADG (kg.day^{-1}); F96, 0.93; F97, 0.93) had significantly ($p<0.05$) superior growth rates when compared to non transgenic littermates (ADG (kg.day^{-1}); F04, 0.78; F09, 0.69). Comparison of P2 values for Northfield transgenic pigs indicated the both transgenic males and females had significantly ($p<0.05$) less body fat than their non transgenic littermates.

Under commercial conditions at Wasleys, transgenic male pigs average daily gain ranged from (0.69-1.06 kg.day^{-1}) compared to non transgenic males (0.64-0.92 kg.day^{-1}) while the transgenic females ranged from (0.71-1.06 kg.day^{-1}) when compared to non transgenic females (0.55-0.99 kg.day^{-1}). Overall the average daily gain of Wasleys transgenic males (15%) and females (24%) was significantly ($p<0.05$) improved when compared with their non transgenic littermates. Comparison of P2 values indicated that Wasleys transgenic males (21%) and females (24%) had significantly ($p>0.05$) less body fat deposition than their non transgenic littermates.

Comparison of mean numbers of transgenic animals is difficult as each animal produced by direct DNA microinjection or by breeding is an individual with respect to its transgene expression status.

TABLE 9.7 MEAN ADG AND P2 VALUES FOR NORTHFIELD AND WASLEYS TRANSGENIC PIGS

Litter I.D.	TM ADG	TM P2	TF ADG	TF P2	NTM ADG	NTM P2	NTF ADG	NTF P2
Northfield Litter 1	0.81		0.76					
Northfield Litter 2	0.58	11.5	0.54	12.7	0.61	13.5		
F10 progeny	0.54	11	0.48	11.5	0.58	11		
F64 progeny	0.84	11.5	0.64	12	0.65	11.4	0.74	13
F68 progeny	0.77	12.3	0.88	11.3	0.77	11.8	0.66	12
Northfield (Mean)	0.71	11.6	0.66	11.9	0.65	11.9	0.70	13
Wasleys 1	0.84	9.5	1.0	7.8	0.88	12.3	0.69	12
Wasleys 2	1.04	8.2					0.57	12
Wasleys 3			1.06	7.5	0.74	13.5		
Wasleys 4	0.84	11	0.85	11.9	0.86	10.6	0.84	11
Wasleys 5	1.06	9.3		0.64	11.5	0.84	13.5	
Wasleys (Mean)	0.98	9.5	0.94	9.07	0.83	11.98	0.71	12

TM Transgenic males
TF Transgenic females
NTM Non transgenic males
NTF Non transgenic females

9.4.4 Transgene Heritability

Table 9.8 shows transgene heritability for several lines of transgenic pigs. Heritability varied on a litter basis from 0% to 75% for each transgene with an overall heritability of 35.6 h^2.

TABLE 9.8 HERITABILITY OF PGH TRANSGENES

Litter I.D.	Transgene	Heritability Estimate T:NT Ratio
F5 Litter 1	$pHMPG_{.01}$	44
F5 Litter 2	$pHMPG_{.01}$	70
F5 Litter 3	$pHMPG_{.01}$	0
F10 Litter	$pHMPG_{.05}$	75
F64 Litter	$pHMPG_{.05}$	25
F68 Litter	$pHMPG_{.05}$	55
EM1027 Litter1	$pHMPG_{.05}$	22
EM1027 Litter2	$pHMPG_{.05}$	14
EM1027 Litter3	$pHMPG_{.05}$	25
EM1027 Litter4	$pHMPG_{.05}$	50
EM1027 Litter5	$pHMPG_{.05}$	11

9.4.5 Zinc Supplmentation

Diets formulated to contain extra zinc were analysed by atomic absorption spectrophotometry (Vetlab). Levels were detected at 9.4 mmol/kg dry weight (940 ppm) for zinc and 1.5 mmol/kg dry weight (150 ppm) for copper. Both components were within expected ranges. Serum zinc levels indicated Zn concentrations were elevated in both control and transgenic animals when fed the Zn supplemented diet. Removal of the Zn supplemented diet resulted in the reduction of the plasma Zn level (see Table 9.9).

TABLE 9.9 MEAN SERUM ZINC LEVELS IN ANIMALS FED ZINC SUPPLEMENTED DIETS

Blood Composition	Transgenic (S.E)(mmol/l) (n=6)	Controls (S.E.)(mmol/l) (n=6)
Zinc added to diet	20.6 (±3.2)	17.3 (±2.9)
Zinc free diet	9.5 (±2.3)	10.6 (±2.3)

Porcine reference 8-15 μmol/l (VetLab)

9.4.6 Health Status

Of the 58 transgenic pigs produced either by direct microinjection or breeding, four animals showed significant pathology that could be directly related to their transgenic status. These included gastric ulcers (F1) (see Plate 6), arthritis (F10, F98) and one prolapse (F72). Of importance was the finding that phenotypic problems only occurred in animals with GH serum levels in excess of 100ng/ml.

9.4.7 Metabolic Profiles

Table 9.10 summarises data on selected physiological profiles in serum from transgenic and non transgenic litter mates. No trends in the data were evident which could be related to transgenic status and no significant differences ($p>0.05$) were observed in any specific parameters examined for differences between transgenic and non transgenic littermates over a variety of ages and zinc supplementations.

9.4.8 Gene Regulation in Wasleys and Northfield Transgenic Litters

Figure 9.1 summarized the data obtained for litters and shows the effects of a zinc supplemented diet on GH levels against ADG in Northfield transgenic pigs compared to non transgenic pigs. Figure 9.2 shows the response of IGF-I after GH levels were elevated following zinc supplementation in Northfield transgenic pigs. Table 9.11 indicates the linear relationships existing between ADG and plasma GH and IGF-I levels in Northfield transgenic pigs.

TABLE 9.11 SUMMARY OF ZINC INDUCED TRANSGENE EXPRESSION IN NORTHFIELD TRANSGENIC PIGS: LINEAR RELATIONSHIPS.

	Feed	Hormone	Litter	Correlation
	Zn-	GH	1	N.S.
			2	N.S.
	Zn-	IGF-I	1	p<0.01
			2	N.S.
	Zn+	GH	1	N.S.
			2	N.S.
	Zn+	IGF-I	1	p<0.05
			2	N.S.
	Zn-	GH	1	N.S.
			2	N.S.
	Zn+	IGF-I	1	p<0.05
			2	p<0.05
Combined				
	Zn-	GH		p<0.01
		IGF-I		N.S.
	Zn+	GH		p<0.01
		IGF-I		p<0.01
	Zn-	GH		N.S.
		IGF-I		p<0.01

Figure 9.3 shows the effects of a zinc supplemented diet on GH levels against ADG in Wasleys transgenic pigs compared to non transgenic littermates. Figure 9.4 shows the response of IGF-I after GH levels were elevated following zinc supplementation in Wasleys transgenic pigs. Table 9.12 indicates the linear relationships existing between ADG and GH and

IGF-I levels in Wasleys transgenic pigs. On a litter basis IGF-I levels correlate significantly ($p<0.05$) with increased ADG. However combining all litters, indicates that a significant correlation exist between GH levels and ADG with a zinc supplemented diet. IGF-I levels also correlate significantly ($p<0.05$) with ADG when combining all litters regardless of Zn supplementation.

TABLE 9.12 SUMMARY OF ZINC INDUCED TRANSGENE EXPRESSION IN WASLEYS TRANSGENIC PIGS: LINEAR RELATIONSHIPS.

	Feed	Hormone	Litter	Correlation
	Zn-	GH	1	N.S.
			2	N.S.
			3	N.S.
			4	N.S.
			5	N.S.
	Zn-	IGF-I	1	N.S.
			2	p<0.01
			3	N.S.
			4	N.S.
			5	p<0.05
	Zn+	GH	1	N.S.
			2	p<0.011
			3	N.S.
			4	N.S.
			5	N.S.
	Zn+	IGF-I	1	N.S.
			2	p<0.05
			3	N.S.
			4	N.S.
			%	N.S.
	Zn-	GH	1	N.S.
			2	N.S.
			3	p<0.01
			4	p<0.05
			5	N.S.
	Zn+	IGF-I	1	N.S.
			2	N.S.
			3	N.S.
			4	p<0.05
			5	N.S.
Combined				
	Zn-	GH		N.S.
		IGF-I		p<0.01
	Zn+	GH		p<0.05
		IGF-I		p<0.01
	Zn-	GH		p<0.05
		IGF-I		N.S.

Figure 9.5 summarizes the effect of zinc on increasing GH level in individual Wasleys and Northfield transgenic pigs and their non transgenic littermates.

Figure 9.6 indicates the effect of elevated GH levels after zinc supplementation on IGF-I levels in Wasleys and Northfield transgenic pigs.

Evidence from both figures indicate that additions of zinc to the diet elevated GH levels and subsequently IGF-I levels in transgenic pigs when compared with non transgenic littermate controls. Removal of zinc from the diet decreased both GH and IGF-I levels in transgenic pigs.

Figure 9.7 shows the overall expected increased efficiency of transgenic animals as determined by improved ADG in both Northfield and Wasleys transgenic pigs when compared with non transgenic littermates.

9.5 Discussion

Transgenic pigs produced using a transgene combining the metallothionien promoter and homologous porcine growth gene are viable, capable of reproducing and show improved production and carcass characteristics (see Plate 6) as a result of regulated transgene expression.

Large variations among the individual performance of Wasleys and Northfield transgenic animals were evident and may reflect the expression status and integration site, evidence for which is discussed later.

The transgenes were inherited with an overall efficiency of 36% which is significantly lower ($p<0.05$) than reported in the review by Pursel *et al.*, (1990a,b). However in some individual lines heritability was as high as 75% and in general there were too few pigs produced to suggest that the transgene was behaving other than as Mendelian dominant. Comparison with other groups is difficult because of the relatively small numbers involved, though expression frequency was similar to the reported in the review by Pursel *et al.*, 1990a,b.

In general the health status of transgenic animals incorporating the homologous growth hormone transgene did not associate with detrimental

effects reported in other GH transgenic pigs worldwide. Transgenic sows were able to maintain pregnancies and offspring born were considered to be of normal size and number for the age of the sow. Transgenic boars were able to mate naturally and displayed normal libido patterns. However, it is significant that those few animals which displayed undesirable phenotypic characteristics had elevated growth hormone serum levels in excess of 100 ng/ml. Therefore to minimise deleterious effects associated with elevated levels of growth hormone it is critical to retain control over gene expression and target expression levels not in excess of 100ng/ml of plasma GH.

Studies indicate that transgene expression could be controlled with additions of zinc to the diet. Supplementation of the diet with 1000 ppm of zinc as sulphate resulted in a two to ten fold increase in GH production followed by a concomitant response by IGF-I and is in support of data reported in the review by Pursel *et al.*, (1990b). Further analysis indicated a correlation between the elevated GH and IGF-I levels against improved ADG. There was a correlation for elevated GH plasma levels and improved ADG when transgenes were induced with zinc. Linear correlations occurred between IGF-I levels and improved ADG regardless of zinc supplementation. Therefore the possibility exists that elevated IGF-I levels may correlate with increased fat deposition and not protein deposition in contrast to GH levels which correlate significantly with ADG only on the zinc supplemented diet. Speculatively then, indices of growth in particular protein accretion are related to GH levels more significantly than IGF-I levels. This effect was not tested and warrants further investigation.

Variation amongst individual litters bred from transgenic animals may be similar to the work of Allen *et al.*, (1990) in transgenic mice where expression and DNA methylation of transgenes at the integration site were controlled by genotype specific modifier genes. The epigenetic modifications at the transgenes locus then continue to be passed on to successive generations. This phenomenon was not investigated in the transgenic pigs and warrants further investigations.

An endless variety of genes for incorporation into the germline of transgenic pigs is possible (Fujii *et al.*, 1991; see review, Hughes *et al.*, 1990; Kopchick *et al.*, 1990). Evidence presented particularly for the pig indicates careful consideration should be given to the origins of the transgenes, as the use of hcterologous genes may result in a more potent action in contrast to homologous derived genes.

In summary, the transgenic pigs studied in this thesis show improved performance and carcass characteristics when compared to non transgenic littermates. Transgenic pigs produced were capable of unassisted breeding and targeted levels of transgene expression of less than 100 ng/ml of GH could be maintained by regulating the transgene with a zinc supplemented diet. The large variation seen in the performance of transgenics bred from one transgenic line indicates that each individual animal transgenes expression status is modified by epigenetic factors at the site of integration. Selected transgenics animals presented in this study have desirable characteristics that would be extremely valuable for incorporation into any commercial arena.

PLATE 6

Photograph 1

Wasleys transgenic boar 2065 and non transgenic littermate 2069 at 134 days old.

Weight (Kgs)

2065 84
2069 63

Photograph 2

Cross section through the loin at the 8th rib of a F2 $HMPG_{0.05}$ boar (2065) from Wasleys line and control (left) sex matched littermate (2069).

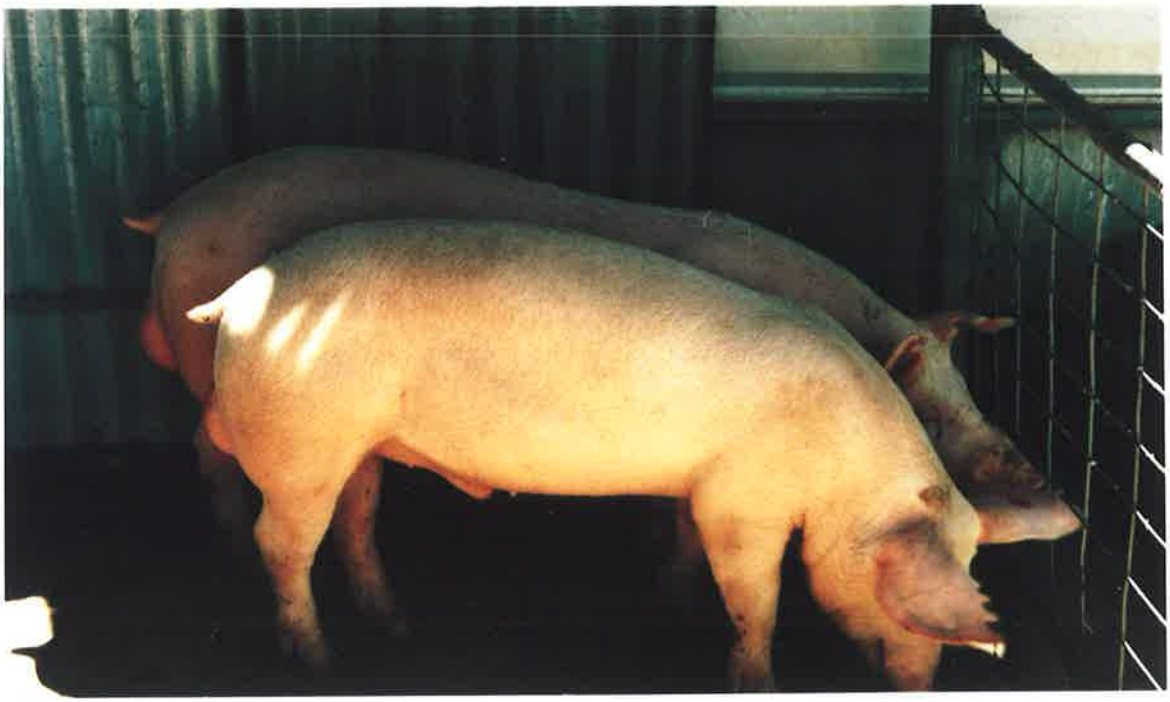
Both animals weighed approximately 90 kg at the time of slaughter.

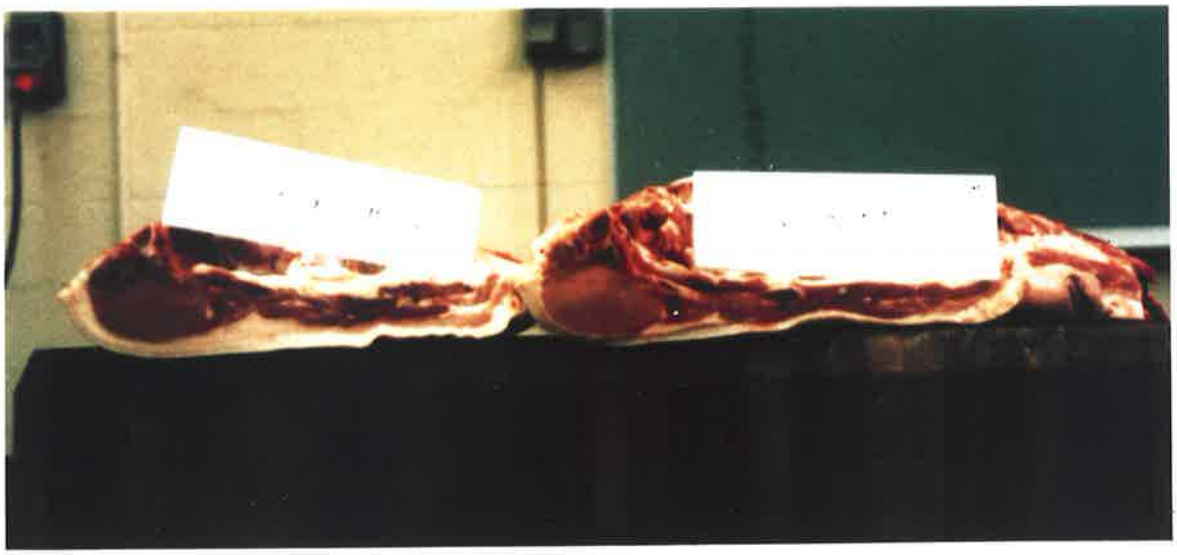
(Source: Bresatec)

Photograph 3

Stomach ulcer (near oesophagus) resulting in the death of transgenic pig F1 on 9/3/1988

(3.2 cm diameter)





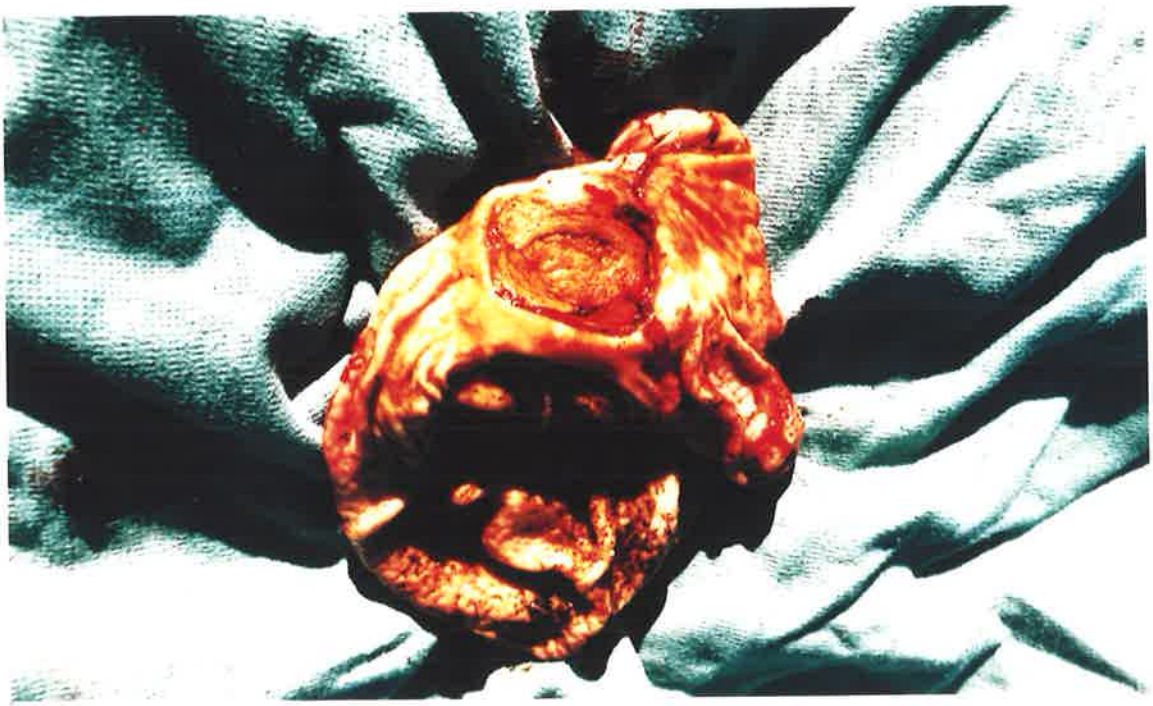


TABLE 9.10 METABOLIC PARAMETERS OF TRANSGENIC PIGS

	Status	Age (Days)	Albumin	Bile Acids	Calcium	Cholest.	Chloride	Creat.	Globulin	Glucose	Potassium	LDH	Sodium	Osmolarity	Phos.	T. Prot.	T. Bili.	Urea
2013 3/2	F NT Zn+	128	34	18	2.3	2.3 -	96	0.12	43	3.5	7.3 +	326 -	138	281 -	3.25	77	0.1	7.6
2013 4/3	F NT Zn-	157	41 +	14	2.67	3.3	112 +	0.14	47	5.4	7.9 +	313 -	162 +	328 +	3.38	88 +	0.1	6.9
2019 4/3	M T Zn-	157	34	39	2.69	2.8	99	0.12	41	4	5.7	256 -	142	285	3.02	75	0.1	6.2
2019 8/2	M T Zn+	133	34	47	2.41	2.3 -	95	0.17	37	3.5	8.9 +	549	133 -	276 -	3.52	72	0.1	9
2041 3/2	M T Zn+	154	29	11	2.48	2.1 -	95	0.13	46	3.1	8 +	440	137	277 -	3.34	75	0.1	4.2
2041 4/3	M T Zn-	183	34	20	2.45	2.6 -	97	0.11	38	4.5	5.9	349 -	140	281 -	3.03	72	0.1	4.9
2049 26/10	F T Zn+	142	32	19	2.74	2.3 -	98	0.1	52	4	6.3	229 -	140	284	3.07	84	0.1	7.8
2049 Last	F T Zn-	182	36	26	2.51	2.2 -	97	0.12	42	4	5.7	235 -	141	285	2.74	77	0.1	8.5
2054 26/10	M T Zn+	133	28	18	2.58	2.7 -	98	0.11	50	4.2	6.6	263 -	142	287	3.24	79	0.1	6.1
2054 Last	M T Zn-	173	37	16	2.34	2.7 -	95	0.1	35	4.1	6.1	291 -	139	283	3	72	0.1	8.7
2058 26/10	F NT Zn+	133	32	18	2.26	2.2 -	97	0.12	40	3.9	6.7	206 -	138	281 -	2.81	72	0.1	7.6
2058 Last	F NT Zn-	173	38	20	2.54	2.6 -	96	0.12	38	4.5	6	423 -	140	286	3.17	76	0.1	10 +
2065 Last	M T Zn-	163	28	11	2.06	2.2 -	85 -	0.11	39	3.3	6.5	481	123 -	247 -	2.43	66	4	3.1 -
2065 26/10	M T Zn+	123	25	35	2.53	2.1 -	96	0.11	42	5	6.8 +	229 -	137	278 -	3.21	68	0.1	5.7
F1 29/9/88	M T Zn-	60	27	45	2.36	2.3 -	95	0.07 -	25	3.8	4.8	172 -	136	270 -	2.43	52 -	0.1	4.6
F1 Zn	M T Zn+	141	33	18	2.49	2.8	95	0.1	33	5.9	4.8	35 -	140	280 -	2.85	67	0.1	4.7
F10 29/9/88	M T Zn-	59	22	98	2.46	3	99	0.08 -	33	6.1	4.4 -	361 -	138	275 -	2.45	54 -	0.1	3.7
F10 Zn	M T Zn+	140	32	22	2.36	2.3 -	103	0.12	41	4.4	5.1	295 -	146	290	2.98	73	0.1	4.8
F2 Zn	F NT Zn+	140	40 +	24	2.53	2.3 -	93 -	0.12	35	4	5.1	283 -	142	283	2.28	75	0.1	5.5
F2 29/9/88	F NT Zn-	60	29	20	1.78 -	2.4 -	98	0.08 -	24	5.4	5.4	818 +	138	277 -	2.9	53 -	0.1	4.9
F40	M T	birth	36	66	2.63	2.7 -	99	0.09 -	28	4.4	6.9 +	300 -	137	275 -	2.59	64 -	0.1	3.1 -
F41	M NT	birth	35	32	2.2	1.9 -	94	0.08 -	20 -	3.5	6.1	652 +	133 -	268 -	2.68	54 -	0.1	6
F8 29/9/88	F NT Zn-	59	25	42	2.43	2.6 -	96	0.07 -	27	5.7	4.5	340 -	136	273 -	2.8	52 -	0.1	5.7
F93	F NT Zn-	141	40 +	15	2.61	2.3 -	94	0.12	35	4.8	5.6	438	143	286	2.64	76	0.1	4.8
F94 Zn	M T Zn+	141	31	37	2.27	2.6 -	98	0.12	32	4.8	5.4	216 -	140	279 -	2.77	63 -	0.1	4.1
F97 Zn	M T Zn+	141	37	34	2.74	1.9 -	94	0.09 -	34	5	5.1	143 -	140	279 -	2.91	70	0.1	3.8

Reference
M/F male or female
T/NT transgenic or non transgenic
Zn+/- 1000 ppm of zinc as sulphate (plus or minus)

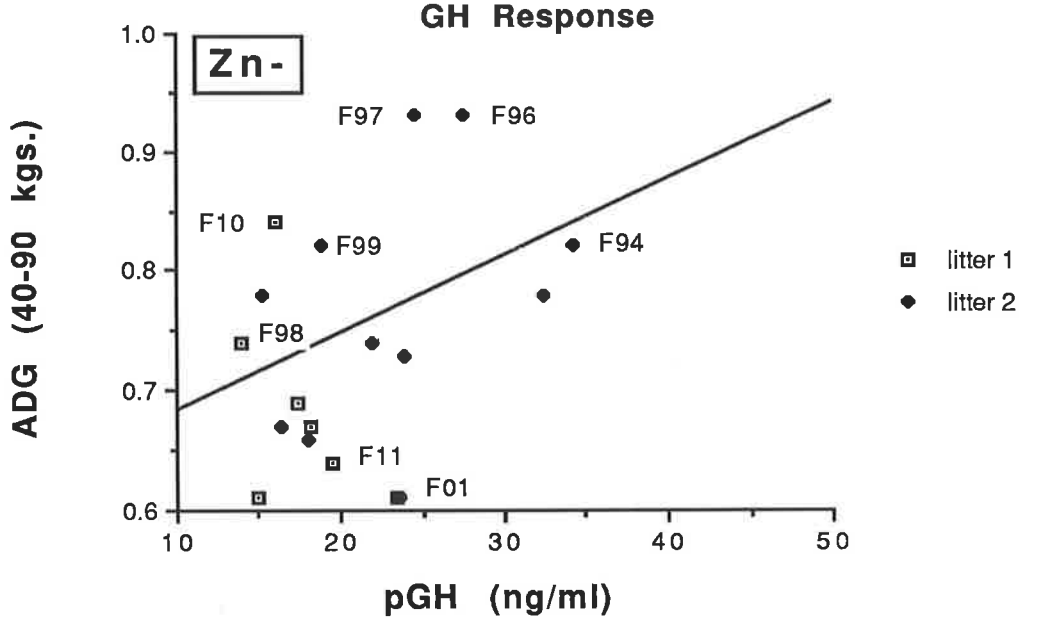
Figure 9.1 Northfield Transgenics: Zinc Induced Transgene Expression. GH Response

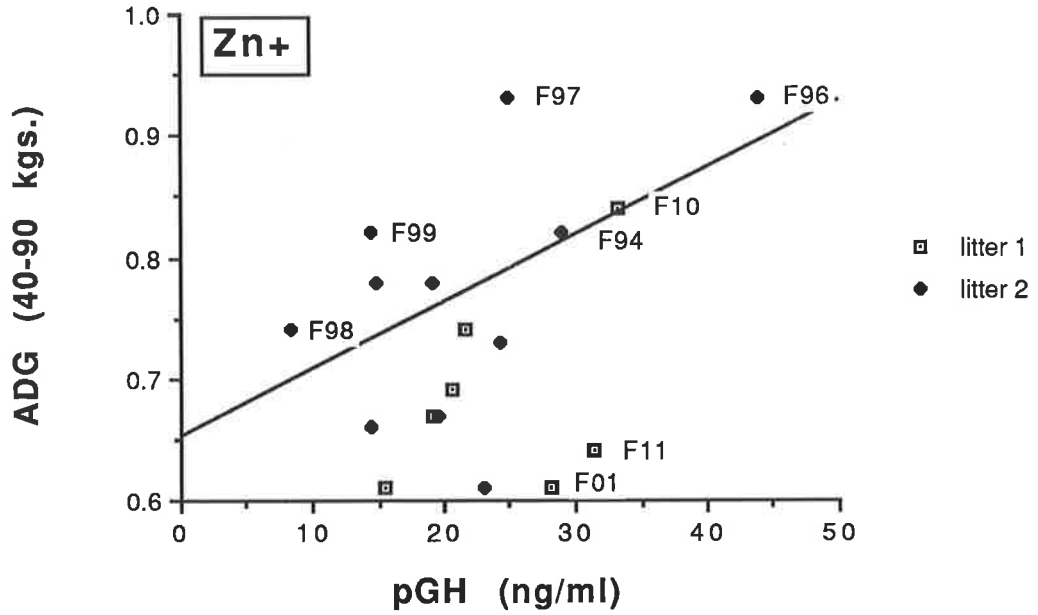
The figures show the ADG (kg/day) between 40 and 90 kgs. and pGH levels in the blood of Northfield Transgenic and Non Transgenic offspring both before and after additions of 1000 ppm of zinc as sulphate to the feed.

(Similar symbols represents animals of the same litter)
(Symbols labelled represent transgenic animals ie. F96)

Figure 9.1 Northfield Transgenics: Zinc Induced Transgene Expression

GH Response





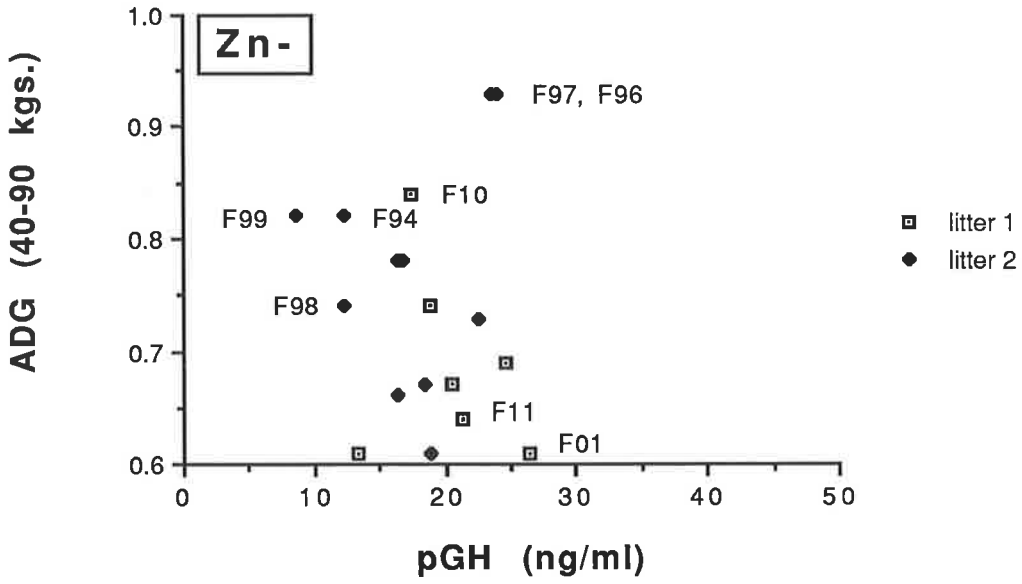


Figure 9.2 Northfield Transgenic: Zinc Induced Transgene Expression IGF-I Response

The figures show the ADG (kg/day) between 40 and 90 kgs. and IGF-I response in the blood of Northfield Transgenic and Non Transgenic offspring both before and after additions of 1000 ppm of zinc as sulphate to the feed.

(Similar symbols represents animals of the same litter)
(Symbols labelled represent transgenic animals ie. F96)

Figure 9.2 Northfield Transgenics: Zinc Induced Transgene Expression

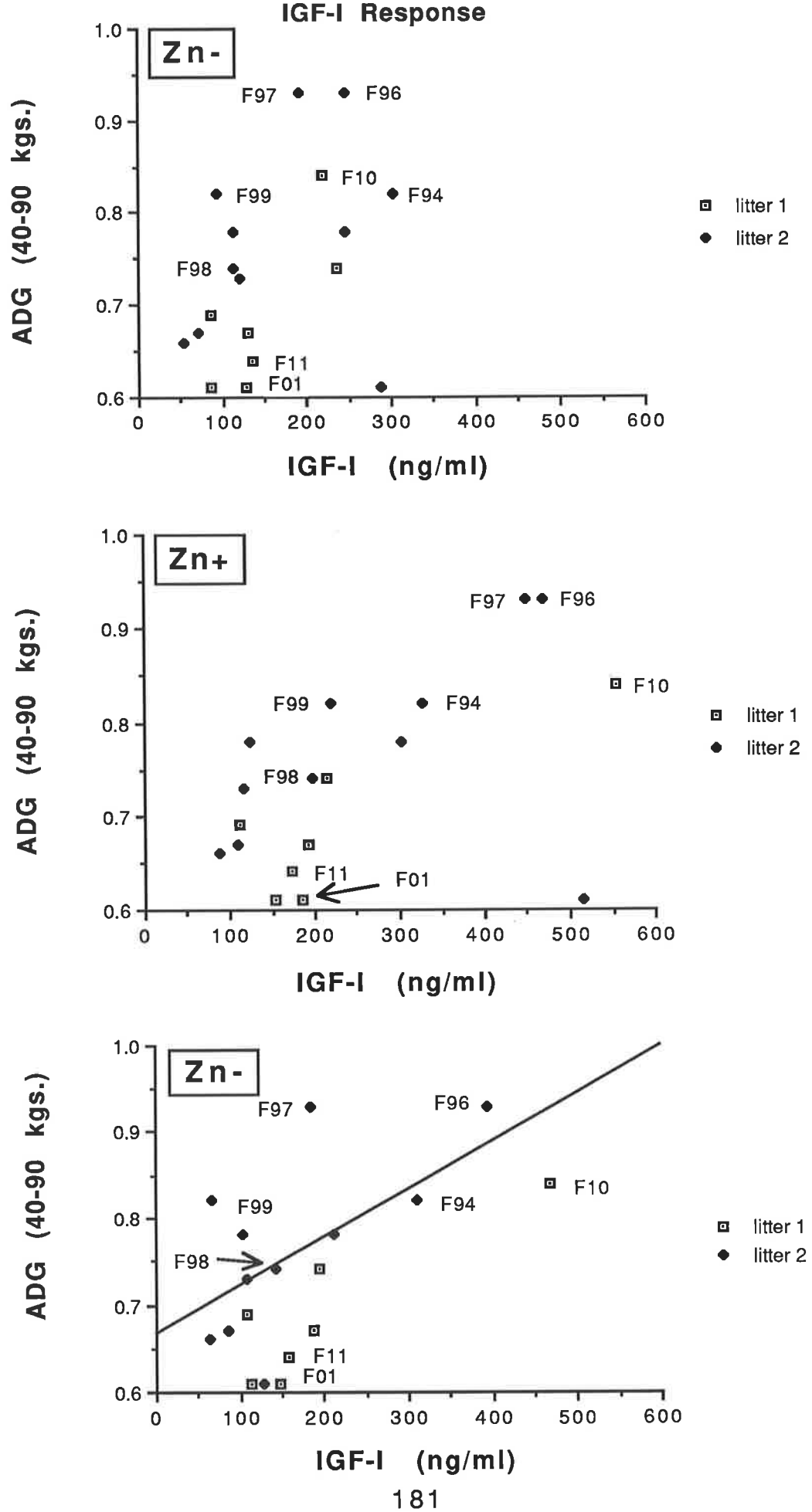


Figure 9.3 Wasleys Transgenics: Zinc Induced Transgene Expression GH Response

The figures show the ADG (kg/day) between 40 and 90 kgs. and pGH levels in the blood of Wasleys Transgenic and Non Transgenic offspring both before and after additions of 1000 ppm of zinc as sulphate to the feed.

(Similar symbols represents animals of the same litter)
(Symbols labelled represent transgenic animals ie. 2031)

Figure 9.3 Wasleys Transgenics: Zinc Induced Transgene Expression

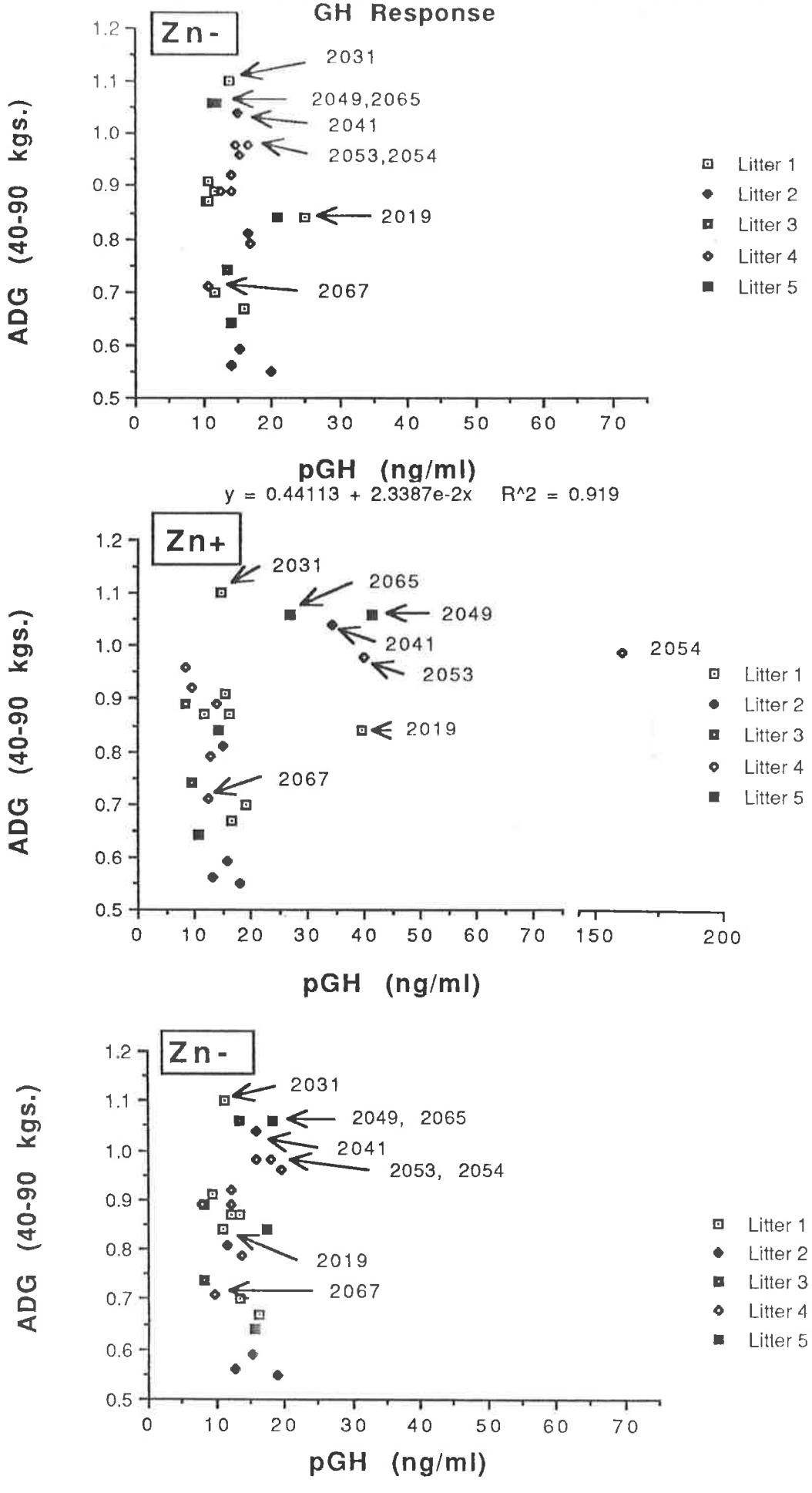


Figure 9.4 Wasleys Transgenics: Zinc Induced Transgene Expression IGF-I Response

The figures show the ADG (kg/day) between 40 and 90 kgs. and IGF-I response in the blood of Wasleys Transgenic and Non Transgenic offspring both before and after additions of 1000 ppm of zinc as sulphate to the feed.

(Similar symbols represents animals of the same litter)
(Symbols labelled represent transgenic animals ie. 2031)

Figure 9.4 Wasleys Transgenics: Zinc Induced Transgene Expression

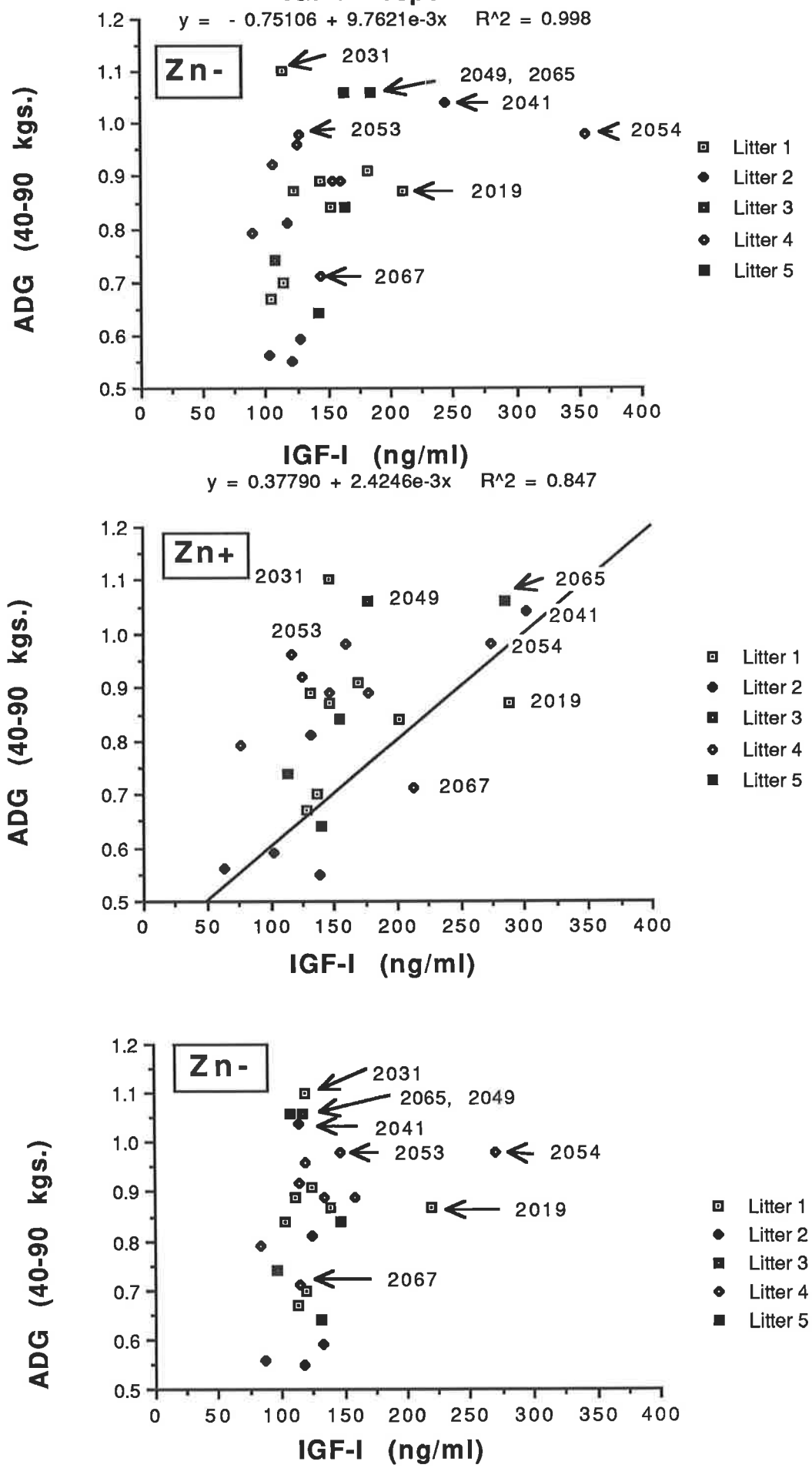


Figure 9.5 GH levels in Northfield and Wasleys Pigs
Transgenics vs Non Transgenics

The figures show the GH levels in Northfield and Wasleys Transgenic and Non Transgenic offspring between 40 and 90 kgs. both before and after additions of 1000 ppm of zinc as sulphate to the feed.

(Closed squares represent Northfield offspring)
(Open circles represent Wasleys offspring)

Figure 9.5 GH Levels in Northfield (—○—) and Wasleys (—■—) Pigs

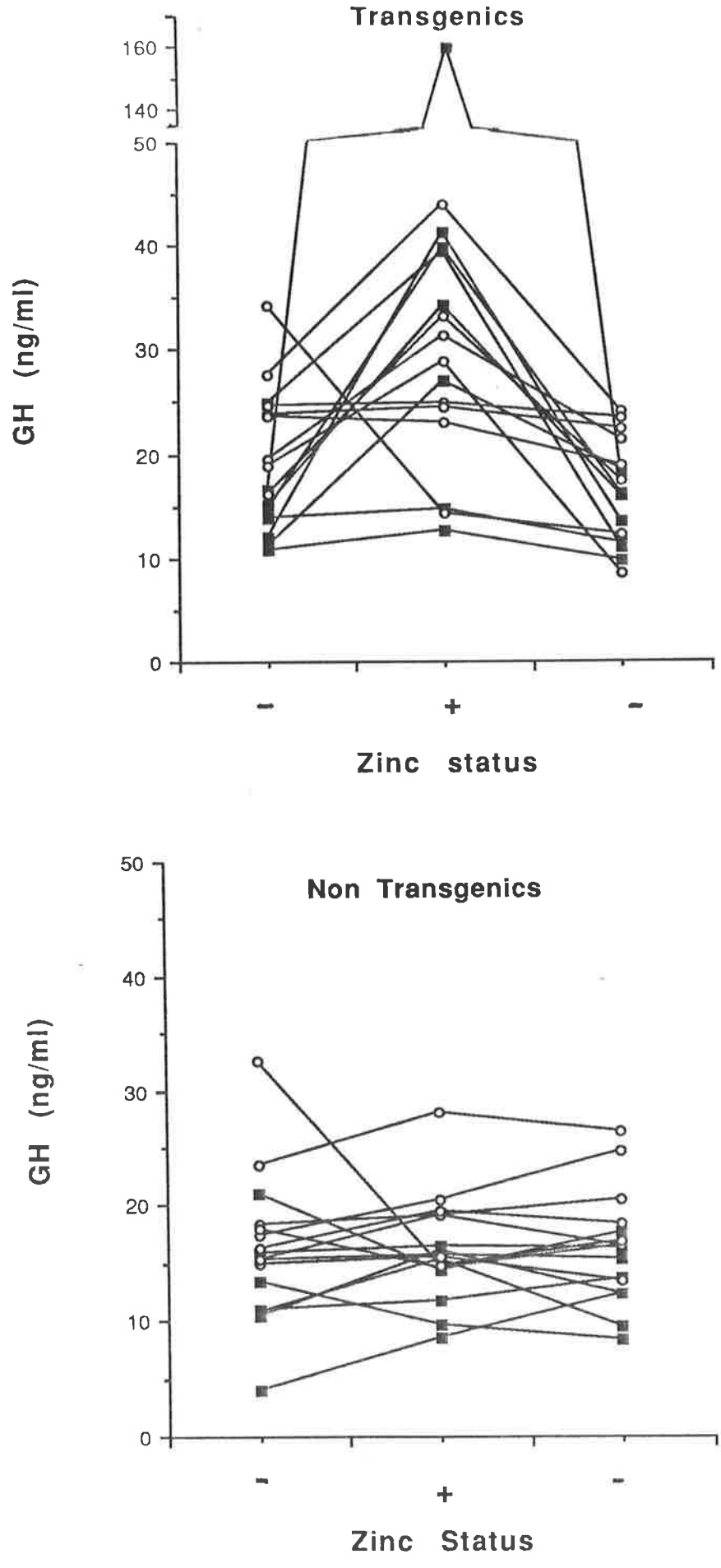
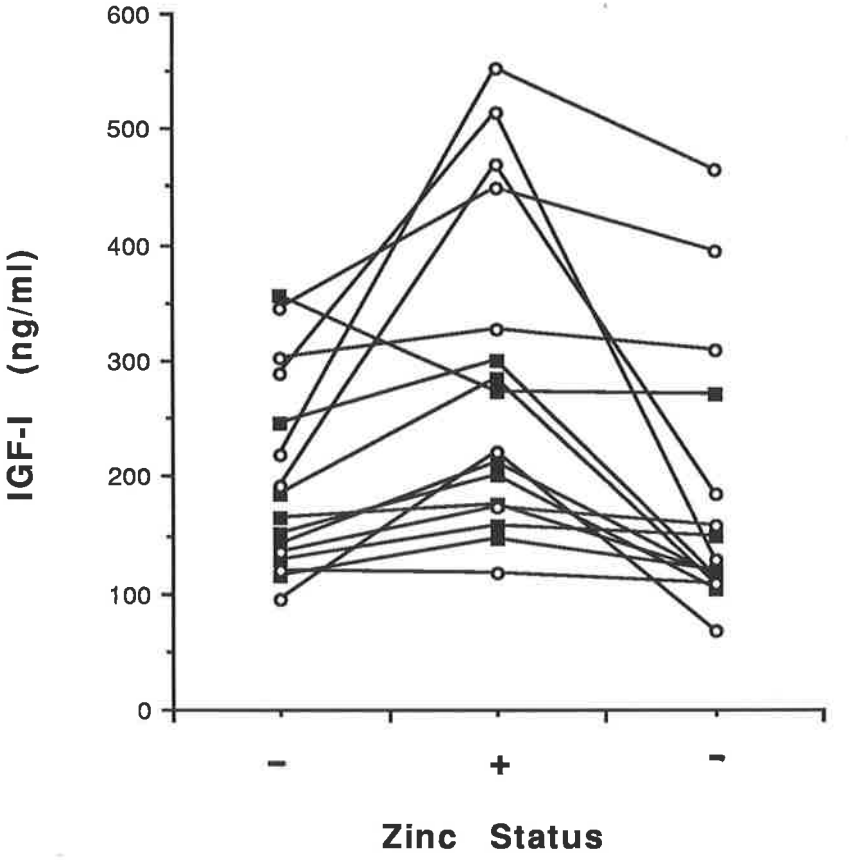


Figure 9.6 IGF-I Response after a Zinc Induced GH Response in Northfield and Wasleys Transgenic Pigs.

The figures show the IGF-I response in Northfield and Wasleys Transgenic and Non Transgenic offspring between 40 and 90 kgs. both before and after additions of 1000 ppm of zinc as sulphate to the feed.

(Closed squares represent Northfield offspring)
(Open circles represent Wasleys offspring)

Figure 9.6 IGF-I Response after a Zinc Induced GH Response
Northfield (—○—) and Wasleys (—■—) Transgenic Pigs



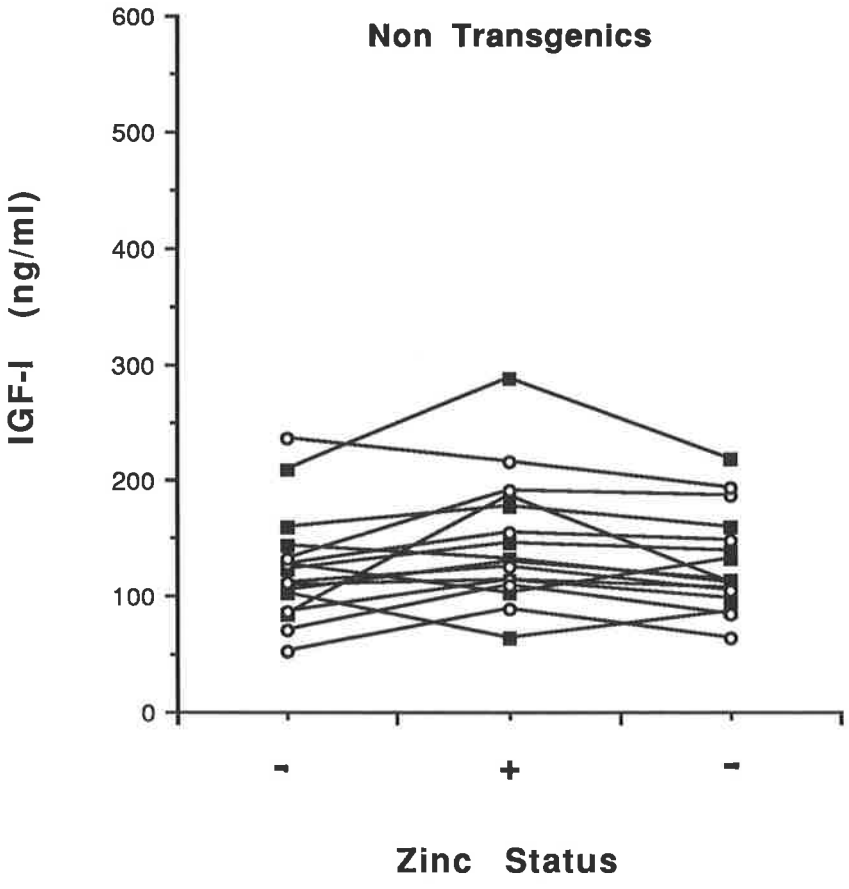


Figure 9.7 Final ADG for Transgenic and Non Transgenic Pigs

Northfield Transgenic Progeny

The figure shows the final ADG between 40 and 90 kgs for Northfield Transgenic and Non Transgenic offspring. Animals are grouped for same sex and litter number.

(Closed triangles represent Northfield Transgenics)
(Open circles represent Northfield Non Transgenics)

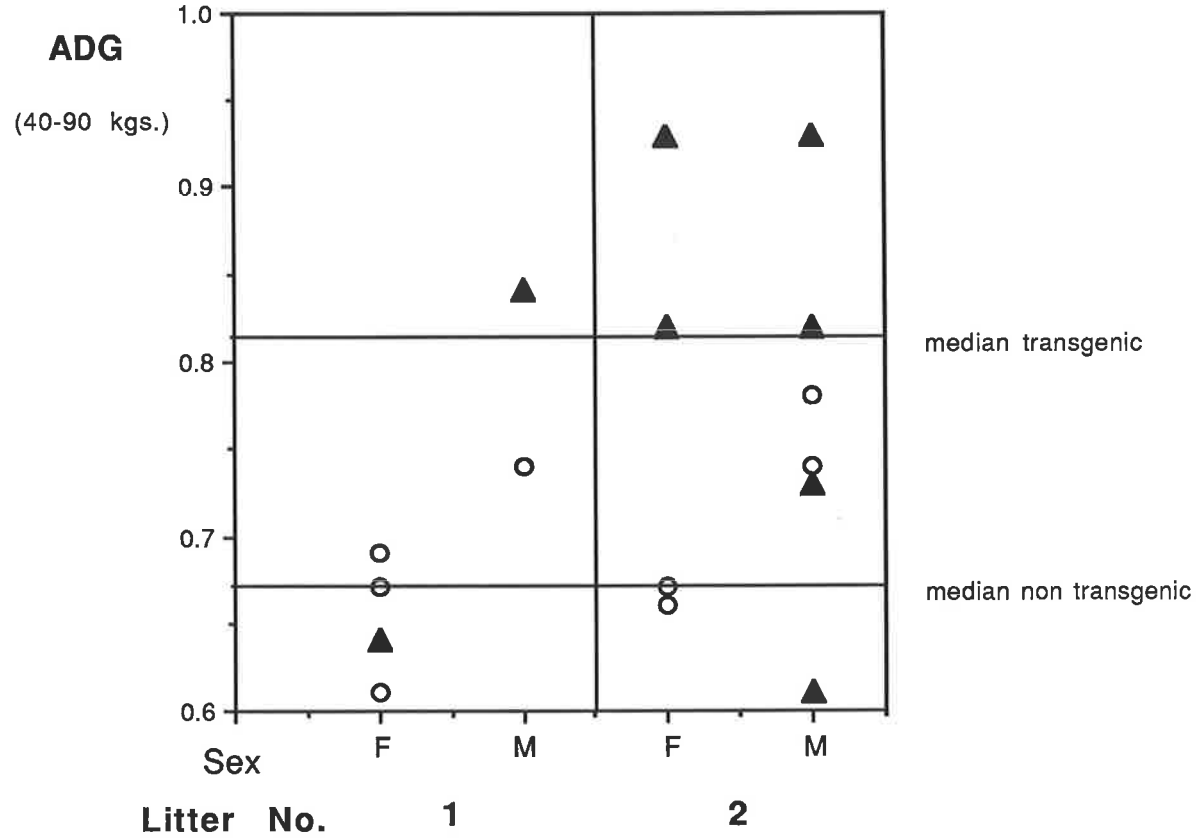
Wasleys Transgenic Progeny

The figure shows the final ADG between 40 and 90 kgs for Wasleys Transgenic and Non Transgenic offspring. Animals are grouped for same sex and litter number.

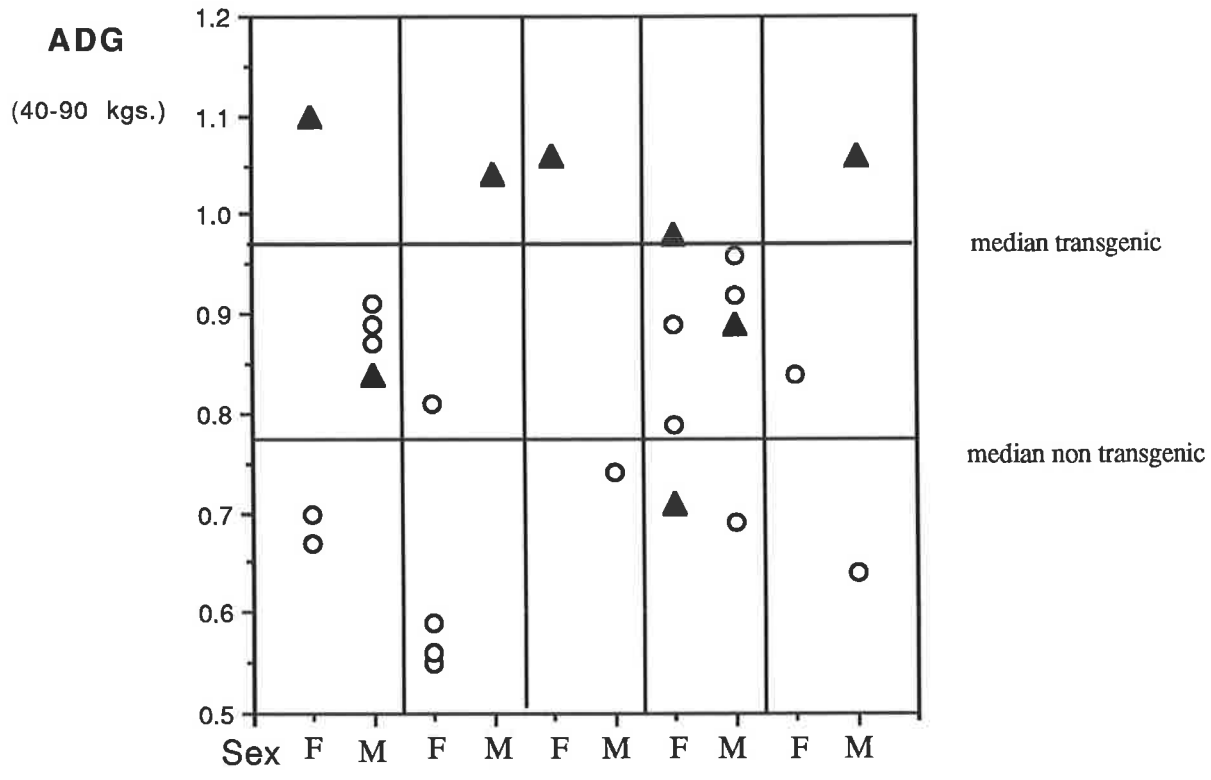
(Closed triangles represent Wasleys Transgenics)
(Open circles represent Wasleys Non Transgenics)

Figure 9.7 Final ADG for Transgenic (▲) and Non Transgenic (O) Pigs

Northfield Transgenic Progeny



Wasleys Trangenic Progeny



SECTION X

GENERAL DISCUSSION

The potential of transgenesis to revolutionize the animal breeding industries is readily appreciated. Initial attempts to adapt this powerful new technology to livestock enterprises have been largely disappointing due in a large part to the failure to surmount numerous biological and technical constraints which reduce the overall efficiency of the procedure. This thesis primarily concerns several studies aimed at increasing the efficiency of producing transgenic pigs using gene transfer techniques based on microinjection.

One crucial limiting factor of transgenesis relates to the low efficiency of gene integration and expression obtained following microinjection. Brinster *et al.*, (1986) suggested that this was due the limited availability of chromosomal breaks and we have explored using a murine model, the possibility of increasing the frequency of transgene integration by experimentally perturbating DNA structure. Pretreatment of embryos with ultraviolet light just prior to gene transfer by microinjection significantly increased integration regardless of the transgene injected whereas pretreatment with Hoechst 33342 stain or restriction enzyme yielded more variable results causing an apparent increase in integration which seemed to be dependant on the type of transgene employed.

An interesting feature of the integration seen following these experimental protocols was that it occurred without increases in the number of integration sites, which raises new questions concerning the determinants regulating transgene insertion and warrants further investigation.

One relevant observation in this regard was the finding of a delay in the cleavage rate of the first cell cycle following ultraviolet light treatment, raising the possibility that integration may be dependant more on the disruption of mechanisms controlling embryo cleavage than an increased number of chromosomal breaks *per se*. For example chromosome repair mechanisms involving the protein kinase $p34^{cdc2}$ and poly (ADP-

ribosylation) complex could deplete NAD(P) and ATP moieties and thus delay signals for activation of the protein kinase and subsequent mitotic events.

As a basis for subsequent pig studies protocols were developed to allow utilization of young gilts as either donors or recipients to overcome previous limitations in the restricted availability of viable zygotes for microinjection. Embryos derived from gilts resulted in the highest yield of fertilized embryos for microinjection. The viability of the embryos was confirmed by the optimal pregnancy rates (100%) and survival rates (29%) whilst retaining integration and expression frequencies of transgenesis similar to those reported by other groups worldwide.

A major factor contributing to the development of the improved procedures was the identification of a simple medium (Whitten's supplemented with BSA) which supported *in vitro* development of porcine zygotes to the hatched blastocyst stage following microinjection. Survival rates of 87% were obtained with 56% yielding young after transfer. The observations were interpreted to indicate that if lethal mutations were induced by transgene integration they had no significant effect on the survival of the preimplantation embryo.

The availability of this simple culture medium also allowed the development of procedures for transfer of embryos between distant sites. It was then shown that collected 1 and 4-8 cell embryos could be successfully transferred between sites during a 30 hour period of extended *in vitro* culture without specialised equipment to yield live offspring. Later stage embryos proved to be more suited to extended *in vitro* culture than early stage embryos. Following transfer pregnancy rates and embryo survival for both 2 and 16-32 cells were similar, indicating that viability was more dependant on uterine components and secretions rather than embryo developmental stage.

Subsequently cryopreservation procedures for peri-hatching stage embryos were developed allowing storage of porcine embryos for indefinite periods. The utilization of the procedures to the trangenesis program was

further advanced when it was shown that hatched blastocysts derived from zygotes using *in vitro* culture could be successfully frozen.

The transgenes utilized in the pig studies were specifically designed to cause only modest increments of circulating homologous GH and IGF-I avoiding health problems found with transgenic pigs with high uncontrolled expression of a potent heterologous transgenes. The data obtained adequately confirmed the potential of homologous growth hormone administered through transgenesis or by injection, to dramatically enhance production characteristics without compromising the health of the animal.

The transgenic animals produced were healthy, reproductively viable and the offspring inherited the transgene in a dominant Mendelian fashion. Transgene expression could be regulated by additions of zinc to the diet. Improved growth (ADG) correlated with plasma GH levels only during the period of exposure to zinc, whereas the correlation between IGF-I and ADG existed regardless of zinc supplementation. A study of the circulating plasma levels of exogenous GH and IGF-I indicated the higher the concentration of circulating growth hormone the more rapid the response of IGF-I. The current models advanced describing the interrelationships between GH and IGF's and their associated binding proteins to growth is becoming markedly complex and warrants further resolution. Speculatively it is suggested that GH, may correlate for protein deposition and IGF-I may correlate with fat deposition, a proposal that warrants further investigation.

The epigenetic and other factors (Allen *et al.*, 1990) acting to cause variation in heritability and expression of transgenes amongst individual transgenic animals are likely to have an important determination in the viability of commercial transgenic programs. Site directed insertion of transgenes using ES technology may allow a degree of control but as indicated by Allen *et al.*, (1990) transgene expression will be dependant on the genetic environment acting upon the transgene.

At the commencement of this project it was anticipated that a large scale (600 sow) experimental piggery would be available to expand

experimental protocols described and allow more detailed investigations, however due to unanticipated community concerns at the site of the proposed piggery the development has been delayed (Seamark, 1991). Nevertheless, the data obtained under less than favourable experimental conditions provides further compelling evidence that transgenesis has a significant place in future livestock production industries.

APPENDIX I

1. Feed Composition for Transgenic Animals

1.1 Pig Weight 5-25 kgs.

Weaner Creep (Commercial Available, Milling Industries, South Australia).

1.2 Pig Weight 25-80 kgs.

Grower Ration were formulated on site at Wasleys and Northfield Piggeries. Formulation varied slightly in composition because of environmental and economic factors influencing the availability of feedstuffs.

Component	Percentage
Meatmeal	4.5
Bloodmeal	1.0
Pea Pollard	5.0
Faba Beans	4.0
Rape Seed Meal	5.0
Cotton Bourke Exp 43%	7.5
Soya Bean Meal	4.2
Wheat/Triticale	28.3
Barley	35.0
Lysine-HCl	0.3
D,L Methionine	0.01
Tallow	3.3
Limestone	0.7
Rock Phosphate	0.7
Salt	0.25
Premix-Grower	0.25

1.2.1 Analysis

Volume	100
Dry Matter	90.3790
DE J	13.8118
DE K	3299.2034
NE-Pig-MJ	9.5765
ME-Poult	11.5978
ME-Rumin	11.7576
NE-M	0.0067
NE-G	0.000
NE-L	0.0180
Protein	19.2811
Fat	5.9003
Fibre	5.4887
NDF	0
ADF	0
Calcium	0.900
T.Phos	0.6379
Av.Phos	0.3990
*Cal/T.P.	1.4110
Lysine	1.1317
Available Lysine	0.9655
Methion	0.2846

1.3 Pig Weight 80-90 kgs and 90+ kgs

Finisher and Dry Sow Feed were similar to the grower ration except amount of protein was reduced from 18 to 15.5 and 14.4 respectively and the DE J was reduced from 13.7 to 12.9 and 12.5 respectively.

APPENDIX II

1. Polymerase Chain Reaction

Perkin Elmer Cetus thermal sequencer(90 cycles, 3 hours)

Cycle	Denaturation	Annealing	Polymerization
First Cycle	94°C for 5 min.	54°C for 2 min.	72°C for 3 min.
Subsequent Cycles	94°C for 1 min.	54°C for 30 sec.	72°C for 1 min.
Last Cycle	94°C for 1 min.	54°C for 2 min.	72°C for 10 min.

APPENDIX III

1. Measurements of IGF-I

Before assay, plasma IGFs were dissociated from binding proteins by acidification. IGFBPs and IGFs in 0.04ml plasma were separated by molecular size under conditions by high-performance liquid chromatography over a Protein-Pak 125 size-exclusion column (Waters/Millipore, Lane Cove, NSW, Australia). IGF-I was measured by radioimmunoassay retained in 0.05 ml of a 3ml pool of acid column fractions containing the IGF region after neutralization with 0.6 vol. of 0.4 mol Tris base/1. Standards and preparation of radioligands were prepared from supplied recombinant human IGF-I(K. Scheibli, Ciba-Geigy, Basel, Switzerland) which has the same amino acid sequence as porcine IGF-I. Sensitivity of the IGF-I assay was 20-30 pg/tube (30-45 μg IGF-I/l plasma) and half-maximal assay response was produced by 180-200 pg IGF-I/tube (270-300 μg IGF-I/l plasma). The intra- and inter-assay coefficients of variation for a pool of pig plasma containing 232 μg IGF-I, which was acid chromatographed five times and measured in triplicate were between 7.8% and 3.1% respectively. IGF-I purified from pig plasma is equipotent with recombinant human IGF-I standard in the IGF-I immunoassay and cross-reaction of plasma purified porcine IGF-II is 0.8% All IGF-I samples were assayed in triplicate (Owens et al., 1991).

2. Measurement of IGF Binding Proteins

Total desaturated IGFBP activity in plasma was measured as 'interference' in the IGF-I RIA as BP have the capacity to complex the radiolabelled IGFs and thus restrict access to other binding sites, such as receptors and antibodies. The ability of 0.05 ml of a 2ml fraction pool of the high molecular weight region of the acid column (IGFBP pool) to inhibit ^{125}I-labelled IGF binding to either anti-IGF-I serum or type-2 IGF receptors (after neutralization, see IGF-I) was compared with the conventional binding inhibition produced by unlabelled IGF standards. The dose response produced by the neutralized acid column IGFBP pool from pig plasma was parallel to that produced by IGF radioligand assays. IGFBP activity is expressed for IGF-I equivalence (Owens et al., 1991).

APPENDIX IV

EXTENSION PAPERS

University of Adelaide

80A:- PRODUCTION OF A HERD OF TRANSGENIC PIGS CONTAINING COPIES OF A PORCINE GROWTH HORMONE CONSTRUCT, 1987.

81C:- GENE INTEGRATION INTO MOUSE EMBRYO GENOME, 1987.

105C:- EVALUATION OF GROWTH HORMONE AND SOMATOMEDIN AS GROWTH PROMOTANTS, 1987.

M/45/87:- BIOASSAY OF PORCINE, BOVINE, OVINE GROWTH HORMONES PRODUCED BY RECOMBINANT DNA TECHNOLOGY, 1987-1989.

M/63/88 & M/55/89:- EMBRYO MANIPULATION AND GENE INJECTION (SHEEP, GOAT, CATTLE AND PIG STUDIES), 1988-1990.

W/37/88 WAITE INSTITUTE:- EVALUATION OF GROWTH HORMONE AND SOMATOMEDIN AS GROWTH HORMONE PROMOTANTS, 1989-1991.

Department of Agriculture.

F4/PR9:- PRODUCTION OF A HERD OF TRANSGENIC PIGS CONTAINING COPIES OF A PORCINE GROWTH HORMONE CONSTRUCT, 1987.

F4/PR14:- PRODUCTION OF A HERD OF PIGS CONTAINING COPIES OF A PORCINE GROWTH HORMONE CONSTRUCT, 1988.

F4/PR15:- EVALUATION OF PHYSIOLOGICAL AND PRODUCTION CHARACTERISTIC IN GROWTH HORMONE TREATED PIGS, 1988.

F4/PR35:- INVESTIGATING TRANSGENE INHERITANCE AND EXPRESSION IN PIGS CONTAINING COPIES OF A PORCINE GROWTH HORMONE GENE CONSTRUCT, 1989.

F4/PR36:- INVESTIGATION INTO PRODUCTION OF LITTERS FROM FROZEN PORCINE EMBRYOS, 1990.

F4/PR37:- PRODUCTION OF PORCINE CLONES BY NUCLEAR TRANSFER USING TECHNOLOGIES ESTABLISHED FOR TRANSGENSIS, 1990.

Non-Prescheduled Research

GENE REGULATION IN TRANSGENIC PIGS, 1989.

INHERITANCE ASSESSMENT OF TRANSGENIC ANIMALS SHOWING ZINC INDUCED PORCINE GROWTH HORMONE EXPRESSION, 1989.

CHAPTER XI

BIBLIOGRAPHY

Aihara, K.Y., Nishi, S., Hatano, M., Kihara, M., Ohta, M., Sakoda, K., Uozumi, T. and Usui, T. (1985). Zinc, copper, manganese, and selenium metabolism in patients with human growth hormone deficiency or acromegaly. *J.Pediatr.Gastroenterol.Nutr.* **4**:610-618.

Ainsworth, L., Tsang, B.K., Downey, B.R. and Marcus, G.J. (1990). The synthesis and actions of steriods and prostaglandins during follicular maturation in the pig. *J.Reprod.Fert.,Suppl.* **40**:137-150.

Allen, N.D., Norris, M.L. and Surani, M.A. (1990). Epigenetic control of trangene expression and imprinting by genotype-specific modifiers. *Cell* **61**:853-861.

Allen, R.L. and Wright, R.W. (1984). *In vitro* development of porcine embryos in co-culture with endometrial cell monolayers or culture supernatants. *J.Anim.Sci.* **59**:1657-1661.

Altszuler, N. (1974). Action of growth hormone on carbohydrate metabolism. In *Handbook of Physiology.* (Eds. Knobil, E. and Sawyer, W.H.). American Physiological Society, Bethesda, MD., pp.233-252.

Andersson, A.M. and Einersson, S. (1980). Studies on the estrus and ovarian activity during five successive estrous cycle in gilts. *Acta Vet.Scand.* **21**:677-681.

Archibong, A.E., England, D.C. and Stormshak, F. (1987a). Ovulation and embryonic survival in pubertal gilts treated with gonadotropin releasing hormone. *J.Anim.Sci.* **65**:752-755.

Archibong, A.E., England, D.C. and Stormshak, F. (1987b). Factors contributing to early embryonic mortality in gilts bred at first estrus. *J.Anim.Sci.* **64**:446-451.

Archibong, A.E., Petters, R.M. and Johnson, B.H. (1989). Development of porcine embryos from one- and two-cell stages to blastocysts in culture medium supplemented with porcine oviductal fluid. *Biol.Reprod.* **41**:1076-1083.

Bachetti, S. and Graham, F.L. (1977). Transfer of the gene for thymidine kinase to thymidine kinase-deficient human cells by purified herpes simplex viral DNA. *Proc.Natl.Acad.Sci.USA* **74**:1590-1594.

Baker, R.D. and Coggins, E.G. (1966). Induced ovulation and fertility in immature gilts. *J.Anim.Sci.* **25**:918. (abstr.).

Baker, R.D. and Coggins, E.G. (1968). Control of ovulation rate and fertilization in prepuberal gilts. *J.Anim.Sci.* **27**:1607-1610.

Baker, R.D. and Dziuk, P.J. (1970). Aerial transport of fertilized pig ova. *Can.J.Anim.Sci.* **50**:226-228.

Baker, R.D. and Downey, B.R. (1975). Induction of estrus, ovulation and fertility in prepubertal gilts. *Annls Biol.Anim.Biochim.Biophys.* **15**:375-382.

Bala, R.M. and Bhaumick, B. (1979). Radioimmunoassay of a basic somatomedin: comparison of various assay techniques and somatomedin levels in various sera. *J.Clin.Endocrinol.Metab.* **49**:770-777.

Barinaga, M., Yamonoto, G., Rivier, G., Vale, W., Evans, R. and Rosenfeld, M.D. (1983). Transcriptional regulation of growth hormone gene expression by growth hormone-releasing factor. *Nature* **306**:84-85.

Barinaga, M. (1989). Making transgenic mice: is it really that easy. *Science* **245**:590-591.

Barsh, G.S., Seeburg, P.H. and Gelinas, R.E. (1983). The human growth hormone gene family: structure and evolution of the chromosomal locus. *Nucleic Acid Res.* **11**:3939-3958.

Barta, A., Richards, R.I., Baxter, J.D. and Shine, J. (1981). Primary structure and evaluation of rat growth hormone gene. *Proc.Natl.Acad.Sci.USA* **78**:4867-4871.

Bass, J.J., Oldham, J.M., Hodgkinson, S.C., Fowke, P.J., Sauerwein, H., Molan, P., Breier, B.H. and Gluckman, P.D. (1991). Influence of nutrition and bovine growth hormone (GH) on hepatic GH binding, insulin-like growth factor-I and growth of lambs. *Journal of Endocrinology* **128**:181-186.

Bauman, D.E., DeGeeter, M.J., Peel, C.J., Lanza, G.M., Gorewit, R.C. and Hammond, R.W. (1982). Effect of recombinantly derived bovine growth hormone (bGH) on lactational performance of high yielding dairy cows. *J.Anim.Sci.* **65(Suppl 1)**:121. (abstr.).

Bauman, D.E., Eppard, J.P., DeGeeter, M.J. and Lanza, G.M. (1985). Responses of high-producing dairy cows to long-term treatment with pituitary somatotropin and recombinant somatotropin. *J.Dairy Sci.* **68**:1352-1362.

Bavister, B.D. (1987). *The Mammalian Preimplantation Embryo: Regulation of Growth and Differentiation In Vitro*, Plenum Press, New York and London,

Bavister, B.D. (1988). Role of oviductal secretions in embryonic growth *in vivo* and *in vitro*. *Theriogenology* **29**:143-154.

Bawden, C.S., Cam, G.R., D'Andrea, R.J., Kuczek, E.S., Powell, B.C., Rogers, G.E. and Sivaprasad, A.V. (1987). A review of the genes for wool keratin and for cysteine biosynthesis: their significance for studies directed towards modified wool production. In *Merino Improvement Programs in Australia.* (Ed. McGuirk, B.J.). Australian Wool Corporation, Melbourne, pp.511-518.

Baxter, R.C. (1991). Physiological roles of IGF binding proteins. In *Modern Concepts of Insulin-like Growth Factors.* (Ed. Spencer, E.M.). Elsevier, New York, pp.371-380.

Bazer, F.W., Geisert, R.D., Thatcher, W.W. and Roberts, R.M. (1982). The establishment of pregnancy. In *Control of reproduction in the pig.* (Eds. Cole, D.J.A. and Foxcroft, G.R.). Butterworths, U.K., pp.227-252.

Bazer, F.W. and Roberts, R.M. (1983). Biochemical aspects of conceptus-endometrial interactions. *J.Exp.Zool.* **228**:373-378.

Beckmann, L.S. and Day, B.N. (1991). Culture of the one- and two- cell porcine embryo: effects of varied osmolarity in Whitten's and Kreb's Ringer Bicarbonate Media. *Theriogenology* **35(1)**:184. (abstr.).

Bianchi, V. and Gelatti, L. (1985). Accuracy of UV-induced DNA repair in v79 cells with imbalance of deoxynucleotides pods. *Mut.Res.* **298**:189-192.

Biery, K.A., Bondioli, K.R. and De Mayo, F.J. (1988). Gene transfer by pronuclear microinjection in the bovine. *Theriogenology* **29**:224. (abstr.).

Biggers, J.D., Gwatkin, R.B.L. and Brinster, R.L. (1961). Development of mouse embryos in organ culture of fallopian tubes on a chemically defined medium. *Nature* **194**:747-749.

Bindon, B.M. and Piper, L.R. (1982). Physiological basis of the ovarian response to PMSG in sheep and cattle. In *Embryo Transfer in Cattle, Sheep and Goats*. (Eds. Shelton, J.N.,Trounson, A.O.,Moore, N.W. and James, J.W.). Sym. Australian Society for Reproductive Biology, Union Offset Company Pty Ltd, ACT Australia, pp.1-5.

Blum, W.F. and Ranke, M.B. (1991). Plasma IGFBP-3 levels as clinical indicators. In *Modern Concepts of Insulin-Like Growth Factors*. (Ed. Spencer, E.M.). Elsevier, New York, pp.381-393.

Bolze, M.S., Reeves, R.D., Lindbeck, F.E. and Elders, M.J. (1987). Influence of zinc growth, somatomedin, and glycosaminoglycan metabolism in rats. *Am.J.Physiol.* **252**:E21-E37.

Boulikas, T. (1986). Protein-protein and protein-DNA interactions in calf thymus nuclear matrix usimg cross-linking by ultraviolet light. *Biochem.Cell Biol.* **64**:474-484.

Boulikas, T. (1990). Poly (ADP-ribosylated) Histones in Chromatin Replication. *J.Biol.Chem.* **265**:14638-14647.

Boyd, R.D., Bauman, D.E., Beermann, D.H., DeNeergaard, A.F., Souza, L. and Butler, R.D. (1986). Titration of the porcine growth hormone dose which maximizes lean deposition in swine. *J.Anim.Sci.* **63(Suppl 1)**:218. (abstr.).

Braam-Markson, J., Jaudon, C. and Krug, R.M. (1985). Expression of a functional influenza viral cap-recognizing protein by using a bovine papilloma virus vector. *Proc.Natl.Acad.Sci.USA* **82**:4326-4330.

Brackett, B.G. (1981). *In vitro* culture of the zygote and embryo. In *Fertilization and Embryonic Development in Vitro*. (Eds. Mastroianni, L.Jr and Biggers, J.D.). Plenum Press, New York, pp.61-79.

Bradley, A., Evans, M.J., Kaufman, M.H. and Robertson, E.J. (1984). Formation of germ-line chimaeras from embryo-derived teratocarcinoma cell lines. *Nature* **309**:255-256.

Braithwaite, G.D. (1975). The effect of growth hormone on calcium metabolism in sheep. *Br.J.Nutr.* **33**:309-316.

Brash, D.E. and Haseltine, W.A. (1982). UV-induced mutation hotspots occur at DNA damage hotspots. *Nature* **298**:189-192.

Brazeau, P., Vale, W., Burgus, R., Ling, N., Butcher, M., Rivier, J. and Guillemin, R. (1973). Hypothalamic polypeptide that inhibits the secretion of immunoreactive pituitary growth hormone. *Science* **179**:77-79.

Brem, G., Brenig, B., Goodman, H.M., Selden, R.C., Graf, F., Kruff, B., Springmann, K., Hondele, J., Meyer, J., Winnacker, E-L. and Kräusslich, H. (1985). Production of transgenic mice and pigs by microinjection into pronuclei. *Zuchthygiene* **20**:251-252.

Brem, G., Brenig, B., Muller, M., Kräusslich, H. and Winnacker, E-L. (1988). Production of transgenic pigs and possible application to pig breeding. *Occ.Publ.Br.Soc.Anim.Prod.* **12**:15-31.

Brinster, R.L. (1963). A method for *in vitro* cultivation of mouse ova from two-cell to blastocyst. *Exp.Cell Res.* **32**:205-208.

Brinster, R.L. (1965). Studies on the development of mouse embryos *in vitro*. *J.Reprod.Fert.* **10**:227-240.

Brinster, R.L., Chen, H.Y. and Trumbauer, M.E. (1981). Mouse ooyctes transcribe injected Xenopus 5S RNA genes. *Science* **211**:396-398.

Brinster, R.L. and Palmiter, R.D. (1982). Induction of foreign genes in animals. *Trends Biochem.Sci.* **7**:438-440.

Brinster, R.L., Chen, H.Y., Trumbauer, M.E., Yagle, M.K. and Palmiter, R.D. (1985). Factors affecting the efficiency of introducing foreign DNA into mice by microinjecting eggs. *Proc.Natl.Acad.Sci.USA* **82**:4438-4442.

Brinster, R.L. and Palmiter, R.D. (1986). Introduction of genes into the germline of animals. *Harvey Lect.* **80**:1-38.

Brinster, R.L., Allen, J.M., Behringer, R.R., Gelinas, R.E. and Palmiter, R.D. (1988). Introns increase transcriptional efficiency in transgenic mice. *Proc.Natl.Acad.Sci.USA* **85**:836-840.

Brinster, R.L., Sandgren, E.P., Behringer, R.R. and Palmiter, R.D. (1989). No simple solution for making transgenic mice. *Cell* **59**:239-241.

Broermann, D.M., Xie, S., Nephew, K.P. and Pope, W.F. (1990). Limitations of oviductal transfer in swine. *Theriogenology* **33**:709-721.

Bryan, K.A., Hammond, J.M., Canning, S., Mondschein, J., Carbaugh, D.E., Clark, A.M. and Hagen, D.R. (1989). Reproductive and growth responses of gilts to exogenous porcine pituitary growth hormone. *J.Anim.Sci.* **67**:196-205.

Burki, K. and Ullrich, A. (1982). Transplantation of the human insulin gene into fertilized mouse eggs. *EMBO J.* **1**:127-131.

Busch, W., Maab, P., Freistedt, R. and Wohlfahrt, E. (1988). The application of allytrembolone (Regumate) for synchronisation of oestrus in gilts under big farm conditions. *Proc. 11th Int. Congr. Anim. Reprod. A.I. Dublin* 465. (abstr.).

Buttle, H.L. and Hancock, J.L. (1967). The control of ovulation in the sow. *J.Reprod.Fert.* **14**:485-487.

Cahill, L.P. (1981). Factors influencing the follicular response of animals to PMSG. In *Embryo Transfer in Cattle, Sheep and Goats.* (Eds. Shelton, J.N.,Trounson, A.O.,Moore, N.W. and James, J.W.). Sym. Australian Society for Reproductive Biology, Union Offset Company Pty Ltd ACT Australia, pp.5-8.

Cameron, R.D.A., Durack, M., Fogarty, R., Putra, D.K.H. and McVeigh, J. (1989). Practical experience with commercial embryo transfer in pigs. *Aust.Vet.J.* **66**:314-318.

Camous, S.Y., Heyman, Y., Meziou, W. and Menezou, Y. (1984). Cleavage beyond the block stage and survival after transfer of early bovine embryos cultured with trophoblastic vesicles. *J.Reprod.Fert.* **72**:479-585.

Campbell, R.G., Steele, N.C., Caperna, T.J., McMurtry, J.P., Solomon, M.B. and Mitchell, A.D. (1988a). Interrelationship between sex and exogenous growth hormone administration on performance, body composition and protein and fat accretion of growing pigs. *J.Anim.Sci.* **67**:177-186.

Campbell, R.G., Steele, N.C., Caperna, T.J., McMurty, J.P., Solomon, M.B. and Mitchell, A.D. (1988b). Interrelationships between energy intake and endogenous porcine growth hormone administration on the performance, body composition and protein and energy metabolism of growing pigs weighing 25 to 55 kilograms live weight. *J.Anim.Sci.* **66**:1643-1655.

Campbell, R.G. and Taverner, M.R. (1988c). Genotype and sex effects on the relationship between energy intake and protein deposition in growing pigs. *J.Anim.Sci.* **66**:676-688.

Campbell, R.G., Steele, N.C., Caperna, T.J., McMurtry, J.P., Solomon, M.B. and Mitchell, A.D. (1989). Interrelationships between sex and exogenous growth hormone administration on performance, body composition and protein and fat accretion of growing pigs. *J.Anim.Sci.* **67**:177-186.

Capecchi, M. (1980). High efficiency transformation by direct microinjection of DNA into cultured mammalian cells. *Cell* **22**:479-488.

Caperna, T.J., Campbell, R.G. and Steele, N.C. (1989). Interrelationships of exogenous porcine growth hormone administration and feed intake level affecting various tissue levels of iron, copper, zinc and bone calcium of growing pigs. *J.Anim.Sci.* **67**:654-663.

Carlson, C.S., Hilley, H.D., Meuten, D.J., Hagan, J.M. and Moser, R.L. (1988). Effect of reduced growth rate on the prevalence and severity of osteochondrosis in gilts. *Am.J.Vet.Res.* **49**:396-402.

Carr, D. and Friesen, H.C. (1976). Growth hormone and insulin binding to human liver. *J.Clin.Endocrinol.Metab.* **42**:484-493.

Casida, L.E. (1935). Prepubertal development of the pig ovary and its relation to stimulation with gonadotrophic hormones. *Anat.rec.* **61**:389-396.

Chang, M.C. (1950). Development and fate of transferred rabbit ova or blastocyst in relation to the ovulation of recipients. *J.Exp.Zool.* **114**:197-226.

Chapman, I.M., Helfgott, A. and Willoughby, J.O. (1991). Disappearance half-life times of exogenous and growth hormone-releasing factor-stimulated endogenous growth hormone in normal rats. *Journal of Endocrinology* **128**:369-374.

Cheek, D.B. and Hill, D.E. (1974). Effect of growth hormone on cell and somatic growth. In *Handbook of Physiology.* (Eds. Knobil, E. and Sawyer, W.H.). American Physiological Society, Bethesda, MD., pp.159-185.

Christenson, R.K., Pope, C.E., Zimmerman-Pope, V.A. and Day, B.N. (1973). Synchronization of estrus and ovulation in superovulated gilts. *J.Anim.Sci.* **36**:914-918.

Christenson, R.K. and Teague, H.S. (1975). Synchronization of ovulation and artificial insemination of sows after lactation. *J.Anim.Sci.* **41**:560-563.

Chung, C.S., Etherton, T.D. and Wiggins, J.P. (1985). Stimulation of swine growth by porcine growth hormone. *J.Anim.Sci.* **60**:118-130.

Church, R.B., Schaufele, F.J. and Meckling, K. (1985). Embryo manipulation and gene transfer in livestock. *Can.J.Anim.Sci.* **65**:527-537.

Clarkson, J.L. and Nancarrow, C.D. (1988). Use of trophoblastic vesicles as models for ovine blastocysts cultured *in vitro*. *Proc.11th Int.Congr.Anim.Reprod.& A.I.Dublin* **2**:148. (abstr.).

Cleaves, J.E. (1983). Inhibition of DNA synthesis by UV light under controlled temperature. *Nature* **209**:1317-1320.

Clemmons, D.R. and Van der Wyk, J.J. (1981). Hormonal control of immunoreactive somatomedin production by cultured human fibroblasts. *J.Clin.Invest.* **67**:10-19.

Clemmons, D.R., Camacho-Hubner, C., Jones, J.I., McCusker, R.H. and Busby, W.H.,Jr, (1991). Insulin-like growth factor binding proteins: mechanisms of action at the cellular level. In *Modern Concepts of Insulin-like Growth Factors*. (Ed. Spencer, E.M.). Elsevier, New York, pp.475-486.

Cloud, J.G. (1990). Strategies for introducing foreign DNA into the germ line of fish. *J.Reprod.Fert.,Suppl.* **41**:107-116.

Coleman, M.E. and Etherton, T.D. (1991). Effects of exogenous porcine growth hormone on serum insulin-like growth factor-binding proteins in growing pigs. *Journal of Endocrinology* **128**:175-180.

Constantini, F. and Lacy, E. (1981). Introduction of a rabbit β-globin gene into the mouse germ line. *Nature* **294**:92-94.

Copeland, K.C., Underwood, L.E. and Van der Wyk, J.J. (1980). Induction of immunoreactive somatomedin C in human serum by growth hormone: dose-response relationships and effect on chromatographic profiles. *J.Clin.Endocrinol.Metab.* **50**:690-697.

Cory, S. (1986). Activation of cellular oncogenes in hemopoietic cells by chromosome translocation. *Adv.Cancer Res.* **47**:189-226.

Cossack, Z.T. (1984). Somatomedin-C in zinc deficiency. *Experientia* **40**:498-507.

D'Ercole, A.J., Stiles, A.D. and Underwood, L.E. (1984). Tissue concentrations of somatomedin C: further evidence for multiple sites of synthesis and paracrine or autocrine mechanisms of action. *Proc.Natl.Acad.Sci.USA* **81**:935-939.

Daughaday, W.H., Hall, K., Raben, M.S., Salmon, W.D.,Jr, Van der Brande, J.L. and Van der Wyk, J.J. (1972). Somatomedin: proposed designation for sulphation factor. *Nature* **235**:107-109.

Daughaday, W.H., Herington, A.C. and Phillips, L.S. (1975). The regulation of growth by endocrines. *Ann.Rev.Physiol.* **37**:211-244.

Daughaday, W.H. (1981). The adenohypophysis. In *Textbook of Endocrinology.* (Ed. Williams, R.H.). W.B. Saunders Company, Philadelphia, pp.73-116.

Daughaday, W.H. (1983). The somatomedin hypothesis: origins and recent development. In *Insulin-Like Growth Factors Somatomedins.* (Ed. Spencer, E.M.). Walter de Gruyter, New York, pp.3-11.

Davis, D.L. and Day, B.N. (1978). Cleavage and blastocyst formation by pig eggs *in vitro. J.Anim.Sci.* **46**:1043-1053.

Davis, D.L. (1985). Culture and storage of pig embryos. *J.Reprod.Fert.,Suppl.* **33**:115-124.

Davis, D.L., Stevenson, J.S. and Schmidt, W.E. (1985). Scheduled breeding of gilts after estrous synchronization with altrenogest. *J.Anim.Sci.* **60**:599. (abstr.).

Davis, D.L., Stevenson, J.S, Pollmann, D.S. and Allee, G.L. (1986). Estrous and litter traits in gilts altered by altrenogest, flushing and pubertal status. *J.Anim.Sci.* **64**:1117-1126.

Davis, R.J., Faucher, J.M., Racaniello, L.K., Carruthers, A. and Czech, M.P. (1987). Insulin-like growth factor-I and epidermal growth factor regulate the expression of transferrin receptors at the cell surface by distinct mechanisms. *J.Biol.Chem.* **262**:13126-13132.

Day, B.N., Neill, J.D., Oxenreider, S.L., Waite, A.B. and Lasley, J.F. (1965). Use of gonadotrophins to synchronize estrous cycles in swine. *J.Anim.Sci.* **24**:1075-1079.

Day, B.N., Longenecker, D.E., Jaffe, S.C., Gibson, E.W. and Lasley, J.F. (1967). Fertility of swine following superovulation. *J.Anim.Sci.* **26**:777-780.

Day, B.N. and Longenecker, D.E. (1968). Synchronization of estrus and superovulation in swine with I.C.I. 33828 and pregnant mare serum. *VI Cong. Intern. Reprod. Anim. Insem. Artif., Paris Vol. II* 1419. (abstr.).

Day, B.N. (1984). Estrous cycle regulation. *Proc.10th Int.Congr.Anim.Reprod.A.I.Uni.of Illinois,* **4**:1-8.

Dayhoff, M.O. (1972). *Atlas of protein sequence and structure,* Ed. 5th National Biomedical Research Foundation, Maryland,

Demple, B. and Linn, S. (1980). DNA N-glycosylases and UV repair. *Nature* **298**:203-207.

DeNoto, F.M., Moore, D.D. and Goodman, H.M. (1981). Human growth hormone DNA sequence and mRNA structure: possible alternative splicing. *Nucleic Acid Res.* **9**:3719-3720.

Dewey, M.J., Martin, D.W., Martin, G.R. and Mintz, B. (1977). Mosaic mice with teratocarcinoma-derived mutant cells deficient in hypoxanthine phosphoribosyltransferase. *Proc.Natl.Acad.Sci.USA* **74**:5564-5568.

Diacumakos, E. (1973). Methods for micromanipulation of human somatic cells in culture. *Methods Cell Biol.* **7**:287-311.

Doetschman, T., Williams, P. and Maeda, N. (1988). Establishment of hamster blastocyst-derived embryonic stem (ES) cells. *Dev.Biol.* **127**:224-227.

Du Mesnil du Buisson, F. (1954). Possibilte d'ovulation et de fecondation chez la truie avant la puberte. *Annls Endocr.* **15**:333-340.

Dubbs, D.R., Rachmeter, M. and Kit, S. (1974). Recombination and temperature-sensitive mutants of simian virus 40. *Virology* **52**:456-461.

Dufour, J.J., Grasso, F., Daviault, E. and Matton, P. (1988). Relationship between two morphological types of ovaries and their follicular microscopic population before puberty and their ensuing follicular development and ovulation rates at puberty in gilts. *J.Anim.Sci.* **66**:932-938.

Duncan, D.L. and Lodge, G.A. (1960). Diet in relation to reproduction and viability of the young. In *Commonwealth Bureau of Animal Nutrition Tech. Comm. No. 21.* Rowett Research Institute, Bucksburn, Aberdeen, Ed. 3rd.

Dyck, G.W. (1982). Pregnancy Diagnosis. In *Control of Pig Reproduction.* (Eds. Cole, D.J.A. and Foxcroft, G.R.). Butterworths Scientific, London, pp.293-308.

Dziuk, P.J. (1960). Influence of orally administered progestin on estrus and ovulation in swine. *J.Anim.Sci.* **19**:1319-1320.

Dziuk, P.J. and Baker, R.D. (1962). Induction and control of ovulation in swine. *J.Anim.Sci.* **21**:697-699.

Dziuk, P.J., Polge, C. and Rowson, L.E.A. (1964). Intra-uterine migration and mixing of embryos in swine following egg transfer. *J.Anim.Sci.* **23**:37-42.

Dziuk, P.J. (1965). Timing of maturation and fertilization of the sheep egg. *Anat.rec.* **153**:211-224.

Dziuk, P.J. and Polge, C. (1965). Fertility in gilts following induced ovulation. *Vet.Rec.* **77**:236-238.

Dziuk, P.J. and Gehlbach, G.D. (1966). Induction of ovulation and fertilization in the immature gilt. *J.Anim.Sci.* **25**:410-413.

Dziuk, P.J. (1969). Effect of number of embryos and uterine space on embryo survival in the pig. *J.Anim.Sci.* **27**:673-676.

Dziuk, P.J. and Dhindsa, D.S. (1969). Induction of heat, ovulation and fertility in gilts with delayed puberty. *J.Anim.Sci.* **29**:39-41.

Dziuk, P.J. (1987). Embryonic loss in the pig: An enigma. In *Manipulating Pig Production.* Proc. Inaugural Conference Australasian Pig Science Association, Albury, NSW, pp.28-29.

Ebert, K.M., Low, M.J., Overstrom, E.W., Buonomo, F.C., Baile, C.A., Roberts, T.M., Lee, A., Mandel, G. and Goodman, R.H. (1988). A moloney MLV-rat somatotropin fusion gene produces biologically active somatotropin in a transgenic pig. *Molec.Endocrinol.* **2**:277-283.

Eden, S., Isaksson, O., Madsen, K. and Friberg, U. (1983). Specific binding of growth hormone to isolated chondrocytes from rabbit ear and epiphysial plate. *Endocrinology* **112**:1127-1129.

Edwards, R.G. and Gates, A.H. (1959). Timing of the stage of maturation, division ovulation, fertilisation and the first cleavage of adult mice treated with gonadotrophin. *Journal of Endocrinology* **18**:292-304.

Ellicott, A.R., Dziuk, P.J. and Polge, C. (1973). Maintenance of pregnancy in prepubertal gilts. *J.Anim.Sci.* **37**:971-973.

Esbenshade, K.L., Paterson, A.M., Cantley, T.C. and Day, B.N. (1982). Changes in plasma hormone concentrations associated with the onset of puberty in the gilt. *J.Anim.Sci.* **54**:320-327.

Esbenshade, K.L., Ziecik, A.J. and Britt, J.H. (1990). Regulation and action of gonadotrophins in pigs. *J.Reprod.Fert.,Suppl.* **40**:19-32.

Estienne, C.E., Rampacek, G.B., Kraeling, R.R., Estienne, M.J. and Barb, C.R. (1988). Luteinizing hormone receptor number and affinity in corpora lutea from prepuberal gilts induced to ovulate and spontaneous corpora lutea of mature gilts. *J.Anim.Sci.* **66**:917-922.

Etherton, T.D. (1982). The role of insulin-receptor interactions in regulation of nutrient utilization by skeletal muscle and adipose tissue: A review. *J.Anim.Sci.* **54**:58-74.

Etherton, T.D. and Kensinger, R.S. (1984). Endocrine regulation of fetal and postnatal meat animal growth. *J.Anim.Sci.* **59**:511-514.

Etherton, T.D., Wiggins, J.P., Chung, C.S., Evock, C.M., Rebhun, J.F. and Walton, P.E. (1986). Stimulation of pig growth performance by porcine growth hormone-releasing factor. *J.Anim.Sci.* **63**:1389-1399.

Etherton, T.D., Wiggins, J.P., Evock, C.M., Chung, C.S., Rebhun, J.F., Walton, P.E. and Steele, N.C. (1987). Stimulation of pig growth performance by porcine growth hormone: Determination of the dose-response relationship. *J.Anim.Sci.* **64**:433-443.

Evans, H.M. and Long, J.A. (1921). The effect of anterior lobe of the hypophysis administered intraperitoneally upon growth, and the maturity and oestrous cycle of the rat. *Anat.Rec.* **21**:62-63.

Evans, M.J. and Kaufman, M.H. (1981). Establishment in culture of pluripotent cells from mouse embryos. *Nature* **292**:154-156.

Evans, M.J. and Simpson, M.E. (1983). Hormones of the anterior hypophysis. *Amer.J.Physiol.* **98**:511-519.

Evans, M.J., Bradley, A., Kuehn, M.R. and Robertson, E.J. (1985). The ability of EK cells to form chimeras after selection of clones in G418 and some observations on the integration of retroviral vector proviral DNA into EK cells. *Cold Spring Harbor Symp.Quant.Biol.* **50**:685-689.

Evock, C.M., Etherton, T.D., Chung, C.S. and Ivy, R.E. (1988). Pituitary porcine growth hormone (pGH) and a recombinant pGH analog stimulate pig growth performance in a similar manner. *J.Anim.Sci.* **66**:1928-1940.

Eyestone, W.H., Vignieri, J. and First, N.L. (1987). Co-culture of early embryos with oviductal epithelium. *Theriogenology* **27**:228. (abstr.).

Eyestone, W.H. and First, N.L. (1989). Co-culture of early cattle embryos to the blastocyst stage with oviduct tissue or in conditioned media. *J.Reprod.Fert.* **85**:715-720.

Fabricant, J.D., Nuti, L.C., Minhas, B.S., Baker, W.C., Capehart, J.S., Marrock, P., Chalmers, J.H., Bradbury, M.W. and Womack, J.E. (1987). Gene transfer in goats. *Theriogenology* **27**:229. (abstr.).

Farzaneh, F., Panayotou, G.N., Bowler, L.D., Hardas, B.D., Broom, T., Walther, C. and Shall, S. (1988). ADP-ribosylation is involved in the integration of foreign DNA into the mammalian cell genome. *Nucleic Acid Res.* **16**:11319-11326.

First, N.L. (1990). New animal breeding techniques and their application. *J.Reprod.Fert.,Suppl.* **41**:3-14.

Flowers, B., Martin, M.J., Cantley, T.C. and Day, B.N. (1989). The effect of pregnant mare serum gonadotropin on follicle stimulating hormone and estradiol secretion in the prepuberal gilt. *Anim.Reprod.Sci.* **21**:93-100.

Foxcroft, G.R. (1982). Endocrine Control of the Oestrous Cycle. In *Control of Pig Reproduction.* (Eds. Cole, D.J.A. and Foxcroft, G.R.). Butterworths Scientific, London, pp.161-178.

Franchimont, P. and Burger, H. (1975). *Human Growth Hormone and Gonadotrophins in Health and Disease*, North-Holland, Amsterdam,

Franklin, W.A., Lo, K.M. and Haseltine, W.A. (1982). Alkaline lability of novel fluorescent photoproducts produced in ultraviolet light irradiated DNA. *J.Biol.Chem.* **257**:13535-13543.

Fraser, A.F. and Robertson, J.G. (1967). The detection of foetal life in ewes and sows. *Vet.Rec.* **80**:528-529.

Frasier, S.D. (1983). Human pituitary growth hormone (hGH) therapy in growth hormone deficient. *Endocrine Rev.* **4**:155-170.

Friesen, H.C. (1980). Raben lecture 1980: A tale of stature. *Endocrine Rev.* **1**:309-318.

Froesch, E.R. (1983). From NSILA to IGF: A look back on the major advances and breakthroughs. In *Insulin-like Growth Factors Somatomedins.* (Ed. Spencer, E.M.). Walter de Gruyter, New York, pp.13-29.

Froesch, E.R., Schmid, Chr., Schwander, J. and Zapf, J. (1985). Actions of insulin-like growth factors. *Ann.Rev.Physiol.* **47**:443-467.

Froesch, E.R., Schmid, C., Sangger, I., Schoenle, E., Eigenmann, E. and Zapf, J. (1986). Effects of IGF/somatomedins on growth and differentiation of muscle and bone. *J.Anim.Sci.* **63 (Suppl 2)**:57. (abstr.).

Frohman, L.A. and Jansson, J-O. (1986). Growth hormone-releasing hormone. *Endocrine Rev.* **7**:223-253.

Fujii, J., Otsu, K., Zorzato, F., de Leon, S., Khanna, V.K., Weiler, J.E., O'Brien, P.J. and MacLennan, D.H. (1991). Identification of a mutation in porcine ryanodine receptor associated with malignant hyperthermia. *Science* **25**:448-450.

Gandolfi, F. and Moor, R.M. (1987). Stimulation of early embryonic development in the sheep by co-culture with oviduct epithelial cells. *J.Reprod.Fert.* **81**:23-28.

Gardner, R.L. (1968). Mouse chimeras obtained by the injection of cells into the blastocyst. *Nature* **220**:596-597.

Gardner, R.L. and Papaioannov, V.E. (1975). Differentiation in trophectoderm and inner cell mass. In *Early Development of Mammals.* (Eds. Balls, M. and Wild, A.E.). Cambridge University Press, Cambridge, U.K, pp.107-132.

Gates, A.H. and Runner, M.N. (1957). Influence of prepubertal age on number of ova that can be superovulated in the mouse. *Anal.Record* **128**:554-558.

Gates, A.H. (1969). Maximizing yield and development uniformity of eggs. *Nature* **177**:754-755.

Geisert, R.D., Renegar, R.H., Thatcher, W.W., Roberts, R.M. and Bazer, F.W. (1982a). Establishment of pregnancy in the pig.I Interrelationships between preimplantation development of the pig blastocyst and endometrial secretions. *Biol.Reprod.* **27**:925-929.

Geisert, R.D., Brookbank, J.W., Roberts, R.M. and Bazer, F.W. (1982b). Establishment of pregnancy in the pig II. Cellular remodeling of the porcine blastocyst during elongation on day 12 of pregnancy. *Biol.Reprod.* **27**:941-946.

Gilbert, D.M. and Cohen, S.N. (1987). Bovine papilloma virus plasmids replicate randomly in mouse fibroblasts throughout the S phase of the cycle. *Cell* **50**:59-68.

Gleeson, A.R. (1974). Luteal function in cyclic sows after infusion of prostaglandin $F_{2\alpha}$ through a uterine vein. *J.Reprod.Fert.* **36**:487-488. (abstr.).

Goodband, R.D., Nelssen, J.L., Hines, R.H., Kropf, D.H., Thaler, R.C., Schricker, B.R. and Fitzner, G.E. (1988). The effect of porcine somatotrophin (PST) and dietary lysine level on growth performance and carcass characteristics of finishing swine. *J.Anim.Sci.* **66 (1)**:95. (abstr.).

Goodman, H.M. and Schwartz, J. (1974). Growth hormone and lipid metabolism. In *Handbook of Physiology.* (Eds. Knobil, E. and Sawyer, W.H.). American Physiological Society, Bethesda, MD., pp.211-231.

Gopinath, R. and Etherton, T.D. (1989a). Effects of porcine growth hormone on glucose metabolism of pigs: I. Acute and chronic effects on plasma glucose and insulin status. *J.Anim.Sci.* **67**:683-688.

Gopinath, R. and Etherton, T.D. (1989b). Effects of porcine growth hormone on glucose metabolism of pigs: II Glucose tolerance, peripheral tissue insulin sensitivity and glucose kinetics. *J.Anim.Sci.* **67**:689-697.

Gordon, I. and Lu, K.H. (1990). Production of embryos *in vitro* and its impact on livestock production. *Theriogenology* **33**:77-87.

Gordon, I. (1991). Potential application of cattle *in vitro* fertilization in commercial practice and research. *Embryo Transfer Newsletter* **9(2)**:4-8.

Gordon, J.W., Scangos, G.A., Plotkin, D.J., Barbosa, J.A. and Ruddle, F.H. (1980). Genetic transformation of mouse embryos by microinjection of purfied DNA. *Proc.Natl.Acad.Sci.USA* **77**:7380-7384.

Gordon, J.W. and Ruddle, F.H. (1981). Integration and stable germ line transmission of genes injected into mouse pronuclei. *Science* **214**:1244-1246.

Gordon, J.W. and Ruddle, F.H. (1983). Gene transfer into mouse embryos: production of transgenic mice by pronuclear microinjection. *Meth.Enzymol.* **101**:411-433.

Gordon, J.W. (1989). Transgenic Animals. *International Review of Cytology* **115**:171-229.

Gordon, L.K. and Haseltine, W.A. (1980). Comparison of the cleavage of pyrimidine dimer by the bacteriophage T4 Micrococcus luteus UV-specific endonucleases. *J.Biol.Chem.* **255**:12047-12050.

Gordon, L.K. and Haseltine, W.A. (1981). Early steps of excision repair of cyclobutane pyrimidine dimers by the Micrococcus luteus endonuclease: a three step incision model. *J.Biol.Chem.* **256**:6608-6616.

Graessman, A., Graessman, M., Topp, W.C. and Botcham, J. (1979). Retransformation of simian virus 40 revertant cell-line, which is resistant to viral and DNA infections, by microinjection of viral DNA. *J.Virol.* **32**:989-994.

Graham, F.L. and Van der Eb, A. (1973). A new technique for the assay of infectivity of human adenoviruses 5 DNA. *Virology* **52**:456-467.

Graham, F.L., Abrahams, P.J., Mudler, C., Heijneker, H.L., Warnaar, S.O., de Viries, F.A.J., Fiers, W. and Van der Eb, A.J. (1975). Studies on *in vitro* transformations by DNA and DNA fragments of human adenoviruses and simian virus 40. *Cold Spring Harbor Symp.Quant.Biol.* **39**:637-650.

Grasso, F., Castonguay, F., Daviault, E., Matton, P., Minvielle, F. and Dufour, J.J (1988). Study of follicular development and heredity of morphological types of ovaries in prepuberal gilts. *J.Anim.Sci.* **66**:923-931.

Groesbeck, M.D., Parlow, A.F. and Daughaday, W.H. (1986). Stimulation of supranormal growth in prepubertal, adult plateaued, and hypophysectomized female rats by large doses of rat growth hormone: physiological effects and adverse consequences. *Endocrinology* **118**:2292-2298.

Guthrie, H.D., Henricks, D.M. and Handlin, D.L. (1972). Plasma estrogen, progesterone and luteinizing hormone prior to estrus and during early pregnancy in pigs. *Endocrinology* **91**:675-682.

Guthrie, H.D. (1975). Estrous synchronization and fertility in gilts treated with estradiol-benzoate and prostaglandin $F_{2\alpha}$. *Theriogenology* **4**:69-75.

Guthrie, H.D. and Polge, C. (1976). Control of oestrus and fertility in gilts with accessory corpora lutea by prostaglandin analogues, ICI 79,939 and ICI 80,996. *J.Reprod.Fert.* **48**:427-430.

Guthrie, H.D. (1977). Induction of ovulation and fertility in prepubertal gilts. *J.Anim.Sci.* **45**:1360-1367.

Guthrie, H.D. and Polge, C. (1978). Treatment of pregnant gilts with a prostaglandin analogue, cloprostenol, to control oestrus and fertility. *J.Reprod.Fert.* **48**:427-430.

Guthrie, H.D. and Bolt, D.J. (1983). Changes in plasma estrogen luteinizing hormone, follicle-stimulating hormone and 13,14-dihydro-15-keto-prostaglandin $F_{2\alpha}$ in pigs with luteolysis blocked pregnancy, estradiol benzoate, or human chorionic gonadotrophin. *J.Anim.Sci.* **57**:993. (abstr.).

Guthrie, H.D. and Bolt, D.J. (1985). Pituitary and ovarian hormone secretion and ovulation in gilts injected with gonadotrophins during and after oral administration of progesterone agonist (Altrenogest). *Biol.Reprod.* **33**:679-689.

Guthrie, H.D., Meckley, P.E., Young, E.P. and Hartsock, T.G. (1987). Effect of altrenogest and lutalyse on parturition control, plasma progesterone, unconjugated estrogen and 13,14-dihydro-15-keto-prostaglandin $F_{2\alpha}$ in sSows. *J.Anim.Sci.* **65**:203-211.

Guthrie, H.D., Bolt, D.J., Kiracofe, G.H. and Miller, K.F. (1988). Ovarian response to injections of charcoal-extracted porcine follicular fluid and porcine follicle-stimulating hormone in gilts fed a progesterone agonist (Altrenogest). *Biol.Reprod.* **38**:750-755.

Guyda, H.J. (1991). Concepts of IGF physiology. In *Modern Concepts of Insulin-like Growth Factors*. (Ed. Spencer, E.M.). Elsevier, New York, pp.99-109.

Gwatkin, R.B.L. and Biggers, J.D. (1963). Histology of mouse fallopian tubes maintained as organ cultures on a chemically defined medium. *Int.J.Fertil.* **8**:453-457.

Hall, J.D., Featherston, J.D. and Almy, R.E. (1980). Evidence for repair of ultraviolet light-damaged herpes virus in human fibroblast by a recombination mechanism. *Virology* **105**:490-500.

Hall, J.D. and Mount, D.W. (1981). Mechanism of DNA replication: mutagenesis in ultraviolet-irradiated bacteria and mammalian cells. *Prog.Nucl.Acid Res.* **25**:53-126.

Hall, K. and Sara, V.R. (1983). Growth and somatomedins. In *Vitamins and Hormones*. (Eds. Aurbach, G.D. and McCormic, D.B.). Academic Press, New York, Ed. 40th pp.175-233.

Hallford, D.M., Wetteman, R.P., Turman, E.J. and Omtvedt, I.T. (1975). Luteal function in gilts after prostaglandin $F_{2\alpha}$. *J.Anim.Sci.* **41**:1706-1710.

Hammer, R.E., Palmiter, R.D. and Brinster, R.L. (1984). Partial correction of murine hereditary disorder by germ-line incorporation of a new gene. *Nature* **311**:65-67.

Hammer, R.E., Pursel, V.G., Rexroad, C.E.,Jr., Wall, R.J., Bolt, D.J., Ebert, K.M., Palmiter, R.D. and Brinster, R.L. (1985a). Production of transgenic rabbits, sheep and pigs by microinjection. *Nature* **315**:680-683.

Hammer, R.E., Brinster, R.L., Rosenfeld, M.G., Evans, R.M. and Mayo, K.E. (1985b). Expression of human growth hormone releasing factor in transgenic mice results in increased somatic growth. *Nature* **315**:413-416.

Hammer, R.E., Brinster, R.L. and Palmiter, R.D. (1985c). Use of gene transfer to increase animal growth. *Cold Spring Harb.Symp.Quant.Biol.* **50**:379-387.

Hammer, R.E., Pursel, V.G., Rexroad, C.E.,Jr., Wall, R.J., Bolt, D.J., Palmiter, R.D. and Brinster, R.L. (1986). Genetic engineering of mammalian embryos. *J.Anim.Sci.* **63**:269-278.

Hancock, J.L. and Hovell, G.J.R. (1961). Egg transfer in the sow. *J.Reprod.Fert.* **4**:195-201.

Hanly, S. (1961). Prenatal mortality in farm animals. *J.Reprod.Fert.* **2**:182-194.

Hanrahan, J.P. and Owens, J.B. (1985). Variation and repeatability of ovulation rate in cambridge ewes. *Proc.Brit.Soc.Anim.Prod., Winter Meeting* 37. (abstr.).

Haseltine, W.A., Gordon, L.K., Lindon, C.P., Grafstom, R.H., Shaper, N.L. and Grossman, L. (1980). Cleavage of pyrimidine dimers in specific DNA sequences by a pyrimidine dimers in specific DNA sequences by a pyrimidine dimer DNA glycosylase of *M. luteus. Nature* **285**:249-244.

Haseltine, W.A. (1983). Ultraviolet light repair and mutagenesis revisited. *Cell* **33**:13-17.

Heape, I. (1981). Preliminary note on the transplantation and growth of mammalian ova within a uterine foster mother. *Proc.R.Soc.* **48**:457.

Henkin, R.I. (1976). Trace metals in endocrinology. *Med.Clin.N.Am.* **60**:779-788.

Herington, A.C., Cornell, H.J. and Kuffer, A.D. (1983). Recent advances in the biochemistry and physiology of the insulin-like growth factor/somatomedin family. *Int.J.Biochem.* **15**:1201-1213.

Hettle, S.J.H., Harvey, M.J.A., Cameron, E.R., Johnston, C.S. and Onions, D.E. (1989). Generation of transgenic sheep by sub-zonal injection of feline leukemia virus. *J.Cell.Biochem.* **13B**:180. (abstr.).

Heyman, Y. and Menezo, Y. (1987). Interaction of trophoblastic vesicles with bovine embryos developing *in vitro*. In *The Mammalian Preimplantation Embryo*. (Ed. Bavister, B.D.). Plenum Press, New York, pp.175-191.

Heyman, Y., Menezo, Y., Chesne, P., Camous, S. and Garnier, V. (1987). *In vitro* cleavage of bovine and ovine early embryos: Improved development using co-culture with trophoblastic vesicles. *Theriogenology* **27**:59-68.

Hilwig, I. and Gropp, A. (1972). Staining of constitutive heterochromatin in mammalian chromosomes with a new fluorochrome. *Exp.Cell Res.* **75**:122-126.

Hintz, R.L., Rosenfeld, R.G., Wilson, D.M., Bennet, A., Finno, J., McClellan, B. and Swift, R. (1982). Biosynthetic methionyl human growth hormone is biologically active in adult man. *Lancet* **1**:1276-1279.

Hizuka, N., Takano, K., Shizume, K., Asakawa, K., Miyakawa, M., Tanaka, I. and Horikawa, R. (1986). Insulin-like growth factor-I stimulates growth in normal growing rats. *Eur.J.Pharmocol.* **125**:143-146.

Holtz, W., Polanco, A., von Kaufmann, F. and Herrmann, H.H. (1977). Induction of precocious ovulation in gilts. *Proceedings Society for the Study of Fertility* 10. (abstr.).

Holtz, W. and Schlieper, B. (1991). Unsatisfactory results with the transfer of embryos from gilts superovulated with PMSG and hCG. *Theriogenology* **35(6)**:1237-1249.

Horne, C. and Dziuk, P.J. (1979). Relationship between level of estrone sulfate and number of fetuses in gilts from day 10 to 32. *Proc. 71st Ann. Meeting, Am. Soc. Anim. Sci.* pp. 304-305 (abstr.).

Hughes, S.H., Petropoulos, C.J., Federspiel, M.J., Sutrave, P., Forry-Schaydies, S.F. and Bradac, J.A. (1990). Vectors and genes for improvement of animal strains. *J.Reprod.Fert.,Suppl.* **41**:39-49.

Hunter, R.H.F. (1964). Superovulation and fertility in the pig. *Anim.Prod.* **6**:189-194.

Hunter, R.H.F. (1966). The effect of superovulation on fertilisation and embryonic survival in the pig. *Anim.Prod.* **8**:457-465.

Illmensee, K., Hoppe, P.C. and Croce, C. (1978). Chimeric mice derived from human-mouse hybrid cells. *Proc.Natl.Acad.Sci.USA* **75**:1914-1918.

Isaksson, O., Jansson, J-O. and Gause, I.A.M. (1982). Growth hormone stimulates longitudinal bone growth directly. *Science* **216**:1237-1239.

Isgaard, J., Nilsson, A., Isaksson, O., Moller, C. and Norstedt, G. (1987). Regulation of IGF-I mRNA in rat growth plate by growth hormone. *Proc.Endocr.Soc.* 97. (abstr.).

Jaenisch, R. and Mintz, B. (1974). Simian virus 40 DNA sequences in DNA of healthy adult mice derived from preimplantation blastocysts injected with viral DNA. *Proc.Natl.Acad.Sci.USA* **71**:1250-1254.

Jaenisch, R. (1976). Germ line integration and mendelian transmission of the exogenous Moloney leukemia virus. *Proc.Natl.Acad.Sci.USA* **73**:1260-1264.

Jaenisch, R., Breindl, M., Harbers, K., Jahner, D. and Lohler, J.R. (1985a). Retroviruses and insertional mutagenesis. *Cold Spring Harbor Symp.Quant.Biol.* **50**:439-45.

Jaenisch, R., Schnieke, A. and Harbers, K. (1985b). Treatemnt of mice with 5-azacytidine efficiently activates silent retroviral genomes in different tissues. *Proc.Natl.Acad.Sci.USA* **82**:1451-1455.

Jahner, D., Haase, K., Mulligan, R. and Jaenisch, R. (1985). Insertion of the bacterial gpt gene into the germ line of mice by retroviral infection. *Proc.Natl.Acad.Sci.USA* **82**:6927-6931.

James, J.E., Reeser, P.D., Davis, D.L., Straiton, E.C., Talbot, A.C. and Polge, C. (1980). Culture and long-distance shipment of swine embryos. *Theriogenology* **14**:463-469.

James, J.E., James, D.M., Martin, P.A., Reed, D.E. and Davis, D.L. (1983). Embryo transfer for conserving valuable genetic material from swine herds infected with pseudorabies. *J.Am.Vet.Med.Ass.* **183**:525-528.

Johnson, I.D., Hart, I.C. and Butler-Hogg, B. (1985). The effect of exogenous bovine growth hormone and bromocriptine on growth, body development, fleece weight and plasma concentrations of growth hormone, insulin and prolactin in female lambs. *Anim.Prod.* **41**:207-217.

Johnson, M.H., Eager, D. and Muggleton-Harris, A. (1975). Mosaicism in organization of concanavalin-A receptors on surface membranes of mouse eggs. *Nature* **257**:321-322.

Johnson, M.H. (1977). *Development in Mammals*, Oxford North Holland, Vol. 1 and 2,

Johnston, N.E. (1988). The benefits of altrenogest in synchronizing oestrus in gilts. *Proc. 10th Congr. Int. Pig Vet. Soc., Rio de Janeiro Brazil* 281. (abstr.).

Jonmundsson, J.V. and Adelsteinsson, S. (1991). Single genes for fecundity in icelandic sheep. In *Genetics of Reproduction in Sheep.* (Eds. Land, R.B. and Robinson, D.W.). Butterworths, London, pp.159-168.

Jorgensen, J.O.L., Moller, N., Lauritzen, T. and Christiansen, J.S. (1990). Pulsatile versus continuous intravenous administration of growth hormone (GH) in GH-deficient patients: Effects on circulating insulin-like growth factor-1 and metabolic indices. *J.Clin.Endocrinol.Metab.* **70**:1616-1623.

Kane, M.T. (1978). Culture of mammalian ova. In *Control of Reproduction in the Cow.* (Ed. Sreenan, M.). Martinus Nijhoff, The Hague, pp.383-387.

Kane, M.T. (1985). A low molecular weight extract of bovine serum albumin stimulates rabbit blastocyst cell division and expansion *in vitro*. *J.Reprod.Fert.* **73**:147-150.

Kane, M.T. (1987). Culture media and culture of early embryos. *Theriogenology* **27**:49-57.

Kaplan, S.L., Underwood, L.E., August, G.P., Bell, J.J., Blethen, S.L., Blizzard, R.M., Brown, D.R., Foley, T.P., Hintz, R.L., Hopwood, N.J., Johansen, A., Kirkland, R.T., Plotnick, L.P., Rosenfeld, R.G. and Van der Wyk, J.J. (1986). Clinical studies with recombinant-DNA-derived methionyl human growth hormone in growth hormone deficient children. *Lancet* **1**:697-700.

Karalus, U., Downey, B.R. and Ainsworth, L. (1990). Maintenance of ovulatory cycles and pregnancy in prepubertal gilts treated with PMSG and HCG. *Anim.Reprod.Sci.* **22**:235-241.

Karin, M., Haslinger, A., Holtgreve, H., Richards, R.I., Krauter, P., Wesphal, H.M. and Beato, M. (1984). Characterization of DNA sequences through which cadium and glucocorticoid hormones induce human metallothionein-IIA gene. *Nature* **308**:513-519.

Kashiwazaki, N., Ohtani, S., Nagashima, H., Yamakawa, H., Cheng, W.T.K., Lin, A-C., Ma, R.C-S. and Ogawa, S. (1991). Production of normal piglets from hatched blastocysts frozen at -196°C. *Theriogenology* **31(1)**:221. (abstr.).

Kaufmann, U., Zapf, J. and Froesch, E.R. (1978). Growth hormone dependence of non-suppressible insulin-like activity (NSILA) and of NSILA-carrier protein in rats. *Acta Endocrinol.* **87**:716-727.

Kineman, R.D., Kraeling, R.R., Rampacek, G.B. and Hausman, G.J. (1987a). Comparison of induced corpora lutea from prepuberal gilts and spontaneous corpora lutea from mature gilts: Hydroxysteroid dehydrogenase activity. *J.Anim.Sci.* **64**:237-240.

Kineman, R.D., Rampacek, G.B., Kraeling, R.R., Fiorello-Stocks, N.A. and Wilson, R.L. (1987b). Comparison of induced corpora lutea from prepuberal gilts and spontaneous corpora lutea from mature gilts: *In-vitro* progesterone production. *J.Anim.Sci.* **64**:526-532.

Klapper, D.G., Svoboda, M.E. and Van der Wyk, J.J. (1983). Sequence of somatomedin-C: confirmation of identity with insulin-like growth factor-1. *Endocrinology* **112**:2215-2217.

Knott, R.E., England, D.C. and Kennick, W.H. (1984). Estrus, ovulation, conception and embryonic survival in confinement-managed gilts of three weight groups. *J.Anim.Sci.* **581**:281-284.

Kopchick, J.L., McAndrew, S.J., Shafer, A., Blue, W.T., Yun, J.S., Wagner, T.E. and Chen, W.Y. (1991). *In-vitro* mutagenesis of the bovine growth hormone gene. *J.Reprod.Fert.,Suppl.* **41**:25-35.

Kostyo, J.L. and Nutting, D.F. (1974). Growth hormone and protein metabolism. In *Handbook of Physiology.* (Eds. Knobil, E. and Sawyer, W.H.). American Physiological Society, Bethesda, MD., pp.187-210.

Kostyo, J.L. and Isaksson, O. (1977). Growth hormone and regulation of somatic growth. *Int.Rev.Physiol.* **13**:255-274.

Kraeling, R.R. and Rampacek, G.B. (1977). Synchronization of estrus and ovulation in gilts with estradiol and prostaglandin $F_{2\alpha}$. *Theriogenology* **8**:103. (abstr.).

Kraeling, R.R., Dziuk, P.J., Pursel, V.G., Rampacek, G.B. and Webel, S.K. (1981). Synchronization of estrus in swine with allyl trenbolone (RU-2267). *J.Anim.Sci.* **52**:831. (abstr.).

Kraft, L.A., Haines, D.R. and DeLay, R.L. (1986). The effects of daily injections of recombinant porcine growth hormone (rpGH) on growth, feed efficiency carcass composition and selected metabolic and hormonal parameters in finishing swine. *J.Anim.Sci.* **63(Suppl 1)**:218. (abstr.).

Kräulich, H. (1986). Criteria for useful gene transfer. *Proc. 3rd World Cong.Genet.Appl.Livestock Prodn.* 22-31.

Krumlauf, R., Chapman, V.M., Hammer, R.E., Brinster, R.L. and Tilghman, S.M. (1985a). Differential expression of α-fetoprotein genes in transgenic mice. *Mol.Cell Biol.* **5**:1639-1648.

Krumlauf, R., Hammer, R.E., Brinster, R.L., Chapman, V.M. and Tilghman, S.M. (1985b). Regulated expression of α-fetoprotein genes in transgenic mice. *Cold Spring Harb.Symp.Quant.Biol.* **50**:371-378.

Kuehn, M.R., Bradley, A., Roberston, E.J. and Evans, M.J. (1987). A potential animal model for Lesch-Nyhan syndrome through introduction of HPRT mutations into mice. *Nature* **326**:295-298.

Kuzan, F.B. and Wright, R.W. (1981). Attachment of porcine blastocysts to fibroblast monolayers *in vitro*. *Theriogenology* **16**:651-658.

Kuzan, F.B. and Wright, R.W. (1982). Blastocyst expansion, hatching and attachment of porcine embryos co-cultured with bovine fibroblasts *in vitro*. *Anim.Reprod.Sci.* **5**:57-63.

Kveragas, C.L., Seerley, R.W., Martin, R.J. and Vandergrift, W.L. (1986). Influence of exogenous growth hormone and gestational diet on sow blood and milk characteristics and on baby pig blood, body composition and performance. *J.Anim.Sci.* **63**:1877-1887.

Lacey, M., Alpert, S. and Hanahan, D. (1986). Bovine papillomavirus genome elicits skin tumours in transgenic mice. *Nature* **322**:609-612.

Lacy, E., Roberts, S., Evans, E.P., Burtenshaw, M.D. and Costantini, F. (1983). A foreign β-globin gene in transgenic mice: Integration at abnormal positions and expression in inappropriate tissues. *Cell* **34**:343-358.

Land, R.B. and Wilmut, I. (1987). Gene transfer and animal breeding. *Theriogenology* **27**:169-179.

Lathe, R., Clark, A.J., Archibald, A.L., Bishop, J.O., Simons, P. and Wilmut, I. (1986). Novel products from livestock. In *Exploiting New Technologies in Animal Breeding: Genetic Developments*. (Eds. King, J.W.B. and McKay, J.C.). Oxford university press, Oxford, pp.91-102.

Lavitrano, M., Camaioni, A., Fazio, V.M., Dolic, S., Farace, M.G. and Spadofora, C. (1989). Sperm cells as vectors for introducing foreign DNA into eggs: genetic transformation of mice. *Cell* **57**:717-723.

Lesniak, M.A., Gorden, P. and Roth, J. (1977). Reactivity of nonprimate growth hormones and prolactins with human growth hormone receptors on cultured human lymphocytes. *J.Clin.Endocrinol.Metab.* **44**:838-849.

Letcher, R., Simmen, R.C.M., Bazer, F.W. and Simmen, F.A. (1989). Insulin-like growth factor-I expression during early conceptus development in the pig. *Biol.Reprod.* **41**:1143-1151.

Lewis, U.J. (1984). Variants of growth hormone and prolactin and their post-translational modification. *Ann.Rev.Physiol.* **46**:33-42.

Li, C.H. and Papkoff, H. (1956). Preparation and properties of growth hormone from human and monkey pituitary glands. *Science* **124**:1293-1294.

Lindahl, A., Isgaard, J. and Isaksson, O. (1987). Growth hormone *in vivo* potentiates the stimulatory effect of IGF-I on colony formation of epiphysial chondrocytes isolated from hypophysectomised rats. *Endocrinology* **121**:1071-1075.

Lindahl, T. (1982). DNA repair enzymes. *Ann.Rev.Biochem.* **51**:61-87.

Linzer, D.I. and Talamantes, F. (1985). Nucleotide sequence of mouse prolactin and growth hormone mRNAs and expression of these mRNAs during pregnancy. *J.Biol.Chem.* **260**:9574-9579.

Lo, C. (1983). Transformation by iontophoretic microinjection of DNA: multiple integrations without tandem insertions. *Mol.Cell Biol.* **3**:1803-1814.

Lo, C. (1986). Localization of low abundance DNA sequences in tissue sections by *in situ* hybridization. *J.Cell Sci.* **3**:1803-1814.

Lohler, J.R., Timpl, R. and Jaenisch, R. (1984). Embryonic lethal mutation in mouse collagen I gene causes rupture of blood vessels and is associated with erythropoietic and mesenchymal cell death. *Cell* **38**:597-605.

Lohse, J.K., Robl, J.M. and First, N.L. (1985). Progress towards transgenic cattle. *Theriogenology* **23**:205. (abstr.).

Longenecker, D.E., Lasley, J.F. and Day, B.N. (1965). Fecundity in gilts and sows admininstered PMS. *J.Anim.Sci.* **24**:924-926.

Longenecker, D.E. and Day, B.N. (1968). Fertility level of sows superovulated at post-weaning estrus. *J.Anim.Sci.* **27**:709-711. (abstr.).

Lutz, J.B., Rampacek, G.B., Kraeling, R.R. and Pinkert, C.A. (1984). Serum luteinizing hormone and estrogen profiles before puberty in the gilt. *J.Anim.Sci.* **58**:686-691.

Lydon, M.J., Keeler, K.D. and Thomas, D.B. (1980). Vital DNA staining and cell sorting by flow microfluorometry. *J.Cell Physiol.* **102**:175-181.

Machlin, L.J. (1972). Effect of porcine growth hormone on growth and carcass composition of the pig. *J.Anim.Sci.* **35**:794-800.

MacPherson, R.M., Hovell, F.D.D. and Jones, A.S. (1977). Performance of sows first mated at puberty or second or third estrus, and carcass assessment of once-bred gilts. *Anim.Prod.* **24**:333-337.

Magness, R.R. and Ford, S.P. (1983). Estrone, estradiol-17b and progesterone concentrations in uterine lymph and systemic blood throughout the porcine estrous cycle. *J.Anim.Sci.* **57**:449-453.

Maitland, H. and McDougall, J. (1977). Biochemical transformation of mouse cells by fragments of herpes simplex virus DNA. *Cell* **11**:233-241.

Mark, W.H., Signorelli, K. and Lacy, E. (1985). An insertional mutation in a transgenic mouse line results in developmental arrest at day 5 of gestation. *Cold Spring Harbor Symp.Quant.Biol.* **50**:453-463.

Market, C.L. (1984). Genetic manipulation of mammalian embryos: Current techniques and their potential usefulness in livestock improvement. *Proc.10th Int.Congr.Anim.Reprod.A.I.Uni.of Illinois,*

Martin, G.R. (1981). Isolation of a pluripotent cell line from early mouse mebryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc.Natl.Acad.Sci.USA* **78**:7634-7638.

Martinat-Botte, F., Bariteau, F., Badouard, B. and Terqui, M. (1985). Control of pig reproduction in a breeding programme. *J.Reprod.Fert.,Suppl.* **33**:211-228.

Martinat-Botte, F., Bariteau, F., Forgerit, Y., Macar, C., Moreau, A., Terqui, M. and Signoret, J.P. (1990). Control of Oestrus in Gilts II.Synchronization of Oestrus with a Progestagen, Altrenogest (Regumate): Effect on Fertility and Litter Size. *Anim.Reprod.Sci.* **22**:227-233.

Massey, J.M. (1990). Animal production industry in the year 2000 A.D. *J.Reprod.Fert.,Suppl.* **41**:199-208.

Mastroianni, L.,Jr., Ursua, M.A., Avalos, M. and Stambaugh, R. (1969). Some observations on fallopian tube fluid in the monkey. *Am.J.Obstet.Gynecol.* **103**:703-709.

Mauleon, P., Martinat-Botte, F. and Scheid, J.P. (1981). Oestrous control in nulliparous gilts with a progestagen treatment (RU-2267). *J.Anim.Sci.* **49**:317. (abstr.).

Maxam, A.M. and Gilbert, W. (1977). A new method for sequencing DNA. *Proc.Natl.Acad.Sci.USA* **72**:560-564.

McConnell, D.J. (1986). Methods and achievements of genetic engineering: prospects in agriculture. A review. *Livestock Prod.Sci.* **15**:109-131.

McCrane, M.M., deVente, J., Yun, J., Bloom, J., Park, E., Wynshaw-Boris, A., Wagner, T., Rottman, F.M. and Hanson, R.W. (1988). Tissue-specific expression and dietary regulation of a chimeric phosphoenolpyruvate carboxykinase/bovine growth hormone gene. *J.Biol.Chem.* **263**:11443-11451.

McCrane, M.M., Yun, J.S., Roesler, W.J., Park, E.A., Wagner, T.E. and Hanson, R.W. (1990). Developmental regulation and tissue-specific expression of a chimaeric phosphoenolpyruvate carboxykinase/bovine growth hormone gene in transgenic animals. *J.Reprod.Fert.,Suppl.* **41**:17-23.

McKnight, G.S., Hammer, R.E., Kuenzel, E.A. and Brinster, R.L. (1983). Expression of the chicken transferrin gene in transgenic mice. *Cell* **34**:335-341.

McLaren, A. and Biggers, J.D. (1958). Successful development and broth of mice cultivated *in vitro* as early embryos. *Nature* **182**:877-878.

McLaren, D.G., Grebner, G.L., Bechtel, P.J., McKeith, F.K., Novakofski, J.E. and Easter, R.A. (1987). Effects of graded levels of natural porcine somatotrophin (PST) on growth performance of 57 to 103 kg pigs. *J.Anim.Sci.* **65(Suppl 1)**:245. (abstr.).

McLaren, A. and Michie, D. (1956). Studies on the transfer of fertilized mouse eggs to uterine foster-mothers I. Factors affecting the implantation and survival of native and transferred eggs. *J.Exp.Biol.* **33**:394-416.

Menino, A.R. and Wright, R.W.Jr (1982). Development of one-cell porcine embryos in two culture systems. *J.Anim.Sci.* **54**:583-587.

Michalska, A.E. (1991). *Production and Characterization of Transgenic Mice and Pigs Carrying the Porcine Growth Hormone Gene*, PhD Thesis University of Adelaide,

Miller, K.F., Bolt, D.J., Pursel, V.G., Hammer, R.E., Pinkert, C.A., Palmiter, R.D. and Brinster, R.L. (1989). Expression of human or bovine growth hormone gene with a mouse metallothionein-I promoter in transgenic swine alters the secretion of porcine growth hormone and insulin-like growth factor-1. *Journal of Endocrinology* **120**:481-488.

Minhas, B.S., Capehart, J.S., Bowen, M.J., Womack, J.E., McCrady, J.D., Harms, P.G., Wagner, T.E. and Kraemer, D.C. (1984). Visualization of pronuclei in living bovine zygotes. *Biol.Reprod.* **30**:687-691.

Mintz, B. (1962). Formation of a genotypically mosaic embryo. *Am.J.Zool.* **2**:432. (abstr.).

Monray, A. and Rosati, F. (1979). Cell surface differentiation during early embryonic development. *Curr.Top.Devel.Biol.* **13**:45-69.

Moor, R.M., Mattioli, M., Ding, J. and Nagai, T. (1990). Maturation of pig ooyctes *in vivo* and *in vitro*. *J.Reprod.Fert.,Suppl.* **40**:197-210.

Moore, N.W. (1985). The use of embryo transfer and steriod hormone replacement therapy in the study of prenatal mortality. *Theriogenology* **23**:121-129.

Muir, L.A., Wien, S., Duquette, P.F., Rickes, E.L. and Cordes, E.H. (1983). Effects of exogenous growth hormone and diethylstilbestrol on growth and carcass compostion of growing lambs. *J.Anim.Sci.* **56**:1315-1323.

Mullis, S.I. and Faloona, F.A. (1987). Specific synthesis of DNA *in vitro* via a polymerase catalysed chain reaction technique. *Meth.Enzymol.* **155**:335-350.

Murai, J.T., Conti, C.J., Gimence-Conti, J.B., Orlicky, D. and Gerschenson, L.E. (1981). Temporal relationship between rabbit uterine epithelium proliferation and uteroglobin production. *Biol.Reprod.* **24**:649-654.

Murray, J.D., Nancarrow, C.D. and Ward, K.A. (1988). Techniques for the transfer of foreign genes into animals. *Proc.11th Int.Congr.Anim.Reprod.& A.I.Dublin* **5**:20-27.

Nakabeppu, Y. and Sekiguchi, M. (1981). Physical association of pyrimidine dimer DNA glycosylase and apurinic-apyrimidine DNA endonuclease essential for repair of ultraviolet-damaged DNA. *Proc.Natl.Acad.Sci.USA* **78**:2472-2746.

Nancarrow, C.D., Marshall, J.T., Murray, J.D., Hazelton, I.G. and Ward, K.A. (1987). Production of transgenic sheep with the ovine growth hormone gene. *Theriogenology* **27**:263. (abstr.).

Nancarrow, C.D., Ward, K.A. and Murray, J.D. (1988). The future for transgenic livestock in australian agriculture. *Aust.J.Biotech.* **2**:39-44.

Newcomb, M.D., Grebner, G.L., Bechtel, P.J., McKeith, F.K., Novakofski, J., McLaren, D.G. and Easter, R.A. (1988). Response of 60 to 100 kg pigs treated with porcine somatotropin to different levels of dietary crude protein. *J.Anim.Sci.* **66 (1)**:281. (abstr.).

Niall, H.D. (1971). Revised primary structure for human growth hormone. *Nature* **230**:90-91.

Niall, H.D., Hogan, M.L., Tregear, G.W., Segre, G.V., Hwang, P. and Friesen, H. (1973). The chemistry of growth hormone and the lactogenic hormones. *Rec.Progress Horm.Res.* **29**:387-416.

Nicolau, C., Le Pape, A., Soriano, P., Fargette, F. and Juhel, M.-F. (1983). *In vivo* expression of rat insulin after intravenous administration of the liposome-entrapped gene for rat insulin 1. *Proc.Natl.Acad.Sci.USA* **80**:1068-1072.

Niemann, H., Wust, A. and Gardon, J.C. (1989). Succesful intercontinental transport of porcine embryos from Europe to South America. *Theriogenology* **31(3)**:525-530.

Notarianni, E., Laurie, S., Moor, R.M. and Evans, M.J. (1990). Maintenance and differentiation in culture of pluripotential embryonic cell lines from pig blastocysts. *J.Reprod.Fert.,Suppl.* **41**:51-56.

Nurse, P. (1990). Universal control mechanism regulating onset of M-phase. *Nature* **344**:503-508.

O'Brien, J.J. (1986). *Diseases of Swine*, Ed. 6th Iowa State Press, Ames,pp. 725-737.

O'Farrell, P.H., Edgar, B.A., Lakich, D. and Lehner, C.F. (1989). Directing cell division during development. *Science* **246**:635-640.

Olson, K.C., Fenno, J., Lin, N., Harkins, R.N., Snider, C., Kohr, W.H., Ross, M.J., Fodge, D., Prender, G. and Stebbing, N. (1981). Purified human growth hormone is biologically active. *Nature* **293**:408-411.

Oner, G., Bhaumick, B. and Bala, R.M. (1984). Effect of zinc deficiency on serum somatomedin levels and skeletal growth in young rats. *Endocrinology* **114**:1860-1869.

Owens, P.C., Johnson, P.C., Campbell, R.G. and Ballard, F.J. (1989). Growth hormone increases insulin-like growth factor-1 (IGF-I) and decreases IGF-II in plasma of growing pigs. *Journal of Endocrinology* **124**:269-275.

Owens, P.C., Conlon, M.A., Campbell, R.G., Johnson, R.J., King, R. and Ballard, F.J. (1991a). Developmental changes in growth hormone, insulin-like growth factors (IGF-I and IGF-II) and IGF-binding proteins in plasma of young growing pigs. *Journal of Endocrinology* **128**:439-447.

Owens, P.C., Moyse, K.J., Walton, P.E., Harrison, D., Luxford, B. and Campbell, R.G. (1991b). Plasma insulin-like growth factor-I (IGF-I) correlates with growth rate of boars. *Aust.Pig Sci.Assoc.* **in press**: (abstr.).

Page, G.S., Smith, S. and Goodman, H.M. (1981). DNA sequence of the rat growth hormone gene: location of the 5' terminus of the growth hormone mRNA and identification of an internal transposon-like element. *Nucleic Acid Res.* **9**:2087-2104.

Palmiter, R.D., Brinster, R.L., Hammer, R.E., Trumbauer, M.E., Rosenfeld, M.G., Birnberg, N.C. and Evans, R.M. (1982). Dramatic growth of mice that develop from eggs microinjected with metallothionein-growth hormone fusion genes. *Nature* **300**:611-615.

Palmiter, R.D., Norstedt, G., Gelinas, R.E., Hammer, R.E. and Brinster, R.L. (1983). Metallothionein-human Gh fusion genes stimulates growth of mice. *Science* **222**:809-814.

Palmiter, R.D. and Brinster, R.L. (1985). Transgenic mice: Minireview. *Cell* **41**:343-345.

Palmiter, R.D., Chen, H.Y., Messing, A. and Brinster, R.L. (1985a). SV-40 enhancer and large-T antigen are instrumental in development of choroid plexus tumours in transgenic mice. *Nature* **316**:457-460.

Palmiter, R.D., Wilkie, T.M., Chen, H.Y. and Brinster, R.L. (1985b). Expression of growth hormone genes in transgenic mice. In *Banbury Report 20: Genetic Manipulation of the Early Embryo.* (Eds. Costantini, F. and Jaenisch, R.). Cold Spring Harbour Laboratory, New York, pp.123-132.

Palmiter, R.D. and Brinster, R.L. (1986). Germ line transformation of mice. *Ann.Rev.Genet.* **20**:465-499.

Papaioannou, V.E., McBurney, M.E., Gardner, R.L. and Evans, M.J. (1975). Fate of teratocarcinoma cells injected into early mouse embryos. *Nature* **258**:70-73.

Papkoff, H. (1981). Variations in the properties of equine chorionic gonadotrophin. *Theriogenology* **15**:1-11.

Paterson, A.M. (1982). The controlled induction of puberty. In *Control of Pig Reproduction.* (Eds. Cole, D.J.A. and Foxcroft, G.R.). Butterworth Scientific, London, pp.139-160.

Pavlakis, G.N., Hizuka, N., Gorden, P., Seeburg, P.H. and Hammer, D.H. (1981). Expression of two human growth hormone genes in monkey cells infected by simian virus 40 recombinant. *Proc.Natl.Acad.Sci.USA* **78**:7398-7402.

Pedersen, R.A. (1986). Potency, lineage and allocation in preimplantation mouse embryos. In *Experimental Approaches to Mammalian Embryonic Development.* (Eds. Rossant, J. and Pedersen, R.A.). Cambridge University Press, Cambridge, London, pp.3-33.

Peel, C.J., Bauman, D.E., Gorewit, R.C. and Sniffen, C.J. (1981a). Effect of exogenous growth hormone on lactational performance in high yielding dairy cows. *J.Nutr.* **11**:1662-1671.

Peel, C.J., Fronk, T.J., Bauman, D.E. and Gorewit, R.C. (1981b). Lactational response to exogenous growth hormone and abomasal infusion of a glucose-sodium caseinate mixture in high yielding dairy cows. *J.Nutr.* **11**:1662-1671.

Peel, C.J., Steinhour, W.D., Baumann, D.E., Tyrrell, H.F., Brown, A., Reynolds, P.J. and Haaland, G.L. (1982). Administration of bovine growth hormone to high yielding Hostein cows II. Influence on irreversible loss and oxidation rate of free fatty acids and glucose. *J.Dairy Sci.* **65(Suppl 1)**:120. (abstr.).

Pellicer, A., Wagner, E.F., Kareh, A.E., Dewey, M.J., Reuser, A.J., Silverstein, S., Axel, R. and Mintz, B. (1980). Introduction of a viral thymidine kinase gene and the human β-globin gene into developmentally multipotential mouse teratocarcinoma cells. *Proc.Natl.Acad.Sci.USA* **77 (4)**:2098-2102.

Perry, J.S. and Rowlands, I.W. (1962). Early pregnancy in the pig. *J.Reprod.Fert.* **4**:175-182.

Perry, J.S., Heap, R.B., Burton, R.D. and Gadsby, J.E. (1976). Endocrinology of the blastocyst and its role in the establishment of pregnancy. *J.Reprod.Fert.,Suppl.* **25**:85-94.

Petters, R.M. (1986). Recombinat DNA, gene transfer and the future of animal agriculture. *J.Anim.Sci.* **62**:1759-1768.

Petters, R.M., Johnson, B.H. and Shuman, R.M. (1989). Gene transfer to swine embryos using an avian retrovirus. *J.Cell.Biochem.* **13B**:182. (abstr.).

Phillipo, M. (1968). Superovulation in the pig. *Adv.Reprod.Physiol.* **3**:147-166.

Pinkert, C.A., Pursel, V.G., Miller, K.F., Palmiter, R.D. and Brinster, R.L. (1987). Production of transgenic pigs harboring growth hormone (MTbGH) or growth hormone releasing factor (MThGRF) genes. *J.Anim.Sci.* **65(Supp. 1)**:260. (abstr.).

Pinkert, C.A., Rampacek, G.B. and Kraeling, R.R. (1988). Serum concentrations of prolactin, oestrogen and LH during the perioestrous period in prepubertal gilts induced to ovulate and mature gilts. *J.Reprod.Fert.* **83**:471-478.

Pinkert, C.A., Kooyman, D.L. and Dyer, T.J. (1989a). Enhanced growth performance in transgenic swine. In *Transgenic Technology in Medicine and Agriculture.* (Eds. First, N. and Hazeltine, F.). Butterworth Scientific, London,

Pinkert, C.A., Kooyman, D.L., Baumgartner, A. and Keisler, D.H. (1989b). *In-vitro* development of zygotes from superovulated prepubertal and mature gilts. *J.Reprod.Fert.* **87**:63-66.

Piper, L.R. and Bindon, B.M. (1985). The single gene inheritance of the high litter size of the booroola merino. In *Genetics of Reproduction in Sheep.* (Eds. Land, R.B. and Robinson, D.W.). Butterworths, London, pp.115-125.

Polge, C., Rowson, L.E.A. and Chang, M.C. (1966). The effect of reducing the number of embryos during early stages of gestation on the maintenance of pregnancy in the pig. *J.Reprod.Fert.* **12**:395-397.

Polge, C., Moor, R.M., Day, B.N., Booth, W.D. and Rowson, L.E.A. (1967). Embryo numbers and luteal maintenance during early pregnancy in swine. *J.Anim.Sci.* **26**:1499. (abstr.).

Polge, C. and Day, B.N. (1968). Pregnancy following non-surgical egg transfer in pigs. *Vet.Rec.* **82**:712. (abstr.).

Polge, C., Day, B.N. and Groves, T.W. (1968). Synchronization of ovulation and artificial insemination in pigs. *Vet.Rec.* **83**:136-142.

Polge, C. (1977). The freezing of mammalian embryos: perspectives and possibilities. In *The Freezing of Mammalian Embryos.* (Eds. Elliott, K. and Whelan, J.). Elsevier/Exerpta Medica, Amsterdam, North Holland, pp.3-13.

Polge, C. (1981). An assessment of techniques for the control of oestrus and ovulation in pigs. In *Steroids in Animal Reproduction.* (Ed. Jasiorowski, H.). Warsaw Agricultural University: SGGW-AR, Roussel-Uclaf, Warsaw, pp.73-84.

Polge, C. (1982). Embryo transplantation and preservation. In *Control of Pig Reproduction.* (Eds. Cole, D.J.A. and Foxcroft, G.R.). Butterworths, London, pp.277-291.

Polge, E.J.C., Barton, S.C., Surani, M.H.A., Miller, J.R., Wagner, T., Elsome, K., Davis, A.J., Goode, J.A., Foxcroft, G.R. and Heap, R.B. (1989). Induced expression of a bovine growth hormone construct in transgenic pigs. In *Biotechnology of Growth Regulation.* (Eds. Heap, R.B.,Prosser, C.G. and Lamming, G.E.). Butterworths, London, pp.189-199.

Pope, C.E., Vincent, C.K. and Thrasher, D.M. (1968). Effects of I.C.I. 33828 and PMS on reproduction in gilts. *J.Anim.Sci.* **27**:303. (abstr.).

Pope, C.E., Christensen, R.K., Zimmerman-Pope, V.A. and Day, B.N. (1972). Effect of number of embryos on embryonic survival in recipient gilts. *J.Anim.Sci.* **35**:805-808.

Pope, C.E. and Day, B.N. (1977). Transfer of preimplantation pig embryos following *in vitro* culture for 24 or 48 hours. *J.Anim.Sci.* **44**:1036-1040.

Pope, W.F., Maurer, R.R. and Stormshak, F. (1981). Intrauterine migration of the porcine embryo: influence of estradiol-17b and histamine. *Biol.Reprod.* **27**:57-64.

Pope, W.F., Maurer, R.R. and Stormshak, F. (1982). Survival of porcine embryos after asynchronous transfer. *Proc.Soc.Exp.Biol.Med.* **171**:179-183.

Postel, E.H. (1985). Enhancement of genetic transformation frequencies of mammalian cell cultures by damage to the cell DNA. *Mol.Gen.Genet.* **201**:136-139.

Powell, B.C. and Rogers, G.E. (1990). Cyclic hair-loss and regrowth in transgenic mice overexpressing an intermediate filament gene. *The EMBO Journal* **9**:1485-1493.

Pressing, A.L., Dial, G.D., Stroud, C.M., Almond, G.W. and Robison, O.W. (1987). Prostaglandin-induced abortion in swine: Endocrine changes and influence on subsequent reproductive activity. *Am.J.Vet.Res.* **48**:45-51.

Puglisi, T.A., Rampacek, G.B. and Kraeling, R.R. (1978). Corpus luteum function following subtotal hysterectomy in the prepuberal gilt. *J.Anim.Sci.* **46**:707-709.

Puglisi, T.A., Rampacek, G.B., Kraeling, R.R. and Kiser, T.E. (1979). Corpus luteum susceptibility to prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) luteolysis in hysterectomized prepuberal and mature gilts. *Prostaglandins* **18**:257-259.

Pursel, V.G., Elliot, D.O., Newman, C.W. and Staigmiller, R.B. (1981). Synchronization of estrus in gilts with allyl trenbolone: Fecundity after natural service and insemination with frozen semen. *J.Anim.Sci.* **52**:130. (abstr.).

Pursel, V.G., Wall, R.J., Rexroad, C.E.,Jr., Hammer, R.E. and Brinster, R.L. (1985a). A rapid whole mount staining procedure for nuclei of mammalian embryos. *Theriogenology* **24**:687-691.

Pursel, V.G., Wall, R.J., Hammer, R.E. and Brinster, R.L. (1985b). Cleavage of pig embryos after labeling with fluorescent dyes. *Theriogenology* **24**:693-700.

Pursel, V.G., Rexroad, C.E.,Jr., Bolt, D.J., Miller, K.F., Wall, R.J., Hammer, R.E., Pinkert, C.A., Palmiter, R.D. and Brinster, R.L. (1987). Progress on gene transfer in farm animals. *Vet.Immunol.Immunopath.* **17**:303-312.

Pursel, V.G., Campbell, R.G., Miller, K.F., Behringer, R.R., Palmiter, R.D. and Brinster, R.L. (1988a). Growth potential of transgenic pigs expressing a bovine growth hormone gene. *J.Anim.Sci.* **66(Suppl. 1)**:267. (abstr.).

Pursel, V.G., Miller, K.F., Pinkert, C.A., Palmiter, R.D. and Brinster, R.L. (1988b). Effect of ovum cleavage stage at microinjection on embryonic survival and gene integration in pigs. *Proc.11th Int.Congr.Anim.Reprod.& A.I.Dublin* **4**:480-483. (abstr.).

Pursel, V.G., Miller, K.F., Bolt, D.J., Pinkert, C.A., Hammer, R.E., Palmiter, R.D. and Brinster, R.L. (1989a). Insertion of growth hormone genes into pig embryos. In *Biotechnology of Growth Regulation.* (Eds. Heap, R.B.,Prosser, C.G. and Laming, G.E.). Butterworths, London, pp.181-188.

Pursel, V.G., Pinkert, C.A., Miller, K.F., Bolt, D.J., Campbell, R.G., Palmiter, R.D., Brinster, R.L. and Hammer, R.E. (1989b). Genetic engineering of livestock. *Science* **244**:1281-1287.

Pursel, V.G., Hammer, R.E., Bolt, D.J., Palmiter, R.D. and Brinster, R.L. (1990a). Integration, expression and germ-line transmission of growth-related genes in pigs. *J.Reprod.Fert.,Suppl.* **41**:77-87.

Pursel, V.G., Bolt, D.J., Miller, K.F., Pinkert, C.A., Hammer, R.E., Palmiter, R.D. and Brinster, R.L. (1990b). Expression and performance in transgenic pigs. *J.Reprod.Fert.,Suppl.* **40**:235-245.

Raben, M.S. (1956). Preparation of growth hormone from pituitaries of man and monkey. *Science* **125**:883-884.

Raben, M.S. (1958). Treatment of a pituitary dwarf with human growth hormone. *J.Clin.Endocrinol.* **18**:901-903.

Radany, E.H. and Freidberg, E.C. (1980). A pyrimidine dimer-DNA glycosylase activity associated with the v gene product of bacteriophage T4. *Nature* **286**:182-185.

Rampacek, G.B., Robison, O.W. and Ulberg, L.C. (1975a). Uterine capacity and progestin levels in superinduced gilts. *J.Anim.Sci.* **41**:564-569.

Rampacek, G.B., Kraeling, R.R. and Ball, G.D. (1976b). Luteal function in the hysterectomized prepuberal gilt. *J.Anim.Sci.* **43**:792-794.

Rampacek, G.B., Schwartz, F.L., Fellows, R.E., Robison, O.W. and Ulberg, L.C. (1976). Initiation of reproductive function and subsequent activity of corpora lutea in prepubertal gilts. *J.Anim.Sci.* **46**:881-887.

Rampacek, G.B., Kraeling, R.R, Kiser, T.E., Barb, C.R. and Benyshek, L.L. (1979). Prostaglandin F concentrations in utero-ovarian vein plasma of prepuberal and mature gilts. *Prostaglandins* **18(2)**:247-249.

Rampacek, G.B., Kraeling, R.R and Pinkert, C.A. (1985). Regression of induced corpora lutea by human chorionic gonadotropin in prepuberal gilts. *J.Anim.Sci.* **60**:1040-1044.

Rattan, S.I.S (1989). DNA damage and repair during cellular aging. *International Review of Cytology* **116**:47-88.

Reiland, S., Ordell, N., Lundeheim, N. and Olsson, S. (1978). Heridity of osteochondrosis, body constitution and leg weakness in the pig. *Acta Radiol.,Suppl.* **358**:113-137.

Renard, J.-P. and Babinet, C. (1987). Genetic engineering in farm animals: the lessons from the genetic mouse model. *Theriogenology* **27**:181-200.

Rexroad, C.E.,Jr. and Wall, R.J. (1987). Development of one-cell fertilized sheep ova following microinjection into pronuclei. *Theriogenology* **27**:611-619.

Rexroad, C.E. and Pursel, V.G. (1988). Status of gene transfer in domestic animals. *Proc. 11th Int. Congr. Anim. Reprod. A.I. Dublin* **5**:28-35.

Rexroad, C.E.,Jr. and Powell, A.M. (1988a). Co-culture of ovine eggs with oviductal cells and trophoblastic vesicles. *Theriogenology* **29**:387-397.

Rexroad, C.E.,Jr. and Powell, A.M. (1988b). Co-culture of ovine ova with oviductal cells in medium 199. *J.Anim.Sci.* **66**:947-953.

Rexroad, C.E.,Jr., Hammer, R.E., Bolt, D.J., Mayo, K.M., Frohman, L.A., Palmiter, R.D. and Brinster, R.L. (1989). Production of transgenic sheep with growth regulating genes. *Molec.Reprod.Develop.* **1**:164-169.

Rexroad, C.E.,Jr., Hammer, R.E., Behringer, R.R., Palmiter, R.D. and Brinster, R.L. (1990). Insertion, expression and physiology of growth-regulating genes in ruminants. *J.Reprod.Fert.,Suppl.* **41**:119-124.

Richards, W.L., Song, M.K., Krutesch, H., Evarts, R.P., Marsdin, E. and Thergeirsson, S.S. (1985). Measurement of cell proliferation in microculture using Hoechst 33342 for rapid semi automated microfluorimetric determination of chromatin DNA. *Exp.Cell Res.* **159**:235-246.

Rivier, J., Spiess, J., Thorner, M. and Vale, W. (1982). Characterization of a growth hormone-releasing factor from a human pancreatic islet tumor. *Nature* **300**:276-278.

Roberts, R.J. (1983). Restriction and Modification Enzymes and Their Recognition Sequences. *Nucleic Acid Res.* **11**:135-167.

Roberts, R.M., Bazer, F.W. and Thatcher, W.W. (1984). Biochemical interactions between blastocyst and endometrium in the large domestic animals. *J.Biosci.* **6 (Suppl. 2)**:63-74.

Roberts, R.M. and Bazer, F.W. (1988). The function of uterine secretions. *J.Reprod.Fert.* **82**:875-892.

Robertson, H.A. and King, G.J. (1974). Plasma concentrations of progesterone, oestrone, oestradiol-17ß and of oestrone sulphate in the pig at implantation, during pregnancy and at parturition. *J.Reprod.Fert.* **40**:133-141.

Robertson, E.J., Bradley, A., Kuehn, M.R. and Evans, M.J. (1986). Germ-line transmission of genes introduced into cultured pluripotential cells by retroviral vector. *Nature* **323**:445-448.

Robertson, E.J. (1986). Pluripotent stem cell lines as a route into the mouse germ line. *Trends Genet.* **2**:9-13.

Robertson, E.J. (1987). Embryo derived stem cells. In *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach.* (Ed. Robertson, E.J.). IRL Press, Oxford, pp.71-112.

Robertson, G.L., Casida, L.E., Grummer, R.H. and Chapman, A.B. (1951). Some feeding and management factors affecting age at puberty and related phenomena in chester white and poland china gilts. *J.Anim.Sci.* **10**:841-846.

Robinson, I.C.A.F. and Clark, R.G. (1989). Growth promotion by growth hormone and insulin-like growth factor-I in the rat. In *Biotechnology of Growth Regulation.* (Eds. Heap, R.B.,Prosser, C.G. and Lamming, G.E.). Butterworths, London, pp.129-140.

Russel, S.M. and Spencer, E.M. (1985). Local injections of human or rat growth hormone or purified somatomedin C stimulate unilateral tibial epiphysial growth in hypophysectomised rats. *Endocrinology* **116**:2563-2567.

Sakkas, D., Batt, P., Cameron, A. and Trounson, A. (1988). *In vitro* development of goat preimplantation embryos in co-culture with oviductal epithelial cells. *Proc.Aust.Soc.Reprod.Biol.* **20**:48. (abstr.).

Salmon, W.D. and Daughaday, W.H. (1957). A hormonally controlled serum factor which stimulates sulphate incorporation by cartilage *in vivo*. *J.Lab.Clin.Med.* **49**:825-836.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1991). *Molecular Cloning: a laboratory manual*, Ed. 2nd Cold Spring Harbour Laboratory Press, New York.

Sara, V.R. and Hall, K. (1990). Insulin-like growth factors and their binding proteins. *Amer.Physiol.Soc.* **70 (3)**:591-614.

Sarasin, A. and Benoit, A. (1980). Induction of an error-prone mode of DNA repair in UV-irradiated monkey kidney cells. *Mutat.Res.* **70**:71-81.

SAS Institute Inc., (1988). *SAS/STAT Users Guide*, Ed. 6th SAS Institute Inc., Cary, NC, USA.

Saumande, J., Chupin, D., Mariana, J.C., Ortavant, R. and Mauleon, P. (1978). Factors affecting the variablity of ovulation rates after PMSG stimulation. In *Current Topics in Veterinary Medicine.* (Ed. Sreenan, J.M.). Nijhoff: The Hague, pp.122-143.

Scangos, G.A. and Ruddle, F.H. (1981). Mechanisms and application of DNA-mediated gene transfer in mammalian cells - a review. *Cell* **14**:1-10.

Schaeffer-Ridder, M., Wang, Y. and Hofschneider, P.H. (1982). Liposomes as gene carriers: efficient transformation of mouse L cells by thymidine kinase gene. *Science* **215**:166-167.

Schalch, D.S. and Reichlin, S. (1966). Plasma growth hormone concentration in rat determined by radioimmunoassay: influence of sex, pregnancy, lactation, anaesthesia, hypophysectomy and extracellular pituitary transplant. *Endocrinology* **79**:275-280.

Schilling, E. and Cerne, F. (1972). Induction and synchronization of oestrus in prepubertal gilts and anoestrous sows by PMS/HCG-compound. *Vet.Rec.* **91**:471-474.

Schlieper, B. and Holtz, W. (1986). Transfer of pig embryos collected by laparotomy or slaughter. *Anim.Reprod.Sci.* **12**:109-114.

Schnieke, A., Harbers, K. and Jaenisch, R. (1983). Embryonic lethal mutation in mice induced by retrovirus insertion into the α(I) collagen gene. *Nature* **304**:315-320.

Schoenle, E., Zapf, J., Humbel, R.E. and Froesch, E.R. (1982). Insulin-like growth factor-I stimulates growth in hypophysectomized rats. *Nature* **296**:252-253.

Seamark, R.F. (1989). Trangenesis: the challenge for the reproductive biologist. *Reprod.Fertil.Dev.* **1**:391-395.

Seamark, R.F. (1991). Transgenic Livestock - A Personal Experience. *Proc.Nat.Conf.Ani.Biotech.Agric.* 8F(i)-8F(ii).

Seamark, R.F. and Wells, J.R.E. (1991). Biotechnology and Reproduction. (Ed. King, G.). Elsevier World Animal Science Series, Amsterdam, Ed. 14th.

Seawell, P.C., Smith, C.A. and Gabesan, A.K. (1980). denV gene of bacteriophage T4 determines a DNA glycosylase specific for pyrimidine dimers in DNA. *J.Virol.* **35**:790-707.

Seeburg, P.H., Shine, J., Martial, J.A., Baxter, J.D. and Goodman, H.M. (1977). Nucleotide sequence and amplification in bacteria of structural gene for rat growth hormone. *Nature* **270**:486-494.

Seeburg, P.H. (1982). The human growth hormone gene family: nucleotide sequences show divergence and predict a new polypeptide hormone. *DNA* **1**:239-249.

Segal, D.H. and Baker, R.D. (1973). Maintenance of corpora lutea in prepuberal gilts. *J.Anim.Sci.* **37**:762-767.

Sekine, S., Mizukami, T., Nishi, T., Kuwana, Y., Saito, A., Sato, M., Itoh, S. and Kawauchi, H. (1985). Cloning and expression of cDNA for salmon growth hormone in *Escherichia coli*. *Proc.Natl.Acad.Sci.USA* **82**:4306-4310.

Shaw, G.A., McDonald, B.E. and Baker, R.D. (1971). Fetal mortality in the prepubertal gilt. *Can.J.Anim.Sci.* **51**:233-236.

Signoret, J.P., Martinat-Botte, F., Bariteau, F., Forgerit, Y., Macar, C., Moreau, A. and Terqui, M. (1990). Control of oestrus in gilts I. Management-induced puberty. *Anim.Reprod.Sci.* **22**:221-225.

Sillence, M.N. and Etherton, T.D. (1987). Determination of the temporal relationship between porcine porcine growth hormone, serum IGF-I and cortisol concentrations in pigs. *J.Anim.Sci.* **64**:1019-1023.

Sim, M.M. and First, N.L. (1987). Laprascopic embryo transfer in the pig. *J.Anim.Sci.* **64(Suppl 1)**:386. (abstr.).

Simons, J.P., Wilmut, I., Clark, A.J., Archibald, A.L., Bishop, J.O. and Lathe, R. (1988). Gene transfer in sheep. *Biotechnology* **6**:179-183.

Sjöberg, J., Wahlstrom, T., Grudzinkas, J.G. and Sinosich, M.J. (1986). Demonstration of pregnancy-associated plasma protein-A (PAPP-A)-like material in the fallopian tube. *Fertil.Steril.* **45**:517-521.

Smith, C., Meuwissen, T.H.E. and Gibson, J.P. (1987). On the use of transgenes in livestock improvement. *Anim.Breed.Abstr.* **55**:1-10.

Smith, H.O. and Wilcox, K.W. (1970). A restriction enzyme from *hemophilus influenzae*. I. Purification and general Properties. *J.Mol.Biol.* **51**:379-391.

Smith, P.E. (1927). The disabilities caused by hypophysectomy and their repair. *J.Am.Med.Assoc.* **88**:158-161.

Smith, R.W., Gaebler, O.H. and Long, C.N.H. (1955). *The Hypophyseal Growth Hormone; Nature and Action*, McGraw-Hill, New York,

Snedecor, G.W, and Cochran, W.G. (1989). *Statistical Methods*, Ed. 8th Iowa State University Press, Ames, Iowa,pp. 374-397.

Soares, E.R. (1972) Estimating the frequency of induced dominant lethals in mice by uterine dissection after weaning progeny. *Mutant Res.* **16**:425-427.

Soriano, P., Dijkstra, J., Legrand, A., Spanjer, H., Londos-Gagliardi, D., Roerdink, F., Scherphof, G. and Nicolau, C. (1983). Targetted and nontargetted liposomes for *in vivo* transfer to rat liver cells of a plasmid containing the preproinsulin 1 gene. *Proc.Natl.Acad.Sci.USA* **80**:7128-7131.

Southern, E.M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J.Mol.Biol.* **98**:503-517.

Souza, L.M., Boone, T.C., Murdock, D., Langley, K., Wypych, J., Fenton, D., Johnson, S., Lai, P.H., Everett, R., Hsu, R-Y. and Bosselman, R. (1984). Application of recombinant DNA technologies to studies on chicken growth hormone. *J.Exp.Zool.* **232**:465-473.

Spindler, S.R., Mellon, S.H. and Baxter, J.D. (1982). Growth hormone gene transcription is regulated by thyroid and glucocorticoid hormones in cultured rat pituitary tumor cells. *J.Biol.Chem.* **257**:11627-11632.

Srikandakumar, A. and Downey, B.R. (1989). Induction of Ovulation in Gilts with Cloprostenol. *Theriogenology* **32 (3)**:445-449.

Steele, N.C. and Elsasser, T.H. (1989). Regulation of somatomedin production, release and mechanism of action. In *Animal Growth Regulation*. (Eds. Campion, D.R.,Hausman, G.J. and Martin, R.J.). Plenum Press, New York, pp.295-315.

Stevenson, J.S. and Davis, D.L. (1982). Estrous synchronization and fertility in gilts after 14 or 18-day feeding of altrenogest beginning at estrus or diestrus. *J.Anim.Sci.* **55**:119. (abstr.).

Stewart, C.L., Stuhlmann, H., Jahner, D. and Jaenisch, R. (1982). De novo methylation, expression and infectivity of retroviral genomes introduced into embryonal carcinoma cells. *Proc.Natl.Acad.Sci.USA* **79**:4098-4102.

Stewart, C.L., Vanek, M. and Wagner, E.F. (1985). Expression of foreign genes from retroviral vectors in mouse teratocarcinoma chimaeras. *The EMBO Journal* **4**:3701-3709.

Stewart, T.A., Pattengale, P.K. and Leder, P. (1984). Spontaneous mammary adenocarcinomas in transgenic mice that carry MTV/myc fusion genes. *Cell* **38**:627-637.

Stone, B.A., Quinn, P. and Seamark, R.F. (1984). Energy and protein sources for development of pig embryos cultured beyond hatching *in vivo*. *Anim.Reprod.Sci.* **7**:405-412.

Stone, B.A., Seamark, R.F., Godfrey, B.M., Quinn, P. and LLoyd, B. (1986). Oestrone sulphate levels in plasma of sows as a basis for prediction of litter size at term. *Anim.Reprod.Sci.* **11**:51-62.

Sussman, P.M., Tushinski, R.J. and Bancroft, F.C. (1976). Pregrowth hormone: product of the translation *in vitro* of messenger RNA coding for growth hormone. *Proc.Natl.Acad.Sci.USA* **73**:29-33.

Takahashi, Y., Ebihara, S., Nakamura, Y. and Takahashi, K. (1981). A model of human sleep-related growth hormone secretion in dogs: effects of 3, 6 and 12 hours of forced wakefulness on plasma growth hormone, cortisol and sleep stages. *Endocrinology* **109**:262-272.

Tarkowski, A.K. (1961). Mouse chimeras developed from fused eggs. *Nature* **190**:857-860.

Thibault, C. (1966). La culture *in vitro* de l'oeuf de vache. *Ann.Biol.Anim.Biochim.Biophys* **6**:159-164.

Thompson, J.G.E. and Wales, R.G. (1987). Observations on the loss of the cellular vestment surrounding superovulated sheep ooyctes. *Anim.Reprod.Sci.* **15**:169-175.

Tillson, S.A., Erb, R.E. and Niswender, G.D. (1970). Comparison of luteinizing hormone and progesterone in blood and metabolites of progesterone in urine of domestic sows during the estrous cycle and early pregnancy. *J.Anim.Sci.* **30**:795-799.

Too, K., Kawata, K., Fukui, Y., Sato, K., Kagoto, K. and Kawabe, K. (1974). Studies on pregnancy diagnosis in domestic animals by an ultrasonic Doppler method: I. Pregnancy diagnosis in the pig and fetal heart rate changes during pregnancy. *Jap.J.vet.Res.* **22**:61-71

Tsunoda, Y., Shioda, Y., Onodera, M., Nakamura, K. and Uchida, T. (1988). Differential sensitivity of mouse pronuclei and zygote cytoplasm to hoechst staining and ultraviolet irradiation. *J.Reprod.Fert.* **82**:173-178.

Unterman, T.G. and Phillips, L.S. (1985). Glucocorticoid effects on somatomedins and somatomedin inhibitors. *J.Clin.Endocrinol.Metab.* **G1(4)**:618-634.

Van der Putten, H., Botteri, F.M., Miller, A.D., Rosenfeld, M.G., Fan, H., Evans, R.M. and Verma, I.M. (1985). Efficient insertion of genes into the mouse germ line via retroviral vectors. *Proc.Natl.Acad.Sci.USA* **82**:6148-6152.

Varley, M.A. (1983). The regulation of the oestrous cycles in groups of post-pubertal female pigs using allyltrembolone. *Anim.Prod.* **36**:211-215.

Vincent, C.K., Robison, O.W. and Ulberg, L.C. (1964). A technique for reciprocal embryo transfer in swine. *J.Anim.Sci.* **23**:1084-1088.

Vize, P.D., Michalska, A.E., Ashman, R., LLoyd, B., Stone, B.A., Quinn, P., Wells, J.R.E. and Seamark, R.F. (1988). Introduction of a porcine growth hormone fusion gene into transgenic pigs promotes growth. *J.Cell Sci.* **90**:295-300.

Voekle, S.A., Amborsk, G.F., Hill, K.G. and Godke, R.A. (1985). Use of uterine-cell monolayer culture system for micromanipulated bovine embryos. *Theriogenology* **24**:271-281.

Wagner, E.F., Stewart, T.A. and Mintz, B. (1981). The human β-globin gene and a functional thymidine kinase gene in developing mice. *Proc.Natl.Acad.Sci.USA* **78**:5016-5020.

Wagner, E.F. and Mintz, B. (1982). Transfer of nonselectable genes into mouse teratocarcinoma cells and transcription of the transferred human beta-globin gene. *Mol.Cell Biol.* **2**:190-198.

Wagner, E.F. and Stewart, C.L. (1985). Integration and expression of genes introduced into mouse embryos. In *Experimental Approaches to Mammalian Embryonic Development.* (Eds. Rossant, J. and Pedersen, R.A.). Cambridge Univ. Press, Cambridge UK,

Wagner, T.E., Hoppe, P.C., Jollick, J.D., Scholl, D.R., Hodinka, R. and Gault, J.B. (1981). Microinjection of a rabbit β-globin gene in zygotes and its subsequent expression in adult mice and their offspring. *Proc.Natl.Acad.Sci.USA* **78**:6376-6380.

Wagner, T.E., Murray, F.A., Minhas, B. and Kraemer, D.C. (1984). The possiblity of transgenic livestock. *Theriogenology* **21**:29-44.

Wagner, T.E. (1985). The role of gene transfer in animal agriculture and biotechnology. *Theriogenology* **21**:29-44.

Wagner, T.E. and Jöechle, W. (1986). Recombinant gene transfer in animals: the potential for improving growth in livestock. In *Control and Manipulation of Animal Growth.* (Eds. Buttery, P.J.,Haynes, N.B. and Lindsay, D.B.). Butterworths, London, pp.293-313.

Wagner, T.E., Chen, X.Z. and Hayes, W.B. (1986). Mammalian gene transfer and gene expression. In *Molecular and Cellular Aspects of Reproduction.* (Ed. Dhindsa, D.S.). Plenum Press, New York.

Walker, S.K. (1989). *Collection, Culture and Pronuclear Microinjection of Sheep Zygotes*, Ph.D. Thesis, University of Adelaide,

Wall, R.J., Pursel, V.G., Hammer, R.E. and Brinster, R.L. (1985). Development of porcine ova that were centrifuged to permit visualization of pronuclei and nuclei. *Biol.Reprod.* **32**:645-651.

Wall, R.J. and Hawk, H.W. (1988). Development of centrifuged cow zygotes cultured in rabbit uteri. *J.Reprod.Fert.* **82**:673-680.

Wallis, M. (1981). The molecular evolution of pituitary growth hormone, prolactin and placental lactogen: a protein family showing variable rates of evolution. *J.Mol.Evol.* **17**:10-18.

Walton, J.R., Murray, J.D., Marshall, J.T. and Nancarrow, C.D. (1987). Zygote viability in gene transfer experiments. *Biol.Reprod.* **37**:957-967.

Walton, P.E., Etherton, T.D. and Chung, C.S. (1987). Exogenous pituitary and recombinat growth hormones induce insulin and insulin-like growth factor-I resistance in pigs adipose tissue. *Domest.Anim.Endocrinol.* **4**:183-189.

Walton, P.E. and Etherton, T.D. (1989). Effects of porcine growth hormone and insulin-like growth factor-1 (IGF-1) on immunoreactive IGF-binding protein concentration in pigs. *Journal of Endocrinology* **120**:153-160.

Wang, S.V. (1976). *Photochemistry and Photobiology of Nucleic Acids*, Academic Press, New York,

Wangsness, P.J., Martin, R.J. and Gahagan, J.H. (1977). Insulin and growth hormone in lean and obese pigs. *Amer.J.Physiol.* **233**:104.

Ward, K.A. (1982). Possible contributions of molecular genetics to animal improvement. In *Future Developments in the Genetic Improvement of Animals.* (Eds. Barker, J.S.F.,Hammond, K. and McClintock, A.E.). Academic Press, Sydney, pp.17-39.

Ward, K.A., Murray, J.D., Marshall, J.T. and Nancarrow, C.D. (1982). The practical aims and recent progress in the transfer of recombinant DNA to ruminants. *Rev. Rural Sci.* **6**:39-47.

Ward, K.A., Murray, J.D., Nancarrow, C.D., Boland, M.P. and Sutton, R. (1984). The role of embryo gene transfer in sheep breeding programs. In *Reproduction in Sheep.* (Eds. Lindsay, D.R. and Pearce, D.T.). Australian Academy of Science, Canberra, pp.279-285.

Warnick, A.C., Wiggins, E.L., Casida, L.E., Grummer, R.H. and Chapman, A.B. (1951). Variation in puberty phenomena in inbred gilts. *J.Anim.Sci.* **10**:479-483.

Webel, S.K., Peters, J.B. and Anderson, L.L. (1970). Control of estrus and ovulation in the pig by ICI 33828 and gonadotrophins. *J.Anim.Sci.* **30**:791-794.

Webel, S.K. (1978). Control of ovulation in the pig. In *Control of Ovulation.* (Eds. Crighton, D.G.,Haynes, N.B.,Foxcroft, G.R. and Lamming, G.E.). Butterworths, London, pp.421-434.

Webel, S.K., Scheid, J.P. and Bouffault, J.C. (1980). Oestrous control in pigs, US field trial results with a new progestin compound, RU-2267, Regumate. *Int. Symp. Steriods in Animal Production*, Roussel UCLAF, Warsaw,

Webel, S.K. and Day, B.N. (1982). The Control of Ovulation. In *Control of Pig Reproduction.* (Eds. Cole, D.J.A. and Foxcroft, G.R.). Butterworths Scientific, London, pp.211-224.

Welp, C. and Holtz, W. (1988). Induction of parturition in pigs by intravaginal application of the prostaglandin analog cloprostenol. *Theriogenology* **29 (5)**:1037-1041.

White, K.L., Hiehnke, K., Rickfords, L.F., Southern, L.L., Thompson, D.L.,Jr. and Wood, T.C. (1989). Early embryonic development *in vitro* by coculture with oviductal epithelial cells in pigs. *Biol.Reprod.* **41**:425-430.

Whitten, W.K. (1957). Culture of tubal ova. *Nature* **179**:1081-1082.

Whitten, W.K. (1971). Nutritional requirements for the culture of preimplantation embryos *in vitro.* In *Advances in Biosciences.* Pergamon Press, New York,

Whittingham, D.G. and Biggers, J.D. (1967). Fallopian tube and early cleavage in the mouse. *Nature* **213**:942-943.

Whittingham, D.G. (1968). Intra- and inter-specific transfer of ova between explanted rat and mouse oviducts. *J.Reprod.Fert.* **17**:575-578.

Whittingham, D.G. (1971). Survival of mouse embryos after freezing and thawing. *Nature* **233**:125-126.

Whittingham, D.G. and Bavister, B.D. (1974). Development of hamster eggs fertilized *in vitro* and *in vivo*. *J.Reprod.Fert.* **38**:489-492.

Wieghart, M., Hoover, J., Choe, S.H., McCrane, M.M., Rottman, F.M., Hanson, R.W. and Wagner, T.E. (1988). Genetic engineering of livestock-transgenic pigs containing a chimeric bovine growth hormone (PEPCK/bGH) gene. *J.Anim.Sci.* **66 (Suppl. 1)**:266. (abstr.).

Wieghart, M., Hoover, J.L., McCrane, M.M., Hanson, R.W., Rottman, F.M., Holtzman, S.H., Wagner, T.E. and Pinkert, C.A. (1990). Production of transgenic pigs harbouring a rat phosphoenolpyruvate carboxykinase-bovine growth hormone fusion gene. *J.Reprod.Fert.,Suppl.* **41**:89-96.

Wigler, M., Silverstein, S., Lee, L.S., Pellicer, A., Cheng, Y.C. and Axel, R. (1987). Transfer of purified herpes virus thymidine kinase gene to cultured mouse cells. *Cell* **11**:223-232.

Wilkie, T.M., Brinster, R.L. and Palmiter, R.D. (1986). Germline and somatic mosaicism in transgenic mice. *Dev.Biol.* **118**:9-18.

Wilmut, I., Archibald, A.L., Harris, S., McClenaghan, M., Simons, J.P., Whitelaw, C.B.A. and Clark, A.J. (1990). Modification of milk composition. *J.Reprod.Fert.,Suppl.* **41**:135-146.

Wolfrom, G.W, Ivy, R.E. and Baldwin, C.D. (1986). Effect of native porcine growth hormone (npGH) injected intramuscularly in barrows. *J.Anim.Sci.* **63(Suppl. 1)**:219. (abstr.).

Wollen, T.S. (1985). Use and handling of drugs and biologicals in embryo transfer. *Theriogenology* **23**:31-42.

Wollenberg, C., Wentz, I., Blum, B. and Holtz, W. (1990). Survival of pig embryos flushed from the reproductive tract immediately or two hours after slaughter of donors. *J.Anim.Sci.* **68**:2023-2026.

Woychik, R.P., Camper, S.A., Lyons, R.H., Horowitx, S., Goodwin, E.C. and Rottman, F.M. (1982). Cloning and nucleotide sequencing of the bovine growth hormone gene. *Nucleic Acid Res.* **10**:7197-7199.

Wright, R.W. (1977). Successful culture *in vitro* of swine embryos to the blastocyst stage. *J.Anim.Sci.* **44**:854-858.

Wright, R.W. and Bondioli, K.R. (1981). Aspects of *in vitro* fertilization and embryo culture in domestic animals. *J.Anim.Sci.* **53**:702-729.

Wright, R.W. and O'Fallon, J.V. (1987). Growth of domesticated animal embryos *in vitro*. In *The Mammalian Preimplantation Embryo.* (Ed. Bavister, B.D.). Plenum Press, New York, pp.251-271.

Yu, L-Y., Tushinski, R.J. and Bancroft, F.C. (1977). Glucocorticoid induction of growth hormone synthesis in a strain of rat pituitary cells. *J.Biol.Chem.* **252**:3870-3875.

Zapf, J., Hauri, C., Waldvogel, M., Futo, E., Hasler, H., Binz, K., Guler, H.P., Schmid, C. and Froesch, E.R. (1989). Recombinant human insulin-like growth factor I induces its own specific carrier protein in hypophysectomized and diabetic rats. *Proc.Natl.Acad.Sci.USA* **86**:3813-3817.

Zar, J.H. (1974). *Biostatistical Analysis*, Ed. 19th Prentice-Hall, Inc., Englewood Cliffs, N.J.,pp. 228-235.

Zezulak, K.M. and Green, H. (1986). The generation of insulin-like growth factor-I sensitive cells by growth hormone action. *Science* **233**:551-553.