PROSPECTS FOR APPLICATION OF MOLECULAR GENETIC MANIPULATION TO IMPROVE REPRODUCTION

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SUMMARY

Prospects for molecular manipulation of animal genomes to improve reproduction are considered with the assumptions that it will become possible to control expression following either transfer of genes or modification of endogenous genes. In the areas of seasonality, number of ovulations, pre- and peri-natal survival and determination of sex there are few projects which could be initiated at present. In the longer term, as understanding of the physiological and molecular control of reproduction advances, there are likely to be opportunities to modify all of these aspects of reproduction.

INTRODUCTION

Revolutionary opportunities for the modification of animal performance are being created by the development of new methods of embryo manipulation and the application of molecular biology. The purpose of this paper is to review the potential application of these procedures for the improvement of reproductive performance in livestock. There are three sections: a consideration of the methods of molecular manipulation that are available at present and those that seem likely to become available; a discussion the modification of hormonal systems and an analysis of candidate genes for manipulation of some aspects of reproduction. It is not possible to include an exhaustive list of references, in some sections reference is made to suitable reviews.

METHODS OF MOLECULAR MANIPULATION

Two types of molecular genetic modification are considered to be feasible. Firstly, gene transfer, the addition of DNA to the genome. Secondly, gene mutation, the modification or ablation of genes already present in the target species. The latter type of manipulation requires precise site directed modifications, and as yet only gene transfer has been achieved in livestock. The usual method of gene transfer involves direct injection of several hundred copies of the gene into a nucleus in the early embryo. This procedure has several limitations. The proportion of injected embryos that survive to become transgenic offspring is low making it expensive; expression of the transgene is variable and in 5-10% of cases an endogenous gene is damaged by the insertion. A variety of approaches is being pursued with the aim of reducing these limitations (see Wilmut and Clark, 1990 for review), but the greatest improvement would be offered by the isolation of embryo stem cells from livestock species.

Stem cells have been isolated from mouse embryos by culture in such a way that when injected into the blastocoel cavity of another embryo they are sometimes able to colonise that embryo and contribute to all of the tissues of the resulting pup, including the germline (Robertson, 1987). While the cells are in culture there is the opportunity to make site directed changes to endogenous genes. Thus difficult changes can be made in cell culture and verified before taking the expensive step of making transgenic animals. A disadvantage in their use follows from the fact that the young are chimeras and a second generation must be produced before the effects of the gene can be assessed, however, there is the possibility, particularly in ruminants, that nuclear transfer from such cells into enucleated oocytes could be used to avoid the chimeric generation (Smith and Wilmut, 1989).

In addition to making precise modifications to the gene, control of expression of modified genes is required. Gene expression involves many steps: transcription; processing of the RNA and export from the nucleus; translation and degradation of the mRNA; post-translational modification and secretion of many proteins also occurs. Gene expression is regulated at each of these levels, although not in every case, and modulation of expression at each level can be envisaged. The types of alteration that can be considered are increasing or decreasing expression, altering the pattern of expression and production of proteins with modified properties. Nevertheless, there will be limits to the opportunity to modify gene expression and these will become apparent as the research progresses.

To date molecular genetic manipulation in livestock species has achieved untargeted insertion of additional genes, with no control over the site of insertion and little control over expression. Our ability to make site directed changes and control expression will increase and this will create new opportunities. It may be possible for gene expression to be down-regulated or switched-off completely. Alternatively expression may be directed to a particular stage of growth or of the reproductive cycle or limited to

certain cell types. A population of cells may be destroyed by production of toxins in the cells.

MODIFICATION OF ENDOCRINE SYSTEMS

One principal will be important in any modification: it should be as specific as possible. In many cases hormones have a variety of effects depending upon the tissue and the concentration of the other hormones. Gene transfer studies have already provided evidence of the potential pleiotropic effects of an increase in concentration of one hormone. Transfer of growth hormone genes into pigs led to some desirable changes in the carcass, but also to a series of problems including arthritis, susceptibility to stress and lowered reproductive performance (Pursel *et al.*, 1989). While mice will be invaluable for assessing proposed manipulations at the molecular level, the physiological differences between species are so great as to require the use of the species of interest to confirm that the desired change has been made. Many aspects of reproduction are governed by either steroid or protein hormones in feedback systems and several points at which these may be modified are shown below.

Points at which hormone action may be manipulated. Details are considered in the text.

At site of production	During transport of hormone	At target tissue
Hormone synthesis, (tissue rate)	Binding protein: (affinity for hormone amount of protein)	Hormone receptor (number affinity)
Hormone structure (affinity stability activity)	Rate of hormone clearance	Chromosome acceptor site (availability)
Hormone release		Product of the effector gene

It may be desirable to increase synthesis of a hormone by a particular cell type. Production of steroid hormones is the result of specific enzyme action. Increasing the level of enzyme present, by transfer of additional copies of the gene, might be expected to lead to greater production of the hormone. However, as increases in the amount of a single enzyme are expected to have only a small effect upon the flux through a pathway (Kacser and Burns, 1979), a successful approach might be complex and involve transfer of the genes for all or many of the enzymes in the chain. Conversely, a reduction in hormone production may be achieved, but this may simply require the reduction of one enzyme of the pathway to make it rate limiting. Production of protein hormone is a more direct consequence of gene expression and may be simpler to manipulate than steroid hormone synthesis. These changes may require placing expression of the gene outside any feedback loops which exist.

The pattern of release of protein hormones is sometimes subject to precise feedback control and these mechanisms may be amenable to manipulation. Regulation of gonadotrophin release will be discussed in a later section, and similar mechanisms may apply to growth factors. By contrast, there is no control of steroid hormone release.

As the characteristics of protein hormones are sometimes dependent upon the post-translational modifications, these modifications may be a target for manipulation. The half-life and binding of many serum proteins is influenced by the presence of carbohydrate side-chains (Rademacher *et al.*, 1988). Change in the amount or nature of the carbohydrate may alter the effect of a hormone. Thus, hCG which is deglycosylated, either chemically or enzymatically, has an increased affinity for the receptor and so is a potent competitive inhibitor of the native hormone. By contrast, it has lost the ability to induce a response in the cell. Evidence has been emerging for tissue and species specificity of these post-translational modifications.

Hormones are transported in complexes with specific binding proteins and modification to the amount or nature of the binding protein may alter the availability of the hormone to the target tissues. Binding protein for growth hormone is particularly well characterised (Baumbach *et al.*, 1989). This protein is generated by alternative splicing of the gene for the receptor protein. Similar proteins for any of the hormones concerned with reproduction could provide a route for modification of the effect of that hormone either by modifying the amount of protein produced or altering the affinity of the protein for the hormone.

The mechanisms of action of steroid and protein hormones are different. While steroid hormones enter the cell before binding to specific receptors, peptide hormones bind to cell surface receptors and exert their effects through second messengers. In both cases there are several possible sites for the modification of their effects. Steroids circulate in the blood and diffuse freely into cells throughout the body (see Rories and Spelsberg, 1989). In target cells the hormone binds to receptor proteins in the nucleus, causing a conformational change in the protein that in turn modifies gene expression. Two

types of acceptor site for receptor proteins have been characterised: specific DNA sequences and protein acceptor sites. In the DNA, sometimes near to the transcription start site, there are specific binding sites that are required for steroid induced (or repressed) expression of the genes. The response to a steroid may involve a cascade of genes, with expression of the later genes being regulated by the product of an early gene. In view of the need for specificity it may be appropriate to induce a mutation in the acceptor site or in a gene later in the cascade of effector genes.

The mechanism of action of GnRH has provided a suitable model for study of the regulation of the effect of protein hormones (Clayton, 1989). The peptide is bound to a specific receptor whose number is regulated by several hormones including GnRH itself and oestradiol. Binding of GnRH induces activation of phospholipase C and both release of intracellular calcium by inositol 1,4,5-triphosphate and entry of calcium through specific channels. A second product of phospholipase C is diacylglycerol which in turn activates protein kinase C which induces LH release. Simultaneous activation of protein kinase C and mobilisation of calcium induces maximal LH release. Secondary effects may depend upon cAMP. Second messengers are not appropriate targets for manipulation as they may have a variety of effects in each cell type and different effects in other cell types, however, regulation of receptor number or of the ultimate target for the second messenger may provide suitable routes for the modification of the effect of protein hormones.

CANDIDATE GENES

The reproductive performance of an animal is the product of a considerable number of processes and improvement would be desirable for all of these. Four aspects considered are: a) seasonal breeding, b) number of ovulations, c) survival (both pre- and post-natal) and d) determination of sex. These are of particular importance in a) sheep, b) cattle and sheep and c) pigs, respectively.

Seasonality

There are two reasons for believing that it may be possible to modify the pattern of seasonal breeding in sheep either by gene manipulation or direct selection. First, there are differences between breeds in the length of their season, ranging from 199 to 277 days in Dorset Horn to 83 to 154 days in Border Leicester ewes in Britain (Hafez, 1952), while in sub-tropical environments Ossimi ewes are almost non-seasonal (Aboul-Naga *et al.*,1985). Variation exists in both the time of the onset and end of the breeding season, (Wheeler and Land, 1977) suggesting that different mechanisms may be responsible for these changes. Secondly, there are reports that the length of the season of Texel ewes was extended by direct selection (Grommers and van der Geer, 1987), although few within breed heritability estimates are available. In this way, selection has greatly reduced the seasonal changes in pig reproduction (Mauget, 1982). The mechanisms that are believed to govern seasonality in ewes will be considered before a discussion of the possible routes for molecular genetic manipulation.

Many aspects of animal life are rhythmical in nature with periodicity ranging from milliseconds for the flight song of Drosophila to annual cycles. While a great deal is known about the physiological and molecular regulation of circadian rhythms much less is known of the circannual cycles (Gwinner, 1981). In addition to the reproductive cycles, animals exhibit annual cycles in appetite, fat deposition, moult, hibernation and migration, all of which contribute to the ability of the species to survive in the wild and exploit predictable changes in the environment, particularly in food supply and temperature. It should not be assumed that all of these circannual rhythms arise from one clock that is able to measure the passage of a year (Gwinner, 1981). There are differences between species and between breeds within a species in the precise pattern of annual changes. In rams of southern breeds of domesticated sheep, such as the Merino, the increase in concentration of FSH in plasma occurs almost 3 months earlier that that in wild type Mouflon sheep (Lincoln *et al.*,1990).

One characteristic of the circannual cycles is that they persist in a constant environment and it may be that the effect of the environmental cues is to entrain the endogenous cycle to the changes in the environment. Sheep that are exposed to constant daylength continue to exhibit both onset and termination of breeding seasons (Ducker *et al.*, 1973). Such endogenous cycles are also observed in ewes that are blind (Legan and Karsch, 1983) or if the nervous pathway from the eye to the pineal gland is interrupted (Lincoln, 1979; Bittman *et al.*, 1983). The most important of the entraining environmental cues are change in day length, but nutrition (Lincoln *et al.*, 1989) and social cues derived from other animals in the flock also exert an influence (Wayne *et al.*, 1989). As sheep begin to breed as day length shortens, it has been assumed that this reduction induces the onset of sexual activity, but recent research emphasises the importance of the increase in day length after the previous winter solstice in determining the time of the onset of breeding (Malpaux *et al.*, 1989). By contrast, the reduction in day length had a particular role in establishing the length of the season (Malpaux *et al.*, 1988).

The period of darkness is indicated to the rest of the body by release of melatonin from the pineal

gland and it is this that provides the photoperiodic cues. The eyes of sheep are required to detect changes in day length (Legan and Karsch, 1983) and the effector pathway from the optic nerve passes through the suprachiasmatic nuclei within the hypothalamus to the superior cervical ganglia in the neck before looping back to the pineal gland. The central role of changes in length of melatonin production in the control of seasonal breeding is confirmed by measurements of melatonin concentration (Ravault and Thimonier, 1988) and by use of melatonin treatment to advance the time of the onset of breeding (Arendt *et al.*, 1988). While the importance of melatonin is established, many questions remain concerning the mechanism and site of action of melatonin, although specific receptors have been identified in several sites in the central nervous system. Recently, melatonin binding to receptors in the pars tuberalis of the pituitary gland has been shown to inhibit the increase in cAMP induced by forskolin, the non-specific activator of adenylate cyclase (Morgan *et al,* 1990).

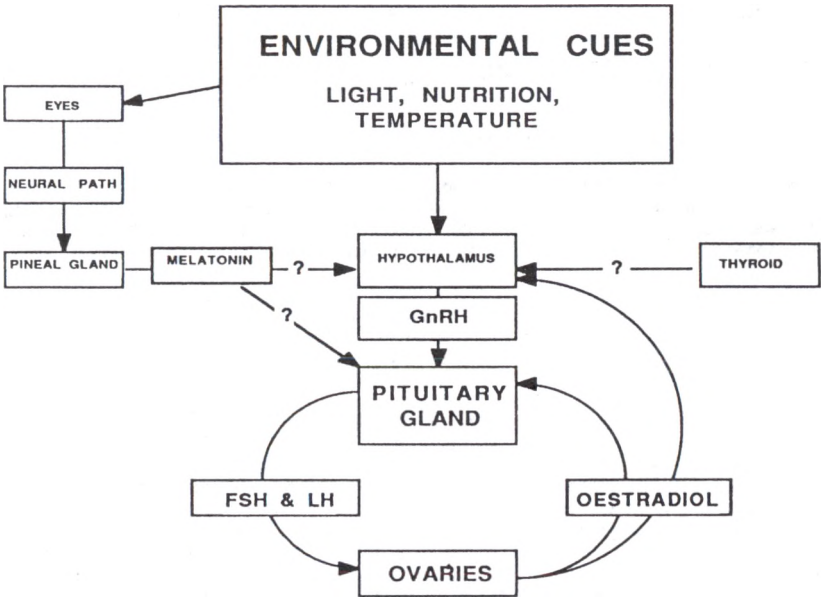

Figure 1. Diagrammatic representation of the mechanisms that regulate seasonal breeding in sheep.

The seasonal patterns of reproduction reflect changes in the frequency of activity of the GnRH pulse generator that in turn causes pulses of LH. Changes in LH pulse frequency seem likely to involve modifications to the putative GnRH pulse generator, while changes in amplitude could result from changes in the quantity of GnRH released or in the response of the pituitary to GnRH. The concept of a pulse generator to govern the pulsatile release of gonadotrophin has long been discussed in the literature (see Lincoln *et al.*, 1985; Dyer and Robinson, 1989). It has been argued that production of a pulse of GnRH is an intrinsic property of the group of LHRH cells, while other inputs (unknown) determine the frequency and amplitude of the pulse (Dyer and Robinson, 1989).

Two concepts have been introduced in attempts to account for the seasonal changes in gonadotrophin concentration: "steroid negative feedback" and "direct photoperiodic drive". In ovariectomised ewes, LH in peripheral blood was at the same elevated concentration throughout the year unless oestradiol was administered at concentrations that mimicked that of the luteal phase of the oestrous cycle (Legan *et al.*, 1977). In the presence of oestradiol, the concentration of LH increased dramatically at the same time that intact ewes began to cycle and decreased at the time of anoestrus. The site of action of oestradiol in exerting this negative feedback effect upon GnRH release is not known, but the GnRH neurones themselves are not sensitive to oestradiol. The second effect is observed in the absence of steroid; during anoestrous in ovariectomised ewes the peaks of LH are large, but infrequent, whereas they become more frequent, but smaller in the breeding season reflecting

changes in the "direct photoperiodic drive" (Karsch *et al.*,1984). A great deal remains to be learned about these changes. Is direct photoperiodic drive a result of changes in light or part of the endogenous rhythm that is entrained by changes in light? Are the two mechanisms "steroid negative feedback" and "direct photoperiodic drive" entirely separate, related or the same?

Two other systems may influence the seasonal pattern of breeding, thyroid hormones and the endogenous opioids. The effects of endogenous opioids are exceptionally difficult to study and there is no clear understanding of their role (Lincoln and Ssewannyana, 1989), however, it has been suggested that they may have a causitive role in the induction of anoestrous. Finally, there is recent evidence to show that thyroid hormone may be required for the onset of anoestrus (Nicholls *et al.*, 1988). When Welsh Mountain ewes were thyroidectomised the length of the breeding season was extended dramatically, in a response comparable to that in birds. Again the mechanism and site of action of the thyroid hormones in this context are unknown.

Although the fundamental mechanisms are not understood there are a number of opportunities to use gene manipulation to change the response to the seasons. There are two potential types of change: release of the endogenous cycles from the influence of the environment or the establishment of animals with continous reproductive activity. The expected effect of modifying the pattern of production of melatonin or of changing the response to melatonin would be to reveal the spontaneous patterns. The endogenous pattern could be entrained by social cues from other animals, but the period of sexual activity would not be changed. Alternatively, the duration of a breeding season might be extended by preventing the effect of thyroid hormones on seasonality and so mimicking the effect of thyroidectomy without the undesirable effects upon health.

Finally there are two approaches that may lead to a continuous breeding season. If the seasonal inhibitory effect of oestradiol is part of the endogenous annual reproductive cycle, prevention of the effect might create an animal without seasonal changes. The animal would be expected to show either continuous breeding or anoestrous depending upon the influence of the other mechanisms. The seasonal inhibitory effect of oestradiol may be exerted in a specific population of cells that are able to modify the function of the GnRH pulse generator. Site directed modification could be addressed at the acceptor site for the oestrogen receptor within these cells to prevent the effect of oestradiol. Alternatively, if they have no other function, genetic ablation by gene transfer could cause the destruction of the cells. Secondly, it may be possible to increase GnRH production by transfer of modified GnRH genes. While transfer of intact genes would not be expected to have any effects, because of the existing homeostatic mechanisms, it may be possible to modify regulatory elements and cause greater expression outside the normal breeding season. One limitation to this approach arises from the ability of excess GnRH to desensitise the pituitary gland and render it unresponsive.

Number of ovulations

Three lines of evidence suggest that it should be possible to increase the number of ovulations in sheep by molecular manipulation. First, there are differences between breeds of sheep in the number of ovulations with means ranging from around 1 to over 3 (Bindon and Piper, 1986). In some cases, but not all, the large number of ovulations reflects the effects of major genes of which the best studied is the Booroola gene (Bindon and Piper, 1986). It is not known whether or not the different genes are allellic. Second, direct selection for number of ovulations induced significant change with a heritability estimates of up to 0.5 in sheep (Hanrahan and Quirke, 1985). Finally, physiological manipulation has led to useful increases in the number of ovulations (see Webb and Morris, 1988). Immunisation against endogenous steroids or inhibin and treatment with an inhibitor of 3-β-hydroxysteroid dehydrogenase have all resulted in useful increases in the number of ovulations in sheep. While the rationale behind their initial use was an expected increase in the concentration of FSH reflecting a change in the feedback mechanisms, there is evidence of local effects and this has led to an intense debate concerning the actual method of action (see Webb and Morris, 1988). Whatever the route, these observations provide every reason to expect that similar changes arising from gene modification will be effective.

While the general pattern of hormone changes in sheep and cattle may be similar there is evidence of important differences between the species in the mechanisms that regulate the number of ovulations. First, there are no prolific breeds of cattle and the frequency of spontaneous twin ovulations is very low. Secondly, some treatments, such as immunisation against ovarian steroids, that lead to an increase in number of ovulations in sheep do not have this effect in cattle (see Webb and Morris, 1988). In this discussion, the mechanisms that are believed to apply in sheep are considered first, before any known species differences.

Complex mechanisms, that are not fully understood, have been shown to regulate recruitment of follicles, their growth, selection for ovulation or atresia. Across breeds, there is no relationship between the number of large antral follicles and the number of ovulations, rather ovulation depends upon selection of a breed specific number of follicles from the growing population (Webb *et al.*, 1989). There appear to be at least two mechanisms that regulate the number of ovulations in ruminants and it may be

possible to increase the number of ovulations by manipulation of either regulatory mechanism. The two mechanisms involve feedback control of gonadotrophin concentration and intra-ovarian regulation of follicle growth (Fig 2). In this context the term "intra-ovarian" is used to include effects of the products of one ovary upon the other ovary.

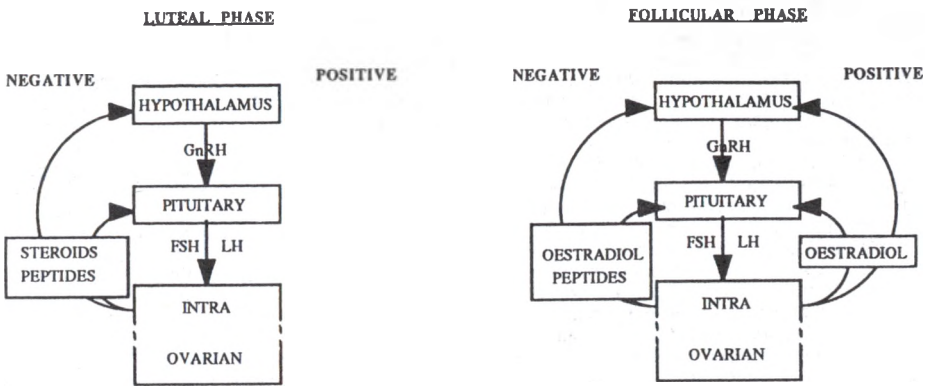

Figure 2. The relationship between hypothalamus, pituitary gland and ovary in the regulation of the number of ovulations; shown during both luteal and follicular phases of the oestrous cycle.

Modification of ovarian feedback

The final stages of follicle growth and ovulation are dependent upon gonadotrophins and increasing the concentration of FSH can result in an increase in the number of ovulations (McNeilly, 1985). However, this often causes an undesirably large increase in the number of ovulations, associated with large variation between females. This relatively large response may reflect the overwhelming of the second regulatory mechanism at the ovarian level. There are then two particular difficulties in attempting to increase the number of ovulations by overriding the feedback system. Any modification must not be so great as to cause superovulation, nor must it interfere with the other effects of ovarian steroids, for example in regulation of seasonal breeding or oestrous behaviour.

Control of gonadotrophin concentration is exerted at the hypothalamus and the pituitary gland, and modification at both sites will be considered. The pattern of GnRH secretion by the hypothalamus is regulated by the negative feedback effects of oestradiol and progesterone, while little is known of the effects of ovarian peptides such as inhibin (Clarke, 1988). An increase in GnRH concentration might be achieved by reduction of negative feedback effects of either progesterone or oestradiol. An appropriate increase in GnRH release through modification of the oestradiol acceptor site of the GnRH gene seems more likely to succeed than a reduction in the negative feedback effects of progesterone which may lead to a premature surge of gonadotrophin. It would be essential not to modify the ability to respond positively to oestradiol by producing a surge of LH capable of inducing ovulation and secondly not to increase the concentration of GnRH to the range where it causes gonadotroph desensitisation (Clayton, 1989).

The synthesis and release of gonadotrophins by the pituitary gland is under the control of GnRH, steroids and peptides from the ovary, including inhibin. In particular, in sheep, oestradiol and inhibin govern FSH release, while in cattle the role of inhibin is not clear (Martin *et al.*, 1988; Price and Webb,1988). Three possible means of modification of FSH release will be considered. Specific changes to the acceptor site of the gene that responds to the negative effects of oestradiol may permit greater synthesis of FSH. A higher concentration of oestradiol, that is more follicles, would then be required for the normal level of inhibition. Again, it would be essential that any such change did not modify the positive feedback loop or cause premature surges of LH. A second similar approach would be to reduce the number of inhibin receptors and so permit increased FSH release. A third, rather different approach would be to modify the ratio of the two sub-units of FSH. The α sub-unit is a component of several hormones whereas the β sub-unit is specific to the particular hormone. As might be anticipated, the amount of LH released into the circulation has recently been shown to be related to the amount of β sub-unit mRNA (J. McNeilly personal communication). Transfer of a fusion gene of α sub-unit promoter with the β sub-unit structural sequences would be expected to increase the production of FSH.

At the ovary the objective would be to reduce the production of oestradiol or inhibin in response

to particular concentrations of gonadotrophin. While treatment with an inhibitor of 3 β hydroxysteroid dehydrogenase leads to an increase in the number of ovulations (Webb, 1987), there are two drawbacks to a reduction in the production of oestradiol. Firstly, there might be side-effects arising from any changes in activity of the enzymes in the adrenal gland. Secondly, as oestradiol is required for normal follicle growth, too great a reduction in the concentration of oestradiol might itself cause a direct reduction in follicle growth.

Modification of intra-ovarian mechanisms

An alternative approach would be to modify intra-ovarian mechanisms that regulate follicle growth. This approach seems inherently more likely to succeed than modification of gonadotrophin secretion, because there may be less risk of superovulation. Although no specific factors have been shown unequivocally to have a direct effect upon the ovary the evidence for such effects is increasing: first, the maintenance of breed differences in the number of ovulations in hypophysectomised ewes given constant doses of gonadotrophin (Fry *et al.*, 1988; Driancourt *et al.*, 1988), second, in some experiments an increase in the number of ovulations was induced by immunisation against steroid, despite the fact that FSH concentration was apparently not increased by the treatment (see Webb and Morris, 1988).

There is a rapidly growing list of compounds that are able either to have an effect *in vivo* or to modify either division or differentiation of follicle cells in culture. Bovine follicular fluid reduced the response to FSH in pituitary stalk sectioned ewes given gonadotrophin (Larson *et al.*, 1987) suggesting the presence of a factor(s) in the follicular fluid capable of acting directly on the ovary. Similar factors capable of inhibiting the response to gonadotrophin have been isolated from human and pig follicular fluid (Kling *et al.*, 1984). A number of known growth factors, particularly IGF, EGF and TGF influence follicle cells in culture and may also act to modify the number of ovulations (see Carson *et al.*,1989). In seeking to modify these mechanisms the amount of peptide factor produced might be diminished by reducing expression of the gene. Although immunisation against inhibin has lead to a greater number of ovulations (Webb and Morris, 1988) in some cases this was not associated with an increase in the concentration of FSH and so may reflect intra-ovarian effects. Alternatively the response to the factors in growing follicles could be reduced. This might be achieved by a reduction in the number of receptors for the factor or by modification to the response within the cells. In particular, the acceptor site of the effector gene could be modified.

Prenatal Survival

Prenatal survival (the proportion of ovulations that are represented by neonates) varies around 0.7 in the major farm species. Within breeds of all species prenatal survival is negatively correlated both phenotypically and genetically with ovulation rate. Success in improving ovulation rate is likely to be accompanied by a decline in prenatal survival. In sheep and cattle at their present level of prolificacy this is not sufficient to negate the effects of increases in ovulation rate. In consequence improved ovulation rate is accompanied by improved litter size, as demonstrated in sheep by the Booroola gene (Piper *et al.*, 1985). In pigs, Johnson *et al*, (1984) demonstrated that selection could be used to increase ovulation rate markedly, but prenatal survival declined and there was little change in litter size. Similar observations, of a response of litter size in cattle and sheep and a limited response of litter size in pigs, are observed when embryo number is manipulated by hormonal treatments or embryo transfer. The challenge that prenatal survival represents is not necessarily to increase its overall mean level, although this would be of some value, particularly in pigs, but to maintain its level when ovulation rate is increased.

It should not be assumed that prenatal survival will have been maximised for any given ovulation rate by natural selection. Prenatal survival has been improved by artificial selection in mice (Bradford, 1969). Furthermore, prenatal survival varies between breeds and there is evidence that it is higher in some prolific sheep breeds, such as the Romanov (Ricordeau *et al.*, 1982). The prolific Chinese Meishan pig has both a higher ovulation rate and higher prenatal survival than the Large White pig. However, adjusting for the difference in ovulation rate, the main cause of the greater litter size in the Meishan is an increase of over 0.2 in the proportion of ova surviving for a given ovulation rate (Haley and Lee, 1990).

The causes of prenatal mortality and its positive correlation with ovulation rate are still uncertain. However, there may be three important components. Firstly, a component associated with chromosomal abnormalities or other genetic lesions, recognizable chromosomal abnormalities may account for losses of 5-10% (e.g. McFeely, 1967; Long and Williams, 1980). Secondly an effect associated with the embryo prior to and during implantation, possibly related to asynchrony between the developing embryo and uterus (Wilmut and Sales, 1981; Wilmut *et al.*, 1985; Pope, 1988; Roberts *et al.*, 1990). Thirdly, a level associated with the foetus and associated with crowding and competition due to limited uterine capacity (e.g. Wu *et al.*, 1987; Bennet and Leymaster, 1989).

Although much work is required before improvement of prenatal survival by molecular genetic manipulation can be attempted, research has indicated some areas of promise. The progesterone profile,

particularly in the period from ovulation to implantation, has been shown to be an important variable in the control of embryo survival and the maintenance of pregnancy (Miller and Moore, 1976). Progesterone has a role in the control of the uterine environment in early pregnancy and asynchrony between the maternal progesterone profile and the developing embryo may put the embryo in a lethal environment. A number of studies have indicated the possibility of associations between progesterone and embryonic survival (e.g. Ashworth *et al.*, 1989) and enhanced survival following exogenous treatment (Davis *et al.*, 1986). Not all studies show the effect, however, and it may be that an association is only present when conditions are sub-optimal. For example, reduced peripheral progesterone concentration was associated with embryo mortality in sheep at the end of the breeding season (Ashworth *et al.*, 1989) and in consequence of overfeeding (Parr *et al.*, 1987). In a selected mouse line, where low litter size was due to prenatal mortality and associated with reduced progesterone concentrations, administration of progesterone restored prenatal survival to normal levels (Michael *et al.*, 1975).

It has been suggested that the time of the maternal recognition of pregnancy may consitute a second critical stage for the maintenance of embryos. Pregnancy may not be recognised, and in consequence embryos lost, if they do not signal their presence at the appropriate time or in sufficient strength. Embryonic products implicated in the recognition of pregnancy are oestradiol and trophoblast interferons. In pigs, the administration of oestradiol on days 12 and 13 of pregnancy may increase the proportion of pregnant females (Pope *et al.*, 1987). Trophoblast interferons are major secretory products of both sheep and cattle concepluses at around the time of maternal recognition of pregnancy (Roberts *et al.*, 1990) and may act to extend corpora lutea function via control of prostaglandin $F_{2}\alpha$ secretion from the uterine endometrium. They could also alter immune function to avoid immunogenic rejection of implantation. Intra-muscular injection of recombinant bovine interferon α_{I} at the time of maternal recognition of pregnancy has been shown to increase conception rate in sheep (Roberts *et al.*, 1990).

The circumstances under which natural progesterone, oestradiol or trophoblast interferon concentrations are a factor limiting prenatal survival or the maternal recognition of pregnancy, and under which their administration is of value have yet to be well defined. It is possible that the molecular genetic manipulation of the pathways producing these hormones or their receptors may be of value. However, the existence of maternal/embryonic synchrony implies a fairly precise relationship between the developing embryo and the uterine environment. Increasing the concentration of hormones, such as progesterone, oestradiol or trophoblast interferons, either via administration or via transgenesis, may be just as likely to be detrimental as beneficial. More must be known about optimum hormonal profiles and about the timing and targeting of gene expression in transgenic animals before molecular genetic manipulation is likely to be of value.

Another area that merits closer study is the reported association between the MHC complex and prenatal survival. A possible major gene within the murine MHC (H-2) which affects the rate of pre-implantation embryonic development has been identified (the PED gene, reviewed in Warner, 1986). This gene has a marked effect on the rate of cell division, may affect embryo survival and overall reproductive performance, appears to act via the embryo, not the mother (Brownell and Warner, 1988) and has been located in the Qa-2 sub-region of the H-2 complex. Associations between porcine MHC (SLA) haplotypes and several reproduction traits in pigs have been reported (reviewed by Vaiman *et al.*, 1988). Traits which have been associated with SLA include litter size, piglet weights, embryonic development and embryo survival. None of the studies have excluded all possible potential biases, such as inbreeding or chance associations, and none of the effects can be unequivocally attributed to the SLA complex, rather than linked loci. However, the possibility exists of a PED-like gene linked to SLA in the pig.

If it were possible to increase the number of embryos that implanted via improved ovulation rate and embryonic survival then uterine capacity might become a limiting factor. At present we have relatively little understanding of the causes of variation in this trait. The case for a relationship between uterine length and capacity has been argued (e.g. Wu *et al.*, 1987) and is intuitively appealing. However, compared to the Large White, the prolific Chinese Meishan pig has a smaller uterus in proportion to its smaller body size, at least in early pregnancy (Bazer *et al.*, 1988a; 1988b). It is possible that the uterus of the Meishan has a greater capacity for growth in later pregnancy to accommodate implanted foetuses or alternatively is able to control the growth of fetuses and minimise competition. In this context, studies of uterine growth factors or receptors (summarized by Brigstock *et al.*, 1989) could identify genes for future manipulation.

The lack of knowledge of ways of increasing prenatal survival indicates the value of the Meishan pig as one of the few models available where prenatal survival is markedly increased (Bolet *et al.*, 1986; Haley and Lee, 1990). Our present studies indicate that the advantage of the Meishan is mediated via the maternal genome (Haley and Lee, 1990). However, we have little knowledge on whether the difference between the Meishan and the Large White is mediated by a few or many genes. The methods

for mapping quantitative trait loci onto a molecular genetic marker map may be of value for answering this question (e.g. Lander and Botstein, 1989). Mapping the loci responsible for the high prenatal survival of the Meishan in an F2 cross with the Large White will not only permit manipulation of those genes by marker assisted selection, but may also lead ultimately to their cloning. The Pig Gene Mapping Project (PiGMaP) recently initiated in Europe (Haley *et al,*. 1990) has the mapping of Meishan prenatal survival genes as one ultimate aim.

Perinatal Mortality

Unlike prenatal mortality, the many causes of peri-natal and post-natal mortality can often be enumerated. Important causes are stillbirth, dystocia, exposure, starvation, disease and injury (e.g. see Cundiff *et al.*, 1982). However, these effects may be interrelated and the primary reason is usually unknown. For example, a difficult birth can lead to a weakly animal which does not get a full ration of colostrum and secumbs to disease, starvation or exposure or a combination of all three. None the less, the between and within breed variation in post-natal survival documented by Cundiff *et al.* (1982) indicates that these traits are amenable to genetic manipulation. The negative associations of postnatal survival with litter size found both within and between breeds again suggest that manipulation of survival will assume greater importance when litter size has been increased.

Manipulation of resistance to particular diseases may have a role to play in some cases, and major genes for resistance are known. For example, a major cause of pre-weaning mortality in pigs is scour, of which one major cause is the K-88 strain of *E. coli.* A major gene controlling the presence of receptors for this strain and thus resistance is known to segregate in some populations (Sellwood *et al.*, 1975; Gibbons *et al.*, 1977). Another route to the manipulation of resistance to specific diseases in the piglet would be to manipulate the spectrum of immunoglobulins in the colostrum. However, in all cases of manipulation of specific disease resistance the pathogen may be able to evolve more quickly than the host can be manipulated. A more agile response to evolving disease might be to use molecular engineering techniques to manufacture specific immunoglobulins in culture for the passive immunization of neonates via injection or ingestion of artificial colostrum.

Manipulation of sex ratio

It would be desirable to be able to manipulate the sex ratio in a number of situations. There are advantages in having male offspring for the production of meat as they are often more efficient than females at the production of lean meat and have more desirable carcase conformation. In breeding programmes, offspring of one sex may be particularly useful, for example in the production of bulls for use in AI centres. Identification of the sex of an embryo by DNA probe is now practical (Bondioli *et al.*, 1989), it may also be possible to use gene transfer to increase the proportion of males.

A gene on the Y-chromosome is believed to cause the differentiation of the fetal gonad to a testis and in turn the secondary sex changes are then initiated (see McLaren, 1988). The identity of the primary sex determining gene (PSDG) and the nature of the cascade of genes that cause the other male characteristics are the subject of intensive research and speculation (Burgoyne, 1989), but the recent hope that the gene had been cloned has not been fulfilled. Whatever the nature of the primary gene, it seems reasonable to expect that manipulation of phenotypic sex might be achieved by transfer of the primary sex determining gene and if necessary, other genes further down the cascade that are Y-linked. Threequarters of the offspring of a male (XY) carrying PSDG on an autosome (PSDG,o) would be phenotypic males (X/X,PSDG/o; X/Y,PSDG/o; X/Y,o/o). Only X/X,o/o would be female. Alternatively, if the gene could be transferred to the X chromosome, then all offspring would be male.

CONCLUSIONS

A number of broad conclusions and a cautionary note arise from this analysis. There are very few situations in which a project could be initiated at present with the aim of using molecular manipulation to improve reproductive performance. Many of the most promising opportunities will depend upon the isolation of embryo stem cells or the establishment of alternative methods of site directed mutation and in most cases the genes of interest have not yet been cloned. However, in the longer term, there seem to be real prospects of being able to modify at least some of these aspects of reproductive performance by molecular means. Much remains to be learned about the molecular regulation of reproduction and a greater understanding seems likely to reveal the inadequacies of some of the present suggestions, but it is also certain to reveal further opportunities.

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