DISSERTATION

PHYSICAL AND MOLECULAR CHARACTERISTICS OF DAY 75 NUCLEAR TRANSFER CLONED BOVINE CONCEPTUSES

Submitted by

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ABSTRACT

PHYSICAL AND MOLECULAR CHARACTERISTICS OF DAY 75 NUCLEAR TRANSFER CLONED BOVINE CONCEPTUSES

This study was designed to measure fetal and placental characteristics in bovine day 75 nuclear transfer and control pregnancies. Responses included mRNA concentration of the insulin-like growth factor (IGF) system [IGF-1, IGF-2, IGF1R, IGF2R, IGFBBP-1, -2, -3] and the vascular endothelial growth factor (VEGF) system [VEGF, PlGF, VEGF1R, and VEGF2R]. Fetal attrition of the cloned pregnancies up to day 75 was high (89%, 63 out of 71 frozen embryos transferred; 8 of 16 cloned conceptuses present on day 30 survived to day 75, as did 5 of 5 controls). No significant differences in mean weights of large and medium placentomes were observed between 8 clones and 5 controls. However, the variance of mean weight of large placentomes was greater in clones than in controls; one gestation had placentomes six standard deviations larger than controls. Interestingly, the mean umbilical cord weight/length ratio was significantly greater for clones ($P < 0.05$). Mean fetal length, fetal weight, fetal weight/length index and mean weights for heart, brain, liver, kidneys and the mean brain/liver index did not differ between cloned and control day 75 conceptuses, but numbers per group were limited.

Northern blot analysis, revealed the presence of three transcripts of 3.7kb, 2.2kb and 1.7kb for VEGF and one 1.7 kb transcript for PlGF mRNA in the cotyledons and allantochorion of day 45 cloned and control gestations. All three VEGF bands were present in both cloned and control day 75 cotyledons and caruncles, but the PlGF transcript was barely detectable, except for the cotyledons of one clone.

mRNA for all of genes studied could be detected with real time PCR in day 75 cotyledons and caruncles, and fetal livers contained mRNA for all IGF's and IGFBP's evaluated. In all placentomal tissues, PlGF mRNA concentration was 100-fold less than VEGF mRNA, which seems to be the driving force for placentomal vascularization at day 75. There was a trend for a reduction by half of the PlGF mRNA concentration in caruncle of clones vs. controls ($P = 0.06$). VEGF2R (KDR) mRNA was abundant, but VEGF1R (Flt-1), was only present in very low concentrations; our primer set did not distinguish between soluble versus membrane bound receptor mRNA for VEGF1R.

Four of the cloned conceptuses contained substantially less cotyledonary IGF1R mRNA than the other clones and controls. IGFBP-3 mRNA concentrations were very high in placentomes; IGFBP-1 and -2 mRNA concentration on the other hand was very low for clones and controls. mRNA for IGFBP-1, -2, -3, however, was abundant in day 75 fetal livers, while IGF-1 mRNA was scarce in this tissue. Fetal livers from cloned pregnancies contained 4-fold more IGF-2 mRNA than controls ($P<0.01$). We observed that liver IGF-2 mRNA concentration and liver weight increased with weight of the largest placentome; in clones these increases were associated with a decrease in cotyledonary IGF-2 mRNA, while the opposite occurred with controls. Interestingly, there was a trend to lower IGF2R mRNA concentrations ($P = 0.09$), and IGF-1 mRNA was twofold higher in cotyledons of clones ($P= 0.03$) compared to controls.

For many measurements, means were not significantly different ($P > 0.1$) between clones and controls. However, unequal variances were common, and data points with statistical outlier behavior were observed for clones, which highlights the heterogeneity of the cloned population. This variation in gene expression may exacerbate abnormal placentation later in gestation, and

might explain some of the increased morbidity and mortality seen in calves resulting from cloning by nuclear transfer.

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CHAPTER I: REVIEW OF THE LITERATURE

INTRODUCTION

In this era of biotechnology, mankind reached a major milestone in history with the successful cloning of Dolly, a sheep generated by nuclear transfer from a differentiated, adult mammary cell. When first announced, the public was shocked and in disbelief. Fifteen years later, cloning is still a controversial issue. Cloning ethics, especially in the context of human applications, are debated on an almost daily basis. As with any new technology, there are flaws and pitfalls, and success rates are low. Many reports of gestational and neonatal abnormalities following in vitro embryo culture and asynchronous embryo transfer have been published. Because of the numerous applications this technology holds, we are driven to not only improve the technique, but also to understand the fundamental physiology behind it.

The basic hypothesis that is tested in this thesis is that gestational aberrancies found in pregnancies originating from cloned embryos are due to an abnormal placenta. Gene knockout studies have revealed the fundamental importance of the insulin-like growth factor (IGF) system in the control of fetal and placental development and growth. Since placental vascularization mediates the exchange of nutrients between mother and fetus, and since we are faced with the intriguing question on how the placenta in certain cloned pregnancies can support overgrowth of the fetus, we also evaluated the vascular endothelial growth factor (VEGF) system.

Biometric measurements and evaluation of mRNA expression for the insulin-like growth factor and vascular endothelial growth factor systems were made in placentomes and fetal livers

of day 75 nuclear transfer cloned and control pregnancies. During the first trimester, pregnancy attrition in cloned gestations is very high (Seidel *et al.*, 1997). At day 75 of gestation, placentomal development is well underway, with the largest placentomes centered around the umbilical cord; these tissues have sufficient tcRNA for valid use of a variety of molecular techniques.

We believe that basic information in this area can be applied to reducing the huge amount of pregnancy attrition that occurs with cloning and in vitro fertilization in a number of species, as well as provide information to decrease fetal and neonatal death that occurs with conventional reproduction.

NUCLEAR TRANSFER CLONING

Definition

Cloning can be defined as generating an animal or a set of genetically identical animals descending from the same parent by asexual reproduction. Etymologically, cloning is derived from the Greek word klon or twig which reminds us of asexual reproduction of certain plants e.g. willow trees, potatoes, etc. Monozygous (identical) twins are naturally occurring clones; they are genetically identical, both in nuclear and mitochondrial DNA. In the 1890's H. Driesch experimented with sea urchin embryos. If he obliterated one of the cells of a two-cell stage embryo, a small but complete sea-urchin developed. Conversely, fusion of two early sea-urchin embryos resulted into one giant sea-urchin (Driesch, 1893). The earliest attempt to induce twinning in vitro was done during the 1920's, by splitting a newt embryo using a baby's hair

(Spemann, 1938). With blastomere separation (Willadsen, 1979), cloned sets were limited to two to four identical animals, depending on the species.

In 1986 a major advance in creating larger cloned sets of mammals was achieved: blastomere cloning via nuclear transplantation (Willadsen, 1986). Initially, this involved fusion of a cleavage-stage blastomere containing the donor nucleus with an enucleated oocyte or zygote. Later, pluripotent blastomeres from up to a 16-cell embryo were used as donors, and clutches of cloned embryos could be serially enlarged when the blastomeres of the first round of cloning were separated again and each fused with an enucleated oocyte (Willadsen *et al.*, 1991; Westhusin *et al.*, 1992; Wilson *et al.*, 1995). At that point however, cloning research came to a plateau: the number of totipotent cells in a preimplantation embryo is limited to those in the inner cell mass, and most cells from more advanced embryos or fetuses are differentiated and lose totipotency; at least this was thought to be the case before Dolly. Progressively, nuclear donation from older embryos was possible. However, to expand the potential of cloning, the limitation in cells available had to be overcome.

Embryonic stem (ES) cells are derived from inner cell mass of the early embryo, are relatively undifferentiated, and divide indefinitely in culture, although female cells tend to become aneuploid. Murine ES cell lines were established for certain inbred strains; however, in other species this is still not a straightforward task. Could one redirect the nucleus of a completely differentiated cell to support the development of a new embryo? Ultimately, one decade later this next milestone in cloning was reached. In 1995, personnel at the Roslin Institute, Scotland, created Megan and Morag by nuclear transfer from nuclei from cell lines derived from fetuses and maintained in long-term culture; the cell lines appeared to have differentiated to fibroblasts (Campbell *et al.*, 1996*b*). Only one year later, the same institute

announced the birth of a live lamb "Dolly" produced by nuclear transfer of a donor nucleus obtained from a mammary gland cell of an adult sheep (Wilmut *et al.*, 1997). Although shocking, this was a breakthrough to the concept of terminal cell differentiation and its reversibility.

Cloning procedures

Despite the fact that animals can be cloned by nuclear transfer (NT) from differentiated cells, success rates are low, rarely more than 2% live offspring per successful NT. Much effort has been invested into improvement of all aspects involved of the cloning procedure. The development of embryos reconstructed by NT is dependent upon the stage of the cell cycle and the interactions between the donor nucleus and the recipient cytoplasm.

The steps to clone an animal are depicted in the following flow chart from Infigen:

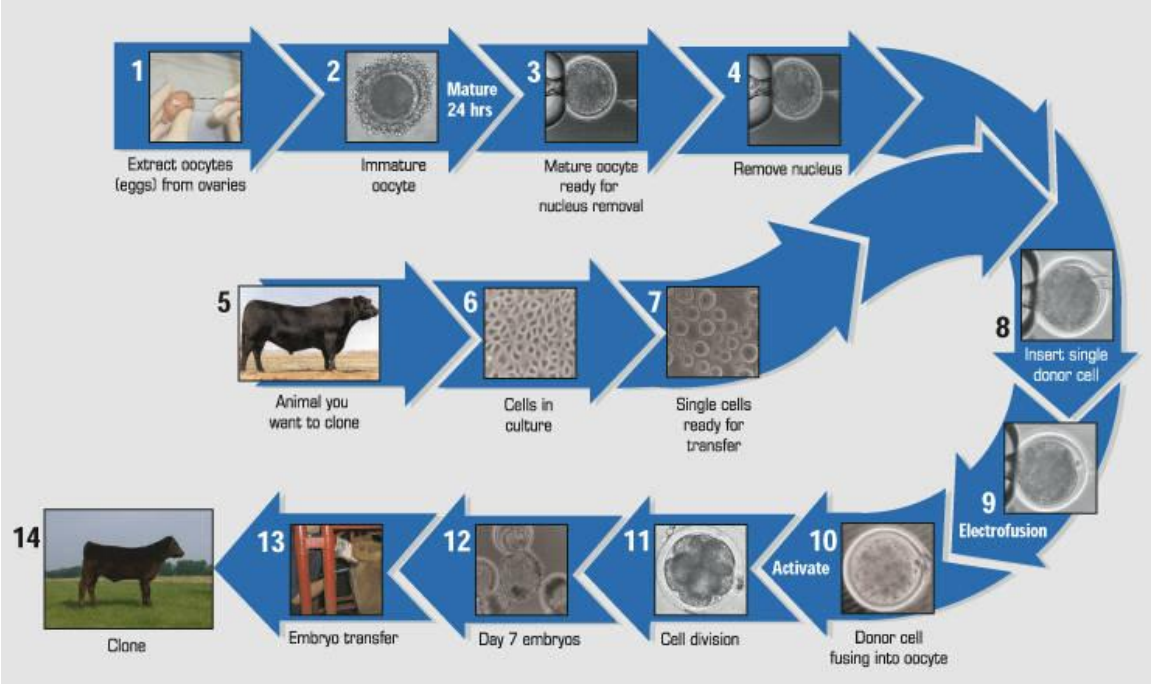

Figure 1.1 Procedures involved in cloning an animal (www.infigen.com)

Understanding these interactions led to two main strategies for cloning. First, when non-activated, enucleated oocytes at metaphase II are used as recipients, the nuclear envelope of the donor nucleus is broken down (NEBD) and the chromatin is prematurely condensed into chromosomes (PCC) due to the high activity of maturation promoting factor (MPF). After parthenogenetic activation, the nuclear membrane reforms, DNA replication begins, and mitosis follows. This technique is only feasible for G_0 or G_1 donor nuclei (2n) since nuclei in G_2 (4n) or M (4n) phases will result in daughter nuclei with 8n DNA content; nuclei in S (2-4n) phase induced to undergo PCC will result in disorganized chromatin. Premature chromosome condensation can cause chromosomal damage or loss and aneuploidy. However, (Cibelli *et al.*, 1998) found that donor cells in G_1 (2n) condense normally in MII oocytes. Metaphase II oocytes have nuclear reprogramming factors present in the cytoplasm; rapid and drastic structural changes including chromosome condensation, spindle formation and protein removal from the DNA helix occur at this time, and allow greater access of these factors. Therefore, sufficient exposure time to the high MPF might be beneficial for nuclear reprogramming (Renard, 1998; Wilmut, 1998).

The second method creates a universal recipient by transferring nuclei into activated ovum cytoplasm, after MPF declines. This oocyte cytoplasm is able to reprogram donor nuclei in any stage of cell cycle. No NEBD and PCC of donor nuclei occur because of the low activity of MPF, and DNA synthesis occurs in relationship to the cell cycle stage (2n or 4n) of the nucleus. This indicates that reprogramming of donor nuclei might occur without NEBD and PCC (Tani *et al.*, 2001). Enucleation of telophase II oocytes is technically easier, removes fewer cytoplasmic factors and organelles, provides a synchronous population of activated recipient cytoplasts, and results in a higher rate of embryonic development (Baguisi *et al.*, 1999).

Cell cycle stage of the donor nucleus and its level of differentiation may facilitate nuclear reprogramming. Quiescent, G_0 donor nuclei have reduced transcriptional activity, and chromatin modifications associated with G_0 may facilitate nuclear reprogramming (Wells *et al.*, 1999). G_0 donor nuclei are compatible with pre-, post or concomitant fusion (Stice *et al.*, 1998). However, Cibelli *et al.*, (1998), suggest that cells should not have ceased dividing (G_0) but be actively dividing, indicating relative undifferentiation. They recommend choosing a cell line that is rapidly dividing but has a long G_1 phase e.g. fetal fibroblasts or artificially arresting cells in G_1. Non-passaged fibroblasts have never successfully generated progeny because: 1) they are nonconfluent; thus fewer cells are in G_0/G_1 phase; 2) the cell population is less homogenous, and cell type affects cloning competence (Dinnyés *et al.*, 2001).

Another major factor in the low efficiency of cloning is incomplete activation of the recipient oocyte. During fertilization, sperm-induced periodic increases in intracellular free Ca^{2+} concentrations activate the oocyte (White & Yue, 1996). Artificial activation methods fail to mimic completely the transient Ca^{2+} oscillations, and usually result in a single intracellular Ca^{2+} rise. A sequential treatment of an intracellular Ca^{2+} transient and a temporary inhibition of protein phosphorylation by 6-dimethylaminopurine (DMAP) effectively activates bovine oocytes. This combination treatment results in a rapid and sustained inactivation of maturation-promoting factor and hence, the activation of the oocyte (Dinnyés *et al.*, 2001).

Developmental potential of reconstituted embryos depends upon reprogramming gene expression by the action of cytoplasmic factors, and this might be enhanced by a prolonged period of exposure (Campbell *et al.*, 1996*a*). According to (King *et al.*, 1996), nuclear reprogramming does not occur immediately following NT, but occurs gradually over the first 2 or 3 cycles. Lavoir et al., (1997) found reprogramming of the transferred nucleus is absent or

incomplete, and aberrant cytokinesis and nucleokinesis, and multinucleated, anucleate and polyploid cells were common. There are different approaches to evaluate nuclear reprogramming, e.g. by examining nuclear lamin epitopes, nucleolar morphology and protein synthesis.

In conclusion: there are two methods to obtain viable cloned offspring by nuclear transfer, and in both cases the cell stage of donor cell and recipient oocyte have to be synchronized.

Identical genetics?

The controversy of cloning from adult cells raised questions and criticism. Was Dolly indeed genetically identical to her mother; and why are clones not identical?

One issue is mitochondrial mosaicism or heteroplasmy. Mitochondrial DNA (mtDNA) encodes genes essential for oxidative phosphorylation and electron transfer, and is inherited maternally (Kaneda *et al.*, 1995). Thus, embryos originating from fusion of a donor cell (containing some cytoplasm) and cytoplasm of an oocyte contain mitochondria from two sources if donor and recipient cells are from different maternal lines. Hiendleder *et al.*, (1999) found that mtDNA haplotype heteroplasmy in adult bovine clones can vary from 21-79%. This suggests that the mtDNA type of donor and recipient cells must be controlled if clones are to have identical nuclear and cytoplasmic genomes. Reports on cytoplasmic genetic effects on phenotypic traits in cattle have been linked to mtDNA polymorphisms (Schutz *et al.*, 1993). Moreover, it has been reported that the in vitro developmental ability of mouse embryos was impaired by heterogenous mtDNA, introduced by nuclear transfer (Mereilles & Smith, 1998; Nagao *et al.*, 1998).

Chromosomal abnormalities, such as mixoploidy and polyploidy, are frequently observed in in vitro produced embryos (Viuff *et al.*, 2001). Pre- and postnatal environment highly affect phenotype. For example, a thoroughbred horse embryo transferred into a cold blood mare will look much more bulky at birth than if born from a thoroughbred mare. Oocyte donor cytoplasmic effects can also play part in the nuclear transfer cloned offspring.

Another question is whether cloning is a "fountain of youth"? Can cloning reverse cellular aging or will nuclear cloned offspring undergo premature senescence? Normal somatic cells of some species display limited division potential. However, germline cells, "immortalized" tissue culture cell lines, and cancer cells have overcome the limitations of cellular replication and aging. For example, aged parents always give birth to offspring containing young cells.

Telomeres are specialized structures at the ends of chromosomes of nearly all eukaryotes, composed of repetitive short DNA sequences and associated telomeric binding proteins, which serve as buffer zones against telomeric attrition and prevent DNA ends from combining with each other and producing aneuploidy. With each round of cell replication, incomplete duplication by DNA polymerase of the 5´ ends of linear DNA molecules occurs; this loss of terminal DNA sequences results in the shortening of chromosomes. The shortening of telomeres may be one of the limiting factors that blocks continued cell division. A common characteristic of cells such as germline cells is enzymatic activity of the ribonucleoprotein complex telomerase that maintains telomeres at full length (review Meeker & Coffey, 1997). Controversy exists whether nuclear transfer to oocytes with non-telomerase expressing donor cells reconstitutes telomeric length. Could the epigenetic change of telomere shortening limit donor success of adult cells (Shiels *et al.*, 1999). The findings of Lanza *et al.*, (2000) suggest that nuclear transfer

extended the replicative life span of senescent cells and telomere length in animals cloned from senescent somatic cells were extended beyond age-matched controls. This ability of cell regeneration has important impact on aging studies and cloning for in vitro tissue and tissue or organ replacement. In this context, it is important to note that nuclear transfer cloning is more efficient with embryonic and fetal cells than with adult cells; this might be due to decreased and variable telomere length. Random selection of nuclei may yield those with sufficiently long telomeres only rarely, which may explain why few adult cells are successful donors (Anderson & Seidel, 1998).

Imprinting

It has been suggested that the success of cloning depends on proper epigenetic reprogramming of transferred nuclei (Santos *et al.*, 2003). Imprinting is an epigenetic mechanism by which the expression of on the order of 100 genes is dependent on whether they are inherited from the mother or father (sex of parent allele-specific expression) (Feil *et al.*, 1998). Evolution of imprinting is most likely linked to intrauterine fetal development since only eutherian mammals distinguish certain genes by imprinting (Killian *et al.*, 2000). Imprinting makes both parental genomes essential for normal embryonic growth. This is proven by many studies with parthenotes and androgenotes. Parthenogenotic embryos have poor trophoblastic tissues, while andrenogenotic conceptuses have underdeveloped embryos and prolific trophoblasts (Kono, 1998). The current theory holds that imprinting has evolved to control a "parental conflict of interest" (Moore & Reik, 1996). When the fetus develops, nutrients are supplied by the mother. It is imperative for the fetus that the mother's well being is not impaired by its growth and it is important for the mother that this pregnancy does not compromise future

gestations. On the other hand, especially in non-monogamous species, the paternally derived alleles in the fetus will demand more resources from the mother since subsequent pregnancies might be from a different father. Therefore, many genes important in fetal and placental growth and development are imprinted. Paternally imprinted genes tend to favor fetal growth while maternally expressed genes will act restrictive (Young *et al.*, 2001). Thus paternally and maternally imprinted genes oppose each others' effects on fetal growth, for example IGF-2 is imprinted paternally, while IGF2R is imprinted maternally.

All epigenetic modifications are erased in primordial germ cells, and new sex specific imprints are established during gametogenesis in the male and female germ-line (Surani, 1998). During oocyte growth, maternal modifications will affect primary imprinting with crucial downstream effects on regulation of expression of maternal alleles during embryogenesis. This was observed in a study with parthenotes from either neonatally derived non-growing oocytes or fully grown oocytes (Obata *et al.*, 1998).

In the zygote, shortly after fertilization and before DNA replication, the paternal genome undergoes genome-wide demethylation. The maternal genome, on the other hand, undergoes a stepwise demethylation during the first mitotic divisions (Dean *et al.*, 2001) that is less radical. De novo methylation of non-imprinted genes occurs in bovine embryos at the 8-16 cell stage (Dean *et al.*, 2001), coinciding with the major wave of transcriptional activation of the embryonic genome (Memili & First, 2000) and the first differentiation event (trophectoderm vs. intracellular mass (ICM) cells). In contrast, with the mouse where de novo methylation is limited to the ICM, bovine extraembryonic tissues are highly methylated (Dean *et al.*, 2001). It is important to keep in mind that primary imprinted genes are not demethylated or de novo remethylated during preimplantation development. This provides a basis for the differential

susceptibility of imprinted and non-imprinted genes to environmental influences in the preimplantation period (Moore & Reik, 1996). Since imprinted genes are expressed mono-allelically, they may be more vulnerable to mutations or epimutations (Young, 2001).

Tucker *et al.*, (1996) have shown that ES cells can reestablish normal methylation and expression patterns of non-imprinted genes, but germ line passage seems to be required to restore those of imprinted genes. It is important to note that imprinting is not entirely uniform. Functionally active epigenetic imprints are under spatiotemporal plasticity, and cell-type specific modification allows for tissue-specific imprinting patterns (Franklin *et al.*, 1996).

Severity of consequences of disruption of imprinting depends on the time point in development and the number and location of cells affected. To determine if early embryonic epigenetic alterations in imprinted genes persist in later development, and are associated with aberrant phenotypes, Dean *et al.*, (1998) established a model based on embryonic stem (ES)-cell derived murine fetuses. ES cell lines were evaluated for altered methylation patterns in four genes (IGF2R, H19, IGF-2 and U2af1-rs1) known to be imprinted. All changes in methylation that occurred during ES cell culture, persisted in the derived ES fetuses, and 2 of the 4 ES-lines resulted in compromised fetal development e.g. polyhydramnios, poor mandibule development and interstitial bleeding. In chimeric fetuses, the degree of chimerism correlated with increased body weight (Dean *et al.*, 1998).

Are the nuclei from some differentiated cells able to regain totipotency? Is the tissue specific imprinting erased when exposed to the cytoplasm of the recipient oocyte? The genomic imprints are not reset as in the germ-line. Does the artificial environment of in vitro culture and manipulation affect the remodeling of the chromosomes? Doherty *et al.*, (2000) found that culture conditions can selectively affect the expression of genomically imprinted genes in

preimplantation mouse embryos. Specifically, the normally hypermethylated and repressed paternal H19 allele lost its methylation and was aberrantly expressed in Whitten's medium, but not so in KSOM medium.

Dean and colleagues (2001) found the global epigenetic reprogramming of bovine somatic nuclei to be aberrant in most preimplantation embryos. In their study, they observed the donor nuclei to be considerably demethylated in the recipient oocyte within hours of introduction and activation. Whether the differential methylation in imprinted genes is protected at that point is unknown. No further passive demethylation during the first mitotic divisions was observed, and many cloned embryos underwent precocious de novo methylation and transcriptional activation at the 4-8 cell stages (Dean *et al.*, 1998, 2001). These observations might be related to improper silencing or non nuclear exclusion of methyltransferase (Dnmt) genes (Dean *et al.*, 2001). A more recent study from the same laboratory showed that fewer blastomeres had a normal methylation pattern in NT embryos from relatively more differentiated fibroblasts compared to those from less differentiated granulosa cells; and this observation correlates with the proportion of NT embryos developing into blastocysts (Santos *et al.*, 2003).

Not only are epigenetic changes with deteriorating developmental consequences detected in the embryonic stage, but also, Young *et al.*, (2001) found that the reduction in expression of IGF2R in day 125 sheep fetuses derived from in vitro culture with an overgrowth phenotype corresponded with an epigenetic change in the gene.

In conclusion, reprogramming by ooplasmic gene modifiers during fertilization and early embryogenesis is quite labile, and its interruption is likely to affect the developmental program (Kono, 1998). Moreover, epigenetic variation in donor cells along with partial reprogramming

results in heterogeneity of epigenetic marking, which is responsible for the developmental potential of cloned embryos (Santos *et al.*, 2003).

Cloning applications and commercial opportunities

The unexpected incidence of abnormal calves from embryos by in vitro manipulation resulted in the swift collapse of agricultural application of cloning. Wide application of IVF in cattle also has stalled in part due to calf abnormalities. The European Community is considering banning IVF and cloning applications with farm animals on the basis of animal welfare considerations (Sinclair, 1998). These events clearly demonstrate that problems with abnormal calves must be solved if these, and related biotechnologies, are to be used in production agriculture. As important as research on this problem is in the context of applying new biotechnologies, obtaining basic physiological understanding from this model may be even more important. Moreover, the gestational abnormalities described in gestations from clones, also occur with natural breeding and artificial insemination, albeit at low incidence, on the order of 1% of calves. However, this equals half a million calves in the US and Canada annually, a severe economic loss. In addition, annual calf death loss approximates 6-10%. Half of the neonatal calf deaths occur during the first two days of life, of which the majority cannot be attributed to infectious disease but rather physiological disturbance (Garry, 1999).

In the cattle industry, artificial insemination (AI) is a multibillion dollar business. With the introduction of this technique, milk production has been genetically increased by 30% over 30 years (Lewis *et al.*, 1998). With cloning, a major change is to come in the artificial reproduction industry (Lewis *et al.*, 1998). The goal in agriculture is to produce clonal lines of

genetic identical animals for increasing genetic gains (Lewis *et al.*, 1998), and therefore improving efficiency of food production.

One envisions that if cloned embryos would be available at $30.00 each with a 50 % calving rate per embryo, it would be economically feasible for farmers to switch to embryo transfer instead of routine AI (McClintock, 1998). Current laboratory costs to produce a large number of genetically identical cloned embryos are $15.00 per blastocyst, but the calving rate is only 17% (Lewis *et al.*, 1998). Therefore, at this point in time, cloning technology in agriculture is only economically feasible for elite, performance tested animals used for cattle shows and AI service. Thus cloning for agricultural purpose will become more attractive when efficiencies increase and costs come down.

Potential limitations to agricultural applications of cloning include: 1) the source of ooplasm and its labor cost to collect oocytes. To generate 100,000 live cloned offspring, one needs ovaries from 650,000 cows (Lewis *et al.*, 1998)! Endeavors to automate oocyte collection are underway (Lewis *et al.*, 1998); 2) Public perception: confusion with transgenics, e.g. public adversity to genetically modified crops in Europe (McClintock, 1998); 3) one needs to eliminate abnormal calves obtain conception rate as with normal breeding without an increase in perinatal mortality (Wilmut, 1998). The use of large scale agricultural cloning implies the risk of inbreeding in future generations. However, McClintock (1998) argues that if done correctly, this large scale control over breeding can actually increase the heterozygosity of the current dairy herd. Only a certain percentage of the herd would carry replacement offspring, while others can be used to produce crossbreds. Furthermore McClintock insists that it is the responsibility of the country to establish gene banks to prevent loss of genetic variation; the farmer should not be expected to be the curator of a museum!

In combination with transgenic procedures, nuclear transfer cloning can more easily generate transgenic farm animals with defined and commercially useful genotypes (Schnieke *et al.*, 1997; Anderson & Seidel, 1998). Cloned transgenic founder animals (nuclear transfer transgenics) can be generated as follows: bovine cell lines derived from embryonic or fetal origin (fibroblasts) would be isolated and propagated, and a gene of interest inserted along with a positive selection gene. Bovine cells expressing the construct would then be selected and propagated. Transgenic cell lines serve as donor nuclei, resulting in cloned transgenic embryos, fetuses and offspring (Stice *et al.*, 1998). This technique can be used to express pharmaceutical proteins in milk; for pharmaceutical protein production, the inefficiency of the process is offset by the high value of pharmaceuticals that can be made from relatively small numbers of animals (McClintock, 1998). Another highly touted commodity is nuclear transfer transgenic pigs for xenotransplantation. Demand greatly exceeds supply for human replacement organs such as heart valves, pancreas, etc. Because of their size, porcine organs are the best fit for humans. A big issue in organ xenotransplantation is tissue rejection because of histocompatibility complex issues. Therefore nuclear transfer knockout pigs for the alpha 1,3-galactosyltransferase epitope, foreign to humans may prevent hyperacute rejection in xenotransplantation (Wilmut *et al*., 1998).

Other benefits for the pharmaceutical industry from nuclear transfer (transgenic) (farm) animals include models for human genetic disease (e.g. cystic fibrosis in sheep, transgenic mice for the evaluation of gene therapy or novel small molecule based therapy) (Wilmut *et al*., 1998). Furthermore, if drugs can be tested in batches of cloned animals, one greatly reduces the genetic variability between individual animals, therefore increasing the power of the study and

decreasing the number of subjects needed. The utility of this approach is clear from use of inbred lines and their crosses in a few species.

Cloning can also benefit human medicine. For gene therapy and organ transplantation we ideally need recipient-specific multipotential cells in order to prevent immunological rejection. Therefore, if one can produce embryonic stem cells from cloning a patient's somatic cell, these cells might be ideal candidates for these new therapeutics (Trounson, 1997). For example, human fetal neural tissue is highly sought after for transplantation therapy for Parkinson's disease (Stice et al., 1998).

One last application of cloning involves reproduction of species threatened by extinction as well as reproduction of precious pets. One just needs to keep in mind that genetic identity by no means ensures identity of personality or temperament.

THE PLACENTA

The placenta has a pivotal role in transfer of essential substrates from the mother to the fetus and removal of waste products; it also is responsible for mediating and/or modulating the maternal environment (Owens, 1991; Bauer *et al.*, 1998). Birth weight depends primarily on the rate of fetal growth, and is highly correlated with placental weight (Alexander, 1964). Phylogenetically, placental membranes (amnion, chorion, allantois, yolk sac) are relatively young structures; appearing with development on land instead of in water. Early in development, certain cells are assigned to the development of these tissues for embryonic nutrition and protection in both oviparous birds and viviparous mammals. Eutherian species develop a placenta in which the maternal and fetal circulations are brought in close juxtaposition

for purposes of exchange (Zumkeller, 2000). Etymologically the word placenta is Latin in origin, and means “flat cake” in reference to the discoidal placenta of humans. However, placental morphology varies greatly between species, and multiple classifications of placental types are described. With deciduata (primates, rodentia, carnivores), interdigitation of fetal and maternal tissues is intense. Upon trophoblastic invasion, the endometrium responds with the formation of large polygonal cells named decidual cells. Outgrowth of fetal tissue (the placenta vera) into maternal tissue allows for an intense exchange in a relative small surface area. At the time of afterbirth expulsion, a great part of maternal endometrium (the decidua) is also expelled. In contrast, in other species, adeciduata appose the chorion to the endometrial tissue without damaging the endometrial epithelium greatly. Therefore, they are named placenta apposita (e.g. placenta diffusa, placenta cotyledonaria). At birth, the pars fetalis separates from the pars maternalis without much loss of maternal tissue with expulsion of the afterbirth. Depending on the intensity of fetal and maternal tissue intergrowth, placentae usually are classified anatomically as follows (Grosser, 1909, 1927):

- Epitheliochorial placenta (e.g. horse, pig): fetal and maternal epithelium remains intact. Nutrients from the maternal circulation are transferred through six layers in order to reach the fetal circulation: maternal capillary endothelium, maternal endometrial connective tissue (stroma) and maternal endometrial epithelium, fetal epithelium (trophoblast), chorionic connective tissue or mesenchyme and endothelium of the fetal capillaries.
- Syndesmochorial placenta (e.g. sheep, goat): in this case there is a disappearance of the endometrial endothelium.
- Endotheliochorial placenta (e.g. carnivores): the fetal villi penetrate to reach the maternal capillaries, endometrial epithelium and stroma are strongly reduced.

- Hemochorial placenta (e.g. humans, rabbits, rats, mice, guinea pig): The trophoblast (of the chorion) is in direct contact with the maternal blood. The trophoblast consists of at least one layer of syntrophoblast.

When based upon size and shape of the contact interface, five types of placentae are described:

- Complete diffuse placenta: the entire chorionic surface covers the total endometrial surface and the chorial villi are diffusely dispersed (e.g. horse).
- Incomplete diffuse placenta: idem supra, except the ends of the gestational sacs are villus free (e.g. pig).
- Cotyledonary placenta: placentation only occurs in specific areas of the chorion at the level of the caruncles in the endometrium. Chorial villi of the cotyledons interdigitate and branch into the caruncular crypts and form placentomes. Depending on the number of placentomes, placentas are termed polycotyledonary (e.g. cow, ewe, goat, respectively 75-120; 80-100; up to 160) or placenta oligocotyledonary (e.g. deer: 10-12 placentomes).
- Zonary placenta: the chorio-endometrial interface forms a girth (e.g. carnivores).
- Discoid placenta: placentation is limited to a disc shaped zone, relatively small compared to the entire chorionic surface (e.g. rodents, primates).

Furthermore placental types can be distinguished based upon the organization of the interdigitation of maternal and fetal blood vessels (folded, lamellar, villous, trabecular, labyrinth), and the relation of blood flow within maternal and fetal capillaries (concurrent, countercurrent, crosscurrent, multivillous) (Benirschke & Kaufmann, 1995; Orbus, 1999).

Bovine placentation and embryonic development

The bovine placenta is apposita, epitheliochorial and polycotyledonaria, and therefore belongs to the adeciduata. In an excellent review, Schlafer *et al.*, (2000) describe bovine placental development. Blastulation occurs, and the outer ectodermic layer of the expanded blastocyst consists of trophoblast cells and is referred to as "trophoblast" or more specifically, trophectoderm. The endodermic cells lining the inner side of the blastocoele will become the yolk sac and the embryonic disc will develop into the embryo. In sheep and cattle, the yolk sac consists of an enlarged sac-like structure below the embryo and two tubal ends that elongate with the trophoblast. At first an open connection between the yolk sac and intestines exists that later closes. The wall of the yolk sac is well vascularized, and primary erythropoeisis occurs within these capillaries (Russe *et al.*, 1992). The vascularized yolk sac supports fetal development until day 35 of gestation ((Thompson & Peterson, 2000). After gastrulation, the outer trophoblast layer combines with the somatic/parietal mesoderm (derived from the embryonic disc) to form the chorion. The amnion is formed by folding of the chorion around the embryo. Upon fusion (by day 16), the embryo is enclosed in a membrane lined by amniotic cells. Part of the amnion dorsal to the developing fetus will gradually fuse with the chorion and form the amniochorion. The allantois develops from a diverticulum of the embryo's hindgut by day 20 and fills the entire exocoelome during the 4^{th} week. Eventually, the chorion and the vascularized allantois appose and become allantochorion or chorioallantois. This apposition will provide vascularization to the chorion which originates from the mesodermal component of the allantois (Green & Winters, 1945; De Sousa *et al.*, 2001).

At about 10 days after fertilization, the embryo starts to elongate rapidly; at 13-14 days it will reach 1-4 cm and by 16 days will be 15-20 cm or more in length. By day 20-22, the

elongating embryo will reach the tip of the non-gravid horn. This elongation is important for communicating pregnancy signals to the mother to prevent luteolysis. On days 17-18 the embryo will become tightly apposed to the uterine endothelium. Raised areas of non-glandular, well vascularized endometrium, termed caruncles, are always present in the uterus of the cow (Orbus, 1999). Between day 22 and 30 (Melton *et al.*, 1951), the chorioallantois will begin to become irregular over the caruncular endometrial zones, and villi will form and protrude and eventually colonize the recesses in the caruncular surface (Schlafer *et al.*, 2000). The caruncle will develop crypts, and the apposing chorioallantoic villi will interdigitate and branch within, thus increasing surface contact area and forming placentomes. Villi in the intercaruncular zones are rudimentary in development but soon disappear (Lauwers, 1991). By 60 days, cotyledons and caruncles are firmly attached (Eley *et al.*, 1978), but will keep developing and become stalk-like halfway through gestation. The placentomes appear in an organized pattern: four rows lengthwise along both horns (Schlafer *et al.*, 2000). At term, the placentomes have a domed-ovoid shape about 10-12 cm in length and 2-3 cm in thickness (Schlafer *et al.,* 2000). The largest and most advanced developed placentomes are found in close proximity to the umbilical cord; smaller placentomes are found in the non-gravid horn and closer to the tip of the uterine horn. Total placentome numbers vary from 70-120. The total contact surface area, taking into account interdigitation, is estimated to be 130 m^2 (Russe & Sinowatz, 1991; Schlafer *et al.*, 2000).

Histologically, the caruncle consists of capillaries and connective tissue surrounding branching crypts. Groups of crypts are separated by thin septa. The crypts are covered with a monolayer of a low cylindrical epithelium and open up to the convex side of the caruncle. The crypt epithelium carries microvilli that interdigitate with the microvilli of the trophoblast (Lauwers, 1991). The cotyledon covers the convex side of the caruncle and carries branched

chorial villi grouped in small clusters on its concave side (Lauwers, 1991). The axis of the chorial villi consists of connective tissue and capillaries covered with a continuous monolayer of trophoblast cells. The trophoblast cells carry microvilli and perform pinocytosis. When maternal erythrocytes accumulate between the endometrium and chorioallantois, they are phagocytosed by trophoblast cells as a significant source of iron (Schlafer *et al.*, 2000). In the ruminant placenta, the vast majority of trophoblastic giant cells are binucleate, and they compromise approximately one fifth of the bovine trophoblast cells (Wooding *et al.*, 1997; Klisch *et al.*, 1999; Schlafer *et al.*, 2000). They are formed in the chorion and are derived from mononuclear trophoblast cells by a single, acytokinetic mitosis (Wooding & Wathes, 1980; Klisch *et al.*, 1999). The binucleate cells are larger than the surrounding cells, carry two nuclei, no microvilli, do not have junctions with surrounding cells, and synthesize numerous granules. Even though undistinguishable microscopically, many subpopulations have been identified by lectin cytochemistry (Munson *et al.*, 1989; Jones, 1994; Schlafer *et al.*, 2000). Binucleate cells produce steroid and protein hormones e.g. progesterone (Reimers *et al.*, 1985); bovine placental lactogen (bPL) (Duello *et al.*, 1986), pregnancy associated glycoproteins (bPAG-1, bPAG-2, bPAG-3) (Roberts *et al.*, 1995), and transforming growth factor beta (Munson *et al.*, 1996). The large granulated binucleate cells appear at first in the chorionic epithelium when the embryo is 16-17 days old ((Greenstein *et al.*, 1958; Bjorkman, 1968; Wooding & Wathes, 1980); at about 20 days, migration of the binucleate cells into the maternal epithelium begins (Wooding & Wathes, 1980). They translocate from the chorionic layer on the fetal side, cross the tight junctions, and penetrate the epithelial monolayer lining the caruncular crypts to reach the basement membrane where they produce transient trinucleate cells and release their granules by exocytosis (Wooding & Wathes, 1980; Wooding, 1992). Subsequently, the cell condenses to a

remnant that is phagocytosed by the chorionic epithelium (Lee *et al.*, 1986). This is in contrast with the sheep where binucleate cells fuse to form a syncitial layer (Lee *et al.,* 1986).

Granule transfer and delivery seems to be the primary function of binucleate cell migration (Wooding & Wathes, 1980; Wooding, 1992), and this mechanism might be required to transfer large and non-diffusible molecules across the placental barrier (Wooding & Wathes, 1980). With the description of fetal binucleate cell migration and fusion and the persistence of a uterine epithelium, the new terminology of "synepitheliochorial" placenta is suggested (Wooding, 1992).

The intercaruncular region of the endometrium is glandular with little vascularization (Amoroso, 1952; Orbus, 1999). The endometrial glands secrete a protein/lipid rich uterine milk which is phagocytosed by trophoblast cells. This histotrophic nutrition is present throughout gestation, but especially significant during the first 40 days of gestation (Melton *et al.*,1951); thereafter, placentomes have established efficient hemotrophic nutrient and waste product exchange.

The umbilical cord in the cow is relatively short and consists only of amniotic tissue. As described by Robinson & Abuhamad (2000), the umbilical cord is established with the formation of the amniotic cavity and the curling of the embryonic disc around the regressing yolk sac. The ectoderm-amnion border becomes the primitive umbilical ring on the ventral surface of the embryo. The umbilical ring contains the allantois, umbilical vessels, extraembryonic coelom, the vitelline duct and the vitelline vessels. As the amniotic cavity enlarges, the contents of the ring are compressed and elongate to form the umbilical cord. Remnants of the yolk sac are incorporated into the umbilical cord and disappear halfway through gestation. Similarly, the allantois, vitelline duct and vitelline vessels become obliterated. Thereafter, the umbilical cord

remains, consisting of 2 umbilical veins and 2 umbilical arteries and the urachus canal covered by amnion and surrounded by Wharton's jelly (a mucopolysaccharide gel). Before entering the navel of the fetus, the 2 umbilical veins become one. At parturition, the umbilical arteries retract to the top of the bladder and close by contraction of the tunica media. The umbilical vein remains in the umbilical remnant and will dry up and fall off (Lauwers, 1991).

Placental vascularization

Uterine vascularization is supplied by the small ovarian arteries and the large middle artery (Hansel & Asdell, 1951). The middle uterine artery ramifies into numerous primary branches in the broad ligament which give rise to a number of arcuate arteries that encircle the uterine horn. "T"-shaped arterioles branched from the arcuate arteries vascularize the outer muscular layer. The endometrium is supplied by complex branches and sub branches of the arcuate arteries. Caruncular arterioles are more tightly coiled and possess unusually thick walls containing many elastic fibrils (Hansel & Asdell, 1951). These same arterioles will be responsible for the maternal vascular system of the placenta (Carter, 1975).

The fetal placental vascularization is derived from the umbilical cord. Two branches, each containing an umbilical artery and vein, extend into respective horns (Steven, 1968) and branches from the umbilical arteries and veins vascularize each placentome (Carter, 1975). The microvascular architecture of the bovine placenta is of the villous type. The placentome of the cow is mushroom shaped, and fetal villi are located within crypts, surrounded by maternal septa. The fetal villous trees are conical in form (wide base, thinning out to the top), creating a relatively short distance of vessels from the chorionic plate to the capillary complex. The maternal blood vessels are enclosed in septa and disperse into capillaries on the surface of the

crypts and caruncle (Tsutsumi & Hafez, 1966). The fetal villous tree is subdivided into segments of stem villi, intermediate villi, and terminal villi (Kaufmann *et al.*, 1979; Kaufmann, 1982; Leiser *et al.*, 1997). In accordance with the villar organization, vessels are described as stem arteries and veins, intermediate arterioles and venules, and terminal capillaries (Leiser, 1987; Leiser *et al.*, 1997). The caruncular stalk consists of narrow arteries and veins spiraling (Leiser *et al.*, 1997) towards the caruncular basal plate where they are interwoven into a dense connecting vasculature. From this basal plate, stem vessels radiate to form septae, which end in peripherally located capillary complexes.

The course of fetal as well as the maternal arteries and arterioles brings the two capillary systems into close apposition. Stem villi consist of one centrally located artery accompanied peripherally by several veins; this pattern is similar for the intermediate villus. In the neck of the terminal villi, capillary loops are arranged asymmetrically. One shorter arterial limb situated centrally of the terminal capillary system meets with a few longer, more convoluted venous capillary limbs in the periphery. Maternal capillaries surround the fetal capillary in a basket-like fashion (Leiser *et al.*, 1997). Within each cryptal/villous unit, blood flow conditions are cross-current, but the overall arrangement is more of a countercurrent flow because maternal as well as fetal stem arteries reach out to the fetal and maternal sides of the placentome, thus orienting both capillaries in opposite ways (Leiser *et al.*, 1997).

Maternofetal exchange occurs in the capillary complexes (Kaufmann *et al.*, 1979; Benirschke & Kaufmann, 1995), and the more voluminous the capillary complex, the more efficient the process. However, the shorter the venules and veins are, the faster oxygenated, nutrient rich blood can be transported to the fetus. Therefore, smaller villous trees are more efficient. And, as Leiser *et al.* (1997) concluded, the larger the term ruminant placentome, the

smaller the effect for substantial exchange. However, the bovine mushroom shaped placentome with conical villous units favors short routes of supply for voluminous capillary beds over the cup-like placentome of goat and sheep (Tsutsumi, 1962). This is also reflected by a weight ratio of neonate to placenta of 13:1 in the cow vs. 10:1 in sheep or goat (Dantzer *et al.*, 1988; Kaufmann, 1990). One interesting phenomenon is that in the intermediate villi, one arteriole is surrounded by up to eight very thin-walled venules in a countercurrent setting. This extensive venular system conspicuously slows down the blood return to the fetus, but allows for reabsorption of substances. This might be of functional importance for distribution of hormones produced by the placenta (Leiser *et al.*, 1997).

Initially, placental growth exceeds that of the fetus. The instantaneous growth rate of the placenta and cotyledons reaches a maximum at 207 and 202.5 days of gestation respectively, while peak growth rate of the bovine fetus occurs at 230 days (Prior & Laster, 1979). The increase in placental weight during gestation is attributed to both the steady rise of uterine tissue weight as well as the rapid expansion of the fetal villi into maternal stroma (Barcroft & Barron, 1946; Davies & Wimsatt, 1966; Stegeman, 1974; Ehrhardt & Bell, 1995; Orbus, 1999). The initial rise and decline in placental wet weight has been attributed to Wharton's jelly (a mucopolysaccharide), which contributes to the dramatic initial expansion of connective tissue in the villi. Throughout gestation, this connective tissue will be replaced by continuous remodeling of vascular architecture of the placentome (Barcroft & Barron, 1946; Wimsatt, 1950; Stegeman, 1974; Ehrhardt & Bell, 1995; Orbus, 1999). Both maternal and fetal genotype (breed, e.g. Charolais vs. Brahman) affect placentomal and caruncular weight, but cotyledonary weight is only influenced by fetal genotype (Ferrell, 1991*a*).

FETAL GROWTH

The availability of nutrients to the fetus is mediated by the placenta and greatly affects fetal growth (Bauer *et al.*, 1998). Birth weight depends primarily on the rate of fetal growth, and is highly correlated with placental weight (Alexander, 1964). Ferrell (1991*b*) suggests that fetal growth may be limited by uterine blood flow and by function of the uteroplacenta, especially approaching term. Birth weight of twin fetuses is reduced in comparison with singletons, partly due to the reduced blood flow per fetus perfusing the placenta (Ferrell & Reynolds, 1992). Both maternal and fetal genotype influence fetal growth (Ferrell, 1991*a*). The maximal rate of increase in bovine fetal weight occurs around 230 days of gestation (200g/day) (Eley *et al.*, 1978; Prior & Laster, 1979). In late gestation, a decrease in bovine fetal growth rate is observed (Ferrell *et al.*, 1976; Eley *et al.*, 1978); either the placenta cannot sustain the absolute growth rate or, alternatively, hormonal signals from the fetus and/or the placenta may decrease fetal growth rate (Eley *et al.*, 1978). After elongation, growth is due initially to an increase in cell numbers (hyperplasia) with a constant individual cell size. As development continues, growth is due to both hyperplasia and hypertrophy, but later primarily to hypertrophy (Winnick & Noble, 1965; Prior & Laster, 1979). In the bovine fetus, hyperplasia occurs throughout gestation based upon increasing total DNA per fetus, protein/DNA and RNA/DNA ratios, near term (Prior & Laster, 1979). Growth by hypertrophy as indicated by protein/DNA and RNA/DNA ratios continue to increase with increasing fetal age (Prior & Laster, 1979). Growth due to hyperplasia is lower than growth due to hypertrophy; as fetal age increases, retardation of fetal growth later in gestation should have less severe effects on subsequent neonatal development (Prior & Laster, 1979).

GESTATIONAL ABNORMALITIES OBSERVED WITH CLONING

What causes pregnancy attrition?

Manipulation of gametes and preimplantation embryos through in vitro culture, in vitro fertilization, nuclear transfer, etc. can influence differentiation and growth at later stages of development and may cause fetal and neonatal abnormalities (Dean *et al.*, 1998). Culture of cloned embryos may cause an imbalance in the distribution of cells to the trophoblast relative to the inner cell mass (Rieger, 1998). The presence and concentration of serum, glucose, ammonium ion and growth factors in the culture medium all may affect one or more factors in embryonic growth: mitosis, apoptosis, mRNA stability, imprinting, transcription, gene expression and protease activity. For example, high levels of glucose or accumulation of ammonium from amino acid breakdown in cell culture medium result in fetal retardation (De Hertogh *et al.*, 1992; Lane & Gardner, 1994). Often, in vitro manipulations result in abnormal embryogenesis, overgrowth, lengthened gestation, increased abortion, and increased perinatal and postnatal mortality (Kono, 1998). However, one can obliterate the gestational abnormalities seen with in vitro produced, but not cloned bovine embryos, if the embryos are first transferred to the oviduct of sheep and then transferred at the blastocyst stage into heifers (Behboodi *et al.*, 1995). On the other hand, when in vivo matured and fertilized ovine eggs were cultured in vitro for 6 days, an increase in mean birth weight was observed, depending on the culture system used (Sinclair, 1998).

In the past decade, several reports of gestational and neonatal abnormalities following in vitro embryo culture, asynchronous embryo transfer, nuclear transfer, early maternal exposure to excessively high progesterone (Kleemann *et al.*, 2001) and changes in protein content of

maternal diet (Perry *et al.*, 1999; Wallace *et al.*, 1999), have been published. The "large offspring syndrome" (LOS) in cattle and sheep, was first identified by Willadsen *et al.*, (1991) who reported that some calves from nuclear transplantation were exceptionally large, up to 80 % of the cloned calves have a pathological metabolism that leads to high neonatal mortality, but if given intensive care at birth, calves become essentially normal (Garry *et al.*, 1996). These data infer a genetically or epigenetically abnormal placenta. The placenta is directly responsible for mediating and/or modulating the maternal environment and has a pivotal role in transfer of essential substrates from the mother to the fetus.

Abnormal placentation

High pregnancy attrition rates during the first trimester in gestations from cloned and in vitro produced bovine and ovine embryos are very likely caused by inadequate placental development. Stice *et al*. (1996) were first to indicate abnormal placentomal development in gestations of clones derived from embryonic stem cells. All pregnancies were lost prior to 60 days of gestation. Most remarkable was the absence of cotyledons but the presence of a hemorrhagic response in the caruncles at 38 days. Fetal organogenesis, completed by day 42, was apparently normal. Until around day 40, the bovine conceptus can survive on histotrophic and yolk sac nutrition; after this point, hemotrophic (placentome) nutrition becomes essential (Melton *et al.*, 1951; Thompson & Peterson, 2000; De Sousa *et al.*, 2001). Pregnancy loss in the study of Stice et al. (1996) coincided with timing at which placentomal functioning becomes essential. The hemorrhaging in the caruncles indicates a maternal response. Therefore, aberrant fetal placental development is most likely to blame for the high loss of early pregnancies. In agreement with these findings, Peterson & McMillan (1998) observed allantoic malformation

characterized by retarded or non-existent growth and by complete lack of vascularization in in vitro derived bovine embryos during the first month of gestation. Since the allantois is responsible for chorionic vascularization, maldevelopment of the cotyledon may be due to a deficient allantois (Wells *et al.*, 1999). In ruminants much of the placenta is derived from trophoblast; however the amnion and allantois are derived primarily from the inner cell mass, so any of these structures might be involved in the etiology of abnormal placental function. Histologically, a reduction in chorio-allantoic epithelial height, cellularity and capillary numbers in placentas from first semester, bovine cloned fetuses, was observed by Hill *et al.*, (1999, 2000). In pregnancies from cloned sheep, De Sousa *et al.*, (2001) observed a high incidence of developmental retardation and chorionic hypoplasia and deficiencies in chorioallantoic vascularization in day 35 sheep gestations. De Sousa *et al.* were unable to determine if the lack of vascularization was due to a lack of allantoic outgrowth or an absence of vasculogenesis. Stice *et al.* (1996) and Kato *et al.* (1999) tried rescuing their NT clones by creating chimeras, this did not improve development to term, suggesting that the placental deficiencies may not depend on the individual cell (i.e. karyotype anomaly) but rather on interaction between cell types (De Sousa *et al.*, 2001). Hill *et al.* (2000) noted a reduction in chorio-allantoic epithelial height, cellularity and capillary numbers in H&E sections of day 90 placentomes from cloned placentae. Later in gestation, Farin *et al.* (2000) evaluated placental morphology in gestations derived from bovine in vitro produced embryos at day 222. They found the percent total superficial caruncular surface, villous volume density and fetal binucleate cell volume density all to be significantly less in IVF than control pregnancies.

In the third trimester, pregnancy loss is often a consequence of hydroallantois. A high incidence of hydrops amnion and hydroallantois has been reported by multiple groups and is

accompanied by a very high rate of mortality of recipient cows (Hasler *et al.*, 1995; Cibelli *et al.*, 1998; van Wagtendonk-de Leeuw *et al.*, 1998; Wells *et al.*, 1999; Zakhartchenko *et al.*, 1999). The incidence of hydroallantois is about 1% for pregnancies from IVF (1,376 pregnancies) (Hasler, 1998). It is suggested that this excessive fluid accumulation might be a consequence of a low number in placentomes, causing placental dysfunction (Wells *et al.*, 1999). Fewer and enlarged placentomes, enlarged umbilical vessels and edematous placental membranes (Hasler *et al.*, 1995; Garry *et al.*, 1996; Kruip & den Daas, 1997; Cibelli *et al.*, 1998; Wells *et al.*, 1999; Van Wagtendonck-de Leeuw *et al.*, 2000) and intercotyledonary areas that are thickened with a gelatinous-like material at parturition (Garry *et al.*, unpublished) are also frequently observed. Parturitions are frequently lengthy, lacking normal signs of impending birth or pronounced uterine contractions (Avery & Greve, 1995).

Ultimately, parturition does not seem to progress, and the mammary gland is underdeveloped. Again this may reflect placental dysfunction, since placental hormones affect mammogenesis during pregnancy (Wells *et al.*, 1999). The rising levels of fetal cortisol will also induce changes in steroidogenic enzyme activity of the placenta, converting progesterone to estradiol 17β, initiating a cascade of events leading to parturition (Nathanielsz, 1996; Wells *et al.*, 1999). Finally, it is important to note that placental abnormalities in clones are not limited to the ruminants, but are also found in cloned mice; e.g. placental weights of twice the normal value (Wakayama *et al.*, 1999), severe placental hypertrophy with a histologically disrupted architecture (Ono *et al.*, 2001) and, midgestational failure with deficiencies in embryo derived placental vasculature (Kato *et al.*, 1999).

In conclusion, there appear to be multiple putative causes for abnormal placentas in cloned pregnancies.

Abnormal fetuses

The incidence of oversize calves from cloning by nuclear transplantation has been documented in a study involving thousands of calves by Wilson et al. (1995). Overgrowth is the most obvious characteristic of cloned calves for the laymen's eye, and likely is caused by an imbalance in contributions of growth promoting genes versus growth inhibitory genes in certain tissues at specific stages of development (Li *et al.*, 1998*a*). It is important, however, to note that most abnormal pregnancies do not in fact result in oversized calves, but are still very abnormal in that up to 80 % of the cloned calves have a pathological metabolism that leads to high neonatal mortality (Garry *et al.*, 1996). Calves derived from IVF or nuclear transplantation seem to require extra, intensive care due to metabolic pathologies.

The Barker hypothesis suggests that certain diseases such as coronary heart disease, stroke, hypertension and non-insulin dependent diabetes originate through impaired growth and development due to nutritional constraints at critical phases of key fetal development; other adult disease states such as cancer may also be related to nutrition during pregnancy (Barker & Clark, 1997). Therefore, the fetus would adapt to a lack of nutrients or oxygen by slowing its rate of cell division and/or changing the proportion of cell types, hormonal feedback, metabolic activity and organ structure. Consequently, this differential constitution results in permanent changes in physiology and metabolism (Young, 2001). Even the peri-conception nutritional status of the mother can affect preimplantational embryonic development, as seen in high yield producing dairy cattle that have a difficult time conceiving (Young, 2001). More extreme effects of preimplantation insults are seen with in vitro manipulation of the early embryo. A high percentage of these pregnancies are lost throughout gestation. In the first trimester, abortion rates are high, later in gestation hydroallantois is lethal to the fetus and usually also the dam.

Ultimately at birth, up to 80% of cloned calves show metabolic disorders and perinatal morbidity is high, unless intensive care is provided. The most striking observation at birth is the Large Offspring Syndrome (LOS). Dystocia associated with increased birth weight often requires delivery by caesarian section (Kruip & den Daas, 1997). LOS has been linked to serum exposure during in vitro culture. However, full sibling embryos, cultured in the same drop result in both developmentally normal and abnormal offspring (Sinclair *et al.*, 2000). Of course a later synergistic effect by the maternal environment has to be taken into consideration. Also Hardy *et al.* (2001) suggested predisposition of the oocyte before embryo manipulation; by means of a mathematical model, they found that the rate of apoptosis might be predetermined by an oocyte factor. Calves with fetal overgrowth, also can have an enlarged heart, changes in liver and kidneys and increased musculature (Young, 2001). Other neonatal problems observed with cloned offspring that are not necessarily related with birth weight, are breathing difficulties, unresponsive calves that are slow to stand and nurse and sudden perinatal death (Garry *et al.*, 1996; Walker *et al.*, 1996 and others).

Garry et al. (1996) reported that calves independent of size were hypoxic, hypoglycemic, and hypothermic, suffered from metabolic acidosis and had abnormalities in metabolic hormones including low thyroxin and T3, and insulin concentrations four times higher than the reference population shortly after birth. They suggested, in line with the Barker hypothesis, that in utero energy supply and utilization might be different in cloned gestations, promoting growth, but predisposing difficulty with adaptation to extrauterine life. One could compare this with human gestational diabetes, except that in that case, the origin of the problem is maternal. Increased muscle mass, changes in muscle fiber composition (Maxfield *et al.*, 1997), cerebral dysplasia (Schmidt *et al.*, 1996) and skeletal and facial malformations (Walker *et al.*, 1996) all have been

reported. Enlarged organs and greater organ allometric growth coefficients for organs (Farin & Farin, 1995; Sinclair *et al.*, 1997, 1998) have been measured, suggesting a disturbed growth rate and development of organs (Young *et al.*, 1998). Interestingly, increased fetal weight is not associated with larger placentae (Sinclair *et al.*, 1997, 1998). This might indicate that overgrowth is driven by the fetus and not the placenta; still placental function might be altered independent of size (Young *et al.*, 1998).

Large offspring syndrome affects both female and male offspring (Wilson *et al.*, 1995; Goodhand *et al.*, 1997; Yazawa *et al.*, 1997) and occurs in a variety breeds (Kruip & den Daas, 1997). Even though large at birth, offspring size evens out with control populations by 1 year of age (Wilson *et al.*, 1995), and apparently normal offspring are produced in the next generation. It has been suggested that the observed fetal hypoxia might be correlated with the observed edematous placenta (Garry *et al.*, 1996; Hill *et al.*, 1999) impairing O_2 exchange between mother and fetus. Hill *et al.*, (1999) suggested that the placental edema may result from elevated systemic pressures, linked to a dysfunctional right heart and congenital pulmonary hypertension. Certain calves also suffer from pulmonary distress due to lack of surfactant, which is common. Two potential causes suggested by Hill *et al.* (1999) are poor production of surfactant by Type II alveolar cells and/or a reduced surfactant concentration due to dilution by excess amniotic fluids. One cloned calf from the Hill study manifested itself with diabetes mellitus, a disease very rare to the bovine species (Mostaghini & Ivoghli, 1977); the underlying cause was attributed to hyperplasia of insulin-producing pancreatic β-cells (Hill *et al.*, 2000). Renard *et al.* (1999), reported the death of a cloned calf with severe anemia, lymphoid hypoplasia (hypoplastic lymph nodes, thymus and spleen) and deficiency in CD45, a common leucocyte antigen. This condition may impair immune response and render individuals more susceptible to infection. Thompson

and Peterson (2000) indicated a link of this phenotype of depressed haematopoiesis with allantoic malformation since both the yolk sac and allantois have been suggested as potential contributors of haematopoiesis (Al Salami *et al.*, 1985).

Oversized umbilical cords develop inexplicably during most cloned pregnancies, sometimes two to three times the normal size (Cibelli *et al.*, 1998). At Cyagra, a company that produces cloned calves commercially, these cords are surgically ligated at birth, to prevent exsanguination (B Henderson, personal communication 2003).

The tremendous variability in anecdotal abnormalities observed in cloned offspring, highlights the complexity of the syndrome. This again shows our lack of understanding of normal fetal development and the need for research in this field.

THE INSULIN-LIKE GROWTH FACTOR SYSTEM

Introduction

Growth hormone (GH), released by the anterior pituitary, is the major regulator of postnatal growth (Spagnoli & Rosenfeld, 1996). In contradiction with the original "somatomedin hypothesis", its biological actions are either mediated by somatomedins, or result from direct action on target cells (Maharajan & Maharajan, 1993). The two somatomedins, insulin-like growth factor 1 and 2 (IGF-1 and –2) are single chain polypeptides of approximately 7 kDa, with structural homology to proinsulin (Adams *et al.*, 1983; Keller, 1996). Most tissue types synthesize IGF's at low levels; however, the liver is their major source (90 %) of secretion, and is responsible for systemic distribution at relatively high levels in serum (1 μg/ml, 1000 fold

higher than that of insulin) (Froesch *et al.*, 1985). Only small quantities of IGF are stored in liver cells; both synthesis and secretion are rapid and relatively constant (Froesch *et al.*, 1985; Keller, 1996). Therefore, IGF concentrations in blood do not fluctuate dramatically.

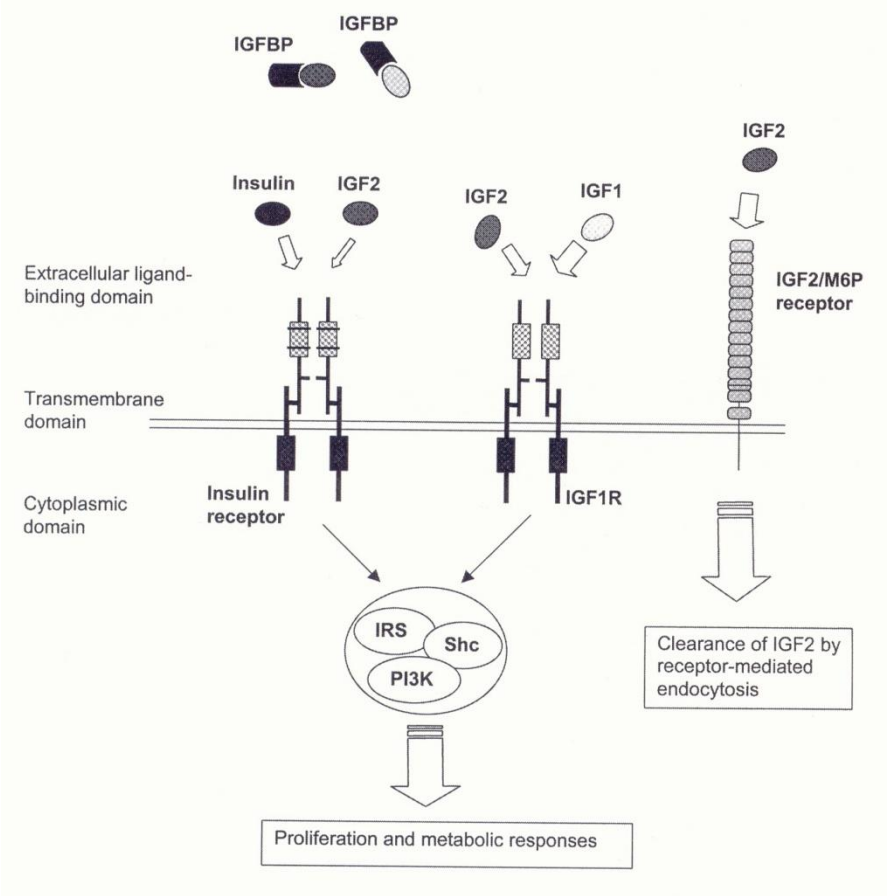

Figure 1.2 The IGFs, their receptors and their binding proteins (Reik *et al.*, 2001).

Although the IGF's exert insulin-like activity, there is a different consequence: cellular hypertrophy. They maintain the basal metabolic rate; promote (but do not control) cell survival, proliferation, differentiation, migration and aggregation; and inhibit apoptosis in a variety of cell types (McCusker, 1998). It is important to keep in mind that IGF's do not direct the cell's fate, but they facilitate and enhance the rate towards this fate, determined by other regulatory factors (Ewton, Falen, & Florini, 1987; McCusker, 1998). For example, IGF-1 seems to function as a

mediator of the mitotic actions of estradiol on the endometrium during the menstrual cycle in women (Giudice *et al.*, 1998; Han & Carter, 2000). Because of the need for cell hypertrophy prior to mitosis, IGF's will stimulate nutrient transport, and DNA, mRNA and protein synthesis (McCusker, 1998). Through these biological effects, IGF-1 and –2 match growth and development to the animals nutritional status (Wathes *et al.*, 1998), and because of the very complex regulation, maximal IGF activity occurs only when the conditions are optimal for growth (McCusker, 1998). Insulin also enhances cell hypertrophy, but does so primarily as a means to increase nutrient stores, and it targets different cell populations (McCusker, 1998). Whether cells respond to insulin or IGF's, is mediated by the presence of the respective receptors i.e. the IR or IGF1R. IR's are abundant on cells with energy storage as their main function (e.g. adipocytes), while the IGF1R will be represented on cells undergoing hyperplasia or differentiation (McCusker, 1998). Expression of receptors can change, e.g. terminal differentiation, and quiescence of cells will decrease the IGF1R's (LeRoith *et al.*, 1995; McCusker, 1998). Insulin secretion varies rapidly, and spikes following nutritional intake. IGF-1 concentrations on the other hand are very steady, and its increased secretion is modulated very slowly to ensure availability of a continued source of energy and protein and stimulation of the IGF1R (McCusker, 1998). This is important since it can take up to several days to complete the cell cycle and differentiation process (Yang & Pardee, 1986; McCusker, 1998).

In the adult, four primary factors control IGF-1 secretion and action in coordinance with nutrient availability and the environment: GH, insulin, glucocorticoids, and thyroid hormone (McCusker, 1998). Growth hormone is released by the anterior pituitary and mediates the secretion of somatomedins by the liver and other organs. Only steady elevation in GH levels will increase IGF-1 secretion. Low insulin results in lowered IGF-1 secretion, but elevated

insulin will not enhance IGF-1 secretion (Thissen *et al.*, 1994; Thrailkill *et al.*, 1997; McCusker, 1998). Normal glucocorticoid concentrations guarantee optimal IGF-1 secretion, and elevated glucocorticoid levels (under stress) will depress IGF-1 secretion (Park *et al.*, 1994*b*; McCusker, 1998). In accordance with temperature, low amounts of thyroid hormone suppress IGF-1 secretion, while high amounts of thyroid hormone enhance IGF-1 secretion (Rodriguez-Arnao *et al.*, 1993; McCusker, 1998).

The growth promoting effects of the IGF's in vivo have been indicated in patients with acromegaly, who have serum levels of IGF-1 elevated three times that of normal patients (Jones & Clemmons, 1995). However these patients also have greater than normal concentrations of growth hormone. African pygmies have low serum concentrations of IGF-1 and do not respond to exogenous administration of growth hormone (Merimee *et al.*, 1981). Another informative example is the dog breeds (e.g. poodles) which have multiple size categories with different levels of IGF-1 but normal concentrations of growth hormone (Eigenmann *et al.*, 1984).

Intra-uterine growth and fetal development seem to be largely growth hormone independent and under direct regulation of the complex insulin/insulin-like growth factor (IGF) system (Young *et al.*, 2001). A positive correlation of IGF-1 and –2 with fetal weight and fetal liver weight in sheep was demonstrated by (Owens *et al.*, 1994). More sophisticated gene deletion studies in mice confirmed the overall control of the IGF system over placental and fetal growth. Genetically engineered mice heterozygous for a disrupted paternal IGF-2 allele or with a homozygous knockout of the IGF-1 gene showed a 40% growth deficiency at birth (DeChiara *et al.*, 1990). Fetal overabundance of IGF-2, by null mutation of the IGF-2/M6PR, causes fetal overgrowth and perinatal lethality in mice (Filson *et al.*, 1993; Lau *et al.*, 1994). In these mutants, the placenta, which normally ceases to grow at day 17, continues to grow until term

(Lau *et al.*, 1994). Placental growth was impaired in the IGF-2(p-) mutation, but neither IGF-1(-/-) nor IGF-1R(-/-) mutations affected placental weight (Baker *et al.*, 1993). These observations suggest that IGF-2 has a relatively greater role in placental development.

IGF action is controlled by a complex system of altered secretion, multiple receptors, and insulin-like growth factor binding proteins (IGFBP). IGF's mediate their effects by binding to specific type 1 and 2 IGF receptors on target cell surfaces. Even though serum levels of IGF's are high, very little free IGF is present since most IGF in circulation is bound to a family of IGFBP's. IGFBP's transport and increase the half-life of IGF's, modulate ligand-receptor interaction and exert IGF-independent actions (Lackey *et al.*, 2000). They are ubiquitous, and their expression is cell and tissue specific (Lackey *et al.*, 2000). They provide a labile pool of IGF's that can temporarily maintain IGF delivery to target cells when IGF secretion is compromised (Hossner *et al.*, 1997; McCusker, 1998). The pool of IGFBP's is large, and growth rate is proportional to flux of IGF's through the pool, not the pool size (Hossner *et al.*, 1997; McCusker, 1998). Trace elements, specifically zinc, have been ascribed a role in targeting IGFBP's to the cell surface, may change the affinity of IGF1R, and optimize IGF activity of cell associated IGFBP's (McCusker, 1998).

IGF-1 and IGF-2 structure

IGF-1 and –2 are members of a gene family that includes insulin and relaxin. They are 60% identical with each other in amino acid sequence, and show 40% sequence homology with insulin. Their structure resembles the pre-pro-insulin pattern but the C-chain is not cleaved off before secretion since the Arg-Arg coding is missing; this makes the IGF's about 50 % longer than insulin. A terminal extension, the D + E-domain, is also attached. The secondary structure

as in the proinsulin protein is preserved by the intrachain disulfide bridges of the cystein groups. The A-domain of IGF-1 is involved with receptor binding, and in some instances, both the A and B domain bind the IGFBP's (Foyt & Roberts, 1991; Keller, 1996). Both IGF-1 and 2 are highly conserved genes; sequence homology across species from xenopus to human is 88% within the A and B domains (Foyt & Roberts, 1991; Keller, 1996). Several upstream regulatory regions exist for both IGF genes, and an array of transcript sizes is produced due to differential splicing, and alternative transcription start sites and polyadenylation sites (Foyt & Roberts, 1991; Keller, 1996).

Insulin-like growth factor 1

The IGF-1 gene spans more than 45 kilobases and consists of 6 exons and 5 introns, while the IGF-2 gene has 9 exons (Foyt & Roberts, 1991). Two predominant transcripts of IGF-1 mRNA, which encode the same IGF-I protein, are approximately 7 and 1 kilobases long and differ in the length of 3'untranslated sequence. The half-lives of these two transcripts are remarkably different; the 1.0 kb transcript has a much shorter decay rate than the longer transcript (Hepler *et al.*, 1990). In addition to multiple transcripts, several protein variants of different lengths have been described. A truncated form of IGF-1, des (1-3)-IGF-I, that lacks the first three N-terminal residues, has been located in fetal and adult human brain (Carlsson-Skwirut *et al.*, 1986; Sara *et al.*, 1986) and bovine colostrum (Francis *et al.*, 1988). This form of IGF-1 has 1.4-10 times higher biological potency than full length IGF-1 (Francis *et al.*, 1988), which may reflect the lack of affinity of this form for IGFBP's. Numerous transcription initiation sites, multiple transcripts of varying stability, and variable post-translational modifications of these genes are responsible for only part of the seemingly overwhelming and complex regulation of

IGF's. IGF-1 regulation is under influence of many factors. Estradiol will upregulate IGF-1 mRNA in the oviduct and uterus at estrus (Murphy & Ghahary, 1990; Wathes *et al.*, 1998). Prolonged environmental changes like stress, nutritional availabilities and temperature will change IGF concentrations (McCusker, 1998). Thissen *et al.*, (1994) thoroughly reviewed the nutritional control of IGF-1 and concluded that in general, a restriction in diet will reduce serum IGF-1 and raise circulation levels of GH. Maternal starvation and hypoglycemia during pregnancy will decrease fetal plasma IGF-1 concentrations (Gallaher *et al.*, 1994), and Fowden (1995) suggested that insulin may affect IGF-1 gene transcription.

Insulin-like growth factor 2

IGF-2 concentrations rarely fluctuate in parallel with changes in IGF-1 concentrations, so they are clearly regulated differently (McCusker, 1998). The concentration of IGF-2 is high in the fetus (Hossner *et al.*, 1997) and has a major role in the control of fetal growth (Jones & Clemmons, 1995). After birth, IGF-2 levels decline and its role at that point remains unclear. Interestingly, IGF-2 expression is tightly regulated by imprinting, which causes IGF-2 to be only expressed from the paternal allele in most tissues in the mouse, human and sheep (Ranier *et al.*, 1993; Feil *et al.*, 1998; Hagemann *et al.*, 1998; McLaren & Montgomery, 1999). In humans and sheep, IGF-2 exhibits a developmental switch from imprinted expression in most fetal tissues to a general silencing in the adult, except for biallelic expression in the liver (McLaren & Montgomery, 1999). IGF-2 is not expressed at all after birth in the mouse. At present genomic imprinting of IGF-2 and IGF2R have not yet been demonstrated in cattle. However, paternal imprinting of IGF-2 has been shown in sheep (Feil *et al.*, 1998).

The delicate control of allele silencing is prone to error and can easily be disrupted with consequent doubling of active copies of the IGF-2 gene (biallelic expression). In humans, relaxation of IGF-2 imprinting, causes pathological phenomena like the Beckwith-Wiedemann overgrowth syndrome, Wilm's tumor and other cancers (O'Dell & Day, 1998). In the liver of the human and sheep, but not the mouse, a physiological switch from imprinted to biallelic IGF-2 expression occurs with development from fetus to adult (McLaren & Montgomery, 1999). Transcription of the IGF-2 gene is under control of four promotors and its imprinting status, which is highly dependent on the methylation of the cluster of genes surrounding the IGF-2 gene (Reik *et al.*, 2000). Because of imprinting, expression from promotor P1 is biallelic, and from P2-P4 is monoallelic (Yun *et al.*, 1998). Methylation patterns of these promotors and multiple regions of the IGF-2 and H19 gene are developmentally regulated, and, therefore, the promotors can also be classified as fetal, fetal-neonatal and adult promotors (de Pagter-Holthuizen *et al.*, 1987). This may well explain the dynamic regulation of the IGF-2 gene in the liver (Li *et al.*, 1998*b*). Just recently, Constância *et al.*, (2002) described a trophoblast-specific transcript P0 of IGF-2 in the mouse. This P0 promotor leads to paternal transcription of the exons 4-6.

IGF-2 expression is possibly regulated by placental lactogen (PL) in the ovine fetal placenta since IGF-2 concentrations correspond to those of PL during gestation (Gluckman *et al.*, 1987). On the other hand, it is the cortisol rise at term that induces the fetus to switch from IGF-2 to IGF-1 production, which is in its turn partly responsible for the switch from proliferation to differentiation at cell cycle levels (Fowden, 1995).

The insulin-like growth factor receptors

Thus far, besides the low affinity insulin receptor, two major types of receptors specific for IGF binding have been described. IGF-2 is known to interact with both the IGF1R and IGF2R. However, it is believed to recognize an additional unknown receptor (XR) because growth retardation of embryos lacking both the IGF1R and IGF-2 (30% of normal birth weight) is more severe than in either individual mutation (45% vs. 60%, respectively) (Louvi *et al.*, 1997). XR is probably not the M6P/IGF2R, since IGF2R mutants are not smaller in size at birth (135%), but suffer high neonatal mortality (Lau *et al.*, 1994; Ludwig *et al.*, 1996), due to their inability to clear IGF-2. In a study by Louvi and others (1997), nullizygous offspring for both IGF1R and the insulin receptor (IR) also results in 30% growth deficiency, indistinguishable from the IGF1R/IGF-2 double mutant phenotype. This finding indicates that the growth promoting function of IGF-2 during fetal life must be partially mediated through IR signaling in the mouse (Louvi *et al.*, 1997).

Insulin-like growth factor 1 receptor

The IGF-1R and the insulin receptor are similar in structure and their intracellular signal transduction is similar. Type 1 IGF receptors are usually expressed on cells in proliferation or differentiation, while insulin receptors will reside on non-mitogenic cells responsible for nutrient storage (McCusker, 1998). Hybrid IGF1R and Insulin receptors do occur in the placenta and bind IGF-1 with preference over insulin (Jones & Clemmons, 1995).

The type 1 IGF receptor (IGF1R) belongs to the family of transmembrane tyrosine kinases. The IGF1R exists as a disulfide linked ($\alpha\beta$-$\alpha\beta$) hetero-heteromer on the cell surface. The α-subunits have binding capacity and the intracellular β-subunits have tyrosine kinase

activity (LeRoith *et al.*, 1995). Monomeric pro-receptor molecules are biosynthesized intracellularly, and are protected by lectin chaperones until correct folding. When the lectin chaperones release the monomers, receptor dimerization occurs, followed by export from the endoplasmic reticulum. The IGF1R mediates most of the biological effects of IGF-1 and IGF-2 (O'Dell & Day, 1998) and preferentially binds IGF-1, has an intermediate affinity for IGF-2 and has low affinity for insulin. IGF1R expression seems to be controlled by IGF-1 and –2 concentrations, but other unidentified tissue specific mechanisms might be involved (Wathes *et al.*, 1998). Interestingly, IGF1R $^{+/-}$ tg mice are long lived and display greater resistance to oxidative stress. Their energy metabolism is normal and their nutrient uptake, physical activity, fertility and reproduction are unaffected. This finding suggests a role for the IGF1R as a central regulator of mammalian lifespan (Holzenberger *et al.*, 2003).

In an excellent review, Adams (2000) states, "given the diverse physiological functions regulated by IGF-1, a diverse (and expanding) repertoire of intracellular proteins has been identified as substrates/effectors of ligand activated IGF-1R signaling". Ligand binding induces auto-phosphorylation of the kinase regulatory domain. Subsequently other tyrosines in the cytoplasmic domain are phosphorylated and become functional docking sites for the recruitment of other proteins, initiating the signaling cascade. Two major pathways have been characterized, involving two distinct adaptor proteins Shc and IRS (insulin receptor substrate). In the first pathway, IGF-1 activates the serine-threonine mitogen-activated protein (MAP) kinases, and the extracellular signal-related kinases ERK1 and ERK2. These in turn phosphorylate a variety of cytoplasmic and nuclear proteins such as growth factor receptors, transcription factors, kinases and other enzymes which mediate a metabolic, mitogenic or differentiative cellular response (Adams, 2000). IGF-1 can also effectively utilize the PI 3-kinase signaling pathway with a

major role in protection of cells and tissues from programmed cell death. Substrates of the IGF-1 / PI 3-kinase cascade are the Akt/PKB serine/threonine kinases. Upon activation, Akt/PKB, a common denominator of antiapoptotic signaling, mediates metabolic responses, proliferation and inhibition of apoptosis (Adams, 2000).

The insulin like growth factor 2 receptor

The type 2 IGF receptor is a monomeric 300 kDa transmembrane protein with no tyrosine kinase activity (Kornfeld, 1992). It is the same molecule as the cation-independent mannose-6-phosphate receptor (Kiess *et al.*, 1988) that binds most lysosomal enzymes and other mannose 6 phosphate containing ligands for delivery to the lysosome (Osipo *et al.*, 2001); but in mammals, it also interacts with IGF-2 (Ludwig *et al.*, 1996). No clear cut downstream pathway has been identified for this receptor, and it is more likely believed to function as a clearance molecule to remove IGF-2 from circulation by internalization and targeting of bound ligand for lysosomal degradation (Kornfeld, 1992; Baker *et al.*, 1993). IGF2R is also thought to exert growth inhibitory effects by participating in the activation of latent transforming growth factor β (TGF-β) (Dennis & Rifkin, 1991). Active TGF-β regulates the synthesis and degradation of extracellular matrix and is a potent growth inhibitor of most normal and transformed cells (Massagué, 1990). Loss of IGF2R in tumor cells supports increased growth and vascularization of tumors by allowing higher local levels of IGF-2 and/or by decreasing the focal activation of latent TGF-β (Osipo *et al.*, 2001). Interestingly, Volpert *et al.* (1996) have shown in vitro that proliferin, a peptide that stimulates endothelial cell migration and neovascularization in vivo, binds to the IGF2R. Apparently, IGF2R plays an essential role in proliferin-induced

angiogenesis; and IGF-2 binding with IGF2R was also shown to have the same angiogenic effects.

In some cell types, IGF2R in the trans Golgi reticulum is essential for transport of newly made lysosomal enzymes to the lysosome (Sahagian *et al.*, 1987). IGF2R in the plasma membrane modulates levels or activities of a variety of ligands that regulate cell growth including TGF-β, IGF-2, retinoic acid, and LIF (Osipo *et al.*, 2001).

The affinity of the type 2 receptor is very selective for IGF-2, 500-fold higher than for IGF-1, and does not bind insulin (Kornfeld, 1992; Wathes *et al.*, 1998). The IGF2R locus is imprinted in a polymorphic fashion in humans and, only a minority of people show monoallelic, maternal expression. Often times, this locus is also found to be inactivated in a variety of human tumors (Murphy & Jirtle, 2000). In mice (Barlow *et al.*, 1991) and sheep (Young *et al.*, 2001), the IGF2R is imprinted and normally only the maternal allele is expressed. When this allele is disrupted, mutant mice have an increase in serum and tissue IGF-2 levels. At birth they exhibit overgrowth (135%), organomegaly, a kinky tail, postaxial polydactyly, heart pathology, and edema. Perinatal death is frequent, which indicates the importance to survival of the M6P/IGF2R lysosomal enzyme trafficking (Lau *et al.*, 1994; Ludwig *et al.*, 1996).

A circulating form of the extracellular domain of the IGF-2R protein exists freely in the plasma and is produced by proteolytic deletion of the transmembrane and intracellular domains of the cellular form (Zaina & Squire, 1998). The soluble form of the IGF2R is believed to down regulate growth. In ovine fetal plasma, high concentrations of this soluble IGF2R are present that can contribute up to 40% of total IGF binding in the blood (Gelato *et al.*, 1989; Gallaher BW, Breier BH, Blum WF, McCutcheon SN, 1995). Transgenic mice with the transmembrane domain sequence of IGF2R deleted, show a reduction in organ size. When this mutation is

crossed with mice overexpressing IGF-2, the decrease in organ growth appears to be exclusively mediated by reducing IGF-2 action in some organs, but must involve other interactions in other tissues (Zaina & Squire, 1998). Loss of IGF2R promotes growth in cancer by sustaining IGF-2 which binds to and activates IGF1R and the IR to increase intracellular growth signals (Osipo *et al.*, 2001).

Insulin receptor

Frasca et al. (1999) identified a new insulin receptor isoform A with high affinity for IGF-2. The insulin receptor (IR) occurs in two isoforms (IR-A and IR-B) resulting from alternative splicing of exon 11 of the gene (Sciacca *et al.*, 1999). The IR-A isoform is predominantly expressed in fetal tissue and malignant cells and binds IGF-2 with high affinity (Frasca *et al.*, 1999). Both Ins and IGF-2 activate IR-A, they activate different intracellular signaling pathways and elicit different biological effects. Activation of IR-A by insulin leads primarily to metabolic effects, whereas activation by IGF-2 leads primarily to mitogenic effects (Frasca *et al.*, 1999). The Ins – PI3-K/Akt pathway, protects cells from apoptosis and the IGF-2- Shc/ERK pathway, stimulates cell migration (Sciacca *et al.*, 2002). Scalia *et al.* (2001) suggest that the coordinated activation or deactivation of Akt, glycogen synthase kinase 3-β (Gsk3β) and Erk may account for the IGF-2 mitogenic effects and support an active role for IR(A) in IGF-2 action. The IR and the IGF1R have a highly homologous structure, but different biological effects. Insulin and IGF-1 half receptors can heterodimerize, leading to the formation of insulin/IGF-1 hybrid receptors that bind IGF-1 with high affinity (Jones & Clemmons, 1995). Hybrid receptors containing IR-A bind to and are activated by IGF-1,-2 and Insulin. Hybrid-Rs containing IR-B bind to and are activated with high affinity by IGF1, with low affinity by IGF-2

and insignificantly by Insulin. Cell proliferation and migration is more effectively stimulated in hybrid-R(A) containing cells (Pandini *et al.*, 2002).

Insulin-like growth factor binding proteins

Unlike insulin, the IGF's are bound in serum and other biological fluids to a family of six structurally and evolutionary related binding proteins, termed IGF-binding proteins (IGFBP-1 to IGFBP-6) (Schneider *et al.*, 2000). Their characteristics in common are: 1) a conservation of gene organization, 2) three structural domains in the mature proteins, with conserved, cysteine rich amino- and carboxy-terminal domains, and 3) high binding affinity for both IGF-1 and –2 (Baxter, 2000). Other proteins e.g. mac25 (sometimes named IGFBP-7 (Oh *et al.*, 1996) that share a structural homology with one domain of the IGFBP's, have been assigned as IGFBP-related proteins and are members of the IGFBP-superfamily because of structural homologies in the amino-terminal end, and low-affinity IGF-binding activity (Hwa *et al.*, 1999). Thus far, 10 IGFBP related proteins have been identified, some of which may exert IGF-independent actions (Hwa *et al.*, 1999). They maintain a labile pool of IGF in circulation and can temporarily maintain IGF delivery to target cells when IGF secretion is compromised (Hossner *et al.*, 1997). All IGFBP's bind the two IGF's with relatively similar affinities, except IGFBP-6 that has a markedly higher affinity for IGF-2 (Baxter, 2000). IGFBP's fail to bind insulin (Jones & Clemmons, 1995). IGFBP's compete with IGF receptors for IGF binding and have a considerably greater affinity than the receptor when they are in solution. Besides the prevention of insulin-like side effects due to the large concentrations of IGF's in circulation, they can modulate IGF action. In general, interaction of an IGFBP with IGF-1 or –2 will block receptor activation (Baxter, 2000). All six IGFBP's have been shown to inhibit IGF action, but

stimulatory effects have also been established for IGFBP-1, -3, and -5 (De Mellow & Baxter, 1988; Clemmons, 1991; Baxter, 2000). The concentration of circulating IGFBP's has been shown to be nutritionally regulated.

An average of 75-80% of the IGF's in circulation are present in a 150 kDa complex, composed of an IGF-1 or IGF-2 molecule, an 85 kDa acid-labile subunit (ALS), and IGFBP-3. This ternary complex has a half life of 6 to 24 times (12h) longer than the binary complexes of IGF with IGFBP-1 or –2 (Jones & Clemmons, 1995). The remaining 20-25% IGF fraction is associated with other IGFBP's, forming a 50 kDa complex. Only less than 1% of the IGF's circulate "free" (7.5 kDa) (Rajaram *et al.*, 1997; Schneider *et al.*, 2000). As a consequence of metabolic stress, the ratio of the IGFBP-3-ALS unit vs. IGFBP-1 plus –2 slowly decreases in serum (McCusker, 1998).

The IGFBP affinity for the IGF's can be modulated by post-translational glycosylation, phosphorylation, proteolysis or by cell or matrix association. IGFBP-1, -3, -5 are also found as phosphoproteins, however this phosphorylation only seems to affect IGFBP-1, increasing its affinity for IGF-1 six-fold (Jones *et al.*, 1991). The state of phosphorylation of IGFBP-1 may be important in the metabolic regulation of the bioavailability of free IGF's and seems to be hormonally regulated (Westwood *et al.*, 1995).

Several proteases can cleave IGFBP's, reducing their ability to bind IGF-1 and -2. Proteolysis occurs mainly at the central domain, leaving the amino- and carboxy-terminals intact (Baxter, 2000). The release of endoproteases for IGFBP degradation is a mechanism by which cells can regulate local IGF activity, freeing IGF's for rapid clearance from the circulation to target cells (McCusker, 1998). This has been observed in cellular differentiation in which the release of IGFBP's and their proteases seems to be temporally regulated. For example, during

follicular atresia in the human, IGFBP-4 will block IGF action, but this will be restored under influence of an IGFBP-4 protease during follicular development (Iwashita *et al.*, 1996; Baxter, 2000).

When IGFBP's attach to a cell membrane or extracellular matrix, their binding affinity for IGF's is reduced. This mechanism could actually allow bound IGF's to be released so they can act upon the cells (Baxter, 2000). Therefore, the presence of bound IGFBP's can actually enhance IGF action by creating a local depot, and therefore increased local availability of IGF's for receptor binding (Han & Carter, 2000). This has been observed in cell culture systems in which IGFBP-3 seems to potentiate IGF activity by this mechanism. All IGF binding proteins except IGFBP-3 can leave the circulation when chelated with IGF's, this may allow for tissue specific targeting (Bar *et al.*, 1990; Wathes *et al.*, 1998).

Interestingly, IGFBP-1, -3, and -5 possess actions independent of the IGF1R. IGFBP-1 stimulates cell migration independently from IGF, through interaction with $\alpha_5\beta_1$-integrin, a fibronectin receptor (Jones *et al.*, 1993), and IGFBP-3 and -5 may have specific cell-surface receptors with serine kinase activity.

Even though all IGFBP's interact with IGF's, their expression seems to be strictly regulated in time and tissue-specific ways. To evaluate specific actions of the individual IGFBP's in vivo, researchers have investigated transgenic mouse models. An excellent review by Schneider *et al.* (2000) summarizes these findings. When the knockout approach was applied to the IGF's and their receptors, very specific phenotypes were induced. In contrast, IGFBP knockouts did not manifest extreme phenotypes, probably due to functional compensation by other members of the IGFBP- superfamily (Schneider *et al.*, 2000). Alternatively, transgenic

overexpression of IGFBP's has successfully induced informative phenotypes (Schneider *et al.*, 2000).

IGFBP-1 was originally isolated from human amniotic fluid and has a molecular weight of 30 kDa. It contains an amino-acid Arg-Gly-Asp integrin recognition sequence for cell surface binding (Wathes *et al.*, 1998). Transcripts for IGFBP-1 are present in the fetal and adult liver. In vitro studies indicate that this BP mainly inhibits IGF-dependent cellular growth and differentiation. Its biological actions are associated with carbohydrate metabolism, female reproductive functions, and fetal growth. IGFBP-1 overexpression reduced brain weight and caused brain growth retardation, and in 50 % of cases, hydrocephalus. In vitro, IGF's stimulate proliferation and survival of neural cells, and in vivo they are essential for normal development of the nervous system (Stewart & Rotwein, 1996; Schneider *et al.*, 2000). Insulin knockout murine fetuses as well as hypoxia will upregulate IGFBP-1 expression (Tazuke *et al.*, 1998). This may support the idea that IGFBP-1 is an important regulator of fetal growth, probably by limiting the availability of IGF's to their target tissues under hypoxia or nutrient deprivation (Schneider *et al.*, 2000). IGFBP-1 overexpression induces hyperglycemia; this may be due to alterations observed in the pancreas, or simply IGFBP-1 inhibiting the hypoglycemic effect of free IGF's. In IGFBP-1 overexpression models, litter size reduction was observed and linked to reduced ovulation and blastocyst implantation (Huang *et al.*, 1997; Schneider *et al.*, 2000). Proteinuria and glomerular lesions were seen; this finding might relate to aberrant expression of the IGF system observed in diabetic renal disease (Schneider *et al.*, 2000).

IGFBP-2 is the second most abundant IGFBP in circulation and preferentially binds IGF-2. The liver and kidney are the major source in adults, but it is expressed in most tissues except muscle. Its protein size is 32 kDa. Just like IGFBP-1, IGFBP-2 has an integrin recognition

sequence to bind cell surfaces (Wathes *et al.*, 1998). Often IGFBP-2 mRNA is found in rapidly proliferating tissues in association with IGF-2 mRNA (Delhanty & Han, 1993; Wathes *et al.*, 1998). Since transgenic mice overexpressing IGF-2 have increased IGFBP-2 serum levels, IGFBP-2 expression seems to be ligand regulated. The inverse phenotype of IGFBP-2 overexpression vis-à-vis IGF-1 overexpression suggests the role of IGFBP-2 is an inhibitor of IGF actions. Some findings indicate the potential involvement of IGFBP-2 with glucose homeostasis and enhanced glucose transport in microvascular endothelial cells (Jones & Clemmons, 1995).

IGFBP-3 is expressed in the fetal liver and widely expressed in the adult, most abundantly in the kidney (Jones & Clemmons, 1995; Baxter, 2000; Schneider *et al.*, 2000). Its molecular weight tends to vary considerably from 16-30 kDa, due to different N-glycosylation. IGFBP-3 cell association involves cell surface glycosaminoglycan interaction (Wathes *et al.*, 1998). Serum concentrations are several fold higher than other IGFBP's with a very high affinity for the IGF's. Therefore, it is a major carrier of IGF's in circulation. IGFBP-3 has both enhancing and inhibitory effects on IGF action and has growth regulatory effects, independently of IGF's (Schneider *et al.*, 2000). The transgenic overexpression models developed a significant organomegaly (Murphy *et al.*, 1995). Involution of the mammary gland after lactation was retarded, and may be due to a decrease in apoptosis, either a direct consequence of IGFBP-3 or mediated by IGF-1, known for its antiapoptotic properties (Neuenschwander *et al.*, 1996).

IGFBP-4 exists as a 24 kDa non-glycosylated or 28 kDa N-glycosylated protein (Schneider *et al.*, 2000). It is again widely expressed in both fetal and adult tissues. During embryonic development it is expressed in the polarizing zone of the limb bud, and the fact that it is present in the interdigital zone might indicate a counteractive role towards the antiapoptotic

effect of IGF-1. IGFBP-4 also plays a major role in bone formation and resorption. Interestingly, the pregnancy associated plasma protein-A (PAPP-A) secreted at high levels by the human placenta, is identical to an IGFBP-4 protease (Lawrence *et al.*, 1999; Han & Carter, 2000). In vitro experiments indicate IGFBP-4 to be consistently inhibitory to IGF actions. However, the IGFBP-4 knockout models are smaller than wild types. This finding might suggest that IGFBP-4 is necessary for optimal IGF-2 action in vivo, instead of being an inhibitor.

IGFBP-5 is very abundant in bone extracts, has a strong affinity for hydroxyapatite and functions as a storage for IGF's, especially IGF-2 in this tissue. IGFBP-5 binds to the extracellular matrix (Wathes *et al.*, 1998). It is universally expressed and involved in ovarian and kidney physiological processes. In circulation it is also found as a ternary complex with IGF-1 or –2 and ALS, and therefore might also have a systemic impact on the regulation of IGF action.

IGFBP-6 has been studied the least. It remarkably prefers to bind IGF-2 over IGF-1 and is widely expressed in adult tissues as a 32 kDa protein. In vitro as well as in vivo IGFBP-6 expression inhibits tumor cell growth, most likely preventing IGF-2 action, an autocrine tumor growth factor (Schneider *et al.*, 2000).

THE IGF SYSTEM IN THE PREGNANT UTERUS

IGF-1 and -2 both participate in the endocrinological regulation of fetal growth (Gluckman, 1995). However, IGF-1 acts exclusively in the fetus (and postnatally), while the IGF-2 system acts both in the fetus and in the placenta (Reik *et al.*, 2001). In Reik's opinion,

IGF-2 seems to act predominantly in an endocrine fashion, coordinating organ growth with the growth of the organism. However in overgrowth and growth deficiency syndromes, imprinted growth factors can act by more local mechanisms, resulting in disproportionate growth (Reik *et al.*, 2001). In contradiction with Reik (cfr. supra), Han *et al.* (1987) believe that IGF's and IGFBP's mainly act in an autocrine/paracrine fashion. Therefore, the identification of their location of synthesis can indicate the potential site of their biological function. In the discussion below, I will repeatably refer to a study by Reynolds *et al.* (1997) with findings on in situ expression of IGF mRNA components in the uterus of the ewe. It is important to keep in mind that these observations were only verified during the first third of gestation, and may differ for other ruminants.

IGF-1, -2, IGF1R and IGF2R mRNA's have been identified to be present from zygote to blastocyst stage in bovine embryos (Watson *et al.*, 1992). IGF-1 and -2 are important regulators of preimplantation and placental development (Wathes *et al.*, 1998). Both stimulate the development of blastocysts (Kaye *et al.*, 1992), while only IGF-2 increases both cleavage rate and yield of in vitro cultured bovine blastocysts (Carolan *et al.*, 1995). Schultz *et al.* (1996) found an upregulation of IGF-2 and IGF-1R mRNA and downregulation of IGF-1 mRNA in bovine nuclear transfer blastocysts. Byrne *et al.* (2002) described how IGF-1 and -2 but not insulin increased the proportion of embryos which form blastocysts. Insulin decreased the incidence of apoptosis without affecting blastocyst cell number, IGF-1 decreased apoptosis and increased total cell number and IGF-2 increased cell number alone. They suggested that IGF-2 might not act directly as a survival factor but rather to advance embryonic development and cell proliferation to a point where apoptosis is downregulated. And Petrik *et al.* (1999) found that mice overexpressing IGF-2 showed decreased apoptosis in islet cells of the pancreas.

Oviductal cells also transcribe IGF-1 and IGF-2 (Watson *et al.*, 1994; Stevenson & Wathes, 1996), but local oviductal production of IGF-1 seems more influential to early embryonic development than IGF-2 in sheep (Wathes *et al.*, 1998). Oviductal IGF-1 may stimulate embryo growth directly or indirectly by stimulation of the secretory activity of the ampulla (Wathes *et al.*, 1998). Similar findings in the mouse and human confirm this theory. In human oocytes and preimplantation embryos, transcripts for IGF-2, IGF1R, IGF2R and IR are present, but not for IGF-1. IGF-2 has been shown to have an autocrine effect on preimplantation development (Lighten *et al.*, 1997). IGF-1 and insulin, on the other hand, are secreted by the maternal reproductive tract in the mouse and act in a paracrine fashion (Lighten *et al.*, 1997).

At days 15 and 18 of the estrous cycle in cattle, IGF-2 expression by the endometrium is higher in pregnant vs. control cows. Therefore, Geisert *et al.*, (1991), suggest that the presence of a conceptus must have a stimulatory effect on this expression. In the ewe, uterine IGF-1 mRNA levels were low following implantation, highest in the maternal stroma surrounding the uterine glands although this declines during early pregnancy, and undetectable in the caruncles after fetal villi invasion (Reynolds *et al.*, 1997). Fetal placental tissue did not appear to contain IGF-1 mRNA (Reynolds *et al.*, 1997). In analogy with murine IGF-1 deletion studies (Baker *et al.*, 1993), these findings suggest that local IGF-1 production might not be important in placental development (Reynolds *et al.*, 1997). Also, in IGF-1 knockout mice, placental growth is normal (Baker *et al.*, 1993).

Insulin-like growth factor 1

Bovine fetal serum IGF-I concentration increases during gestation (Holland *et al.*, 1989), and in the ewe, there is a positive correlation between fetal and maternal IGF-1 concentrations in

late gestation and placental weight. In humans and sheep, cord blood IGF-I concentration positively correlates with fetal size and birth weight and placental weight (Carr *et al.*, 1995; Ostlund *et al.*, 1997). In sheep, both plasma IGF-1 and -2 levels correlate with fetal weight and fetal liver weight (Owens *et al.*, 1994). These findings suggest that maternal nutrition may influence placental growth via an alteration in circulating concentrations of IGF-1 (Carr *et al.*, 1995). However, Putney *et al.* (1990) thought the fetus regulates placental IGF-1 secretion via secretion of estrogen precursors in the baboon. Both IGF-1 and IGFBP-1 stimulate trophoblast proliferation in early human pregnancy (Hills *et al.*, 1999). Infusion of 50 μg/h/kg of IGF-1 increases sheep fetoplacental amino acid uptake, whereas placental lactate production decreased significantly; therefore, IGF-1 has anabolic effects on fetoplacental protein and carbohydrate metabolism (Harding *et al.*, 1994). Concentrations of IGF-1 and IGF-2 are significantly lower in arterial blood of late-term cloned pregnancies than controls (Garry *et al.*, 1998). Immediately after birth, (Matsuzaki & Shiga, 2002) found the plasma concentrations of cortisol and IGF-1 to be lower in somatic cell cloned cattle. They suggest that due to an insufficient prepartum rise in plasma cortisol, the cloned calves failed to initiate the switch to an adult mode of the IGF system during late gestation. Therefore, inappropriate developmental changes in endocrine system may be partly responsible for the fetal overgrowth and perinatal complications associated with cloning.

Insulin-like growth factor 2

IGF-2 is secreted in large quantities in the fetus (Holm *et al.*, 1996) and serves as a constant signal for growth in fetal circulation (Kruip & den Daas, 1997); there is also considerable expression of IGF-2 in the placenta throughout gestation (Wakayama *et al.*, 1998).

IGF-2 has a major effect on tissue composition and cell biomass; it also has effects on fluid dynamics and might control fluid uptake by direct action on maternal capillaries (Gallaher *et al.*, 1994). IGF-2 mRNA was found abundantly throughout gestation in ovine fetal mesodermal allantochorion (Reynolds *et al.*, 1997). In early gestation, IGF-2 is most abundantly expressed at the invading front of intermediate trophoblast in the anchoring villi, stimulating migration (Han *et al.*, 1996; Hamilton *et al.*, 1998). In late pregnancy in humans, IGF-2 binds mainly at the syncytiotrophoblast, but IGF-2 mRNA is only expressed in trophoblasts and chorionic mesoderm, suggesting the syncytiotrophoblast as the target of IGF-2 action (Han *et al.*, 1996). Lower levels were present in the caruncles, placentome capsules and endometrial stroma (Reynolds *et al.*, 1997). The day 114 (gestational age) ovine placentome shows IGF m-RNA is present in the endothelium of fetal vessels, the mesoderm of the fetal villi and is abundant below the trophoblast, which does not express IGF-2 mRNA (Han & Carter, 2000). Surgical reduction of placental size in sheep causes intrauterine growth retardation and is accompanied by an increase of serum IGF-2 (Jones *et al.*, 1988). IGF-2 stimulates extravillous trophoblast migration and invasion through the IGF2R and MAPK pathway (McKinnon *et al.*, 1999), indicating a crucial role of IGF-2 in trophoblast migration and invasion. In preeclampsia, increased IGF-2 mRNA is found in intermediate trophoblasts of peri-infarct regions, suggesting a role for IGF-2 in placental repair or remodeling (Gratton *et al.*, 2002). In diabetic pregnancies fetuses receive a large influx of glucose; this may stimulate the expression of IGF-2 in the placenta, resulting in higher utilization of glucose and overgrowth of the placenta (Shen *et al.*, 1986).

Genetically engineered mice heterozygous for a disrupted IGF-2 allele showed a 40% growth deficiency at birth (DeChiara *et al.*, 1990). From the same laboratory: placental growth was impaired in the IGF-2(p-) mutation, but neither IGF-1(-/-) nor IGF-1R(-/-) mutations

affected placental weight (Baker et al., 1993). Fetal overabundance of IGF-2, by disruption of the IGF-2/M6PR, causes fetal overgrowth and perinatal lethality in mice (Filson *et al.*, 1993; Lau *et al.*, 1994). IGF-2 has been shown to possess local growth promoting effects in fetal tissues, as demonstrated in H19 null mice (Eggenschwiler *et al.*, 1997) in which pups were 30% overgrown at birth, and IGF-2 was only elevated in tissues, not in circulation.

When Constancia *et al.* (2002) abolished the P0 promotor transcript, a marked reduction in IGF-2 signal was observed specifically in the labyrinth trophoblast of the mutant placentae. The absence of the P0 transcript of the IGF-2 gene resulted in 30% reduction of placental and fetal weights near term. Therefore, eliminating this IGF-2 transcript exclusively from the placenta has a profound growth retarding effect. Levels of IGF-2 peptide in the fetal circulation were not affected, and IGF-2 transcripts from other promoters were expressed at wild-type levels. The restriction in growth was as great as with complete lack of placental IGF-2 even though P0 constitutes only 10% of total IGF-2 transcripts in the placenta (but maybe differential translatability of IGF-2 transcripts), suggesting that the P0 transcript is the principal determinant of action of this growth factor in the placenta. Mutant placentae had the same morphological organization as wild type, and volume fraction determinations were proportionally smaller for all areas. Reduction of organ size - with exception of the brain – was largely proportionate. Therefore, the IGF seems to coordinate growth effects (demand and supply) on organ systems, suggesting that brain growth is apparently independent of nutritional demand and supply (Reik *et al.*, 2001), or that a preferential distribution of nutrients towards the brain exists.

Because of the observed growth restriction later in gestation in this knockout, a likely hypothesis is that lack of IGF-2 mRNA affects placental function in the supply of nutrients. Either placental size became limiting for fetal growth only at later stages of gestation, or at

earlier stages of gestation, the smaller placenta compensated by an increased efficiency of nutrient transfer. In order to evaluate placental transport, Constancia *et al*. (2002), tested the P0 knockout model by placental function assays. The system A transporter has been shown to be a determinant of fetal growth (Cramer *et al.*, 2002). Unidirectional maternal-fetal transfer was measured with ^{51}Cr-EDTA as a measure for passive permeability to a hydrophilic solute (Atkinson *et al.*, 1991). With the non-metabolizable amino acid analogue ^{14}C-methyl-aminoisobutyric acid (^{14}C-MeAIB), which is specifically transported by the system A amino acid transporter in the placenta (Johnson & Smith, 1988), secondary active transport capacity was evaluated. Passive permeability to the hydrophilic solute decreased with increasing length of gestation. At earlier stages of gestation, the efficiency of the active transfer was increased and seemed to compensate for reduced placental size and passive permeability. The decrease in passive permeability may involve changes in placental exchange barrier paracellular pore dimensions. At the later stage the increase in efficiency became insufficient to meet requirements, resulting in fetal growth restriction. However, depressed placental system A transporter activity is observed in human FGR in vitro in placental vesicle studies. This may be an attempt to upregulate total placental system A activity (Regnault *et al.*, 2002*b*). It was concluded by Constancia *et al.* (2002) that reduced fetal size can be the outcome of reduced supply or reduced demand, and that the mouse IGF-2 gene is remarkable in combining the control of both supply and genetic demand for maternal nutrients in a single gene!

In humans and mice, fetal overgrowth results from altered expression of several imprinted genes (H19, IGF-2, IGF2R). For example, in the Beckwidth Wiedemann Syndrome (in humans), the mechanism of overgrowth is induced by the loss of imprinting and overexpression of IGF-2. In sheep on the other hand, from in vivo fertilized eggs that were

cultured in vitro for 5 days with co-cultured granulosa cells and/or serum before transfer into ewes, 25% of the fetuses that were recovered at d125 of gestation (term =d147) were defined as large offspring (LO). RT-PCR showed no difference in steady state transcription levels of IGF-2 mRNA in liver, kidney, muscle and heart. By contrast, IGF2R expression was reduced by 30-60% in these LO fetuses. (Young *et al.*, 2001). Then again, in cattle at Day 70 of gestation, Blondin *et al*., found that fetuses originating from in vitro production systems possessed higher levels of IGF-2 mRNA in the liver and a trend to lower levels in skeletal muscle (Blondin *et al.*, 2000). Schrader *et al.* (2003) compared differential gene expression of two day 45 cloned bovine fetal livers to the original nuclear transfer cell donor day 45 fetus fetal liver by DDRT-PCR. cDNA's of 39 genes/Expressed Sequence Tags were found to be differentially abundant. When corresponding mRNA's were verified by semi-quantitative RT-PCR, no significant differences were detected. Moreover, IGF-2 specific primers used in the RT-PCR reaction did not reveal differential expression in the liver.

Insulin-like growth factor 1 receptor

The IGF1R and IGF2R are both present in bovine embryos (Watson *et al.*, 1992). Bovine placental membranes are rich in both type 1 and type 2 IGF receptors, while there is a preponderance of type 1 in humans and type 2 in sheep (Van Buul-Offers *et al.*, 1988). Term placentas from pregnancies complicated with intrauterine growth restriction have significantly higher levels of IGF-2 and IGF1R expression; this may represent a counter regulatory mechanism in response to the growth restriction (Abu-Amero *et al.*, 1998). In the ewe expression of the IGF1R was high in deep intrauterine glands. This high concentration of IGF-1R on uterine endothelial glands suggests a role for IGF-1 and –2 in regulating their secretory activity (Wathes

et al., 1998). Secretion of uterine histotroph by endometrial glands in the intercotyledonary regions is thought to continue to provide an important source of nutrients to the developing fetus throughout gestation in the ewe (Moffat *et al.*, 1987; Roberts & Bazer, 1988). Before implantation, moderate IGF1R levels were present in the caruncular stroma. However, after day 30 of gestation when fetal-maternal interdigitation is established, no more IGF1R could be detected in either the fetal or maternal parts of the sheep placentome (Reynolds *et al.*, 1997). Local IGF-2 expression present at villi interdigitation raises questions about which receptor the local IGF-2 binds. Based upon these data, any putative actions of the high IGF-2 concentrations within the placentomes cannot be mediated via the IGF-1R (Wathes *et al.*, 1998); this and other strong evidence from murine knockout studies again raises the question of the existence of an unknown XR. IGF-1 or IGF1R murine knockouts do not affect placental weight, while IGF-2 knockout of the paternal allele in mice severely diminishes placental development (Baker *et al.*, 1993). The placentas of murine IGF-2/IGF1R double mutants were equally affected by growth retardation as seen in the IGF-2 single mutants. In vitro studies with human placental tissues have indicated the presence of an unknown high affinity IGF receptor with equal affinity for IGF-1 and –2, and only slightly less affinity for insulin (Hintz *et al.*, 1984). LeRoith and others (1995) detected IGF1R discrepancies in molecular mass and antibody neutralizing binding activity, and therefore suggested the existence of another receptor; however, this also might have represented IR-IGF1R heterodimers (Jones & Clemmons, 1995). Lacroix *et al.* (1995), however, identified IGF1R protein in the trophoblast cells of ovine cotyledons at days 40, 50 and 75 of gestation.

Insulin-like growth factor receptor 2

IGF-2/M6P receptor content is differentially regulated during bovine fetal development (Pfuender *et al.*, 1995). In ovine fetal plasma, the circulating form of the IGF-2/M6PR is extremely high (Gelato *et al.*, 1989; Gallaher *et al.*, 1994), but placental IGF-2/M6PR decreases in ewes during pregnancy (Lacroix *et al.*, 1995). IGF2R mRNA in the ewe was localized to the caruncular-like stroma lining the luminal epithelium (Reynolds *et al.*, 1997). Lacroix and his group (1995) immunohistochemically located IGF2R protein in the fetal placental mesoderm. The IGF2R gene is imprinted, and normally only the maternal allele is expressed. When this allele is disrupted, mutant mice have an increase in serum and tissue IGF-2 levels. At birth they exhibit overgrowth (135%), organomegaly, a kinky tail, postaxial polydactyly, heart pathology, and edema. Perinatal death is frequent, which indicates the importance to survival of the M6P/IGF2R lysosomal enzyme trafficking (Lau *et al.*, 1994; Ludwig *et al.*, 1996). Disruption of IGF2R has also been shown to indirectly affect placental neovascularization (Young & Fairburn, 2000). Large sheep fetuses born after in vitro culture of in vivo fertilized eggs to the blastocyst stage, showed a decrease in IGF2R transcript and circulating IGF2R protein in late gestation, but no corresponding increase in circulating IGF-2 or tissue IGF-2 expression (Young *et al.*, 2001). Therefore, IGF2R may also affect fetal growth in IGF-2-independent mechanisms, and epigenetic change in the imprinted IGF2R locus might explain a genetic component of the "Large Offspring Syndrome" (Young *et al.*, 2001).

Insulin-like growth factor binding proteins

All IGFBP's studied so far are synthesized either in the uterine luminal epithelium or in underlying subepithelial stromal cells. This suggests that the IGFBP's are important for

regulating the transfer of IGF-1 and –2 between fetal and maternal compartments (Wathes *et al.*, 1998).

IGF binding protein -1 is a secretory product of decidualized endometrium and a major constituent of amniotic fluid (Crossey *et al.*, 2002). Phosphorylated IGFBP-1 has been shown to inhibit metabolic actions of IGF-1 on trophoblast cells in vitro. Conversely, non-phosphorylated BP-1 had the opposite effect, enhancing IGF activity (Yu *et al.*, 1998). In vivo, an inverse correlation between fetal growth and BP-1 levels in fetal circulation exists. Small for gestational age babies have elevated BP-1 levels in fetal cord serum (Langford, 1994).

IGFBP-1 stimulates trophoblast proliferation in early human pregnancy (Hills *et al.*, 1999). The BP-1 gene has putative progesterone response elements that mediate endometrial stromal cell BP-1 gene expression early in gestation (Crossey *et al.*, 2002). Therefore, the migration of first trimester invasive trophoblast in vitro is stimulated by IGFBP-1, likely by interaction with the RGD-binding site of the $\alpha_5\beta_1$-integrin (Irving & Lala, 1995). Gleeson *et al.* (1999), furthermore, indicated that IGFBP-1 seems to stimulate extravillous trophoblast migration and invasion by signaling through $\alpha_5\beta_1$-integrin, leading to signal transduction via FAK and MAPK. IGFBP-1 also increases the activity of gelatinases and tissue inhibitor of metalloproteinase 1 (TIMP-1) in first trimester cytotrophoblastic cells independently from IGF-1 (Bischof *et al.*, 1998). Even though IGFBP-1 is abundant in baboon uteri during implantation, this is not the case in ruminants (Tarantino *et al.*, 1992; Han *et al.*, 1996). This might be explained by the fact that IGFBP-1 appears to facilitate trophoblast penetration of the maternal interface, a much more invasive process in primate vs. ruminant placental development (Tarantino *et al.*, 1992; Wathes *et al.*, 1998). At birth, fetal weight and IGFBP-1 concentration was inversely correlated in humans (Ostlund *et al.*, 1997), and there is a slight negative correlation between serum IGFBP-1

levels and placental weight (Rutanen *et al.*, 1984). High IGFBP-1 levels in amniotic fluid are associated with lower placental weight (Verhaeghe *et al.*, 1999).

In primates, during implantation, the cell-cell communication between fetal trophoblasts and maternal decidual cells at the maternal-fetal interface is mediated by interactions between IGF-2 and IGFBP-1 (Hamilton *et al.*, 1998). BP-1 is expressed abundantly in the maternal decidua basalis throughout pregnancy (Han *et al.*, 1996; Giudice, 1997), and BP-1 facilitates normal invasion into the maternal decidua (Han *et al.*, 1996; Hamilton *et al.*, 1998) since BP-1 may modulate the action of IGF-2 on villous trophoblast proliferation, syncitial differentiation or apoptosis (Gratton *et al.*, 2002). In preeclampsia, trophoblast invasion into the maternal decidua and the spiral arteries at the maternal-fetal interface are impaired (Bronsens *et al.*, 1973; Zhou *et al.*, 1993, 1997). This results in shallow placental invasion and reduced perfusion, which may reduce oxygen tension in the placenta, and alter trophoblast differentiation and function (Redman, 1991; Zhou *et al.*, 1993; Genbacev *et al.*, 1996), suggesting cell specific altered growth processes in the placenta. In preeclampsia, IGF-2 and IGFBP-1 expression is altered at the maternal-fetal interface (Gratton *et al.*, 2002). Low levels of circulating BP-1 in the second trimester predict onset and are a marker of pre-eclampsia (De Groot *et al.*, 1996; Giudice *et al.*, 1997; Hietala *et al.*, 2000). At term, maternal circulating levels of BP-1 are elevated, but basal plate decidua has significantly decreased BP-1 mRNA in preeclampsia (Gratton *et al.*, 2002). Therefore, the increased level of BP-1 in circulation in women with preeclampsia is most likely not from decidual origin, and the conflict in levels is most likely because of sources of BP-1: maternal liver, decidua, or fetal BP-1 transported into maternal circulation (Gratton *et al.*, 2002). In women with severe late second and early third trimester preeclampsia, the elevated BP-1 is most likely due to increased hepatic production, which is consistent with the correlation with

hepatic transaminases (Giudice, 1997; Gratton *et al.*, 2002). Even though increase in circulating BP-1 is seen in women with preeclampsia, there is no change in IGF-2 levels (Giudice, 1997). The decrease in circulating BP-1 is the biochemical proof/evidence of impaired trophoblast invasion and decidual dysfunction, and decreased BP-1 at materno-fetal interface may contribute to abnormal trophoblast invasion in preeclampsia (Gratton *et al.*, 2002). Crossey *et al.* (2002), modeled the effects of excess IGFBP-1 on fetoplacetal growth in transgenic mice overexpressing human IGFBP-1. As explained in a recent comment by de Vrijer *et al.* (2002), we will mention their findings with caution, since it is important to keep in mind that in wild-type rodents, no IGFBP-1 is expressed in the decidua, only in the myometrium. In mice, however, BP-1 like actions may be ascribed to decidual alpha 2 macroglobulin which can bind IGF's (Han & Carter, 2000). In the BP-1 overexpression model Crossey *et al.* (2002) found marked effects early in pregnancy such as early placental failure. Effects on later placental development seemed rather minimal since normal term placental and fetal weights were obtained; suggesting other factors may overcome the initial BP-1 related deficiencies (Regnault *et al.*, 2002*c*).

In conclusion, Crossey *et al.* (2002) found that: decidual BP-1 excess was associated with increased placental mass (mainly in the labyrinth zone). Placental IGF-2 expression was unchanged, but IGF-2 bioavailability at the fetomaternal interface might be altered. In this study phosphorylated BP-1 predominated, which would suggest reduced IGF bioavailability. In the placentomegaly of the transgenic dams, trophoblast invasion of the decidualized endometrium was less pronounced. BP-1 was only present in amniotic fluid of transgenic mothers, independent of fetal genotype, indicating that IGFBP-1 would be mainly derived from maternal deciduas. Crossey *et al.* (2002) suggested the following potential explanatory mechanisms: BP-1 excess might attenuate trophoblast invasion and proliferation as a consequence of reduced local

IGF bioavailability. Alternatively, reduced IGF might influence trophoblast differentiation, perhaps favoring the growth of noninvasive over invasive trophoblast lineages. On the other hand, BP-1 may have direct IGF-independent effects on trophoblast invasion. Irwin & Giudice (1998) reported that BP-1 inhibited trophoblast invasion of endometrial stromal cells in vitro. In contrast, Gleeson *et al.* (2001) showed increased trophoblast invasion in response to BP-1. Both studies show that these effects are mediated through binding of BP-1 to the α5β1 integrin. In comparison with IGF-2 deficient mice, in both cases, spongiotrophoblast cells account for a greater proportion of the junctional zone. However, in the IGF-2 null mice, placental mass is reduced, whereas there is a large increase in labyrinthine zone and therefore increased placental mass in IGFBP-1 mutants. The exchange functions of the rodent placenta are largely confined to the labyrinthine zone. Thus, compensatory changes in fetal vascularization could result in an increase in labyrinthine tissue volume.

Regnault *et al.* (2002*c*) studied fetal gene expression in a placental insufficiency - intrauterine growth retardation (PI-IUGR) model. With ribonuclease protection assays, they found fetal liver IGFBP-1 to be upregulated in the model, but no significant differences were seen for IGF-1, IGF-2 and IGFBP-3 prior to maximal growth.

Endometrial expression of IGFBP-2 occurs in early pregnancy, and in the control non-gravid estrous cycle in the cow but not the sheep; this may be mediated by the rise in progesterone (Geisert *et al.*, 1991). From day 29 on in ovine gestation, IGFBP-2 is exclusively detected in the dense caruncular-like stroma underlying the luminal epithelium (Reynolds *et al.*, 1997). This finding agrees with a previous study by Delhanty & Han, (1992), which showed IGFBP-2 was present in close proximity to IGF-2 in rapidly proliferating tissues. Plasma

concentrations of BP-2 were higher in somatic cell cloned cattle, immediately after birth (Matsuzaki & Shiga, 2002).

In pregnant ewes, IGFBP-3 is present in the caruncles, endometrial stroma, placentome capsule and some allantochorion (Reynolds *et al.*, 1997). IGFBP-3 was not detected in fetal tissue by in situ hybridization (Reynolds *et al.*, 1997). Remarkably high levels of IGFBP-3 mRNA were localized to maternal blood vessel walls of the caruncles in the region of fetal villi penetration (Reynolds *et al.*, 1997). This might be important in regulation of placental blood flow and in the rate of transfer of IGF-1 and –2 from the maternal circulation to the uterus (Wathes *et al.*, 1998). It is important to remember that both IGF-1 and –2 when complexed to IGFBP-3 cannot cross the placental epithelium (Bar *et al.*, 1990). There is a strong correlation of IGF-1 and IGFBP-3 serum levels and birth weight as well as placental weight (Osorio *et al.*, 1996). Serum from pregnant women and rats contains a placental protease that cleaves IGFBP-3, reducing its affinity for IGF's (Davenport *et al.*, 1992; Han & Carter, 2000). In cases of utero-placental insufficiency, IGFBP-3 protease activity was increased (Langford *et al.*, 1995; Zumkeller, 2000). Zumkeller (2000) suggests that decidual IGFBP protease activity may be modulated by IGF-1 and progesterone.

During pregnancy in the ewe, IGFBP-4 mRNA expression is co-localized with IGFBP-2 in the caruncular stroma lining the luminal space, and is also abundant in the placentome capsule and somewhat less in the caruncular stroma (Reynolds *et al.*, 1997). This localization creates a barrier of IGFBP-4 surrounding the placentome and might help concentrate IGF-2 within the placentome (Wathes *et al.*, 1998). In the ovine fetus, levels of IGFBP-1 and -2 are higher than in the adult, while IGFBP-3 concentrations are lower (Wathes *et al.*, 1998).

Starvation of the ewe will cause a fall in insulin, while IGFBP-1 and -2 levels will go up, fetal glucose will drop, and IGFBP-3 and -4 concentrations will decrease (Gallaher BW, Breier BH, Blum WF, McCutcheon SN, 1995). This rise in fetal plasma IGFBP-1 was linked to a fetal hepatic, but not placental origin (Osborn *et al.*, 1992). Ligation of the uterine artery in the rat (Unterman *et al.*, 1990; Price *et al.*, 1992) and reduction of uterine blood flow in sheep (McLellan *et al.*, 1992) induced IGFBP-1 upregulation in the fetal liver. In newborn cloned vs. control calves, there was no difference in GH concentrations, while IGF-1 levels were lower, IGFBP-1 and -2 were higher, and IGFBP-3 and -4 seemed to be lower (Chavatte-Palmer *et al.*, 2000). At 3 weeks of age, patterns for these factors balanced out between both groups. Interestingly, in human intrauterine growth restriction (IUGR), IGFBP-1 is also elevated; this may reflect fetal suffering due to placental insufficiency (Popovici *et al.*, 2001), even though one pathology represents undergrowth and the other overgrowth (Chavatte-Palmer *et al.*, 2000). In accordance with these data, Garry *et al.* (1998), reported that concentrations of IGF-1 and IGF-2 were significantly lower in arterial blood of late-term cloned pregnancies. Also preimplantation blastocysts cloned by nuclear transfer indicated an upregulation of IGF-2 and IGF-1R mRNA, and downregulation of IGF-1 mRNA (Schultz *et al.*, 1996).

Exogenous administration of bGH to pregnant ewes stimulates fetal growth in the last third of gestation, but has little effect on placental size. This suggests a role for growth hormone in regulation of fetal growth due to changes in maternal nutrient partitioning or placental function (Jenkinson *et al.*, 1999).

There are other imprinted genes that influence fetal growth, however; their mechanistic effect on the insulin/IGF system is unknown:

1) Meg1/Grb10 (growth factor receptor bound protein 10) is maternally expressed and may inhibit signal transduction of the IGF's as well as insulin to direct fetal growth (Murphy & Jirtle, 2000; Reik & Walter, 2001). In both mice and man, 2) the insulin gene is imprinted and can have fetal growth consequences. It is chromosomally adjacent to the IGF-2 gene and is also paternally expressed in the placenta, but not normally in the pancreas (Giddings *et al.*, 1994). 3) Peg3 (paternally expressed gene 3) null mice exhibit fetal growth retardation, suggesting a role of this gene in fetal growth. 4) Peg1/Mest (paternally expressed gene 1/mesoderm specific transcript) has a growth-promoting role. 5) Deletion of Snrpn (small nuclear ribonucleoprotein polypeptide N) and its flanking sequence yields smaller progeny (Vrana *et al.*, 1998; Tilghman, 1999). 6) Gpc3 (glypican 3) may bind and affect IGF-2. It is not imprinted, but because of its location on the X-chromosome it is probably subjected to haploid dosage control. 7) Tsix, is the antisense gene reciprocally imprinted to the X-inactivation gene, Xist (Lee, 2000). Mice with a Tsix deletion feature IUGR. 8) Mash2 is an essential regulator of trophoblast giant cell differentiation and is embryologically lethal (Guillemot, 1995). 9) Other gene mutations associated with placental development that are not lethal but result in fetal growth retardation are, for example: Esx1, PdgfB, Wnt2 (Efstratiadis, 1998; Ihle, 2000).

From the preceding review, it is obvious that IGF regulation is exceedingly complex. During pregnancy this complexity is even more difficult to unravel due to interaction among fetal, maternal, and placental compartments. Nonetheless these complexities must be dealt with if one is to understand what goes wrong in pregnancies from embryos cloned via nuclear transplantation.

THE VASCULAR ENDOTHELIAL GROWTH FACTOR SYSTEM

Development of an adequate placental blood supply is imperative for normal fetal growth and developmental rates (Barcroft & Barron, 1946; Rosenfeld *et al.*, 1974; Reynolds, 1986; Reynolds *et al.*, 1987). About 40 days after conception in cattle, placental functioning becomes a sine qua non since hemotrophic exchange of substrates from the mother to the fetus is mediated by the placenta, an organ in which the fetal and maternal circulation are brought in close apposition.

During placentation, fetal chorionic villi invade the maternal crypts. New blood vessels sprout and branch abundantly, forming a complex architecture. This process is induced and controlled by complex interactions of numerous angiogenic factors. Moreover, growth and remodeling of the placental vascular beds takes place throughout gestation in concert with increasing rates of uterine and umbilical blood flow rate as well as fetal oxygen and nutrient uptake (Rosenfeld *et al.*, 1974; Reynolds, 1986; Reynolds *et al.*, 1987). The underlying mechanism of control of this vascular architecture is delicately balanced, and can easily be disrupted by environmental stressors (e.g. hypoxia) which induce structural adaptations in an attempt to enhance the capacity for exchange (Penninga & Longo, 1998). In this chapter, the complex interactions of the most important factors influencing vasculogenesis and angiogenesis will be discussed.

Vasculogenesis versus angiogenesis

Vasculogenesis occurs solely in the embryo and pertains to the initial differentiation of mesenchymal cells into angioblasts, the precursors of endothelial and haematopoietic cells from

the mesoderm (Poole & Coffin, 1989; Sherer & Abulafia, 2001), and the subsequent establishment of a primitive vascular network. Angiogenesis, on the other hand, is defined as development of new capillaries from pre-existing vessels (Folkman, 1985). Physiologically angiogenesis is observed during the ovulatory cycle in the follicle, placentation, and embryonic and fetal development. Pathologically, it is induced by inflammation, wound healing, immune reactions and neoplasia (Sherer & Abulafia, 2001).

Vascularization of the placental villi is the result of de novo formation of capillaries rather than protrusions of embryonic vessels (Ahmed *et al.*, 2000). Villi are made up of a solid trophoblast with a loose mesenchymal center derived from the extra-embryonic coelomic cavity (Benirschke & Kaufmann, 1995; Ahmed *et al.*, 2000). Before actual formation of blood vessels, macrophages (Hofbauer cells) (Demir *et al.*, 1989) migrate into the mesenchyme of the villi, and present angiogenic growth factors. These growth factors will stimulate the haemangioblastic cell cords, which are precursors for fetal endothelium, to form capillary lumens (Ahmed *et al.*, 2000; Breier, 2000).

Angiogenesis on the other hand, begins with a focal degradation of the basement membrane of a pre-existing capillary. This defect allows endothelial cells to migrate, proliferate and assemble into new tubes. Subsequently, peri-endothelial support cells are recruited to support further vessel maturation (Klagsbrun & D'Amato, 1991; Hanahan, 1997; Koblizek *et al.*, 1998; Sherer & Abulafia, 2001).

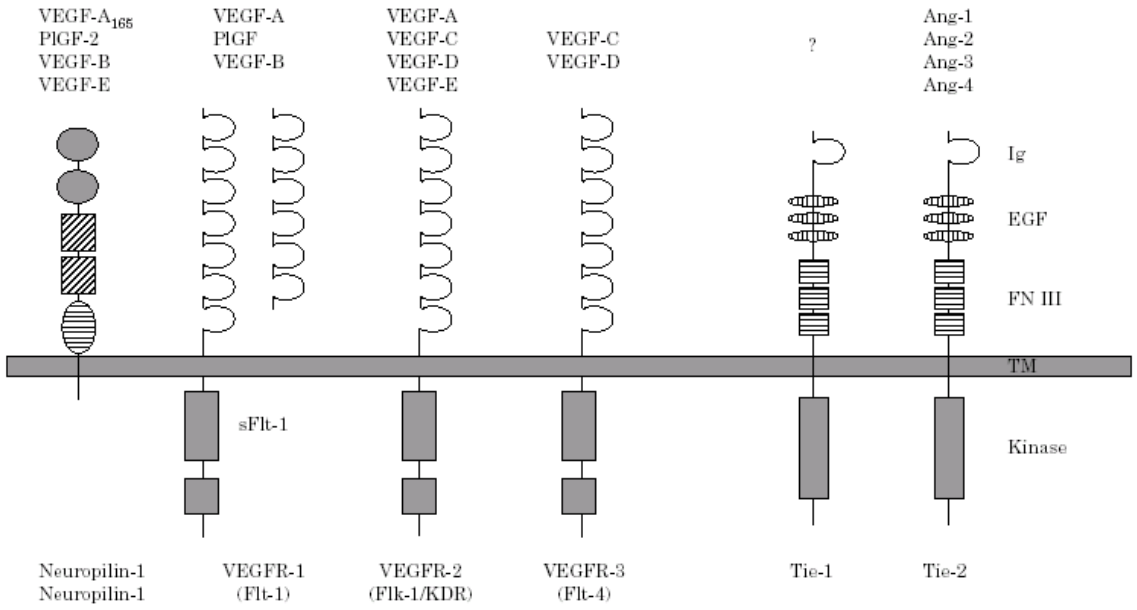

Figure 1.3 The endothelial signaling system. TM: is transmembrane domain, FN: fibronectin-like domain, Ig: immunoglobulin-like domain, Kinase: intracellular tyrosine kinase domain, Tie receptors express epidermal growth factor-like repeats (EGF). (Breier, 2000)

Based upon these excellent reviews (Hanahan, 1997; Ahmed *et al.*, 2000; Breier, 2000; Sherer & Abulafia, 2001), I will discuss the current consensus on vascular morphogenic signaling and placental angiogenesis. Regulation of angiogenesis depends on a fine balance of numerous promoting and inhibiting paracrine signals, modulating the activity of certain receptor kinases. Promotors of angiogenesis include vascular endothelial growth factor (VEGF), placental growth factor (PlGF), angiopoietins (Ang-1, Ang-2, Ang-3 and Ang-4), acidic and basic fibroblast growth factors (FGF); platelet-derived growth factors (PDGF), transforming growth factor α (TGF-α), transforming growth factor β (TGF-β), tumor necrosis factor α (TNF-α), folliculostellate-derived growth factor, angiotropin, and angiogenin. Non-specific angiogenesis inhibitors include interferons and platelet factor 4. Specific anti-angiogenic factors include angiostatin and endostatin (Sherer & Abulafia, 2001).

Vascular endothelial growth factor

Endothelial cell motility is promoted by binding of extracellular matrix proteins such as vitronectin and fibronectin to integrins expressed on the plasma membrane of endothelial cells (Eatock *et al.*, 2000). VEGF, also VEGF-A, is a potent-endothelium specific mitogen with a key role in the induction of angiogenesis in the placenta (Cheung, 1997); it also is important in development and maintenance of placental vascular function during gestation. Initially the molecule was identified as a potent permeability factor (VPF) (Senger *et al.*, 1983; Connolly *et al.*, 1989), and therefore VEGF might be involved in the regulation of amniotic fluid volume and composition (Cheung, 1997).

The active form of VEGF is a 40-45 kDa disulphide-linked homodimeric glycoprotein that is structurally related to the platelet-derived growth factor family. This growth factor is highly conserved across species. Thus far five isoforms derived from a single gene by alternative splicing of the 8 exons, have been found, resulting in proteins of 121,145,165,189 and 206 amino acids in length, respectively (Tischer *et al.*, 1991; Ferrara *et al.*, 1992; Dvorak *et al.*, 1995). The larger forms ($VEGF_{189}$ and $_{206}$) contain a unique heparin binding domain which affects their diffusibility because of cell membrane/matrix association (Torry & Torry, 1997). $VEGF_{165}$ is the most abundant isoform, including in the sheep placenta (Cheung & Brace, 1998); it is both diffusible and heparin binding. $VEGF_{145}$ is specific to the human endometrium (Charnock-Jones *et al.*, 1994). VEGF $_{189}$ and $_{206}$ maintain full biological activity and can be released from the extracellular matrix by proteolysis.

Cheung *et al.* (1995) have demonstrated expression and localization of VEGF mRNA in ovine placenta and fetal membranes and defined its possible role in fetal development and placental function (Cheung, 1997). In the pig, the VEGF system distribution is spatio-temporally

regulated throughout gestation. VEGF-KDR interaction is the main signal during initial placental angiogenesis, whereas the VEGF-Flt-1 interaction is more important in regulation and maintaining vessel integrity during late pregnancy (Dantzer *et al.*, 2000).

Athanassiades *et al.* (1998) have shown that VEGF will stimulate proliferation but not migration or invasiveness, in human extravillous trophoblast isolated from 1st trimester placentae. Ablation of VEGF alleles in mice results in abnormal blood vessel development and is lethal by mid-gestation (Carmeliet *et al.*, 1996). With VEGF deficiency, in situ differentiation of blood islands (vasculogenesis), sprouting from pre-existing vessels (angiogenesis), lumen formation, formation of large vessels, establishment of interconnections, and the spatial organization of intra- and extra-embryonic vessels are impaired (Carmeliet *et al.*, 1996). VEGF and VEGF receptors are expressed in all developing organs, and the high affinity VEGF receptors are localized primarily in the developing vessels, whereas VEGF is secreted from cells in the vicinity. In sheep, expression of VEGF by trophoblast cells probably allows for paracrine effects on both maternal and fetal endothelial cells in the vicinity of the fetal villi, causing vascular remodeling and controlling permeability to increase the surface area and rate of nutrient exchange between mother and fetus (Torry & Torry, 1997; Orbus, 1999). This indicates the importance of VEGF in stimulation of growth and survival of blood vessels in a paracrine fashion (Breier, 2000). Levels of VEGF are increased in many tumors and are elevated in women with pre-eclampsia (Clark *et al.*, 1998*b*).

VEGF activity is stimulated by hypoxia, cytokines and various other hormones (Neufeld *et al.*, 1994). The hypoxia inducibility of VEGF transcription seems to be largely mediated by HIF-1 (hypoxia-inducible factor) activity (Semenza *et al.*, 1999) a heterodimeric transcription factor tightly regulated by oxygen concentration (Richard *et al.*, 1999). This transcription factor

interacts with the VEGF hypoxia response element (HRE), activating transcription (Ikeda *et al.*, 1995; Levy *et al.*, 1995; Breier, 2000). In addition to transcriptional upregulation induced by hypoxia, VEGF-A mRNA is stabilized by specific proteins binding to the 3'-untranslated region of the transcript (Claffey *et al.*, 1998; Levy *et al.*, 1998). Apparently, VEGF mRNA contains destabilizing elements in its 3'untranslated region (3'UTR), 5'UTR and coding region. Each region can independently promote mRNA degradation under normoxic conditions. In contrast, under hypoxia mRNA stabilization depends on the cooperation of all three elements (Dibbens *et al.*, 1999). Both IGF-1 and -2 are also potent inducers of VEGF expression (Punglia *et al.*, 1997; Kim *et al.*, 1998). In vitro, IGF-1 regulates VEGF expression in endometrial adenocarcinoma cells at the post-transcriptional level by enhancing the stabilization of $VEGF_{121}$ and $VEGF_{165}$ (Bermont *et al.*, 2000). And in the eye, elevated IGF-1 levels have been shown to promote neovascularization through increased retinal VEGF gene expression (Punglia *et al.*, 1997). On the other hand, IGF-2 induced by hypoxia may contribute to the angiogenesis of hepatocellular carcinomas (Kim *et al.*, 1998). Steroids induce endometrial expression of various growth and angiogenic factors (Eatock *et al.*, 2000), and in fact, VEGF may serve as a local mediator of the effects of reproductive hormones on the endometrial vasculature (Jaffe, 2000). Estrogens upregulate VEGF expression in uterine tissue from rats (Cullinan-Bove & Koos, 1993) and women (Ahmed *et al.*, 1995). VEGF is also known to increase endothelial nitric-oxide synthase (eNOS) expression via activation of the KDR receptor tyrosine kinase (Shen *et al.*, 1999).

In the past few years, at least six more proteins have been identified as members of the VEGF family (Veikkola & Alitalo, 1999) because of sequence similarity and interaction with receptors of the VEGF family. The VEGF-B variant has 45 % amino acid homology to VEGF-A and has a mitotic effect on endothelial cells and a regulatory role in regulation of extracellular

matrix degradation and cell adhesion (Olofsson *et al.*, 1998). VEGF-B is particularly abundant in heart and skeletal muscle (Olofsson *et al.*, 1996). Clark *et al.* (1998) were unable to detect mRNA for either VEGF-C or VEGF-B in the human placenta by in situ hybridization, but Vuorela *et al.*, (1997), identified large amounts of mRNA for both VEGF-B and –C in human placenta by Northern blotting. VEGF-C functions as a regulator of lymphangiogenesis (Olofsson *et al.*, 1999).

VEGF-D is structurally most closely related to VEGF-C by virtue of the presence of N- and C- terminal extensions not found in the other VEGF family members (Farnebo *et al.*, 1999). Large amounts of VEGF-D are found in lung tissue, and this gene is strongly upregulated in embryonic lung prior to birth. Therefore VEGF-D may be of importance to lung vascularization during the last trimester of gestation (Farnebo *et al.*, 1999). Thus far, VEGF-D mRNA has not been detected in placental tissue (Fairbrother *et al.*, 1998).

Interestingly, VEGF's –A, -B and -C appear to be differentially regulated. Among them, only VEGF-A is induced by hypoxia (Enholm, 1997; Yonekura *et al.*, 1999). Other potent inducers of VEGF-A, like Ras oncoprotein and mutant p53 tumor suppressor, also do not increase VEGF-B and –C mRNA levels (Enholm, 1997). Growth factors such as PDGF, epidermal growth factor (EGF), TGF-β, and the tumor promotor phorbol myristate 12,13-acetate (PMA), on the other hand, stimulate VEGF-C but not –B mRNA expression (Enholm, 1997). These differential findings in regulation led to the suggestion by Enholm (1997) that the members of the VEGF family may serve distinct functions in vivo.

Placental Growth Factor

Placental growth factor (PlGF) is another angiogenic factor expressed in the placenta (Maglione *et al.*, 1991; Persico *et al.*, 1999). Structurally, PlGF shares 53% homology (71% similarity) with the PDGF-like region of VEGF. PlGF is a homodimeric glycoprotein of 46-50 kDa in size. Three isoforms of PlGF have thus far been described and are generated by alternative splicing of the mRNA for PlGF (Cao *et al.*, 1997; Ziche *et al.*, 1997). PlGF-2 has a 21-amino acid insertion not present in PlGF-1 coding for a highly basic region near the C-terminus (Hauser & Weich, 1993) that appears to be a heparin-binding domain (Athanassiades *et al.*, 1998). PlGF-3 is 70 amino acids longer than PlGF.

Ziche *et al.* (1997) have shown with in vitro studies that PlGF has a comparable effect to VEGF on cultured microvascular endothelium, and demonstrated that PlGF-1 can induce angiogenesis in vivo and stimulate migration and proliferation of endothelial cells in vitro. Despite this finding, PlGF is a very weak stimulator of endothelial cell chemotaxis and proliferation (Hauser & Weich, 1993). (Park *et al.* (1994), indicated that PlGF has no effect alone, but potentates the action of low doses of VEGF on microvascular endothelial cells both in vitro, and even more strikingly, in vivo. Murine Gene knock-outs for PlGF, however, develop normally (Carmeliet & Collen, 1999), probably because of compensation by other VEGF family members; also, PlGF might act primarily on trophoblast cells (Athanassiades *et al.*, 1998) that express Flt-1 (Breier, 2000). PlGF mRNA has been reported in the sheep fetal placenta (Regnault *et al.*, 2003). In that report, pregnant sheep were exposed to heat-stress to induce intra-uterine growth restriction (IUGR). They found no effect of tissue or treatment on oPlGF expression, but cotyledonary oVEGF mRNA content increased during the active stage of placental development, and oVEGF mRNA was elevated following heat exposure during the

period of maximal development. Ahmed *et al.*, (2000) obtained different results: PlGF mRNA and protein increased in IUGR placentae.

In vitro exposure of human trophoblast cell cultures to low oxygen tension downregulates PlGF expression, while increasing VEGF expression (Shore *et al.*, 1997). In human placentae, PlGF levels rise during the second trimester and peak at the beginning of the third trimester before falling to low levels at delivery (Torry *et al.*, 1998). In vitro, PlGF promotes extravillous trophoblast cell proliferation without influencing migratory or invasive behavior (Athanassiades *et al.*, 1998). In conclusion, PlGF appears to function in a paracrine mode on vascular endothelial cells in placental angiogenesis, and as an autocrine mediator of trophoblast function (Sherer & Abulafia, 2001)

PLGF and VEGF dimers

Both PlGF and VEGF form homodimers as well as heterodimers with each other (DiSalvo *et al.*, 1995; Cao *et al.*, 1996). Homodimers of PlGF bind to Flt-1 with high affinity, to Flt-1 but not to KDR (Park *et al.*, 1994*a*). PlGF/VEGF heterodimers on the other hand do bind KDR and elicit a mitogenic response in vitro, but with a 20- to 50-fold decrease in activity compared to homodimers (Cao *et al.*, 1996). These PlGF/VEGF heterodimers stimulate a strong chemotaxis in endothelial cells similar to that elicited by VEGF homodimers; PlGF homodimers, however do not influence chemotaxis by these cells. Two possible conclusions are deduced from these findings. PlGF expression may function to down-regulate the mitogenic effects of VEGF by formation of heterodimers, while up-regulating the chemotactic effects of VEGF on endothelial cells, possibly through various ligand-induced receptor dimerizations (Cao *et al.*, 1996). On the other hand DiSalvo *et al.* (1995), found PlGF/VEGF heterodimers to be equally

potent endothelial cell mitogens as VEGF homodimers, and therefore PlGF would only serve to enhance the mitogenic activity of VEGF. As Cao *et al*. (1996) point out, it is important to keep in mind that different levels, or types of receptors expressed by the endothelial cells used in each assay may have a large impact on the outcome.

Receptors

Thus far two major receptors for the VEGF family have been identified; the kinase domain containing region (KDR) and the fms-like tyrosine kinase (Flt-1); both are found in human placental tissues (Terman *et al.*, 1992; de Vries *et al.*, 1992). They belong to a family of transmembrane receptor tyrosine kinases (RTK's), and are endothelial cell specific (Terman *et al.*, 1992; de Vries *et al.*, 1992), each with distinct signals. Several other reports, however, have also demonstrated functional VEGF receptor expression in non-endothelial cells (Charnock-Jones *et al.*, 1994; Ahmed *et al.*, 1995). These receptors are widely expressed in vascular endothelial cells, and are also found in placental tissues where VEGF is localized (Cheung, 1997). Basic receptor tyrosine kinase (RTK) signaling is mediated through ligand-dependent autophosphorylation and activation of Ras pathways (Hanahan, 1997). Both receptors have an extracellular region containing seven immunoglobulin (Ig)-like domains, a single transmembrane domain, and a cytoplasmic consensus tyrosine kinase sequence (Banks *et al.*, 1998) (figure 1.3).

The first receptor, KDR (kinase-insert domain-containing receptor) or VEGF-R2 is homologous to the murine Flk-1 (fetal liver kinase-1) (Terman *et al.*, 1992). Mice homozygously deficient for Flk-1 show failure of blood-island formation and vasculogenesis, and severe reduction in haematoangioblast progenitors and then endothelial cells; they die mid-gestation (Shalaby *et al.*, 1995). Heterozygous mutants are essentially normal. In a study of

VEGF binding to its receptors in ovine placenta and fetal membranes, the predominant placental site of KDR was the maternal vascular endothelium, but there was a weaker signal in the maternal epithelium at 62, 103 and 142 days. At 103 and 142 days but not at 62 days, KDR was abundantly present in villous blood vessel endothelium. In fetal membranes, at the same days of gestation, KDR was expressed in the amniotic epithelium and intramembranous blood vessel endothelium; there was no KDR or VEGF binding in the chorionic cytotrophoblast (Bogic *et al.*, 2001).

Flt-1 (fms-like tyrosine kinase) or VEGF-R1 is the other major receptor for the VEGF family proteins (Quinn *et al.*, 1993). Homodimers of PlGF bind with high affinity to Flt-1, but not to KDR (Park *et al.*, 1994*a*). Embryos lacking the Flt-1 gene homozygously, have normal hematopoietic progenitors and abundant endothelial cells that migrate and proliferate; however, there is a failure to assemble these cells into normal vascular cells (Fong *et al.*, 1995). Again, heterozygous deletions are normal. Flt-1 appears to be the predominant receptor in humans, whereas KDR is the major receptor in sheep (Cheung, 1997).

Bogic *et al.*, (2001) could not detect Flt-1 expression in sheep placentae or fetal membranes by Northern-blots at 62, 103 and 142 days of gestation. However, Regnault *et al.* (2002*a*), did identify Flt-1 mRNA by ribonuclease protection assay and reverse transcription PCR in sheep placentomes.

A pregnancy-associated soluble variant of the flt-1 receptor (sFlt-1) has also been described (Kendall & Thomas, 1993; Kendall *et al.*, 1996; Banks *et al.*, 1998; Clark *et al.*, 1998*a*). The protein has a subunit size of 150 kDa. Normally it is present as a multimer of 400-550 kDa, and complexes of 600-700 kDa are formed after binding multiple VEGF molecules (Banks *et al.*, 1998). So far the Flt-1 mRNA's identified arise by alternative splicing (Kendall &

Thomas, 1993). Flt-1 is secreted by the human placenta and expected to function as a VEGF antagonist, suggesting that regulation of VEGF action is essential to successful pregnancy (Clark *et al.*, 1998*a*). Regnault *et al.* (2002*a*) were unable to identify sFlt-1 in ovine placental membranes.

In conclusion, KDR appears to be required for early development of the endothelial cell lineage and the mitogenic and chemotactic responses of endothelial cells. Flt-1 may be important for regulation of normal endothelial cell adhesion and interaction with the extracellular matrix (Bogic *et al.*, 2001), and the maintenance of mature blood vessels (Waltenberger *et al.*, 1994; Fong *et al.*, 1995; Orbus, 1999). In situ binding studies with recombinant VEGF in ovine placenta and fetal membranes suggest KDR plays a key role in maintaining vascularity and permeability (Bogic *et al.*, 2001).

More recently two other receptors, neuropilin-1 (NRP-1) and neuropilin-2 (NRP-2), were identified. The ligands VEGF-A_{165} (Soker *et al.*, 1998), PlGF-2 (Migdal *et al.*, 1998), VEGF-B (Makinen *et al.*, 1999) and the virus-encoded VEGF-E (Meyer *et al.*, 1999) bind to NRP-1. $VEGF_{165}$, PLGF-2 and VEGF-C bind to NRP-2. $VEGF_{121}$ does not bind to either of these receptors (Neufeld *et al.*, 2002). Originally, neuropilins were identified as receptors for semaphorins with a function in axonal guidance (Chen *et al.*, 1997; He & Tessier-Lavigne, 1997). The intracellular domains of the neuropilins are short and for signal transduction to occur, they complex with other receptors. Co-expression of KDR and neuropilin-1 on endothelial cells causes an increase in binding affinity of KDR for $VEGF_{165}$ (Soker *et al.*, 1998); however, controversy exists whether KDR complexes to NRP-1 (Neufeld *et al.*, 2002). In contrast, it has been shown that Flt-1 can form complexes with the neuropilins (Neufeld *et al.*,

2002). Neuropilin-1 knockout mice die in utero due to abnormal development of the cardiovascular system (Kitsukawa *et al.*, 1997).

Furthermore, VEGFR-3 or Flt-4 has been identified as the receptor for VEGF-C and –D; it does not play a role in vasculogenesis, but is important in vascular remodeling and the formation of lymphatic vessels (Veikkola & Alitalo, 1999).

In a model of placental insufficiency and intrauterine growth restriction, both caruncular and cotyledonary VEGF and PlGF mRNA concentrations increase with gestational age, but the respective protein concentration would only increase in the cotyledon. Furthermore, VEGF mRNA is elevated in hyperthermic ewes at 55 days of gestation. Later at 90 days, no changes in VEGF and PlGF mRNA were observed by hyperthermia. For the receptors, Flt-1 and KDR, levels rose over time in this model. However, receptor mRNA concentration was significantly reduced in the cotyledon of placentas from sheep exposed to hyperthermia (Regnault *et al.*, 2002*a*).

Angiopoietins and their receptors

To make the story on vasculogenesis and angiogenesis more complete, it is important to discuss two other major regulators: angiopoietin-1 and –2 (Ang-1 and Ang-2) and their receptor Tie-2. The Ang-1 and Ang-2 proteins are both about 75 kDa in size and are fairly homologous in sequence (Hanahan, 1997). They both contain a coiled-coil and a fibrinogen-like domain and bind to Tie-2 with similar affinity; neither binds the Tie-1 receptor. VEGF stimulates Ang-2 expression (Oh *et al.*, 1999). Ang-2 mRNA transcription is increased under reduced oxygen pressure, and the stability of Ang-1 mRNA is reduced under this condition (Charnock-Jones, 2002). Ang-1, Ang-2 and VEGF may affect the presence of occludin and plakoglobin. These

two proteins modulate tight junction stability, and therefore permeability of the materno-fetal endothelial barrier (Leach, 2002). Ang-1 induces autophosphorylation of Tie-2 in vitro. Ang-2 however does not induce receptor phosphorylation upon binding, and seems to competitively inhibit Ang-1-induced kinase activation of the Tie-2 receptor (Hanahan, 1997). Tie-2 is expressed on trophoblast, and its activation induces trophoblast proliferation, migration and production of nitric oxide (Ahmed & Perkins, 2000). Tie-2 knockout mice die later in embryogenesis; endothelial cells are present and organized into tubes, but the vessels are immature, lacking branching and organization into large and small vessels (Dumont *et al.*, 1994; Sato *et al.*, 1995). In these mice, the neurectoderm lacks vascularization, the endocardium and myocardium are mal-developed at the cellular level, and defects in vessel architecture are evident in multiple other tissues. Therefore, Tie-2 appears to control recruitment of stromal cells to stabilize the structure of endothelial tubes and modulate the function of blood vessels. Tie-1 has a similar structure to Tie-2, and the null phenotype is lethal with edema and hemorrhage as the major pathological finding, implicating Tie-1 control in fluid exchange across capillaries and in hemodynamic stress resistance (Puri *et al.*, 1995; Sato *et al.*, 1995).

In conclusion, Hanahan (1997) suggests a model for control of vasculogenesis, vessel maturation and maintenance, angiogenesis, and regression, based upon gene-knockout phenotypes. Early in embryogenesis, VEGF binds VEGFR-2, inducing the differentiation and proliferation of endothelial cells. In contrast VEGF binding VEGFR-1 elicits endothelial cell-cell interactions and capillary tube formation, a process that closely follows proliferation and migration of endothelial cells. Ang-1 binding to the Tie-2 receptor recruits and maintains the association of peri-endothelial support cells (pericytes, smooth muscle cells, myocardiocytes), thus stabilizing newly formed blood vessels. Ang-2 does not activate Tie-2, but binds and blocks

kinase activation in endothelial cells. This Ang-2 negative signal loosens vessel structure, reducing endothelial cell contact with surrounding matrix and support cells. In presence of VEGF and other inducers, this renders the endothelial cells more accessible and responsive towards angiogenesis. In absence of these angiogenic inducer proteins, Ang-2 elicits endothelial cell death, likely by apoptosis (Hanahan, 1997).

CHAPTER II: CHARACTERISTICS OF PLACENTAL AND FETAL TISSUES FROM NUCLEAR CLONED BOVINE PREGNANCIES

INTRODUCTION

Recent scientific breakthroughs in nuclear transplantation with fetal and adult somatic cells highlight the need to investigate associated pregnancy anomalies, such as excessive abortion rates, hydroallantois that usually is lethal to the recipient, stillbirths and metabolically abnormal neonates. The incidence of oversize calves from cloning by nuclear transplantation has been best documented in a study involving thousands of calves by Wilson *et al.* (1995). Most of these abnormal pregnancies did not in fact result in oversize fetuses, but were still very abnormal, resulting in neonatal death. Calves derived from IVF or nuclear transplantation require extra, intensive care due to metabolic pathologies. This is best documented by Garry *et al.* (1996) who reported that calves independent of size, were hypoxic, hypoglycemic, and hypothermic, and had abnormalities in metabolic hormones including low thyroxin and T3, and insulin concentrations four times higher than the reference population shortly after birth. With intensive care at birth, calves become essentially normal, and as determined from other studies (Wilson *et al.*, 1995), such offspring have normal pregnancies and offspring.

The placenta has a pivotal role in transfer of essential substrates from the mother to the fetus and is responsible for mediating and/or modulating the maternal environment. Placental and parturitional abnormalities have been observed directly with pregnancies from nuclear transplantation and IVF (Holm *et al.*, 1996; Kruip & den Daas, 1997). Parturition is frequently

lengthy, lacking normal signs of impending birth or pronounced uterine contractions (Avery & Greve, 1995). The incidence of hydroallantois is about 1% for pregnancies from IVF (1,376 pregnancies) (Hasler, 1998), which is about 10 times the normal incidence and is accompanied by a very high rate of mortality among recipient cows. Furthermore, cotyledons from cloned pregnancies are edematous, and intercotyledonary areas are thickened with a gelatinous-like material at parturition (Garry *et al.*, unpublished). Enlarged umbilical vessels, edematous membranes, and a greater than usual allantoic volume also were observed by Wells *et al.*, (1999). Peterson & McMillan (1998) observed impaired allantoic development and vascularization in 24% of in vitro-derived embryos at day 25. Allantochorion colonizes the maternal caruncles between days 20 to 35 (Melton *et al.*, 1951), so growth restriction during this period can limit numbers of placentomes. Later in pregnancy, size and morphology rather than number of placentomes are affected (Melton *et al.*, 1951). Nuclear transfer pregnancies from pluripotent embryonic cell lines were all lost before 60 days of gestation in one study (Stice *et al.*, 1996). These fetuses developed through organogenesis (by day 42); however, a deficiency in placentome development occurred where cotyledons and villi were absent, and a hemorrhagic response was noted in the caruncles void of crypts. Timing of pregnancy loss in this study coincided with timing of placentome formation in normal conceptuses. The caruncular hemorrhaging of nuclear transfer pregnancies indicated a maternal response; however, proper placentome formation did not ensue (Stice *et al.*, 1996). Since the bovine conceptus can survive on uterine milk until approximately 40 days of gestation (Melton *et al.*, 1951), fetal-maternal exchange via the placenta only becomes necessary thereafter. These data thus infer a genetically or epigenetically abnormal placenta, and we hypothesize that aberrant placental development may explain much of the attrition and pathologies of nuclear-cloned pregnancies.

Our objective was to characterize and compare fetal and placental tissues from nuclear-cloned and control day 75 bovine pregnancies. In a previous study with nuclear transfer embryos from the same origin, we showed that most recipient cows pregnant at day 70, would carry pregnancies to term (Seidel *et al.*, 1997). By this time point, gestational losses due to embryonic failure or severely underdeveloped placentation are eliminated. However in such pregnancies stillbirth, hydroallantois, dystocia or perinatal morbidity still occur, and many times this seems related to placental pathology (Cibelli *et al.*, 1998; Hill *et al.*, 1999). Basic information in this area might be applied to reducing the huge amount of pregnancy attrition in cloning and in vitro fertilization experiments, as well as provide information to decrease fetal and neonatal death that occurs with conventional reproduction.

MATERIALS AND METHODS

Cloned embryos

Frozen, cloned bovine Brangus embryos produced by nuclear transplantation were kindly donated to us by Dr. JM Wilson from Granada Biosciences, Inc. Embryos were cloned as described by Willadsen *et al.*, (1991), Westhusin *et al.*, (1992) and Wilson *et al.*, (1995). Oocytes were bisected, and each half was fused with a blastomere from a 16+ -cell embryo (Westhusin *et al.*, 1992). In most cases, cloned clutches were enlarged by serial nuclear transplantation; when embryos of the first round of cloning reached the morula stage, blastomeres were again separated, and each was individually fused with a bisected oocyte to enlarge the clutch of cloned embryos. After fusion, embryos were transferred surgically to

ligated sheep oviducts and recovered 6-7 days later. Those embryos developing normally (late morulae and blastocysts) were frozen in 0.25cc French straws using 1.5 M ethylene glycol in modified Dulbecco's phosphate-buffered saline (Voelkel & Hu, 1992). Straws were seeded at –7°C and cooled to –35°C at 0.5°C/min. Embryos were then held at –35°C for 15 min before plunging in LN2. Thawing was in air for 12 sec followed by 12 sec in a 35°C water bath. Even though these embryos can be transferred directly, without the need for removal of the ethylene glycol, we expelled the thawed embryos in PBS in order to evaluate embryo quality (1=excellent…4=poor). Subsequently, embryos of quality 1 or 2 were loaded pair wise into straws, and transferred, one or two per recipient, ipsilateral to the corpus luteum 6 to 8 days post-estrus. All gestations of clones in this study were from different cloned sets and of different parental origin, except within twin pregnancies.

Procedures with animals

All heifers were kept at the Animal Reproduction and Biotechnology Laboratory and fed 10 kg alfalfa hay once daily. All procedures with animals were approved by the University Animal Care and Use Committee. Estrous cycles of heifers were synchronized by injection of 25 mg PGF2α i.m. For day 45 tissue sample collections, eight control Angus heifers were artificially inseminated with Brangus semen 12 hours after first signs of estrus. Seven days after the first signs of estrus, twenty-nine frozen-thawed nuclear cloned embryos of grade 1 or 2 and from different clonal sets were transferred nonsurgically into 15 beef heifers. Recipient heifers received caudal epidural anesthesia prior to nonsurgical transcervical transfers (Elsden & Seidel, 1995); after local clipping and scrubbing the tailhead with betadine soap and 70% isopropyl alcohol, 6 ml of 2% lidocaine was injected into the epidural space at the 1st intercoccygeal joint.

45 days post-estrus, heifers again received epidural anesthesia and placental membranes were collected nonsurgically by transcervical, nonsurgical aspiration (Glover & Seidel, 2003). A stainless steel 30 cm tube fitted around a solid cervical round tip expander of 5mm diameter (Fig. 2.1) was gently manipulated through the cervix. Once the expander-tube communicated with the uterine lumen, the expander was removed and plastic tubing was hooked up to the stainless steel tube. About 100 ml 0.4% bovine serum albumin fraction V (USB - Amersham Life Science, 70195 Albumin fraction five CAS 9048-46-8) in phosphate- buffered saline (PBS) (Elsden & Seidel, 1995) were infused by gravity into the uterine cavity, and the uterus was gently massaged transrectally.

Once membranes ruptured, the intrauterine content was aspirated under negative pressure with a 100 ml syringe. After aspiration, all heifers received 25 mg ProstaglandinF2α (Lutalyse, Upjohn, Michigan) i.m.

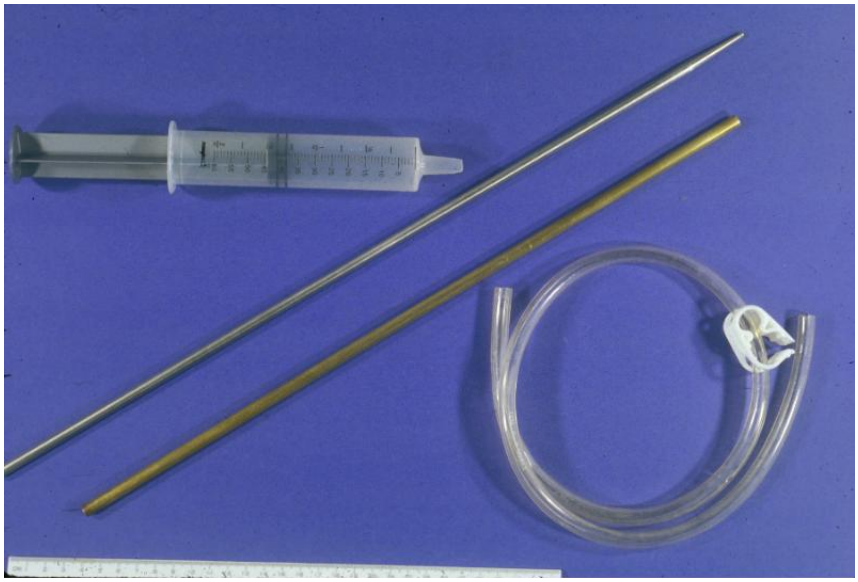

Figure 2.1 Equipment for transcervical nonsurgical aspiration.

For the day 75 tissue collections: ten control Angus heifers were bred by artificial insemination with Brangus semen, 12 hours after the first signs of estrus. Seven days after the first signs of estrus, Seventy-one frozen-thawed, cloned embryos of grade 1 or 2 and from different clonal sets were transferred nonsurgically into 41 beef heifers as described above. All heifers were checked by ultrasound for a fetal heartbeat at 30, 45, 60 and 75 days of gestation.

Tissue Collection

For the day 45 tissue collections, the aspirated fetal placental membranes were identified and separated into intercotyledonary allantochorion, cotyledons and amnion. The cotyledons were dissected from the allantochorion with microsurgical scissors under a stereoscope at 7-10 X. All tissues were snap frozen and stored in liquid nitrogen.

For day 75 tissue collections, the uterus was removed from the 75 ± 1 day pregnant heifers by means of mid-ventral hysterectomy. Heifers were fasted 24 hours prior to surgery and then underwent a midventral laparatomy under general anesthesia. Anesthesia was induced by intravenous injection of sodium thiopental (~5 mg/lb), and heifers were intubated and maintained on 2-4 % halothane gas for the duration of surgery. Analgesia was started intra-operatively (1 mg/kg i.v. banamine at surgery and daily for 3 days); beginning analgesia pre-operatively could have compromised the experiment since pharmacological effects on the tissues being collected are unknown.

The anesthetized heifers were placed on their backs and tied to the surgical table upside down. The surgical area was clipped and prepared using multiple alternate scrubs of betadine soap and 70% isopropyl alcohol, and then sprayed with betadine. Plastic booties were placed on the hind feet, the area flanking the incision was covered with sterile towels; a sterile surgical

drape was placed over the entire cow, except the head. A 25 cm incision was made between the navel and udder, and the reproductive tract was exposed. The ovarian artery and vein on both sides were isolated, clamped and double ligated. The uterus was removed starting with the apex of the horns, and the vessels within the broad ligament were ligated as removal of the horns proceeded. The uterus was amputated through the uterine body by double clamping and a double ligation with sterile umbilical tape caudal to the line of transaction. The clamp cranially to the line of transection was to avoid peritoneal spillage of uterine contents. The stump was oversewn with an inverting suture pattern. The abdominal incision was closed in three layers; individual chromic catgut sutures were used for the first layer, continuous sutures with chromic catgut for the second layer, and individual Braunamid sutures for the outer layer. The heifers were monitored for surgical complications at least twice daily for the week following surgery. The braunamid skin sutures were removed 10 days after surgery.

Once the uterus was collected, it was kept on ice, and weighed after the mesometrium was removed. The intact feto-placental unit was exposed by cutting through the uterus along the mesometrial surface from the tubo-uterine junctions to the uterine body. Gross anatomy was observed, and specific attention was paid to development, length and vascularization of amnion, allantois and chorion; abnormalities were recorded. Allantoic and amniotic fluids were aspirated by using sterile 20 gauge needles and plastic syringes and aliquots of fluids were stored at -20^{o}C. The entire amnion was dissected, and the fetus and umbilical cord were removed. The fetus was dissected free of membranes, and fetal wet weight and crown-rump length (curved) were recorded. The crown rump length was measured from a point midway between the orbits of the eyes following the vertebral ridge to the base of the tail, i.e., approximately the first coccygeal vertebra (Hubbert *et al.*, 1972). The length and weight of the umbilical cord were measured.

Two large placentomes in the umbilical area, 2 medium and 2 small placentomes were kept intact, dissected, weighed and measured. The remainder of the placentomes were manually separated into fetal cotyledonary and maternal caruncle components, cotyledons were counted, and samples of cotyledons and caruncles classified as large, medium or small were measured and weighed. The fetus was dissected, and weights of the brain, heart, kidneys and liver were documented. Intercaruncular endometrium and myometrium, and intercotyledonary allantochorion were also biopsied. All tissues were rinsed in physiological saline and respective samples were snap frozen in liquid nitrogen for tcRNA or protein isolation, or fixed in 4% paraformaldehyde (PFA) in PBS, and embedded in OTC for potential studies of histology, in situ hybridization and immunocytochemistry.

Data analysis

No physical measurement data were obtained from day 45 pregnancies. For day 75, physical measurement values are given as mean ±SEM. Due to the low sample number and heterogenous variance, we opted to analyze data from physical measurements by the non-parametric Wilcoxon rank sum test of SAS (SAS institute, Cary, NC). We compared placentome weight (of the largest placentome), fetal length, fetal weight, fetal weight/length index, umbilical cord weight/length index, heart, brain, liver and kidneys weights and the organ/bodyweight indexes and brain/liver index between treatment groups (controls vs. clones), sex and twinning. To evaluate twin effects, we also analyzed data including and excluding the measurements from twin pregnancies (recipient numbers 61, 82 and 39 – see table 2.2.). Equality of variances were evaluated by the 2-tailed F-statistic (SAS institute, Cary, NC). Furthermore, Pearson correlation

coefficients were determined among all physical measurements, including the weight of the largest placentome.

RESULTS

Day 45 tissue collections

Five of eight inseminated control heifers were diagnosed pregnant at 45 days. Six of 15 recipient heifers were pregnant with a live cloned pregnancy at day 45 as determined by ultrasonography. The nonsurgical, transvaginal aspiration was effective for obtaining adequate amounts of fetal membranes at this stage of gestation. Placentomes are very primitive in their development at this point of gestation, with a range in diameter of 3-10 mm, and we usually could dissect about 7-15 cotyledons from the tissues obtained. In one case (out of eleven) we retrieved the fetus.

Day 75 tissue collections

Five of 10 control heifers inseminated with Brangus semen, remained pregnant through day 75. From the 71 embryos transferred into 41 heifers, 16 day 30 pregnancies were obtained, and as determined by ultrasound of a fetal heartbeat, 13, 8, 8 conceptuses remained viable at 45, 60, 75 days of gestation respectively. Thus, there was a higher pregnancy attrition between day 30 and day 75 for clones than controls (figure 2.2).

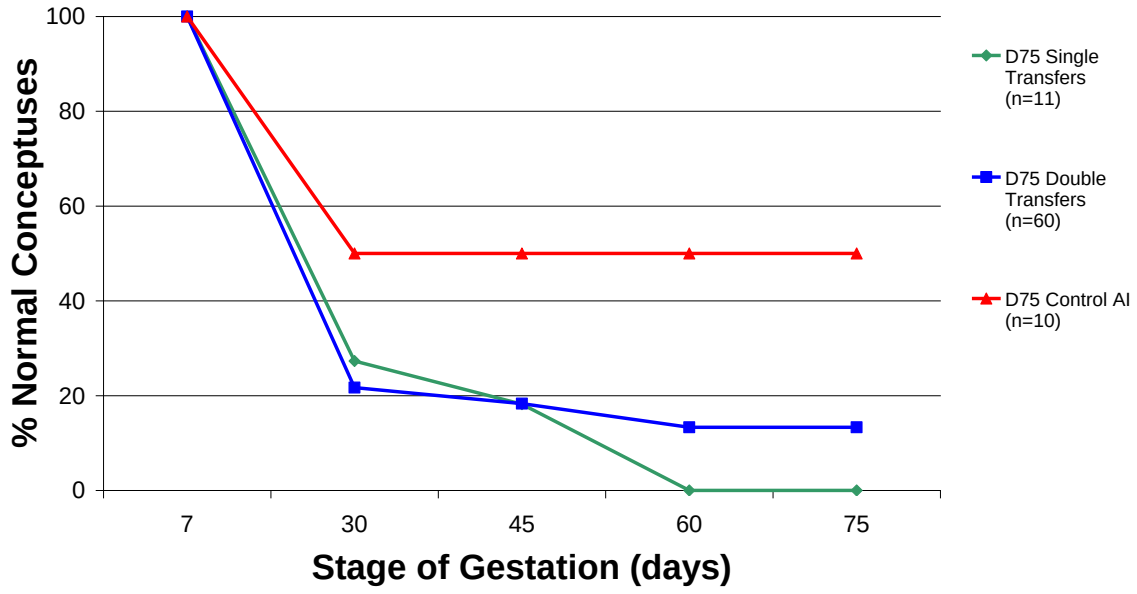

Figure 2.2 Conceptus attrition.

One fetus died between 45 and 60 days, but spontaneous abortion did not occur by day 75. At surgical recovery, we found jellied allantoic fluid and a decomposing, brown colored fetus in a brownish amniotic fluid. In one of the three twin pregnancies, one of the fetuses died between 30 and 45 days; its regressing gestational sac occupied one uterine horn without placentomes, but the counterpart was alive with no obvious abnormality, except for its rather small size (45 g) at Day 75 (see table 2.1., 2.2 and Fig 2.3.a.)

Even though placentomes seemed generally larger in the cloned gestations, no significant difference in large medium and small placentomes was found between treatment groups, although one cloned gestation had placentomes six standard deviations larger than the mean control placentomes (see table 2.1., 2.2 and Fig 2.3.b). However, we should keep in mind that sampling and categorization by size was somewhat subjective. Because of the amputation of the placenta through the body, and since many placentomes are still tiny and underdeveloped at day

75 of gestation, we were unable to count all placentomes. Nevertheless, there were clearly fewer placentomes when very large ones were present.

Interestingly, the mean weight to length ratio (g/cm) of the umbilical cord was significantly greater for clones (table 2.2, fig 2.3.c). Mean weights of heart, brain, liver, kidneys did not differ between groups (table 2.1). Neither did organ/bodyweight and the brain/liver indexes. However, numbers per group were limited, and the cloned population is very heterogenous (table 2.2, Fig 2.3 a, b, c).

Also, when removing the three cloned twin gestations from the data set, findings were only altered slightly (table 2.1). When the physical measurements were evaluated by fetal sex, ignoring treatment, only the brain weight stood out as different, with males having heavier brains than females at this point in gestation ($P < 0.08$ with twins, $P < 0.07$ without twins). When the weight of the largest placentome of the gestation was correlated with fetal weight, length and liver weight, positive correlations with p-values of $r = 0.45$ ($P = 0.09$), $r = 0.50$ ($P = 0.06$) and $r = 0.27$ ($P = 0.34$) were found when including twins; excluding twins the following p-values were obtained $r = 0.82$ ($P = 0.004$), $r = 0.77$ ($P = 0.01$) and $r = 0.93$ ($P < 0.0001$), respectively.

Table 2.1 Physical Measurements of gestational tissues collected on day 75 of gestation

	Controls (N=5) (Mean ± SEM)	**Clones** (N=10) (Mean ± SEM)	**P variance**	**w/o Twins** (N=5) (Mean ± SEM)	**P variance**
Mean Placentome weight (g):					
Large	2.41 ± 0.24	4.25 ± 0.98	0.004	3.25 ± 0.43	0.29
Medium	0.86 ± 0.13	1.23 ± 0.19	0.26	1.08 ± 0.22	0.33
Small	0.18 ± 0.03	0.21 ± 0.02	0.12	0.21 ± 0.02	0.12
Fetal weight (g)	59.5 ± 2.23	59.8 ± 3.04	0.22	60.9 ± 5.25	0.13
Fetal length (mm)	129 ± 5.63	135.9 ± 2.65	0.29	134.4 ± 4.68	0.73
Umbilical cord (g/cm)	0.35 ± 0.03*	0.46 ± 0.03*	0.67	0.45 ± 0.02*	0.25
Brain (g)	2.33 ± 0.07	2.30 ± 0.08	0.37	2.41 ± 0.09	0.55
Heart (g)	0.48 ± 0.07	0.50 ± 0.04	0.40	0.50 ± 0.06	0.78
Kidneys (g)	0.34 ± 0.06	0.45 ± 0.04	0.82	0.49 ± 0.06	0.83
Liver (g)	2.48 ± 0.09	2.64 ± 0.15	0.10	2.68 ± 0.25	0.07

* $P < 0.05$

Table 2.2 Variability in day 75 cloned pregnancies.

Cow No.	Fetus[b]	Fetal weight[a](g)	Placenta	L Placentome Weight[a] (g)	Umbilical Cord[a] (g)
61	2 L	+ 1 SD (65.3)	Very large placentomes	+ 6 SD (9.9)	- 1 SD (0.30)
39	1 D, 1 S	- 2 SD (45.4)	38 placentomes in one horn only	< 1 SD (2.6)	+ 2SD (0.48)
49	1 S	- 2 SD (50.2)	Gravid horn: wart-like placentomes; non-gravid horn: regular placentomes but poorly vascularized	< 1 SD (2.3)	+ 1 SD (0.44)
31	1 L	+ 2 SD (69.5)	Large placentomes	+ 2 SD (4.0)	+ 1 SD (0.46)
82	2 N	< 1 SD (58.9)	Normal placentomes	< 1 SD (1.9)	+ 2 SD (0.63)
54	1 S	- 2 SD (47.1)	Normal placentomes	< 1 SD (2.4)	+ 1 SD (0.42)
29	1 N	< 1 SD (63.9)	Uterus: size of twin pregnancy, lots of allantoic fluid, large placentomes, non gravid horn, few placentomes	+ 1 SD (3.2)	+ 2 SD (0.51)
79	1 L	+ 2 SD (73.7)	Large placentomes	+ 2 SD (4.4)	+ 1 SD (0.44)

[a] Number of standard deviations from controls. [b] L=large, S=small, D=death, N=normal, for twins, fetal weight and umbilical cord w/l were averaged, since they were almost equal.

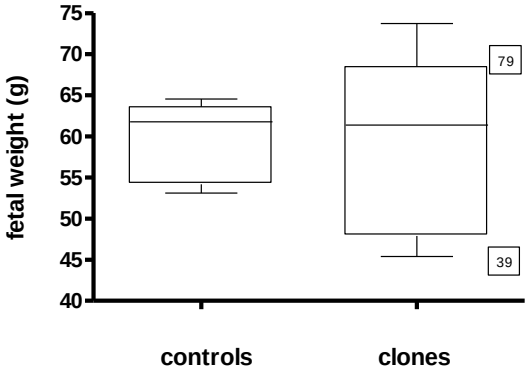

Fig 2.3.a. Fetal weight by treatment

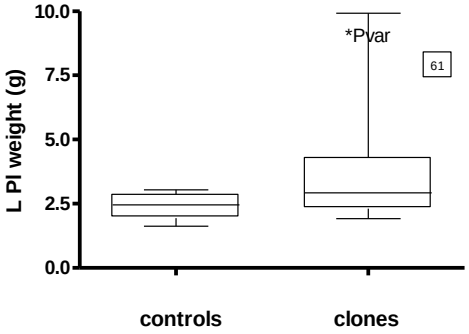

Fig 2.3.b. Large placentome weight by treatment

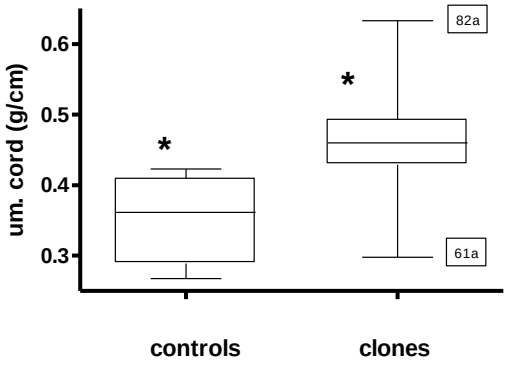

Fig 2.3.c. Umbilical cord size by treatment

Figure 2.3 Means, standard errors and range of physical measurements in day 75 placentomes. Boxed numbers are recipient numbers (see table 2.2.).
* $P < 0.05$

DISCUSSION

Our population of cloned embryos has previously been shown to result in gestational abnormalities typically observed with cloning. Field data from the company Granada International show that about 10% of these frozen, cloned embryos develop to term. Embryos from the same source were used by Garry *et al.* (1996) and resulted in 73 cloned calves, many of which were severely abnormal metabolically. In another study in our laboratory, seven recipients were flushed nonsurgically 8 days after transfer. From these, 3 of 14 (21%) embryos transferred were recovered and appeared to be developing normally (Seidel *et al.*, 1997). Seventy more of these day-7 cloned embryos were thawed and transferred; 13 (19%) appeared normal ultrasonographically at 4 weeks of gestation. However there was a linear attrition up till day 70, and only 5 (7%) went to term (Seidel *et al.*, 1997). In the present study, we also demonstrated severe gestational loss. Initially, more than 75% failed. And from the cloned gestations alive at 30 days (22.5%), we lost another half by day 60 (11.3%). Around day 35 is when placentation becomes imperative for gestational survival in the cow (Thompson & Peterson, 2000). At that point organogenesis is completed, and the embryo is now a fetus; its further growth and development can no longer be supported by histotroph or vascularized yolk sac nutrition. Therefore hemotrophic nutrition becomes essential (Melton *et al.*, 1951; Thompson & Peterson, 2000; De Sousa *et al.*, 2001). If the primitive establishment of placentomes fails at this time, gestational death will follow. This has been well documented in cloning experiments by Stice *et al.*, 1996; Peterson & McMillan, 1998 and Thompson & Peterson, 2000. Therefore, we suspect that most of the lost gestations seen in this experiment between days 30 and 60 were probably caused by severely impaired placentation. The vital

pregnancies that we collected at day 75 all had placentomes established. However, in one twin pregnancy, cotyledons were absent on the gestational sac of the dead fetus, and no caruncles were seen in that horn of the uterus; the other horn contained 38 placentomes and a viable, but small fetus. As described in table 2.2., many macroscopic aberrancies such as large placentomes, irregularly shaped, wart-like placentomes, and pale, poorly vascularized placentomes were observed. This is an early indication of high variability in cloned gestations. If gestation would have continued, some of these placentae would most likely have caused fatal pathologies manifesting either later in gestation (e.g. hydroallantois) or perinatally (e.g. edematous placenta with impaired oxygen transfer to the fetus).

Besides the large variance in fetal weight, only one fetus (#54) seemed to have macroscopically abnormal organs in that these seemed jellied even though the fetus was alive. To our knowledge, this has not been reported in the literature previously.

The one gestation with a fetus that died at day 55 did not abort by day 75, but why is unknown. Somehow the allantoic but not the amniotic fluid had jellified. This prevented separation of the placentomes into cotyledons and caruncles and might have prevented uterine prostaglandin release, luteolysis and abortion.

Of extreme interest is the observation of enlarged umbilical cords so early in gestation. Anecdotal reports of huge umbilical cords at birth in clones are numerous. Many times, if not taken care of surgically at birth, the umbilical will rupture at birth causing perinatal death due to exsanguination. Thus far, no explanation has been offered for this phenomenon. Molecular research on vascular and other growth factors and the functional role of Wharton's jelly are imperative for elucidation of this problem.

None of the mean organ weights or organ/bodyweight ratios differed between controls and clones; however, numbers were limited in this study. Also the brain/liver index did not deviate between treatment groups. When an embryo is nutritionally deprived, organs such as the liver will grow relatively smaller in comparison with the brain, which will be normal in size; this probably involves a brain saving mechanism. Day 75 might be too early in gestation to see energy depriviation effects.

Fetal sex did not seem to affect our findings. It has been shown previously that fetal gender has no effect on cotyledon weight, cotyledon area, placental weight (Prior & Laster, 1979). In a study by Szuba *et al.* (1988), no statistically significant differences between male and female fetuses in weight and length were revealed. Hubbert *et al.* (1972) showed that as fetal weight increased, the changes in organ weight: fetus weight ratio varied between organs. Organ weights did not significantly differ for breed or sex differences at respective fetal ages. Certain organs maintained a relative constant relationship with fetal weight throughout the fetal period (heart 0.8%, pancreas 0.07%, spleen 0.3%), while other organs changed in relation to fetal weight by 50 % or more. Interestingly, we observed a tendency to heavier brains in males at this point in gestation independent of treatment. When twins were excluded from the data set, a high positive correlation between size of the largest placentome and fetal weight, length and liver weight was observed, regardless of treatment. Early data on fetal growth and placental development indicated that birth weight depends primarily on fetal growth rate, and is highly correlated with placental weight (Alexander, 1964). Interestingly, (Sinclair *et al.*, 1997, 1998) found that overgrowth of cloned fetuses at term was not associated with larger placentae. However, at Day 75 of gestation, larger placentomes supported larger fetuses, both in control and cloned pregnancies. It makes sense that twin pregnancies bias this finding since placentomes

from these gestations actually have to support 2 fetuses. Most likely, the larger liver size is a consequence of the larger fetal size.

We conclude that there is marked attrition in cloned pregnancies between day 30 and 75 of gestation. Placentation appears necessary to support fetal growth at day 75. Placentomes from clones were not enlarged significantly at this point of gestation, but aberrancies in morphology and vascularization were observed. This is the first study identifying enlarged umbilical cords of cloned pregnancies as early as Day 75 of gestation; in contrast, cloned fetuses and their organs were not enlarged in this study. These observations support the hypothesis that abnormal placental development in clones leads to abnormal fetal development or abortion.

CHAPTER III: PLACENTAL AND FETAL LIVER EXPRESSION OF INSULIN-LIKE GROWTH FACTOR SYSTEM mRNA IN NUCLEAR CLONED BOVINE PREGNANCIES

INTRODUCTION

Recent scientific breakthroughs in nuclear transplantation with fetal and adult somatic cells highlight the need to investigate associated gestational and neonatal anomalies such as excessive abortion rates, hydroallantois lethal to the recipient, stillbirth, high birth weights and metabolically abnormal neonates (Willadsen *et al.*, 1991; Behboodi *et al.*, 1995; Wilson *et al.*, 1995; Garry *et al.*, 1996; Walker *et al.*, 1996; Kruip & den Daas, 1997; Garry, 1999; Farin *et al.*, 2001; Bertolini & Anderson, 2002). Findings of abnormal placentation in the first trimester (Stice *et al.*, 1996; Peterson & McMillan, 1998; Thompson & Peterson, 2000) and morphological abnormalities of placentation at birth such as an edematous placenta, few but very large placentomes, and pale, poorly perfused placentomes (Farin & Farin, 1995; Garry *et al.*, 1996) all infer a genetically or epigenetically abnormal placenta, and may explain much of the attrition and pathologies of nuclear-cloned pregnancies. The placenta has a pivotal role in transfer of essential substrates between the mother and the fetus and is responsible for mediating and/or modulating the maternal environment. Abnormal expression of one or more genes almost certainly is the cause of the abnormal pregnancies described.

Insulin-like growth factors (IGF's) are amongst the logical candidate genes for aberrant expression in cloned pregnancies. IGF-1 and IGF-2 and their receptors and binding proteins are involved with transport and metabolism of glucose and other carbohydrates and lipids. The IGF

system has especially profound effects on cell proliferation and differentiation during fetal development; its control over placental growth has been confirmed by gene deletion studies in mice (Baker et al., 1993). IGF-2 is secreted in large quantities in the fetus (Hossner *et al.*, 1997) and serves as a constant signal for growth in fetal circulation (McCusker, 1998); there is also considerable expression of IGF-2 in the placenta throughout gestation (Wathes *et al.*, 1998). More recently, Young *et al.* (2001) described a correlation between epigenetic modifications in the ovine IGF2R gene and fetal overgrowth in IVF gestations. IGF2R is maternally imprinted to be inactive. The imprinting process seems vulnerable to environmental effects that can deregulate methylation patterns and result in abnormal phenotypes (Reik *et al.*, 2001).

Despite the abundance of information on placental function, data on the expression and interactions of IGF's and their binding proteins during early bovine gestation and in placentas and fetuses derived from cloning and IVF are scarce, and no convincing explanation of the molecular cause of the observed abnormalities is available. Based on our data of lower IGF-1 and IGF-2 concentrations in arterial blood of late- term clone fetuses (Garry *et al.*, 1998), and the publication of Blondin *et al.* (2000) indicating a 2 fold rise in IGF-2 levels in the fetal liver of IVF pregnancies at day 70 of gestation, we collected tissues after hysterectomy of Day 75 control and nuclear-cloned bovine pregnancies. In a previous study with nuclear transfer embryos from the same origin, we identified that most recipient cows pregnant at day 70, would carry pregnancies to term (Seidel *et al.*, 1997). Day 75 bovine pregnancies have a fully functional placenta. By this time point, gestational losses due to embryonic failure or severely underdeveloped placentation have been eliminated. However in these pregnancies stillbirth, hydroallantois, dystocia or perinatal morbidity still occur frequently.

In this study we evaluated relative mRNA concentrations for the insulin-like growth factor family; IGF-1, IGF-2, IGF1R, IGF2R, IGFBP1, IGFBP2, IGFBP3, by means of real time RT-PCR in the fetal cotyledon and maternal caruncle of large placentomes, and the fetal liver of day 75 cloned and control gestations.

MATERIALS AND METHODS

RNA isolation

Total cellular RNA was isolated from frozen tissues (-80^{o}C) previously collected, (cfr. Chapter 2) by means of the RNeasy Maxi Kit (Qiagen, Valencia, CA). To disrupt cells, frozen tissues were ground under LN_2 using a mortar and pestle. One gram of powdered tissue was subsequently added to a lysing buffer (RLT) containing 4M guanidium isothiocyanate and 0.1M β-mercaptoethanol and homogenized with a rotor-stator homogenizer for 45-90 sec. An equal volume of 70% ethanol was then added to the lysate, creating conditions that promote selective binding of RNA to the RNeasy membrane in the spin column. After centrifugation, total cellular RNA was bound to the membrane, contaminants were washed away, and RNA was eluted in RNase-free water. DNase digestion was not required since the RNeasy silica membrane efficiently removes the DNA. To concentrate the RNA eluates, we added 1/10th volume of 8M LiCl and 2.5x volumes of ice cold 100% EtOH and mixed these thoroughly. This mixture was chilled for 20 min at –80^{o}C, and subsequently centrifuged for 15 min at 10,000 x g at 4^{o}C. Supernatant was removed, and the pellet was dissolved at the desired concentration. To evaluate the concentration and quality of the isolated tcRNA, a 1 μg aliquot of each sample was separated

by gel electrophoresis on a denaturing formaldehyde gel as per instructions of the manufacturer (NorthernMax kit, Ambion, TX). Samples were electrophoresed at 75 volts initially; once the samples had migrated out of the wells, the voltage was increased to 100V. Electrophoresis continued until the bromophenol blue dye front had migrated three quarters of the length of the gel. To evaluate the tcRNA integrity, the gel was exposed with UV light and photographed. If the RNA was not degraded, the 18S and 28S ribosomal bands were distinct and sharp without smearing.

Oligonucleotide primer design for real time PCR

β-actin primer design was based upon the cloned sheep cDNA sequence provided by R.V. Anthony (Colorado State University). The 5' primer was GGGACCTGACCGACTACC and the 3' sequence was GACAGCGAGGCAGGATGG. This resulted in a 519 bp cDNA with an annealing temperature of 55^{o}C, corresponding to bp 634-1152 of complete coding sequence of the ovine beta actin mRNA (accession number gi 2182268).

IGF-1 primer design was based upon the published sequence of bovine mRNA for the insulin-like growth factor I (accession number X15726). The 5' primer was CACATCCTCGCATCTCTT, and the 3' primer was TTGAGAGGCGCGCAGTACATC. This resulted in a 260 bp cDNA with an annealing temperature of 57^{o}C, corresponding to bp 78-338 in the protein coding sequence.

We designed two primer sets for IGF-2. The first set was used to identify IGF-2 in the fetal liver. The 5' primer sequence was ACTTCAGCCGACCATC CAGCC, and the 3' primer sequence was TCAGCGGACGGTGACTCTTGG. This corresponds to bp 66-389 of the partial coding sequence of bovine IGF-2 mRNA (accession number X53553); the optimal annealing

temperature was 60°C. Because of unclear results with the above mentioned primers in the cotyledon and caruncle, we designed a new set of primers. This 5' primer sequence was CGGCGGGGAGCTGGTGGA and the 3' primer sequence was TGGCGGGGGTGGCACAGT. This corresponds to bp 13-179 of the same coding sequence with an optimal annealing temperature of 61°C.

Primers for the IGF1R were based upon the published sequence of bos taurus mRNA for the insulin-like growth factor I receptor (accession number X54980). The 5' primer GCTGTAACCATGAGGCTGAGA and 3' primer AGAGCGATCAGGTGGAT correspond to bp 1038-1517 of the protein coding sequence creating a 405 bp cDNA with an annealing temperature of 60°C.

IGF2R primer design was based upon the coding sequence of bovine cation independent mannose 6-phosphate receptor protein (accession numbers J03527, M15869). The resulting 5' primer GTGCCGTGGATAAGAATG and the 3' primer CTGCCTGGGAGTCAAAGA gave a 291bp cDNA with an optimal annealing temperature of 56°C, corresponding to bp 1792-2065 of the protein coding sequence.

We also evaluated amounts of mRNA for the IGFBP's. The primer sequences for BP 1 through 3 were provided by B. De Vrijer (Health Sciences Center, Denver, CO).

The 5' GCCCATCCTCTGGAATGC and 3' TTGTTGCAGTTTGGCAGAT primer sequences were derived from the bovine IGFBP-1 sequence (accession number X54979). For IGFBP-2 the 5' primer GCGAGGGCACTTGCGAA*A and 3' primer CTTGCCCATCTGCCGGTGCTG were based upon the ovine IGFBP-2 sequence (accession number S44612). However, we had to modify one nucleotide (*) in the 5' primer since the published Bos taurus BP2 sequence indicated a mismatch. The IGFBP-3 primers were designed

from the bovine mRNA sequence for IGFBP-3 (accession number M76478). The 5' primer was CTCAGAGCACAGACACCCA and the 3' primer GGCATATTTGAGCTCCAC. Resulting cDNAs were 180 bp, 233 bp, and 335 bp long, respectively; optimal annealing temperatures were 57, 62 and 57^{o}C, respectively.

Reverse transcription

After tcRNA isolation of the tissues as described above, 2.5 μg of tcRNA quantified by spectrophotometry and gel electrophoresis, was reverse transcribed to synthesize first strand cDNA for PCR by use of the Superscript TM First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA). The tcRNA, 0.5mM dNTP, 0.5μg Oligo(dT)12-18 and DEPC-treated water were mixed and incubated for 5 min at 65^{o}C and placed on ice for 1 min. A mixture of 5 mM $MgCl_2$, 0.01mM DTT and 40 units of RNaseOUT recombinant RNase inhibitor in a final volume of 20 μl (20mM Tris-HCl (pH 8.4), 50mM KCl) was added and incubated for 2 min at 42^{o}C. Finally, 50 units of Superscript II RT were added and the reaction was incubated for 50 min at 42^{o}C. The reaction was terminated at 70^{o}C for 15 min, and then chilled on ice and stored at –20^{o}C.

Real Time PCR

For each gene of interest: IGF-1, IGF-2, IGF1R, IGF2R, IGFBP-1, -2, -3, and β-actin, we determined the optimal annealing temperature of the specific primers empirically by conventional PCR over a gradient of annealing temperatures. cDNA was generated by RT from cotyledon, caruncle or liver mRNA, as described above. A 50 μl PCR reaction consisted of 2μl of this RT product, water, 2.5 U Taq polymerase, and appropriate volumes of the following to

obtain concentrations of 20 mM Tris-HCl (pH 8.0), 50 mM KCl, 1.5mM $MgCl_2$, 0.2 mM dNTP mix, and 0.2 μM of each primer. The reaction was then overlayed with 20 μl mineral oil. Subsequently, four vials of 50 μl of PCR product were generated by conventional PCR under optimal conditions, pooled and electrophoresed on a 1% agarose gel, and purified by QIAquick Gel Extraction Kit (Qiagen, Valencia, CA) following the instructions of the manufacturer.

The cDNA product from the PCR reaction was sequenced at our University Macromolecular Resources Center by use of the specific primers, or cloned into a vector and sequenced by means of the T3 and T7 promotors (PCR-ScriptTM Amp Cloning Kit, Stratagene, La Jolla, CA). Purified amplicon was serially diluted over a 10ng/μl – 10^{-10}ng/μl range, for standard curve purposes.

To determine intra- and interassay variation, 10 parallel reactions were run with the same cDNA (intra-assay) and the same cDNA was run in 10 independent assays (interassay) (Leutenegger *et al.*, 2001; Bertolini *et al.*, 2002). Each assay was evaluated by determination of amplification efficiency on standard curve DNA dilutions, correlation coefficients, and melting curve analysis. Because of the good reproducibility, one assay was run per transcript, provided that the correlation coefficient of the standard curve was ≥ 0.99.

The initial lightcycler run was used to determine the optimal $MgCl_2$ concentration, and the optimal range of standards for both cotyledon and caruncle. Final Mg^{2+} concentrations of 1,2,3,4 and 5 mM were evaluated with 2μl of RT cDNA in Lightcycler-DNA Master SYBR Green I ready-to-use mix for PCR, which contained *Taq* DNA polymerase, reaction buffer, dNTP mix, SYBR Green I dye and $MgCl_2$ (Roche, Mannheim, Germany), 0.5μM of each primer, 0.16μl anti-*Taq* antibody and water to obtain a 20μl reaction volume. For example, a reaction was cycled 45 times with a 95°C denaturation step for 0 sec, a 60°C annealing step for 5 sec and

72°C extension step for 20 sec with slopes of 20°C/s each. The number of cycles necessary depends on the abundance of template copy number; the annealing temperature is dependent on the primer sequence; and the length of the extension phase depends on the length of the amplicon with 1 sec / 25 bp. Fluorescence was measured at the end of the extension phase. The melting curves were obtained at the end of the amplification by cooling the sample at 20°C/sec to 60°C and then increasing the temperature to 95°C at 0.1°C/s. Fluorescence was determined every 0.1°C.

After identification of the optimal conditions for PCR for each gene of interest, initial concentrations of mRNA were determined in the cotyledon and caruncle of five control and eight cloned day 75 pregnancies. Every lightcycler run included a negative control in which template was replaced by water, 5 standard samples with known concentrations, and the respective samples of day 75 cotyledons and caruncles. All genes of interest were evaluated from the same batch of RT product generated for each tissue sample. Quantification data was analyzed using the Lightcycler analysis software. Background fluorescence was removed by setting a noise band. The standard curve was prepared as described above, and by determining the intersection point (expressed as the cycle number) of an amplification signal on the standard curve, the initial template concentration could be derived. Values were normalized with those generated for β-actin.

The specificity of the amplification reactions detected with SYBR Green I dye was determined by performing a melting curve analysis. The products of a PCR reaction can be melted by increasing the temperature of the sample. At the T_m of the product, a sharp reduction in the level of fluorescence is seen. Non-specific products tend to melt at a much lower temperature than the longer specific products.

Southern Blotting

The cDNA generated from each real time PCR reaction was also separated on a 1% agarose gel with EtBr to verify product specificity by Southern blotting. After gel electrophoresis, the gel was exposed to UV light and photographed. Subsequently, the DNA was denatured by soaking the gel in 1.5 M NaCl/0.5 M NaOH for 1 hour, neutralizing in 1M Tris (pH 8) /1.5M NaCl for 1 hour, and capillary transferring to Zetabind® nylon membrane (CUNO Laboratory Products, Meriden, CT) overnight by upward capillary transfer using 10X SSC as the transfer buffer. After transfer, the membranes were rinsed 5min in 6X SSC to remove salt and agarose and cross-linked by UV irradiation. Membranes were wrapped in Saran wrap and stored at –20^{o}C until hybridization.

The membranes were prehybridized in 15 ml of prehybridization buffer containing 50% formamide, 5X Denhardt's solution, 6X SSPE, 1% SDS, 200μg/ml herring sperm DNA and 50μg/ml yeast t RNA, for 5 hours to overnight at 42^{o}C. After incubation, the matching cDNA fragment (519bp β-actin, 260 bp IGF-1, 323bp IGF-2, 405bp IGF1R, 291bp IGF2R, 180bp IGFBP1, 233bp IGFBP-2, 335bp IGFBP-3) was random prime labeled (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, England or Ambion, Austin, TX) with [α-^{32}P-deoxycytidine-5'-triphosphate (ICN Pharmaceuticals, Costa mesa, CA) to a specific activity ≥1 x 10^{8} cpm/μg DNA. Radiolabeled cDNA was denatured by boiling for 5 min followed by 5 min on ice, and then added to the prehybridization solution at 2 x 10^{6} cpm/ml. After overnight hybridization at 42^{o}C, the hybridization buffer was discarded, and the membranes were rinsed for 15 min at room temperature in 2X SSC/0.1% SDS, 15 min at 42^{o}C in 2X SSC/0.1% SDS, and twice for 30 min in 0.1X SSC/0.1% SDS at 60^{o}C to remove non-specifically bound probe.

Membranes were then exposed to radiographic film (Sterling™ Brand X-Ray Film, Life Science Products, Denver, CO) until sufficient band intensity was established for analysis.

Data analysis

For quantitative analysis of real time PCR data, the initial template concentration of the target mRNA was directly derived from its measured fluorescence signal and the standard curve. Relative levels of mRNA for the gene of interest within each sample were calculated as a ratio of the initial template concentration of the gene of interest to the initial β-actin concentration of the sample. Values are expressed as mean arbitrary units ± standard error of the mean (SEM).
Due to the low number of samples and non normality, we analyzed data by the non-parametric Wilcoxon rank sum test. We compared treatment groups (controls vs. clones) for the cotyledon, caruncle of large placentomes and for the liver. To evaluate whether twin effects confounded our findings, we also analyzed data leaving out the measurements from twin pregnancies. Preliminary least-squares analyses of variance indicated no significant effects of sex of fetus or twinning for any response.

Furthermore, since fetal sexes were reasonably balanced in both clones and controls, sex of fetus was ignored in the analyses presented. Equality of variances was evaluated by 2 tailed F-tests. Amounts of mRNA transcripts were also compared in the cotyledon vs. the caruncle within treatment groups. Pearson correlation coefficients were determined among all mRNA signals from all genes and fetal weight, placental weight, liver weight and umbilical cord weight/length ratio.

RESULTS

Relative gene expression patterns in the cotyledon and caruncle

In cotyledons and caruncles, levels of IGF-1 mRNA were low, about 100- fold less than β-actin. There was significantly less IGF-1 mRNA in cotyledons of control pregnancies ($P < 0.05$) compared to clones. In caruncles, however, no such difference was observed (tables 3.1, 3.2, 3.3, Fig. 3.1a). However, the variance was significantly more variable in clones in both cotyledons and caruncles ($P = 0.03$; $P = 0.0003$).

IGF-2 mRNA levels were high in cotyledons with concentrations similar to the amount of β-actin mRNA copies. In contrast to IGF-1, IGF-2 mRNA was higher in controls than clones, although of borderline significance ($P = 0.065$). In the caruncle, levels were 10 fold less IGF-2 mRNA for both clones ($P < 0.001$) and controls ($P < 0.001$) (tables 3.1, 3.2, 3.3, Fig 3.1b). In caruncles, variances for IGF-2 mRNA were significantly more variable in clones ($P = 0.026$).

mRNA levels for the IGF1R were quantified in both parts of the placentome, the cotyledon and caruncle; concentrations of mRNA were 100-fold less than β-actin. The cotyledons of four day 75 cloned pregnancies had a weak signal for IGF1R and showed double peaking in the melting curve. When the real time PCR products were evaluated by EtBr gel and Southern blotting, very faint signals for these samples were observed in comparison with others (table 3.1, 3.2, Fig 3.1c). When mRNA concentrations for the IGF2R were measured, a trend towards higher concentrations of mRNA for IGF2R in control cotyledon was observed ($P = 0.9$). Copy numbers were about 10-fold less abundant than β-actin, and there was slightly more IGF2R message in the caruncle than in the cotyledon ($P = 0.056$ (controls); P = 6.00E-0.4 (clones) (table 3.1, 3.2, 3. 3, Fig 3.1d).

mRNA for the IGFBP's 1, 2 and 3 was measured in cotyledons, caruncles and fetal livers of Day 75 pregnancies. IGFBP-1 transcripts were scarce in the placentome, 1000 fold less than β-actin. In the cotyledons of two pregnancies, one control and one clone, there was a double peak in the melting curve. This was manifest by two PCR products on an EtBr stained gel. The very low concentrations might be nearing the limits of the sensitivity of the assay. When twin pregnancies were left out, a tendency to higher levels of BP-1 mRNA in the caruncles of control pregnancies was observed (P = 0.056). IGFBP-2 mRNA copy levels in cotyledons and caruncles also were very low. In caruncles, message for BP-2 was substantially (10 fold) higher in control pregnancies than in most cloned pregnancies (P = 0.02), regardless of twinning.

The most abundantly expressed gene of those studied in both cotyledons and caruncles was IGFBP-3. mRNA concentration for BP-3 was 10-fold higher than β-actin in the placentome. No significant differences were observed between treatment groups, nor were gender effects observed.

When considering the hierarchy of mRNA abundance in cotyledons, BP-3 was the most abundant, 10-fold higher than for IGF-2 and β-actin. IGF2R transcripts were present about 10 fold less than β-actin, and IGF-1, IGF1R, BP-1 and BP-2, 100-fold less. In caruncles BP-3 transcripts were again the most abundant, 10 fold more than β-actin. For the IGF2R, mRNA content was about 10-fold less, and for IGF-1 and -2 there was about a 100-fold less of these gene products in comparison to β-actin. For IGF1R, there was a 1000-fold difference in the number of mRNA molecules compared to β-actin, while BP-2 and BP-1 message is very low, about 10,000-fold less than for β-actin.

Table 3.1 Relative mRNA concentrations of IGF family genes in cotyledons

	(n=5)	**w/ twins** (n=8)			**w/o twins** (n=5)		
Gene	**mean cont (SE)**	**mean clone (SE)**	**Pmean**	**Pvar**	**mean clone (SE)**	**Pmean**	**Pvar**
β-actin	282.98 (40.61)	281.33 (36.89)	0.72	0.83	295.02 (59.42)	1.00	0.48
IGF1/Act	0.012 (0.003)	0.020 (0.0086)	0.03	0.03	0.034 (0.007)	0.016	0.13
IGF2/Act	1.45 (0.16)	0.97 (0.14)	0.065	0.89	1.02 (0.13)	0.095	0.70
1R/Act	0.0086 (0.0013)	0.0044 (0.002)	0.13	0.23	0.0063 (0.003)	0.55	0.16
2R/Act	0.10 (0.015)	0.071 (0.0076)	0.09	0.29	0.063 (0.009)	0.095	0.36
BP1/Act	0.0025 (0.0006)	0.020 (0.016)	0.72	<0.0001	0.0033 (0.0009)	1.00	0.53
BP2/Act	0.0032 (0.0015)	0.023 (0.02)	0.94	<0.0001	0.0033 (0.0024)	1.00	0.40
BP3/Act	8.21 (1.22)	8.18 (0.92)	0.95	0.85	7.51 (1.04)	1.00	0.77

Table 3.2 Relative mRNA concentrations of IGF family genes in caruncles

	(n=5)	**w/ twins** (n=8)			**w/o twins** (n=5)		
Gene	**mean cont (SE)**	**mean clone (SE)**	**Pmean**	**Pvar**	**mean clone (SE)**	**Pmean**	**Pvar**
β-actin	227.4 (56.98)	245.13 (49.53)	0.83	0.90	177.4 (28.02)	0.84	0.0002
IGF1/Act	0.054 (0.0048)	0.13 (0.04)	0.13	0.0003	0.17 (0.06)	0.10	0.04
IGF2/Act	0.12 (0.016)	0.17 (0.04)	0.4	0.026	0.21 (0.06)	0.31	0.024
1R/Act	0.01 (0.0016)	0.0091 (0.0016)	0.4	0.76	0.0098 (0.0024)	0.69	0.50
2R/Act	0.32 (0.067)	0.21 (0.057)	0.28	0.94	0.17 (0.02)	0.15	0.04
BP1/Act	0.0075 (0.0025)	0.0033 (0.0008)	0.13	0.05	0.0024 (0.0009)	0.056	0.08
BP2/Act	0.011(0.0021)	0.004 (0.0013)	0.02	0.51	0.0034 (0.0012)	0.03	0.30
BP3/Act	14.92 (1.9)	13.1 (1.4)	0.62	0.78	14.77 (1.5)	1.00	0.63

Table 3.3 Probabilities of mean differences in mRNA content of cotyledons vs. caruncles within treatments. (includes twin data)

	Controls	**Clones**
Gene	**P-value (cot/car)**	**P-value (cot/car)**
IGF1/Act	0.008* (<)	0.03* (<)
IGF2/Act	0.008* (>)	< 0.001* (>)
1R/Act	0.54 (=)	0.13 (=)
2R/Act	0.056 (<)	< 0.001* (<)
BP1/Act	0.032* (<)	0.96 (=)
BP2/Act	0.032* (<)	0.44 (=)
BP3/Act	0.032* (<)	0.015* (<)

* P < 0.05

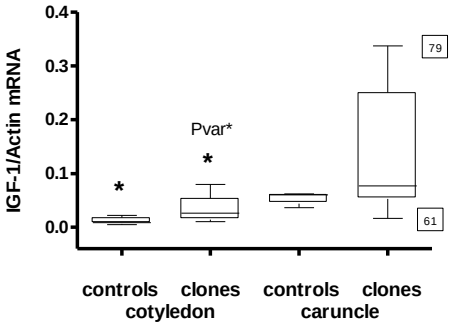

Fig 3.1.a. IGF-1 mRNA placentomes

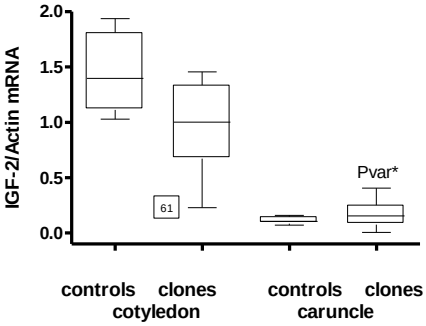

Fig 3.1.b. IGF-2 mNRA placentomes

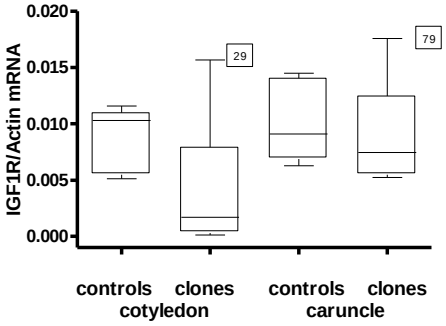

Fig 3.1.c. IGF1R mRNA placentomes

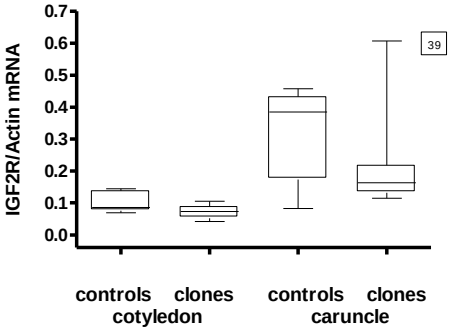

Fig 3.1.d. IGF2R mRNA placentomes

Figure 3.1 Median, 25-75th percentile and ranges of mRNA concentrations for IGF-1 and IGF-2 and their receptors in day 75 placentomes.
Boxed numbers are recipient numbers (see table 2.2)
* $P < 0.05$

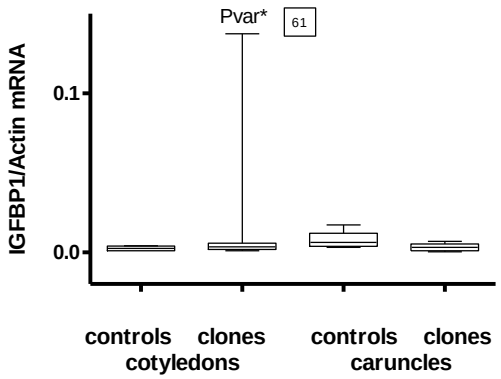

Fig 3.2.a. IGFBP-1 mRNA placentomes

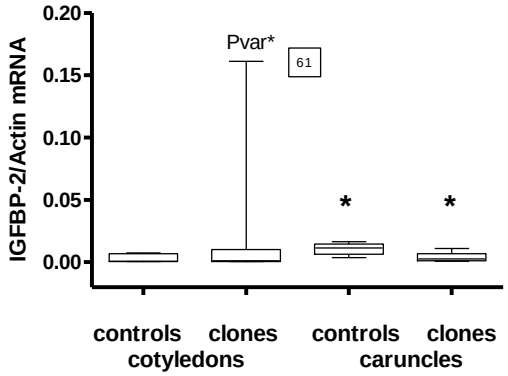

Fig 3.2.b. IGFBP-2 mRNA placentomes

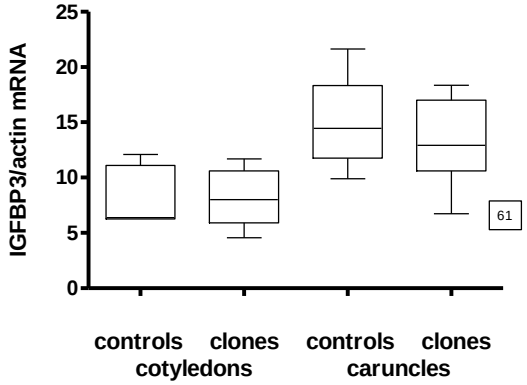

Fig 3.2.c. IGFBP-3 mRNA placentomes

Figure 3.2 Median, 25-75th percentile and ranges of mRNA concentrations for the IGFBP's in day 75 placentomes.
Boxed numbers are recipient numbers (see table 2.2.)
* $P < 0.05$

Relative mRNA concentrations in fetal liver

In day 75 fetal livers, IGF-1 mRNA levels were a 1000-fold less abundant than β-actin, and no differences were seen between fetuses from cloned or control pregnancies (table 3.4). IGF-2 mRNA levels were high in the liver, at the same relative concentration as β-actin mRNA. Fetal livers from cloned pregnancies contained more (3-4 fold) IGF-2 mRNA than controls, whether twin gestations were taken into consideration or not ($P = 0.008$ vs. $P = 0.019$).

In contrast to the placentome, IGFBP-1 mRNA was abundant in the fetal liver and expressed at the same level as β-actin. IGFBP-2 mRNA was also found in the liver, in relative abundance at about 10-fold less than β-actin. mRNA concentration for IGFBP-3 was high in fetal liver, at the same level of expression as β-actin.

Considering the overall hierarchy of abundance for the genes of interest in the fetal liver, BP-1, BP-3 and IGF-2 transcripts were present in similar amounts as β-actin, BP-2 about 10 fold less, but IGF-1 message on the other hand, was relatively scarce at 1000-fold less than β-actin.

Table 3.4 Relative mRNA concentrations of IGF family genes in the liver

	(n=4)	**w/ twins** (n=8)			**w/o twins** (n=5)		
Gene	**mean cont (SE)**	**mean clone (SE)**	**Pmean**	**Pvar**	**mean clone (SE)**	**Pmean**	**Pvar**
β-actin	569.75(69.76)	576 (70.63)	1.0	0.19	519.8 (65.8)	0.73	0.97
IGF1/Act	0.0008 (0.0002)	0.0008 (0.0001)	0.93	0.26	0.0007 (0.0001)	1.0	0.46
IGF2/Act	0.24 (0.03)	0.8 (0.29)	0.008	0.0014	1.01 (0.44)	0.03	0.0008
BP1/Act	0.87 (0.2)	0.95 (0.14)	0.57	0.82	0.92 (0.16)	0.41	0.84
BP2/Act	0.037 (0.014)	0.078 (0.022)	0.21	0.22	0.079 (0.033)	0.41	0.14
BP3/Act	0.41 (0.06)	0.59 (0.12)	0.57	0.12	0.59 (0.11)	0.56	0.26

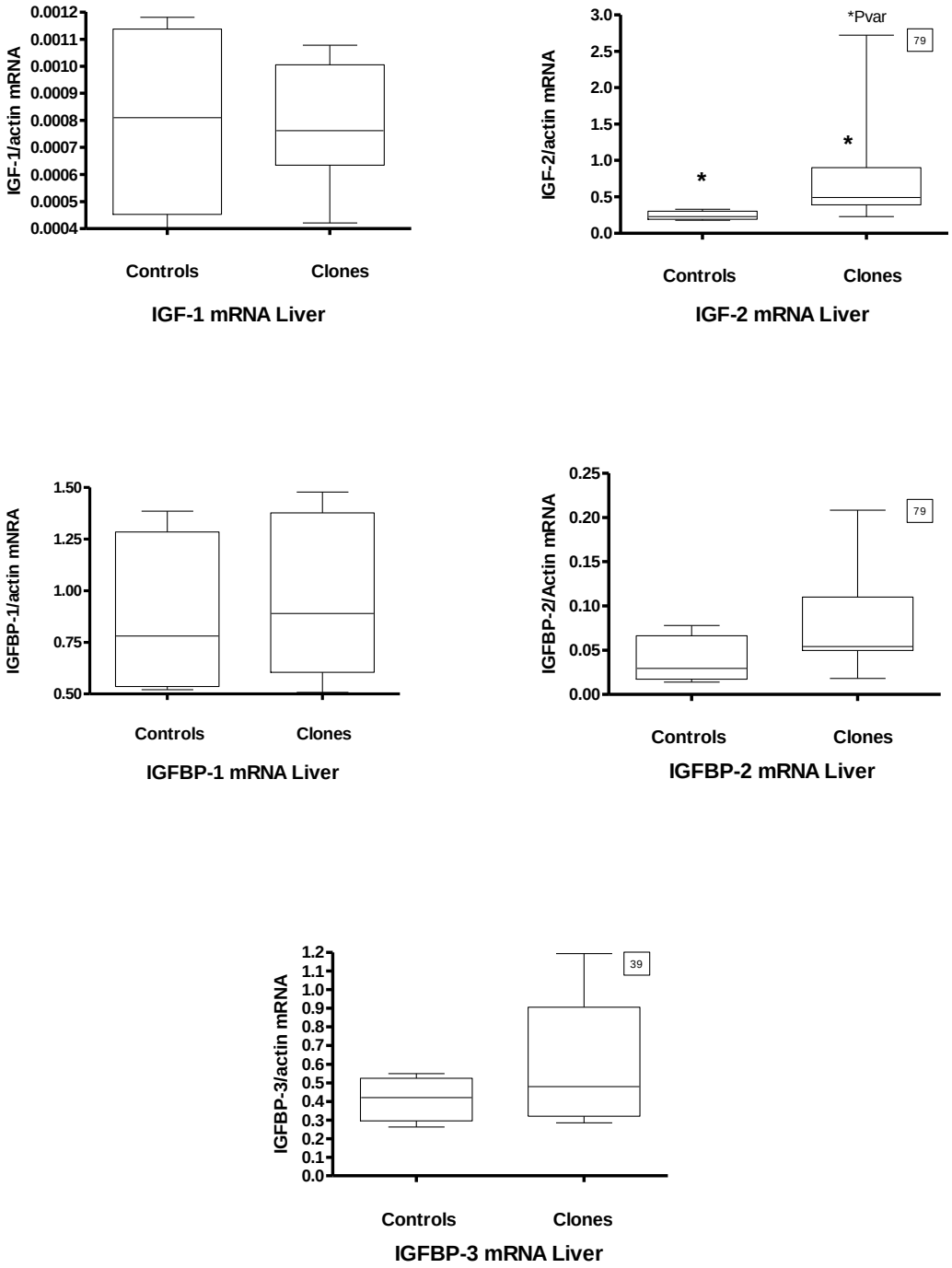

Figure 3.3 Median, 25-75th percentile and ranges of mRNA concentrations for the genes of the IGF system in day 75 fetal liver.
Boxed numbers are recipient numbers (see table 2.2.)
* $P < 0.05$

Correlation analyses between and within physical and molecular traits

Correlation coefficients were calculated using all data and by treatment to evaluate whether different trends existed for each group. Interestingly, clones occasionally revealed correlation coefficients with an opposite sign (negative vs. positive or vice versa) in comparison to controls. It was of utmost importance to graph the data, since distortion sometimes occurred due to outlier effects. These outlier effects were mostly due to extreme variability of cloned gestations. In controls, a positive correlation was observed between largest placentome weight and cotyledonary IGF-2 mRNA ($r = 0.90$, $P = 0.03$), while the correlation was opposite in clones for these traits ($r = -0.81$, $P = 0.015$). Because of the low numbers, correlations were usually not significant in the control group. A positive correlation between cotyledonary BP-3 and IGF-2 content was observed in clones, pooled data, and controls, ($r = 0.75$, $P = 0.03$; $r = 0.65$, $P = 0.016$; $r = 0.8$, $P = 0.1$, respectively). No significant correlation coefficients were observed between umbilical cord weight length ratio and gene expression levels in the fetal liver.

Individual gestations

Four pregnancies, numbers 61, 39, 49 and 31, had very faint signals for IGF1R mRNA in cotyledons. The singleton cloned pregnancy number 79 had the largest fetus (+ 2SD) and largest fetal liver as well as large placentomes (+ 2SD) and a slightly enlarged umbilical cord (+1SD). Fetal liver mRNA concentrations for this pregnancy were extremely high for IGF-2, BP-1, BP-2 and BP-3; IGF-1 mRNA content on the other hand was normal. In the placentome, in contrast, this gestation had very high concentrations of mRNA for IGF-1 and IGF1R in caruncles. Clone 39, a twin gestation with one dead and one live conceptus, has very high IGF2R mRNA but low IGF1R mRNA in caruncles. Clone 61 had aberrant mRNA concentrations for multiple genes.

The cotyledon had increased mRNA for the BP1, BP2 (10 -fold) and PLGF genes; however, IGF-2 and IGF1R mRNA levels were low in the cotyledon. This twin pregnancy had the largest placentomes (+6SD) in the study, and both fetuses were relatively large (+ 1SD). However, the umbilical cord of one of the fetuses was the smallest one of the clones. mRNA for BP-3 was very low in the caruncles of this pregnancy. Unfortunately, livers were not saved for this pregnancy.

DISCUSSION

In vitro manipulations of bovine embryos have been associated with detrimental consequences during gestation. Abortion rates are above 50% early in gestations resulting from cloning, and further pregnancy attrition occurs throughout gestation at higher than normal rates. Despite the abundance of information on placental function, data on the expression and interactions of IGF's and their binding proteins after implantation in natural bovine gestations and in cloned or IVF placentas and fetuses is scarce, and no convincing explanation of the molecular cause of observed abnormalities is available. In this study, we evaluated if disturbances in the pattern of gene expression in the IGF system could be detected in the placentomes and fetal liver of nuclear transfer clones.

Insulin-like growth factor 1

In the ewe, Reynolds *et al.* (1997) found that uterine IGF-1 mRNA levels were low following implantation, highest in the maternal stroma surrounding the uterine glands (although

this declined during early pregnancy), and undetectable in the caruncles after fetal villus invasion (Reynolds *et al.,* 1997). Fetal placental tissues did not hybridize for IGF-1 mRNA (Reynolds *et al.*, 1997). These findings suggest that local IGF-1 production might not be important in placental development (Reynolds *et al.*, 1997). Also, in IGF-1 knockout mice (Baker *et al.*, 1993), placental growth is normal. In this study we also found IGF-1 mRNA levels to be relatively low in both cotyledon and caruncle. However, in cotyledons of cloned pregnancies, relative mRNA for IGF-1 was double ($P = 0.03$) that of controls. The fetal livers produced little IGF-1 mRNA, and no difference was observed between clones and controls. If IGF-1 production is indeed upregulated, then one wonders what its paracrine effect on placental development might be, since both IGF-1 and IGFBP-1 stimulate trophoblast proliferation in early human pregnancy (Hills *et al.*, 1999).

Insulin-like growth factor 2

Our studies revealed relatively high amounts of IGF-2 mRNA in the cotyledon, at a similar level to the housekeeping gene β-actin. In the caruncle, levels were about 10 fold less. There was a trend towards reduced IGF-2 mRNA in the cotyledon of the cloned pregnancies ($P = 0.065$). However, livers of cloned fetuses produced 3-4- fold higher concentrations of IGF-2 mRNA than controls. IGF-2 is secreted in large quantities in the fetus (Hossner *et al.*, 1997) and serves as a constant signal for growth in the fetal circulation (McCusker, 1998); there is also considerable expression of IGF-2 mRNA in the placenta throughout gestation (Reynolds *et al.*, 1997; Wakayama *et al.*, 1998; Wathes *et al.*, 1998). In early gestation, IGF-2 mRNA is most abundantly expressed at the invading front of intermediate trophoblast in the anchoring villi, stimulating migration through the IGF2R and MAPK pathway (Han *et al.*, 1996; Hamilton *et al.*,

1998; McKinnon *et al.*, 2001). In late human pregnancy, IGF-2 is found mainly at the syncytiotrophoblast (Han *et al.*, 1996). Surgical reduction of placental size in sheep causes intrauterine growth retardation and is accompanied by an increase of maternal plasma IGF-2 (Jones *et al.*, 1988). In preeclampsia in humans, increased IGF-2 mRNA is found in trophoblasts of peri-infarct regions, suggesting a role for IGF-2 in placental repair or remodeling (Gratton *et al.*, 2002). In human diabetic pregnancies, fetuses receive a large influx of glucose; this may stimulate expression of IGF-2 in the placenta, resulting in higher utilization of glucose and overgrowth of the placenta (Shen *et al.*, 1986).

Genetically engineered mice heterozygous for a disrupted IGF-2 allele showed a 40% growth deficiency at birth (DeChiara *et al.*, 1990). Fetal overabundance of IGF-2, by disruption of the IGF-2/M6PR, causes fetal overgrowth and perinatal lethality in mice (Filson *et al.*, 1993; Lau *et al.*, 1994). IGF-2 also possesses local growth promoting effects in fetal tissues, as demonstrated in H19 null mice (Eggenschwiler *et al.*, 1997) in which pups were 30% overgrown at birth, and IGF-2 was only elevated in tissues, not in circulation.

When Constancia *et al.* (2002) abolished the IGF-2 promotor (P0) transcript in mice, a marked reduction in IGF-2 signal was observed specifically in the labyrinth trophoblast of the mutant placentae. The absence of the P0 transcript of the IGF-2 gene resulted in 30% reduction of placental and fetal weights near term. Therefore, eliminating this IGF-2 transcript exclusively from the placenta has a profound growth retarding effect. Mutant placentae had the same morphological organization as wild type, but volume fraction determinations were proportionally smaller for all areas. Reduction of organ size - with exception of the brain – was largely proportionate. Therefore, the IGF system seems to coordinate growth effects (demand and supply) on organ systems, suggesting that brain growth is apparently independent of nutritional

demand and supply (Reik *et al.*, 2001) and/or that a preferential distribution of nutrients towards the brain exists.

Because of the observed growth restriction later in gestation in this knockout, a reasonable hypothesize is that lack of IGF-2 transcript affects placental function via the supply of nutrients. Either placental size became limiting for fetal growth only at later stages of gestation, or at earlier stages of gestation, the smaller placenta compensated by increased efficiency of nutrient transfer. To evaluate placental transport, Constancia et al. (2002), tested the P0 knockout model by placental function assays. They concluded that reduced fetal size can be the outcome of reduced supply or reduced demand. And, that the mouse IGF2 gene is remarkable in combining the control of both supply and genetic demand for maternal nutrients in a single gene! In humans and mice, fetal overgrowth can result from altered expression of several imprinted genes (H19, IGF-2, IGF2R). For example, in the human Beckwidth Wiedemann Syndrome, the mechanism of overgrowth is induced by the loss of imprinting and overexpression of IGF-2 (O'Dell & Day, 1998). In sheep, on the other hand, in vivo fertilized embryos that were cultured in vitro for 5 days with co-cultured granulosa cells and/or serum before transfer into ewes, led to interesting results after embryo transfer; 25% of the fetuses were defined as large offspring (LO), and IGF-2R expression was reduced by 30-60% in these LO fetuses (Young *et al.*, 2001). Then again, in cattle at day 70 of gestation, Blondin *et al.* (2000) found that fetuses originating from in vitro production systems possessed higher levels of IGF-II mRNA in the liver and a trend to lower levels in skeletal muscle. Different results were obtained by Schrader *et al.* (2003), who compared differential gene expression of two day 45 cloned bovine fetal livers to the original nuclear transfer cell donor day 45 fetal liver. IGF-2-specific primers used in the RT-PCR reaction did not reveal differential expression in these livers.

From this information we might conclude that IGF-2 can act both in a paracrine and endocrine function. Even though the underlying cause for overexpression of IGF-2 mRNA by the fetal liver of clones is unknown, this presence of extra IGF-2 in fetal circulation might affect placental development. Perhaps the cotyledon expresses less IGF-2 for paracrine action in clones in a compensatory way.

Insulin-like growth factor receptors

In day 75 cloned and control gestations, placentomal IGF1R mRNA was low, 10- fold less than the IGF2R. Interestingly, four cloned pregnancies had only a very faint signal in the cotyledon in comparison with other clones and controls. Bovine placental membranes contain both type 1 and type 2 IGF receptors, while there is a preponderance of type 1 receptor in humans and type 2 in sheep (Van Buul-Offers *et al.*, 1988). Term placentas from pregnancies complicated with intrauterine growth retardation have significantly higher levels of IGF-2 and IGF1R expression; this may represent a counter regulatory mechanism in response to the growth retardation (Abu-Amero *et al.*, 1998). Since IGF2R receptor mainly acts as a scavenger of IGF-2, most growth effects of IGF-2 are mediated by the IGF1R. In the placenta it remains a conundrum why local IGF-2 mRNA expression is abundant but IGF1R mRNA is rather scarce; this raises questions about which receptor binds the local IGF-2. McKinnon (2001) suggested that human trophoblast migration is mediated by the IGF2R – MAPK pathway. However, strong evidence from murine knockout studies raises the question of the existence of an unknown XR. In vitro studies with human placental tissues have indicated the presence of an unknown, high affinity IGF receptor, XR, with equal affinity for IGF-1 and –2, and only slightly less affinity for insulin (Hintz *et al.*, 1984). LeRoith and others (1995) detected IGF1R discrepancies in

molecular mass and antibody neutralizing binding activity, and therefore suggested the existence of another receptor. IGF-1 or IGF1R murine knockouts have little effect on placental weight, while IGF-2 knockout of the paternal allele in mice severely diminishes placental development (Baker *et al.*, 1993). The placentas of murine IGF-2/IGF1R double mutants were equally affected by growth retardation as seen in the IGF-2 single mutants. Four of our eight cloned conceptuses had extremely low concentrations of mRNA for the IGF1R gene; this might be a compensatory mechanism against the high levels of fetal IGF-2 in circulation. If the XR exists, and it has been suggested that this is the insulin receptor A (IR-A) (Vella *et al.*, 2002), then maybe this XR is upregulated in the cotyledon of these gestations.

IGF2R mRNA copies were present in the placentome, about 10-fold more than the IGF1R. No differences in relative amounts of mRNA for these genes were observed between the treatment groups. One twin pregnancy (#39) featured a 3 fold higher level of IGF2R mRNA. In this gestation, one dead fetus occupied one horn deprived of placentomes; therefore, the other fetus was supported by few placentomes. One would expect lower levels of the scavenger receptor, unless the IGF2R mediates IGF-2 induced trophoblast invasion as suggested by McKinnon (2001).

Insulin-like growth factor binding proteins

All IGFBP's studied so far are synthesized either in the uterine luminal epithelium or in underlying subepithelial stromal cells. This suggests that the IGFBP's are important for regulating transfer of IGF-1 and –2 between fetal and maternal compartments (Wathes *et al.*, 1998).

IGFBP-1 transcripts are scarce in the placentome, 1000 fold less than the housekeeping gene β-actin. In the cotyledons of two gestations, one control and one clone, BP-1 was barely detectable. When twin pregnancies were left out, a tendency to lower levels of BP-1 mRNA in the caruncles of cloned pregnancies was observed (p=0.056). On the other hand, one cloned pregnancy (#61) with the largest placentomes revealed 15 fold higher BP-1 and BP-2 mRNA in cotyledons in comparison to cotyledons of all other clones and controls, a finding confirmed by a repeated measurement.

Phosphorylated IGFBP-1 inhibits metabolic actions of IGF-1 on trophoblast cells in vitro. Conversely, non-phosphorylated BP-1 has the opposite effect, enhancing IGF activity (Yu *et al.*, 1998). IGFBP-1 stimulates trophoblast proliferation in early human pregnancy (Hills *et al.*, 1999). The BP-1 gene has putative progesterone response elements that mediate endometrial stromal cell BP-1 gene expression early in gestation (Crossey *et al.*, 2002). Therefore, migration of first trimester human invasive trophoblast in vitro is stimulated by IGFBP-1, likely by interaction with the RGD-binding site of the $\alpha_5\beta_1$-integrin (Irving & Lala, 1995). Gleeson *et al.* (1999), furthermore indicated that IGFBP-1 seems to stimulate extravillous trophoblast migration and invasion by signaling through $\alpha_5\beta_1$-integrin, leading to signal transduction via FAK and MAPK.

IGFBP-1 also increases the activity of gelatinases and tissue inhibitor of metalloproteinase 1 (TIMP-1) in first trimester cytotrophoblastic cells independently of IGF-1 (Bischof *et al.*, 1998). Even though IGFBP-1 is abundant in baboon uteri during implantation, this is not the case in ruminants (Tarantino *et al.*, 1992; Han *et al.*, 1996). This might be explained by the fact that IGFBP-1 appears to facilitate trophoblast penetration of the maternal

interface, a much more invasive process in primate vs. ruminant placental development (Tarantino *et al.*, 1992; Wathes *et al.*, 1998).

Crossey *et al.* (2002) modeled the effects of excess IGFBP-1 on fetoplacetal growth in transgenic mice overexpressing human IGFBP-1. As explained in a comment by Regnault et al. (2002*b*), their findings need cautious interpretation, since it is important to keep in mind that no IGF binding proteins are expressed in the deciduas of wild-type rodents, only in the myometrium. In mice, however, BP-1 - like actions may be ascribed to decidual alpha 2 macroglobulin, which can bind IGF's (Han & Carter, 2000). In the BP-1 overexpression model, Crossey *et al.* (2002) found marked effects early in pregnancy such as early placental failure. Effects on later placental development seemed rather minimal since normal term placental and fetal weights were obtained, suggesting that other factors may overcome the initial BP-1 related deficiencies (Regnault *et al.*, 2002*b*). In conclusion, Crossey *et al.* (2002) found that excess decidual BP-1 was associated with increased placental mass (mainly in the labyrinth zone). Placental IGF-2 expression was unchanged, but IGF-2 bioavailability at the fetomaternal interface might have been altered. In this study phosphorylated BP-1 predominated, which would suggest reduced IGF bioavailability. In the placentomegaly of the transgenic dams, trophoblast invasion of the decidualized endometrium was less pronounced. Crossey *et al.* (2002) suggested the following potential explanatory mechanisms: BP-1 excess might attenuate trophoblast invasion and proliferation as a consequence of reduced local IGF bioavailability. Alternatively, reduced IGF might influence trophoblast differentiation, perhaps favoring growth of noninvasive over invasive trophoblast lineages. On the other hand, BP-1 may have direct IGF-independent effects on trophoblast invasion. Irwin & Giudice (1998) reported that BP-1 inhibited trophoblast invasion of endometrial stromal cells in vitro. In contradiction, however

Gleeson *et al.* (2001) showed increased trophoblast invasion in response to BP-1. Both studies show that these effects are mediated through binding of BP-1 to α5β1 integrin. In comparison with IGF-2 deficient mice, in both cases spongiotrophoblast cells account for a greater proportion of the junctional zone. However, in the IGF-2 null mice, placental mass is reduced, whereas there is a large increase in labyrinthine zone, and therefore an increase in placental mass in IGFBP-1 mutants. The exchange functions of the rodent placenta are largely confined to the labyrinthine zone. Thus, compensatory changes in fetal vascularization could result in an increase in labyrinthine tissue volume.

DeVrijer *et al.* (2002) studied fetal gene expression in a placental insufficiency - intrauterine growth retardation (PI-IUGR) model. With ribonuclease protection assays, they found fetal liver IGFBP-1 to be upregulated in the model, but no significant differences were seen for IGF-1, IGF-2 and IGFBP-3 prior to maximal growth.

Apparent controversy exists about the function of BP-1. Phosphorylated BP-1 inhibits IGF-1 action; dephosphorylation favors it. Opposing in vitro results on the IGF independent effect on trophoblast proliferation and invasion have been published (Irwin & Giudice, 1998; Gleeson *et al.*, 2001), and what happens in a mouse overexpression model is not necessarily transposable to other species. However, our clone twin pregnancy (#61) that was largely overexpressing BP-1 and BP-2 mRNA in the cotyledon had the largest placentomes, 6 standard deviations higher than the mean placentomal size of the controls. This pregnancy however also featured the lowest IGF-2 mRNA levels in the cotyledon and the lowest IGF-1 mRNA levels in the caruncle. The umbilical cord of one of the fetuses had the lowest weight/length ratio in our study. Unfortunately we do not have any liver mRNA data for this gestation.

IGFBP-2 mRNA concentrations were low in day 75 placentomes. It is, however, remarkable that cloned caruncles had a 10 fold lower BP-2 mRNA concentration in comparison with controls (P = 0.02). From day 29 on in ovine gestation, IGFBP-2 is exclusively detected in the dense caruncular-like stroma underlying the luminal epithelium (Reynolds *et al.*, 1997). This finding agrees with a previous study by (Delhanty & Han, 1992), which showed IGFBP-2 was present in close proximity to IGF-2 in rapidly proliferating tissues. Plasma concentrations of BP-2 were higher in somatic cell cloned cattle immediately after birth (Chavatte-Palmer *et al.*, 2000; Matsuzaki & Shiga, 2002).

We found message copy number for IGFBP-3 mRNA to be extremely high in the day 75 placentome, 10 fold higher than the housekeeping gene, β-actin. IGFBP-3 is a major carrier of IGF's in circulation. In pregnant ewes, IGFBP-3 is present in the caruncles, endometrial stroma, placentome capsule and some allantochorion (Reynolds *et al.*, 1997). Remarkably high levels of IGFBP-3 mRNA were localized to maternal blood vessel walls of the caruncles in the region of fetal villi penetration (Reynolds *et al.*, 1997). This might be important in regulation of placental blood flow and in the rate of transfer of IGF-1 and –2 from the maternal circulation to the uterus (Wathes et al., 1998). It is important to remember that both IGF-1 and –2, when complexed to IGFBP-3, cannot cross the placental epithelium (Bar *et al.*, 1990). There is a strong correlation of IGF-1 and IGFBP-3 serum levels with birth weight as well as placental weight (Osorio *et al.*, 1996). Serum from pregnant women and rats contains a placental protease that cleaves IGFBP-3, reducing its affinity for IGF's (Davenport *et al.*, 1992; Han & Carter, 2000). The liver of day 75 fetuses produce large amounts of mRNA for IGFBP's one, two and three.

Gender effects

We did not observe any gender effects, most likely due to the small number of individuals per treatment group. Two out of five control fetuses were female and three out of eight cloned fetuses were female. Removing twins from data analyses, did not have a major effect on means or P-values. Removing twin data increased the P-value for IGFBP-1 mRNA concentration in the caruncle from P = 0.13 to P = 0.056.

Correlations

In controls a positive correlation was observed between weight of the largest placentome and cotyledonary IGF-2 mRNA concentration ($r = 0.90$, $P = 0.03$). Interestingly, this relationship was reversed in clones ($r = -0.81$, $P = 0.015$). A trend towards a negative correlation ($r = -0.55$, $P = 0.077$) was observed between IGF-2 mRNA levels in the fetal liver and cotyledon, and larger livers did express more IGF-2 mRNA ($r = 0.63$, $P = 0.03$). Fetal liver weight also positively correlated with largest placentome weight if twins were excluded ($r = 0.03$, $P < 0.0001$). In conclusion, this means that when the weight of the largest placentome goes up, liver weight and liver IGF-2 mRNA follows this trend; however, in clones this will lead to a decrease in cotyledonary IGF-2, but an increase in controls. If placentome size in clones is aberrant, then the decrease in cotyledonary IGF-2 mRNA is probably an indication of a homeostatic effort. This might also be the explanation why a positive correlation between IGFBP-3 and IGF-2 mRNA was observed in the cotyledon of clones, since BP-3 has a high affinity for IGF-2, which was overexpressed by the fetal liver.

When considering two populations, controls and clones, we observed numerous differences and trends in aberrant gene expression in the IGF system. When we paid close

attention to individual gestations, it was clear that the clones in this data set are very heterogenous, with extremes in both directions for mRNA of certain genes and physical measurements.

In conclusion, even though these day 75 gestations survived embryonic death and were able to develop a vascularized placenta, aberrancies in the gene transcription of the insulin-like growth factor system were observed. These underlying variations in gene expression, may well be exacerbated later in gestation and cause morbidity and mortality.

CHAPTER IV: mRNA FOR VASCULAR ENDOTHELIAL GROWTH FACTOR FAMILY GENES IN PLACENTAS OF NUCLEAR CLONED BOVINE PREGNANCIES

INTRODUCTION

Development of an adequate placental blood supply is imperative for normal fetal growth and developmental rates (Barcroft & Barron, 1946; Rosenfeld *et al.*, 1974; Reynolds, 1986; Reynolds *et al.*, 1987). About 40 days after conception in cattle, placental functioning becomes a sine qua non, since hemotrophic exchange of substrates from the mother to the fetus is mediated by the placenta, an organ in which the fetal and maternal circulation are brought into close apposition. During the process of placentation, fetal chorionic villi invade the maternal crypts. New blood vessels sprout and branch abundantly, forming a complex structural architecture. This process is induced and controlled by complex interactions among numerous angiogenic factors. Moreover, growth and remodeling of the placental vascular beds takes place throughout gestation in correspondence with increasing rates of uterine and umbilical blood flow as well as fetal oxygen and nutrient uptake (Rosenfeld *et al.*, 1974; Reynolds, 1986; Reynolds *et al.*, 1987). The underlying mechanism of control of this vascular architecture is delicately balanced, and can easily be disrupted by environmental stressors (e.g. hypoxia) which induce structural adaptations in an attempt to enhance the capacity for exchange (Penninga & Longo, 1998).

In gestations from in vitro produced embryos, numerous problem related to abnormal placentation have been published. Parturition is frequently lengthy, lacking normal signs of

impending birth or pronounced uterine contractions (Avery & Greve, 1995). In the last trimester, the incidence of hydroallantois is about 1% for pregnancies from IVF (1,376 pregnancies) (Hasler, 1998), about 10 fold higher than with conventional reproduction, and is accompanied by a very high rate of mortality among recipient cows. Furthermore, at birth, cotyledons from cloned pregnancies often are edematous, and intercotyledonary areas are thickened with a gelatinous-like material at parturition (Garry *et al.*, unpublished). Enlarged umbilical vessels, edematous membranes, and a greater than usual allantoic volume also were observed by Wells *et al.* (1999). In the first trimester, Peterson & McMillan (1998) observed impaired allantoic development and vascularization in 24% of in vitro derived embryos at day 25. Allantochorion colonizes the maternal caruncles between day 20 to 35 (Melton *et al.*, 1951) so that growth restriction during this period can limit numbers of placentomes. Later in pregnancy, size and morphology rather than number of placentomes are affected (Melton *et al.*, 1951).

Nuclear transfer pregnancies from pluripotent embryonic cell lines were all lost before 60 days of gestation in one study (Stice *et al.*, 1996). These fetuses developed through organogenesis (by day 42); however, a deficiency in placentome development occurred where cotyledons and villi were absent, and a hemorrhagic response was noted in the caruncles void of crypts, indicating a maternal response, but aberrant fetal interaction (Stice *et al.*, 1996). Abortion in the first trimester coincides with timing of normal placentome formation. These data infer a genetically or epigenetically abnormal placenta, and we believe that aberrant placental development may explain much of the attrition and pathologies of nuclear-cloned pregnancies.

Our objective was to compare gene expression for the vascular endothelial growth factor system in nuclear-cloned and control day 45 and day 75 bovine pregnancies. Up to day 30,

embryonic loss is not related to placentation. Tissue collection at day 45 should provide insight in primitive placentation. In a previous study with nuclear transfer embryos from the same origin, we determined that most recipient cows pregnant at day 70, would carry pregnancies to term (Seidel *et al.*, 1997). By this time point, gestational losses due to embryonic failure or severely underdeveloped placentation have been eliminated. However, in these pregnancies stillbirth, hydroallantois, dystocia or perinatal morbidity still occur, and this usually seems related to placental pathology. Therefore, although day 75 placentas might be grossly normal, underlying molecular abnormalities may be present. Basic information in this area can be applied to reducing the huge amount of pregnancy attrition in cloning and in vitro fertilization gestations, as well as provide information to decrease the substantial incidence of fetal and neonatal death that occurs with conventional reproduction.

MATERIALS AND METHODS

RNA isolation

Total cellular RNA was isolated from frozen tissues (-80^{o}C) previously collected, (cfr. chapter 2) by means of the RNeasy Maxi Kit (Qiagen, Valencia, CA) as described in detail in chapter 3.

cDNA preparation for Northern Blotting

Ovine VEGF cDNA specific for mRNA encoding for the $VEGF_{120}$ isoform (exons 1-5 and 8) was kindly provided by D.A. Redmer (Redmer *et al.*, 1996). Ovine PlGF cDNA was

kindly provided by T. Regnault (University of Colorado Health Sciences Center) and ovine β-actin cDNA was previously cloned by R.V. Anthony (Colorado State University). Each cDNA was digested from respective vectors and separated from plasmid DNA in a 1% (w/v) agarose gel. The cDNA was then isolated from the gel by binding to DEAE cellulose membrane (Schleicher and Schuell NA45, Keene, NH), and was recovered by the methods outlined by the manufacturer. Purified cDNA concentration was determined by spectrophotometry absorbance at 260 nm and agarose gel electrophoresis and stored at –20^{o}C until radiolabeled.

Oligonucleotide primer design for PCR

Primers for β-actin were designed as described in chapter 3. Primers designed for the VEGFR1 (Flt-1) were based upon the cloned ovine cDNA sequence from T. Regnault (University of Colorado health sciences center), and the published inside primers from the nested design from Redmer *et al.* (1996). This 5' primer sequence was CAGCGCACGGCAG TGATA and the 3'primer sequence was CCGCAGTAAAATCCAGGTAA. This corresponds to bp 1518-1779 (262 bp) of the complete coding sequence of human Flt-1 mRNA (accession number AF063657).

Primers for VEGFR2 (KDR) were based upon the cloned ovine cDNA sequence and the primers designed by T. Regnault (University of Colorado health sciences center). The 5' primer ATTTATGATG TGGTTCTGAG and 3' primer GGGATTGGTAAGGATGAC correspond to bp 1038-1517 of the complete coding sequence human KDR mRNA (accession number AF035121), creating a 479 bp cDNA with an annealing temperature of 58^{o}C.

VEGF primers were designed based upon the published sequence for bovine VEGF (accession number M32976) by means of the Oligo program. The primers were designed to fall

within the exon 1-5 region, since this section of the coding sequence is present in all isoforms. The 5' primer TACCCAGATGAGATTGAGTTC falls within exon 3 and the 3' primer CTGGCTTTGGTGAGGTTTGAT falls within exon 4, creating a 153bp cDNA with an annealing temperature of 52.4^{o}C, corresponding to bp 735 – 887 of the published sequence.

PlGF primer design was based upon the primers used to clone the ovine cDNA from T. Regnault (University of Colorado health sciences center), but corrected for sequence incongruency with the published bovine PlGF sequence (accession number AB004272). The resulting 5' primer TTCATCGGAGGTGGAAGTGG falls within the coding sequence, and the 3'primer CTGAGAACGTGACGGTAATA falls within the 3'untranslated region, creating a 415bp cDNA with an optimal annealing temperature of 55.4^{o}C, corresponding to bp 410 – 824 of the published sequence.

VEGF-B primers were designed from the published sequence of bos taurus mRNA for vascular endothelial growth factor B186 precursor, partial coding sequence (accession number AB004274) with 93% sequence homology to the human vascular endothelial growth factor B (VEGFB), mRNA (accession number NM003377). The resulting 5' primer GTGGTGGTGCCCCTGAAC and 3' primer GAGTGGAAGCCCTGTCTG created a 268bp cDNA with an optimal annealing temperature of 61^{o}C, corresponding with bp 114-381 of the published sequence.

All primers were prepared by One Trick Pony Oligos (Ramona, CA).

Northern Blotting

Northern analyses for VEGF, PlGF and β-actin mRNA were performed on 15 μg samples of tcRNA electrophoresed in a 1% (w/v) agarose, 1M formaldehyde and 1X MOPS gel

(NorthernMax, Ambion, Austin, TX). For day 45 pregnancies, allantochorion and cotyledons from 4 control and 6 cloned pregnancies, and for day 75 pregnancies, caruncles and cotyledons from 5 controls and 8 cloned pregnancies were evaluated. After electrophoresis, the separated RNA was transferred to Zetabind® nylon membrane (CUNO Laboratory Products, Meriden, CT) by means of downward capillary action for 1.5 hours using transfer buffer (NorthernMax, Ambion, Austin, TX). After transfer, the membranes were rinsed briefly (10 sec) in MOPS to remove salt and agarose, and cross-linked by UV irradiation. To evaluate efficiency of transfer, RNA integrity and even loading, the membranes were placed in 5% acetic acid for 10 min, and then stained 10 min with 0.04% methylene blue (Sigma Chemical, St. Louis, MO) in 0.5 M sodium acetate, pH 5.2. Destaining was performed with DEPC-treated water until the 18S and 28S ribosomal bands were clearly distinguished. Membranes were wrapped in Saran wrap and stored at –20^{o}C until hybridization.

The membranes were prehybridized in 15 ml of Ultrahyb ^{TM} (Ambion, Austin, TX) in hybridization tubes in a roller hybridization oven at 42^{o}C for at least 60 min. Ultrahyb is a complete prehybridization/hybridization buffer, and it is not necessary to add any additional blocking agents. The 409bp oPLGF, 479bp oVEGF, and the 519bp oβ-actin cDNA fragments were random prime labeled (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, England or Ambion, Austin, TX) with [α-^{32}P-deoxycytidine-5'-triphosphate (ICN Pharmaceuticals, Costa Mesa, CA) to a specific activity ≥1 x 10^{8} cpm/μg DNA. Radiolabeled cDNA was denatured by boiling for 5 min followed by 5 min on ice; it then was added to the prehybridization/ hybridization solution at 2 x 10^{6} cpm/ml. Hybridization was carried out for 22-24 hours at 42^{o}C. Thereafter, the hybridization buffer was discarded, and the blot washed 2 x 5 min in 25 ml 2X SSC/ 0.1% SDS at 42^{o}C and 2 x 15 min in 0.1X SSC/0.1%SDS at 42^{o}C.

Membranes were then exposed to radiographic film (Sterling™ Brand X-Ray Film, Life Science Products, Denver, CO) until sufficient band intensity was established. Subsequently, membranes were exposed to a phosphorimager screen. Intensities of respective bands were quantified using phosphorimage analysis (ImageQuant, Molecular Dynamics, Sunnyvale, CA) and normalized relative to band intensities of β-actin to correct for differences of initial sample tcRNA amount. Results were used to quantify differences in mRNA content between clone and control day 75 tissues. Membranes were stripped of radiolabeled cDNA by incubation with boiling 0.5% SDS on a shaker for 30 min. Subsequently membranes were hybridized with another ^{32}P-labeled cDNA fragment following the same procedure as above.

Reverse transcription – Real time PCR – Southern blotting

Techniques used are described in detail in chapter 3.

Data analysis

Northern blot data are expressed as relative band intensity and quantified by phosphorimage analysis (ImageQuant, Molecular Dynamics, Sunnyvale, CA), and data were analyzed by the Wilcoxon rank sum test (SAS institute, NC).

For quantitative analysis of real time PCR data, the initial template concentration of the target mRNA was directly derived from its measured fluorescence signal and the standard curve. Relative levels of mRNA for each gene of interest within each sample were calculated as a ratio of the initial template concentration of the gene of interest to the initial β-actin concentration of the sample. Values are expressed as mean arbitrary units ± standard error of the mean (SEM).

Due to the low sample number and non normality, we analyzed data by the non-parametric Wilcoxon rank sum test. We compared treatment groups (controls vs. clones) within the cotyledon, caruncle of large placentomes and within the liver. To evaluate whether twin effects confound our findings, we also analyzed data leaving out the measurements from twin pregnancies. Preliminary least-squares analyses of variance indicated no significant effects of sex of fetus and twinning for any response. Furthermore, since fetal sexes were reasonably balanced in both clones and controls, sex of fetus was ignored in the analyses presented. Equality of variances was evaluated by the 2 tailed F-test. Amounts of mRNA transcripts in the cotyledon vs. the caruncle were also compared within treatment groups. Pearson correlation coefficients were calculated to test relationships among all mRNA signals from all genes and fetal weight, placental weight, liver weight and umbilical cord weight/length ratio.

RESULTS

Northern Blotting

Northern analysis of day 45 bovine allantochorion and cotyledon resulted in three transcripts of 3.7kb, 2.2kb and 1.7kb for VEGF and one 1.7 kb band for PlGF mRNA (figure 4.1). The 2.2 kb band of VEGF mRNA is very faint, while the 3.7kb band was the most prominent. Both VEGF and PlGF mRNA were present, but no significant differences in mRNA concentration were detected between cloned and control tissues for day 45 allantochorion and

cotyledon respectively; data follow: VEGF 3.7 kb: P = 0.17, P=0.91; VEGF 2.2kb P = 0.48, P = not measurable; VEGF 1.7 kb P = 0.35, P = 0.35; PlGF 1.7 kb: P = 0.61, P = 0.76.

Subsequently, we detected VEGF and PlGF mRNA in bovine placentomes that were further advanced in development (day 75). PlGF mRNA was barely detectable by Northern analysis at this stage, with the exception of the cotyledon of one clone (#61) which had a clear, distinguished band for PlGF. In the day 75 placentome, all three VEGF bands were present, but shifts in concentration between the transcripts were observed. Cloned pregnancies had less VEGF mRNA of the 3.7kb band (P = 0.0016) in the cotyledon than controls. In the caruncles however, we could detect the 2.2kb VEGF mRNA band in most clones, but this band was hard to detect in most controls (figure 4.1).

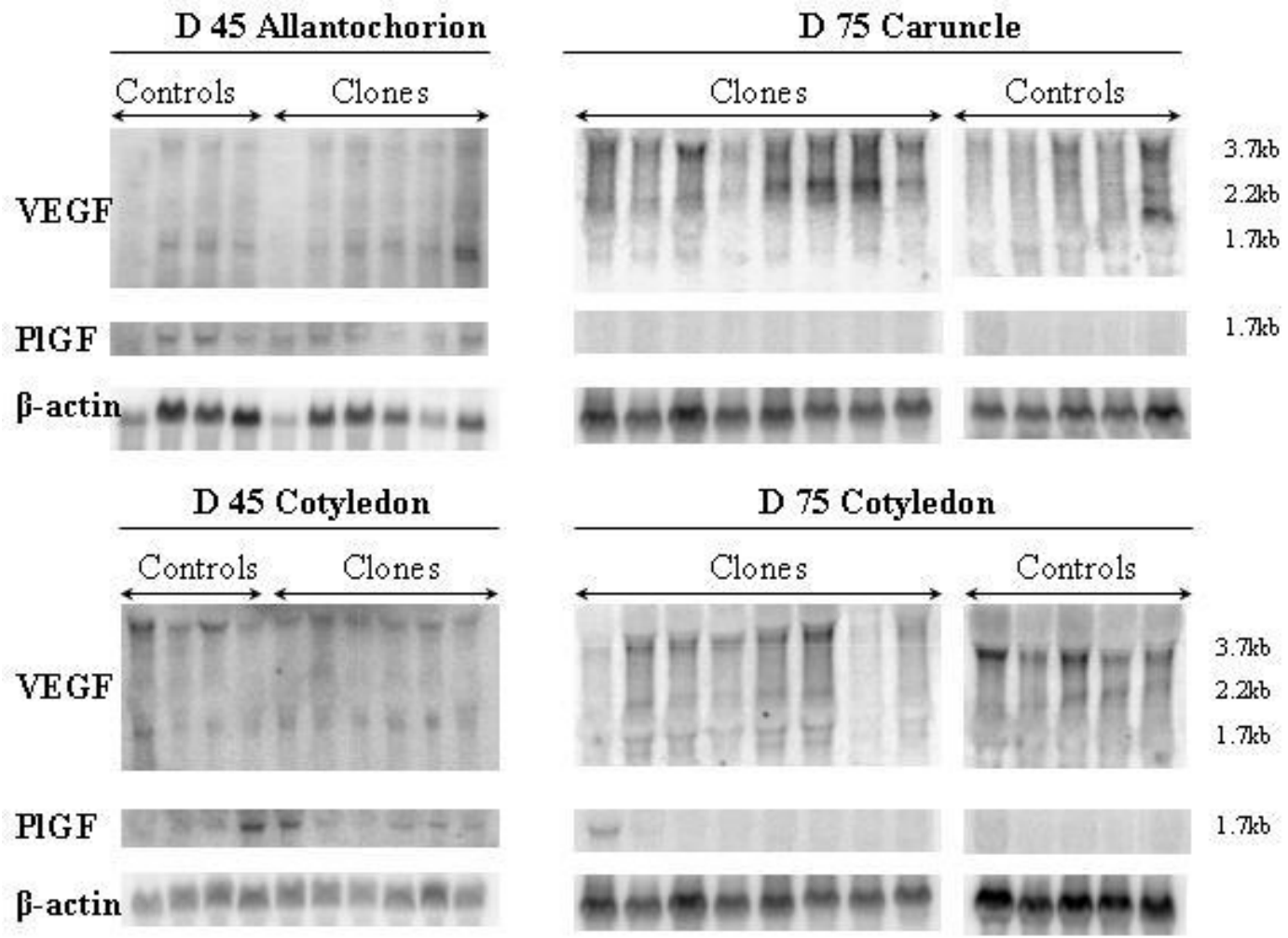

Figure 4.1 Northern blot analyses of VEGF and PlGF mRNA

Real time PCR

We measured transcriptional activity for the VEGF, PLGF, KDR and Flt-1 genes in bovine placentomes of day 75 cloned and control gestations (tables 4.1, 4.2, 4.3; figure 4.2 a, b, c, d). VEGF mRNA was fairly abundant in both cotyledons and caruncles, while about a hundred fold less message for PlGF was detected; in caruncles a trend occurred in which cloned gestations had about half the amount of mRNA for PlGF in comparison to matching control gestations, including and excluding twins (P = 0.06, P = 0.056). Interestingly, clone number 61, a twin pregnancy with extremely large placentomes (+ 6 SD) had about a 10 fold stronger than average signal for PlGF in the cotyledon in comparison with controls as well as other clones. PlGF signal for the caruncles of this pregnancy were normal. This is in agreement with our findings from Northern analysis. As mentioned in chapter 3, this clone had very aberrant gene expression for multiple genes.

The KDR receptor seems to be the primary receptor in the VEGF system in the placentome of bovine day 75 gestations. In the cotyledon, the Flt-1 receptor was barely detectable, and was expressed a 100 fold less than the KDR receptor (table 4.1). Both KDR and Flt-1 were more abundant in the caruncle than in the cotyledon (table 4.3). Clone 29 had 10 fold more expression of Flt-1 mRNA than the average clone. For Flt-1, it is important to mention that our primer set spans a region of the extracellular ligand binding domain present in the coding sequence for both the membrane bound and soluble form of Flt-1. We detected VEGF-B mRNA expression in bovine placentomes by reverse transcription PCR. No relative quantification by real time PCR was performed.

Clones had a significantly higher variance for Flt-1 mRNA than controls in cotyledons, both when results from twin gestations were incorporated or not (P = 0.0035, P = 0.0017) (table

4.1). One singleton cloned gestation (#29, see figure 4.2.d) had a Flt-1 mRNA concentration in the cotyledon 5-fold higher than others; this of course contributed to variability of the clone population. We also observed one cloned twin conceptus with extremely high concentrations of PlGF mRNA in cotyledons. When twins were removed from the data set, the variance did not differ significantly from the control population. This is an example of how individual pregnancies can exhibit statistical outlier behavior. In the caruncle, PlGF mRNA concentration was 2-fold higher in controls versus clones, and variability was significantly higher in the control population; melting curves indicated that we neared detection limits, therefore preventing detection of variability in the clones. No significant correlations were observed between or within placentome and liver mRNA concentrations and weight measurements.

Table 4.1 Relative mRNA concentrations of VEGF family genes in day 75 cotyledons

	(n=5)	**w/ twins** (n=8)			**w/o twins** (n=5)		
Gene	**mean cont (SE)**	**mean clone (SE)**	**Pmean**	**Pvar**	**mean clone (SE)**	**Pmean**	**Pvar**
VEGF	0.15 (0.03)	0.12 (0.02)	0.62	0.44	0.11 (0.02)	0.54	0.36
PlGF	0.0063 (0.0022)	0.0095 (0.0046)	1.00	0.08	0.005 (0.0016)	0.69	0.51
KDR	0.0072 (0.0034)	0.0087 (0.0013)	0.28	0.09	0.0072 (0.0014)	0.55	0.10
Flt-1	3.9E-05 (1.3E-05)	0.0001 (6.1E-05)	0.21	0.0035	0.0002 (0.0001)	0.25	0.0017

Table 4.2 Relative mRNA concentrations of VEGF family genes in day 75 caruncles

	(n=5)	**w/ twins** (n=8)			**w/o twins** (n=5)		
Gene	**mean cont (SE)**	**Mean clone (SE)**	**Pmean**	**Pvar**	**mean clone (SE)**	**Pmean**	**Pvar**
VEGF	0.3038 (0.08)	0.27 (0.045)	0.72	0.38	0.21 (0.04)	0.84	0.15
PlGF	0.0119 (0.0027)	0.0056 (0.0007)	0.06	0.016	0.0047 (0.0006)	0.056	0.011
KDR	0.049 (0.017)	0.062 (0.014)	0.62	0.98	0.084 (0.01)	0.15	0.47
Flt-1	0.0002 (4.5E-05)	0.0003 (5.4E-05)	0.62	0.45	0.0003 (5.7E-05)	0.42	0.66

Table 4.3 Probabilities of differences in mRNA concentration in cotyledons vs. caruncles within treatments (includes twins)

	Controls	**Clones**
Gene	**P-value (cot vs. car)**	**P-value (cot vs. car)**
VEGF	0.01* (<)	0.01* (<)
PlGF	0.15 (=)	0.57 (=)
KDR	0.01* (<)	0.01* (<)
Flt-1	0.008* (<)	0.01* (<)

* $P < 0.05$

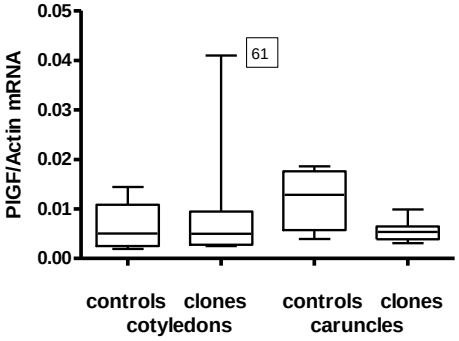

Fig 4.2.a. PlGF mRNA in placentomes

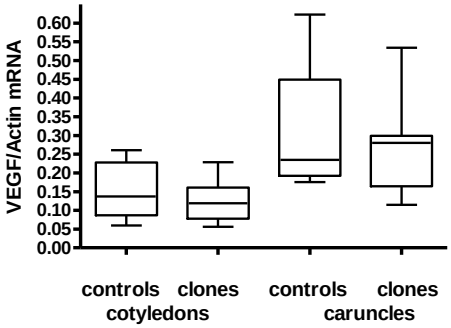

Fig 4.2.b. VEGF mRNA in placentomes

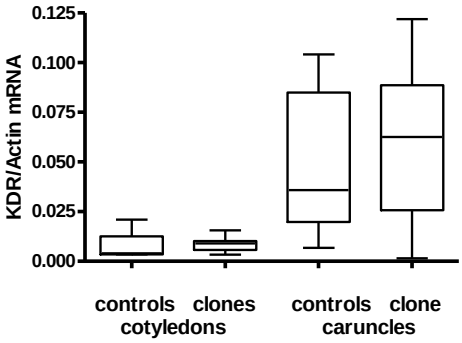

Fig 4.2.c. KDR mRNA in placentomes

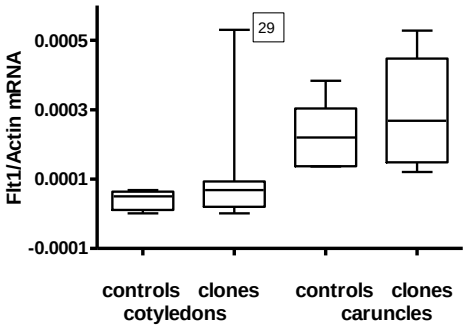

Fig 4.2.d. Flt1 mRNA in placentomes

Figure 4.2 Median, 25-75th percentile and ranges of mRNA concentrations for genes of the VEGF system in day 75 placentomes

DISCUSSION

During placentation, new blood vessels sprout and branch abundantly, and growth and remodeling of the placental vascular beds takes place throughout gestation, corresponding to increasing rates of uterine and umbilical blood flow rate as well as fetal oxygen and nutrient uptake (Rosenfeld *et al.*, 1974; Reynolds *et al.*, 1987). Secretion of angiogenic activity by bovine placental tissues was observed by Reynolds & Redmer, (1988). In their study, coculture of placental tissue explants was mitogenic to bovine aortic endothelial cells (BAEC), but no angiogenic growth factors were identified.

VEGF is a potent-endothelium specific mitogen with a key role in the induction of angiogenesis in the placenta (Cheung, 1997); it also is important in development, continuous remodeling and maintenance of placental vascular function during gestation. Even though transcriptional activity for the VEGF family genes has been demonstrated in sheep placental tissues, this is the first such report in bovine placentomes (Cheung *et al.*, 1995; Cheung & Brace, 1998; Bogic *et al.*, 2000, 2001; Regnault *et al.*, 2002*a*). Although VEGF and PlGF mRNA were present in day 45 bovine allantochorion and cotyledon, it may be too early to detect differences between clones and controls due to the primitive development of placentome microvilli at this stage. At day 75 of bovine gestation, placentome development is well underway. At this point in gestation, fetal villi infiltration into caruncular crypts has established a tight attachment of the cotyledon and caruncle within the placentomes surrounding the umbilical cord.

Data obtained from Northern blot analyses in this study revealed less VEGF mRNA (3.7 kb) in the cotyledons of day 75 clones than in controls, while more of the 2.2kb band was present in caruncles of clones than controls. However, in our findings from real time PCR, no

significant differences in VEGF mRNA concentration were detected. This discrepancy may be explained by the fact that our PCR primer set for VEGF generates a cDNA fragment that is present in all five splice forms of the VEGF gene. Therefore, transcripts from differential splicing are not distinguished in this assay. Northern analyses, in contrast, will separate transcripts by size and reveal information on differential splicing or mRNA protection. Initially VEGF was identified as a potent permeability factor (VPF) (Senger *et al.*, 1983; Connolly *et al.*, 1989). Therefore VEGF might be involved in the regulation of amniotic fluid volume and composition (Cheung, 1997) and have a causative role in the manifestation of hydroallantois in the last trimester of gestation.

Placental growth factor (PlGF) is another angiogenic factor expressed in the placenta (Maglione *et al.*, 1991). Ziche *et al.*, (1997) have shown that PlGF can induce angiogenesis in vivo and stimulate migration and proliferation of endothelial cells in vitro. PlGF appears to function in a paracrine manner on vascular endothelial cells in placental angiogenesis, and as an autocrine mediator of trophoblast function (Sherer & Abulafia, 2001). Concentrations for PlGF mRNA were about a hundred fold less than for VEGF at Day 75. In caruncles, cloned gestations only expressed about half the amount of mRNA for PlGF in comparison to matching control gestations both in twins and singletons ($P = 0.06$, $P = 0.056$), whereas PlGF mRNA concentrations in the cotyledon were unaltered. If aberrant vascularization is developing, this finding may suggest a homeostatic response from the maternal component. Interestingly, clone number 61, a twin pregnancy with extremely large placentomes (+ 6 SD), overexpressed PlGF mRNA in the cotyledon, while VEGF mRNA concentrations were within normal ranges. In sheep, VEGF and PlGF mRNA concentration increases with gestational length (Regnault *et al.*, 2002*a*). One could consider the placentomes in this particular gestation advanced in

development because of their size; twinning may have a similar effect. However, one would then also expect VEGF mRNA to be more abundant. No elevated levels of PlGF were observed in other cloned twin gestations. Athanassiades *et al.* (1998) suggested that PlGF might act primarily on migration of trophoblast cells that express Flt-1. Even though Flt-1 mRNA expression was not abnormally high in this gestation, the PlGF stimulatory effect on trophoblast migration might be the culprit for these abnormally large placentomes. Therefore, we conclude that in this gestation, cloning induced overexpression of PlGF mRNA, and together with other aberrancies observed for gene expression levels in this gestation, contributed to a remarkable increase in placentome size.

To evaluate our findings further, we quantified mRNA of the receptors for VEGF and PlGF (Flt-1 or VEGF1R and KDR or VEG2R). VEGF interacts with both VEG1R and VEGF2R (Terman *et al.*, 1992), while PlGF interacts only with VEGF1R (Park *et al.*, 1994*a*). Upon ligand binding, both receptors autophosphorylate and activate Ras pathways (Hanahan, 1997). VEGF2R seems to be the primary receptor in the VEGF system in the placentome of day 75 bovine gestations. Flt-1 appears to be the predominant receptor in human pregnancies, whereas in sheep, KDR is the major receptor (Cheung, 1997). Bogic *et al.*, (2001) could not detect Flt-1 mRNA in sheep placentas or fetal membranes by methods of in situ hybridization. However, Regnault *et al.* (2002*a*) did identify Flt-1 mRNA in sheep placentomes, and levels of mRNA were similar as for KDR mRNA.

In the cotyledon, the VEGF1R was barely detectable and expressed 100-fold less than the KDR receptor. Both VEGF2R and VEGF1R were more abundant in the caruncle than in the cotyledon. VEGF2R appears to be required for the early development of the endothelial cell lineage and the mitogenic and chemotactic responses of endothelial cells, while Flt-1 may be

important for regulation of normal endothelial cell adhesion and interaction with the extracellular matrix (Bogic *et al.,* 2001), and the maintenance of mature blood vessels (Waltenberger *et al.,* 1994; Fong *et al.,* 1995; Dumont *et al.,* 1995; Orbus, 1999). In situ binding studies with recombinant VEGF in ovine placentas and fetal membranes suggest KDR plays a key role in maintaining vascularity and permeability (Bogic *et al.,* 2001).

Mice homozygously deficient for VEGF2R show failure of blood-island formation and vasculogenesis, and severe reduction in haematoangioblast progenitors and then endothelial cells; they die mid-gestation (Shalaby *et al.,* 1995). Embryos lacking the VEGF1R gene homozygously have normal hematopoietic progenitors and abundant endothelial cells that migrate and proliferate; however, there is a failure to assemble these cells into normal vascular cells (Fong *et al.,* 1995). For both knockouts, heterozygous mutants are essentially normal. Therefore, other genes or mechanisms seem to be able to correct aberrant levels of these receptors to a certain extent. In one clone (#29) there were 10-fold higher concentrations of VEGF1R mRNA in the cotyledon. This pregnancy seemed normal in other aspects, except for a large umbilical cord.

In conclusion, since both VEGF and VEGF2R have the highest mRNA concentrations, they are most likely the primary modulators of placental vascularization at this early stage in placentation. Subtle changes in gene expression of the VEGF system might exacerbate into an abnormal placenta or change fluid dynamics later in gestation resulting in morbidity or mortality.

REFERENCES

Abu-Amero SN, Ali Z, Bennett P, Vaughan JI & Moore GE (1998). Expression of the insulin-like growth factors and their receptors in term placentas: a comparison between normal and IUGR births. *Molecular Reproduction & Development* **49,** 229–235.

Adams SO, Nissley SP, Handwerger S & Rechler MM (1983). Developmental patterns of insulin-like growth factor-I and -II synthesis and regulation in rat fibroblasts. *Nature* **302,** 150–152.

Adams TE (2000). Structure and function of the type 1 insulin-like growth factor receptor. *Cellular & Molecular Life Sciences* **57,** 1050–1093.

Ahmed A & Perkins J (2000). Angiogenesis and intrauterine growth restriction. *Best Practice & Research in Clinical Obstetrics and Gynaecology* **14,** 981–998.

Ahmed A, Dunk C, Ahmad S & Khaliq A (2000). Regulation of placental vascular endothelial growth factor (VEGF) and placenta growth factor (PIGF) and soluble Flt-1 by oxygen--a review. *Placenta* **21,** S16–S24.

Ahmed A, Li XF, Dunk C, Whittle MJ, Rushton DI & Rollason T (1995). Colocalisation of vascular endothelial growth factor and its Flt-1 receptor in human placenta. *Growth Factors* **12,** 235–243.

Al Salami M, Simpson-Morgan MW & Morris B (1985). *Immunology of the sheep*. F. Hoffman-La Roche and Co., Basle.

Alexander G (1964). Studies on the placenta of sheep (Ovis aries L.): effect of surgical reduction in the number of caruncles. *Journal of Reproduction & Fertility* **7,** 307–322.

Amoroso EC (1952). Placentation. *In: Parkes AS, ed Marshall's Physiology of Reproduction London: Longmans Green and Co*127–311.

Anderson GB & Seidel GEJ (1998). Cloning for profit. *Science* **280,** 1400.

Athanassiades A, Hamilton GS & Lala PK (1998). Vascular endothelial growth factor stimulates proliferation but not migration or invasiveness in human extravillous trophoblast. *Biology of Reproduction* **59,** 643–654.

Atkinson DE, Robinson NR & Sibley CP (1991). Development of the passive permeability characteristics of the rat placenta during the last third of gestation. *American Journal of Physiology* **261,** R1461–R1464.

Avery B & Greve T (1995). Apparent abnormalities of in vitro produced bovine embryos. *In: Reproduction and Animal Breeding Advances and Strategy (eds G Enne, GF Greppi and A Lauria), Elsevier, Amsterdam*171–183.

Baguisi A *et al.* (1999). Production of goats by somatic cell nuclear transfer. *Nature Biotechnology* **17,** 456–461.

Baker J, Liu JP, Robertson EJ & Efstratiadis A (1993). Role of insulin-like growth factors in embryonic and postnatal growth. *Cell* **75,** 73–82.

Banks RE, Forbes MA, Searles J, Pappin D, Canas B, Rahman D, Kaufmann S, Walters CE, Jackson A, Eves P, Linton G, Keen J, Walker JJ & Selby PJ (1998). Evidence for the existence of a novel pregnancy-associated soluble variant of the vascular endothelial growth factor receptor, Flt-1. *Molecular Human Reproduction* **4,** 377–386.

Bar RS, Boes M, Dake BL, Sandra A, Bayne M, Cascieri M & Booth BA (1990). Tissue localization of perfused endothelial cell IGF binding protein is markedly altered by association with IGF-I. *Endocrinology* **127,** 3243–3245.

Barcroft J & Barron DH (1946). Observations upon the form and relation of the maternal and fetal capillaries of the placenta of the rabbit. *American Journal of Anatomy* **94,** 569–595.

Barker DJP & Clark PM (1997). Fetal undernutrition and disease in later life. *Reviews of Reproduction* **2,** 105–112.

Barlow DP, Stoger R, Hermann BG, Saito K & Schweifer N (1991). The mouse insulin-like growth factor type-2 receptor is imprinted and closely linked to the Tme locus. *Nature* **349,** 84–87.

Bauer MK, Harding JE, Bassett NS, Breier BH, Oliver MH, Gallaher BH, Evans PC, Woodall SM & Gluckman PD (1998). Fetal growth and placental function. *Molecular and Cellular Endocrinology* **140,** 115–120.

Baxter RC (2000). Insulin-like growth factor (IGF)-binding proteins: interactions with IGFs and intrinsic bioactivities. *American Journal of Physiology Endocrinology Metababolism* **278,** E967–E976.

Behboodi E, Anderson GB, BonDurant RH, Cargill SL, Kreuscher BR, Medrano JF & Murray JD (1995). Birth of large calves that developed from in vitro-derived bovine embryos. *Theriogenology* **44,** 227–232.

Benirschke K & Kaufmann P (1995). Placental types. In: Anonymous Pathology of the human Placenta.

Bermont L, Lamielle F, Fauconnet S, Esumi H, Weisz A & Adessi GL (2000). Regulation of vascular endothilial growth factor expression by insulin-like growth factor-I in endometrial cells. *International Journal of Cancer* **85,** 117–123.

Bertolini M & Anderson GB (2002). The placenta as a contributor to production of large calves. *Theriogenology* **57,** 181–187.

Bertolini M, Beam SW, Shim H, Bertolini LR, Moyer AL, Famula TR & Anderson GB (2002). Growth, development, and gene expression by in vivo- and in vitro-produced day 7 and 16 bovine embryos. *Molecular reproduction and development* **63,** 318–328.

Bischof P, Meisser A, Campana A & Tseng L (1998). Effects of decidua-conditioned medium and insulin-like growth factor binding protein-1 on trophoblastic matrix metalloproteinases and their inhibitors. *Placenta* **19,** 457–464.

Bjorkman NH (1968). Fine structure of cryptal and trophoblastic giant cells in the bovine placentome. *Journal of Ultrastructure Research* **24,** 249–258.

Blondin P, Farin PW, Crosier AE, Alexander JE & Farin CE (2000). In vitro production of embryos alters levels of insulin-like growth factor-II messenger ribonucleic acid in bovine fetuses 63 days after transfer. *Biology of Reproduction* **62,** 384–389.

Bogic LV, Brace RA & Cheung CY (2000). Cellular localization of vascular endothelial growth factor in ovine placenta and fetal membranes. *Placenta* **21,** 203–209.

Bogic LV, Brace RA & Cheung CY (2001). Developmental expressionn of vascular endothelial growth factor (VEGF) receptors and VEGF binding in ovine placenta and fetal membranes. *Placenta* **22,** 265–275.

Breier G (2000). Angiogenesis in Embryonic development - A Review. *Placenta* **21,** S11–S15.

Bronsens IA, Robertson WB & Dixon HG (1973). The role of spiral arteries in the pathogenesis of preeclampsia. *Obstetrical Gynecology Annual* **1,** 177–181.

Van Buul-Offers S, Hoogerbrugge CM & de Poorter TL (1988). The bovine placenta: a specific radioreceptor assay for both insulin-like growth factor I and insulin-like growth factor II. *Acta Endocrinologica* **118,** 306–313.

Byrne AT, Southgate J, Brison DR & Leese HJ (2002). Regulation of apoptosis in the bovine blastocyst by insulin and the insulin-like growth factor (IGF) superfamily. *Molecular reproduction and development* **62,** 489–495.

Campbell KHS, Loi P, Otaegui PJ & Wilmut I (1996*a*). Cell cycle co-ordination in embryo cloning by nuclear transfer. *Reviews of Reproduction* **1,** 40–46.

Campbell KHS, McWhir J, Ritchie WA & Wilmut I (1996*b*). Sheep cloned by nuclear transfer from a cultured cell line. *Nature* **380,** 64–66.

Cao Y, Chen H, Zhou L, Chiang MK, Anand-Apte B, Weatherbee JA, Wang Y, Fang F, Flanagan JG & Tsang ML (1996). Heterodimers of placenta growth factor/vascular endothelial growth factor. Endothelial activity, tumor cell expression, and high affinity binding to Flk-1/KDR. *Journal of Biological Chemistry* **271,** 3154–3162.

Cao Y, Ji WR, Qi P & Rosin A (1997). Placenta growth factor: identification and characterization of a novel isoform generated by RNA alternative splicing. *Biochemical & Biophysical Research Communications* **235,** 493–498.

Carlsson-Skwirut C, Jornvall H, Holmgren A, Andersson C, Bergman T, Lundquist G, Sjorgen B & Sara VR (1986). Isolation and characterization of variant IGF-1 and IGF-2 from adult human brain. *FEBS Letters* **201,** 46–50.

Carmeliet P & Collen D (1999). Role of vascular endothelial growth factor and vascular endothelial growth factor receptors in vascular development. *Current Topics in Microbiology & Immunology* **237,** 133–158.

Carmeliet P, Ferrera V, Breier G, Pollefeyt S, Kieckens L, Gertsenstein M, Fahrig M, Vandenbroek A, Harpal K, Eberhardt C, Declercq C, Pawling J, Moons L, Colle D, Risau W & Nagy A (1996). Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature* **380,** 435–439.

Carolan C, Lonergan P, Monget P & Mermillod P (1995). Role of insulin family of growth factors in bovine oocyte matruation (IVM) and embryo development (IVC) in vitro. *Journal of Reproduction & Fertility*.

Carr JM, Owens JA, Grant PA, Walton PE, Owens PC & Wallace JC (1995). Circulating insulin-like growth factors (IGFs), IGF-binding proteins (IGFBPs) and tissue mRNA levels of IGFBP-2 and IGFBP-4 in the ovine fetus. *Journal of Endocrinology* **145,** 545–557.

Carter AM (1975). Placental circulation. *In: Steven DH, ed Camparative Placentation Essays in Structure and Function New York Academic Press*108–160.

Charnock-Jones DS (2002). Soluble Flt-1 and the angiopoietins in the development and regulation of placental vasculature. *Journal of Anatomy* **200,** 607–615.

Charnock-Jones DS, Sharkey AM, Boocock CA, Ahmed A, Plevin R, Ferrara N & Smith SK (1994). Vascular endothelial growth factor receptor localization and activation in human trophoblast and choriocarcinoma cells. *Biology of Reproduction* **51,** 524–530.

Chavatte-Palmer P, Heyman Y, Monget P, Richard C, Kahn G, Le Bourhis D, Vignon X & Renard JP (2000). Plasma IGF-I, IGF-BP and GH concentrations in cloned neonatal calves from somatic and embryonic cells: preliminary findings. *Theriogenology* **53,** 213 (abstr.).

Chen H, Chedotal A, He Z, Goodman CS & Tessier-Lavigne M (1997). Neuropilin-2, a novel member of the neuropilin family, is a high affinity recepetor for the semaphorins Sema E and Sema IV but not in Sema III. *Neuron* **19,** 547–559.

Cheung CY (1997). Vascular endothelial growth factor: possible role in fetal development and placental function. *Journal of the Society for Gynecologic Investigation* **4,** 169–177.

Cheung CY & Brace RA (1998). Ovine vascular endothelial growth factor: nucleotide sequence and expression in fetal tissues. *Growth Factors* **16,** 11–22.

Cheung CY, Singh M, Ebaugh MJ & Brace RA (1995). Vascular endothelial growth factor gene expression in ovine placenta and fetal membranes. *American Journal of Obstetrics & Gynecology* **173,** 753–759.

Cibelli JB, Stice SL, Golueke PJ, Kane JJ, Jerry J, Blackwell C, Ponce de Leon FA & Robl JM (1998). Cloned transgenic calves produced from nonquiescent fetal fibroblasts. *Science* **280,** 1256–1258.

Claffey KP, Shih SC, Mullen A, Dziennis S, Cusick JL, Abrams KR, Lee SW & Detmar M (1998). Identification of the human VPF/VEGF 3' untranslated region mediating hypoxia-induced mRNA stability. *Molecular Biology of the Cell* **9,** 469–481.

Clark DE, Smith SK, He Y, Day KA, Licence DR, Corps AN, Lammoglia R & Charnock-Jones DS (1998*a*). A vascular endothelial growth factor antagonist is produced by the human placenta and released into the maternal circulation. *Biology of Reproduction* **59,** 1540–1548.

Clark DE, Smith SK, Licence D, Evans AL & Charnock-Jones DS (1998*b*). Comparison of expression patterns for placenta growth factor, vascular endothelial growth factor (VEGF), VEGF-B and VEGF-C in the human placenta throughout gestation. *Journal of Endocrinology* **159,** 459–467.

Clemmons DR (1991). Insulin-like growth factor binding proteins: roles in regulating IGF physiology. *Journal of Developmental Physiology* **15,** 105–110.

Connolly DT, Heuvelman DM & Nelson R (1989). Tumor vascular permeability factor stimulates endothilial cell growth and angiogenesis. *Journal of Clinical Investigation* **84,** 1470–1478.

Constância M, Hemberger M, Hughes J, Dean W, Ferguson-Smith A, Fundele R, Stewart F, Kelsey G, Fowden A, Sibley C & Reik W (2002). Placental-specific IGF-II is a major modulator of placental and fetal growth. *Nature* **417,** 945–948.

Cramer S, Beveridge M, Kilberg M & Novak D (2002). Physiological importance of system A-mediated amino acid transport to rat fetal development. *American journal of physiology Cell physiology* **282,** C153–C160.

Crossey PA, Pillai CC & Miell JP (2002). Altered placental development and intrauterine growth restriction in IGF binding protein-1 transgenic mice. *The Journal of Clinical Investigation* **110,** 411–418.

Cullinan-Bove K & Koos RD (1993). Vascular endothelial grwoth factor/vascular permeability factor expression in the rat uterus: rapid stimulation by estrogen correlates with estrogen-induced increases in uterine capillary permeability and growth. *Endocrinology* **133,** 829–837.

Dantzer V, Leach L & Leiser R (2000). Angiogenesis and placental vasculature - A workshop report. *Placenta* **21,** S69–S70.

Dantzer V, Leiser R, Kaufmann P & Luckhardt M (1988). Comparative morphological aspects of placental vascularization. *Trophoblast Research* **3,** 221–244.

Davenport ML, Pucilowska J, Clemmons DR, Lundblad R, Spencer JA & Underwood LE (1992). Tissue-specific expression of insulin-like growth factor protein-3 protease activity during rat pregnancy. *Endocrinology* **130,** 2505–2512.

Davies J & Wimsatt WA (1966). Observation on the fine structure of the sheep placenta. *Acta Anatomia (Baswl)* **65,** 182–223.

DeChiara TM, Efstratiadis A & Robertson EJ (1990). A growth-deficiency phenotype in heterozygous mice carrying an insulin-like grwoth factor II gene disrupted by targeting. *Nature* **345,** 78.

Dean W, Bowden L, Aitchison A, Klose J, Moore T, Meneses JJ, Reik W & Feil R (1998). Altered imprinted gene methylation and expression in completely ES cell-derived mouse fetuses: association with aberrant phenotypes. *Development* **125,** 2273–2282.

Dean W, Santos F, Stojkovic M, Zakhartchenko V, Walter J, Wolf E & Reik W (2001). Conservation of methylation reprogramming in mammalian development: Aberrant reprogramming in cloned embryos. *Proceedings of the National Academy of Sciences of the United States of America* **98,** 13734–13738.

Delhanty PJ & Han VK (1992). The characterization and expression of ovine insulin-like growth factor-binding protein-2. *Journal of Molecular Biology* **9,** 31–38.

Delhanty PJD & Han VKM (1993). The expression of insulin-like growth factor (IGF) binding protein-2 and IGF-II genes in the tissues of the developing ovine fetus. *Endocrinology* **132,** 41–52.

Demir R, Kaufmann P, Castellucci M, Erbengi T & Kotowski A (1989). Fetal vasculogenesis and angiogenesis in human placental villi. *Acta anatomica* **136,** 190–203.

Dennis PA & Rifkin DB (1991). Cellular activation of latent transforming growth factor beta requires binding to the cation-independent mannose-6-phosphate/insulin-like grwoth factor type II receptor. *Proceedings of the National Academy of Sciences of the United States of America* **88,** 580–584.

DiSalvo J, Bayne ML, Conn G, Kwok PW, Trivedi PG, Soderman DD, Palisi TM, Sullivan KA & Thomas KA (1995). Purification and characterization of a naturally occurring vascular endothelial growth factor.placenta growth factor heterodimer. *Journal of Biological Chemistry* **270,** 7717–7723.

Dibbens JA, Miller DL, Damert A, Risau W, Vadas MA & Goodall GJ (1999). Hypoxic regulation of vascular endothelial growth factor mRNA stability requires the cooperation of multiple RNA elements. *Molecular Biology of the Cell* **10,** 907–919.

Dinnyes A, Dai Y, Barber M, Liu L, Xu J, Zhou P & Yang X (2001). Development of Cloned Embryos from Adult Rabbit Fibroblasts: Effect of Activation Treatment and Donor Cell Preparation. *Biological Reproduction* **64,** 257–263.

Doherty AS, Mann MRW, Tremblay KD, Bartolomei MS & Schultz RM (2000). Differential effects of culture on imprinted H19 Expression in the preimplantation mouse embryo. *Biology of Reproduction* **62,** 1526–1535.

Driesch H (1893). No Title. *Anat Anz* **8,** 348.

Duello TM, Byatt JC & Bremel RD (1986). Immunohistochemical localization of placental lactogen in binucleate cells of bovine placentomes. *Endocrinology* **119,** 1351–1355.

Dumont DJ, Fong GH, Puri MC, Gradwohl G, Alitalo K & Breitman ML (1995). Vascularization of the mouse embryo: a study of flk-1, tek, tie, and vascular endothelial growth factor expression during development. *Developmental dynamics : an official publication of the American Association of Anatomists* **203,** 80–92.

Dumont DJ, Gradwohl G, Fong GH, Puri MC, Gertsenstein M, Auerbach A & Breitman ML (1994). Dominant-negative and targeted null mutations in the endothelial receptor tyrosine kinase, tek, reveal a critical role in vasculogenesisof the embryo. *Genes & Development* **8,** 1897–1909.

Dvorak HF, Brown LF, Detmar M & Dvorak AM (1995). Vascular permeability factor/vascular endothilial growth factor, microvascular hyperpermeability, and angiogenesis. *American Journal of Pathology* **146,** 1029–1039.

Eatock MM, Schatzlein A & Kaye SB (2000). Tumor vasculature as a target for anticancer therapy. *Cancer Treatment Reviews* **26,** 191–204.

Efstratiadis A (1998). Genetics of mouse growth. *International Journal of Developmental Biology* **42,** 955–976.

Eggenschwiler J, Ludwig T, Fisher P, Leighton PA, Tilghman SM & Efstratiadis A (1997). Mouse mutant embryos overexpressing IGF-II exhibit phenotypic features of the Beckwith-Wiedemann and Simpson-Golabi-Behmel syndromes. *Genes & Development* **11,** 3128–3142.

Ehrhardt RA & Bell AW (1995). Growth and metabolism of the ovine placenta during mid-gestation. *Placenta* **16,** 727–741.

Eigenmann JE, Patterson DF & Froesch ER (1984). Body size parallels insulin-like growth factor I levels but not growth hormone secretory capacity. *Acta Endocrinologica* **106,** 448–453.

Eley RM, Thatcher WW, Bazer FW, Wilcox CJ, Becker RB, Head HH & Adkinson RW (1978). Development of the coneptus in the bovine. *Journal of Dairy Science* **61,** 467–473.

Elsden RP & Seidel GEJ (1995). Procedures for recovery, bisection, freezing, and transfer of bovine embryos. *In: 6th ed, Anim Reprod Biotechnol Lab Bulletin No 09*p 41.

Enholm B (1997). Comparison of VEGF, VEGF-B, VEGF-C and Ang1 mRNA regulation by serum, growth factor, oncoproteins and hypoxia. *Oncogene* **14,** 2475–2483.

Ewton DZ, Falen SL & Florini JR (1987). The type II insulin-like growth factor (IGF) receptor has low affinity for IGF-I analogs: pleiotypic actions of IGFs on myoblasts are apparently mediated by the type I receptor. *Endocrinology* **120,** 115–123.

Fairbrother WJ, Champe MA, Christinger HW, Keyt BA & Starivasnik MA (1998). Solution structure of the heparin-binding domain of vascular endothelial growth factor. *Structure* **6,** 637–648.

Farin PW & Farin CE (1995). Transfer of bovine embryos produced in vivo or in vitro: survival and fetal development. *Biology of Reproduction* **52,** 676–682.

Farin PW, Crosier AE & Farin CE (2001). Influence of in vitro systems on embryo survival and fetal development in cattle. *Theriogenology* **55,** 151–170.

Farin PW, Stockburger EM, Rodriguez KF, Crosier AE, Blondin P, Alexander JE & Farin CE (2000). Placental morphology following transfer of bovine embryos produced in vivo or in vitro. *Theriogenology* **53,** 474 (abstr.).

Farnebo F, Piehl F & Lagercrantz J (1999). Restricted expression pattern of vegf-d in the adult and fetal mouse: high expression in the embryonic lung. *Biochemical & Biophysiology Research Communication* **21,** 891–894.

Feil R, Khosla S, Cappai P & Loi P (1998). Genomic imprinting in ruminants: allele-specific gene expression in parthenogenetic sheep. *Mammalian Genome* **9,** 831–834.

Ferrara N, Houck K, Jakeman LB & Leung DW (1992). Molecular and biologicaql properties of the vascular endothelial growth factor family of proteins. *Endocrine Reviews* **13,** 18–32.

Ferrell CL (1991*a*). Maternal and fetal influences on uterine and conceptus development in the cow.2. Blood flow and nutrient flux. *Journal of Animal Science* **69,** 1954–1965.

Ferrell CL (1991*b*). Maternal and fetal influences on uterine and conceptus development in the cow. 1. Growth of tissues of the gravid uterus. *Journal of Animal Science* **69,** 1945–1953.

Ferrell CL & Reynolds LP (1992). Uterine and umbilical blood flows and net nutrient uptake by fetuses and uteroplacental tissues of cows gravid with either single or twin fetuses. *Journal of Animal Science* **70,** 426–433.

Ferrell CL, Garrett WN & Hinman N (1976). Growth, development, and comparison of the udder and gravid uterus of beef heifers during pregnancy. *Journal of Animal Science* **42,** 1477–1489.

Filson AJ, Louvi A, Efstratiadis A & Robertson EJ (1993). Rescue of the T-associated maternal effect in mice carrying null mutations in Igf-2 and Igf2r, two reciprocally imprinted genes. *Development* **118,** 731–736.

Folkman J (1985). Tumor angiogenesis. *Advances in Cancer Research* **43,** 175–203.

Fong GH, Rossant J, Gertsenstein M & Breitman ML (1995). Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. *Nature* **376,** 66–70.

Fowden AL (1995). Endocrine regulation of fetal growth. *Reproduction, Fertility, & Development* **7,** 351–363.

Foyt HL & Roberts CTJ (1991). The IGF-1 gene: structure, expression, and gene products. *In: LeRoith D (ed) Insulin-like Growth factors: Molecular and Cellular Aspects CRC Press, Boca Raton, 1991:* 49–85.

Francis GL, Upton FM, Ballard FJ & McNeil KA (1988). Purification and partial sequence analysis of insulin-like growth factor I from bovine colostrum. *Biochemical Journal* **251,** 95–103.

Franklin GC, Adam GIR & Ohlsson R (1996). Genomic imprinting and mammalian development. *Placenta* **17,** 3–14.

Frasca F, Pandini G, Scalia L, Sciacca L, Mineo R, Constantino A, Goldfine ID, Belfiore A & Vigneri R (1999). Insulin receptor isoform A, a newly recognized, high-affinity insulin-like growth factor II receptor in fetal and cancer cells. *Molecular & Cellular Biology* **19,** 3278–3288.

Froesch ER, Schmid C, Schwander J & Zapf J (1985). Actions of insulin-like growth factors. *Annual Review of Physiology* **47,** 443–467.

Gallaher BW, Breier BH, Blum WF, McCutcheon SN GP (1995). A homologous radioimmunoassay for ovine insulin-like growth factor-binding protein-2: ontogenesis and the respose to growth hormone, placental lactogen and insulin-like growth factor-1 treatment in sheep. *Journal of Endocrinology*75–82.

Gallaher BW, Oliver MH, Eichhorn K, Kessler U, Kiess W, Harding JE, Gluckman PD & Breier BH (1994). Circulating insulin-like growth factor II/mannose-6-phosphate receptor and insulin-like growth factor binding proteins in fetal sheep plasma are regulated by glucose and insulin. *European Journal of Endocrinology* **131,** 398–404.

Garry F (1999). Physiolobical problems of newborn calves. *Proceedings of the 18th Annual Convention of the American Embryo Transfer Association*pp5–pp9.

Garry F, Adams R, Holland MD, Hay WW, McCann JP, Wagner AP & Seidel GE (1998). Arterial oxygen, metabolite and energy regulatory hormone concentrations in cloned bovine fetuses. *Theriogenology* **49,** 321(abstr.).

Garry FB, Adams R, McCann JP & Odde KG (1996). Postnatal characteristics of calves produced by nuclear transfer cloning. *Theriogenology* **45,** 141–152.

Geisert RD, Lee C, Simmen FA, Zavy MT, Fliss AE, Bazer FW & Simmen RCM (1991). Expression of messenger RNA's encoding insulin-like growth factor-I, -II, and insulin-like growth factor binding protein-2 in bovine endometrium during the estrous cycle and early pregnancy. *Biology of Reproduction* **45,** 975–983.

Gelato MC, Rutherford C, Stark RI & Daniel SS (1989). The insulin-like growth factor/mannose-6-phosphate receptor is present in fetal and maternal sheep serum. *Endocrinology* **124,** 2935–2943.

Genbacev O, Joslin R, Damsky CH, Pillotti BM & Fisher JS (1996). Hypoxia alters early gestation human trophoblast differentiation/invasion in vitro and models the placental defects that occur in preeclampsia. *Journal of Clinical Investigation* **97,** 540–551.

Giddings SJ, King CD, Harman KW, Flood JF & Carnaghi LR (1994). Allele specific inactivation of insulin 1 and 2, in mouse yolk sac indicates imprinting. *Nature Genetics* **6,** 310–313.

Giudice LC (1997). Multifaceted roles for IGFBP-1 in human endometrium and pregnancy. *Annual New York Academy of Science* **828,** 146–156.

Giudice LC, Mark SP & Irwin JC (1998). Paracrine actions of insulin-like growth factors and IGF binding protein-1 in non-pregnant endometrium and at the decidual-trophoblast interface. *Journal Reproductive Immunology* **39,** 133–148.

Giudice LC, Martina NA, Crystal RA, Tazuke SI & Druzin M (1997). Insulin-like growth factor binding protein-1 at the maternal fetal interface and insulin-like growth factor-1 and insulin-like growth factor-II, and insulin-like growth factor binding protein-1 in the circulation of womenwith severe preeclampsia. *American Journal of Obsterics & Gynecology* **176,** 751–757.

Gleeson LM, Chakraborty C, McKinnon T & Lala PK (1999). Insulin-like growth factor binding protein (IGFBP)-1 stimulates extravillous trophoblast (EVT) migration and invasion by signalling through a5b1 integrin. *Placenta* **20,** A.26.

Gleeson LM, Chakraborty C, McKinnon T & Lala PK (2001). Insulin-like growth factor-binding protein 1 stimulates human trophoblast migration by signaling through alpha 5 beta 1 integrin via mitogen-activated protein kinase pathway. *Journal of Clinical Endocrinology and Metabolism* **86,** 2482–2493.

Glover MD & Seidel GEJ (2003). Increased mRNA for allograft inflammatory factor-1, LERK-5, and a novel gene in 17.5-day relative to 15.5-day bovine embryos. *Biology of Reproduction* **69,** 1002–1012.

Gluckman PD (1995). Clinical review 68: The endocrine regulation of fetal growth in late gestation: the role of insulin-like growth factors. *Journal of Clinical Endocrinology & Metabolism* **80,** 1047–1050.

Gluckman PD, Breier BH & Davis SR (1987). Physiology of the somatotropic axis with particular reference to the ruminant. *Journal of Dairy Science* **70,** 442–466.

Goodhand KL, Dolman DF, Watt RG, Higgins LC, Staines ME & Broadbent PJ (1997). Calving outcome following transfer of in vivo and in vitro produced bovine embryos. *Proceedings of the British Society of Animal Science*.

Gratton RJ, Asano H & Han VKM (2002). The regional expression of insulin-like growth factor II (IGF-II) and insulin-like growth factor binding proetin-1 (IGFBP-1 in the placentae of women with pre-eclampsia.q. *Placenta* **22,** 303–310.

Green WW & Winters LM (1945). Prenatal development of sheep. *University of Minnisota Agricultural Experimental Station Technical Bulletin* **169,** 3–36.

Greenstein JS, Murray RW & Foley RC (1958). Observatioons on the morphogenesis and histochemistry of the bovine pre-attachment placenta between 16 and 33 days of gestation. *Anatomical Record* **132,** 321–341.

De Groot CJ, O'Brian TJ & Taylor RN (1996). Biochemical evidence of impaired trophoblastic invasion of decidual stroma in women destined to pre-eclampsia. *American Journal of Obsterics & Gynecology* **175,** 24–29.

Grosser O (1909). Vergleichende Anatomie und Entwicklungsgeschichte der Eihaute und der Placenta mit besonderer Beruckschtigung des Menschen. *Vienna: Braumuller*.

Grosser O (1927). Fruhentwickluing, Eihautbildung und Placentation des Menschen un der Saugetiere. *Deutsche Frauenheilkunde, Geburtshilfe, Gynakologie und Nachbargebiete in Einzeldarstellungen*.

Guillemot E (1995). Genomic imprinting of Mash2, a mouse gene required for trophoblast development. *Nature Genetics* **9,** 235–242.

Hagemann LJ, Weilert LL, Beaumont SE & Tervit HR (1998). Development of bovine embryos in single in vitro production (sIVP) systems. *Molecular Reproduction & Development* **51,** 143–147.

Hamilton GS, Lysiak JJ, Han VKM & Lala PK (1998). Autocrine-paracrine regulation of human trophoblast invasiveness by Insulin-Like Growth Factor (IGF)-II and IGF-Binding protein-1 (IFGBP-1). *Experimental Cell Research* **243,** 1–10.

Han VK, Bassett N, Walton J & Challis JR (1996). The expression of insulin-like growth factor (IGF) and IGF-binding protein (IGFBP) genes in the human placenta and membranes: evidence for IGF-IGFBP interactions at the feto-maternal interface. *Journal of Clinical Endocrinology & Metabolism* **81,** 2680–2693.

Han VKM & Carter AM (2000). Spatial and temporal Patterns of expression of messenger RNA for insulin-like growth factors and their binding proteins in the placenta of man and laboratory animals. *Placenta* **21,** 289–305.

Han VKM, D'Ercole AJ & Lund PK (1987). Cellular localization of somatonedin (insulin-like growth factor) messenger RNA in the human fetus. *Science* **236,** 193–197.

Hanahan D (1997). Signaling vascular morphogenesis and maintenance. *Science* **277,** 48–50.

Hansel W & Asdell SA (1951). The effects of estrogen and progesterone on the arterial system of the uterus of the cow. *Journal of Dairy Science* **34,** 37–44.

Harding JE, Liu L, Evans PC & Gluckman PD (1994). Insulin-like growth factor 1 alters feto-placental protein and carbohydrate metabolism in fetal sheep. *Endocrinology* **134,** 1509–1514.

Hardy K, Spanos S, Becker D, Iannelli P, Winston RML & Stark J (2001). From cell death to embryo arrest: Mathematical models of human preimplantation embryo development. *Proceedings of the National Academy of Sciences of the United States of America* **98,** 1655–1660.

Hasler JF (1998). The current status of oocyte recovery, in vitro embryo production, and embryo transfer in domestic animals, with an emphasis on the bovine. *Journal of Animal Science* **76,** 52–74.

Hasler JF, Henderson WB, Hurtgen PJ, Jin ZQ, McCauley AD, Mower SA, Neely B, Shuey LS, Stokes JE & Trimmer SA (1995). Production, freezing and transfer of bovine embryos and subsequent calving results. **43,** 141–152.

Hauser S & Weich HA (1993). A heparin-binding form of placenta growth factor (PlGF-2) is expressed in human umbilical vein endothelial cells and in placenta. *Growth Factors* **9,** 259–268.

He Z & Tessier-Lavigne M (1997). Neuropilin is a receptor for the axonal chemorepellent semaphorin III. *Cell* **90,** 739–751.

Hepler JE, Van Wyck JJ & Lund PK (1990). Different half-lives of insulin-like growth factor I mRNA's that differ in length of 3' untranslated sequence. *Endocrinology* **127,** 1550–1552.

De Hertogh R, Vanderheyden I, Pampfer S, Robin D & Delcour t J (1992). Maternal insulin treatment improves pre-implantation embryo development in diabetic rats. *Diabetologia* **35,** 406–408.

Hiendleder S, Schmutz SM, Erhardt G, Green RD & Plante Y (1999). Tranmitochondrial differences and varying levels of heteroplasmy in nuclear transfer cloned cattle. *Molecular Reproduction & Development* **54,** 24–31.

Hietala R, Pohja-Nylander P, Rutanen EM & Laatikainen T (2000). Serum insulin-like growth factor binding protein-1 at 16 weeks and subsequent preeclampsia. *Obstetrics & Gynecology* **95,** 185–189.

Hill JR, Burghardt RC, Jones K, Long CR, Looney CR, Shin T, Spencer TE, Thompson JA, Winger QA & Westhusin ME (2000). Evidence for Placental Abnormality as the Major Cause of Mortality in First-Trimester Somatic Cell Cloned Bovine Fetuses. *Biology of Reproduction* **63,** 1263–1787.

Hill JR, Roussel AJ, Cibelli JB, Edwards JF, Hooper NL, Miller MW, Thompson JA, Looney CR, Westhusin ME, Robl JM & Stice SL (1999). Clinical and pathologic features of cloned transgenic calves and fetuses (13 case studies). *Theriogenology* **51,** 1451–1468.

Hills FA, Elder MG, Chard T & Sullivan MHF (1999). Regulation of human primary trophoblast growth by insulin-like growth factors (IGF) and IGF binding protein-1. *Placenta* **20,** A.29.

Hintz RL, Thorsson AV, Enberg G & Hall K (1984). IGF-II binding on human lymphoid cells: demonstration of a common high affinity receptor for insulin like peptides. *Biochemical & Biophysical Research Communications* **118,** 774–782.

Holland MD, Williams SE, Godfredson JA, Field TG, Young JW, Tatum JD, Hossner KL & Odde KG (1989). Insulin-like growth factor (IGF-1) and bovine fetal growth. *Animal Science* **67 (Suppl ,** 199 (abstr.).

Holm P, Walker SK & Seamark RF (1996). Embryo viability, duration of gestation and birth weight in sheep after transfer of in vitro matured and in vitro fertilized zygotes cultured in vitro or in vivo. *Journal of Reproduction & Fertility* **107,** 175–181.

Holzenberger M, Dupont J, Ducos B, Leneuve P, Geloen A, Even PC, Cervera P & Le Bouc Y (2003). IGF-1 receptor regulates lifespan and resistance to oxidative stress in mice. *Nature* **421,** 182–187.

Hossner KL, McCusker RH & Dodson MV (1997). Insulin-like growth factors and their binding proteins in domestic animals. *Animal Science* **64,** 1–15.

Huang H, Rajkumar K & Murphy LJ (1997). Reduced fecundity in insulin-like growth factor binding protein-I transgenic mice. *Biology of Reproduction* **56,** 284–289.

Hubbert WT, Stalheim OH & Booth GD (1972). Changes in organ weights and fluid volumes during growth of the bovine fetus. *Growth* **36,** 217–233.

Hwa V, Oh Y & Rosenfeld RG (1999). The insulin-like growth factor-binding protein (IGFBP) superfamily. *Endocrine Reviews* **20,** 761–787.

Ihle JM (2000). The challenges of translating knockout phenotypes into gene function. *Cell* **102,** 131–134.

Ikeda E, Achen MG, Breier G & Risau W (1995). Hypoxia-induced transcriptional activation and increased mRNA stability of vascular endothelial growth factor in C6 glioma cells. *Journal of Biological Chemistry* **270,** 19761–19766.

Irving JA & Lala PK (1995). Functional role of cell surface integrins on human trophoblast cell migration: regulation by TGF-beta, IGF-II, and IGFBP-1. *Experimental Cell Research* **217,** 419–427.

Irwin JC & Giudice LC (1998). Insulin-like growth factor binding protein-1 binds to placental cytotrophoblast alpha5beta1 integrin and inhibits cytotrophoblast invasion into decidualized endometrial stromal cultures. *Growth Hormone & Igf Research* **8,** 21–31.

Iwashita M, Kudo Y, Yoshimura Y, Adachi T, Katayama E & Takda Y (1996). Physiological role of insulin-like-growth-factor-binding protein-4 in human folliculogenesis. *Hormone Research* **46,** 31–36.

Jaffe RB (2000). Importance of angiogenesis in reproductive physiology. *Seminars in Perinatology* **24,** 79–81.

Jenkinson CM, Min SH, Mackenzie DD, McCutcheon SN, Breier BH & Gluckman PD (1999). Placental development and fetal growth in growth hormone-treated ewes. *Growth hormone & IGF research : official journal of the Growth Hormone Research Society and the International IGF Research Society* **9,** 11–17.

Johnson LW & Smith CH (1988). Neutral amino acid transport systems of microvillous membrane of human placenta. *American Journal of Physiology* **251,** C773–C780.

Jones CJP (1994). Lectin-histochemical analysis of glycans in ovine and bovine near-term placental binucleate cells. *Cell Tissue Research* **278,** 601–610.

Jones CT, Gu W, Harding JE, Price DA & Parer JT (1988). Studies on the growth of the fetal sheep. Effects of surgical reduction in placental size, or experimental manipulation of uterine blood flow on plasma sulphation promoting activity and on the concentration of insulin-like growth factors I and II. *Journal of Developmental Physiology* **10,** 179–189.

Jones JI & Clemmons DR (1995). Insulin-like growth factors and their binding proteins: biological actions. *Endocrine Reviews* **16,** 3–34.

Jones JI, D'Ercole AJ, Camacho HC & Clemmons DR (1991). Phosphorulation of insulin-like growth factor (IGF)-binding protein 1 in cell culture and in vivo: effects on affinity for IGF-I. *Proceedings of the National Academy of Sciences of the United States of America* **88,** 7481–7485.

Jones JI, Gockerman A, Busby WJ, Wright G & Clemmons DR (1993). Insulin-like growth factor binding protein 1 stimulates cell migration and binds to the alpha 5 beta 1 intergrin by means of ite ARg-Gly-Asp sequence. *Proceedings of the National Academy of Sciences of the United States of America* **90,** 10553–10557.

Kaneda H, Hayashi J, Takahama S, Taya C, Lindahl KF & Yonekawa H (1995). Elimination of paternal mitochondrial DNA in intraspecific crosses during early mouse embryogenesis. *Proceedings of the National Academy of Sciences of the United States of America* **92,** 4542–4546.

Kato Y, Yabuuchi A, Motosugi N, Kato J-ya & Tsunoda Y (1999). Developmental Potential of Mouse Follicular Epithelial Cells and Cumulus Cells After Nuclear Transfer. *Biology of Reproduction* **61,** 1110–1114.

Kaufmann P (1982). Development and differentiation of the human placental villous tree. *Bibliotheca Anatomica* **22,** 29–39.

Kaufmann P (1990). Placentation und Placenta. *In: Human embryologie KV Hinrichsen, ed Springer-Verlag, Berlin*159–204.

Kaufmann P, Sen DK & Schweikhart G (1979). Classification of human placental villi. I. Histology. *Cell & Tissue Research* **200,** 409–423.

Kaye PL, Bell KL, Beebe LF, Dunglison GF, Gardner HG & Harvey MB (1992). Insulin and the insulin-like growth factors (IGFs) in preimplantation development. *Reproduction, Fertility, & Development* **4,** 373–386.

Keller ML (1996). The insulin-like growth factor system in the bovine uterus and elongating embryo. *PhD thesis, Colorado State University*.

Kendall RL & Thomas AL (1993). Inhibition of vascular endothelial growth factor activity by an endogenously encoded soluble receptor. *Proceedings of the National Academy of Sciences of the United States of America* **90,** 10705–10709.

Kendall RL, Wang G & Thomas KA (1996). Identification of a natural soluble form of the vascular endothelial growth factor receptor, Flt-1, and its heterodimerization with KDR. *Biochemical & Biophysical Research Communications* **226,** 324–328.

Kiess W, Blickenstaff GD, Sklar MM, Thomas CL, Nissley SP & Sahagian GG (1988). Biochemical evidence that the type II insulin-like growth factor receptor is identical to the cation-independent mannose 6-phosphate receptor. *Journal of Biological Chemistry* **263,** 9339–9344.

Killian JK, Byrd JC, Jirtle JV, Munday BL, Stoskopf MK, MacDonald RG & Jirtle RL (2000). M6P/IGF2R imprinting evolution in mammals. *Molecular Cell* **5,** 707–716.

Kim KW, Bae SK, Lee OH, Bae MH, Lee MJ & Park BC (1998). Insulin-like growth factor II induced by hypoxia may contribute to angiogenesis of hepatocellular carcinoma. *Cancer Research* **58,** 348–351.

King WA, Shepherd DL, Plante L, Lavoir MC, Looney CR & Barnes FL (1996). Nucleolar and mitochondrial morphology in bovine embryos reconstructed by nuclear transfer. *Molecular Reproduction & Development* **44,** 499–506.

Kitsukawa T, Shimuzu M, Sanbo M, Hirata T, Taniguchi M, Bekku Y, Yagi T & Fujisawa H (1997). Neuropilin-1-semaphorin III/D-mediated chemorepulsive signals play a crucial role in peripheral nerve projection in mice. *Neuron* **19,** 995–1005.

Klagsbrun M & D'Amato RJ (1991). Regulation of angiogenesis. *Annual Review of Physiology* **53,** 217–239.

Kleemann DO, Walker SK, Hartwich KM, Fong L, Seamark RF, Robinson JS & Owens JA (2001). Fetoplacental growth in sheep administered progesterone during the first three days of pregnancy. *Placenta* **22,** 14–23.

Klisch K, Hecht W, Pfarrer C, Schuler G, Hoffmann B & Leiser R (1999). DNA content and ploidy level of bovine placentomal trophoblast giant cells. *Placenta* **20,** 451–458.

Koblizek TI, Weiss C, Yancopoulos GD, Deutsch U & Risau W (1998). Angiopoietin-1 induces sprouting angiogenesis in vitro. *Current Biology* **8,** 529–532.

Kono T (1998). Influence of epigenetic changes during oocyte growth on nuclear reprogramming after nuclear transfer. *Reproduction, Fertility & Development* **10,** 593–598.

Kornfeld S (1992). Structure and function of the mannose 6-phosphate/insulinlike growth factor II receptors. *Annual Review Biochemistry* **61,** 307–330.

Kruip TAM & den Daas JHG (1997). In vitro produced and cloned embryos: effects on pregnancy, parturition and offspring. *Theriogenology* **47,** 43–52.

Lackey BR, Gray SLL & Henricks DM (2000). Physiological basis for use of Insulin-like growth factors in reproductive applications: a review. *Theriogenology* **53,** 1147–1156.

Lacroix MC, Servely JL & Kann G (1995). IGF-I and IGF-II receptors in the sheep placenta: evolution during the course of pregnancy. *Journal of Endocrinology* **144,** 179–191.

Lane M & Gardner DK (1994). Increase in postimplantation development of cultured mouse embryos by amino acids and induction of fetal retardation and exencephaly by ammonium ions. *Journal of Reproduction & Fertility* **102,** 305–312.

Langford K (1994). The pathophysiology of the insulin-like growth factor axis in fetal growth failure: a basis for programming by undernutrition? *European Journal of Clinical Investigation* **24,** 851–856.

Langford KS, Nicolaides KH, Jones J, Abbas A, McGregor AM & Miell JP (1995). Serum insulin-like growth factor-binding protein-3 (IGFBP-3) levels and IGFBP-3 protease activity in normal, abnormal, and multiple human pregnancy. *Journal of Clinical Endocrinology & Metabolism* **140,** 5587–5597.

Lanza RP, Cibelli JB, Blackwell C, Cristofalo VJ, Francis MK, Baerlocher GM, Mak J, Schertzer M, Chavez EA, Sawyer N, Lansdorp PM & West MD (2000). Extension of cell life-span and telomere length in animals cloned from senescent somatic cells. *Science* **288,** 665–669.

Lau MM, Stewart CE, Liu Z, Bhatt H, Rotwein P & Stewart CL (1994). Loss of the imprinted IGF2/cation-independent mannose 6-phosphate receptor results in fetal overgrowth and perinatal lethality. *Genes & Development* **8,** 2953–2963.

Lauwers (1991). Cursus embryologie, Universiteit Gent, Fakulteit Diergeneeskunde.

Lavoir MC, Kelk D, Rumph N, Barnes F, Betteridge KJ & King WA (1997). Transcription and translation in bovine nuclear transfer embryos. *Biology of Reproduction* **57,** 204–682.

Lawrence JB, Oxvig C, Overgaard MT, Sottrup-Jensen L, Gleich GJ, Hays LG, Yates JR 3rd & Conover CA (1999). The insulin-like growth factor (IGF)-dependent IGF binding protein-4 protease secreted by human fibroblasts is pregnancy-associated plasma protein-A. *Proceedings of the National Academy of Sciences of the United States of America* **96,** 3149–3153.

LeRoith D, Werner H, Beitner-Johnson D & Roberts Jr. CT (1995). Molecular and cellular aspects of the insulin-like growth factor 1 receptor. *Endocrine Reviews* **16,** 143–163.

Leach L (2002). The phenotype of th human materno-fetal endothelial barrier: molecular occupancy of paracellular junctions dictate permeability and angiogenic plasticity. *Journal of Anatomy* **200,** 599–606.

Lee CS, Wooding FB & Brandon MR (1986). Ultrastructural immunogold investigation of the function and diversity of binucleate cells in the ovine placenta using a monoclonal antibody. *Placenta* **7,** 495–504.

Lee JT (2000). Disruption of imprinted X inactivation by parent - of origin effects at Tsix. *Cell* **103,** 17–27.

Leiser R (1987). Mikrovaskularisation der Ziegenplazenta dargestellt mit rasterelektronish untersuchten Gefassausgussen. *Schw Arch Tierheilk* **129,** 59–74.

Leiser R, Krebs C, Klisch K, Ebert B, Dantzer V, Schuler G & Hoffmann B (1997). Fetal villosity and microvasculature of the bovine placentome in the second half of gestation. *Journal of Anatomy* **191,** 517–527.

Leutenegger CM, Higgins LC, Matthews DL, Tarantal AF, Luciw P, Pedersen NC & North TW (2001). Real-time TaqMan PCR as a specific and more sensitive alternative to the branched-chain DNA assay for quantitation of simian immunodeficiency virus RNA. *AIDS Research Human Retroviruses* **17,** 243–251.

Levy AP, Levy NS, Wegner S & Goldberg MA (1995). Transcriptional regulation of the rat vascular endothelial growth factor gene by hypoxia. *Journal of Biological Chemistry* **270,** 13333–13340.

Levy NS, Chung S, Furneaux H & Levy AP (1998). Hypoxic stabilization of vascular endothelial growth facotr mRNA by the RNA-binding protein HuR. *Journal of Biological Chemistry* **273,** 6417–6423.

Lewis IM, Peura TT & Trounson AO (1998). Large-scale applications of cloning technologies for agriculture: an industry perspective. *Reproduction, Fertility & Development* **10,** 677–681.

Li M, Squire JA & Weksberg R (1998*a*). Overgrowth syndromes and genomic imprinting: from mouse to man. *Clinical Genetics* **53,** 165–170.

Li X, Gray SG, Flam F, Pietsch T & Ekstrom TJ (1998*b*). Developmental-dependent DNA methylation of the IGF2 and H19 promoters is correlated to the promoter activities in human liver development. *International Journal of Developmental Biology* **42,** 687–693.

Lighten AD, Hardy K, Winston RM & Moore GE (1997). Expression of mRNA for the insulin-like growth factors and their receptors in human preimplantation embryos. *Molecular Reproduction & Development* **47,** 134–139.

Louvi A, Accili D & Efstratiadis A (1997). Growth-promoting interaction of IGF-II with the insulin receptor during mouse embryonic development. *Developmental Biology* **189,** 33–48.

Ludwig T, Eggenschwiler J, Fisher P, D'Ercole AJ, Davenport ML & Efstratiadis A (1996). Mouse mutants lacking the type 2 IGF receptor (IGF2R) are rescued from perinatal lethality in Igf2 and Igf1r null backgrounds. *Developmental Biology (Orlando)* **177,** 517–535.

Maglione D, Guerriero V, Viglietto G, Delli-Bovi P & Persico MG (1991). Isolation of a human placenta cDNA encoding for a protein related to the vascular permeability factor. *Proceedings of the National Academy of Sciences of the United States of America* **88,** 9267–9271.

Maharajan P & Maharajan V (1993). Growth hormone signal transduction. *Experientia* **49,** 980–987.

Makinen T, Olofsson B, Karpanen T, Hellman U, Soker S, Klagsbrun M, Eriksson U & Alitalo K (1999). Differential binding of vascular endothelial growth factor B splice and preteolytic isoforms to neuropilin-1. *Journal of Biological Chemistry* **274,** 21217–21222.

Massagué J (1990). The transforming growth factor-beta family. *Annual review of cell biology* **6,** 597–641.

Matsuzaki M & Shiga K (2002). Endocrine characteristics of cloned calves. *Cloning and stem cells* **4,** 261–267.

Maxfield EK, Sinclair KD, Dolman DF, Staines ME & Maltin CA (1997). In vitro culture of sheep embryos increases weight, primary fiber size and secondary to primary fiber ratio in fetal muscle at day 61 of gestation. *Theriogenology* **47,** 376.

McClintock AE (1998). Impact of cloning on cattle breeding systems. *Reproduction, Fertility & Development* **10,** 667–669.

McCusker RH (1998). Controlling insulin-like growth factor activity and the modulation of insulin-like growth factor binding protein and receptor binding. *Journal of dairy science* **81,** 1790–1800.

McKinnon T, Chakraborty C, Gleeson LM, Chidiac P & Lala PK (2001). Stimulation of human extravillous trophoblast migration by IGF-II is mediated by IGF type 2 receptor involving

inhibitory G protein(s) and phosphorylation of MAPK. *Journal of Clinical Endocrinology & Metabolism* **86,** 3665–3674.

McKinnon T, Gleeson LM, Chakraborty C LP (1999).) Insulin like growth factor (IGF)-II stimulates extravillous trophoblast (EVT) migration and invasion by signalling through type 2 IGF receptor. *Placenta* **20,** 47.

McLaren RJ & Montgomery GW (1999). Genomic imprinting of the insulin-like growth factor 2 gene in sheep. *Mammalian Genome* **10,** 588–591.

McLellan KC, Hooper SB, Bocking AD, Delhanty PJ, Phillips ID, Hill DJ & Han VK (1992). Prolonged hypoxia induced by the reduction of maternal uterine blood flow alters insulin-like growth factor-binding protein-1 (IGFBP-1) and IGFBP-2 gene expression in the ovine fetus. *Endocrinology* **131,** 1619–1628.

Meeker AK & Coffey DS (1997). Telomerase: a promising marker of biological immortality of germ, stem, and cancer cells. *Biochemistry (Moscow)* **62,** 1323–1331.

De Mellow JS & Baxter RC (1988). Growth hormone-dependent insulin-like growth factor (IGF) binding protein both inhibits and potentiates IGF-I-stimulated DNA synthesis in human skin fibroblasts. *Biochemical & Biophysical Research Communications* **156,** 199–204.

Melton AA, Berry RO & Butler OD (1951). The interval between the time of ovulation and attachment of the bovine embryo. *Journal of Animal Science* **10,** 993–1005.

Memili E & First NL (2000). Zygotic and embryonic gene expression in cow: a review of timing and mechanisms of early gene expression as compared with other species. *Zygote* **8,** 87–96.

Mereilles F & Smith LC (1998). Mitochondrial genotype segregation during preimplantation development in mouse heteroplasmic embryos. *Genetics* **148,** 877–883.

Merimee RJ, Zaph J & Froesch ER (1981). Dwarfism in the pygmy: An isolated deficiency of insulin-like growth factor I. *New England Journal of Medicine* **305,** 965–968.

Meyer M, Clauss M, Lepple-Wienhues A, Waltenberger J, Augustin HG, Ziche M, Lanz C, Buttner M, Rziha HJ & Dehio C (1999). A novel vascular endothelial growth factor encoded by Orf virus, VEGF-E, mediates angiogenesis via signalling through VEGFR-2 (KDR) but not VEGFR-1 (Flt-1) receptor tyrosine kinases. *EMBO Journal* **18,** 363–374.

Migdal M, Huppertz B, Tessler S, Comforti A, Shibuya M, Reich R, Baumann H & Neufeld G (1998). Neuropilin-1 is a placenta growth factor-2 receptor. *Journal of Biological Chemistry* **273,** 22272–22278.

Moffat J, F.W. B, P.J. H, P.W. C & R.M. R (1987). Purification, secretion and immunocytochemical localization of the uterine milk proteins, major progesterone-induced proteins in secretions of the sheep. *Biology of Reproduction*419–430.

Moore T & Reik W (1996). Genetic conflict in early development: parental imprinting in normal and abnormal growth. *Reviews of Reproduction* **1,** 73–77.

Mostaghini K & Ivoghli B (1977). Diabetes mellitus in the bovine. *Cornell Vet* **1977,** 24–28.

Munson L, Kao JJ & Schlafer DH (1989). Characetrization of glycoconjugates in the bovine endometrium and chorion by lectin histochemistry. *Journal of Reproduction & Fertility* **87,** 509–517.

Munson L, Wilhite A, Boltz VF & Wilkinson JE (1996). Transforming growth factor beta in bovine placentas. *Biology of Reproduction* **55,** 748–755.

Murphy LJ & Ghahary A (1990). Uterine insulin-like growth factor-1: regulation of expression and its role in estrogen-induced uterine proliferation. *Endocrine Reviews* **11,** 443–453.

Murphy LJ, Molnar P, Lu X & Huang H (1995). Expression of human insulin-like growth factor-binding protein-3 in transgenic mice. *Journal of Molecular Endocrinology* **15,** 293–303.

Murphy SK & Jirtle RL (2000). Imprinted genes as potential genetic and epigenetic toxicologic targets. *Enviormental Health Perspectives* **108,** 5–11.

Nagao Y, Totsuka Y, Atomi Y, Kaneda H, Lindahl KF, Imai H & Yonekawa H (1998). Decreased physical performance of congenic mice with mismatch between the nuclear and the mitochondrial genome. *Genes and Genetic Systems* **73,** 21–27.

Nathanielsz PW (1996). The timing of birth. *American Scientist* **84,** 562–569.

Neuenschwander S, Schwartz A, Wood TL, Roberts CTJ & Henninghausen L (1996). Involution of the lactating mammary gland is inhibited by the IGF system in a transgenic mouse model. *Journal of Clinical Investigation*.

Neufeld G, Kessler O & Herzog Y (2002). The interaction of Neuropilin-1 and Neuropilin-2 with tyrosine kinase receptors for VEGF. *Advances in Experimental Medicine & Biology* **515,** 81–90.

Neufeld G, Tessler S, Gitay-Goren H, Cohen T & Levi BZ (1994). Vascular endothilial growth factor and its receptors. *Progressive Growth Factor Research* **5,** 89–97.

Obata Y, Kaneko-Ishino T, Koide T, Takai Y, Ueda T, Domeki I, Shiroishi T, Ishino F & Kono T (1998). Disruption of primary imprinting during oocyte growth leads to the modified expression of imprinted genes during embryogenesis. *Development* **125,** 1553–1560.

Oh H, Takagi H, Suzuma K, Otani A, Matsumura M & Hoonda Y (1999). Hypoxia and vascular endothelial growth factor selectively up-regulated angiopoietin-2 in bovine microvascular endothelial cells. *Journal of Biological Chemistry* **274,** 15732–15739.

Oh Y, Nagalla SR, Yamanaka Y, Kim H-S, Wilson E & Rosenfeld RG (1996). Synthesis and Characterization of Insulin-like Growth Factor-binding Protein (IGFBP)-7. RECOMBINANT HUMAN mac25 PROTEIN SPECIFICALLY BINDS IGF-I AND -II. *Journal of Biological Chemistry* **271,** 30322–30325.

Olofsson B, Jeltch M, Eriksson U & Alitalo K (1999). Current biology of VEGF-B and VEGF-C. *Current Opinion in Biotechnology* **10,** 528–535.

Olofsson B, Korpelainen E, Pepper MS, Mandriota SJ, Aase K, Kumar V, Gunji Y, Jeltsch MM, Shibuya M, Alitalo K & Eriksson U (1998). Vascular endothelial growth facotr B (VEGF-B) binds to VEGF receptor-1 and regulates plasminogen activator activity in endothelial cells. *Proceedings of the National Academy of Sciences of the United States of America* **95,** 11709–11714.

Olofsson B, Pajusola K, Kaipainen A, von Euler G, Juokov V, Saksela O, Orpana A, Pettersson RF, Alitalo K & Eriksson U (1996). Vascular endothelial growth factor B, a novel growth factor for endothelial cells. *Proceedings of the National Academy of Sciences of the United States of America* **93,** 2576–2581.

Ono Y, Shimozawa N, Ito M & Kono T (2001). Cloned Mice from Fetal Fibroblast Cells Arrested at Metaphase by a Serial Nuclear Transfer. *Biology of Reproduction* **64,** 44–50.

Orbus R (1999). Placental function in placental insufficiency-intrauterine growth restricted pregnancies. *Master thesis, Colorado State University*.

Osborn BH, Fowlkes J, Han VK & Freemark M (1992). Nutritional regulation of insulin-like growth factor-binding protein gene expression in the ovine fetus and pregnant ewe. *Endocrinology* **131,** 1743–1750.

Osipo C, Dorman S & Frankfater A (2001). Loss of insulin-like growth factor II receptor expression promotes growth in cancer by increasing intracellular signaling from both IGF-I and insulin receptors. *Experimental Cell Research* **264,** 388–396.

Osorio M, Torres J, Moya F, Pezzullo J, Salafia C, Baxter R, Schwander J & Fant M (1996). Insulin-like growth factors (IGFs) and IGF binding proteins-1, -2, and -3 in newborn serum: relationships to fetoplacental growth at term. *Early Human Development* **46,** 15–26.

Ostlund E, Bang P, Hagenas L & Fried G (1997). Insulin-like growth factor I in fetal serum obtained by cordocentesis is correlated with intrauterine growth retardation. *Human Reproduction* **12,** 840–844.

Owens JA (1991). Endocrine and substrate control of fetal growth: placental and maternal influences and insulin-like growth factors. *Reproduction, Fertility, & Development* **3,** 501–517.

Owens JA, Kind KL, Carbone F, Robinson JS & Owens PC (1994). Circulating insulin-like growth factors-I and -II and substrates in fetal sheep following restriction of placental growth. *Journal of Endocrinology* **140,** 5–13.

O'Dell SD & Day IN (1998). Insulin-like growth factor II (IGF-II). *International Journal of Biochemistry & Cell Biology* **30,** 767–771.

de Pagter-Holthuizen P, Jansen M, van Schaik FM, van der Kammen R, Oosterwijk C, Van den Brande JL & Sussenbach JS (1987). The human insulin-like growth factor II gene contains two development-specific promoters. *FEBS Letters* **214,** 259–264.

Pandini G, Frasca F, Mineo R, Sciacca L, Vigneri R & Belfiore A (2002). Insulin/insulin-like growth factor I hybrid receptors have different biological characteristics depending on the insulin receptor isoform involved. *Journal of Biological Chemistry* **277,** 39684–39695.

Park JE, Chen HH, Winer J, Houck KA & Ferrara N (1994*a*). Placental growth factor. Potentiation of vascular endothelial growth factor bioactivity, in vitro and in vivo, and high affinity binding to Flt-1 but not Flk-1/KDR. *Journal of Biological Chemistry* **269,** 25646–25654.

Park JH, McCusker RH, Mohammadpour H, Blackwood DJ, Hrbek M & Vanderhoof JA (1994*b*). Dexamethasone inhibits mucosal adaptation after small bowel resection. *American Journal of Physiology* **266,** G497–G503.

Penninga L & Longo LD (1998). Ovine placentome morphology: effect of high altitude, long-term hypoxia. *Placenta* **19,** 187–193.

Perry VEA, Norman ST, Owens JA, Daniel RCW & Phillips N (1999). Low dietary protein during early pregnancy alters bovine placental development. *Animal Reproduction Science* **55,** 13–21.

Persico MG, Vinenti V & DiPalma T (1999). Structure, expression and receptor-binding properties of placental growth factor (PlGF). *Current Topics of Microbiology & Immunology* **237,** 31–40.

Peterson AJ & McMillan WH (1998). Variation in the rate of emergence of the bovine allantois. *In: Proceedings of the 29th annual Conference of the Australian Society for Reproductive Biology Abstract 23*.

Petrik J, Pell JM, Arany E, McDonald TJ, Dean WL, Reik W & Hill DJ (1999). Overexpression of insulin-like growth factor-II in transgenic mice is associated with pancreatic islet cell hyperplasia. *Endocrinology* **140,** 2353–2363.

Pfuender M, Sauerwein H, Funk B, Kessler U, Barenton B, Schwarz HP, Hoeflich A & Kiess W (1995). The insulin-like growth factor-II/Mannose-6-phosphate receptor is present in fetal bovine tissues throughout gestation. *Domestic Animal Endocrinology* **12,** 317–324.

Poole TJ & Coffin D (1989). Vasculogenesis and angiogenesis: two distinct morphogenetic mechanisms establish embryonic vascular pattern. *Journal of Experimental Zoology* **251,** 224–231.

Popovici RM, Lu M, Bhatia S, Faessen GH, Giaccia AJ & Giudice LC (2001). Hypoxia regulates insulin-like growth factor-binding protein 1 in human fetal hepatocytes in primary culture: suggestive molecular mechanisms for in utero fetal growth restriction caused by uteroplacental insufficiency. *The Journal of clinical endocrinology and metabolism* **86,** 2653–2659.

Price WA, Rong L, Stiles AD & D'Ercole AJ (1992). Changes in IGF-I and -II, IGF binding protein, and IGF receptor transcript abundance after uterine artery ligation. *Pediatric Research* **32,** 291–295.

Prior RL & Laster DB (1979). Development of the bovine fetus. *Journal of Animal Science* **48,** 1546–1553.

Punglia RS, Lu M, Hsu J, Kuroki M, Tolentino MJ, Keough K, Levy AP, Levy NS, Goldberg MA, D'Amato RJ & Adamis AP (1997). Regulation of vascular endothelial growth factor expression by insulin-like growth factor I. *Diabetes* **46,** 1619–1626.

Puri MC, Rossant J, Alitalo K, Bernstein J & Partanen (1995). The receptor tyrosine kinase TIE is required for integrity and survival of vascular endothelial cells. *EMBO Journal* **14,** 5884.

Putney DJ, Pepe GJ & Albrecht ED (1990). Influence of the fetus and estrogen on serum concentrations and placental formation of insulin-like growth factor I during baboon pregnancy. *Endocrinology* **127,** 2400–2407.

Quinn TP, Peters KG, de Vries C, Ferrara N & Williams LT (1993). Fetal liver kinase 1 is a receptor for vascular endothelial growth factor and is selectively expressed in embryyonic vascular endothelium. *Proceedings of the National Academy of Sciences of the United States of America* **90,** 7533–7537.

Rajaram S, Baylink DJ & Mohan S (1997). Insulin-like growth factor-binding proteins in serum and other biological fluids: regulation and functions. *Endocrine Reviews* **18,** 801–831.

Ranier S, Johnson LA & Dobry CJ (1993). Relaxation of imprinted genes in human cancer. *Nature* **362,** 747–749.

Redman CW (1991). Current Topic: pre-eclampsia and the placenta. *Placenta* **12,** 304–310.

Redmer DA, Dai Y, Li J, Charnock-Jones DS, Smith SK, Reynolds LP & Moor RM (1996). Characterization and expression of vascular endothelial growth factor (VEGF) in the ovine corpus luteum. *Journal of reproduction and fertility* **108,** 157–165.

Regnault TR, Orbus RJ, de Vrijer B, Davidsen ML, Galan HL, Wilkening RB & Anthony RV (2002*a*). Placental expression of VEGF, PlGF and their receptors in a model of placental insufficiency-intrauterine growth restriction (PI-IUGR). *Placenta* **23,** 132–144.

Regnault TR, de Vrijer B & Anthony RV (2002*b*). The IGF-II-deficient placenta: aspects of its function. *Trends in Endocrinology & Metabolism* **13,** 410–412.

Regnault TRH, Galan HL, Parker TA & Anthony RV (2002*c*). Placental development in normal and compromised pregnancies - A Review. *Placenta* **23,** S119–S129.

Regnault TRH, de Vrijer B, Galan HL, Davidsen ML, Trembler KA, Battaglia FC, Wilkening RB & Anthony RV (2003). The relationship between transplacental O2 diffusion and placental expression of PlGF, VEGF and their receptors in a placental insufficiency model of fetal growth restriction. *The Journal of physiology* **550,** 641–656.

Reik W & Walter J (2001). Genomic imprinting: Parental influence on the genome. *Nature Reviews Genetics* **2,** 21–32.

Reik W, Constancia M, Dean W, Davies K, Bowden L, Murrell A, Feil R, Walter J & Kelsey G (2000). Igf2 imprinting in development and disease. *International Journal of Developmental Biology* **44,** 145–150.

Reik W, Davies K, Dean W, Kelsey G & Constancia M (2001). Imprinted genes and the coordination of fetal and postnatal growth in mammals. *In: The cell cylce and development Wiley, Chichester (Novartis Foundation Symposium 237)*19–35.

Reimers TJ, Ullman MB & Hansel W (1985). Progesterone and prostanoid production by bovine binucleate trophoblast cells. *Biology of Reproduction* **33,** 1227–1236.

Renard JP (1998). Chromatin remodelling and nuclear reprogramming at the onset of embryonic development in mammals. *Reproduction, Fertility, & Development* **10,** 573–580.

Renard JP, Chastant S, Chesne P, Richard C, Marchal J, Cordonnier N, Chavatte P & Vignon X (1999). Lymphoid hypoplasia and somatic cloning. *Lancet* **353,** 1489–1491.

Reynolds LP (1986). Utero-ovarian interactions during early pregnancy: Role of conceptus-induced vasodilation. *Journal of Animal Science* **62,** 47–61.

Reynolds LP & Redmer DA (1988). Secretion of angiogenic activity by placental tissues of cows at several stages of gestation. *Reproduction and Fertility* **83,** 497–502.

Reynolds LP, Millaway DS, Kirsch JD, Infeld JE & Redmer DA (1987). Angiogenic activity of placental tissues of cows. *Journal of Reproduction & Fertility* **81,** 233–240.

Reynolds TS, Stevenson KR & Wathes DC (1997). Pregnancy-specific alternations in the expression of the insulin-like growth factor system during early placental development in the ewe. *Endocrinology* **138,** 886–897.

Richard DE, Berra E & Pouyssegur J (1999). Angiogenesis: how a tumor adapts to hypoxia. *Biochemical & Biophysical Research Communications* **266,** 718–722.

Rieger D (1998). Effects of the in vitro chemical environment during early embryogenesis on subsequent development. *Archives of toxicology* **(Suppl. 20,** 121–129.

Roberts RM & Bazer FW (1988). The functions of uterine secrections. *Journal of Reproduction and Fertility* **82,** 875–892.

Roberts RM, Xie S, Nagel RJ, Low B, Green J & Beckers JF (1995). Glycoproteins of the aspartyl proteinase gene family secreted by the developing placenta. *Advanced Experimental Medical Biology* **362,** 231–240.

Robinson JN & Abuhamad AZ (2000). Abdominal wall and umbilical cord anomalies. *Clinics in Perinatology* **27,** 947–978.

Rodriguez-Arnao J, Miell JP & Ross RJM (1993). Influence of thyroid hormones on the GH-IGF-1 axis. *Trends Endocrinological Metabolism* **4,** 169–173.

Rosenfeld CR, Morriss FH, Makowski EL, Meschia G & Battaglia FC (1974). Circulatory changes in teh reproductive tissues of ewes during pregnancy. *Gynecologic Investigation* **5,** 252–268.

Russe I & Sinowatz F (1991). *Embryologie der Haustiere*ed. Imogen Russe PP. Berlin.

Russe I, Sinowatz F, Richter L, Lehmann M & Schallenberger E (1992). The development of the yolk sac in ruminants (sheep and cattle). *Anatomia, Histologia, Embryologia: Veterinary Medicine Series C* **21,** 324–347.

Rutanen EM, Koistinen R, Wahlstrom T, Bohn H & Seppala M (1984). The content of placental protein12 in decidua and fetal membranes is greater than placenta. *British Journal of Obstetrics & Gynaecology*1240–1244.

Sahagian GG, Distler JJ & Jourdian GW (1987). Characterization of a membrane-associated receptor from bovine liver that binds phosphomannosyl residues of bovine testicular beta-galactosidase. *Proceedings of the National Academy of Sciences of the United States of America* **78,** 4289–4293.

Santos F, Zakhartchenko V, Stojkovic M, Peters A, Jenuwein T, Wolf E, Reik W & Dean W (2003). Epigenetic marking correlates with developmetal potential in cloned bovine preimplantation embryos. *Current Biology* **13,** 1116–1121.

Sara VR, Carrlsson-Skwirut C, Andersson C, Hall E, Sjorgen B, Homgren A & Jornvall H (1986). Characeterization of somatomedins from human fetal brain: identification of a variant from insulin-like growth factor I. *Proceedings of the National Academy of Sciences of the United States of America* **83,** 4904–4907.

Sato T, Tozawa Y, Deutsch U, Wolburg-Bucholz K, Fujiwara Y, Gendron-Maguire M, Gridley T, Wolburg H, Risau W & Qin Y (1995). Distinct roles of the receptor tyrosine kinases Tie-1 and Tie-2 in blood vessel formation. *Nature* **376,** 70–74.

Scalia P, Heart E, Comai L, Vigneri R & Sung CK (2001). Regulation of the Akt/Glycogen synthase kinase-3 axis by insulin-like growth factor-II via activation of the human insulin receptor isoform-A. *Journal of cellular biochemistry* **82,** 610–618.

Schlafer DH, Fisher PJ & Davies CJ (2000). The bovine placenta before and after birth: placental development and function in health and disease. *Animal Reproduction Science* **60-61,** 145–160.

Schmidt M, Greve T, Avery B, Beckers JF, Sulon J & Hansen HB (1996). Pregnancies, calves and calf viability after transfer of in vitro produced bovine embryos. *Theriogenology* **46,** 527–539.

Schneider MR, Lahm H, Wu M, Hoeflich A & Wolf E (2000). Transgenic mouse models for studying the functions of insulin-like growth factor-binding proteins. *FASEB Journal* **14,** 629–640.

Schnieke AE, Kind AJ, Ritchie WA, Mycock K, Scott AR, Ritchie M, Wilmut I, Colman A & Campbell KH (1997). Human factor IX transgenic sheep produced by transfer of nuclei from transfected fetal fibroblasts. *Science* **278,** 2130–2133.

Schrader AD, Iqbal MJ & Jones KL (2003). Gene expression in cloned bovine fetal liver. *Cloning & Stem Cells* **5,** 2003.

Schultz GA, Harvey MB, Watson AJ, Arcellana-Panlilio MJ, Jones K & Westhusin ME (1996). Regulation of early embryonic development by growth factors: Growth factor gene expression in cloned bovine embryos. *Journal of Animal Science* **74,** 50–57.

Schutz M, Freeman A, Lindberg G & Beitz D (1993). Effects of maternal lineages grouped by mitochondrial genotypes on milk yield and composition. *Journal of Dairy Science* **76,** 621–629.

Sciacca L, Constantino A, Pandini G, Mineo R, Frasca F, Scalia P, Sbraccia P, Goldfine ID, Vigneri R & Belfiore A (1999). Insulin receptor activation by IGF-II in breast cancers: evidence for a new autocrine/paracrine mechanism. *Oncogene* **18,** 2471–2479.

Sciacca L, Mineo R, Pandini G, Murabito A, Vigneri R & Belfiore A (2002). In IGF-I receptor-deificient leiomyosarcoma cells autocrine IGF-II induces cell invasion and protection from apoptosis via the insulin receptor isoform A. *Oncogene* **21,** 8240–8250.

Seidel GE, Brink Z, Holland MD & Wilson JM (1997). Pregnancy attrition from frozen bovine embryos cloned by nuclear transplantation. *Theriogenology* **47,** 236 (abstr.).

Semenza GL, Agani F, Iyer N, Kotch L, Laughner E, Leung S & Yu A (1999). Regulation of cardiovascular development and physiology by hypoxia-inducible factor 1. *Annual New York Academy of Science* **874,** 262–268.

Senger DR, Galli SJ & Dvorak AM (1983). Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. *Science* **219,** 983–985.

Shalaby F, Rossant J, Yamaguchi TP, Gertsenstein M, Wu XF, Breitman ML & Schuh AC (1995). Failure of blood island formation and vasculogenesis in Flk-1 deficient mice. *Nature* **376,** 62–66.

Shen BQ, Lee DY & Zioncheck TF (1999). Vascular endothelial growth factor governs endothelial nitric-oxide synthase expression via a KDR/Flk-1 receptor and a protein kinase C signaling pathway. *Journal of Biological Chemistry* **274,** 33057–33063.

Shen SJ, Wang CY, Nelson KK, Jansen M & Ilan J (1986). Expression of insulin-like growth factor II in human placentas from normal and diabetic pregnancies. *Proceedings of the National Academy of Sciences of the United States of America* **83,** 9179–9182.

Sherer DM & Abulafia O (2001). Angiogenesis during implantation, and placental and early embryonic development. *Placenta* **22,** 1–13.

Shiels PG, Kind AJ, Campbell KH, Waddington D, Wilmut I, Colman A & Schnieke AE (1999). Analysis of telomere lengths in cloned sheep. *Nature* **399,** 316–317.

Shore VH, Wang TH, Wang CL, Torry RJ, Caudle MR & Torry DS (1997). Vascular endothelial growth factor, placental growth factor and their receptors in isolated human trophoblast. *Placenta* **18,** 657–665.

Sinclair KD (1998). Workshop on Embryo Development In Vitro: Current Challenges and Future Concepts. *Proceedings IETS Satelite Symposium*28–29.

Sinclair KD, Maxfield EK, Robinson JJ, Maltin CA, McEvoy TG, Dunne LD, Young LE & Broadbent PJ (1997). Culture of sheep zygotes can alter fetal growth and development. *Theriogenology* **47,** 380 (Abstr.).

Sinclair KD, McEvoy TG, Carolan C, Maxfield EK, Maltin CA, Young LE, Wilmut I, Robinson JJ & Broadbent PJ (1998). Conceptus growth and development following in vitro culture of ovine embryos in media supplemented with bovine sera. *Theriogenology* **49,** 218 (Abstr.).

Sinclair KD, Young LE, Wilmut I & McEvoy TG (2000). In-utero overgrowth in ruminants following embryo culture: lessons from mice and a warning to men. *Human reproduction (Oxford, England)* **15 Suppl 5,** 68–86.

Soker S, Takashima S, Miao G, Neufeld G & Klagsbrun M (1998). Neuropilin-1 is expressed by endothelial and tumor cells as an isoform-specific receptor for vascualr endothelial growth factor. *Cell* **92,** 735–745.

De Sousa PA, King T, Harkness L, Young LE, Walker SK & Wilmut I (2001). Evaluation of gestational deficiencies in cloned sheep fetuses and placentae. *Biology of reproduction* **65,** 23–30.

Spagnoli A & Rosenfeld RG (1996). The mechanisms by which growth hormone brings about growth. The relative contributions of growth hormone and insulin-like growth factors. *Endocronology & MetabolismClinics of North America* **25,** 615–631.

Spemann H (1938). Embryonic development and induction. *Yale University Press, New Haven, CT*.

Stegeman JHJ (1974). Placental development in sheep and its relation to fetal development. *Bijdragen tot de Dierkunde* **44,** 3–72.

Steven DH (1968). Placental vessels of the foetal lamb. *Journal of Anatomy* **103,** 539–551.

Stevenson KR & Wathes DC (1996). Insulin-like growth factors and their binding proteins in the ovine oviduct during the oestrous cycle. *Journal of Reproduction & Fertility* **108,** 31–40.

Stewart CEH & Rotwein P (1996). Growth, differentiation and survival: multiple physiological functions for insulin-like growth factors. *Physiology Review* **76,** 1005–1026.

Stice SL, Robl JM, Ponce de Leon FA, Golueke PJ, Cibelli JB & Kane JJ (1998). Cloning: new breakthroughs leading to commercial opportunities. *Theriogenology* **49,** 129–138.

Stice SL, Strelchenko NS, Keefer CL & Matthews L (1996). Pluripotent bovine embryonic cell lines direct embryonic development following nuclear transfer. *Biology of Reproduction* **54,** 100–465.

Surani MA (1998). Imprinting and the initiation of gene silencing in the germ line. *Cell* **93,** 309–312.

Szuba Z, Kalisinska E & Babula Z (1988). Growth of body weight and length of bovine fetuses. *Anatomia, Histologia, Embryologia: Veterinary Medicine Series C* **17,** 53–59.

Tani T, Kato Y & Tsunoda Y (2001). Direct exposure of chromosomes to nonactivated ovum cytoplasm is effective for bovine somatic cell nucleus reprogramming. *Biology of Reproduction* **64,** 324–330.

Tarantino S, Verhage HG & Fazleabas AT (1992). Regulation of insulin-like growth factor-binding proteins in the baboon (Papio anubis) uterus during early pregnancy. *Endocrinology* **130,** 2354–2362.

Tazuke SI, Mazure NM, Sugawara J, Garland G, Faessen GH, Suen LF, Irwin JC, Powell DR, Giacca AJ & Giudice LC (1998). Hypoxia stimulates insulin-like grwoth factor binding protein-I (IGFBP-I) gene expression in HepG2 cells: a possible model for IGFBP-1 expression in fetal hypoxia. *Proceedings of the National Academy of Sciences of the United States of America* **95,** 10188–10193.

Terman BI, Dougher-Vermazen M, Carrion ME, Dimitrov D, Armellino DC, Gospodarowicz D & Bohlen P (1992). Identification of the KDR tyrosine kinase as a receptor for vascular endothelial cell growth factor. *Biochemical & Biophysical Research Communications* **187,** 1579–1586.

Thissen JP, Ketelslegers JM & Underwood LE (1994). Nutritional regulation of the insulin-like growth factors. *Endocrine Reviews* **15,** 80–101.

Thompson JG & Peterson AJ (2000). Bovine embryo culture in vitro: new developments and post-transfer consequences. *Human Reproduction* **15,** 59–67.

Thrailkill K, Quattrin T, Baker L, Litton J, Dwigun K, Rearson M, Poppenheimer M, Kotlovker D, Giltinan D, Gesundheit N & Martha P (1997). Dual hormonal replacement therapy with insulin and recombinant human insulin-like growth factor (IGF)-I in insulin-dependent diabetes mellitus: effects on the growth hormone/IGF/IGF-binding protein system. *The Journal of clinical endocrinology and metabolism* **82,** 1181–1187.

Tilghman SM (1999). The sins of the fathers and mothers: Genomic imprinting in mammalian development. *Cell* **96,** 185–193.

Tischer E, Mitchell R, Hartman T, Silva M, Gospodarowicz D, Fiddes JC & Abraham JA (1991). The human gene for vascular endothelial growth factor. *Journal of Biological Chemistry* **266,** 11947–11954.

Torry DS & Torry RJ (1997). Angiogenesis and the expression of vascular endothelial growth factor in endometrium and placenta. *American Journal of Reproductive Immunology (Copenhagen)* **37,** 21–29.

Torry DS, Wang HS, Wang TH, Caudle MR & Torry RJ (1998). Preeclampsia in associated with reduced serum levels of placental growth factor. *American Journal of Obstetrical Gynecology* **179,** 1539–1544.

Trounson AO (1997). Cloning: potential benefits for human medicine. *Medical Journal of Australia* **167,** 568–569.

Tsutsumi Y (1962). The vascular pattern of the placenta in farm animals. *Journal of Faculty Agriculture Hokkaido University Sapporo* **52,** 408–420.

Tsutsumi Y & Hafez ESE (1966). Vascular architecture of the placentome in the cow and rabbit. *Cornell Veterinarian* **56,** 527–543.

Tucker KL, Beard C, Dausmann J, Jackson-Grusby L, Laird PW, Lei H, Li E & Jaenisch R (1996). Germ-line passage is required for establishment of methylation and expression patterns of imprinted but not of nonimprinted genes. *Genes & Development* **10,** 1008–1020.

Unterman T, Lascon R, Gotway MB, Oehler D, Gounis LC, Simmons RA & Ogata ES (1990). Circulating levels of insulin-like growth factor binding protein-1 (IGFBP-1) snf heptic mRNA are increased in the small for gestational age (SGA) fetal rat. *Endocrinology* **127,** 2035–2037.

Veikkola T & Alitalo K (1999). VEGFs, receptors and angiogenesis. *Seminars in Cancer Biology* **9,** 211–220.

Vella V, Pandini G, Sciacca L, Mineo R, Vigneri R, Pezzino V & Belfiore A (2002). A novel autocrine loop involving IGF-II and the insulin receptor isoform-A stimulates growth of thyroid cancer. *The Journal of clinical endocrinology and metabolism* **87,** 245–254.

Verhaeghe J, Coopmans W, van Herck E, van schoubroeck D, Deprest JA & Witters I (1999). IGF-I, IGF-II, IGF binding protein 1, and C-peptide in second trimester amniotic fluid are dependent on gestational age but do not predict weight at birth. *Pediatric Research* **46,** 101–108.

Viuff D, Hendriksen PJ, Vos PL, Dieleman SJ, Bibby BM, Greve T, Hyttel P & Thomsen PD (2001). Chromosomal abnormalities and developmental kinetics in in vivo-developed cattle embryos at days 2 to 5 after ovulation. *Biology of Reproduction* **65,** 204–208.

Voelkel SA & Hu YX (1992). Direct transfer of frozen-thawed bovine embryos. *Theriogenology* **37,** 23–37.

Volpert O, Jackson D, Bouck N & Linzer DI (1996). The insulin-like growth factor II/mannose 6-phosphate receptor is required for proliferin-induced angiogenesis. *Endocrinology* **137,** 3871–3876.

Vrana PB, Guan XJ, Ingram RS & Tilghman SM (1998). Genomic imprinting is disrupted in interspecific Peromyscus hybrids. *Nature Genetics* **20,** 362–365.

de Vries C, Escobedo JA, Ueno H, Houck K, Ferrara N & Williams LT (1992). The fms-like tyrosine kinase, a receptor for vascular endothelial growth factor. *Science* **255,** 989–991.

de Vrijer B, Anthony RV & Regnault TR (2002). Placental development in normal and compromised pregnancies-- a review. *Trends in Endocrinology & Metabolism* **13,** 410–412.

Vuorela P, Hatva E, Lymboussaki A, Kaipainen A, Joukov V, Persico MG, Alitalo K & Halmesmaki E (1997). Expression of vascular endothelial growth factor and placenta growth factor in human placenta. *Biology of Reproduction* **56,** 489–494.

Van Wagtendonck-de Leeuw AM, Mullaart E, de Roos APW, Merton JS, den Daas JHG, Kemp B & de Ruigh L (2000). Effect of different reproduction techniques: AI, MOET or IVP, on health and welfare of bovine offspring. *Theriogenology* **53,** 575–599.

van Wagtendonk-de Leeuw AM, Aerts BJ & den Daas JH (1998). Abnormal offspring following in vitro production of bovine preimplantation embryos: a field study. *Theriogenology* **49,** 883–894.

Wakayama T, Perry AC, Zuccotti M, Johnson KR & Yanagimachi R (1998). Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei. *Nature* **394,** 369–374.

Wakayama T, Rodriguez I, Perry AC, Yanagimachi R & Mombaerts P (1999). Mice cloned from embryonic stem cells. *PNAS* **96,** 14984–14989.

Walker SK, Hartwich KM & Seamark RF (1996). The production of unusually large offspring following embryo manipulation: concepts and challenges. *Theriogenology* **45,** 111–120.

Wallace JM, Bourke DA, Aitken RP & Cruickshank MA (1999). Switching maternal dietary intake at the end of the first trimester has profound effects on placental develpment and fetal growth in adolescent ewes carrying singleton fetuses. *Biology of Reproduction* **61,** 101–110.

Waltenberger J, Claesson-Welsh L, Siegbahn A, Shibuya M & Heldin CH (1994). Different signal transduction properties of KDR and Flt1, two receptors for vascular endothelial growth factor. *Journal of Biological Chemistry* **269,** 26988–26995.

Wathes DC, Reynolds TS, Robinson RS & Stevenson KR (1998). Role of the insulin-like growth factor system in uterine function and placental development in ruminants. *Journal of Dairy Science* **81,** 1778–1789.

Watson AJ, Hogan A, Hahnel A, Wiemer KE & Schultz GA (1992). Expression of growth factor ligand and receptor genes in the preimplantation bovine embryo. *Molecular Reproduction & Development* **31,** 87–95.

Watson AJ, Watson PH, Arcellana-Panlilio M, Warnes D, Walker SK, Schultz GA, Armstrong DT & Seamark RF (1994). A growth factor phenotype map for ovine preimplantation development. *Biology of Reproduction* **50,** 725–733.

Wells DN, Misica PM & Tervit HR (1999). Production of Cloned Calves Following Nuclear Transfer with Cultured Adult Mural Granulosa Cells. *Biology of Reproduction* **60,** 996–1005.

Westhusin ME, Levanduski MJ, Scarborough R, Looney CR & Bondioli KR (1992). Viable embryos and normal calves after nuclear transfer into Hoechst stained enucleated demi-oocytes of cows. *Journal of Reproduction and Fertility* **95,** 475–480.

Westwood M, Gibson JM, Williams AC, Clayton PE, Hamberg O, Flyvbjerg A & White A (1995). Hormonal regulation of circulating insulin-like growth factor-binding protein-1 phosphorylation status. *Journal Clinical Endocrinalolgy Metabolism* **80,** 3520–3527.

White KL & Yue C (1996). Intracellular receptors and agents that induce activation inbovine oocytes. *Theriogenology* **45,** 91–100.

Willadsen SM (1979). A method for culture of micromanipulated sheep embryos and its use to produce monozygotic twins. *Nature* **277,** 298–300.

Willadsen SM (1986). Nuclear transplantation in sheep embryos. *Nature* **320,** 63–65.

Willadsen SM, Janzen RE, McAlister RJ, Shea BF, Hamilton G & McDermand D (1991). The viability of late morulae and blastocysts produced by nuclear transplantation in cattle. *Theriogenology* **35,** 161–170.

Wilmut I (1998). Embryonic and somatic cell cloning. *Reproduction, Fertility, & Development* **10,** 639–643.

Wilmut I, Schnieke AE, McWhir J, Kind AJ & Campbell KH (1997). Viable offspring derived from fetal and adult mammalian cells. *Nature* **385,** 810–813.

Wilson JM, Williams JD, Bondioli KR, Looney CR, Westhusin MR & McCalla DF (1995). Comparison of birth weight and growth characteristics of bovine calves produced by nuclear transfer (cloning), embryo transfer and natural mating. *Animal Reproduction Science* **38,** 73–84.

Winnick M & Noble A (1965). Quantitative changes in DNA, RNA, and protein during prenatal and postnatal growth in the rat. *Developmental Biology* **12,** 451.

Wooding FB (1992). Current topic: the synepitheliochorial placenta of ruminants: binucleate cell fusions and hormone production. *Placenta* **13,** 101–113.

Wooding FB & Wathes DC (1980). Binucleate cell migration in the bovine placentome. *Journal of Reproduction & Fertility* **59,** 425–430.

Wooding FB, Morgan G & Adam CL (1997). Structure and function in the ruminant synepitheliochorial placenta: central role of the trophoblast binucleate cell in deer. *Microscopy Research & Technique* **38,** 88–99.

Yang HC & Pardee AB (1986). Insulin-like growth factor I regulation of transcription and replicating enzyme induction necessary for DNA synthesis. *Journal of Cellular Physiology* **127,** 410–416.

Yazawa S, Aoyagi Y, Konishi M & Takedomi T (1997). Characterization and cytogenetic analysis of Japanese black calves produced by nuclear transfer. *Theriogenology* **48,** 641–650.

Yonekura H, Sakurai S, Liu X, Migita H, Wang H, Yamagishi S, Nomura M, Abedin MJ, Unoki H, Yamamoto Y & Yamamoto H (1999). Placental growth factor and vascular endothelial growth factor B and C expression in microvascualr endothelial cells and pericytes. Implication in autocrine and paracrine regulation of angiogenesis. *Journal of Biological Chemistry* **49,** 35172–35178.

Young LE (2001). Imprinting of genes and the Barker hypothesis. *Twin Research* **4,** 307–317.

Young LE & Fairburn HR (2000). Improving the safety of embryo technologies: Possible role of genomic imprinting. *Theriogenology* **53,** 627–648.

Young LE, Fernandes K, McEvoy TG, Butterwith SC, Gutierrez CG, Carolan C, Broadbent PJ, Robinson JJ, Wilmut I & Sinclair KD (2001). Epigenetic change in IGF2R is associated with fetal overgrowth after sheep embryo culture. *Nature Genetics* **27,** 153–154.

Young LE, Sinclair KD & Wilmut I (1998). Large offspring syndrome in cattle and sheep. *Reviews of Reproduction* **3,** 155–163.

Yu J, Iwashita M, Kudo Y & Takeda K (1998). Phosphorylated insulin growth factor (IGF)-binding protein -1 (IGFBP-1) inhibits while non-phosphorylated IGFBP-1 stimulates IGF-1-induced amino acid uptake by cultured trophoblast cells. *Growth Hormone & Igf Research* **8,** 65–70.

Yun K, Jinno Y, Sohda T, Niikawa N & Ikeda T (1998). Promoter-specific insulin-like growth factor 2 gene imprinting in human fetal liver and hepatoblastoma. *Journal of Pathology* **185,** 91–98.

Zaina S & Squire S (1998). The soluble type 2 insulin-like growth factor (IGF-II) receptor reduces organ size by IGF-II-mediated and IGF-II-independent mechanisms. *Journal of Biological Chemistry* **273,** 28610–28616.

Zakhartchenko V, Alberio R, Stojkovic M, Prelle K, Schernthaner W, Stojkovic P, Wenigerkind H, Wanke R, Düchler M, Steinborn R, Mueller M, Brem G & Wolf E (1999). Adult cloning in cattle: potential of nuclei from a permanent cell line and from primary cultures. *Molecular reproduction and development* **54,** 264–272.

Zhou J, Damsky CH & Fisher SJ (1997). Preeclampsia is associated with failure of human cytotrophoblasts to mimic a vascular adhesion phenotype. One cause of defective endovascular invasion in this syndrome? *Journal of Clinical Investigation* **99,** 2152–2164.

Zhou Y, Damsky CH, Chui K, Roberts JM & Fisher SJ (1993). Preeclampsia is associated with abnormal expression of adhesion molecules by invasive trophblasts. *Journal of Clinical Investigation* **91,** 950–960.

Ziche M, Maglione D, Ribatti D, Morbidelli L, Lago CT, Battaglia FC, Paoletti I, Bana A, Tucci M, Parise G, Vincenti V, Granger HJ, Viglietto G & Persico MG (1997). Placental growth factor-1 is chemotactic, mitogenic, and angiogenic. *Laboratory Investigation* **76,** 517–531.

Zumkeller W (2000). Current topic: the role of growth hormone and insulin-like growth factors for placental growth and development. *Placenta* **21,** 451–467.