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A molecular biological study of the preimplantation of insulin-like growth factor genes and their receptors in in vitro produced bovine embryos to improve in vitro culture systems and embryo quality

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A Molecular Biological Study of the Preimplantation Expression of Insulin-Like Growth Factor Genes and their Receptors in *In Vitro* Produced Bovine Embryos to Improve *In Vitro* Culture Systems and Embryo Quality

Mohamed A. Yaseen

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1. INTRODUCTION

Reproductive biotechnologies have made great progress during the past 15 years. In vitro production (IVP) of bovine embryos has emerged as a reliable alternative method to conventional ovulation induction techniques and as important tool to study preimplantation embryo development. A variety of embryo culture systems has been developed for the production of viable embryos (Bavister, 1995). Most of culture media are supplemented with serum or serum albumin as protein sources, frequently together with somatic cells in co-culture (Bavister, 1995). However, despite the recent improvements in bovine IVP, in vitro generated embryos display a number of marked differences compared to their in vivo counterparts (for review see Thompson, 1997; Holm and Callesen, 1998; Niemann and Wrenzycki, 2000). Moreover, extended in vitro culture seems to be associated with the 'large calf syndrome' (Kruip and denDaas, 1997).

Niemann and Wrenzycki (2000) reported that transcription of several developmentally important genes in preimplantation embryos is affected by the in vitro environment, possibly leading to perturbation of differentiation and organogenesis, and contributing to the 'large offspring syndrome'. Bovine embryos respond to changes in their environment very early on in development by modifying the synthesis of specific mRNAs (Wrenzycki et al., 1999).

Insulin and insulin-like growth factors (IGFs) are a group of structurally related polypeptides that regulate the growth of many types of mammalian cells. Growth factors have been identified in follicular fluid (Hammond et al., 1988) and in the female reproductive tract (Buhi et al., 1997). Expression of mRNA transcripts for the IGF family has been detected in embryos of a variety of species. Therefore, several lines of evidence indicate that the polypeptide growth factors of the IGF family have an important role in early development.

Messenger-RNA expression of IGFs, their receptors and IGFBPs during bovine preimplantation development were shown to vary qualitatively (Schultz et al., 1992; Watson et al., 1992; Winger et al., 1997; Yoshida et al., 1998a; Lonergan et al., 2000) and the changes in the abundance of IGF-I mRNA and IGF-IR in relation to culture medium composition have not yet been investigated. The results obtained by the use of a semi-quantitative RT-PCR assay provide insight into the regulation of early bovine development at the molecular level and will aid to characterize the transcriptional activity of bovine embryos to optimize the in vitro culture systems with the final goal to improve the quality of the embryos generated in vitro.

The aim of this study was to determine the relative abundance of mRNAs for the IGF-I and IGF-II ligands and their receptors (IGF-IR and IGF-IIR) in preimplantation bovine development up to the hatched blastocyst stage using two different in vitro culture systems. These two contrasting culture systems (complex and undefined versus simple and defined) were used to determine effects on mRNA expression originating from the in vitro production process.

2. REVIEW OF LITERATURE

2.1. Development of mammalian oocytes and preimplantation embryos

2.1.1. Development and maturation of mammalian oocytes in vivo

Oocyte maturation is traditionally defined as those events associated with the initiation of germinal vesicle breakdown (GVBD) and completion of the first meiotic divition, referred to here as nuclear maturation. More correctly, oocyte maturation should be defined as those events that render the oocyte capable for fertilization and initiate the program that directs preimplantation embryonic development (Leibfried et al., 1987).
As reviewed by Dieleman et al. (2002), final maturation of the ovulatory follicle in the cyclic cow is initiated by the preovulatory LH surge. During the following 24-h period, the oocyte undergoes progression of meiosis to metaphase II and several changes in cytoplasmic organization, such as a continued development of the lipid store, reduction of the Golgi compartment, rearrangement of mitochonodria and alignment of the cortical granules along the oolemma (De Loos et al., 1991; Hyttel et al., 1986; Hyttel et al., 1997; Kruip et al., 1983). The increased lipid compartment probably forms an essential energy pool for the oocyte to support development after fertilization up to the blastocyst stage (Betteridge and Fléchon, 1988). Further, numerous ribosomes appear especially adjacent to the chromosomes (Hyttel et al., 1997; Kruip et al., 1983). This redistribution of the ribosomes is propably related to inactivity of the site for ribosome synthesis, the nucleolus, already at the start of final maturation (Hyttel et al., 2001). The preovulatory follicle also undergoes a series of changes during final maturation. The membrana granulosa cells cease to synthesize estradiol and the follicular wall luteinizes shortly before ovulation, coinciding with a marked increase of progestrone production and extensive expansion of the cumulus (Dieleman et al., 1983a; Dieleman et al., 1983b; Dieleman and Blankenstein, 1985; Dieleman et al., 1986). The oocyte develops largely on its own after receiving the signal to resume meiosis as mediated by the follicular cells directly or indirectly via the cumulus cells. Extensions of the corona cells penetrating the zona and intruding the oocyte, which are already retracted to a more superficial position before the LH surge, are retracted further (Hyttel et al., 1997), indicating

that communication between the oocyte and its surrounding cumulus cells decreased soon after onset of maturation and is ultimately lost (Dieleman et al., 2002). The existence of an oocyte-granulosa cell regulatory loop, essential for normal follicular differentiation as well as for the production of an oocyte competent to undergo fertilization and embryogenesis, is proposed. Although gonadotrophins are essential for driving the differentiation of granulosa cell phenotypes, within its sphere of influence, the oocyte is probably the dominant factor determining the direction of differentiation and the function of the granulosa cells associated with it (Eppig, 2001). Granulosa cells participate in global suppression of transcription in oocyte prior to nuclear maturation (De la Fuente and Eppig, 2001). On the other hand, newly synthesized proteins are observed throughout maturation in vivo in oocytes incubated as COCs with major changes in protein patterns occuring around germinal vesicle breakdown (Kastrop et al., 1991a; Kastrop et al., 1992), and, possibly, part of the peptides originate from the cumulus cells. Also, transcription in the cumulus cells might be needed for final maturation in vivo (Dieleman et al., 2002). During the first 2 h of IVM of COCs from 4- to 8-mm follicles, transcription is necessary to drive resumption and completion of meiosis (Kastrop et al., 1991b), although the immature oocyte itself at the germinal vesicle (GV) stage is capable of incorporating uridine, suggesting synthesis of mRNA (Memili et al., 1998). Many of the maternally derived products needed to prepare the biological machinary, such as re-assembly of the nucleolus as factory of ribosomes necessary for protein synthesis (Hyttel et al., 1998; Hyttel et al., 2001), are stored in the oocyte during its growth. To prevent degradation of the mRNAs and proteins during storage in the oocyte, post-transcription and post-translation mechanisms, such as described for oocytes of lower organisms, will also be essential in the cow in vivo (Brevini-Gandolfi and Gandolfi, 2001). The role of the stored mRNA does not end with pronucleus formation (Sirard, 2001).

Final maturation in vivo (see Fig. 1) appears to include a complex series of processes that equip the oocyte with the necessary cell biological tools and stores to successfully undergo fertilization and further embryonic and fetal development. It is suggested that in vivo maturation provides the prematured oocyte with additional traits that may augment the capacity of the blastocyst to develop to term (Dieleman et al., 2002).

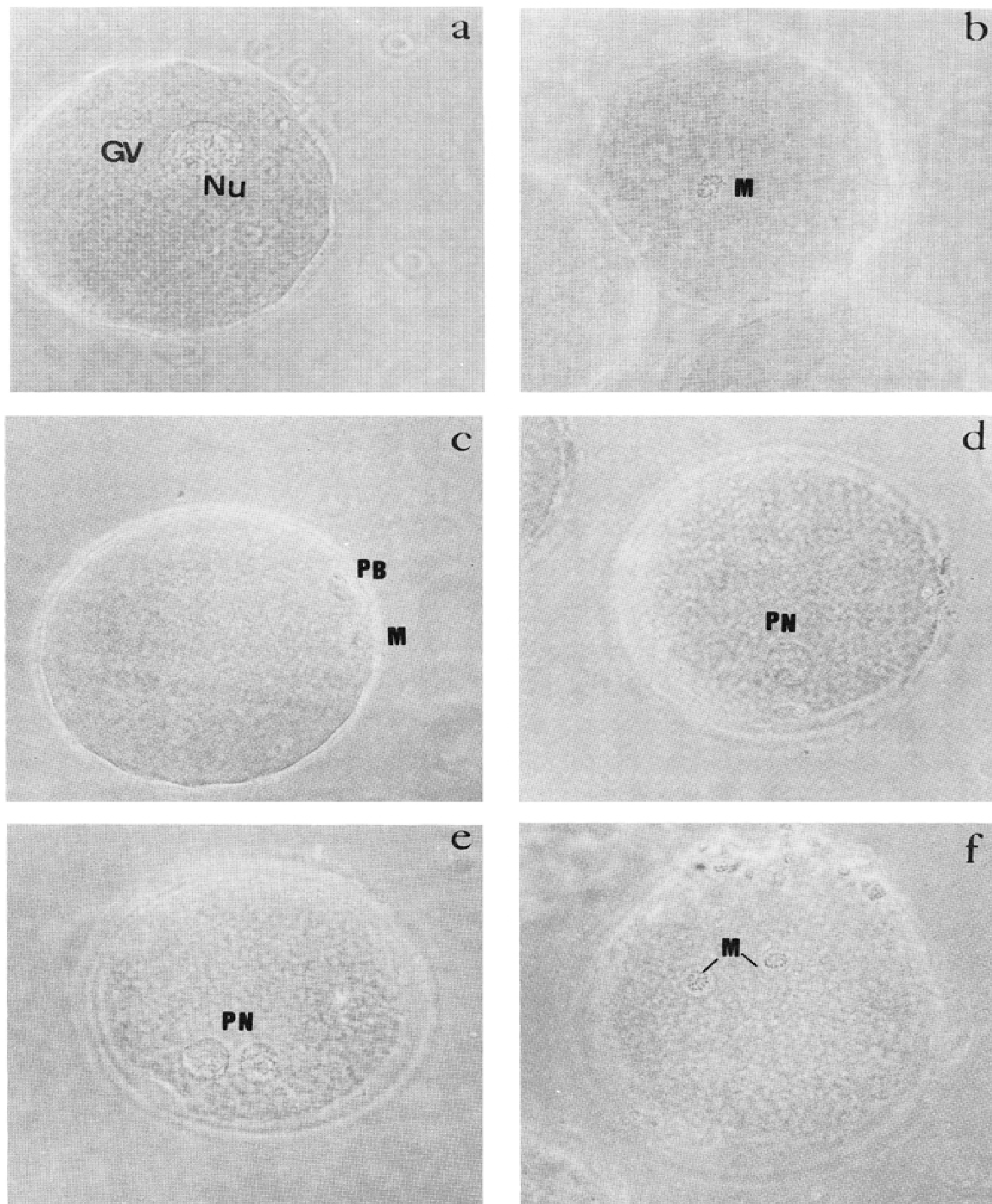


Fig. 1 Photomicrographs of fixed and stained whole mounts of bovine oocytes representing the various nuclear configuration. (a) bovine oocyte at the germinal vesicle stage (GV: Germinal vesicle, Nu: nucleolus), (b) bovine oocytes showing a metaphase I, (c) bovine oocyte at the metaphase II stage, (d) bovine oocyte showing one pronuclear-like (PN) structure and a polar body, (e) bovine oocyte showing two pronuclear-like (PN) and (f) bovine oocyte showing two metaphase plates (M) (Sirard et al., 1989).

2.1.2. Fertilization in vivo

Before implantation, the mammalian embryo has to pass certain critical milestones in embryogenesis under the conditions of the female reproductive tract i.e. the oviduct is the site of fertilization and the first steps of early embryo development (Van Soom et al., 1996; Enright et al., 2000). New life starts at the moment of fertilization when the zygote is formed. Penetration of the ovum may be assumed to happen very soon after ovulation, probably within about 2 hours. Shortly after penetration through the zona pellucida and fusion of the gametes, the non-membrane bound sperm head decondenses within the oocyte cytoplasm. Subsequently, a nuclear envelope forms and swelling of the male pronucleus is observed. Simultaneously, meiosis resumes with the extrusion of the second polar body and formation of a nuclear envelope around the decondensing female chromatin (Crozet et al., 1984; Hyttel et al., 1988). During the process of fertilization in mammals the genetic sex of the future embryo is fixed. (see Lawrence and Fowler, 1997).

In the cow, transcription and proteins from the oocyte govern initial embryonic development after fertilization until during the 4th cell cycle when embryonic control of development becomes evident (De Sousa et al., 1998b). Although transcription has been observed in vivo as early as the 1-cell stage albeit at low levels (Hay-Schmidt et al., 2001), it seems to be driven during the first cell cycle mainly by transcription factors originating from the oocyte (Dieleman et al., 2002).

2.1.3. Cleavage and embryonic cell cycles in vivo

Cleavage is a process of several successive cell divisions which occur without any increase in the total mass of the very young embryo. The rate at which cleavage proceeds varies both between and within species and among the individual cells, known as blastomeres, of a single embryo. In consequence, after the first cleavage division of the cytoplasm gives a two-cell egg, the two- and four-cell stages of cleavage are more often encountered than are the three- and five-cell stages, with the eight-cell stage predominating on the following day. The synthesis of DNA in the daughter cells succeeds each mitosis during the first cleavage division (see Lawrence and Fowler, 1997).

In cow, the time required for progression to the first cleavage ranges from 23-31 hours post fertilization (Hyttel et al., 1988) and 20 hours after the estimated time of ovulation was also suggested (Laurincik et al., 1994). Thus, the duration of the first cell cycle is 7-9 hours (Laurincik et al., 1994). Cleavage to the 4- and 8-cell stage occurs at approximately 36-50 and 56-64 hours post fertilization, respectively. When the mean time period for this cleavage is considered, the second and the third cell cycles are 13 and 24 hours long, respectively (Eyestone and First, 1988; Sirard and Lambert, 1985; First and Barnes, 1989). Cleavage to the 16-cell stage occurs at approximately 80-86 hours post fertilization with a fourth cell cycle duration of 21-30 hours (Sirard and Lambert, 1985).

2.1.4. Blastocyst formation and hatching in vivo

By the processes of mitotic division embryos continue to develop after entering the uterus. When the embryo has reached the stage of containing 16 or more cells it is termed a morula (see Lawrence and Fowler, 1997). In cattle, compaction starts 5 days post ovulation at the 32-cell stage. Morulae remain firmly compact until the seventh cell cycle is almost completed (Van Soom et al., 1997). Compaction is generally regarded as a prerequisite for the formation of the blastocoele and then cavitation occurs after 6 days post insemination (Betteridge and Fléchon, 1988). Subsequently, individual blastomeres secrete fluid into the intercellular spaces, after which they become arranged around a central fluid-filled space known to become the blastocoele (see Lawrence and Fowler, 1997). Blastocyst formation starts between the 64- and 128-cell stage at days 6, 7, and 8 post ovulation (Van Soom et al., 1997). At the blastocyst stage the group of cells destined to form the embryo proper (the inner cell mass, ICM) becomes distinguishable from those that will form embryonic membranes (the trophoblast). The inner cell mass appears as a knob to one side of the central cavity and these cells are the progenitors of the ultimate adult oganism. The trophoblast layer is a single peripheral layer of large flattened cells which are the progenitors of the placenta and the embryonic membranes (see Lawrence and Fowler, 1997). The slow in vivo transition from the morula to the blastocyst stage allows sufficient time for allocation of inner cells to the ICM of embryo (Van Soom et al., 1997).

Differentiation into trophoblast and inner cell mass is followed by shedding of the protective zona pellucida. The shedding process is known as hatching which occurs between days 9 and 11 post fertilization in cattle, days 7 and 8 in sheep and days 6 and 7 in pig (see Lawrence and

Fowler, 1997). Hatching was predominant at day 9 post ovulation in cattle (Van Soom et al., 1997). These rapid changes in morphology are associated with alterations in metabolism (Barnett and Bavister, 1996) and expanding and hatching blastocysts occur through continued activity of different sodium pumps (Betteridge and Fléchon, 1988).

As already mentioned, the initial period of mammalian preimplantation development is regulated by gene transcripts and polypeptides produced by and stored in the oocyte during its development (Schultz, 1993). However, following one to three cleavage divisions, control of development is taken over by expression of portions of the embryonic genome and the maternally derived transcripts and proteins are gradually degraded (Telford et al., 1990; Watson et al., 1996). It is clear that the messenger RNAs (mRNAs) include transcripts for several growth factors and their receptors as well as for a number of cell adhesion and cell junction proteins, together with transcripts for the subunits of the Na/K-ATPase, these specific transcripts are produced for blastocyst formation (for review see Watson et al., 1996; Hyttel et al., 1998).

2.2. In vitro production of bovine embryos (IVP)

Mammalian preimplantation embryos normally develop within the protected environment of the female reproductive tract. In vitro systems are commonly used for the production of bovine embryos. In vitro systems try to mimic the conditions of the female reproductive tract. The in vitro production of embryos consists of four steps where the success of a particular step depends on the success of the previous step. These steps are: harvesting of oocytes, in vitro maturation (IVM) of recovered oocytes, in vitro fertilization (IVF) of IVM oocytes and in vitro culture (IVC) of the IVM/IVF zygotes for development to the desired stage.

2.2.1. Harvesting of oocytes

2.2.1.1. Recovering oocytes from live animals

Oocytes may be obtained from a wide range of mammalian species by flushing the reproductive tract or produced by the recovery of mature or immature oocytes from antral

follicles in the ovary (Trounson, 1992). The treatment of clinical cases of infertility arising from oviductal obstruction or infundibular adhesions (Sirard, 1989) also requires collection of oocytes from the live animals. To obtain the oocytes from live animals, there are two different techniques: surgical technique with recovery rates 53-79% (Sirard and Lambert, 1985; Lambert et al., 1986) and non-surgical techniques with recovery rates 71% for normal superovulated donors and 11% for unsuperovulated donoers with known fertility problems (Elsden et al., 1976).

An efficient exploitation of the female gamete pool and a shortening of the generation interval are important aims in modern animal husbandry involving advanced reproductive technologies such as in vitro production of embryos. This requires the abundant availability of developmentally competent cumulus oocytes complex (COC) from valuable donor cows (Bungartz et al., 1995). In vitro embryo production from live donors by using ultrasound-guided technique (ovum pick up, OPU) is an extremely versatile technique because it can be applied to donors of all ages from two-months-old calves to very old cows (Galli et al., 2001). It can be used for dry and lactating donors and even during pregnancy up to the third or fourth month (Van der Shans et al., 1991; Klossok, 1997; Kuwer, 1997; Eikelmann, 1999; Frank, 1999; Rick et al., 1996; Rust et al., 1999; Galli et al., 2001). It is normally applied in a regime of two ultrasound-guided oocyte collection sessions per week (Bungartz et al., 1995; Galli et al., 2001). Ultrasound-guided technology for the aspiration of small and preovulatory follicles has been successfully employed in humans (Lenz et al., 1987), cattle (Simon et al., 1993; Bungartz et al., 1995) and horses (Brück and Greve, 1994; Meintjes et al., 1994). This technology allows for repeated recovery of oocytes from the same animals without causing reduction in fertility attributed to the formation of adhesions or scars, as can be observed with laparascopic techniques (Reichenbach et al., 1993; Bungartz et al., 1995; Rick et al., 1996; Galli et al., 2001). Thus, OPU could be an important tool for the genetic improvement and for assisted reproduction of infertile females (Galli et al., 2001).

2.2.1.2. Recovering oocytes from abattoir ovaries

2.2.1.2.1. Collection of ovaries

Although abattoir ovaries are an inexpensive and easily available source of oocytes, it is unlikely that high producing cows will be slaughtered to obtain oocytes. A temperature above 30°C during collection and transportation of ovaries was considered essential for maintaining the oocytes developmental capabilities (First and Parrish, 1987). However, recent studies have demonstrated that ovaries can be held at 24 to 25°C for at least 11 hrs without compromising the development potential of the recovered oocytes (Yang et al., 1990). Phosphate buffered saline and normal saline (0.9% NaCl) are the commonly used transportation media (Khurana, 1992; Eckert and Niemann, 1995; Wrenzycki et al., 1996).

2.2.1.2.2. Isolation of oocytes

Aspiration with an 18-20 G needle attached to a disposable syringe has been widely used to recover oocytes from 1-8 mm follicles. This method is simple, fast and practical. Aspiration with the aid of a suction pump at a reduced pressure of 200 to 250 mm Hg has also been reported (Sirard and Lambert, 1986; Sirard et al., 1988; Berg and Brem, 1989). Recovery of oocytes by aspiration technique was confirmed in many recent investigations (Yoshida et al., 1998a; Bieser et al., 1998; Blondin et al., 2000; Enright et al., 2000; Guixue et al., 2001; Wrenzycki et al., 2001b). Dissection of intact follicles followed by rupture to release the oocytes, has also been used in several studies (Lonergan et al., 1991; Pavlok et al., 1992; Blondin and Sirard, 1995). In a comparative study, Lonergan et al. (1991) reported that while aspiration of the follicle in situ was faster, the follicle dissection method provided a higher number of good quality oocytes.
Slicing and mincing of the ovaries were used by some researchers (Takagi et al., 1991; Xu et al., 1992; Eckert and Niemann, 1995; Wrenzycki et al., 1996).

2.2.1.2.3. Selection of oocytes

Visual assessment of the compactness and the extent of cumulus investment has been the most widely used criterion to select bovine immature oocytes for their in vitro maturation and developmental potential (Wiemer et al., 1991; Hazeleger and Stubbings, 1992; Madison et al., 1992; Lonergan et al., 1994; Eckert and Niemann, 1995; Hyttel et al., 1997; Sirard and Blondin, 1998). These studies have univocally demonstrated that the extent and the quality of the cellular investments surrounding the oocyte is the best guide of its development ability. Other studies have suggested a relationship between the size of follicle and the oocyte recovered from it, a higher yield of good quality oocytes obtained from larger follicles (Tan and Lu, 1990; Lonergan et al., 1992; Pavlok et al., 1992). Along this line, recent data have demonstrated a clear relationship between oocyte diameter and developmental competence (Lonergan et al., 1994; Arlotto et al., 1996; Blondin and Sirard, 1995; Fair et al., 1995; Hyttel et al., 1997; Sirard and Blondin, 1998).

2.2.2. In vitro maturation (IVM) of oocytes

In vitro maturation of bovine follicular oocytes has become one of the core technologies for producing a large number of embryos for embryo transfer (Lim et al., 1999). Recent progress in optimizing this system has made it possible to develop approximately 30% of the oocytes to the morula and blastocyst stages (Hyttel et al., 1997).
Complex culture medium as TCM199 buffered with bicarbonate or HEPES and supplemented with various sera or bovine serum albumin (BSA), and/or gonadotropines (e.g., FSH and LH), and/or steroids (Estradiol-17β) and/or Suigonan^{R} (hCG and eCG) have been widely used for IVM of bovine oocytes (Khurana, 1992; Nagao et al., 1994; Eckert and Niemann, 1995; Keskintepe et al., 1995; Wrenzycki et al., 1996; Yoshioka et al., 1998a; Holm et al., 1999; Lonergan et al., 1999; Jacobsen et al., 2000; Raina et al., 2001). On the other hand, Ham's F-12 (Sanbuissho and Threlfall, 1989), Waymouth's medium MB 752/1 (Rose and Bavister, 1992) and MEM (Bavister et al., 1992) were also used for IVM. Of the sera used, fetal calf serum is the most common, but sera from steers or oestrous cows are also used (Thompson, 1997). More attention is necessary for determining the specific metabolic needs to choose optimal culture conditions required for maturing bovine oocytes (Brackett and Zuelke, 1993).

It is well established that the culture conditions employed for IVM of mammalian oocytes can significantly influence in vitro fertilization (IVF) rates and subsequent embryonic development (Brackett et al., 1989; Rose and Bavister, 1992).
Immature bovine oocytes recovered from ovarian follicles are capable of resuming meiosis in the absence of serum (Süss et al., 1988; Eckert and Niemann, 1995; Wrenzycki et al., 1999).
Interaction between the cumulus cell vestments and the oocyte appears important for subsequent development competence, which is commonly referred to as cytoplasmic maturation (Moor and Osborn, 1983; Staigmiller and Moor, 1984). Therefore, cumulus cells surrounding the oocytes are often not removed prior to IVF. Their presence appears to be beneficial for the acrosomal reaction of the sperm cells, and mechanical or enzymatic removal of the cumulus cells may damage the oocyte as well as increase the incidence of polyspermy (see Gordon, 1994). Durnford and Stubbing (1992) reported that ECS can be replaced by bovine oviductal epithelial cells for maturation of the bovine COCs in vitro without affecting their subsequent development to blastocysts.
As reported in many studies, bovine IVM culture media are commonly supplemented with gonadotophins to provide a beneficial effect on subsequent fertilization and embryonic development competence (Brackett and Zuelke, 1993).
The utility of defined IVM conditions has been extended to investigations of the effects of FSH and growth factors on bovine IVM (Harper and Brackett, 1992 a; b), and to biochemical characterization of hormonal effects on oocyte metabolism during IVM (Zuelke and Brackett, 1992). Lowering the high gonadotrophin concentrations required for in vitro efficacy, i.e. 50 µg LH/ml or 10 µg FSH/ml to near physiological levels (500 ng/ml) was achieved by combination of the respective gonadotrophins with physiological concentrations (10 ng/ml) of EGF (Harper and Brackett, 1992a) or IGF-1 (Harper and Brackett, 1992b) in defined IVM media.
Several studies have attempted maturation of the oocytes in chemically defined media (Ectors et al., 1992; Nagao et al., 1994; Eckert and Niemann, 1995; Keskintepe et al., 1995; Keskintepe and Brackett, 1996; Lonergan et al., 1999; Wrenzycki et al., 1999; Hashimoto et al., 2000). These studies found that the rate of cleavage and the yield of embryos from the oocytes matured with or without the presence of macromolecular fraction (PVA or ECS) were similar but the presence of serum improved in vitro hatching of the blastocyst. Gardner et al. (2001) developed a basal maturation medium (G-Mat) for bovine embryos around the requirements of the oocyte and embryo. Rather than using serum they have supplemented medium G-Mat with recombinant human albumin together with hyaluronan (produced by

fermentation). Bovine embryos derived from oocytes matured in G-Mat developed significantly better in culture than embryos from oocytes matured in TCM-199 with serum.
Recently, the addition of growth factors, in particular epidermal growth factor (EGF) and insulin-like growth factor-I (IGF-I), has also been shown to stimulate maturation and reduce the requirement for pharmacological doses of gonadotrophins during maturation (Harper and Brackett, 1993a; 1993b; Gandolfi et al., 1996; Rieger et al., 1998; Guler et al., 2000; Carneiro et al., 2001; Wei et al., 2001).
The pH of the commonly used media varies between 7.1 to 7.4 (Wright and O'fallon, 1987). However, it has been reported that the inclusion of Hepes in the culture medium reduced the proportion of sheep zygotes forming blastocysts (Walker et al., 1989). Most of the culture media contain bicarbonate to regulate the pH, but some media have been supplemented with 25 mM Hepes to achieve greater stability of the pH (Walker et al., 1989).
The osmolarity of the commonly used maturation media varies between 276-316 mOs (Wright and O'fallon, 1987). Early studies with mouse embryos indicated that the osmotic pressure of the culture medium was not a critically important epigenetic regulator (Bavister, 1995). In general, preimplantation embryos appear to be very adjustable to a wide range of osmotic pressures. It seems a good precaution to include amino acids in the medium to serve as osmolytes in order to help embryos cope with variations in osmotic pressure (Bavister, 1995).
A maturation period of 22-24 hrs is used by most of the laboratories (as reviewed by Khurana, 1992). Bovine oocytes can undergo nuclear maturation in vitro at temperatures ranging from 35 to 39°C (Katska and Smorag, 1985; Kim and Park, 1990; Khurana, 1992). Since most of the media are bicarbonate buffered, an atmosphere of CO_2 in air (20% O_2) has been a common practice (Khurana, 1992; Eckert and Niemann, 1995; Wrenzycki et al., 1996). However, some workers have used a low oxygen level (5%) for the maturation of oocytes (Coskun et al., 1991; Younis and Brackett, 1991; Gandhi et al., 2000; Hashimoto et al., 2000) without any noticeable benefits.
In general, the rate of maturation reported in many studies has been below 80% but in some studies, high rates of 90 to 98% have been achieved (Sirard et al., 1988; Kim and Park, 1990; Eckert and Niemann, 1995).

2.2.3. Sperm preparation, sperm capacitation and oocyte fertilization in vitro

Several techniques have been used to isolate high motility spermatozoa through the removal of abnormal and dead spermatozoa prior to in vitro fertilization. These include, swim-up separation (Parrish et al., 1986), passing through a glass wool column (Fukui et al., 1983) or percoll gradient solution (Saeki et al., 1991a). Stubbings and Wosik (1991) found no differences between glass wool and swim-up separation techniques with respect to the mean number of spermatozoa recovered per straw and the rates of fertilization and embryonic development of inseminated oocytes. However, most of workers prefer the swim-up method to separate the highly motile population of spermatozoa (see Khurana, 1992).

Sperm capacitation and the acrosome reaction are necessary physiological events that enable the sperm cell to penetrate the zona pellucida for the ovum to initiate the fertilization process (Chang, 1984). Capacitation involves biochemical changes in the outer sperm membranes that allow the morphologically observable acrosome reaction to occur. The acrosome reaction includes fusion and breakdown of the sperm plasma and outer acrosomal membranes progressing to exocytosis that enables enzyme release (Brackett and Zuelke, 1993). In vitro conditions that alter the sperm surface, elevate the internal pH of the sperm cell which results in a release of internal calcium (Ca_i) stores, or agents that promote rapid uptake of calcium to result in Ca_i increase, e.g. the calcium ionophore A23187, have effectively prepared ejaculated and/or frozen bull sperm for IVF (Brackett and Zuelke, 1993; Yang et al., 1993).

Various approaches used to stimulate sperm capacitation in vitro include, bovine follicular fluid (Fukui et al., 1983), oviductal fluid (Parrish et al., 1989), high ionic strength medium (Brackett et al., 1982; Bondioli and Wright, 1983), calcium ionophore (Kim and Park, 1990; Aoyagi et al., 1990; Kajihara et al., 1991), heparin (Parrish et al., 1986; 1988; Fukui et al., 1990), caffeine (Aoyagi et al., 1990; Funahashi et al., 1991; Takagi et al., 1991) and cAMP (Critser et al., 1984).

Simple, chemically defined media such as modified Tyrode's albumin lactate pyruvate medium supplemented with several capacitating and motility stimulating agents such as heparin, epinephrine, hypotaurine and penicillamine TALP (Parrish et al., 1986; 1988; Choi et al., 1991), modified BO medium (Goto et al., 1988; Funahashi et al., 1991; Suzuki et al., 1991) or SOF (Choi et al., 1991) supplemented with BSA as protein source have been used for in vitro fertilization of bovine oocytes. Choi et al. (1991) noted that the rates of fertilization, cleavage or development to the blastocyst were similar when using SOF or

Tyrodes medium for fertilization. Most bovine IVF procedures employ sperm concentrations of approximately 10^6/ml and insemination is most frequently carried out in 50 µl drops containing 5 to 40 oocytes per drop with several drops under mineral oil in each petri dish.
A 5% CO_2 atmosphere is necessary to maintain proper pH with bicarbonate buffering but a lowered O_2 tension may not be essential (Wang et al., 1992). Simulation of in vitro conditions including 39°C, sterile and compatible milieux for gametes and embryos, must be approximated for success in embryo production (Brackett and Zuelke, 1993).
While the majority of investigations have reported penetration rates between 60 to 80%, occasionally rates approaching 90% have also been recorded (Utsumi et al., 1988; Saeki et al., 1991b).

2.2.4. In vitro culture

In vitro culture systems must be designed to provide conditions closely resembling those found in vivo. Of the stages involved in in vitro production, embryo culture varies and is likely to have a pronounced effect on development, morphology, metabolism and gene expression, as embryos are held in media for up to nine days (Gardner et al., 1994; Thompson et al., 1995; Thompson, 1997; Eckert et al., 1998; Wrenzycki et al., 1999; Niemann and Wrenzycki, 2000). Previously, in embryos of most species, development in vitro was arrested at a stage which corresponds to the transition from maternal to zygotic control (see also 2.3.5.1) of development (First and Parrish, 1987). In cattle, development is arrested at 8- to 16-cell stage, commonly referred to as ‘critical’ or ‘block’ stage (Telford et al., 1990). This promoted many workers to propose that the oviduct secreted factors are conducive to early embryonic development and providing a specific environment essential for certain developmental events taking place before the 16-cell stage (First and Parrish, 1987). The oviduct in situ had been used as a temporary surrogate in vivo incubator to circumvent the problem of an arrested development in vitro (Utsumi et al., 1988; Sirard et al., 1988; Greve et al., 1989).
There are many bovine embryo culture systems which support development to post-compaction stages. These can de defined broadly as either co-culture, cell-conditioned or cell-free systems (Thompson and Duganzich, 1996). Usually the basic culture media (TCM199 and SOF) are supplemented with serum or bovine serum albumin (BSA), which are complex undefined and semi-defined mixture (Gardner and Lane, 1993). When the undefined

components (sera and BSA) are replaced by synthetic macromolecules, such as polyvinyl alcohol (PVA), resulting in chemically defined media (Pinyopumminter and Bavister, 1991; Eckert and Niemann, 1995; Keskintepe and Brackett, 1996; Wrenzycki et al., 1999; 2001a). The major difference between these systems and co-culture is that the concentration of each major component is known before the addition of embryos. This enables the evaluation of effects of new or changing concentrations of components on embryo development (Bavister, 1995; Leese et al., 1995; Thompson, 1996). Thus defined embryo culture systems allow for specific investigation of energy substrates, growth factors, and other factors capable of influencing embryonic development, thus providing the insight with which to tailor culture conditions to specific embryonic requirements (Brackett and Zuelke, 1993; Gandhi et al., 2000). Usually the rates of development are considerably lower in defined media than in BSA (semi-defined) or serum (undefined) supplemented in vitro culture systems (Eckert and Niemann, 1995; Keskintepe and Brackett, 1996; Wrenzycki et al., 1999; 2001a).

Co-culture of bovine oocytes is normally performed with either TCM199 or Ménézo's B2 medium, usually supplemented with serum, the most common used one is fetal calf serum (Thompson and Duganzich, 1996). The somatic cells of choice are usually bovine granulosa, bovine oviduct epithelial (BOEC) or buffalo rat liver cells (as reviewed by Brackett and Zuelke, 1993; Thompson, 1997). Co-culture techniques stem primary from work described by Rexroad and Powell (1986) and Gandolfi and Moor (1987). Conditioning of medium is a frequently used alternative that involves harvesting medium from somatic cell cultures, then using the cell-free 'conditioned' medium to support embryo development (Eyestone and First, 1989). Jiang et al. (1991) found no differences of zygotes progressing to morula, blastocysts or hatched blastocysts when co-cultured with granulosa, uterine or oviductal cell monolayers. Vergos et al. (1991) compared the effectiveness of in vivo culture in the ovine oviduct with that of in vitro culture either on a monolayer of BOEC or in media conditioned by BOEC to support development of the bovine zygotes to morulae/blastocysts and found that the yield of embryos was similar in all three treatments but the use of conditioned media or ovine oviductal cells increased the proportion of good quality embryos. A combination of co-culture with BOEC for 72h followed by co-culture of IVF embryos with uterine cells may yield better quality blastocysts than after co-culture of bovine IVM/IVF embryos with granulosa cells (Goto and Iritani, 1992). Advocates of both co-culture and conditioning argue that facilitative growth-promoting factors (most likely peptide growth factors and/or cytokines) are added to the medium, and there is evidence to support this (Gandolfi, 1994; Satoh et al., 1994).

A standard medium for in vitro culture of bovine zygotes is tissue culture medium (TCM199). Bavister et al. (1992) did not observe any block at the 8- to 16-cell stage when IVF derived zygotes were cultured in TCM199 containing no serum and/or somatic cells. It is indicated that the presence of serum had a biphasic effect, inhibiting the first cleavage and promoting the compaction of the morula (see also Pinyopummintr and Bavister, 1994; Bavister, 1995). It has been shown that the replacement of serum with PVP/PVA (defined medium) did not affect the frequencies of cleavage or blastocyst formation (Nagao et al., 1991; Yang and Seidel, 1992). On the contrary, some studies reported that omission of protein supplement from the culture medium reduced the yield of blastocyst (Chen-Lu and Lu, 1990; Eckert and Niemann, 1995; Wrenzycki et al., 1999). It has also been argued that coculture and conditioning simply reduce or remove inhibitory components, such as glucose and oxygen (Bavister, 1992; Watson et al., 1994).

Synthetic oviductal fluid (SOF) is one medium commonly used for bovine embryo culture in vitro. This medium was originally based upon the biochemical analysis of ovine oviduct fluid (Tervit et al., 1972). Synthetic oviductal fluid has subsequently been modified by the addition of amino acids (Gardner et al., 1994). Other modifications have been included, the removal of glucose (Takahashi and First, 1992), the addition of citrate (Keskintepe et al., 1995) and the addition of EDTA for the initial 72 hr of the culture period (Gardner et al., 1997). Fukui et al. (1991) obtained 31% blastocysts from IVF bovine zygotes using SOF supplemented with FCS but without somatic cells. Lonergan et al. (1999) demonstrated that the presence of FCS in the SOF medium stimulates blastocyst development and increases the quality of the blastocysts produced (based on hatching rate and the cell number). Holm et al. (1999) used SOF with amino acids, sodium citrate and myo-inositol (SOFaaci) for production of bovine embryos and suggested that they are of a quality similar to that of in vivo embryos. Gandhi et al. (2000) have shown that SOF medium can be used successfully throughout maturation, fertilization and preimplantation embryo development. Wrenzycki et al. (2001a) have investigated the developmental rates of bovine embryos generated in two different systems (either TCM or SOF), each supplemented with either serum, BSA or PVA. No significant differences were detected for the two basic culture media with regard to cleavage rates, developmental rates to the morula and blastocyst stage. Also, no significant difference was found for the cleavage rate in terms of protein supplementation. However, a significantly higher percentage of morulae and blastocyst stage was obtained in serum-enriched medium than in medium supplemented with BSA and PVA. Of the components added to medium to increase development, protein (usually serum albumin) and serum appear to have the most

influence on embryo development, morphology, metabolism and gene expression (see Thompson, 1997; Eckert et al., 1998; Niemann and Wrenzycki, 2000; Wrenzycki et al., 2001a). With regard to effects of in vitro conditions on gene expression, the SOF system provides an environment in which preimplantation development of bovine embryos is more similar to that occurring in vivo than in the TCM system (Wrenzycki et al., 2001a).
The important components of the incubation environment are: humidity, temperature and gas phase. While the embryo develops at a constant temperature in vivo, it appears to be able to tolerate some degree of fluctuating of temperature in vitro during collection and handling. It has been demonstrated that various aspects of in vitro production of bovine embryos are temperature dependent and that the best results are obtained at 39°C, the body temperature of the cow (Lenz et al., 1983). Nitrogen, the main component of the gas used for in vitro culture, is considered inert and its concentration can be adjusted to accommodate changes in the concentrations of the other two components. The amount of carbon dioxide in the culture atmosphere is determined by the CO_2 requirement of the cells in culture and by the buffer system employed. Wang et al. (1992) demonstrated that raising the concentration of CO_2 from 5% to 10% did not influence the proportion of the IVF derived 2- to 8-cell bovine embryos reaching the blastocyst stage or hatching in vitro. Khurana and Wales (1989) demonstrated that the reduction of the oxygen concentration during in vitro culture of mouse embryos stimulated catabolic utilization of glucose and suggested that the discrepancy in the glycogen levels of the blastocysts derived in vitro or obtained in vivo could result from differences in the oxygen levels of the two systems. Oxygen radicals have been proposed as a common factor between the apparent beneficial effects of glutamine and the detrimental effects of glucose on early stage embryo development (Rieger, 1992). Hashimoto et al (2000) demonstrated that low oxygen tension and high concentration of glucose during in vitro maturation support the developmental competence of bovine oocytes by means of decreasing intracellular reactive oxygen species (ROS) content. The results of several studies (Thompson et al., 1990; Fukui et al., 1991; Voelkel and Hu, 1992; Nagao et al., 1994; Lonergan et al., 1999; Alexopoulos et al., 2001) concluded that embryo culture under reduced O_2 (5%) result in significantly improved embryo development.

2.2.5. Large Offspring Syndrome (LCS)

In general, the in vitro culture of preimplantation stage embryos from the 1-cell to the blastocyst stage is a very sensitive phase of development. The development of embryos in vitro is slower than in vivo. This slowing of the developmental rate is manifested by reduced cell numbers, which is also consistent with a progressive loss of viability and reduced metabolism (McKiernan and Bavister, 1994). The abnormally large fetuses or offspring (Large Calf Syndrome) often occuring from embryos that have been cultured in vitro a few days indicate the long-term media effects carried into gestation (Walker et al., 1992; Thompsone et al., 1992). IVP embryos result in both calves and lambs that have increased birth weight (Farin and Farin, 1995; Hasler et al., 1995; van Wagtendonk-de et al., 1998), a longer gestation period (Kruip and den Daas, 1997; Sinclair et al., 1995), an increased incidence of abortions (Hasler et al., 1995), a higher perinatal mortality (Behboodi et al., 1995; Kruip and den Daas, 1997; van Wagtendonk-de et al., 1998), more congenital abnormalities (Schmidt et al., 1996), more hydro-allantois (Hasler et al., 1995; van Wagtendonk-de et al., 1998), relatively more bull calves and more problems during calving compared to calves born after artificial insemination (AI) or after multiple ovulation and embryo transfer. Upon cloning (nuclear transfer), these problems seem to be even more pronounced: calves often show breath problems and symptoms of acidosis, are lethargic, slow and do not suckle spontaneously immediatly after birth (Garry et al., 1996; Wilson et al., 1995).

It is not yet clear what factor(s) cause(s) the “Large Offspring Syndrome” in sheep and cattle. Factors at any stage of the sequential process (maturatuion, fertilization amd culture) may play a decisive role (van Wagtendonk-de et al., 2000). One of the factors most often mentioned in relation to the “Large Offspring Syndrome” is serum added to the culture medium. Serum contains numerous components (e.g. hormones) and as such provides a rich but undefined environment for embryo development (Thompson, 1996). In addition, the production of embryotrophic growth factors by co-culture cells, the high oxygen tension in which embryos are cultured (20% vs 6-7% in the uterus), the static culture system and the high ammonium concentrations (waste product of protein metabolism) in the culture medium, are often mentioned as the causes of the unusually large offspring (van Wagtendonk-de et al., 2000). Recently, from the perspective of the large offspring syndrome as well as from a quality control point of view, (semi-) defined culture media were developed (CR1aa

(Rosenkrans et al., 1991); KSOM (Erbach et al., 1994); SOF (Tervit et al., 1972); G1/2 medium (Gardner and Lane, 1998). So far, most studies about birth weights of calves and lambs used synthetic oviduct fluid (SOF), to which BSA and/or amino acids are added as an alternative protein source (SOFaaBSA) under low (5%) oxygen tension. It can be concluded that using a semi-defined medium for in vitro culture (SOF) may improve characteristics of IVP calves born (for review see van Wagtendonk-de et al., 2000) and with regard to the relative abundance of mRNA transcribed by embryos produced in different medium systems, the SOF system provides an environment in which preimplantation development of bovine embryos is more similar to that occuring in vivo than the TCM system (Wrenzycki et al., 2001a).

Several imprinted genes (i.g. genes which are expressed only from the maternal or paternal allele) are known to have significant effects on fetal size and survival in other species and are possible candidates for involvement in livestock LOS (see Young et al., 1997; 1998). Imprinted genes are defined as those genes for which expression is determined by their parental origin. Imprinted genes have been identified in mice, rats, sheep, horses and humans with differences among species, but not yet in cattle (Morison et al., 2001). Aberrant expression patterns of imprinted genes have been found in mice and humans (Moore and Reik, 1996) and were related to specific phenotypes, e.g. Beckwith-Wiedemann Syndrome (BWS; a congenital overgrowth disorder) in humans. Imprinting is usually associated with allele-specific methylation of DNA sequences, which interacts with modifications in chromatin structure and acetylation of the chromatin-associated histone proteins. Almost all imprinted genes have differentially methylated regions (DMRs; Reik and Walter, 2001). DNA methylation is normally associated with gene silencing (Bird and Wolffe, 1999). However, seven of 18 imprinted genes have DMRs that are methylated on the active allele (Reik and Walter, 2001). Presumably, methylation of imprinted genes can be either associated with an induction or a silencing of gene expression, depending on the gene (Barlow, 1997). Changes in the pattern of DNA methylation with a de novo methylation and induction of specific genes are commonly observed in human cancer (Jones, 1996; Baylin et al., 1998). Major changes in putative imprinting mechanisms such as DNA methylation of imprinted genes occur in the mouse embryo during preimplantation development. Alterations in DNA methylation are stably transmitted through repeated cell cycles such that changes in the embryo may still act at the fetal stages. Thus any distruption in establishment and/or maintenance of imprinting during the vulnerable periods of embryo culture or manipulation is a plausible candidate mechanism for inducing fetal loss and Large Offspring Syndrome. Identification of these

disruptions may provide crucial means to improve the success of current procedures (for review, see Young et al., 1998; Young and Fairburn, 2000). Thompson (1997) suggested that LOS may be caused by differences in gene expression induced by these manipulations. Evidence suggesting that the expression of several growth factor genes differs between nuclear transfer-derived embryos and those produced either in vivo or in vitro has been presented (Westhusin et al., 1995; Wrenzycki et al., 2001b; 2002). Loss of imprinted genes like IGF-II with a subsequent increased IGF-II expression has been suggested to be a possible candidate for the Large Offspring Syndrome (Young et al., 1997). Young et al. (2000) found the IGF-II gene to be monoallelic expressed in both normal and oversized sheep fetuses. It has not been documented that increased fetal levels of IGF-II are responsible for increased fetal growth seen in oversized sheep and bovine fetuses derived from in vitro produced embryos (Jacobsen et al., 2000).

2.3. Gene expression

2.3.1. Gene expression in the eukaryotic cells

Gene expression is the process by which the genetic code contained in the nucleus is read and used by the cell. The expression of genes begins with the production of a single stranded RNA copy of one strand of the gene's double helix. This copying process is called transcription and is carried out by a RNA polymerase. RNA has many uses in the cell. The five major classes of RNA are ribosomal RNA (rRNA 71%), small stable RNAs (15%) including transfer RNAs (tRNA), nuclear hnRNA (7%), nuclear rRNA precursors (4%) and cytoplasmic mRNA (3%)(Alberts et al., 1994c; Lewin et al., 2000b).

There are three different RNA polymerases in eukaryotic cells, polymerase I, II, and III. RNA polymerase I is used to produce the 45S ribosomal RNA precursor. RNA polymerase II makes the mRNAs which will be used for protein production in the cytoplasm. RNA polymerase III produces the small stable RNAs including the tRNAs and the 5S ribosomal RNA. Transcription is initiated when a RNA polymerase binds to the DNA in the region upstream (5') from the region which actually encodes the functional RNA product. This region is called the promoter sequence because it is the site where various control factors are

able to bind to prevent or promote access by the RNA polymerase. It often is a sequence known as the TATA box which is rich in the nucleotides T and A (Alberts et al., 1994c; d; Lewin et al., 2000b).

Regulation of transcription is complex and depends on the presence of many factors. The factor which binds the TATA box is known as the TATA binding protein (TBP). It is just one subunit of the transcription factor, known as TFIID, which is the first to bind the promoter. Once TFIID is bound, other transcription factors TFIIB, TFIIE, TFIIF, TFIIH act together to bring RNA polymerase II into the position where it can initiate transcription. The complexity of this multicomponent system affords rich possibilities for controlling the expression of each gene in a time and tissue specific manner (Alberts et al., 1994d).
After initiation of transcription, the RNA polymerase moves downstream along one strand of the DNA building a complementary sequence to that strand from RNA nucleotide monomers until reaching a stop signal. The first modification to the single stranded RNA is the creation of a protective cap on its 5' end, which occurs almost immediately. Eventually the polymerase II passes over a signal region containing the cleavage signal AAUAAA. While polymerase II continues to transcribe RNA, another enzyme cuts the completed RNA copy at a point 10 to 30 nucleotides downstream from the cleavage signal. Poly(A) polymerase adds a "tail" of 100 – 200 adenosine (A) nucleotides which serve to protect the 3' end of the mRNA from degradation with the help of poly (A)-binding protein (PABP) which binds to the poly(A) tail. The poly (A) tail is useful for laboratory purification of mRNA from the other types of RNA. Complementary strands of poly (T) can be produced synthetically and used to coat solid surfaces such as magnetic beads or synthetic fibres so that they are able to hybridise to the poly(A) tail of messenger RNA and immobilize it. This technique is described in detail in the following chapters (Alberts et al., 1994c; Lewin et al., 2000a).

When mRNA is initially produced, it includes segments called exons which are eventually translated into protein sequence and other segments (introns) which may have regulatory functions but do not contain sequences information for construction of the protein. These intron sequences are only found on recently transcribed mRNA in the nucleus and must be edited out of the primary transcript by cutting at both ends of each intron and splicing the remaining exon ends together. After this modification, the mRNA can be exported to the cytoplasm where it can be used by the ribosomes to produce proteins in the process called

translation. Once the introns have been removed, the finished mRNA molecule is exported to the cytoplasm where it can be translated into protein by a ribosome (Alberts et al., 1994c).

Translation is the process by which the mRNA sequence is read and translated into an amino acid polymer (protein) by structures called ribosomes which are made up of both RNA and protein subunits. A group of three RNA nucleotides in the mRNA called a codon is the signal to add a specific amino acid. Translation normally begins at the first AUG sequence after the 5' cap. The first amino acid added is methionine (because AUG codes for methionine) and forms the amino terminal (N terminal) end of the new protein. For each codon, a new amino acid must be transported to the ribosome by a special form of RNA known as transfer RNA (tRNA). As described above, the complete set of tRNA molecules is produced in the nucleus by RNA polymerase III. The tRNA serves to transfer a specific amino acid into a place in the ribosome where it is added to the growing protein polymer. The mRNA sequence eventually contains signals to stop with one of three sequences UAA, UAG or UGA. This prevents further addition of amino acids and produces the C terminal end of the protein (Alberts et al., 1994a; b).

The spectrum of mRNA within the nucleus and the cytoplasm is unique for each cell type and for each point in development. It is estimated that a typical cell contains as many as 10,000 different RNA transcripts at any point in time. Regulation of the amount of each RNA is the fundamental process of differentiation. This can take place at various points beginning with modification of the structure of the DNA helix to permit interaction of transcription factors at the promoter which initiate the binding of RNA polymerase II. The next opportunity for control is during the process of editing out the introns and splicing the exons together. Transportation of the edited mRNA from the nucleus to the ribosomes is the third control point. The rate of degradation of mRNA within the nucleus and also in the cytoplasm is the fourth important means of regulation. Control of degradation begins with protection by the poly (A) tail. When the tail is longer than 15 residues, the protein factor [poly(A) binding protein: PABP] can bind the tail. PABP then binds to the cap at the 5' end of the RNA and protects that end as well. One mechanism for degrading such RNA is inclusion of several repeats of the targeting sequence AUUUA which enhances loss of residues in the poly (A) tail which in turn causes loss of PABP protection. The cytoplasm contains several enzymes which can rapidly degrade unprotected RNA from both the 5' and the 3' ends. A final point of control in gene expression is at the point of translation initiation. For example, the oocyte

contains many well protected “maternal” mRNA molecules which are inactive but ready to be used quickly during development when released and transported to the ribosomes (Alberts et al., 1994d; Lewin et al., 2000a; c).

2.3.2. Detection of gene expression

The detection of gene expression can be accomplished in several ways. “Classical” methods relied on the observation of biochemical or phenotypic changes in cells or organisms in order to determine the expression of a specific gene. Later, advances in macromolecular separation technology made it feasible to identify and isolate a particular gene product or protein molecule. With the advent of recombinant DNA techniques, it is now possible to detect and analyze the transcriptional product of any gene (Erlich, 1989).

2.3.2.1. General methods for detection of mRNA

The regulation of gene expression in higher eukaryotes continues to be a primary area of investigation in molecular biology. Detection of gene expression is essential to understanding basic cellular and molecular mechanisms in the control of embryonic gene expression (Wrenzycki and Niemann, 2002). Key to the study of regulation of gene expression is the ability to isolate, analyze and quantify RNA molecules, specifically messenger RNA molecules which code for proteins of interest (Flick and Anson, 1995). As shown by Kawasaki (1990) sensitive methods for the detection and analysis of RNA molecules are an important aspect of most cell/molecular biology studies. Methods commonly in use include in situ hybridization, Northern gels, dot or slot blots, S- 1 nuclease assays, and RNase A protection studies. Detailed descriptions of these techniques can be found in several laboratory manuals (Davis et al., 1986; Ausubel et al., 1987; Berger and Kimmel, 1987). The most sensitive of these methods is in situ hybridization, in which 10 to 100 molecules can be detected in a single cell. However, the in situ hybridization method can be technically difficult and does not lend itself to the processing of a large number of samples. With other techniques, the level of detection is about 0.1 to 1.0 pg of the target sequence. For an average-sized mRNA this translates to 10^5 to 10^6 target sequence molecules, and for most practical purposes

the detection limit has been reached. A number of methods have been developed to quantitate, measure the size of, and map the 5' and 3' termini of specific mRNA molecules in preparations of cellular RNA. These include:

- Northern hybridization (RNA blotting), in which the size and amount of specific mRNA molecules in preparations of total or poly(A)$^+$ RNA are determined (Alwine et al., 1977) The RNA is separated according to size by electrophoresis through a denaturing agarose gel and is then transferred to activated cellulose (Alwine et al., 1977), nitrocellulose (Goldberg, 1980; Thomas, 1980), or glass or nylon membranes (Bresser and Gillespie, 1983). The RNA of interest is then located by hybridization with radiolabeled DNA or RNA followed by autoradiography.

Northern blotting is one of the more commonly used techniques for RNA analysis and quantification, referring to the separation of a total RNA preparation by denaturing gel electrophoresis, transfer of the RNA onto a membrane by capillary blotting, and detection of sequences of interest via hybridization to a specific labeled probe. DNA probes labeled with ^{32}P via random primed labeling are typically used such that the final signal is detected by autoradiography. However, in recent years non-radioactive methods for Northern blotting have been developed in which probes labeled with biotin or fluorescein are hybridized to the blot which is subsequently detected on X-ray film via chemiluminescence with the appropriate substrate. Both methods are capable of yielding similar detection limits when proper procedures are followed. Northern blotting has the advantage of permitting the visualization of intact RNAs, providing information on size and integrity.

- Dot and slot hybridization, in which an excess of radiolabeled probe is hybridized to RNA that has been immobilized on a solid support (Kafatos et al., 1979; Thomas, 1980; White and Bancroft, 1982). Densitometric tracings of the resulting autoradiographs can allow comparative estimates of the amount of the target sequence in various preparations of RNA.

- Mapping RNA using nuclease S1 or ribonuclease, in which the precise positions of the 5' and 3' termini of the mRNA and the locations of splice junctions can be rigorously determined (Berk and Sharp, 1977; Weaver and Weissmann, 1979). Labeled or unlabeled RNA or DNA probes derived from various segments of the genomic DNA are hybridized to mRNA, often under conditions favoring the formation of DNA:RNA hybrids (Casey and Davidson, 1977). The products of the hybridization are then digested with nuclease S1 or RNAase under conditions favoring digestion of single stranded nucleic acids only.

Analysis of the digestion products by gel electrophoresis yields important quantitative and qualitative information about the mRNA structure.

- Primer extension, in which a small radiolabeled fragment of DNA is hybridized to the mRNA and used as a primer for reverse transcriptase. The resulting product should extend to the extreme 5' terminus of the mRNA, and thus the size of the product reflects the number of nucleotides from the position of the label to the 5' terminus of the mRNA.
- Solution hybridization, in which the absolute concentration of the sequence of interest is calculated from the rate of hybridization of a small amount of a specific radioactive probe with a known quantity of purified cellular RNA (see, Roop et al., 1978; Durnam and Palmiter, 1983). Alternatively, an excess of a radiolabeled probe is incubated with a known amount of RNA. The concentration of the RNA of interest can then be estimated from the amount of radioactivity that becomes resistant to nuclease S1 (Favaloro et al., 1980; Beach and Palmiter, 1981; Williams et al., 1986).
- Filter hybridization, in which purified cellular RNA is end-labeled with ^{32}P and hybridized to a large excess of the homologous DNA that has been immobilized on a solid support (Williams et al., 1986).

2.3.3. Reverse Transcription-polymerase chain reaction (RT-PCR)

Analysis of gene expression requires accurate determination of mRNA levels. First, mRNA converted into DNA using the well-known process of reverse transcription, which is used by RNA viruses to convert their genomic RNA into a DNA within the host cell and second, perform PCR amplification on the resulting complementary DNA (cDNA).

Standard RT-PCR offers a rapid, versatile and extremely sensitive way of analyzing whether a target gene is being expressed and can provide a semi-quantitative information about expression levels. Theoretically RT-PCR should be able to amplify one single mRNA molecule, although in practice this is not likely to be a realistic goal. However, RT-PCR is an extremely valuable tool when limited material, such as specific differentiated cells, is available. In this context RT-PCR can be used either to detect specific transcripts by using sequence-specific primers, or can be used to create cDNA libraries by using generic primers such as oligo-dT and either random oligonucleotides (McPherson and Møller, 2000).

The polymerase chain reaction (PCR) is an in vitro method which can amplify small amounts of a specific DNA fragment to levels that are readily detectable. The method involves the use

of two synthetic oligodeoxynucleotide primers, 20 to 30 nucleotides in length, that hybridize to opposite strands of the target sequence, followed by amplification through repeated cycles of heat denaturation of DNA, annealing of the primers to complementary sequences, and extension by a thermostable DNA polymerase (Saiki et al., 1988). Because the extension product of each primer can serve as a template for the other primer, each cycle effectively doubles the amount of the target DNA sequence produced in the previous cycle, resulting in its exponential accumulation. By combining the reverse transcription (RT) of RNA with the PCR amplification process, it is possible to detect RNA sequences present at low copy number in very small amounts of material. Because several mRNA species can be assayed within the same RNA preparation through the use of appropriate primer pairs, RT-PCR has been termed “mRNA phenotyping” (Rappolee et al., 1988b). This is particularly applicable to the analysis of gene expression in oocytes and preimplantation embryos, where it is not feasible to obtain large numbers for study (Arcellana-Panlilio and Schultz, 1993).

2.3.3.1. Reverse Transcription (RT)

First-strand cDNA synthesis can be achieved by reverse transcription of RNA primed with oligo(dT), random hexanucleotides, or a specific downstream sequence (Kawasaki, 1990). The use of primers other than the customary oligo(dT) appears to be helpful in improving efficiency of reverse transcription where unresolved secondary structure in the RNA is a problem (Rappolee, 1990). However, in many instances, the choice of RT primer is not critical, and equal success can be achieved with the use of either primer, some workers have found that the random hexamer approach is the most consistent and results in the highest amplification of target sequence (Kawasaki, 1990). Reverse transcriptases available commerc-ially are of two types: cloned mouse Moloney leukemia virus (MMLV) reverse transcriptase and avian myeloblastosis virus (AMV) reverse transcriptase.

RT-PCR is based on the ability of the enzyme reverse transcriptase, an RNA-dependent DNA polymerase, to generate a complementary strand of DNA (first-strand cDNA) using the mRNA as a template. The reverse transcriptase reaction can be performed on either total cytoplasmic RNA or on purified mRNA. It is important that no genomic DNA is present, as this will also provide a template for the PCR amplification step. An appropriate control for any contaminating DNA is a control reaction in which the reverse transcriptase step is omitted. Many commercial kits generate high-quality DNA-free total or mRNA preparations

or an RNAse-free DNAse I digestion step can be included in the RNA extraction protocol (McPherson and Møller, 2000).

2.3.3.2. Polymerase chain reaction (PCR)

As mentioned in brief above, the PCR is an in vitro method for producing large amounts of a specific DNA fragment of defined length and sequence from small amounts of a complex template (Saiki et al., 1985; Mullis and Foloobna, 1987; White et al., 1989) (Fig. 2). In 1971, an idea was published for using two oligonucleotide primers with their 3' ends pointing towards each other in a repair replication reaction to synthesize two new strands of duplex DNA from a double-stranded template (Kleppe et al., 1971). However, the crucial idea and experimental conditions for using the products of each round of synthesis as the templates for each further cycle (instead of removing the products and reusing the original template in a linear rather than exponential synthesis) were not manifest until the publication of PCR in 1985.

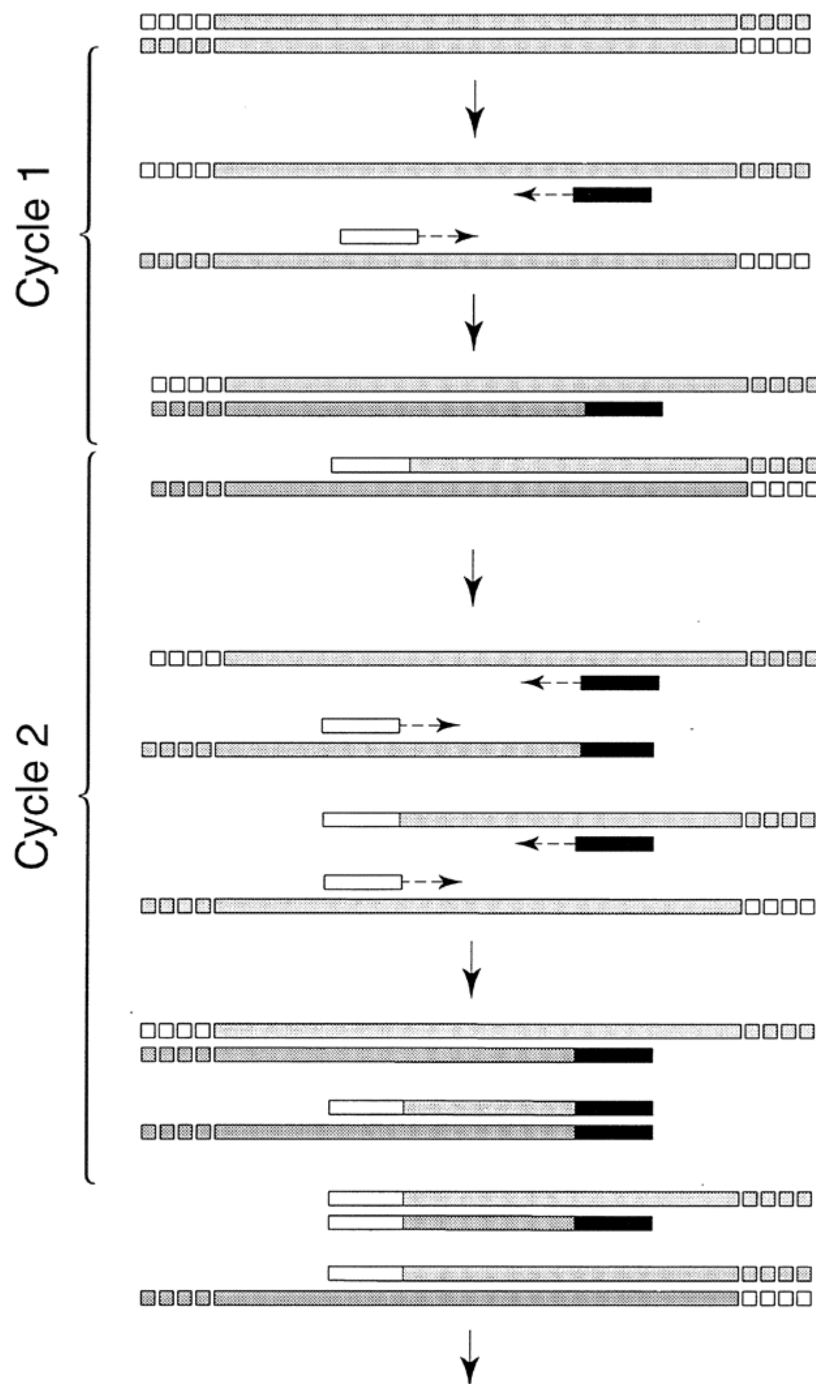


Fig. 2 PCR theoretically doubles the amount of target DNA at each cycle. The lengths of products generated from template DNA during the first two cycles are not defined. In cycle 2 the first single-strand products of defined length are produced because of priming on products generated during cycle 1 (adapted from McPherson and Møller, 2000).

Since then, the PCR method has had a major impact on molecular biology and many other fields, and several accounts of the concept and its development from an idea into a routine technique have appeared (Mullis, 1990; Rabinow, 1996). This evolution involved a series of innovations in PCR technology and instrumentation (White, 1996). These include:

- Thermostable DNA polymerases
- Thermocyclers
- Thermostable reverse transcriptases
- Enzymatic control of contamination
- Hot start for increased specificity
- Quantitative PCR
- Internal controls to detect inhibition
- RNA-selective PCR
- Improved enzymes for DNA sequencing
- Homogenous detection systems

PCR is a technique to amplify specific stretches of DNA and consists of several cycles of three steps (Fig. 2): template denaturation, primer annealing and primer extension (appropriate upstream and dounstream primers are used and can either be specific to the target gene). First, the complementary DNA strands are separated by heat denaturation (>90°C). Upon cooling two designed primers (short synthesized oligonucleotides) will specifically anneal to the target DNA, one on each strand. The temperature is then raised to 72°C, the optimal temperature for heat resistant polymerases. Using free single nucleotides, the polymerases start to synthesize complementary DNA from the primer-template binding sites (primer extension). The primers are oriented so that extension of one creates a new binding site for the other. Thus, the amplification is exponential: in each cycle the amount of template (PCR product) is doubled. In theory, 30 cycles result in a billionfold amplification. Traditionally the PCR products are separated and identified by electrophoresis using agarose gels stained with a DNA-specific dye, ethidium bromide (as reviewed by Bredbacka, 2001).

The optimal temperature of annealing is primer specific, usually about 60°C for a 20-mer oligonucleotide. At lower temperatures primers will anneal to targets that do not perfectly match, resulting in nonspecific amplification products and decline in specific amplification. It is also noteworthy that in PCR the DNA template molecule must be intact (without breaks) to be a target for exponential amplification (see Bredbacka, 2001). In some cases, particularly

when transcript levels are low, some optimization of PCR conditions will probably be necessary to obtain a convincing result (McPherson and Møller, 2000).
The potential of PCR in preimplantation diagnostics in cow and mouse embryos was demonstrated by King and Wall (1988), who successfully amplified single copy genes and transgenes. Soon thereafter commercial PCR-sexing of bovine embryos was implemented (Herr and Reed, 1991; Thibier and Nibart, 1992) as well as other bovine embryo assays of potential commercial interest (Schwerin et al., 1994).
With the use of nested PCR many optimization problems can be solved. Nested PCR is a two-step amplification consisting of a first round PCR with "outer" primers flanking the primer target sequences to be used in the second round. Thus the resulting products from the first round serve as template for the second round where the inside primers are used. Nested PCR is originally designed to increase specificity and sensitivity: any nonspecific products emerging from the first amplification step are unlikely to be suitable targets for the second round of PCR. In other words, specificity is defined by four primers instead of two. For a multiplex-PCR approach this would mean that the first step could be performed with the same amplifying parameters using nested primer pairs for each locus. The products from the first step are then aliquoted into individual PCR amplifications, which can then be carried out using optimized parameters for each loci. The increased overall specificity of this approach allows moderate non-specific amplification in the first step. To allow target annealing of all primers the first step can thus be performed at a low temperature (reviewed by Bredbacka, 2001).

2.3.3.2.1. Primer design for the PCR

Although the PCR is undoubtedly a very powerful method for the analysis of specific nucleotide sequences, each application must be optimized for the particular target sequence and its primer set.
There are several computer programs that can be used to assist primer design, for example the OLIGO™-program. However, in practice it is important to design primers by following some simple rules. Some of the more critical guidelines are listed below:
1. Oligonucleotide primers should be 20 to 30 nucleotides in length with a melting temperature (T_m) near 72°C (55°C to 75°C), so that the PCR annealing step has optimal stringency. The G/C and A/T contents of the primers should be balanced, and the primers

themselves should lack secondary structure. An empirical formula to define the temperature at which 50% of short duplexes (14-20 bp) dissociate (T_m) has been determined:

$T_m = 4(G + C) + 2(A + T)$

where G, C, A, and T are the number of corresponding nucleotides, and denaturation is at 0.9 M NaCl.

Wu et al. (1991) reported a formula for primers 20-35 nt long to calculate T_p, the optimization annealing temperature ± 2-5°C:

$T_p = 22 + 1.46$ [(2 × number of G+C) + (number A+T)].

There are useful software packages which calculate T_m based on nearest-neighbor thermodynamic properties.

2. The primers should not have complementarity, and 3' sequence overlaps should be avoided to prevent primer-dimer formation.

3. Generally, primers should bracket a sequence of 200 to 600 nucleotides in length, though longer targets have been amplified efficiently.

4. When possible, primers should be designed to span an intron so that amplification from contaminating genomic DNA can be distinguished from the cDNA of interest.

5. Primers should span a sequence with a diagnostic restriction site or a sequence that spans a cDNA clone possessed by the laboratory, so as to provide tools for the verification of the identity of the PCR product by restriction analysis or Southern blot (see Arcellana-Panlilio and Schultz, 1993).

2.3.3.2.2. Factors affecting specificity of the PCR (Optimization of PCR)

For dNTP, a low concentration was recommended by Innis et al. (1988) whereas, high dNTP concentrations by Rappolee (1990) to improving the fidelity and specificity of amplification. In any case, it is important to keep the dNTPs concentrations balanced. Taq polymerase tends to misincorporate or even to terminate prematurely when any one nucleotide concentration is significantly different from the others (Innis et al., 1988).

The free magnesium ion (Mg^{2+}) concentration is critical, since it is required for Taq DNA polymerase activity. For most applications, a range of concentrations from 1 to 4 mM $MgC1_2$ (Arcellana-Panlilio and Schultz, 1993) and 0.5 to 2.5 mM (Innis and Gelfand, 1990) were found to be useful. Concentrations of 0.24 to 2.4 µM of each primer per 50 µl reaction mix

were recommended. Too high concentration of primers in the reaction may promote mispriming and subsequent accumulation of nonspecific products (Saiki, 1989).

It is important that the denaturation step in each cycle accomplishes its purpose. Incomplete denaturation of the target template and/or the PCR product is the most likely cause for failure of a PCR. On the other hand, excessively long denaturation times or too high temperatures lead to unnecessary loss of enzyme activity. The half-life of Taq DNA polymerase activity is greater than 2 hr at 92.5°, 40 min at 95° and 5 min at 97.5°, respectively (Innis and Gelfand, 1990).

The annealing temperature (see the formula above) is dependent on the T_m of the primers, which, in turn, is a function of length and of base composition. Higher annealing temperatures favor correct priming and therefore increase specificity. The optimal annealing temperature can be determined by testing a range of annealing temperatures from 55° to 72°, up to about 5° below the T_m of the primers or trying a touch down program. The 72°C extension temperature is optimum for Taq polymerase. The extension time is primarily dependent on the length of the target sequence, although the rate of nucleotide incorporation can be affected by buffer, pH, salt concentration and the nature of the template (Innis et al., 1990). For the final extension, a temperature 72°C also was recommended.

The cycle number is dependent on the initial concentration of the template DNA. For the detection of messages of low copy number, intuitively one would try to execute as many cycles as possible. Arcellana-Panlilio and Schultz (1993) observed higher background owing to nonspecific amplification at too many cycles (>40 cycles). In this case, it is often better to increase the initial template concentration. For example, for routine analysis, cDNA equivalents of 30 embryos are enough for one reaction. For rarer mRNAs amount (< 200 copies per cell), needs more embryos for one reaction. Optimizing cycle number becomes most important in quantitation to determine the range of cycle number in which linear fragment production occurd (Arcellana-Panlilio and Schultz, 1993: Temeles et al., 1994; Wrenzycki et al., 1999).

Holding the product at 4°C after the last cycle essentially inactivates the enzyme and is a good temperature at which to store the amplified product for the short term until analysis. (Arcellana-Panlilio and Schultz, 1993).

Following amplification, 5 to 10 µl of each reaction is taken for analyze by gel electrophoresis with appropriate molecular weight markers. Amount of agarose in gel [% (w/v)] ranged from 0.3 to 2.0 for separation dependent on the products (see Sambrook et al., 1989). Ethidium bromide staining was recommended for visualizing the PCR products and a restriction

enzyme was used to verify identity the PCR products (Arcellana-Panlilio and Schultz, 1993). Recently, the RT-PCR products can be verify by sequenced and identified the product from each primer pair (Wrenzycki et al., 1999).

2.3.3.2.3. The problem of contamination in PCR and RT-PCR

To perform a PCR and to optimize the reactions to reach the highest specificity and product yield, it is important to understand the potential problems of contamination. Great care should be taken to avoid contaminating the PCRs since this can be wrong in terms of wasted time and reagents and potentially, in laboratories where clinical diagnosis or forensic analyses are performed, it could affect people's lives. Some major sources of DNA contamination are bench surfaces, laboratory equipment, pipettors, air-borne particles and debris such as skin or hair, or contaminated solutions. The greatest contamination dinger comes from previously PCR-amplified molecules, cloned DNA molecules carrying the target gene and original template DNAs (McPherson and Møller, 2000).

In RT-PCR, both nucleic acid and ribonuclease contamination must be controlled. As a general rule, gloves should always be worn and changed frequently, and semi-sterile technique should be adopted. Milli-Q TOC (Total Organic Carbon) water used in RNA extraction solutions, in reverse transcriptase reactions and PCR amplification. Many of these precautions. are common practice to avoid ribonuclease contamination when handling RNA. Carry-over of PCR products from previous amplifications must also avoided. It is a good habit to handle pre- and post-PCR solutions with separate, dedicated pipettors. Whenever possible, perform pre- and post-PCR procedures in separate laboratory rooms.

Another potential problem during RT-PCR is genomic DNA contamination in the RNA preparation. This is particularly relevant when the target mRNA is expressed at low levels, thus requiring large numbers of amplification cycles. While addition of DNAse to completely eliminate genomic DNA may be impractical, there are means to differentiate between amplified cDNA and genomic DNA products. The easiest way is to design the primers such that they span one or more introns within the gene. Thus, PCR products generated from contaminating genomic DNA will be larger than products from cDNA.

Even under carefully optimized conditions, PCR can generate non-specific products. This is also true for RT-PCR. Different products may result from amplification of alternatively spliced transcripts or from non-specific primer annealing. Several chemicals, when included

in the PCR reaction, have been found to increase specificity by reducing non-specific primer annealing. These include dimethylsulfoxide (DMSO) (Bookstein et al., 1990), tetramethyl-ammonium chloride (TMAC) (Hung et al., 1990), and formamide (Sarkar et al., 1990).

2.3.4. Semi-quantitative and quantitative RT-PCR

While use of RT-PCR can detect the presence or absence of mRNA species it does not provide a quantitative measurement of levels of gene expression principally due to the 'plateau effect'. However, by modifying the standard method, RT-PCR can be used to quantify the levels of mRNA in a sample or provide insight into the relative expression levels between different cell types or in response to external stimuli (McPherson and Møller, 2000).
It is relatively easy to amplify rare mRNA transcripts to detectable limits by RT-PCR. However, because PCR amplification is an exponential process, particularly in early cycles, small differences in any of the parameters that control the efficiency of the reaction can substantially affect the final yield of PCR product, making it difficult to quantitate the amount of mRNA in the original material (Gilliland et al., 1990). If relative differences in transcript levels are to be compared between different cell types, a semi-quantitative approach may be sufficient. The simplest way of performing such analysis is to determine the amounts of the PCR product during the exponential phase of the PCR but before the plateau phase. This method can be useful for analyzing changes in the level of a target transcript in identical tissue or cells in response to external stimuli (McPherson and Møller, 2000). Because the total efficiency of amplification for each set of primers during each cycle is not known, semi-quantitative RT-PCR used to compare relative abundance of one mRNA among different samples (Temeles et al., 1994; Wrenzycki et al., 1999). Of course valid comparisons are only possible when the same primer combinations and reaction conditions are used for all samples. The PCR experiments should be performed in parallel at least four to five times to ensure that the results obtained are consistent and reproducible. Since every PCR displays different reaction dynamics it is difficult to compare semi-quantitative data from separate experiments and comparisons of mRNA transcript levels from amplified genes using different primer pairs cannot be made. More robust and reliable methods for mRNA quantitation rely on the use of internal standards and quantitative competitive RT-PCR (Temeles et al., 1994; Wrenzycki et al., 1999; McPherson and Møller, 2000).

Two basic strategies for qPCR have been employed: (1) the use of an internal standard of known copy number, which employs the same primers as those of the cDNA of interest but can be distinguished from the target cDNA after coamplification; and (2) the comparison of sample PCR yields against a calibration curve of external standards (Arcellana-Panlilio and Schultz, 1993).

2.3.4.1. Internal Standards method

Regardless of the quantitative PCR (qPCR) strategy, the standards largely determine the validity as well as the accuracy of the method. Thus, great care must be taken in the choice/design of the standard, its production, its purification, and its own quantitation. Although cDNA standards can be utilized directly to coamplify with reversed-transcribed sample RNA (Gilliland et al., 1990). Arcellana-Panlilio and Schultz (1993) recommended the use of RNA standards, which can be added to the sample RNA, reverse transcribed together with the sample RNA, and then coamplified. A known amount of rabbit globin-mRNA/embryo was added prior to RNA isolation, this mRNA which was not present in the embryo at this time, served as an internal standard to correct for differences between samples in RNA recoveryed and efficiency of the RT-PCR and both the globin and mRNA under study were coamplified (Temeles et al., 1994; Wrenzycki et al., 1999; 2001a).

2.3.4.2. External Standards method

The PCR amplified product itself cloned into a transcription vector makes a useful external standard which can be produced, purified and quantitated in the same manner as the internal standards (Arcellana-Panlilio and Schultz, 1993).

2.3.5. Gene expression in the preimplantation embryos

2.3.5.1. Activation of the embryonic genome

In most animal species, early development is controlled by gene transcripts and polypeptides produced by and stored in the oocyte during its development (Schultz, 1993). The sequential activation of the embryonic genome is crucial for normal embryonic development. However, following one to three cleavage divisions, control of development is taken over by expression of portions of the embryonic genome and the maternally derived transcripts and proteins are gradually degraded (Telford et al., 1990; Watson et al., 1996). This transition from maternal to embryonic control of development is referred to as the maternal-embryonic transition (MET) or maternal to zygotic transition (MZT) (First and Barnes, 1989; Barnes and Eystone, 1990; Telford et al., 1990). There are species differences in the developmental period at which the transition from control by maternally inherited molecules to that of embryonic genome-derived transcription products occurs (Telford et al., 1990). MET occurs during the 8- to 16-cell stage in cattle, as demonstrated by a marked incorporation of ^{3}H-uridine into RNA following short term (20 min) incubation (Camous et al., 1986; Kopecny et al., 1989) and resulting qualitative changes in pattern of embryonic protein synthesis (Frei et al., 1989). Furthermore, Barnes and First (1991) reported that amanitin sensitive protein synthesis occurs in bovine embryos as early as the third cell cycle (4-cell) stage and recently, it was demonstrated that a long term (6-10 h) incubation with ^{3}H-uridine resulted in incorporation into RNA in in vitro produced bovine 2- to 4-cell embryos (Plante et al., 1994; Viuff et al., 1996; Hyttel et al., 1996). As the same Hay-Schmidt et al. (1997) demonstrated that incubation of in vivo produced bovine zygotes and embryos for 10 h with ^{3}H-uridine results in dense autoradiographic labelling of the pronuclei in zygotes and the nuclei in 2-, 4- and early 8-cell embryo.

There are indications that some of the transcripts produced by the bovine embryo during the initial cell cycles code for transcription factors. Thus, transcription during the first two cell cycles may be driven by transcription factors of maternal heritage. During the third cycle there may be a shortage of these molecules before the embryonic transcription results in their renewal (see Hyttel et al., 1998).

Overall, maternal-embryonic transition appears to include at least two phases: the first phase includes a quantitatively low rate of transcription during the first two cell cycles and an even lower rate during the third. The second phase consists of an emphatic trancription initiated during the fourth cycle (see Hyttel et al., 1998). Initial and major activation of embryonic genome in different species are summarized in Table 1.

MET is very important for the large number of genes that must be activated and the pattern of embryonic gene expression that follows. It is this gene expression that sets the stage for later events associated with differentiation and successful embryo implantation. These characteristics make the MET important for understanding how the subsequent gene expression pattern unfolds. Furthermore, embryos cannot proceed through development if they do not properly undergo MET (Memili and First, 1999).

Another recent study explored embryonic genome activation for rRNA genes in cattle and pig embryos (Hyttel et al., 2000). The focus of the research was to correlate aberrations in the genome activation machinery, the nucleoli, with cell cycle arrest at later developmental stages. The results showed that the nucleoli became structurally recognizable with rRNA gene activation occurring in blastomeres of in vivo-derived embryos at the end of the third- and fourth-cell cycles for pigs and cattle, respectively. Interestingly, in vitro-derived cattle and pig embryos displayed abnormal nucleolus formation, which was associated with deficient rRNA gene activation. In turn, these problems with rRNA gene activation result in developmental abnormalities later, which may include cleavage arrest or certain deleterious phenotypes associated with offspring derived from in vitro produced embryos.

Table 1: The timing of embryonic genome activation in different species embryos

Species	Activation of embryonic genome			
	Initial	References	Major	References
Humans	4-cell	Telford et al. (1990)	4-8-cell	Telford et al. (1990)
Cattle	2-cell	Plante et al. (1994)	8-cell	King et al. (1989)
Sheep	2-cell	Kelk et al. (1994)	8-cell	Telford et al. (1990)
Goats	2-cell	Kelk et al. (1994)	8-cell	Kelk et al. (1994)
Pigs	Early 4-cell	Prather (1993)	Late 4-cell	Prather (1993)
Horses	4-cell	Ball et al. (1993)	8-16-cell	Grøndahl et al. (1993)
Rabbits	2-cell	Telford et al. (1990)	16-cell	Telford et al. (1990)
Mice	1-2-cell	Christians et al. (1995)	2-cell	Telford et al, (1990)

2.3.5.2. Expression of genes throughout preimplantation development

Preimplantation development is characterized by three major developmental transitions that occur following fertilization. The first is the maternal-to-zygotic transition, which is also referred to as zygotic gene activation (ZGA) or embryonic genome activation (EGA), in which maternal transcripts that direct early development are replaced by transcripts expressed from the zygotic/embryonic genome (see 2.3.5.1.). The ZGA represents the beginning of the transition from maternal to embryonic control of development. During this transition the embryo begins to synthesize mRNA and then protein. The expression of these embryonic transcripts is essential for further development, since inhibiting their expression (e.g., the addition of α-amanitin) results in cleavage arrest usually within one cleavage division following addition of the inhibitor of transcription. The second transition is compaction. Compaction results in the formation of a communicating polarized epithelium. It is followed by cavitation which is characterized by the rapid accumulation of fluid between the blastomeres leading to the formation of blastocoelic cavity. Finally, the third transition is the differentiation of the morula into the blastocyst (blastocyst formation), which is composed of totipotent cells of the inner cell mass (ICM) that will give rise to the embryo proper, and the specialized cells of the trophectoderm (TE), which is a fluid-transporting epithelium that will give rise to extraembryonic tissue. Each of these developmental transitions is accompanied by major changes in the pattern of gene expression (for review seeDe Sousa et al, 1998a; Schultz et al., 1999). There is now sufficient information about the temporal pattern of expression of individual genes in mouse preimplantation development to provide a reasonably accurate view of the genetic programme underlying cleavage, compaction, and blastocyst formation (Kidder, 1992; 1993; Schultz et al., 1999). Some very new techniques, most notably mRNA detection by means of the RT-PCR (see **2.3.3.**), have increased analytical sensitivity to the point where experiments can be performed with one (single) instead of thousands of embryos. The majority of the results from the genetic program for preimplantation development, which will present, come from the mouse model, but the bovine model has generated increasing interest in the past years.

2.3.5.2.1. Genes Encoding Growth Factors and Receptors

Polypeptide growth factors have attracted considerable attention recently as mediators of cell interactions in a wide range of mammalian embryos. The structure of growth factors and their genes is analyzed in the next section (see **2.3.6.**).

2.3.5.2.2. Genes involved in nuclear functions

Construction of the zygotic nucleus, DNA replication, chromatin assembly, activation of embryonic transcription, and processing of primary transcripts are all functions that must be fully operational within the first one to three cleavage cycles of mammalian development. Research on the mouse has begun to supply some of the details concerning the development of these functions.

- Transcription factors

Several such transcription factors and the sequence elements they recognize have been identified (Schöler et al., 1989a; b; Dooley et al., 1989). One of these, termed oct-3 by some investigators and oct-4 by others (it belongs to a family of transcription factors that bind to a spccific octamer of bases in DNA), is expressed only in the germ line and in totipotent or pluripotent stem cells, including the preimplantation embryos (see Kidder, 1993). Transcripts of this gene have been detected in ovarian oocytes, fertilized eggs, morulae, and blastocysts of the mouse by in situ hybridization; in late blastocysts the transcripts become restricted to the inner cell mass (Rosner et al., 1990; Schöler et al., 1990). Since oct-3 is expressed in oocytes as well as in preimplantation stages, this transcription factor is a good candidate for an oogenetic gene product required for postfertilization development (Rosner et al., 1991). Oct-3 is required not only for transcription beginning in the two-cell stage, but also for, initiating DNA replication (Rosner et al., 1991). In addition to maternal factors, it is likely that some nuclear functions associated with the early cleavages in the mouse are encoded by genes activated in the two-cell stage. Because of the sensitivity of their synthesis to α-amanitin, they have been called the transcription-requiring complex (TRC). Their synthesis is limited to the two-cell stage. TRC may be products of a family of regulatory genes involved in the burst of transcription that occurs in the late two-cell stage (for review see Kidder, 1993). Kirchhof et al. (2000) have shown that Oct-4 regulation differs between mice, pigs and cattle embryos.

They detected Oct-4 protein in the ICM but not in the trophectoderm in in vivo murine blastocysts, whereas in porcine and bovine blastocysts, derived in vivo or in vitro, Oct-4 protein was detected in both the ICM and the trophectoderm. Thus, in pigs and cattle, Oct-4 expression from the endogenous gene was clearly not restricted to the pluripotent cells of the early embryo. Van Eijk et al. (1999) demonstrated Oct-4 in bovine oocytes and in vitro preattachment-stage embryos (in both the ICM and trophectoderm cells of the blastocyst).

- Small nuclear RNAs (snRNAs)

Another early postfertilization nuclear function that relies on oogenetic gene products is the processing (splicing) of primary transcripts. Since fully functional mRNAs resulting from embryonic transcription enter the cytoplasm beginning in the two-cell stage in the mouse (Schultz, 1986), the nuclear RNA processing machinery must be operational from the moment embryonic transcription is activated. RNA processing is carried out by small nuclear ribonucl-eoproteins (snRNPs), that consist of a specialized class of small nuclear RNAs (snRNAs) and their associated proteins (Maniatis and Reed, 1987). According to in situ hybridization analysis, the snRNAs are concentrated in the germinal vesicle of the primary oocyte. They disperse through the cytoplasm upon germinal vesicle breakdown, to become reincorporated in the pronuclei of the zygote and later the diploid nuclei after the first cleavage. After genomic activation the snRNAs increase continuously, on a per-embryo basis, but by the second or third cleavage they reach a plateau on a per-cell basis (see Kidder, 1993).

- Histones

These proteins constitute another component of nuclei that must accumulate continuously throughout cleavage (for a review of the contribution of histones to chromatin structure, see Svaren and Chalkley, 1990). The synthesis of all four types of nucleosomal core histone (H2A, H2B, H3, and H4) as well as H1 has been detected during preimplantation development of the mouse from fertilization onward, and measurement of the absolute rates of synthesis of three of them suggested a balance between histone and DNA synthesis in each cell cycle (Kaye and Wales, 1981). Histone mRNAs reach high levels during cleavage and are therefore relatively easy to quantify. The three types of mRNA are abundant and roughly equal in number in ovulated, unfertilized oocytes, where mRNA for core histones has been estimated to make up about 3% of the total mRNA pool. These mRNAs decline by 89%-90% after fertilization, reaching a low point in the late two-cell stage. They subsequently increase again, maintaining a constant number of mRNAs per cell between the four-cell and blastocyst stages and reaching a total core histone mRNA content equivalent to about 1.6% of the total pool (Graves et al., 1985). In cattle, H3 was detected from the matured oocyte up to the

blastocyst stage. Only a twofold increase of the relative abundance of histone H3 mRNA was observed between the 6-8 cell stage and the blastocyst stage. H3 mRNA levels at the blastocyst stage were only 55% of the level found in mature oocytes. This lower levels of H3 mRNA appears to be sufficient to produce enouph histone H3 protein to package newly synthesized DNA for each cell division (Bilodeau-Goeseels and Schultz, 1997a).

2.3.5.2.3. Genes encoding cytoskeletal elements

Genes encoding the subunits of cytoskeletal elements are of interest because of the involvement of these elements in cytoarchitectural changes associated with fertilization, compaction, and cavitation (reviewed by Fleming and Johnson, 1988).

- Actins

These constitute a multigene family, with six isoforms having been characterized in mammals: two cytoplasmic isoforms (β and γ) that are present in most if not all cell types, two sarcomeric (α). isoforms unique to skeletal or cardiac muscle, and two smooth muscle isoforms (Taylor and Pikó, 1990). The mRNAs encoding these isoforms share extensive sequence identity in their coding regions but can be distinguished by probes specific for their 3′ untranslated regions. Although the synthesis of α, β, and γ isoforms had been reported in preimplantation stages of the mouse (Abreu and Brinster, 1978), only β and γ mRNAs could be detected by quantitative hybridization with isoform-specific riboprobes (Taylor and Pikó, 1990). The actin mRNAs increase in excess of cell number. This presumably reflects the importance of the actin microfilamcnt system, and the need for accelerated actin synthesis, in the morphogenetic events leading to blastocyst formation (Nisson et al., 1989). β-actin was detected from bovine mature oocytes up to blastocysts stage. Bovine 6-8-cell embryos were found to contain about 10% of the amount of β-actin mRNA contained in mature oocytes. By the morula stage, bovine embryos were observed to contain about half of the β-actin mRNA amount of the mature oocytes but blastocysts contained 110 times more β-actin mRNA than 6-8-cell embryos (Bilodeau-Goeseels and Schultz, 1997a). The gene products encoding E-cadherin, β-actin and zonula occludens protein 1 are expressed and maintain cellular distribution patterns consistent with its predicted role in mediating trophectoderm differentiation in in vitro produced bovine embryos (Barcroft et al., 1998).

- Tubulins

There is much less information on the accumulation of mRNAs encoding other cytoskeletal proteins in the mouse. Both α and β tubulins are synthesized in ovulated oocytes and through preimplantation development (Abreu and Brinster, 1978), but only α mRNA has been analyzed by hybridization with a cDNA. By the late two-cell stage this mRNA has declined to 14% of its level in the unfertilized oocytes; it then increases through cleavage to reach a maximum in blastocysts (Paynton et al., 1988).

- Cytokeratins

Cytokeratin filaments and the synthesis of cytokeratins are markers of trophectoderm differentiation (Chisholm and Houliston, 1987). Two intermediate filament proteins, cytokeratins Endo A. and Endo B, have been shown to be synthesized and assembled into filaments by the eight-cell stage (Emerson, 1988).

2.3.5.2.4. Genes encoding membrane channels and ion transporters

Several plasma membrane functions are known to play important roles in specific morphogenetic events in preimplantation development, and in the past few years the genes encoding these functions have been identified and their patterns of expression elucidated. These include genes encoding connexins (the protein subunits of gap junction channels) and the subunits of Na^+, K^+-ATPase (the plasma membrane sodium pump) (Kidder, 1993).

- Connexins

Gap junctions are aggregations of intramembranous particles (connexons) that can be found in regions of closely apposed plasma membranes in many tissues; they are morphological manifestations of intercellular communication. Each connexon is a hexameric assembly of protein subunits called connexins. Gap junctional intercellular communication has been implicated in metabolic coupling, cell growth control, and cell patterning (for reviews of the structure and function of gap junctions, see Bennett et al., 1991; Wrenzycki, 1995). Gap junctions are assembled de novo during the eight-cell stage of mouse development, during compaction, providing channels for embryo-wide intercellular coupling that is maintained for the remainder of the preimplantation period (Kidder, 1987). Three independent lines of evidence have indicated that gap junctional communication between the blastomeres is essential for maintaining the compacted state, and, thus for subsequent blastocyst formation (Kidder, 1993). Using RT-PCR, connexin 43 transcripts were detected in bovine morulae and

blastocysts grown in vivo (Wrenzycki et al., 1996). The gap junctions play a direct (although unspecified) role in compaction in the mouse (Bennett et al., 1991) and in bovine embryos (Wrenzycki et al., 1996). The connexins constitute a family of related proteins, each of which has a particular tissue distribution in adults (Bennett et al., 1991). The most common nomenclature uses the predicted molecular mass of each polypeptide to distinguish it: Connexin32 is 32 kD, connexin43 is 43 kD, and so on. Connexin32 appears to be a persistent oogenetic product, because a polypeptide of the same size and immunoreactivity can be detected at roughly the same level in all preimplantation stages. Connexin32 mRNA, however, could not be detected in any stage of preimplantation development (Barron et al., 1989). Transcripts of connexin26 were not detected either. Connexin43 has also been detected by immunoblotting in all mouse preimplantation stages, but in this case it clearly increases after the second cleavage. The accumulation of connexin43 is driven by embryonic transcription (Valdimarsson et al., 1991).

- Na^+, K^+-ATPase

The plasma membrane sodium pump has long been assumed to be a principal mediator of transtrophectodermal fluid transport during cavitation (Benos and Balaban, 1990). The currently favored hypothesis is that this sodium flux, driven by Na^+, K^+ -ATPase, drives a corresponding flow of water across the trophectoderm into the blastocele. Results showing that Na^+, K^+-ATPase is localized in membranes lining the blastocele cavity (i.e., in the basolateral plasma membranes of trophectoderm cells) are consistent with this view (Benos and Balaban, 1990). According to immunocytochemical evidence, Na^+, K^+-ATPase in the mouse is concentrated in the basolateral plasma membranes of mural trophectoderm, including its extensions covering the inner cell mass (Watson and Kidder, 1988; Kidder and Watson, 1990). Na^+, K^+-ATPase consists of two types of subunits, a catalytic (α) subunit and a noncatalytic, glycosylated (β) subunit (Geering, 1990). One explanation for this might be that much of the α-subunit content and enzymatic activity of the conceptus during this period is of oogenetic origin. The β-subunit content, however, does increase markedly during the blastocyst stage following the rapid increase in $\beta1$ mRNA content (Gardiner et al., 1990; Watson et al., 1990). The β-subunit is known to be required for processing, maturation, and transport of the α-subunit (McDonough et al., 1990) and may be acting during preimplantation development to trigger an influx of new (embryonically coded) Na^+, K^+-ATPase molecules during cavitation (for review see Kidder, 1993). Transcripts encoding the Na-K-ATPase $\alpha1$, $\alpha2$, $\alpha3$ and $\beta2$ isoforms were detected throughout bovine preattachment

development. β1-subunit transcripts were not detected until the morula stage but were present in blastocysts. Transcripts encoding α4 isoforms were not detected in bovine embryos (Betts et al., 1997).

2.3.5.2.5. Genes Encoding Cell Surface and Secreted Proteins

Proteins expressed externally on the surfaces of embryonic cells or secreted into extracellular spaces are good candidates for agents involved in interactions between blastomeres or signalling between the conceptus and the uterus. Many of the proteins in this category belong to several families of growth factors and their receptors, which are described separately in the next section (see **2.3.6.**).

- Alkaline phosphatase

The enzyme alkaline phosphatase is a prominent cell surface protein, one that has received recent attention. It is expressed on the plasma membranes in regions of close cellular apposition and is thus a marker of membrane regionalization (Sepúlveda and Izquierdo, 1990). In mouse, its activity increases continuously from the two-cell stage onward, eventually disappearing from trophectoderm and becoming restricted to the inner cell mass in the late blastocyst (Barron et al., 1989; Hahnel et al., 1990). Using PCR technique, transcripts of two different alkaline phosphatase genes in the mouse were detected and found to increase from the two-cell stage to the blastocyst (Hahnel et al., 1990). In cattle, TSAP2 mRNA was detected first at the four-cell stage prior to the major burst of embryonic transcription and TSAP3 at the eight-cell stage with the major burst in transcription. Furthermore, the transcription of TSAP2 and TSAP3 displays a curious “on-off” pattern during early clavages between 40 and 120 hr after insemination (McDougall et al., 1998).

- Extracellular matrix components

Proteinaceous components of the extracellular matrix constitute another class of gene products that are involved in mediating cell interactions. Fibronectin is just one of several extracellular

matrix components identified in preimplantation stages of the mouse; the list includes laminin, nidogen, thrombospondin, collagen, and heparan sulfate proteoglycan (O’Shea et al., 1990).

- Secreted proteinases

In contrast to their role in mediating cell interactions, extracellular matrix proteins in some situations pose barriers to cell migration or morphogenesis. Thus they sometimes serve as substrates for extracellular proteinases. A family of metalloproteinases has been implicated as key agents in extracellular matrix degradation (Brenner et al., 1989), and the timing of expression of the genes encoding two of these has been investigated in preimplantation mouse development. PCR amplification of reverse-transcribed RNA preparations revealed that transcripts of the stromelysin and collagenase genes are present in unfertilized eggs and in all preimplantation stages, increasing quantitatively through time. These transcripts do not appear to suffer the precipitous decline that most oogenctic mRNAs undergo between ovulation and the late two-cell stage. This is a group of genes expressed in association with cavitation but whose functions are primarily related to the initial stages of implantation, as the trophoblast invades the uterine lining (Brenner et al., 1989).

2.3.5.2.6. Genes encoding “housekeeping” functions

- Metabolic enzymes

Despite the importance of intermediary metabolism in regulation embryonic development, relatively little attention has been devoted to genes encoding metabolic enzymes. Oocyte-encoded glucose phosphate isomerase (GPI-1) activity was found to persist through the first 4-5 days of development in the mouse, depending on the amount of enzyme present in the unfertilized egg in different strains, and embryo-encoded enzyme can first be detected after 3.5 days (late morulae) (West and Flockhart, 1989). Because of their ample oogenetic store of GPI, zygotes homozygous for a null allele of Gpi-1 were able to develop beyond implantation (West et al., 1990). Another gene activated late in preimplantation development is Pgk-1, which encodes phosphoglycerate kinase (PGK-1). The maternally inherited Pgk-1 gene is activated in the blastocyst stage, whereas the paternally encoded allozyme was not detected until day 6 i.e. just after implantation (Krietsch et al., 1982). In cattle, Lequarre et al. (1997) studied some genes involved in glucose metabolism. These genes were glucose-transporter-1, hexokinase (HK), glucose-6-phosphate-dehydrogenase (G6PDH), and glucose-phosphate-isomerase (GPI). They have shown that GLUT-1 mRNA level was reduced by half during maturation and fertilization, HK mRNA levels decreased during the first cleavages but

increased significantly at the morula and GPI transcript remained stable throughout development whereas there was a significant rise for G6PDH at the 4-cell stage.

- Metallothioneins

The metallothioneins are small metal-binding proteins that are considered to be important for buffering cells against toxic levels of metals such as zinc, copper, or cadmium. RT/PCR was used to show that two metallothionein genes, MT-1 and MT-II, are constitutively transcribed at low levels throughout preimplantation development of the mouse. From the eight-cell stage onward the number of transcripts of MT-I could be increased by exposure to zinc or cadmium.

- rig (rat insulinoma gene) gene

The rig gene codes for a protein that contains a nuclear location signal and a DNA-binding domain. Relatively high levels of rig gene transcripts are expressed in growing oocytes, cleavage-stage embryos, embryonic carcinoma cells and mouse myeloma cells (Taylor and Pikó, 1991).

2.3.5.2.7. Genes Associated With Endogenous Retroviruses

Endogenous retroviruses, known as intracisternal A particles (IAP) to reflect the fact that viruses form by budding an RNA nucleoid core into the cisternae of the endoplasmic reticulum, account for a significant fraction of the mRNA present in the cytoplasm during preimplantation mouse development (Pikó et al., 1984). Two subfamilies of IAP genes, type 1 and type II, have been distinguished, and both are transcribed throughout this period (Poznanski and Calarco, 1991).

The genes involved in bovine preimplantation embryos (in total: 72 genes) and mRNA expression patterns are summarized in Table 2.

Table 2: Overview on mRNA expression patterns in bovine preimplantation embryos as detected by various modifications of RT-PCR.

Gene	Pattern of accumulation[a]	RT-PCR method	Expression patterns *			References
			In vivo	In vitro	Cloned	
Genes involved in nuclear function						
12S rRNA	mat. + embr.	semi-quantitative		+		Bilodeau-Goeseels and Schultz, 1997a
U_2, U_{3sn}RNA	mat. + embr.	semi-quantitative		+		Bilodeau-Goeseels and Schultz, 1997a
H3	mat. + embr.	semi-quantitative		+		Bilodeau-Goeseels and Schultz, 1997a
Oct-4	mat. + embr.	nested PCR		+		Van Eijk et al., 1999
Genes encoding cytoskeletal elements						
Cytochrome B	mat. + embr.	semi-quantitative		+		Bilodeau-Goeseels and Schultz, 1997a
β-actin	mat. + embr.	semi-quantitative		+		Bilodeau-Goeseels and Schultz, 1997a, Peippo et al., 2000
Genes encoding membrane channels and ion transporters						
Na^+, K^+-ATPase subunits (α_1, α_2, α_3)	mat. + embr.	qualitative		+		Betts et al., 1997
β1	Embryonic	qualitative		+		Betts et al., 1997
β2	mat. + embr.	qualitative/semi-quantitative		+		Betts et al., 1997
Cx43	mat. + embr.	qualitative/semi-quantitative	+	-		see Wrenzycki et al., 1996, Wrenzycki et al., 1999
Genes encoding cell surface and secreted proteins						
AP2	mat. + embr.	qualitative		+		McDougall et al., 1998
AP3	embryonic	qualitative		+		McDougall et al., 1998
DcII	embryonic	semi-quantitative	+	+	+	Wrenzycki et al., 1999, Wrenzycki et al., 2001b
DcIII	embryonic	semi-quantitative		+		Wrenzycki et al., 1999
pan ZO-1 (α, β)	mat. + embr.	emi-quantitative		+		Miller et al., 2001
pan ZO-2 (α, β)	mat. + embr.	semi-quantitative		+		Miller et al., 2001
Occludin	mat. + embr.	semi-quantitative		+		Miller et al., 2001
JAM	mat. + embr.	semi-quantitative		+		Miller et al., 2001
Genes encoding growth factors and receptors						
BFGF	blastocyst				+	see Schultz et al., 1996

Table 2: Overview on mRNA expression patterns in bovine preimplantation embryos as detected by various modifications of RT-PCR (Continued)

Gene	Pattern of accumulation[a]	RT-PCR method	Expression patterns *			References
			In vivo	In vitro	Cloned	
IGF-I	mat. + embr.	qualitative	+	+	-	see Schultz et al., 1996; Watson et al., 1992a
IGF-IR, IGF-II	mat. + embr.	qualitative	+	+	+	see Schultz et al., 1996; Watson et al., 1992a
IGF-IIR	mat. + embr.	qualitative/ semi-quantitative		+	+	Watson et al., 1992a, Wrenzycki et al., 2001b
TGF-α	mat. + embr.	qualitative	+	+	+	see Schultz et al., 1996; Watson et al., 1992a
IL6	morula	qualitative		+	-	see Daniels et al., 2000
FGF4	morula, blastocyst	qualitative		+	-	see Daniels et al., 2000
FGFr2	morula, blastocyst	qualitative		+	+	see Daniels et al., 2000
TGF-β_2, PDGF-α, PDGF-αr, insulin-r	mat. + embr.	qualitative		+		Watson et al., 1992a
IGFBP-2, -3, -4	mat. + embr.	qualitative		+		Winger et al., 1997
IGFBP-5	embryonic	qualitative		+		Winger et al., 1997
IGFBP-1, -6	-----------	qualitative		+		Winger et al., 1997
bLIF, LR-β, gp130	mat. + embr.	qualitative	-	+		see Eckert and Niemann, 1998
Genes encoding "housekeeping" functions						
GPX, GCS	mat. + embr.	qualitative		+		Harvey et al., 1995
G6PDH, GPI	mat. + embr.	semi-quantitative		+		Lequarre et al., 1997
Glut-1	mat. + embr.	semi-quantitative and qualitative	+	+	+	Wrenzycki et al., 1999, 2001b, Augustin et al., 2001
HK	mat. + embr.	semi-quantitative		+		Lequarre et al., 1997
Glut3, Glut8, SGLT-1	mat. + embr.	qualitative		+		Augustin et al., 2001
Glut4	At blastocyst	qualitative		+		Augustin et al., 2001
Glut5	embryonic	qualitative		+		Augustin et al., 2001
Genes involved in antioxidative defence, chromosome inactivation, stress adaptation, trophoblastic function, DNA methylation, compaction, cavitation and differentiation						
Plako	mat. + embr.	semi-quantitative		+		Wrenzycki et al., 1999

Table 2: Overview on mRNA expression patterns in bovine preimplantation embryos as detected by various modifications of RT-PCR (Continued)

Gene	Pattern of accumulation[a]	RT-PCR method	Expression patterns *			References
			In vivo	In vitro	Cloned	
Hsp70.1	mat. + embr.	semi-quantitative	+	+	+	Wrenzycki et al., 1999; Wrenzycki et al., 2001b
IF τ	embryonic	semi-quantitative	+	+	+	Wrenzycki et al., 1999; Wrenzycki et al., 2001b
Poly(A)	mat. + embr.	semi-quantitative		+		Wrenzycki et al., 1999
β-catenin	mat. + embr.	qualitative	+	+		Barcroft et al., 1998
E-cadherin	mat. + embr.	qualitative		+	+	Barcroft et al., 1998, Wrenzycki et al., 2001b
Catalase	mat. + embr.	qualitative		+		Harvey et al., 1995
CuZn-SOD	mat. + embr.	qualitative		+		Harvey et al., 1995, Lequarre et al., 2001
Mn SOD	maternal	qualitative		+		Lequarre et al., 2001
Xist	mat. + embr.	nested PCR/ semi-quantitative	+	+	+	see De La Fuente et al., 1999, Wrenzycki et al., 2002
G6PD, PGK	mat. + embr.	qualitative/ semi-quantitative	+	+	+	see Peippo et al., 2000, Wrenzycki et al., 2002
HPRT, ZFX	mat. + embr.	qualitative				
DNMT	blastocyst	semi-quantitative	+	+	+	Wrenzycki et al., 2001b
Mash2	blastocyst	semi-quantitative		+	+	Wrenzycki et al., 2001b

[a] maternal = expression detected prior to the burst of embryonic genomic activity at the 8-16-cell stage (Telford et al., 1990);

embryonic = expression after embryonic genomic activation;

qualitative RT-PCR = presence or absence of a specific transcript;

semi-quantitative RT-PCR = determination of relative abundance of a gene specific transcript.

* + : transcript present;

\- : transcript absent (adapted from Niemann and Wrenzycki, 2000; Wrenzycki and Niemann, 2002).

2.3.6. Growth factor genes

Growth factors can be broadly defined as multifunctional, locally acting, intercellular signalling polypeptides which control both the ontogeny and maintenance of tissue form and function. A multicellular organism can be considered as a community of different types of cells whose individual proliferation, differentiation and physiological function must, in some way, be coordinated for the overall function of both individual tissues and the whole organism. This is achieved by specific intercellular signals which control cell multiplication, differentiation and behaviour. These intercellular signals can be of two types: long-range (or endocrine) signals which are released into the circulation or other body fluids, and which modify the behaviour of physically remote cell types; and short-range (or paracrine) signals which act locally within tissues. Classical hormones, such as insulin or glucagon, are examples of long-range signalling molecules. Growth factors are, by contrast, predominantly short-range, locally acting, intercellular signalling molecules (Heldin and Westermark, 1984).
Growth factors share a number of common biological properties apart from their predominantly local mode of action. They often exert their biological actions at very low (typically 10^{-9} – 10^{-11} M) concentrations. This is because their action is mediated by their association with specific, high affinity receptors expressed by the target cell type (for review see Gullick, 1994).

The function of the growth factor receptor is not only to interact specifically with the ligand on the outside of the cell but also to generate an intracellular signal on the inside of the cell (Heldin and Westermark, 1984). It is this generation of growth factor receptor-mediated intracellular signals, and their “interpretation” by the responding cell that leads to the modification of target cell behaviour. As a result, the biological actions of growth factors are not (as their name might suggest) confined to the regulation of cell multiplication but can extend into a wide variety of aspects of cell function including differentiation, migration and gene expression. In addition, growth factors exhibit cell-type specificity in their action. In other words, the same growth factor can have very different biological effects depending on the type of cell with which it interacts. In this case it is not surprising that some growth factors at least (in contrast to endocrine hormones) are found to be widespread in their expression and distribution in the embryo and adult (see Gullick, 1994).

2.3.6.1. Growth factor families

There are currently about 80 or more known genes in both vertebrates and invertebrates, encoding proteins which can be considered, on the basis of their biological function, to be growth factors. It is also very likely that more growth factor genes remain to be discovered. Growth factors therefore represent a large set of polypeptides. These proteins, as a whole, do not share any common structural features but many can be grouped, on the basis of amino-acid sequence and tertiary structure, into multigene families. It is likely that, as new growth factors are discovered, the size and extent of growth factor multigene families will increase (reviewed by Gullick, 1994). In the following we will focus attention on the most prominent growth factor families and their involvement in preimplantation development.

2.3.6.1.1. Platelet-derived growth factor

Platelet derived growth factor (PDGF) was discovered as the major component of serum required for the multiplication of a variety of cell types in culture (Ross et al., 1986). PDGF was first purified from platelets and is a heterodimeric molecule composed of two disulphide-linked polypeptide chains [each of M_r 12 500 (12.5K)] -PDGF-A and PDGF-B. Subsequent purification of PDGF-like activities from a number of different sources showed that PDGF exists in three forms: an AB heterodimer and AA and BB homodimers. PDGF-A and PDGF-B are widespread in their expression, being particularly prominent in the early embryo, placenta and central nervous system. PDGF-A -and PDGF-B exhibit distinct patterns of expression in vivo suggesting that each form of PDGF may have distinct biological functions. Although PDGF was first identified by its ability to induce the multiplication of fibroblasts and smooth muscle cells, it has many other biological activities including chemotactic effects and regulation of differentiation of glial cells in the optic nerve. The PDGF family has been extended by the discovery of vascular endothelial cell growth factor (VEGF) which was identified as a mitogen for cultured endothelial cells. VEGF proves to be related to PDGF in sequence and has the characteristic two-chain structure, although it exhibits different biological functions and target cell specificity in vitro (see Gullick, 1994). PDGF-A were detected throughout bovine early development (Watson et al., 1992a; Yoshida et al., 1998a). Bovine preimplantation development in vitro was enhanced in the presence of PDGF (Eckert and Niemann, 1996; Lim and Hansel, 1996).

2.3.6.1.2. Epidermal growth factor family

Epidermal growth factor (EGF) was one of the first growth factors to be discovered, as a result of its striking effects on the maturation of various epithelia in the newborn mouse (Cohen, 1962). EGF is a polypeptide of M_r 6000 which is cleaved from a much larger transmembrane precursor protein in the submaxillary gland (Carpenter and Cohen, 1979). Guler et al. (2000) suggested that EGF may play an important role in the nuclear and cytoplasmic maturation of sheep oocytes in vitro, in which addition of EGF to IVM medium improved significantly the resumption of meiosis, and upon fertilization cleavage and blastocyst rates. The addition of EGF to the IVM-IVF media with the presence of a reduced amount of confined BSA (> 97% albumin) improved the developmental rates of porcine embryo (Wei et al., 2001).

Transforming growth factor alpha (TGFα) was isolated from the conditioned media of virus-transformed cell lines as an EGF-like bioactivity (Derynck, 1988). TGFα is also cleaved from a larger transmembrane precursor to yield the mature protein (M_r 6K) which is related in both primary sequence and three-dimensional structure to EGF. It is of interest that the transmembrane precursor of TGFα is also biologically active; the 'tethering' of TGFα precursor in the membrane may therefore represent a mechanism for confining TGFα action to cells in immediate physical contact. Unlike EGF, however, TGFα is relatively widespread in its expression, being found in a wide variety of foetal and adult tissues. TGFα seems to bind to the same receptor as EGF and is, to all appearances, identical to EGF in biological function. A number of additional cellular proteins have been isolated which resemble EGF/TGFα in sequence. These include amphiregulin (AMPH) and heparin-binding epidermal growth factor (HB-EGF). These molecules both have a long N-terminal extension which appears to confer affinity for heparin on the protein, suggesting that both AMPH and HB-EGF may be bound to heparin sulphate-containing glycosaminoglycans within the extracellular matrix (ECM) in vivo. The localization of the growth factor in the ECM by glycosaminoglycans seems to be another common mechanism for confining the biological action of growth factors to a strictly local mode of action (for review see Gullick, 1994; Lennard et al., 1998).

2.3.6.1.3. Fibroblast growth factor family

Fibroblast growth factor (FGF) was discovered, as its name suggests, from its ability to induce the proliferation of fibroblast cells in vitro. A key feature of the purification of FGF was the exploitation of the fact that FGF (like AMPH and HB-EGF) binds avidly to immobilized heparin. Initial purification of FGF bioactivity from brain revealed two closely related molecules, with very similar biochemical and biological properties, termed (on the basis of their pH and affinity for heparin) basic FGF (bFGF) and acidic FGF (aFGF). aFGF and bFGF are powerful mitogens for a wide variety of cell types, including fibroblasts, neurectodermal cells and, most notably, capillary and large vessel endothelial cells. Expression studies revealed that bFGF exhibits extremely widespread expression in both adult and foetal tissues as well being found in a large number of both normal and tumorigenic cell lines (including large vessel endothelial cells). The expression of aFGF is, by contrast, restricted principally to cells of the central and peripheral nervous system. An additional feature of both aFGF and bFGF is that they lack a characteristic secretory signal sequence at their N termini and neither are, in fact, usually secreted from cells. This would tend to indicate that they are not normally made available to responsive cells unless released by mechanisms such as cell damage. Cloning and sequencing of the int-2 gene revealed a molecule with significant sequence similarity to the aFGF and bFGF prototype genes. Further studies of genes capable of inducing the proliferation of fibroblasts led to the discovery of K-FGF/Hst (FGF-4) and FGF-5. Molecular cloning of genes related to FCF-4 led to the discovery of FGF-6, a molecule sharing 80% identity in aminoacid sequence with FGF-4. An important feature of these other FGF family members is that they all contain functional secretory signal sequences, and are therefore readily exported from cells. This suggests that at least one rationale for the existence of multiple related family members may be, in this case, to provide a means of controlling the dissemination and export of these molecules in vivo, by means of selective expression of individual family members (for review see Gullick, 1994).

Aside from their mitogenic properties, FGF family members have striking effects in early development. Some studies suggest that FGF-like growth factors may be ‘natural’ signals involved in the control of cell differentiation and pattern formation in early development (see Gullick, 1994). However, transcripts encoding bFGF were detected up to the bovine 8-16-cell stage and then declined following the 8-16-cell stage, bFGF were detected throghout early ovine development (Watson et al., 1992; 1994; Yoshida et al., 1998a). The supplementation of FGF, EGF or both in a defined culture medium can improve the in vitro development of

bovine embryos to morula and blastocyst stages; FGF and EGF may act synergistically on bovine embryo development in vitro (Lee and Fukui, 1995). Lim and Hansel (1996) suggested that the action of PDGF at the time of activation of the embryonic genome is independent of the presence of bFGF in the culture environment.

2.3.6.1.4. Transforming growth factor beta (TGFβ) family

The members of the transforming growth factor beta (TGFβ) family have multiple effects on cell function and are widespread in expression (Massagué, 1990). This polypeptide-induced 'anchorage independent growth' results from the combined action of TGFα and a second, structurally unrelated molecule, TGFβ. TGFβ is a homodimeric, disulphide-bonded protein of M_r 25K made up from two 12.5K polypeptide chains. Three closely related TGFβ genes exist (TGFβs 1, 2 and 3), creating three distinct homodimeric proteins and the hypothetical possibility of creating additional species by heterodimeric combinations of individual monomeric species. TGFβs 1-3 are widespread in their expression, TGFβ1 being almost ubiquitous, and in many tissues the different TGFβ family members are coexpressed. A very important feature of the TGFβs is that they are secreted from cells in latent form, as a biologically inactive complex formed from one molecule of TGFβ and an additional 'latency-associated protein'. The consequence of latency is that TGFβ is biologically inactive until the latent complex is broken down. A significant feature of TGFβ action is that its biological effects are often most clearly manifest in the presence of other growth factors. In other contexts, however, TGFβ can block the mitogenic action of other growth factors. An important target of TGFβ action is the extracellular matrix. TGFβ alone, or in combination with other growth factors, has striking effects on the promotion of ECM deposition in a wide variety of cell types. This is brought about both by enhanced expression of ECM structural components, such as fibronectin and integrins. and by inhibition of enzymes involved in ECM turnover. Again the effects of TGFβ on ECM deposition are most clearly seen in the presence of other growth factors. TGFβs 1-3 are members of an extended superfamily of structurally related molecules, many of which have important biological activities. The activin/inhibin family is composed of homo- and heterodimers of two protein chains derived from three genes α, βA and βB. Activins act on isolated animal pole cells to induce mesodermal differentiation including the production of cell types (such as notochord) which are not

generated in the presence of FGFs. Other TGFβ superfamily polypeptides include the family of morphogenetic proteins BMPs 2-7 which induce bone deposition in vivo and mullerian inhibitory substance (MIS) which is a major mediator of secondary sexual differentiation in mammals (for review see Gullick, 1994).

TGFβ and bFGF act synergistically to promote development of bovine embryos beyond the 8-cell block observed in vitro (Larson et al., 1992). Additional TGFβ-like genes have been isolated from other species such as flies which may well also prove to have significant functions as intercellular signalling molecules in development (Heath, 1994). Lim and Hansel (2000) concluded that embryo development to the blastocyst stage is regulated by exogenous PDGF, FGF and TGF.

2.3.6.1.5. Insulin-like growth factors (IGFs)

With the insulin-like growth factors (IGFs) the distinction between growth factors and endocrine hormones becomes blurred. The existence of IGFs was first predicted in the form of the 'somatomedin hypothesis', which argued that the effects of pituitary growth hormone on skeletal growth were mediated by means of an intermediate class of bioactive peptides, the somatomedins. Both IGF-I and IGF-II are made as secreted prohormones (M_r 9K and 14K respectively) and require proteolytic cleavage to achieve their 6K form. Although similar in their biological function, IGF-I and IGF-II exhibit significant differences in their pattern of expression in vivo. In particular, IGF-I is expressed in juvenile life and is almost exclusively synthesized in the liver under the control of growth hormone (as predicted by the original hypothesis). IGF-II, by contrast, is expressed predominantly in the embryonic and foetal stages of mammalian development in a wide variety of different tissues. This suggests that the IGFs may have both paracrine and endocrine functions in controlling the growth of many tissue types in vivo (Froesch et al., 1985).

Unlike many of the molecules considered so far, both IGF-I and ICF-II are present in the circulation and can be readily detected in plasma. As might be predicted from their patterns of synthesis, circulating IGF-I levels rise during juvenile life and then decline after puberty, whereas circulating ICF-II levels are highest in the foetal circulation and decline after birth. The biological importance of IGF-II for growth of the whole organism has been dramatically demonstrated in the living animal (Gullick, 1994).

The concept of classical endocrine control of reproductive function has been now extended to a more complex regulatory system, including paracrine and autocrine modulating mechanisms (Giudice, 1992; 1994; Katz et al., 1993; Jones and Clemmons, 1995). As the significance of putative intraovarian regulators became increasingly recognized, much of the attention has centered on insulin-like growth factors (IGFs). Experimental evidence has shown that IGFs exert a variety of effects at the level of murine, rabbit, porcine, and human ovarian cells, thereby raising the possibility of a meaningful in vivo role (Adashi et al., 1985; Giudice 1992; 1994; Katz et al., 1993; Jones and Clemmons, 1995). Cyclic follicular development is dependent on the precise cascade of gonadotropin stimulation to regulate subsequent granulosa cell ontogeny (Adashi et al., 1985; Giudice 1992; 1994). Although IGFs can act alone, their most important role may act synergistically with gonadotropins in the stimulation of a variety of granulosa cell function.

The use of homologous recombination technology proved conclusively that IGF-II was required for normal embryonic development, as IGF-II null mice (requiring only the disruption of the paternal allele due to parental imprinting) were 60% smaller than their wild-type littermates (DeChiara et al., 1990; 1991). These growth-deficient animals were otherwise apparently normal and fertile, demonstrating that IGF-II protein is not essential for development and survival. The phenotype of the null mutant mice was surprising and difficult to reconcile with the fact that IGF-II had been shown to have a specific expression pattern in many different tissues throughout the embryo (Wood et al., 1990; Pintar et al., 1991; Streck et al., 1992). However, later experiments showed that mice with both copies of the IGF-I gene knocked out were not only reduced in size to a similar extent, but also displayed severe muscle dystrophy and most (> 95%) of these mice died at birth (Powell-Braxton et al., 1993). These findings clearly demonstrated that IGF-I is essential for correct embryonic development in mice. The reduction in size of both types of IGF knockout mice indicates a role for IGFs as cell survival factors. In support of this contention, studies in vitro with cells derived from null mutants for IGF-II (Lamm and Christofori, 1998) and the type-I IGF receptor (Cui et al., 1997) demonstrated an increased number of apoptotic cells, whereas animals transgenic for IGF-I (Neuenschwander et al., 1996; Leri et al., 1999), IGF-II (Petrik et al., 1999) and the type-I IGF receptor (Steller et al., 1996) demonstrated a reduced number of apoptotic cells.

An important feature of the circulating IGFs is that they are not found free in plasma but are associated with a specific set of binding proteins. The function of the IGF-binding proteins (IGFBPs) is obscure but their primary function may be to block the bioavalability of circulating IGFs, thereby providing an additional mechanism for local control of growth factor

action. (IGFBPs) regulate the availability of the IGFs to their target cells (Jones and Clemmons, 1995; Sara and Hall, 1990). Advances in molecular biology open the way for understanding regulation and function of these peptides and proteins in ovarian physiology. The components of the IGF-IGFBP systems play an important role via autocrine/paracrine control in the process of cyclic ovarian follicular development (Yoshimura, 1998).

The IGF-IGFBP system is comprised of the IGF peptides, IGF-I and IGF-II, specific receptors, and a family of proteins, called IGFBPs. This system constitutes a family of cellular modulators that play essential roles in the regulation and development (Baxter and Martin, 1989; Jones and Clemmons, 1995; Nissley and Lopaczynski, 1991; Rechler, 1993; Rosenfeld et al., 1990; Sara and Hall, 1990). The biological functions of the IGFs are mediated by a family of transmembrane receptors, which includes the insulin, IGF-I, and IGF-II/ mannose-6-phosphate receptors. The biological actions are also modulated by a family of at least six IGFBPs that are found in the circulation and in extracellular compartments and are produced by most tissues.

2.3.6.1.5.1. IGFs action and roles

IGFs are involved in several biological processes, such as growth, development and metabolism (Stewart and Rotwein, 1996). Peptide growth factors interact with receptors located on the cell membrane which are usually glycoproteins and which communicate with secondary messenger systems by conformational changes. This often involves the autophosphorylation of tyrosine residues located on the intracellular domain of the receptor. The second messenger systems utilized are diverse and include changes to intracellular calcium levels, cyclic AMP, cellular alkalization, and phosphoinositol metabolites. The net biological events induced by growth factors are, however, remarkably consistent and include a rapid stimulation of amino acid transport, glucose uptake and utilization, and RNA and protein synthesis (Fig. 3). This is followed by DNA synthesis (Hill, 1989). Since expression of IGF-I receptors is widespread, it is not surprising that IGF-I actions can be identified in many types of cells. The best charachterized of the actions of the IGFs are their stimulatory actions on cell proliferation. Complementary to their effects on cell proliferation, the IGFs can also inhibit cell death. The IGFs induce differentiation and stimulate differentiated functions in several cell types (for review see Jones and Clemmons, 1995). For more about IGFs actions and functions see also 2.3.6.5.2. and 2.3.6.1.5.3..

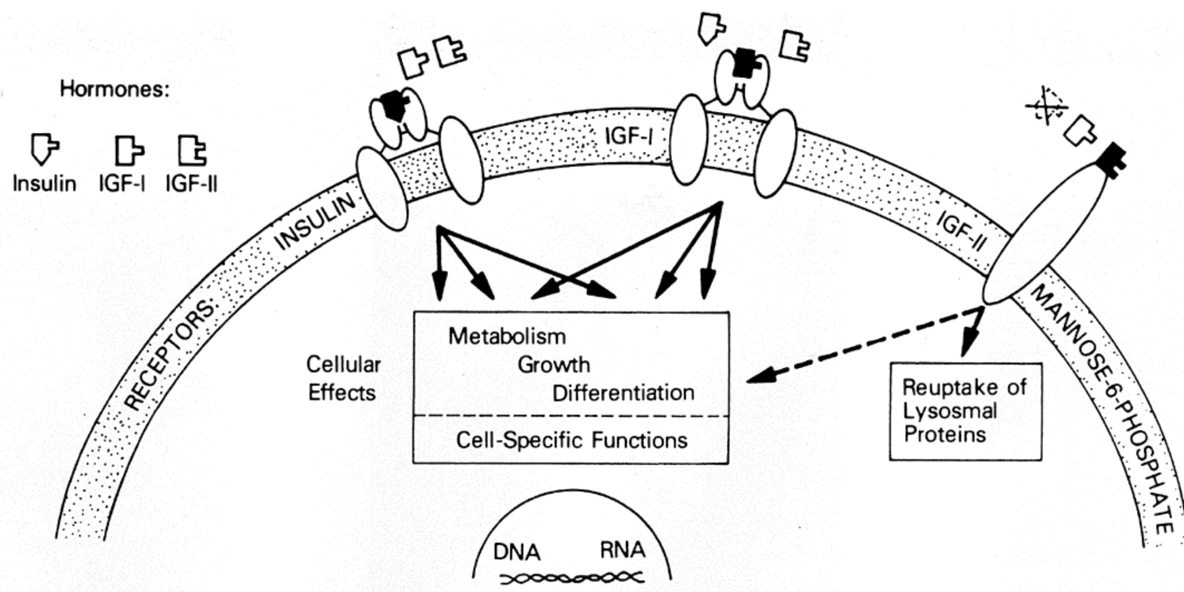


Fig. 3 Schematic representation of insulin and IGFs, their receptors, and biological actions. The insulin receptor and the IGF-I receptor have similar heterotetrameric structures. The insulin receptor binds insulin with high affinity and IGF-I and II with lower affinities while the IGF-I receptor binds IGF-I with high affinity and IGF-II and insulin with lower affinity. Each of these two receptors, activated by any of the three peptides, mediates a broad range of intracellular effects specific for that receptor in that particular cell type, and typically including stimulation (or inhibition) of metabolic events, growth and differentiation, as well as cell-specific processes. The function of the third receptor, now known as IGF-II/mannose-6-phosphate receptor, monomeric in structure, is much less well understood. Besides its ability to bind IGF-II and IGF-I (but not insulin), it also binds, at another site, proteins that have mannose-6-phosphate (M6P) residues. Among these proteins are lysosomal enzymes that are secreted and then internalized via these receptors and thereby targeted to the lysosomes. The IGF-II/M6P receptor is more abundant intracellularly than in the cell-membrane (adapted from De Pablo et al., 1990).

2.3.6.1.5.2. Insulin-like growth factor-I

Bovine IGF-I is a 70 amino acid, basic, single chain polypeptide, with a molecular mass of 7649 daltons. The bovine cDNA is 93% identical to the human sequence, and the amino acid sequence is 96% conserved (Fotsis et al., 1990). IGF-I and IGF-II amino acid sequences share identical residues in 45 positions, giving a sequence homology of 62% (Jones and Clemmons, 1995). Three disulfide bridges maintain the tertiary structure of the molecule (Watson et al., 1999). Bovine, porcine and human IGF-I are identical, where as rat and mouse IGF-I differ from human by three and four amino acid residues, respectively (Jones and Clemmons, 1995). Both of the IGF genes span a considerable length of genomic DNA. A single gene locus for IGF-I has been mapped to bovine chromosome 5 and the long arm of human chromosome 12 (Brissenden et al., 1984). The IGF-I gene consists of six exons, five introns and at least two promoters (de Pagter-Holthuizen et al., 1987; Holthuizen et al., 1990; Rotwein, 1991).
The finding from Palma et al. (1997) support the hypothesis that culture media containing high concentrations of IGF-I combined with estrous cow serum and granulosa cells can improve the development of embryos produced in vitro. IGF-I and EGF can stimulate cumulus expansion, oxidative metabolism, nuclear maturation and cleavage after fertilization of bovine oocytes in vitro (Rieger et al., 1998). Supplementation of culture medium with IGF-I increased the proportion of human embryos developing to the blastocyst stage from 35% to 60% (Lighten et al., 1998). Addition of IGF-I to the IVM medium stimulating oocyte maturation in the buffalo at a concentration of IGF-I 100 ng/ml (Pawshe et al., 1998). However, addition of IGF-I at similar concentration did not improve nuclear or cytoplasmic maturation of sheep oocytes (Guler et al., 2000). IGF-I stimulates equine oocyte maturation in a dose-dependent manner with highest nuclear maturation rate at a high concentration (200 ng/ml) in 36 hours but no significant effect was observed after 48 hours (Carneiro et al., 2001). The addition of IGF-I to the bovine IVM medium (TCM199 + 10% FCS) did not affect the cleavage rate but blastocyst yields were significantly increased at a low and high concentration (50 or 500 ng/ml) of IGF-I (Markkula and Makarevich, 2001).

2.3.6.1.5.3. Insulin-like growth factor-II

The IGF-II protein is highly conserved between species (the 180 amino acids that encode the mature bovine and ovine IGF-II clones are identical) and rat, human, bovine and ovine forms differ at only one amino acid (Brown et al., 1990). The precursor molecule contains a 24-residue amino-terminal signal peptide, a 67 (M_r 7,471) amino acid, mature IGF-II polypeptide and an 89 amino acid carboxyl terminal. Bovine IGF-II has over 60% identity with IGF-I (Brown et al., 1990; Fotsis et al., 1990). Human and bovine IGF-II differ by three amino acids. Rat and mouse IGF-II differ by two amino acids, and they have four and six amino acid residue differences, respectively, compared with human IGF-I (Jones and Clemmons, 1995). A single gene locus for IGF-II has been mapped to bovine chromosome 29 and the short arm of human chromosome 11 (Brissenden et al., 1984). The IGF-II gene consists of nine exons and contains four promoter elements (de Pagter-Holthuizen et al., 1987; Holthuizen et al., 1990; Rotwein, 1991). The IGF-II gene is located in proximity to the insulin gene, there being a promoter region located only 1.4 kilobases downstream of the insulin gene (for review see Yoshimura, 1998).

For many years it was considered that there was a change from IGF-II to IGF-I during fetal to adult growth, as IGF-II is expressed predominantly in the embryo (Bhaumick and Bala, 1987). The IGF-II gene is also a member of a small family of genes that have been shown to be subject to genomic imprinting (Reik et al., 1996; Moore et al., 1997). An imprinted gene is expressed primarily from one specific parental allele and such genes have been shown to exert important effects, primarily on fetal development.

2.3.6.1.5.4. Insulin-like growth factor binding proteins (IGFBPs)

The IGFs are almost entirely bound in vivo to high-affinity IGF-binding proteins of which there are at least six members (see Clemmons, 1993; Murphy and Barron, 1993; Jones and Clemmons, 1995). All IGFBPs display structural homology, bind IGF-I and IGF-II specifically and have a negligible affinity for insulin. Sequence alignments of IGFBPs reveal regions of homology within the amino- and carboxyl-terminal regions. The positions of 18 cysteines, which participate in the formation of disulfide bridges and contribute to three-dimensional structure, are conserved in IGFBPs 1-5. The rat IGFBP-6 sequence lacks

two and the human IGFBP-6 sequence lacks four of the 18 conserved cysteines found in the other IGFBPs. In serum, approximately 75% of the circulating IGF is complexed with IGFBP-3, and an 88 kDa glycoprotein, the acid labile subunit (ALS), forming a 150 kDa protein complex. This 150 kDa complex prolongs the half-life of IGFs in serum to 12-15 h, which is considerably longer than the 10 min half-life of free IGFs. The half-life of free binding proteins is between 30 and 90 min. IGFBPs can inhibit or potentiate IGF action under various conditions (see Clemmons, 1993; Murphy and Barron, 1993; Jones and Clemmons, 1995). The characterization of specific proteases for the IGFBPs has further complicated the situation as these proteases cleave binding proteins into forms with altered affinity for the IGFs. IGFBPs are subject to post-translational modifications, and direct cellular effects, in which binding to IGF ligand is not necessary, have been described (Jones and Clemmons, 1995). The affinity of IGFBPs for IGFs is controlled by phosphorylation, glycosylation and specific proteolysis (Clemmons, 1998).

The IGFBPs have been proposed to have four major functions that are essential to coordinate and regulate the biological activities of the IGFs. These are 1) to act as transport proteins in plasma and to control the efflux of IGFs from the vascular space; 2) to prolong the half-lives of the IGFs and regulate their metabolic clearance; 3) to provide a means of tissue- and cell type-specific localization; 4) to directly modulate interaction of the IGFs with their receptors and thereby indirectly control biological actions. In addition, recent evidence has emerged that the IGFBPs can have direct effects on cellular functions, and at least one of the IGFBPs is a ligand for a specific cell surface receptor (see Jones and Clemmons, 1995). There is increasing evidence that IGFBPs not only regulate IGF bioavailability, but that they also have their own receptors that mediate IGF-independent actions. Cell surface receptors for IGFBP-1 (Jones et al., 1993), IGFBP-2 (Rauschnabel et al., 1999), IGFBP-3 (Oh et al., 1993a) and IGFBP-5 (Andress, 1995; 1998) have been described, although, to date, none of these proteins have been cloned. Direct IGF-independent effects of lGFBPs include modulation of bone cell proliferation (Mohan et al., 1995) and growth arrest of breast and prostate cancer cells (Oh et al., 1993a; b; 1995; Rajah et al., 1997). However, owing to the diverse range of properties imparted by the IGFs and IGFBPs, their role during organ and whole animal development has been difficult to unravel (Allan et al., 2001).

2.3.6.2. Growth factor receptors

Growth factor receptors are cell-surface proteins whose purpose is to receive information from outside cells and to convey it across the cell membrane (signal transduction). The information resides in the concentration of a peptide or protein called a growth factor which binds to the receptor and converts the receptor to an active form. The receptor then interacts with proteins on the inner surface of the membrane and alters their properties or subcellular location resulting in changes in the cell's behaviour. These changes are various and occur at different times after receptor activation. Some alterations such as ion fluxes occur in fractions of seconds, others such as changes in the cell's shape take several minutes. The most fundamental consequence of receptor activation is to stimulate a programme of events which results in cell division. This process may take 18 hours or longer to be completed. Three classes of growth factor receptors have been described (Ullrich and Schlessinger, 1990; Aaronson, 1991). One consists of proteins formed of a core structure of seven transmembrane α-helical sequences. The next family is formed of large glycoproteins which generally possess a single transmembrane sequence and tyrosine kinase activity (Ullrich and Schlessinger, 1990). A third receptor family consists of molecules which span the cell membrane once, but do not have kinase activity themselves (Aaronson, 1991). These three superfamilies are characterized both by their structure and by their mechanism of signal transduction.

2.3.6.2.1. Insulin-like growth factor receptors

There are two known receptors (see Fig. 3) that specifically recognize the IGFs (Jones and Clemmons, 1995; Nissley and Lopaczynski, 1991; Steele-Perkings et al., 1988). The IGF-I receptor (also known as the type I IGF receptor) has a high degree of homology with the insulin receptor and is the only IGF receptor to definitively have IGF-mediated signaling functions. The IGF-II receptor (also known as the type 2 IGF receptor) is identical to the cation-independent mannose 6-phosphate receptor, which functions in the trafficking of lysosomal enzymes but has no known IGF signaling function. The IGF-I receptor has, in most tissues, a higher affinity for IGF-I compared to IGF-II, and it binds insulin with moderate

affinity. The IGF-II receptor has a high affinity for IGF-II and a lower affinity for IGF-I, and does not bind insulin (see De Pablo et al., 1990; Jones and Clemmons, 1995).

2.3.6.2.1.1. Insulin-like growth factor-I receptor (IGF-IR)

The actions of IGF-I and IGF-II are mediated largely through the IGF-I receptor (LeRoith et al., 1995). The IGF-I receptor is synthesized as a single chain polypeptide. Post-translational modifications include cleavage of a signal polypeptide and further cleavage into a 707 amino acid, extracellular α-subunit and a 626 amino acid, transmembrane β-subunit. The α- and β-subunits are linked by disulfide bonds. Two αβ complexes are joined by additional disulfide bonds creating the mature $\alpha_2\beta_2$ receptor. Binding of IGF ligands is mediated by the extracellular α-subunit within a cysteine-rich region. Tyrosine kinase activity occurs in the cytoplasmic β-domain. Binding of IGF ligand to the α-subunit stimulates phosphorylation of both tyrosine and serine residues (LeRoith et al., 1995). Autophosphorylation of the IGF-I receptor results in multiple signalling pathway cascades leading to the stimulation of cell growth (LeRoith et al., 1995; Jones and Clemmons, 1995).

2.3.6.2.1.2. Insulin-like growth factor-II receptor (IGF-IIR)

The IGF-II-mannose-6-phosphate receptor (IGF-II/M6P) receptor is a monomeric 215 kDa glycoprotein with high IGF-II binding affinity, binding IGF-I at 500-fold lower level than IGF-II, and with no affinity for insulin (Schultz and Heyner, 1993; Jones and Clemmons, 1995). Sequence comparisons of the IGF-II receptor and the cation-independent mannose-6-phosphate receptor revealed identical molecules. The binding sites for IGF-II and M6P are distinct and both ligands can bind simultaneously to the receptor (Morgan et al., 1987). The IGF-II receptor protein contains a large extracellular domain, comprising 93% of the total receptor, a single transmembrane domain and a small cytoplasmic tail. Fifteen repeat sequences of eight conserved cysteine residues, a single fibronectin type II repeat and 19 N-linked glycosylation sites are located on the extracellular domain (Morgan et al., 1987). The binding of IGF-II to the receptor results in internalization and degradation of IGF-II (Morgan et al., 1987). It is still unclear whether the IGF-II receptor has a biological role beyond regulating free concentrations of IGF-II. A soluble form of the IGF-II/M6P receptor

generated by proteolytic cleavage of the membrane bound receptor has been identified in rats (Jones and Clemmons, 1995).

2.3.6.3. Growth factors and binding proteins throughout preimplantation development

There are many studies in which RT-PCR and immunolocalization were applied to characterize the expression of the growth factor genes during mammalian preimplantation development (see Watson et al., 1992; 1994; Schultz et al., 1993; Schultz and Heyner, 1993; Kaye and Harvey, 1995). These include mRNAs encoding for TGF-α, TGF-βl, TGF-β2, TGF-β3, PDGF-A, Kaposi´s sarcoma-type growth factor (kFGF) , IGF-I and IGF-II. Transcripts encoding cytokines such as interleukin 3 (IL-3), interleukin 6 (IL-6) and leukaemia inhibitory factor (LIF) also are expressed by the blastocyst during early mouse development. The activation of growth factor ligand and receptor genes is selective, as transcripts encoding several factors including EGF, nerve growth factor (NGF) and insulin have not been detected during the first week of development for any mammalian species (Watson et al., 1992; 1994). It was thought that IGF-I was not produced by the preimplantation mouse embryo until the eight-cell stage. However, the use of RNA extracts from larger embryo pools has led to the detection of IGF-I mRNAs in all murine preimplantation stages (Doherty et al., 1994). IGF-I mRNAs were not detected in mice (Rappolee et al., 1992), rat embryos (Zhang et al., 1994), water buffalo (Daliri et al., 1999) and human (Lighten et al., 1997a). IGF-I mRNAs were detected during both bovine and ovine preimpalntation development using qualitative RT-PCR (Watson et al., 1992; 1994). RT-PCR amplicons for TGF-β2, TGF-α, PDGF-A and IGF-II were detected throughout bovine early development (Watson et al., 1992) and transcripts encoding IGF-II and TGF-α throughout ovine in vitro development (Watson et al., 1994). Similar to mouse and rat early embryos, products encoding EGF, NGF, or insulin were not detected in bovine or ovine early embryos. However, transcripts encoding bFGF were detected only up to the 8- to l6-cell stage in bovine embryos, and then declined markedly following the 8- to l6-cell stage during early ovine development (Watson et al., 1992; 1994). TGF-α, TGF-β and EGF mRNA transcripts were not detected in any of the preimplantation water buffalo stage, however transcripts encoding IGF-II were detected, except in 2-4-cell (Daliri et al., 1999).

A number of receptor genes are expressed in the early mouse embryo including the insulin-r, IGF-Ir, IGF-IIr, EGFr, PDGF-α r, and colony-stimulating factor lr (CSF-I) (see Schultz and

Heyner, 1993; Kaye and Harvey, 1995). In the mouse, the IGF type 1 receptor was detected by cell surface binding of 1GF-I and IGF-II in morula and blastocyst stages (Mattson et al., 1988) and by gold-labelled IGF-I binding as early as the 8-cell stage (Smith et al., 1993). The IGF-II/M6P receptor was first detected in 2-cell mouse embryos (Schultz and Heyner, 1993; Kaye and Harvey, 1995). Bovine and ovine early embryos express transcripts encoding PDGF α-r, insulin-r, and IGF-I-r and IGF-II-r throughout the first week of development (Watson et al., 1992; 1994). Insulin- and IGF-I-r transcripts were detected at each step of water buffalo embryonic development (Daliri et al., 1999). Growth factor ligand mRNAs during preimplantation development are summarized in Table 3.

Transcripts encoding IGFBPs 2-4 were detected throughout early bovine development while IGFBP-5 mRNAs were detected only weakly in bovine blastocysts (Winger et al., 1997). mRNAs encoding IGFBPs 1 and 6 were not detected during this developmental interval in bovine embryos. In the mouse embryo, mRNAs encoding IGFBP-6 were detected only in blastocysts, while transcripts encoding IGFBP-2, -3 and -4 were detected throughout murine preimplantation development (Hahnel and Schultz, 1994). Transcripts encoding IGFBP-5 were not detected in any preimplantation stage in mice (Hahnel and Schultz, 1994).

Table 3: Growth factor ligand mRNAs during preimplantation development

Factor	Mouse*	Cow	Sheep[a]	Buffalo	Human
Insulin	-	-	-	+	-
IGF-I	+	+	+	-	-
IGF-II	+	+	+	+	+
TGF-α	+	+	+	-	n.a.
EGF	-	-	-	-	n.a.
NGF	-	-	-	n.a.	n.a.
bFGF	-	+	+	n.a.	n.a.
TGF-β1	+	+	+	-	n.a.
PDGF-α	+	+	n.a.	n.a.	n.a.

* +: transcript present -: transcript absent [a] n.a.: not analysed

Data from Schultz and Heyner (1993); Kay and Harvey (1995); Lighten et al. (1997); Daliri et al. (1999).

3. MATERIALS AND METHODS

3.1. In vitro production of bovine embryos

3.1.1. Preparation of the media

3.1.1.1. PBS medium

Dulbecco´s phosphate buffered saline (PBS; # D6650, Sigma Chemical Co., St. Louis, MO, USA) was used as the basic medium for transportation and washing of ovaries. For slicing as shown in Table 1, it was supplemented with 2 % heat-inactivated newborn calf serum (NBCS, Boehringer, 295957, Mannheim, Germany) and 2 IU heparin (Serva, Heidelberg Germany). PBS medium was prepared in double distilled water 1-3 days before use and stored at 4°C.

3.1.1.2. Collection medium for cumulus-oocyte complexes

TCM199 (Tissue culture medium) containing L-glutamine and 25 mmol Hepes / l (# M2520; Sigma) supplemented with 22 µg pyruvate / ml, 350 µg $NaHCO_3$ / ml, 50 µg gentamicin / ml and 1 mg bovine serum albumin / ml (BSA; # A-9647 fraction V, Sigma) for the TCM-system or 1 mg polyvinyl alcohol / ml (PVA; P8136, Sigma) for the SOF-system (Table 4) was prepared in double distilled water (Ampuwa[R], Fresenius AG, Bad Homburg, Germany). The pH was adjusted at 7.2 and the medium was stored at 4°C for 2 weeks after passing through a 0.22 µm filter for sterilization.

3.1.1.3. Maturation media

TCM199 containing L-glutamine and 25 mmol Hepes / l (# M2520; Sigma) supplemented with 22 µg pyruvate / ml, 2.2 mg $NaHCO_3$ / ml, and 50 µg gentamicin / ml was prepared once in two weeks, the pH adjusted at 7.4, and stored at 4°C after passing through a 0.22 µm filter

for sterilization. For oocyte maturation (Table 4), this medium was supplemented with hormones and 10% estrous cow serum (ECS) for the TCM-system or polyvinyl alcohol for the SOF-system respectively, as described below.

Table 4: Composition of TCM-system and SOF-system used in this experiment

Systems of media used in this study	**The TCM-System Co-culture with COC; complex, undefined medium (supplemented with serum-ECS)**	**The SOF-System Simple chemically defined medium (supplemented with polyvinyl alcohol-PVA)**
Slicing medium	PBS + NBCS	PBS + PVA
Collection medium	TCM air + BSA	TCM air + PVA
Maturation medium	TCM + ECS	TCM + PVA
Fertilization medium	Modifications of Tyrode's medium (Fert-TALP with BSA)	
Culture medium	TCM + ECS	SOF + PVA
Culture conditions:		
Volume of droplet:	200 µl	30 µl
Numbers of IVF oocytes/droplet:	20	6-8
Culture atmosphere:	5% CO_2 in air in high humidity atmosphere at 39°C	5% CO_2, 7% O_2 and 88% N_2 in high humidity atmosphere at 39°C

3.1.1.3.1. Hormones

One dose Suigonan^R (200 IU hCG and 400 IU eCG, Intervet, Tönisvorst, Germany) was prepared in 0.9% sodium chloride, aliquoted and stored at –20°C in small sterile plastic tubes. Stock solutions were prepared fresh every 2-3 months. Before use, 1 ml of the maturation medium was added to each tube to get concentrations of 5 IU/ml hCG and 10 IU/ml eCG.

3.1.1.3.2. Serum

Estrous cow serum (ECS) was collected from cows at the Institute farm during standing estrus and blood was allowed to stand at 4°C overnight after initial clotting at room temperature. The following day, serum was separated, pooled and centrifuged at 500 g for 20 min. Serum was heat inactivated at 56°C for 30 min before it was passed through a 0.22 µm filter for sterilization. It was aliquoted into 1 ml lots and stored in sterile plastic tubes at –20°C.

3.1.1.3.3. Polyvinyl alcohol (PVA)

PVA is a linear polymer (inter polymer) of high molecular weight for the fractionation of complex protein mixtures (Polson et al., 1964). It is used in protein-free (chemically defined) media instead of the serum or other protein source. PVA appears to have no biological activity, it is inert i.e. it does not stimulate the embryo development (Bavister et al., 1995).

3.1.1.4. Fertilization medium

The composition of IVF media is shown in Table 5. Two modifications of Tyrode's medium (Parrish et al., 1988) were used. Sperm-TALP was employed for swim-up separation of the motile fraction of semen and subsequent washing of sperm. It was supplemented with pyruvate and BSA (# A-9647 fraction V, Sigma) on the day of use. The other modification, Fert-TALP, was used for washing of the IVM oocytes before they were placed into the fertilization drops made from Fert-TALP medium. This medium was supplemented with gentamicinsulfat, pyruvate and BSA (# A-9647 fraction V, Sigma) on the day of use. The IVF media were prepared in double distilled water (Ampuwa^R, Fresenius AG) bimonthly, the pH adjusted at 7.4, and stored at 4°C after passing through a 0.22 µm cellulose filter for sterilization. The fertilization medium was prepared fresh by supplementing Fert-TALP with the capacitation inducing agents consisting of hypotaurine, epinephrine and heparin.

3.1.1.4.1. Capacitation agents

The stock solution of epinephrine (# 4250, Sigma) was prepared by mixing 1.83 mg in 40 ml of the solution containing 50 mg sodium metabisulphite and 165 mg sodium lactate (60%) in

Table 5: Concentration of the IVF media and SOF medium

Component	Unit	Sperm-TALP*	Fert-TALP*	SOF-system**
NaCl	mM	100,0	114,0	108,0
KCL	mM	3,1	3,2	7,2
$NaHCO_3$	mM	25,0	25,0	25,0
NaH_2PO_4	mM	0,3	0,3	-
KH_2PO_4	mM	-	-	1,2
$CaCl_2$	mM	2,0	2,0	1,7
$MgCl_2$	mM	0,4	0,5	0,5
HEPES	mM	10,0	-	-
Na-Laktat	mM	21,6	10,0	3,3
Na-Pyruvat	mM	1,0	0,2	0,33***
Glucose	mM	-	-	1,5
Glutamin	mM	-	-	1,0***
Penicillamin	µM	-	20,0	-
Phenolrot	µg/ml	0,01	0,01	0,01
Gentamicin	µg/ml	50,0***	50,0***	50,0***
Heparin	IU	-	0,01***	-
Hypotaurin	µM	-	10,0***	-
Epinephrin	µM	-	1,0***	-
Non-essential amino acids	µl/ml	-	-	10,0***
Essential amino acids	µl/ml	-	-	20,0***
BSA	mg/ml	6,0***	6,0***	-
PVA	mg/ml	-	-	6,0***

* Based on media described by Parrish et al., (1988).

** Based on media described by Tervit et al., (1972).

*** Added freshly on the day of use.

50 ml bidistilled water (Ampuwa^{R}). The pH was adjusted to 4.0. The stock solution of hypotaurine (# H1384, Sigma) was prepared by dissolving 1.09 mg in 10 ml of 0.9% sodium chloride. The final working stock was prepared by mixing 4 ml of the epinephrine stock and 10 ml of the hypotaurine stock with 26 ml of physiological saline and then filtered for sterilization. Aliquots of 80 µl were stored frozen at –20°C and mixed with 2 ml of the Fert-TALP medium before use. Fresh stocks were made every two months. One mg heparin sodium salt (Serva, Heidelberg, Germany) containing 177 IU was dissolved per ml physiological saline. Before use, 4 µl of this stock (aliquoted and stored at –20°C) was mixed with 2 ml of the Fert-TALP to give a working concentration of 0.56 µg/ml. Fresh stocks were also made every to months.

3.1.1.5. Culture media

Two culture systems (TCM-system & SOF-system) were used for the in vitro production of embryos (Table 3).

3.1.1.5.1. TCM-system

The maturation medium, TCM199 containing L-glutamine and 25 mmol Hepes / l supplemented with 22 µg pyruvate / ml, 2.2 mg $NaHCO_3$ / ml, 50 µg gentamicin / ml and 10% ECS for the TCM-system, (as described above) without hormones was used for in vitro culture of the IVM/IVF zygotes to produce all embryonic stages up to the hatched blastocyst. The embryos were equilibrated at 39°C, in an atmosphere of high humidity and 5% CO_2 in air for 8-10 days.

3.1.1.5.2. Synthetic oviduct fluid (SOF) medium

The SOF-medium (Tervit et al., 1972; Gardner et al., 1994) supplemented with PVA was used in this work as simple, defined medium as shown in Table 5. The stock solutions were prepared in double distilled water (Ampuwa^{R}) monthly and stored at 4°C after passing

through a 0.22 µm cellulose filters for sterilization. On the day of use, 10 ml of stock solution was supplemented with 0.33 mM sodium pyruvate, 1.0 mM glutamin, 0.5 mg gentamicin, 0.1 ml non-essential amino acids MEM (# M7145, Sigma), 0.2 ml essential amino acids BME (# B6766, Sigma) and 60 mg PVA (Keskintepe and Brackett, 1996; Eckert et al., 1998).

3.1.2. Collection of ovaries

Ovaries were recovered from local slaughterhouse (Lübbecke: app. 100 km from the lab in Mariensee) from cows and heifers, mostly of Holstein origin within 5 min of evisceration. No selection with respect to the stage of estrous cycles was done. However, ovaries from cows with uterine pathology such as pyometra, abnormal cervical discharge or ovarian cysts were not collected. Following incision, each pair of ovaries was rinsed free of blood and extraneous material with PBS before being placed in an insulated flask (thermos bottle) containing PBS medium and being transported to the laboratory within 2-3 hr at ambient temperature 25-30°C. Upon arrival in the laboratory, the transportation medium was removed and the ovaries were washed two times in fresh PBS medium before the slicing began.

3.1.3. Recovery of oocytes

The oocytes were recovered from the ovaries by the slicing method. The slicing units consist of 6-8 razor blades (0.15 mm, Romi, Solingen, Germany) which joined together in a metal skeleton. The slicing device cuts the surface of the ovaries in various dimensions for ~3 mm distance. Ovaries were held in PBS medium supplemented with 2 IU heparin and 2% NBCS (TCM-system) or 1 mg/ml PVA (SOF-system). Following slicing, the resulting fluid was passed through a filter in glass beaker and allowed to stand ~15 min for sedimentation of cumulus oocyte complexes. The suspension was transferred to 15 ml centrifuge tubes (Greiner, Nürtingen, Germany) and then the sediment was removed and diluted with PBS medium [supplemented with 2 IU heparin and 2% NBCS (TCM-system) or 1 mg/ml PVA (SOF-system)] in 60 mm plastic dishes (# 628102, Greiner GmbH, Nürtingen, Germany) before being viewed under a stereomicroscope. Only oocytes with non expanded cumulus cells and a homogeneous evenly granulated cytoplasm were selected and transferred to warm

collection medium at 39°C. Collection of the oocytes was accomplished within 2-3 hr of the arrival of the ovaries at the laboratory. The oocytes were transferred to the maturation medium.

3.1.4. Classification of cumulus-oocyte complexes

Cumulus-oocyte complexes were examined under a stereomicroscope at 35× magnification and classified into two categories based upon the character of the cellular investments (Pavlok et al., 1992).

Category I : Oocytes with a homogeneous evenly granulated cytoplasm possessing at least three layers of compact cumulus cells (see Fig. 4).

Category II : Oocytes with fewer than three layers of cumulus cells or partially denuded but also with a homogeneous evenly granulated cytoplasm (see Fig. 4).

Regardless of the category, oocytes with degenerated cytoplasm, or surrounded by expanded, degenerating, dark looking cumulus cells were not used in this study.

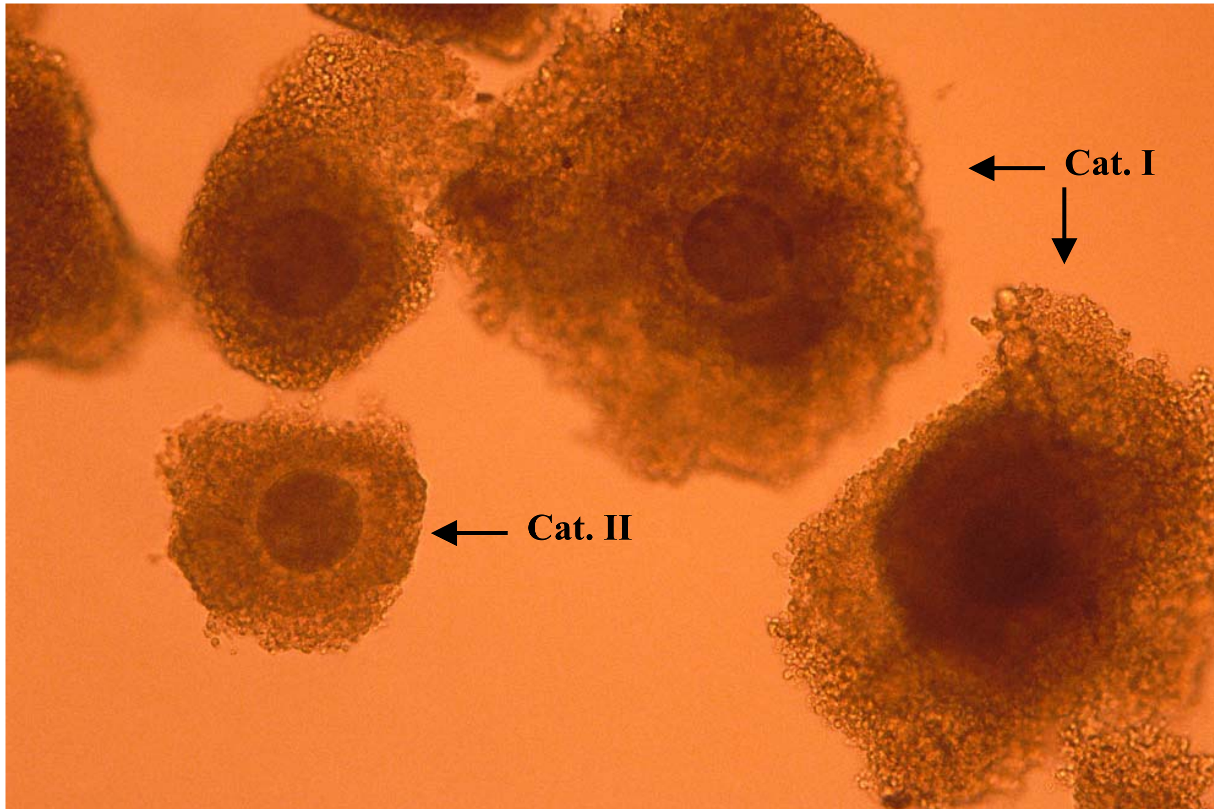


Fig. 4 In vitro bovine cumulus oocyte complex, categories I and II.

3.1.5. Removal of cumulus cells

Immature oocytes, matured oocytes and fertilized oocytes were denuded from the cumulus cells by vortexing for 3 min in collection medium (TCM-air) followed by gentle pipetting and collection the denuded ova under a stereomicroscope.

3.1.6. Fixation and staining of oocytes

Denuded oocytes were washed three times in PBS medium supplemented with 1 mg/ml PVA and mounted on grease free slides in a small quantity of medium. A mixture of vaseline and paraffin wax (9:1 w/w) was used to support the coverslip in contact with the oocytes without excessive pressure. The slides were fixed in acetic-alcohol (acetic acid : ethanol = 1:3) for at least 24 hr and were stained with 1% (w/v) aceto-lacmoid solution before evaluation under a phase contrast microscope at 400 × magnification.

3.1.7. In vitro maturation

On the day of use, stock TCM199 medium, that had been prepared as described above, was supplemented with 10% estrous cow serum (TCM-system) or 1 mg/ml PVA (SOF-system) to produce washing medium. A portion of this medium was supplemented with hCG and eCG to serve as maturation medium. For maturation, four 100 µl droplets were covered with silicone oil (Serva, Heidelberg, Germany) in 35 mm sterile polystyrene culture dishes (# 627170, Greiner GmbH, Nürtingen, Germany) and equilibrated in the culture environment for 2-3 hr. Immature oocytes were washed three times in washing medium before being transferred in groups of 20-25 to the maturation drops. Equilibration and incubation were carried out at 39°C an atmosphere of high humidity and 5% CO_2 in air for 23-24 hr.

3.1.7.1. Determination of maturation rate

For determination of the maturation rate, a random sample of the oocytes at the end of the 24 hr incubation was denuded of the cumulus cells, fixed and stained (as described above) for cytological examination to assess the status maturation. In vitro maturation was confirmed by the presence of a metaphase II (M II) spread with the first polar body. The maturation rate was expressed as the percentage of matured versus the total number evaluated.

3.1.8. In vitro fertilization (IVF)

On the day of use, the already prepared Fert-TALP was supplemented with BSA (# A-9647 fraction V, Sigma), gentamicine and pyruvate. In vitro matured oocytes were washed three times in 100 µl droplets under silicone oil. The IVF medium was prepared by adding hypotaurine, heparin and epinephrine to the Fert-TALP medium. Four 100 µl droplets of this medium were placed in a 35 mm petri culture dish and overlaid with silicone oil. Washing and fertilization media were equilibrated in the culture environment for 2-3 hr. Prior to insemination, the IVM oocytes were washed three times in washing medium and transferred in groups of 20-25 to the fertilization droplets. The oocytes were then returned to the incubator for at least 20 min until sperm preparation was accomplished. The co-incubation of oocytes and sperms lasted 18-20 hr at 39°C under 5% CO_2 in air with high humidity.

3.1.8.1. Preparation of sperm

For in vitro fertilization, frozen semen from one bull with a proven history of successful IVF was used throughout the study. Sperm was prepared as described by Parrish et al., (1988). Two straws were thawed in a water bath at 38°C for 1 min. For swim-up separation of the motile fraction, the thawed semen (0.2 ml) was layered under 1 ml of Sperm-TALP (Table 5) that was freshly supplemented with BSA (# A-9647 fraction V, Sigma) and pyruvate in sterile glass held at an angle of 45°. The motility of the sperm after thawing was determined under a phase contrast microscope (200 ×). After 1 hr at 39°C under 5% CO_2 in air, 850 µl from the top of the medium from each vial was collected and pooled in a sterile centrifuge tube. Following the addition of ~3 ml Sperm-TALP medium, the swim-up separated sperm were centrifuged at 350 × g (36°C) for 10 min. The sperm pellet was resuspended in fresh 5 ml of Sperm-TALP medium and centrifuged again. The final sperm pellet was suspended to ~200 µl with IVF medium and incubated for 15 min at 39°C under 5% CO_2 in air. During this time sperm concentration was determined using a counter slide (Thoma; Superior, Omnilab, Gehrden, Germany). The concentration was adjusted to 50 million sperm/ml using the fertilization medium for dilution. A ~2 µl aliquot of this sperm suspension was introduced to each 100 µl fertilization droplet containing ~20 oocytes to give a final sperm concentration of 1 million sperm per ml.

3.1.8.2. Determination of fertilization rate

For determination of the fertilization rate, after 18 hr from start of the oocyte-sperm co-incubation, some of the oocytes were picked up randomly, denuded, fixed and stained (as described above). Oocytes containing two polar bodies, two pronuclei and a residual sperm tail were considered to be normally fertilized. Oocytes with more than two pronuclei and decondensed sperm heads were evaluated as polyspermic. Fertilization of oocytes was evaluated under a phase contrast microscope at 400 × magnification.

3.1.9. In vitro culture of embryos

3.1.9.1. Culture of embryos in TCM-system

On the day of use, TCM199 was supplemented with 10% ECS for wash and culture media. Following fertilization, presumptive zygotes with surrounding cumulus cells were washed three times and then transferred in groups ~20 zygotes into 0.8 ml culture medium placed under silicone oil in a 4-well Nunclon petri dish. Prior to use, the wash and culture dishes were equilibrated in the culture environment for 2-3 hr. The culture conditions were the same as for IVM and IVF. According to the requirements of the embryonic stages, the culture was either continued for 8-9 days to determination of the proportion of expanded/hatched blastocysts stage or terminated at appropriate time points to obtain embryos of pre-defined developmental stages for analysis of gene expression. Day 0 is defined as the day of fertilization. The culture medium was not replaced during the 8-9 days culture period.
The cumulus cells attached to the bottom of the dish within 2-3 days of culture to form a monolayer. Cleavage rate was evaluated on day 3 of the culture under a stereomicroscope at 45 × magnification.

3.1.9.2. Culture of embryos in SOF-system

On the day of use, the stock solution of SOF medium was supplemented with Na-pyruvate, glutamine, gentamicine, non-essential amino acids, essential amino acids and PVA (Table 5). This medium was used as wash and culture medium. Prior to use, the wash and culture dishes were equilibrated in the culture environment for 2-3 hr. Following fertilization, presumptive zygotes were denuded of cumulus cells, washed three times in 100 µl droplets of washing medium and then transferred in groups of 6-8 zygotes into 30 µl of culture medium. Zygotes were cultured under silicone oil in 5% CO_2, 5% O_2 and 90 %N_2 (Air Product, Hattingen, Germany) in a humidified atmosphere in Modular incubator (ICN Biomedicals, Inc., Aurora, No. 615300, Ohio, USA) at 39°C (Fig. 5). Culture was either continued for 8-9 days when expanded/hatched blastocysts stage were produced or terminated at appropriate time points to obtain embryos of pre-defined developmental stages for analysis of gene expression. The

culture medium was not replaced during the 8-9 days culture period. Cleavage rate was evaluated under a stereomicroscope at 45 × magnification on day 3.

Fig. 5 Modular incubator chamber (closed and opened).

3.1.10. Morphology of the embryonic stages

The embryonic stages in this study were assessed under a stereomicroscope at 45 × magnification after denudation. Early cleavage stage embryos were referred to by the number of cells, such as, 1-cell, 2-cell, 8-cell and 16-cell stages. COC, IVM oocytes and stage of embryonic development in relation to the numbers of the cells is given below according to Robertson and Nelson (1998).

COC: Category I and II of COC (Fig. 4)

Immature oocytes: Oocytes without cumulus cells (Fig. 6)

Mature oocytes: Oocytes after IVM for 23-24 hr without cumulus cells (Fig. 7)

Zygotes: Zygotes after IVF for 19 hr without cumulus cells (Fig. 8)

2-4-cell: Embryos with 2-4 cells after IVC for 2 days (Fig. 9)

8-16-cell: Embryos with 8-16 cells after IVC for 3 to 4 days (Fig. 10)

Morula: Embryos with more cells than 16 cells after IVC for 5 to 7 days (Fig. 11). Morula is a 'ball of cells' with individual blastomeres not further to identify. The cellular mass of the embryo filled most of the space.

Blastocyst (expanded): The embryo after IVC for 8 to 9 days (Fig. 12) with the initiation of blastocoel formation and the appearance of a ring. Expanding

blastocysts had a clearly defined blastocoel with a pronounced differentiation between the outer trophoblast layer and the darker, inner cell mass. Expanded blastocyst had a larger diameter than the other embryonic stages with a concurrent thinning of the zona pellucida.

Blastocyst (hatched): The embryos which had completely shed the zona pellucida after IVC for 8 to 11 days (Fig. 13). It looked like either a spherical mass with a prominent blastocoel (floating).

The cumulus cells were removed from the embryonic stages (as described above). All oocytes or embryos were washed extensively and the absence of cumulus cells was verified at × 200 magnification to ensure that the transcripts did not originate from residual cumulus cells. Pools of oocytes or embryonic stages were collected at appropriate stages of development (see above). After washing three times in PBS containing 0.1% PVA, embryonic stages were stored in pools of 20-25 at −80°C in a minimum volum (5 µl or less) of medium until use for the analysis of gene expression.

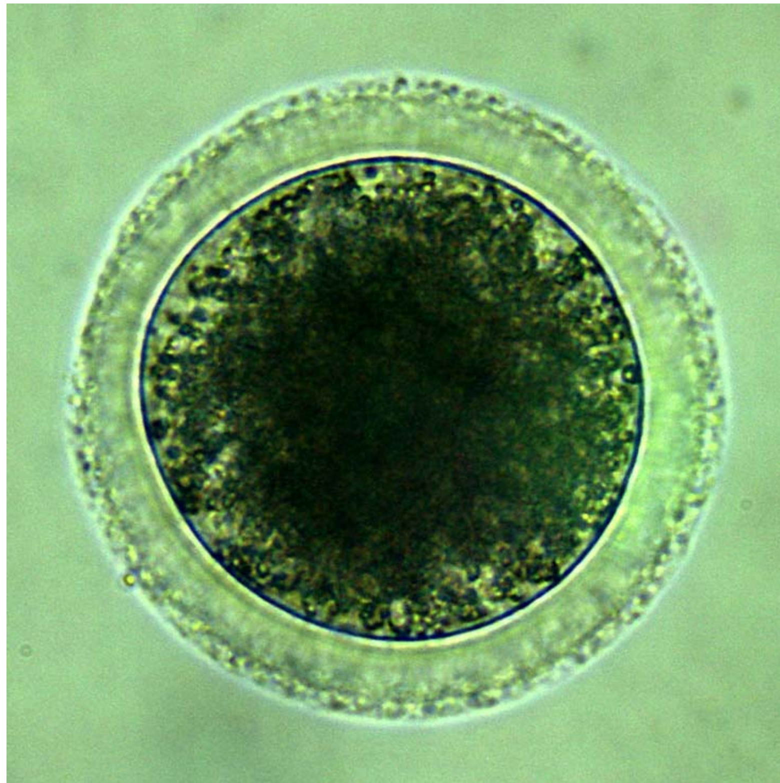


Fig. 6 In vitro bovine immature oocyte without cumulus cells.

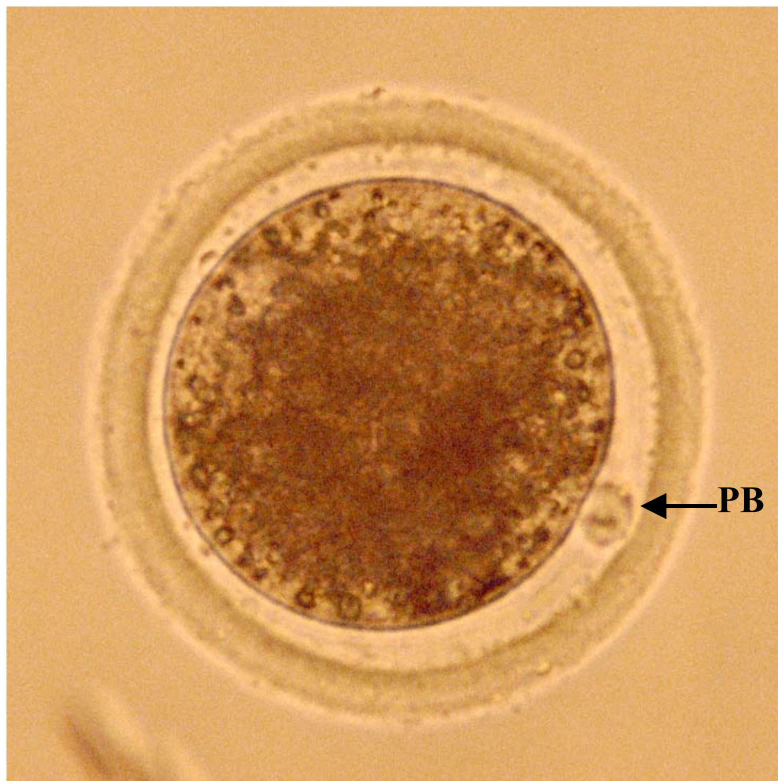


Fig. 7 In vitro bovine matured oocyte without cumulus cells after 24 hr in IVM.

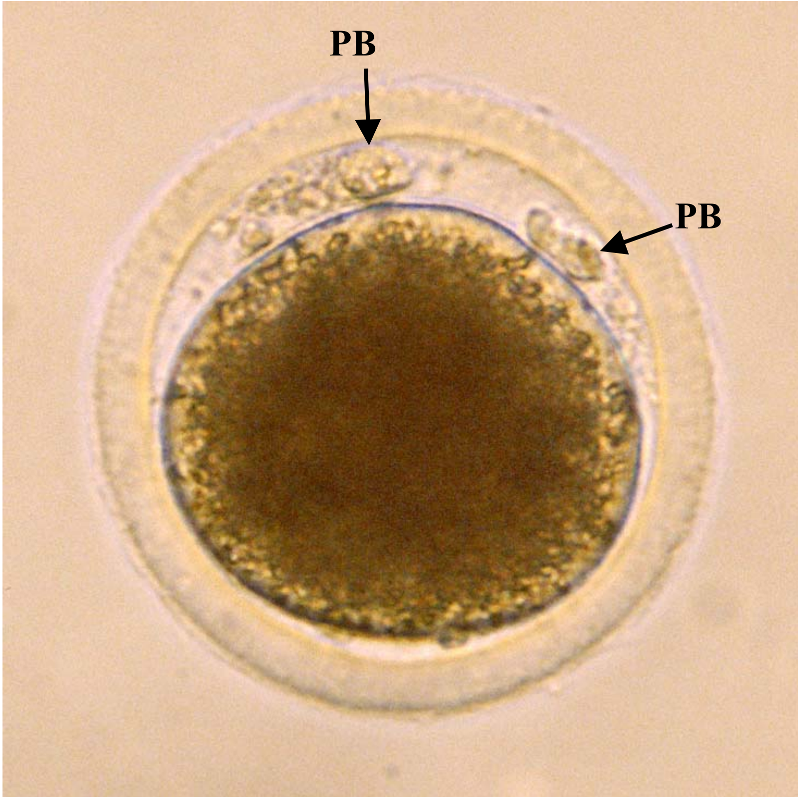


Fig. 8 In vitro bovine zygote after 19 hr in IVF.

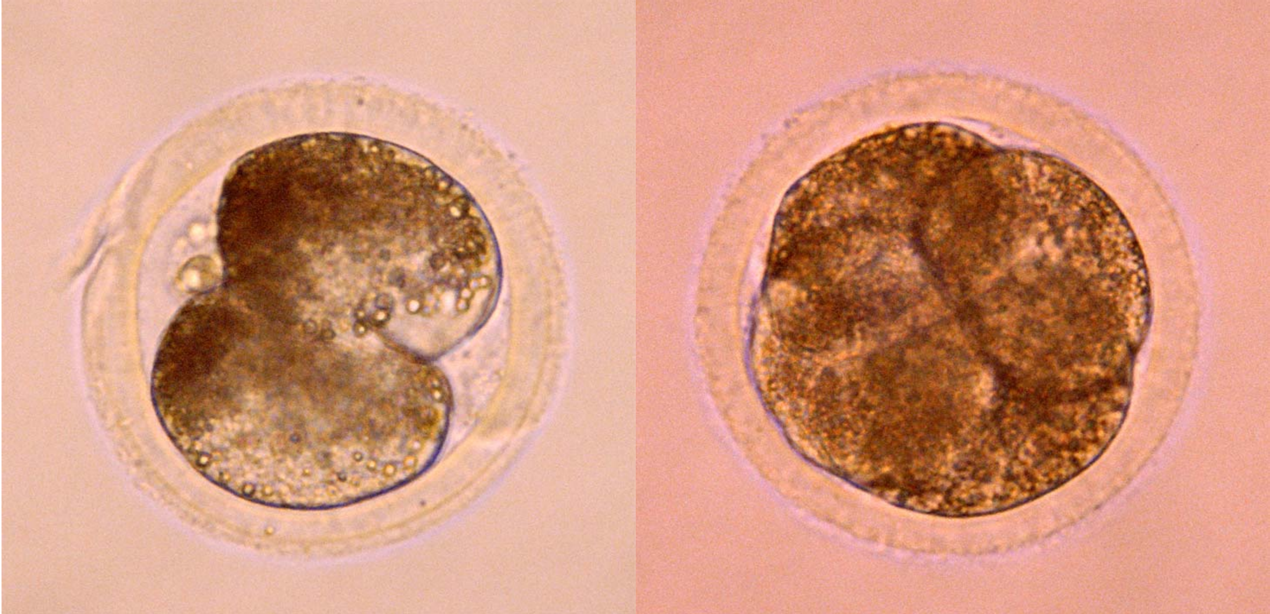


Fig. 9 In vitro derived bovine 2-4-cell embryos after two days in IVC.

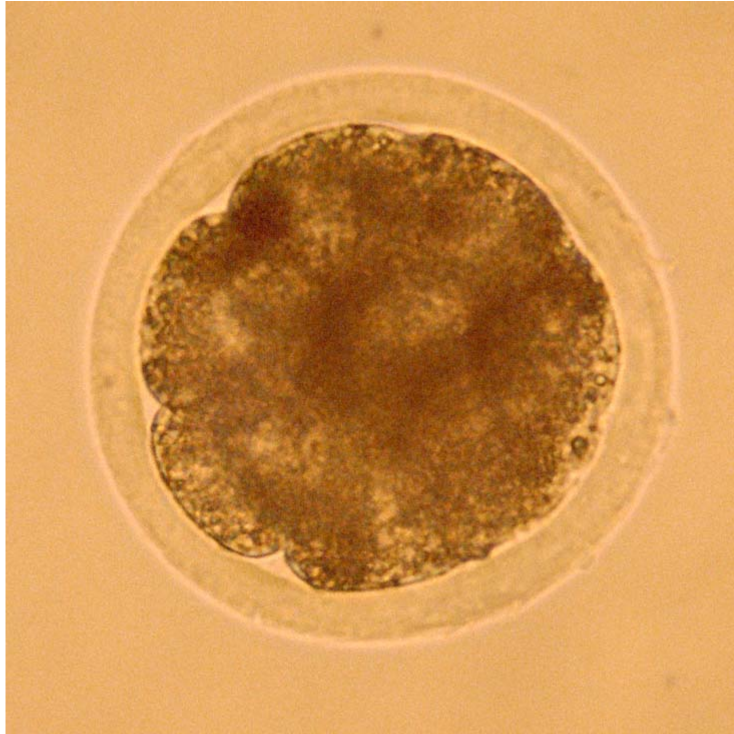


Fig. 10 In vitro derived bovine 8-16-cells embryo after 3 days in IVC.

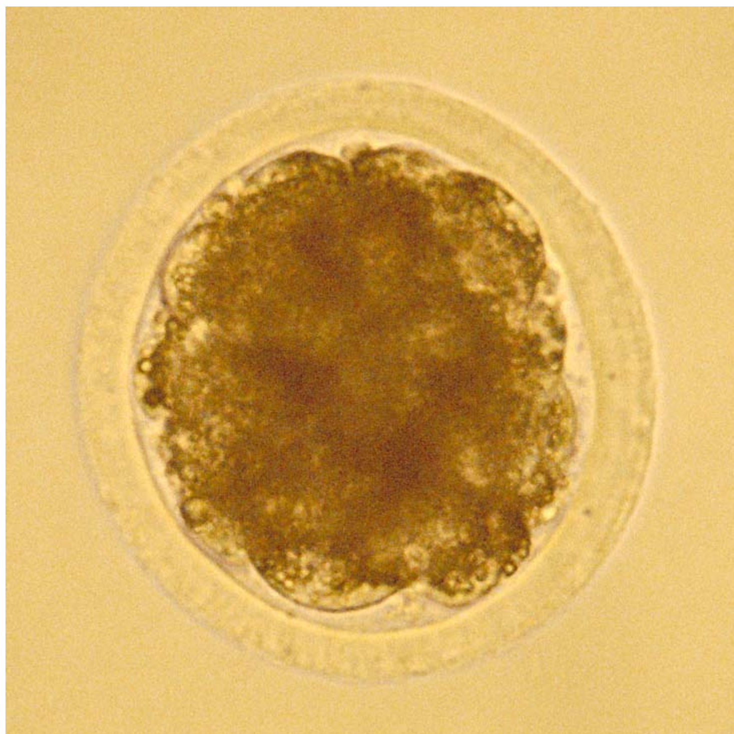


Fig. 11 In vitro derived bovine morula after 6 days in IVC.

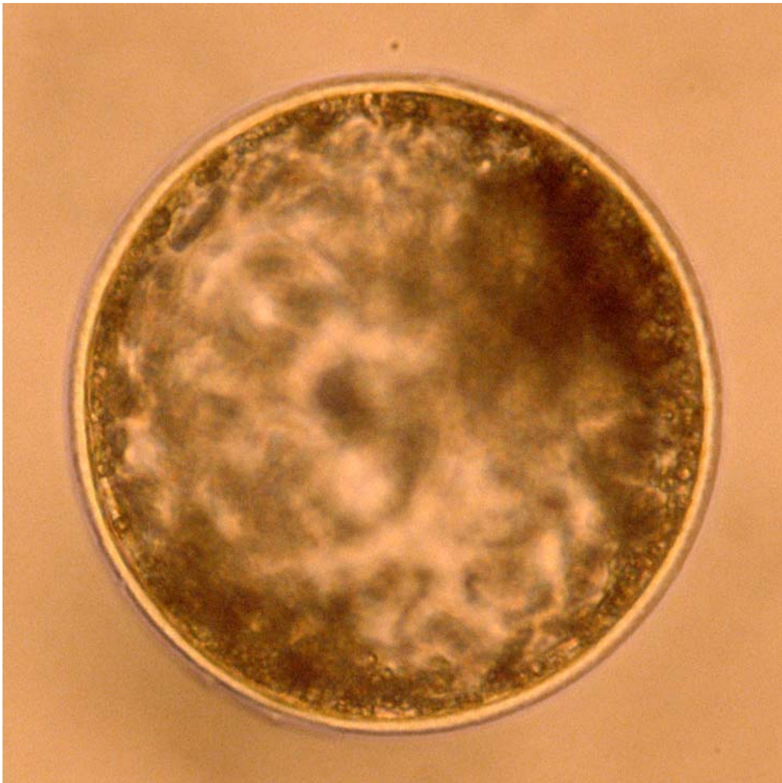


Fig. 12 In vitro bovine derived expanded blastocyst after 8 days in IVC.

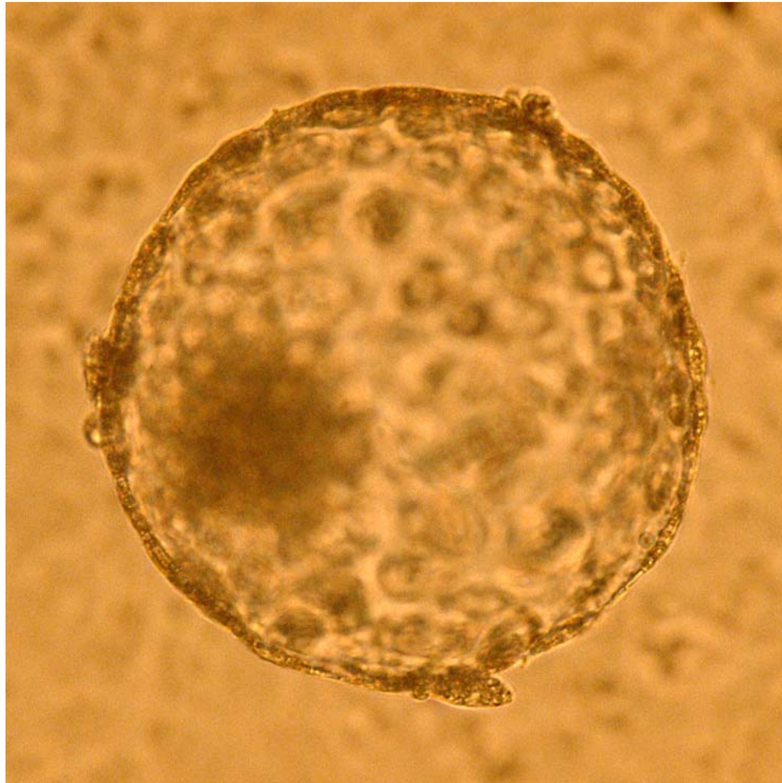


Fig. 13 In vitro bovine derived hatched blastocyst after 9 days in IVC.

3.2. Determination of the relative abundance of IGF-I, IGF-IR, IGF-II and IGF-IIR gene transcripts in bovine embryos

3.2.1. Isolation of RNA

3.2.1.1. Solutions used to isolate RNA from tissue

- TRIZOL Reagent (U.S.Patent No.5,346,994):
  - a mono-phasic solution of phenol and guanidine isothiocyanate
- Chloroform
- Isopropanol alcohol
- 75% Ethanol
- Autoclaved water

3.2.1.2. Solutions used to isolate RNA from bovine embryos

- Phosphate-buffered saline (PBS) pH 7.4:

  1 ml Dynabeads Oligo$(dT)_{25}$ is supplied in the kit as a suspension of 5 mg beads/ml PBS, containing 0.05% Tween-20 and 0.02% NaN_3 as a preservative.
  - 137 mM NaCl
  - 2.7 mM KCl
  - 4.3 mM $Na_2HPO_4.7H_2O$
  - 1.4 mM KH_2PO_4
- Lysis/binding buffer (l):

  (30 ml, supplied)
  - 100 mM Tris-HCl, pH 8.0
  - 500 mM LiCl
  - 10 mM EDTA, pH 8.0
  - 1% LiDS (SDS)
  - 5 mM dithiothreitol (DTT)

- Washing buffer with LiDS (2):
  (2 x 30 ml, supplied)
  - 10 mM Tris-HCl, pH 8.0
  - 0.15 M LiCl
  - 1 mM EDTA
  - 0.1% LiDS
- Washing buffer (3):
  (30 ml, supplied)
  - 10 mM Tris-HCl, pH 8.0
  - 0.15 M LiCl
  - 1 mM EDTA

3.2.1.3. Isolation of RNA from the tissues

As a positive control, total RNA was extracted from bovine oviduct and uterus by an improvement of the single-step RNA isolation method developed by Chomczynski and Sacchi (1987). Bovine tissue samples (0.2-0.3 g) were homogenized with 3 ml TRIZOL reagent in a 50 ml centrifuge tube (Greiner Labortechnik, Frickenhausen, Germany) and incubated for 5 min at room temperature, 200 µl chloroform was added to an 1 ml aliqot of the homogenate, shaken vigorously for 10 sec and incubated for 2 min at room temperature. Samples were centrifuged at 11. 000 × ***g*** for 15 min at 4°C. After centrifugation, the mixture separates into a lower red, phenol-chloroform phase, an interphase and a colorless upper aqueous phase. The aqueous phase (500 µl) was transferred to a fresh tube, mixed with 500 µl isopropanol alcohol, and then held at room temperature for 10 min. The samples were centrifuged again at 14, 000 × ***g*** for 15 min at 4°C. The resulting RNA pellet was washed by addition of 1 ml 75% ethanol, shaken vigorously and then centrifuged at 7500 × ***g*** for 5 min at 4°C. After centrifugation, ethanol was removed, the RNA pellet was dried (air-dry for 15 min), and then the pellet was dissolved in 100 µl autoclaved water. Contaminating genomic DNA was digested by incubating the samples with 10 IU DNase (Boehringer, Mannheim, Germany) for 30 min at 37°C.

DNase was inactivated by heating the samples to 80°C for 10 min. RNA concentration and purity were determined by spectrophotometry.

3.2.1.4. Isolation of RNA from the embryos

Poly(A)$^+$ RNA was isolated using a Dynabeads mRNA DIRECT Kit (Dynal, Oslo, Norway) according to the manufacturers instructions with minor modifications (Wrenzycki et al., 1999). Prior to use, 10 µl of Dynabeads oligo $(dT)_{25}$ were washed twice by lysis/binding buffer l (see above). Pools of oocytes or embryos (20-25) in 0.6 ml siliconized polyprobyl tube (No. 710136, Biozym Diagnostic GmbH, Hess Oldendorf, Germany) were lysed by adding 150 µl of lysis/binding buffer, vortexed, and centrifuged briefly. As an internal standard, 0.1 pg rabbit globin mRNA (BRL, Gaithersburg, MD) per oocyte or embryo was added to the tube. After vortexing for 10 sec, brief centrifugation and incubation at room temperature for 10 min, 10 µl of prewashed Dynabeads oligo $(dT)_{25}$ were pipetted into the tube. After 5 min incubation at room temperature, these were shaken vigorously for binding poly(A)$^+$ RNAs to the oligo(dT) Dynabeads, the beads were separated by placing the tube in a Dynal MPC-E-1 magnetic separator for 2 min. After removal of the supernatant, poly(A)$^+$ RNAs was washed once in 100 µl washing buffer with LiDS 2 (see above) and twice in 100 µl washing buffer 3 by adding the washing buffer, followed by brief shaking, 30 sec in the Dynal MPC-E-1 magnetic separator, and removal of the washing buffer by gentle pipetting. Poly(A)$^+$ RNAs were then eluted from the beads by incubation in 22 µl sterile water at 65°C for 2 min, and aliquots were used immediately for reverse transcription (RT).

3.2.2. Reverse transcription-polymerase chain reaction

3.2.2.1. Solutions and reagents

- 10 × RT buffer (Perkin-Elmer, Vaterstetten, Germany):
  - 100 mM Tris-HCL, pH 8.3
  - 500 mM KCl
- 50 mM $MgCl_2$ (Gibco BRL, Eggenstein, Germany)
- dNTPs (Amersham, Braunschweig, Germany)

  100 mM stock solution of dNTPs was diluted in water (1:10) and aliquoted in stock solution (10 mM) for using in Reverse transcriptase.
- Random hexamers (Perkin-Elmer, Vaterstetten, Germany)

50 µM supplied in 10 mM Tris-HCl, pH 8.3.

- RNAse-inhibitor (Perkin-Elmer, Vaterstetten, Germany): 20 IU / µl.
- MuLV (Murine Leukemia Virus) reverse transcriptase (Perkin-Elmer, Vaterstetten): 50 IU / µl.
- Autoclaved water

3.2.2.2. Reverse transcription

The reverse transcription reaction was carried out in a final volume of 20 µl. A master mix (MM) was used for reverse transcription to minimize pipetting errors. It was prepared by adding the reagents in 0.6 ml siliconized polyprobyl tubes (# 710136, Biozym Diagnostic GmbH) as shown in Table 6 and after brief centrifugation, MM was divided into 0.2 ml PCR tubes (# 711080, Biozym Diagnostic GmbH). Prior to use, total RNA from bovine tissue was thawed on ice or at 65°C for 30-60 sec, poly(A)$^{+}$ RNA isolated from the oocytes or embryos was reverse transcribed after the isolation. Solutions and reagents of reverse transcription were thawed at 65°C for 30-60 sec, and then placed on ice throughout the preparation of the MM. Through preparation the MM, sterile tubes were used and also the pipette tips were changed for each pipetting. After addition of poly(A)$^{+}$ RNA from the oocytes or embryos and total RNA from the tissue to the reverse transcription reaction, the combination was mixed, centrifuged briefly and then transferred to the PCR machine (PTC-2000, Peltier Thermal Cycler, MJ Research, INC. Watertown, Massachusetts, U.S.A.).

The RT reaction was carried out at 25°C for 10 min and 42°C for 1 hr followed by a denaturation step at 95°C for 5 min, and cooling to 4°C. After termination of the RT program, the reaction mixture was diluted to get a final concentration of 0.5 oocyte or embryo equivalent/µl and 50 fg globin RNA/µl.

3.2.3. Polymerase chain reaction (PCR)

3.2.3.1. Solutions and reagents

- 10 × PCR buffer (Gibco BRL, Eggenstein, Germany):

- 200 mM Tris-HCL, pH 8,4
- 500 mM KCl

- 50 mM $MgCl_2$ (Gibco BRL, Eggenstein, Germany)
- dNTPs (Amersham, Braunschweig, Germany)
  See RT (3.2.1.2.1)
- Taq-polymerase (Gibco BRL, Eggenstein, Germany): 5 IU/µl.
- Bovine specific primers (MWG-Biotech, Ebersberg, Germany) for IGF-I, IGF-IR, IGF-II, and IGF-IIR: The sequence and positions of the primers used, the annealing temperature, the fragment sizes, and the sequences reference of the expected PCR products are shown in Table 7.
- Rabbit globin primer: see also Table 7.
- Autoclaved water.

Table 6: Composition of the Master mix for reverse transcription

Component	Sample poly(A)$^+$RNA from oocytes/embryos	Globin-RNA without preparation	Negative sample Without reverse transcription	Negative sample without RNA
$MgCl_2$: (50 mM)	2 µl	2 µl	2 µl	2 µl
10× RT-buffer	2 µl	2 µl	2 µl	2 µl
dNTPs: (10 mM)	2 µl	2 µl	2 µl	2 µl
Hexamers Primer [Oligo d(T) 50µM]	1 µl	1 µl	1 µl	1 µl
RNase inh.: (20 U/µl)	1 µl	1 µl	-	1 µl
Rev. Trans.: (50 U/µl)	1 µl	1 µl	-	1 µl
Poly(A)$^+$ RNAs	11 µl	-	11 µl	-
Globin-RNA*	-	2-2.5 µl	-	-
H_2O	-	8.5-9 µl	2 µl	11 µl

* Globin RNA: 1 pg/µl

Table 7: Primers used for PCR

Genes Sequences references [EMBL accession no.]	Primer sequences and positions	Annealing Temperature (°C)	Fragment Size (bp)
Globin Cheng et al. (1986) [X04751]	GCAGCCACGGTGGCGAGTAT (241-260) GTGGGACAGGAGCTTGAAAT (555-657)	60	257
IGF-I Fotsis et al. (1990) [X15726]	GATGGGCATTTCCCCCAATGAA- ATAAGTAA (580-609) CTGTAAAACAAACAGCCTGTGT -TGCGTAGA (884-913)	55	334
IGF-IR Sneyers et al. (1991) [X54980]	CATCTCCAACCTCCGGCCTTTT- ACTCT (186-213) CCCAGCCTGCTGCTATTTCTTTT -TCTAT (695-722)	59	538
IGF-II Brown et al. (1990) [X53553]	CTTCAGCCGACCATCCAGCCGC- ATAAAC (67-96) TCAGCGGACGGTGACTCTTGGC- CTCTCT (362-389)	64	323
IGF-IIR Lobel et al. (1987) & (1988) [J03527 & M15869]	CGCCTACAGCGAGAAGGGGTTA -GTC (4799-4823) AGAAAAGCGTGCACGTGCGCTT -GTC (5067-5091)	62	293

3.2.3.2. Preparation of the reaction mixture and PCR

PCR was performed in a final volume of 50 µl PCR buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl, Gibco BRL, Eggenstein, Germany), 1.5 mM $MgCl_2$, 200 µM of each dNTP, 0.5 µM of each sequence-specific primer (except for IGF-II primer which was used at a concentration of 1.0 µM) as shown in Table 8. A master mix (MM) was used to minimize pipetting errors. It was prepared by collecting the reagents in a 1 ml eppendorf tube (Greiner Labortechnik,

Frickenhausen, Germany). Prior to use, cDNA from bovine tissue was thawed on ice or at 65°C for 30-60 sec but, cDNAs of oocytes or embryos were used directly for PCR after reverse transcription. Solutions and reagents of MM-PCR were thawed at 65°C for 30-60 sec, mixed and then placed on ice throughout the preparation of the MM. To eliminate contamination, rubber gloves were worn throughout the work, sterile tubes were used, the pipette tips were changed for each pipetting. MM was prepared in a special room which was cleaned by 50 mM NaOH and 70% ethanol. After brief centrifugation, MM was divided into 0.2 ml PCR tubes (No. 711080, Biozym Diagnostic), cDNAs from oocytes or embryos and bovine tissues were added and the tubes were placed into a PTC-200 thermocycler (MJ Research, Watertown, MA). The polymerase chain reaction (PCR) was performed with cDNA equivalents corresponding to 4 oocytes or embryos (IGF-II: 8 embryos equivalents) from different pools of oocytes or embryos generated in different IVP runs and 100 fg of globin RNA by employing a PCR program as described in Table 8. Throughout hot starts to obtain specific amplification, 0.2µl Taq-polymerase was added to each PCR tube.

Table 8: Master mix reactions of PCR

Component	1× MM	Concentration
Primer: (20 µM)	1.3 or 2.5 µl	0.5 or 1 µM
dNTP's: (10 mM)	1 µl	0.2 µM
$MgCL_2$: (50 mM)	1.5 µl	1.5 mM
10× PCR-buffer	5 µl	1×
Taq polymerase: (5U/µl)	0.2 µl	1 U
H_2O	depend on cDNA value	-

3.2.3.3. Temperature of the PCR-program

Temperature profile of the PCR-program to assess the relative abundance of IGFs is described in Table 9.

Table 9: Temperature profile of the PCR program to assess the relative abundance of IGFs

Hot start:	97°C	2 min	
	72°C	2 min	Addition of Taq DNA-polymerase
35 cycles (IGF-I)	95°C	15 sec	Denaturation of DNA
37 cycles (IGF-II)	* d.t.	15 sec	Annealing of the primer
35 cycles (IGF-IR)	72°C	15 sec	Extension of DNA
35 cycles (IGF-IIR)			
27 cycles (globin)			
	72°C	5 min	Extension
	4°C	Forever	

* d.t.; different temperature, where the annealing temperatures are different for each primer dependent on sequences of the primer and each primer had a optimal annealing temperature (60°C: Globin, 55°C: IGF-I, 59°C: IGF-IR, 64°C: IGF-II and 62°C: IGF-IIR).

3.2.3.4. Optimization of the PCR parameters for each gene

PCR parameters were optimized for some genes, at first employing general conditions, which had usually provided satisfactory results. These general conditions are done in a 50 µl volume and, in addition to the sample of cDNA, containing 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 0.5 µM of each sequence-specific primer and 1 unit of taq DNA polymerase. The temperature of the PCR-program is described in Table 8. These general conditions were used in this work except for IGF-II primer which was used at a concentration of 1.0 µM to obtain good results.

3.2.3.5. Determination the linear range of amplification for each gene

For each pair of gene-specific primers, semilog plots of the fragment intensity as a function of cycle number were used to determine the range of cycle numbers in which linear fragment production occurred and the number of PCR cycles was kept within this range. These preliminary amplification experiments were performed on a fixed amount of RNA (Wrenzycki et al., 1999). The point at which an exponential accumulation plateau is reached,

estimated by noting the point at which continued cycles do not produce a doubling in products. For the insulin-like growth factor genes and their receptors, a linear range of amplifications was observed from 31 to 39 amplification cycles. Linear fragment means that, the PCR products (output) depend on the value of cDNA (input).

3.2.3.6. Controls in RT-PCR

As negative controls, tubes were prepared with water instead of RNA. To control water, solutions and reagents were prepared in which reverse transcriptase was omitted during the RT-reaction to control for genomic DNA contamination.

3.2.4. Analysis of the RT-PCR products by agarose gel electrophoresis

3.2.4.1. Solutions and reagents

- Electrophoresis buffer is Tris-Acetate-EDTA (TBE-)-buffer.
  Stock solutions of 5× TAE-buffer containing
  - 90 mM Tris (# T 1503, Sigma)
  - 90 mM borate pH 8,3
  - 2 mM EDTA solutions
  - in 1 litre autoclaved distilled water

Prior to use, stock solution was diluted by ratio 1:5 to prepare 1× TAE-buffer.

- Loading buffer: 5× stock solution was contained
  - 50% glycerol (Merck, Darmstadt, Germany)
  - 25 mM EDTA (Merck, Darmstadt, Germany)
  - 0,25% Xylenecyanol (Serva, Heidelberg, Germany)
- Ethidium bromide: 10 mg/ml in distilled water

To determine amplification product size and quantity, a 1.5% agarose gel (10 cm long by 15 cm wide with wells) was employed. It was produced by dissolving 1.5 g agarose (Gibco/BRL, Eggenstein, Germany) in 100 ml 1× TAE-buffer by boiling in microwell,

cooling to ~65°C, adding 2µl EtBr-solution, mixing by swirling and pouring into gel electrophoresis tank for 45 min at room temperature. This gel can be stored in plastic cover at 4°C.

3.2.4.2. Gel electrophoresis

After addition of 5 µl of 10× loading buffer, RT-PCR products were subjected to electrophoresis on a 1.5% agarose gel in lx TBE buffer containing 0.2 pg / ml EtBr with further addition of EtBr in the same concentration to the running buffer. After running at 80 V for 45 min, the fragments were visualized on a 312 nm UV-transilluminator. The image of each gel was recorded with CCD camera (Quantix, Photometrics, München, Germany) and the IP Lab Spectrum program (Signal Analytics Corporation, Vienna, VA). The intensity of each band was determined by densitometry using an image analysis program (IP Lab Gel). The relative amount of the mRNA of interest was calculated by dividing the intensity of the band for each developmental stage by the intensity of the globin band for the corresponding stage. For each mRNA, experiments were repeated with a minimum of four or five separate oocyte or embryo batches, each derived from different IVP runs.

3.2.5. Verification of RT-PCR products

RT-PCR products were verified by comparing the size of the PCR fragment obtained with the expected size. The RT-PCR products from each primer pair were sequenced and identified by a commercial sequencing service (Toplab GmbH, Martinsried, Germany).

3.3. Experimental design

The relative abundance of IGF-I and IGF-II and their receptors (IGF-IR & IGF-IIR) gene transcripts in bovine immature and matured oocytes, zygotes, 2-4-cell, 8-16-cell stages, morulae, expanded blastocysts and hatched blastocysts produced in vitro using two different culture systems (TCM-system/SOF-system) were determined. Factors compared were the two different culture systems (TCM199 supplemented with serum & SOF supplemented with PVA) and the different embryonic stages (Fig. 14 & Fig. 15).

3.4. Statistical Analysis

Data were analyzed using the SigmaStat 2.3 (Jandel Scientific, San Rafael, CA) software package. After testing for normality (Kolmogorov-Smirnov test with Lilliefor correction) and equal variance (Levene Median test); a one-way ANOVA (or a one-way ANOVA on ranks) followed by multiple pairwise comparisons using either Tukey test or Duncan's method was employed to determine differences between both treatments at the identical developmental stage and in the temporal expression patterns within one treatment group. Differences of $P \leq 0.05$ were considered to be significant.

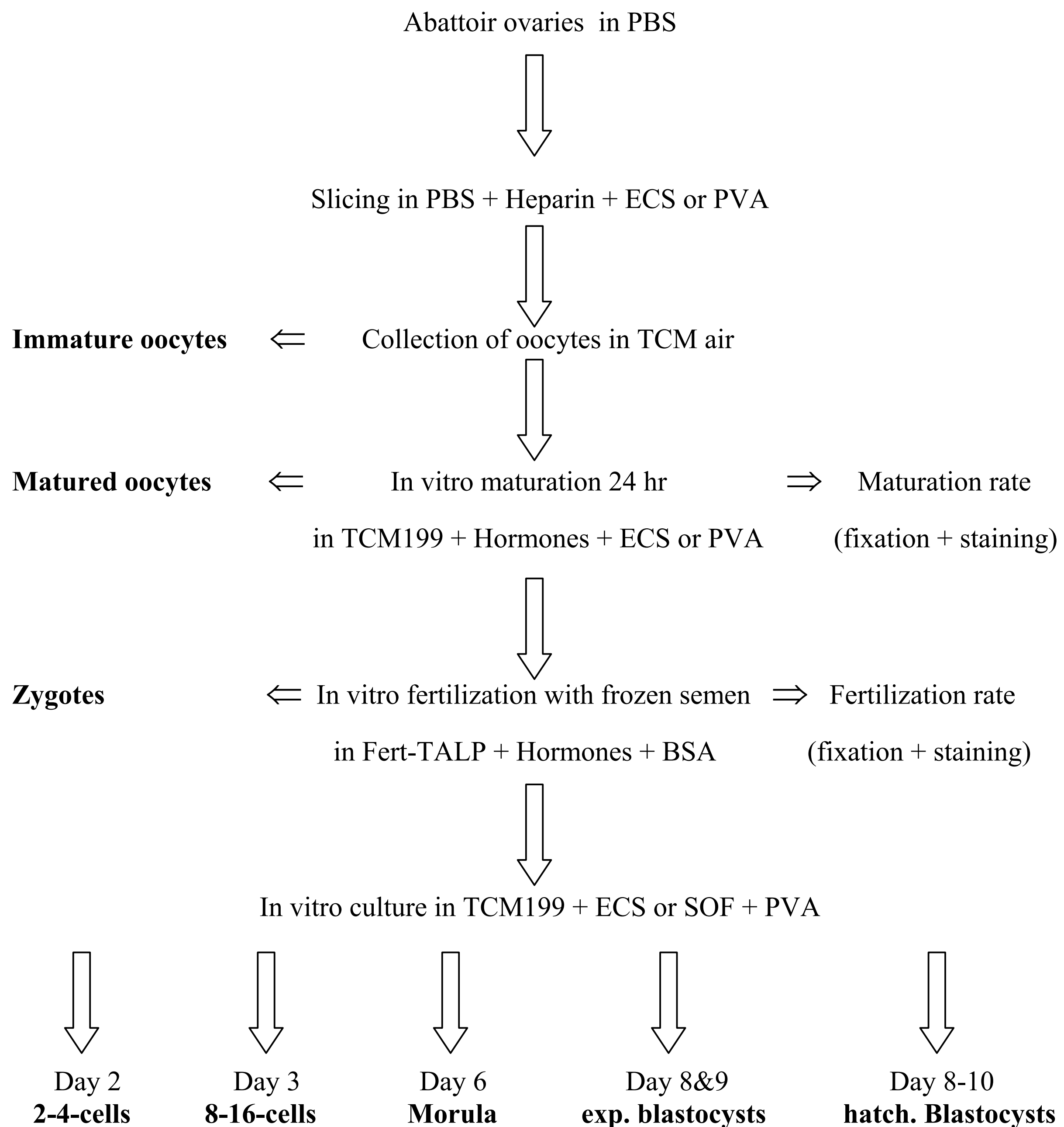


Fig. 14 Overview on experimental design for in vitro production of embryos from different culture systems.

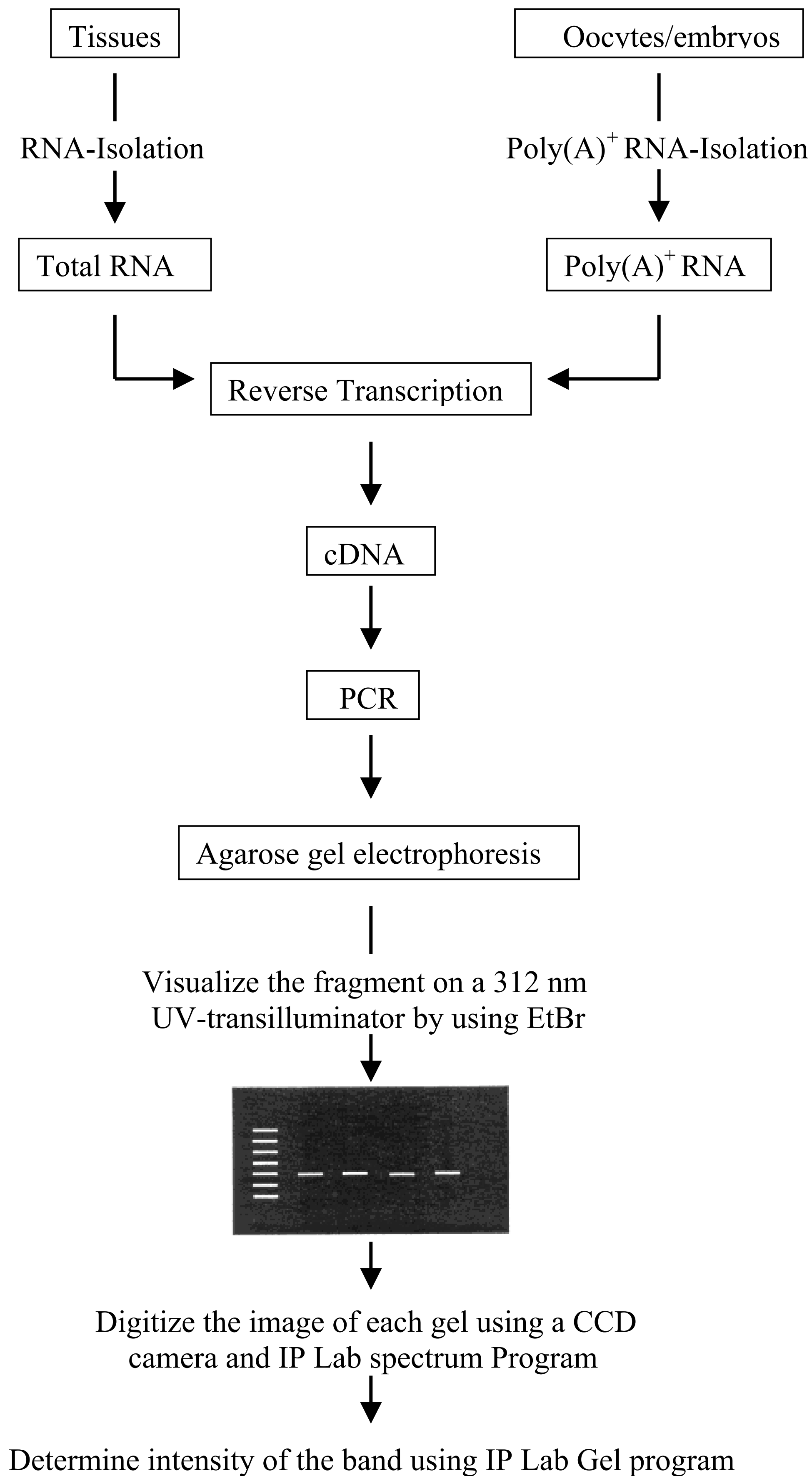


Fig. 15 Experimental design for detection of gene transcription.

4. RESULTS

4.1. Effect of the in vitro-culture systems on the embryonic development

This study was conducted in the period from July 1998 to October 2000. A total of 6325 oocytes (category I & II) from 997 ovaries (6.7 ± 1.7 oocytes/ovary) were used for this study. Immature oocytes, matured oocytes, zygotes, 2-4-cells, 8-16-cells, morulae, expanded blastocysts and hatched blastocysts were produced using two different in vitro culture systems (TCM-system and SOF-system). The TCM-system is a complex, undefined medium and the SOF-system is a simple, chemically defined medium. The developmental rates of bovine embryos generated in the two medium systems are summarized in Table 10. Maturation and fertilization rates tended to be higher in the TCM-system than in the SOF-system. A significantly higher percentage of 2-4-cell and blastocysts was obtained in the TCM-system than in SOF-system. Embryos cultured in the presence of serum appeared dark with numerous "granules", whereas the serum-free system yielded much lighter embryos with apparently fewer "granules."

4.2. Optimization of the PCR parameters

PCR parameters consisted of 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 0.5 µM of each sequence-specific primer and 1 unit of Taq DNA polymerase in addition to the sample of cDNA. The temperature cycles of the PCR-program are described in Table 8. These conditions provided satisfactory results except for the IGF-II primers which were used at a concentration of 1.0 µM to obtain a reproducible bands. This concentration was determined after testing (Fig. 16).

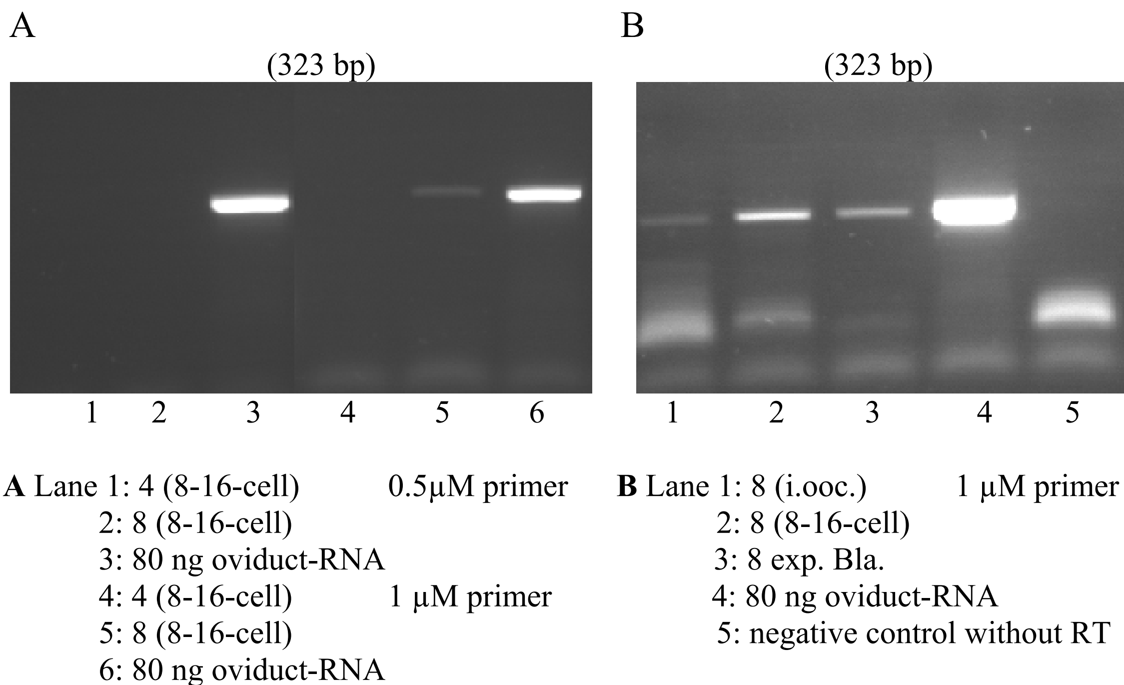
4.3. Optimization of the semi-quantitative RT-PCR assay

To optimize the amplification reaction for each pair of gene-specific primers, semilog plots of the fragment intensity as a function of cycle number. A cycle numbers in which linear fragment

Table 10: Maturation, Fertilization and Developmental Rates (Means ± SEM) of Bovine Embryos Produced in Vitro

Developmental stage	Percentage of embryos (relative to absolute number of oocytes)			
	TCM-System	No. of replicates	SOF-System	No. of replicates
Maturation rate	91 ± 4.9 (24/26)	3	76.5 ± 2.4 (33/43)	3
Fertilization rate	70.9 ± 8.3 (17/24)	3	64.2 ± 3.4 (23/36)	3
2-4-cell embryos (day 2)	60.7 ± 1.0[a] (281/468)	6	54.8 ± 1.8[b] (447/828)	8
8-16-cell embryos (day 3-4)	47.0 ± 1.7 (167/371)	8	46.8 ± 2.3 (236/514)	6
Morulae (day 6)	33.1 ± 0.7 (135/410)	7	28.3 ± 2.8 (198/523)	7
Expanded Blastocysts (day 8/9)	25.0 ± 1.2[a] (220/819)	11	16.5 ± 0.6[b] (174/1061)	12

∗Numbers with different superscripts within one line are significantly different (a:b $P \leq 0.05$). The total numbers vary for each developmental stage since the aim was to produce all developmental stages in one IVP run.



A Lane 1: 4 (8-16-cell) 0.5µM primer
2: 8 (8-16-cell)
3: 80 ng oviduct-RNA
4: 4 (8-16-cell) 1 µM primer
5: 8 (8-16-cell)
6: 80 ng oviduct-RNA

B Lane 1: 8 (i.ooc.) 1 µM primer
2: 8 (8-16-cell)
3: 8 exp. Bla.
4: 80 ng oviduct-RNA
5: negative control without RT

Fig. 16 Optimization of IGF-II gene bands by using two different concentrations of IGF-II primers (0.5 µM vs 1 µM).

production occurred was determined and the number of PCR cycles was kept within this range. These preliminary amplification experiments were performed on a fixed amount of RNA (Wrenzycki et al., 1999). A linear range was obtained when the amount of PCR product was approximately doubled upon doubling of the initial RNA input. For the IGF genes and their receptors, a linear increase in the PCR products was observed from 31 to 39 amplification cycles (figures 17-19).

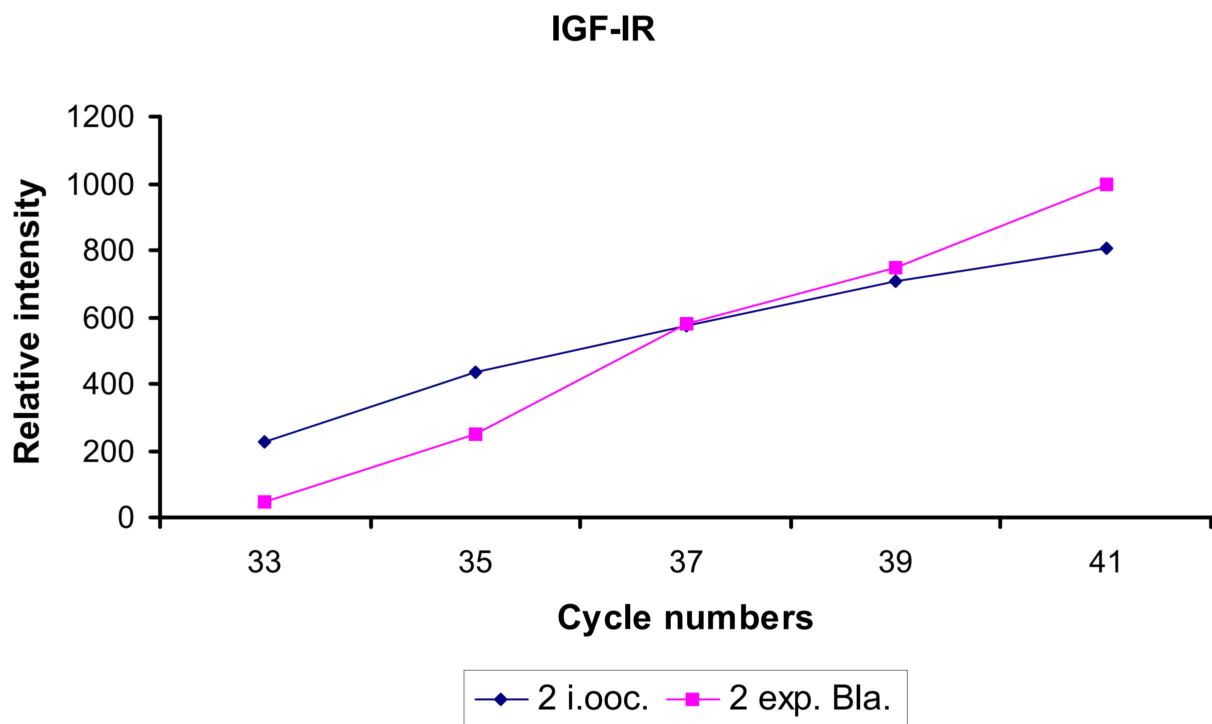


Fig. 17 Validation of the semi-quantitative RT-PCR assay regarding the number of cycles using a gene specific primer pair to detect IGF-IR RNA.

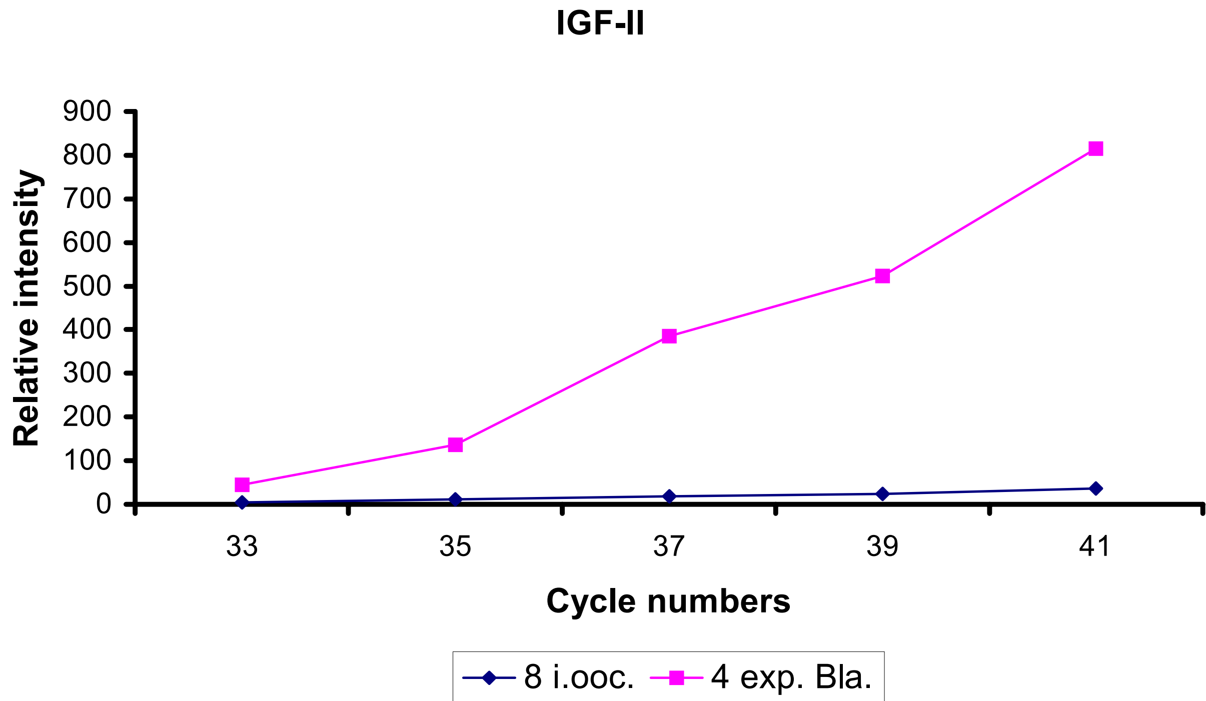


Fig. 18 Validation of the semi-quantitative RT-PCR assay regarding the number of cycles using a gene specific primer pair to detect IGF-II RNA.

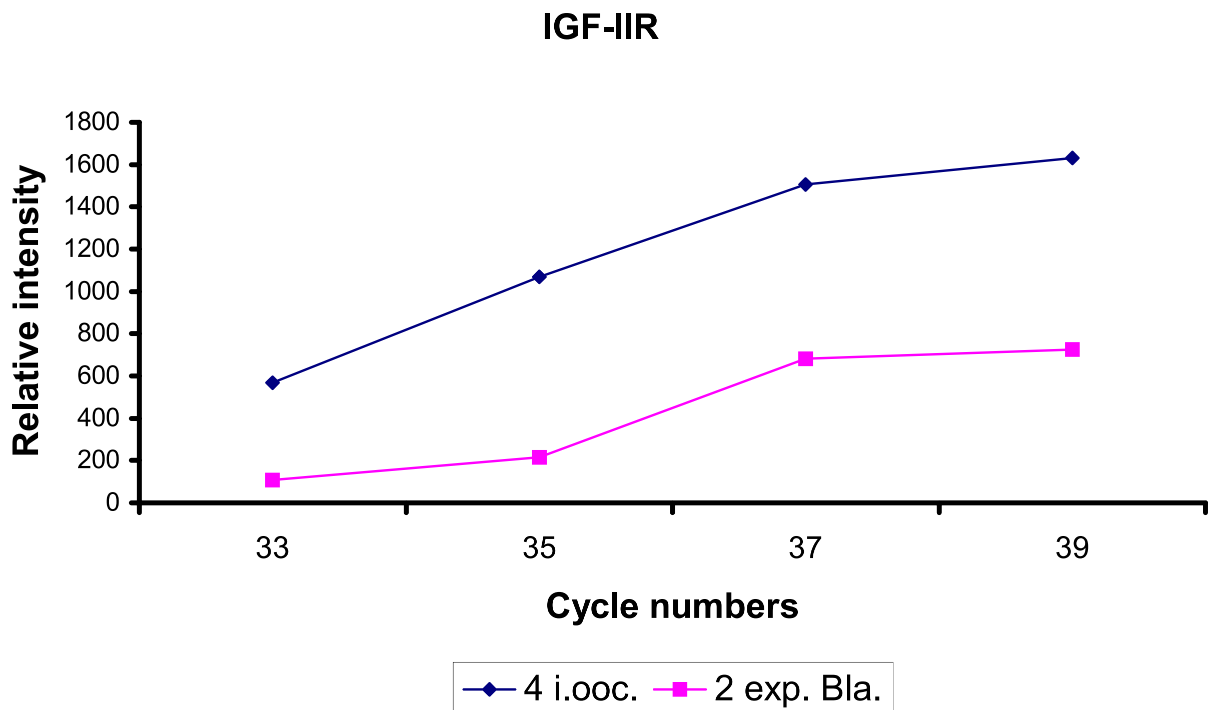


Fig. 19 Validation of the semi-quantitative RT-PCR assay regarding the number of cycles using a gene specific primer pair to detect IGF-IIR RNA.

At the same time, the fixed cycle number was used to prove that the amount of added RNA gave a proportional output of RT-PCR product (Fig. 20). At 37 cycles (IGF-I and IGF-II) and 35 cycles (IGF-IR and IGF-IIR), a linear increase in the PCR products as a function of RNA input was found (Figure 20A example for IGF-IR). Globin RNA, added as an internal standard, was effectively amplified at 27 PCR cycles (Figure 20B), and showed a linear increase in the amount of PCR products as a function of RNA input from 50 fg up to 400 fg (Fig. 20B).

The RNA recovery rate was estimated as the ratio between the intensity of the globin fragment with and without RNA preparation procedure, starting with an equivament of 50 fg in the PCR reaction (Wrenzycki et al., 1998b; 1999). On the average, 66% of poly(A)-tailed RNA was recovered using our Dynabeads oligo d(T) mRNA isolation method.

Quantification of the product abundance and calculation of the coefficint of variation (CV: the standard deviation expressed as a percentage of the mean) revealed that the variation in the amount of one gene transcript ranged from 24-58% among experiments (interassay CV). The intraassay-CV was estimated from replicated PCR amplifications of the same globin cDNA template to detecte differences caused by the PCR reaction starting with an equivalent of 50 fg. This intraassay-CV amounted to 8 ± 6% (globin without preparation) and 12 ± 5% (globin with preparation). The CV for the relative abundance (RA-CV) calculated from the values of the relative amount of each transcript obtained from the different experiments ranged from 37-47%.

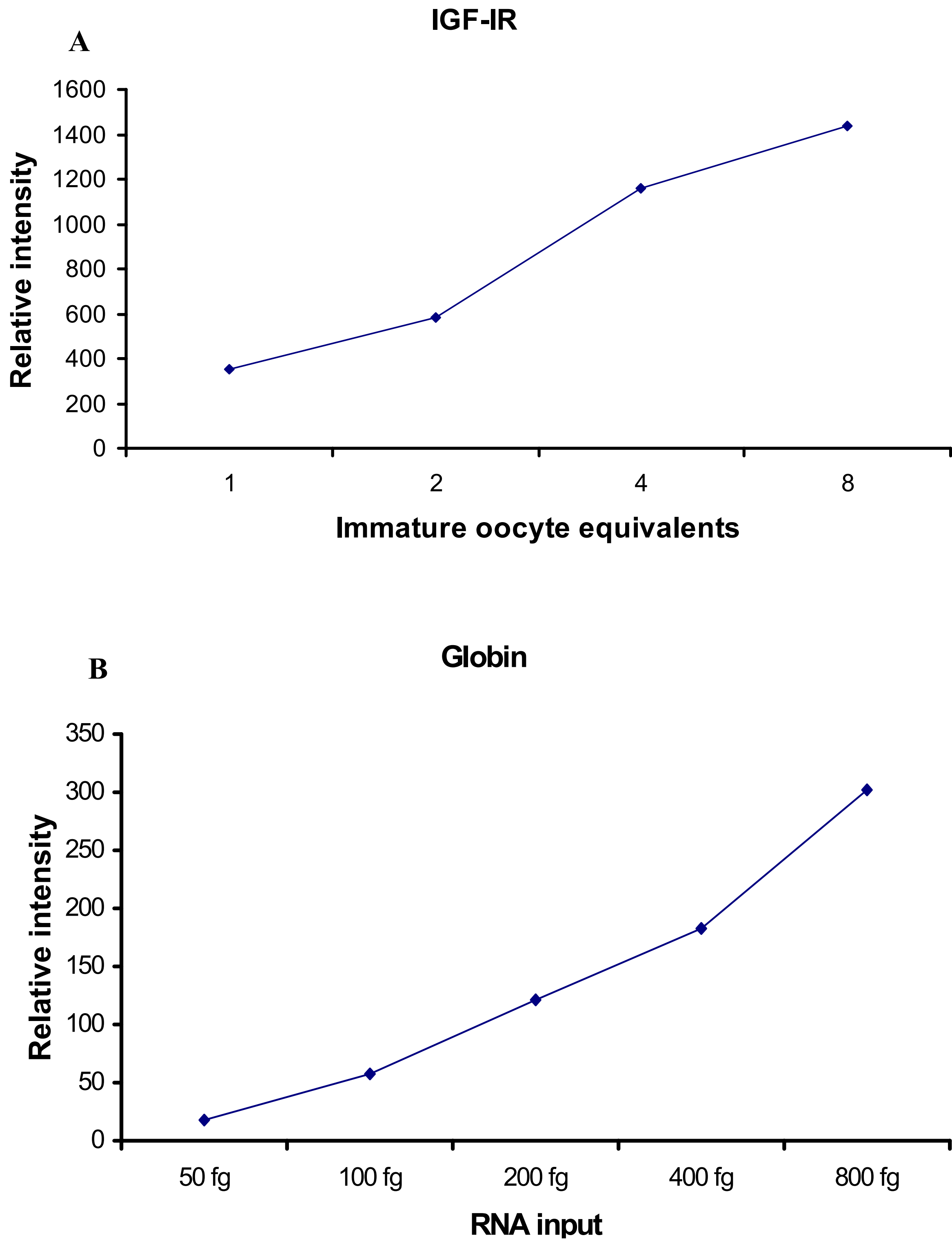


Fig. 20 Validation of the semi-quantitative RT-PCR assay regarding the amount of RNA input (A: variable amounts of immature oocytes equivalents using a gene specific primer pair to detect IGF-IR RNA; B: variable amounts of globin RNA). PCR cycle number was fixed to 35 (A) or 27 (B).

4.4. Ontogenetic expression pattern of IGFs

IGF-I mRNA-transcripts were not detected at any stage of preimplantation bovine development in both culture systems even when the PCR was performed with cDNA equivalents corresponding to 16 oocytes or embryos as shown in Figures 21 and 22. As positive control, IGF-I was detected in bovine oviduct- and uterus-RNA (Fig. 23).

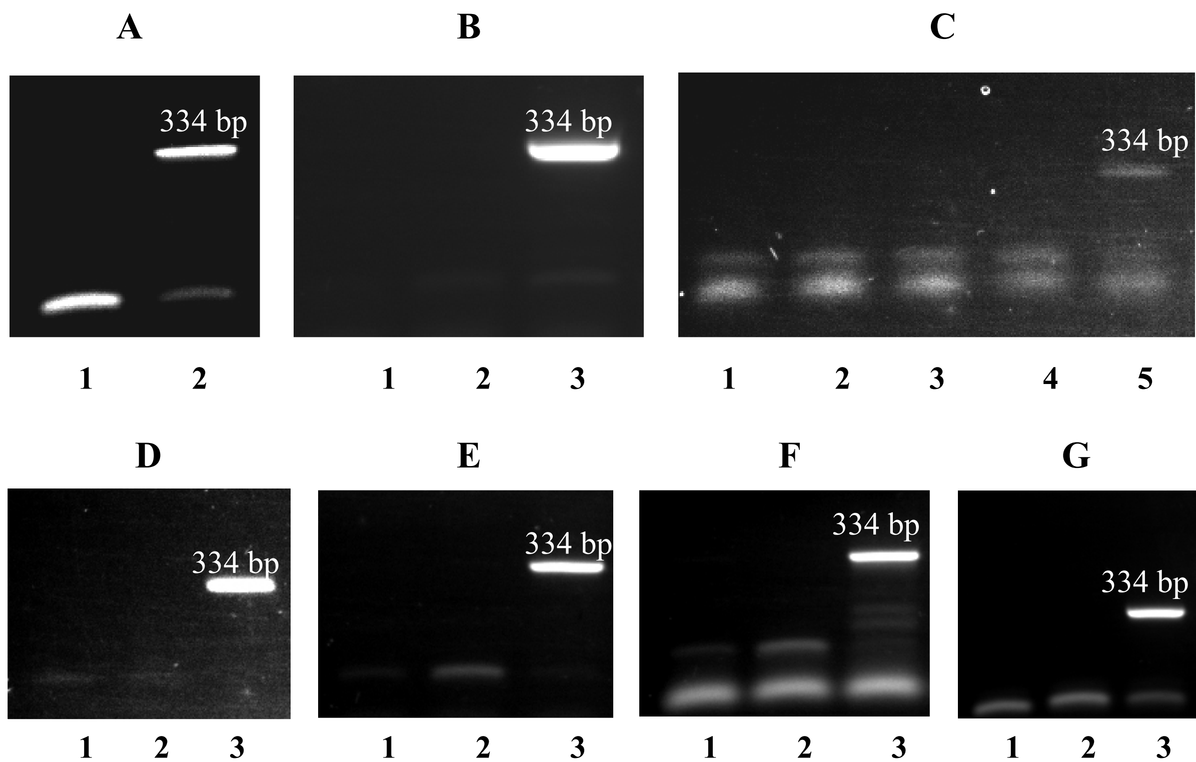


Fig. 21 Analysis of IGF-I transcripts from bovine oocytes and embryos produced in vitro in the SOF-system and from bovine oviduct RNA as positive control. Lane (A1) 16 i.ooc., (A2) 80 ng oviduct RNA, (B1) 8 m.ooc., (B2) 16 m.ooc.,(B3) 80 ng oviduct RNA, (C1) 8 zyg., (C2) 16 zyg., (C3) 8 2-4-cell, (C4) 16 2- 4- cell, (C5) 10 ng oviduct RNA, (D1) 8 8-16-cell, (D2) 16 8-16-cell, (D3) 80 ng oviduct RNA, (E1) 8 morulae, (E2) 16 morulae, (E3) 80 ng oviduct RNA, (F1) 8 exp.bla., (F2) 16 exp.bla., (F3) 80 ng oviduct RNA, (G1) 8 hbla., (G2) 16 hbla. And (G3) 80 ng oviduct RNA.

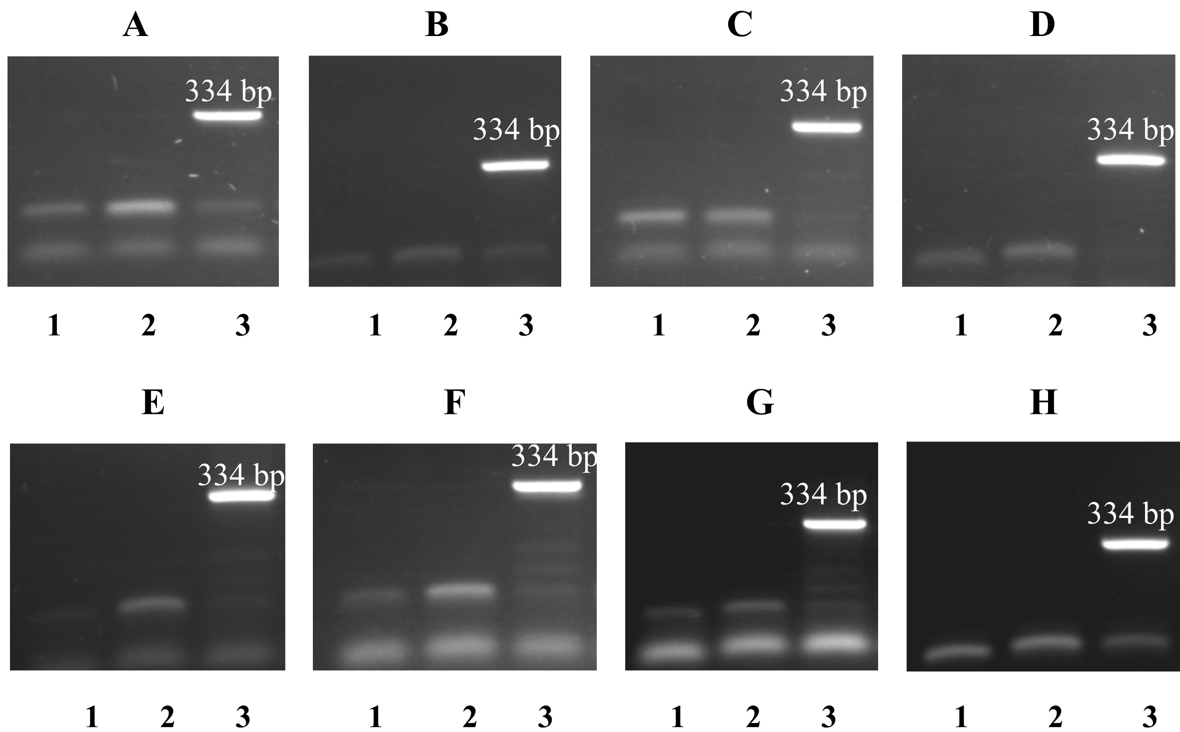


Fig. 22 Analysis of mRNA IGF-I transcripts from bovine oocytes and embryos produced in vitro in the TCM-system and also from bovine oviduct RNA as positive control. Lane (A1) 8 i.ooc., (A2) 16 i.ooc., (A3) 80 ng oviduct RNA, (B1) 8 m.ooc., (B2) 16 m.ooc., (B3) 80 ng oviduct RNA, (C1) 8 zyg., (C2) 16 zyg., (C3) 80 ng oviduct RNA, (D1) 8 2-4-cells, (D2) 16 2-4-cells, (D3) 80 ng oviduct RNA, (E1) 8 8-16-cells, (E2) 16 8-16-cells, (E3) 80 ng oviduct RNA, (F1) 8 morulae, (F2) 16 morulae, (F3) 80 ng oviduct RNA, (G1) 8 exp.bla., (G2) 16 exp.bla., (G3) 80 ng oviduct RNA, (H1) 8 hbla., (H2) 16 hbla. and (H3) 80 ng oviduct RNA.

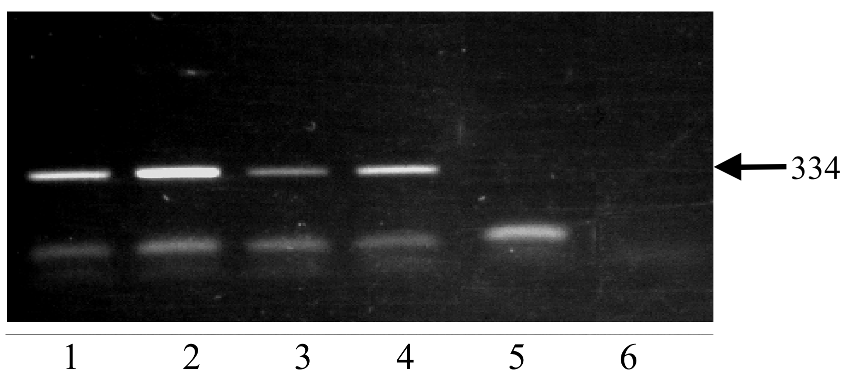


Fig. 23 Detection of IGF-I transcripts in RNA from bovine oviduct and uterus. Lane (1) 20 ng oviduct-RNA, (2) 40 ng oviduct-RNA, (3) 20 ng uterus-RNA, (4) 40 ng uterus-RNA, (5) negative control without RT and (6) negative control without RNA. Arrow: expected size of the fragment in bp.

IGF-IR was detected in the bovine embryonic stages from immature oocytes up to hatched blastocysts (Fig. 24 to 31).

Similarly, IGF-II and IGF-IIR were detected from the immature oocyte stage to the hatched blastocyst stage as shown in Fig. 24 to 31.

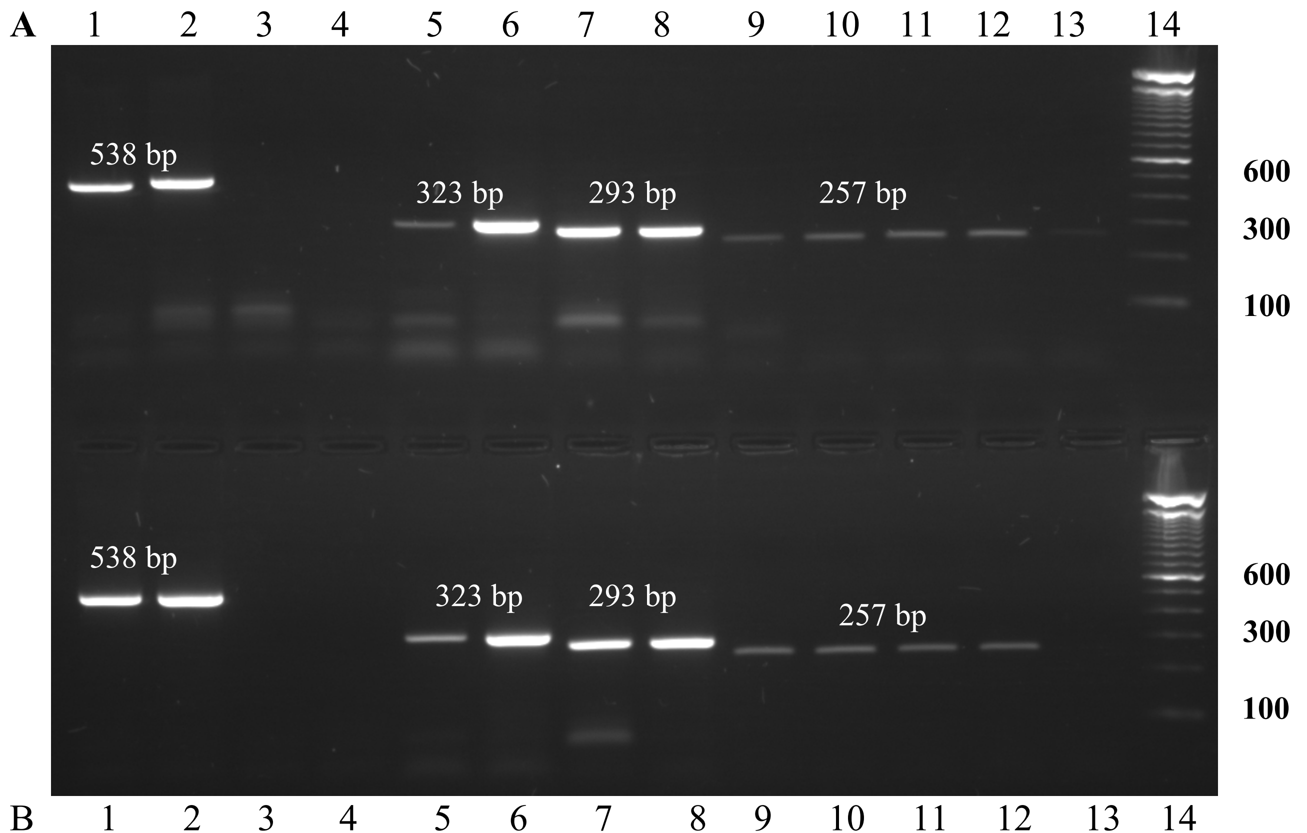


Fig. 24 Semi-quantitative RT-PCR analysis of mRNA IGF-IR, IGF-II and IGF-IIR in in vitro produced bovine immature oocytes (A: oocytes collected in SOF-systems and B: oocytes collected in TCM-systems, see Materials and Methods for details). Each lane represents the RT-PCR products derived from polyA$^+$ RNA from the equivalent of 4 oocytes (IGF-II:8 oocytes). Lanes (A1) oocytes-IGF-IR, Lane (A2) 80 ng oviduct RNA as positive control, (A3) negative control without RT, (A4) negative control without RNA, (A5) oocytes-IGF-II, (A6) 80 ng oviduct RNA, (A7) oocytes-IGF-IIR, (A8) 80 ng oviduct RNA, (A9-10) 100 fg globin RNA without preparation, (A11-12) 100 fg globin RNA with preparation, (A13) 50 fg globin RNA and (A14) MW marker. Lanes (B1) oocytes-IGF-IR, (B2) 80 ng oviduct RNA as positive control, (B3) negative control without RT, (B4) negative control without RNA, (B5) IGF-II, (B6) 80 ng oviduct RNA, (B7) oocytes-IGF-IIR, (B8) 80 ng oviduct RNA, (B9-10) 100 fg globin RNA without preparation, (B11-12) 100 fg globin RNA with preparation, (B13) negative controls without RT for globin and (A14) MW marker. The RNA recovery rate was estimated as the ratio between the intensity of the fragment from globin with preparation and without preparation procedure.

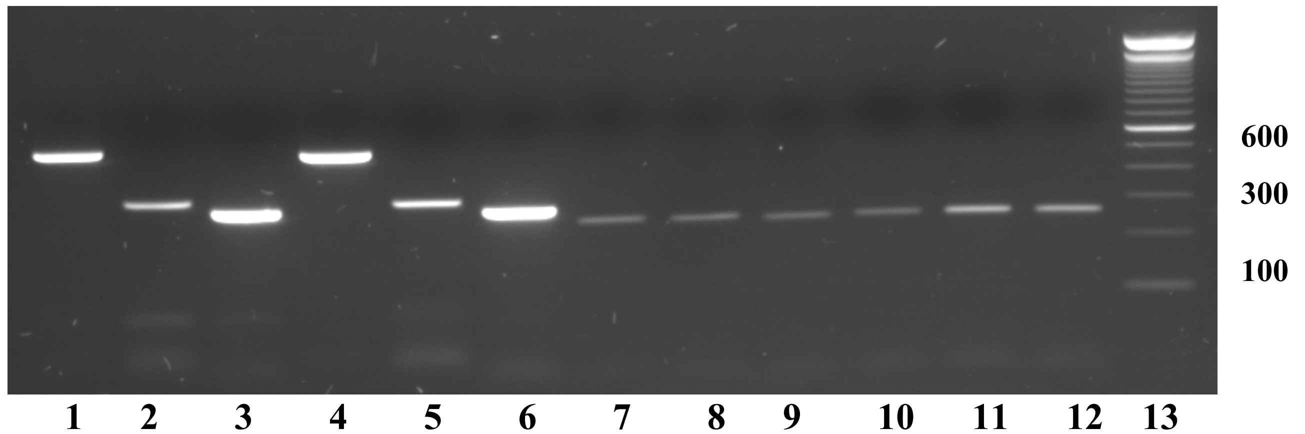


Fig. 25 Semi-quantitative RT-PCR analysis of mRNA IGF-IR, IGF-II and IGF-IIR in in vitro matured bovine oocytes. Each lane represents the RT-PCR products derived from polyA$^+$ RNA from the equivalent of 4 oocytes (IGF-II:8 oocytes). Lanes (1-3) IGF-IR, IGF-II and IGF-IIR in the SOF-system, respectively, lanes (4-6) IGF-IR, IGF-II and IGF-IIR in the TCM-system, respectively, lanes (7-8) 100 fg globin RNA with preparation in the SOF-system, lanes (9-10) 100 fg globin RNA with preparation in the TCM-system, lanes (11-12) 100 fg globin RNA without preparation and lane (13) MW marker. The RNA recovery rate was estimated as the ratio between the intensity of the fragment from globin with preparation and without preparation procedure (expected fragment sizes in brackets).

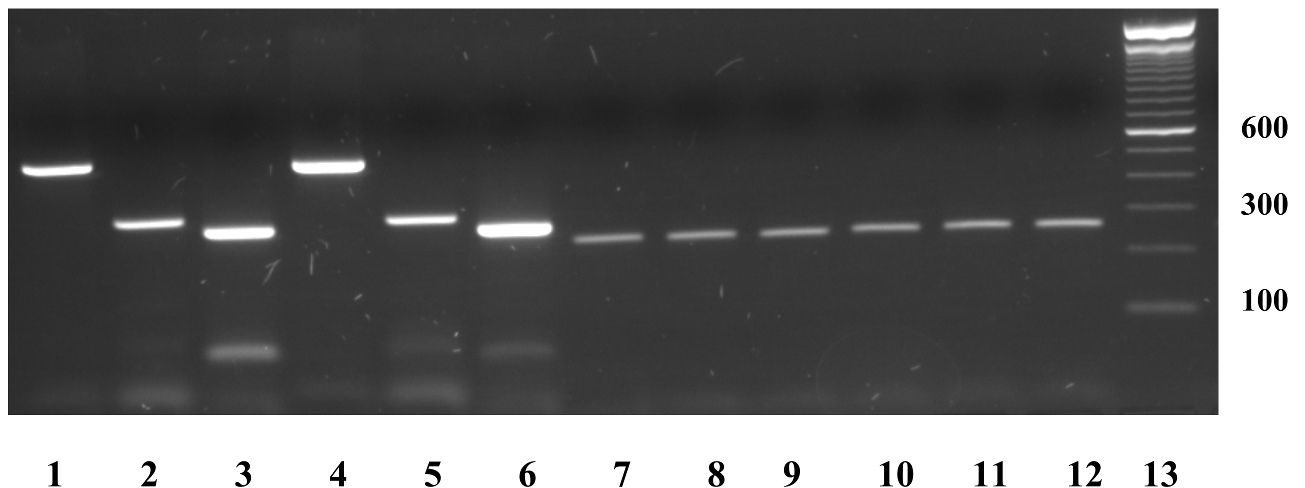


Fig. 26 Semi-quantitative RT-PCR analysis of mRNA IGF-IR, IGF-II and IGF-IIR in in vitro produced bovine zygotes. Each lane represents the RT-PCR products derived from polyA$^+$ RNA from the equivalent of 4 zygotes (IGF-II:8 zygotes). Lanes (1-3) IGF-IR, IGF-II and IGF-IIR in the SOF-system, respectively, lanes (4-6) IGF-IR, IGF-II and IGF-IIR in the TCM-system, respectively, lanes (7-8) 100 fg globin RNA with preparation in the SOF-system, lanes (9-10) 100 fg globin RNA with preparation in the TCM-system, lanes (11-12) 100 fg globin RNA without preparation and lane (13) MW marker. The RNA recovery rate was estimated as the ratio between the intensity of the fragment from globin with preparation and without preparation procedure (expected fragment sizes in brackets).

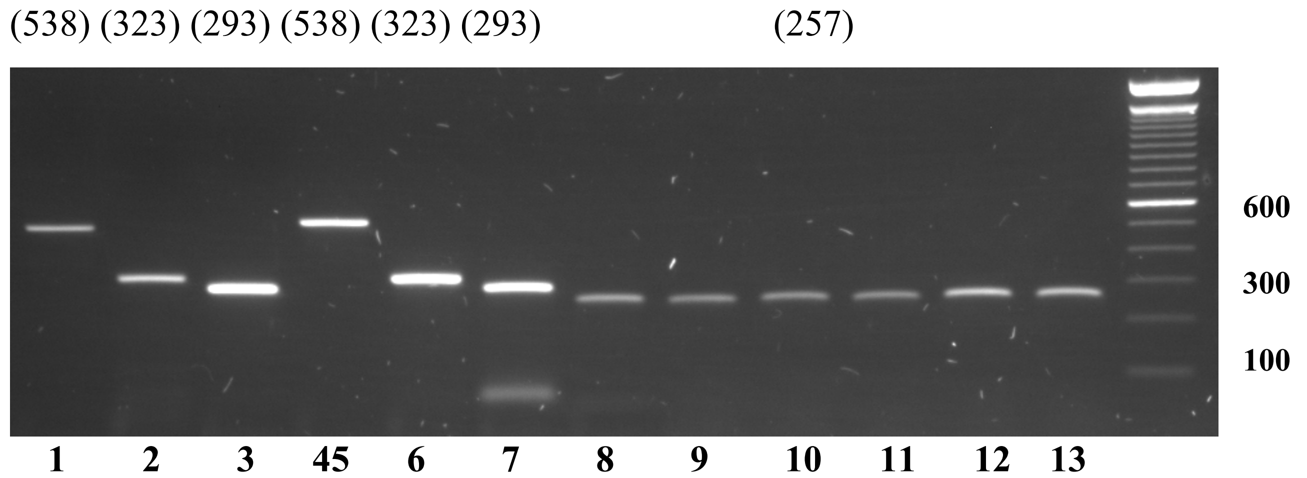


Fig. 27 Semi-quantitative RT-PCR analysis of mRNA IGF-IR, IGF-II and IGF-IIR in in vitro produced bovine 2-4-cells. Each lane represents the RT-PCR products derived from polyA$^+$ RNA from the equivalent of 4 embryos (IGF-II:8 embryos). Lanes (1-3) IGF-IR, IGF-II and IGF-IIR in the SOF-system, respectively, lanes (4-6) IGF-IR, IGF-II and IGF-IIR in the TCM-system, respectively, lanes (7-8) 100 fg globin RNA with preparation in the SOF-system, lanes (9-10) 100 fg globin RNA with preparation in the TCM-system, lanes (11-12) 100 fg globin RNA without preparation and lane (13) MW marker. The RNA recovery rate was estimated as the ratio between the intensity of the fragment from globin with preparation and without preparation procedure (expected fragment sizes in brackets).

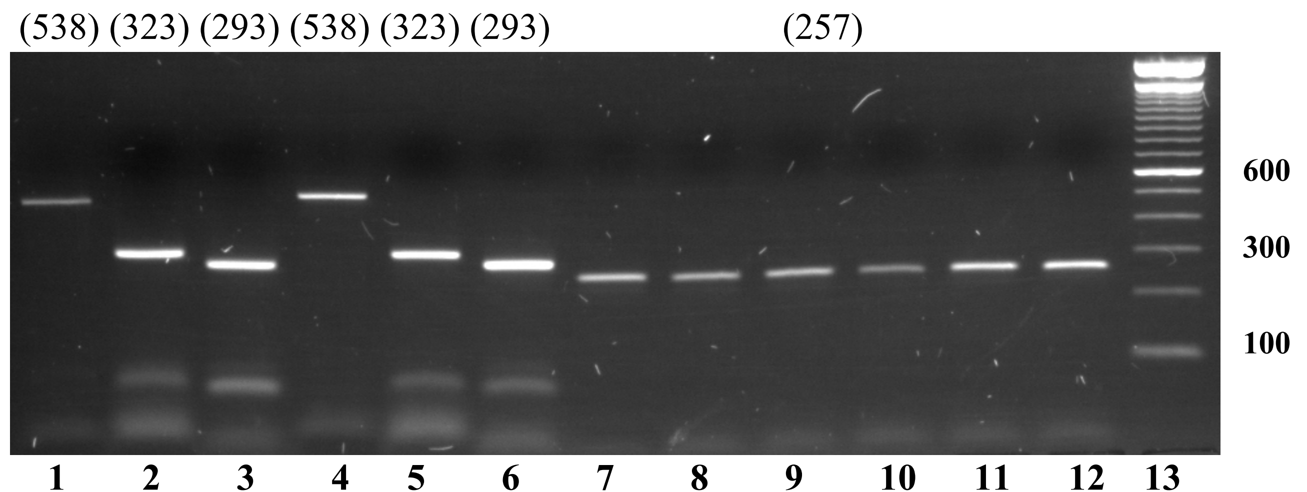


Fig. 28 Semi-quantitative RT-PCR analysis of mRNA IGF-IR, IGF-II and IGF-IIR in in vitro produced bovine 8-16-cells. Each lane represents the RT-PCR products derived from polyA$^+$ RNA from the equivalent of 4 embryos (IGF-II:8 embryos). Lanes (1-3) IGF-IR, IGF-II and IGF-IIR in the SOF-system, respectively, lanes (4-6) IGF-IR, IGF-II and IGF-IIR in the TCM-system, respectively, lanes (7-8) 100 fg globin RNA with preparation in the SOF-system, lane (9-10) 100 fg globin RNA with preparation in the TCM-system, lanes (11-12) 100 fg globin RNA without preparation and lane (13) MW marker. The RNA recovery rate was estimated as the ratio between the intensity of the fragment from globin with preparation and without preparation procedure (expected fragment sizes in brackets).

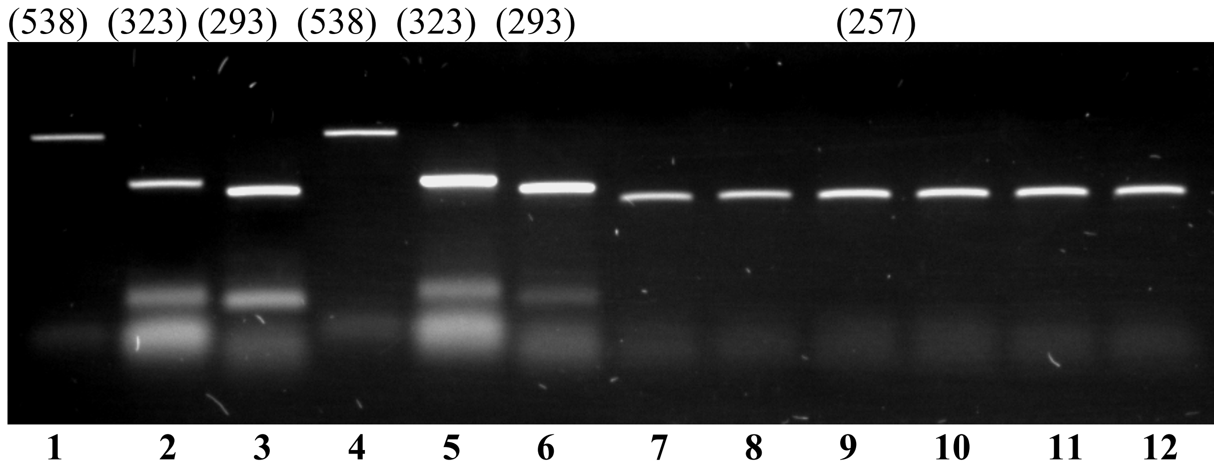


Fig. 29 Semi-quantitative RT-PCR analysis of mRNA IGF-IR, IGF-II and IGF-IIR in in vitro produced bovine morulae. Each lane represents the RT-PCR products derived from polyA$^+$ RNA from the equivalent of 4 embryos (IGF-II:8 embryos). Lanes (1-3) IGF-IR, IGF-II and IGF-IIR in the SOF-system, respectively, lanes (4-6) IGF-IR, IGF-II and IGF-IIR in the TCM-system, respectively, lanes (7-8) 100 fg globin RNA with preparation in the SOF-system, lanes (9-10) 100 fg globin RNA with preparation in the TCM-system and lanes (11-12) 100 fg globin RNA without preparation. The RNA recovery rate was estimated as the ratio between the intensity of the fragment from globin with preparation and without preparation procedure (expected fragment sizes in brackets).

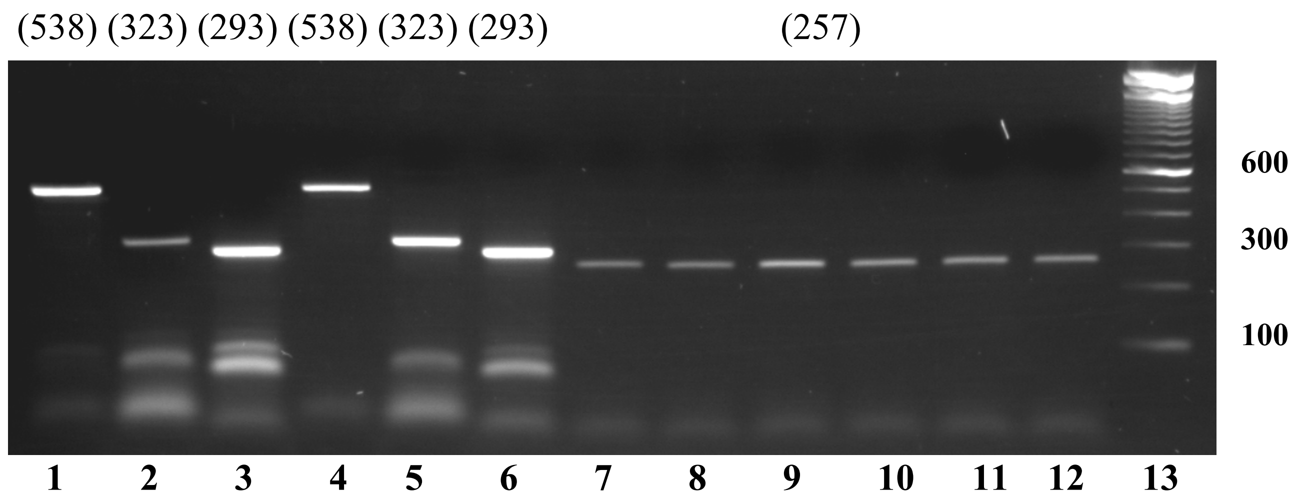


Fig. 30 Semi-quantitative RT-PCR analysis of mRNA IGF-IR, IGF-II and IGF-IIR in in vitro produced bovine expanded blastocysts. Each lane represents the RT-PCR products derived from polyA$^+$ RNA from the equivalent of 4 embryos (IGF-II:8 embryos). Lanes (1-3) IGF-IR, IGF-II and IGF-IIR in the SOF-system, respectively, lanes (4-6) IGF-IR, IGF-II and IGF-IIR in the TCM-system, respectively, lanes (7-8) 100 fg globin RNA with preparation in the SOF-system, lanes (9-10) 100 fg globin RNA with preparation in the TCM-system, lanes (11-12) 100 fg globin RNA without preparation and lane (13) MW marker. The RNA recovery rate was estimated as the ratio between the intensity of the fragment from globin with preparation and without preparation procedure (expected fragment sizes in brackets).

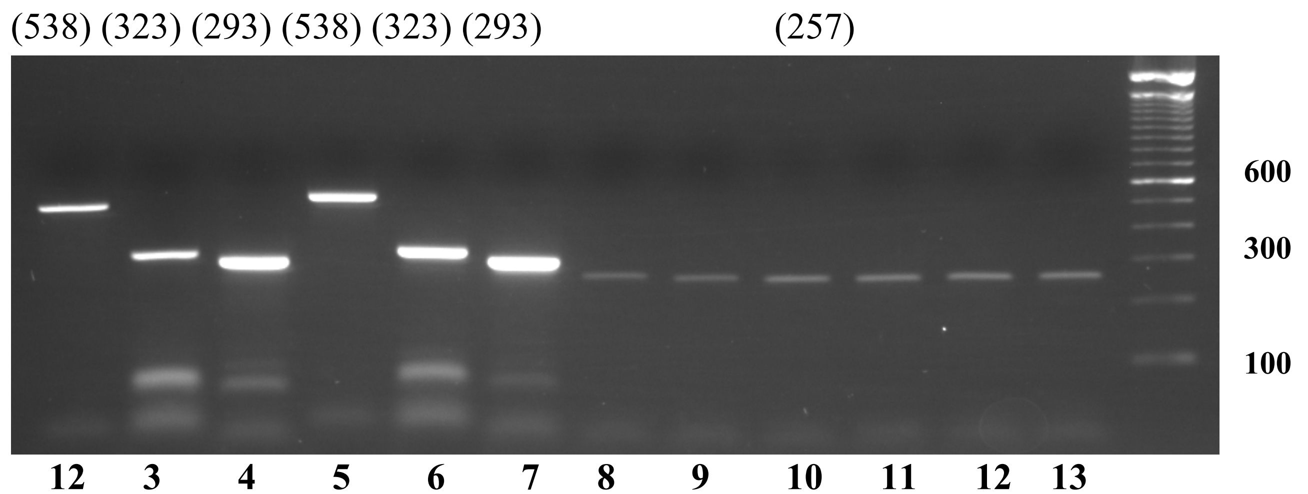


Fig. 31 Semi-quantitative RT-PCR analysis of mRNA IGF-IR, IGF-II and IGF-IIR in in vitro produced bovine hatched blastocysts. Each lane represents the RT-PCR products derived from polyA$^+$ RNA from the equivalent of 4 embryos (IGF-II:8 embryos). Lanes (1-3) IGF-IR, IGF-II and IGF-IIR in the SOF-system, respectively, lanes (4-6) IGF-IR, IGF-II and IGF-IIR in the TCM-system, respectively, lanes (7-8) 100 fg globin RNA with preparation in the SOF-system, lanes (9-10) 100 fg globin RNA with preparation in the TCM-system, lanes (11-12) 100 fg globin RNA without preparation and lane (13) MW marker. The RNA recovery rate was estimated as the ratio between the intensity of the fragment with preparation and without preparation procedure (expected fragment sizes in brackets).

Matured oocytes generally had more mRNA for IGF-IR, IGF-II and IGF-IIR than immature oocytes (except for IGF-IR oocytes in TCM-system). Following maturation, mRNA expression of IGFs and their receptors decreased significantly ($P < 0.05$) during early development. There was a significant ($P < 0.05$) increase in mRNA expression of IGFs and their receptors at the hatched blastocyst stage (Fig. 32).

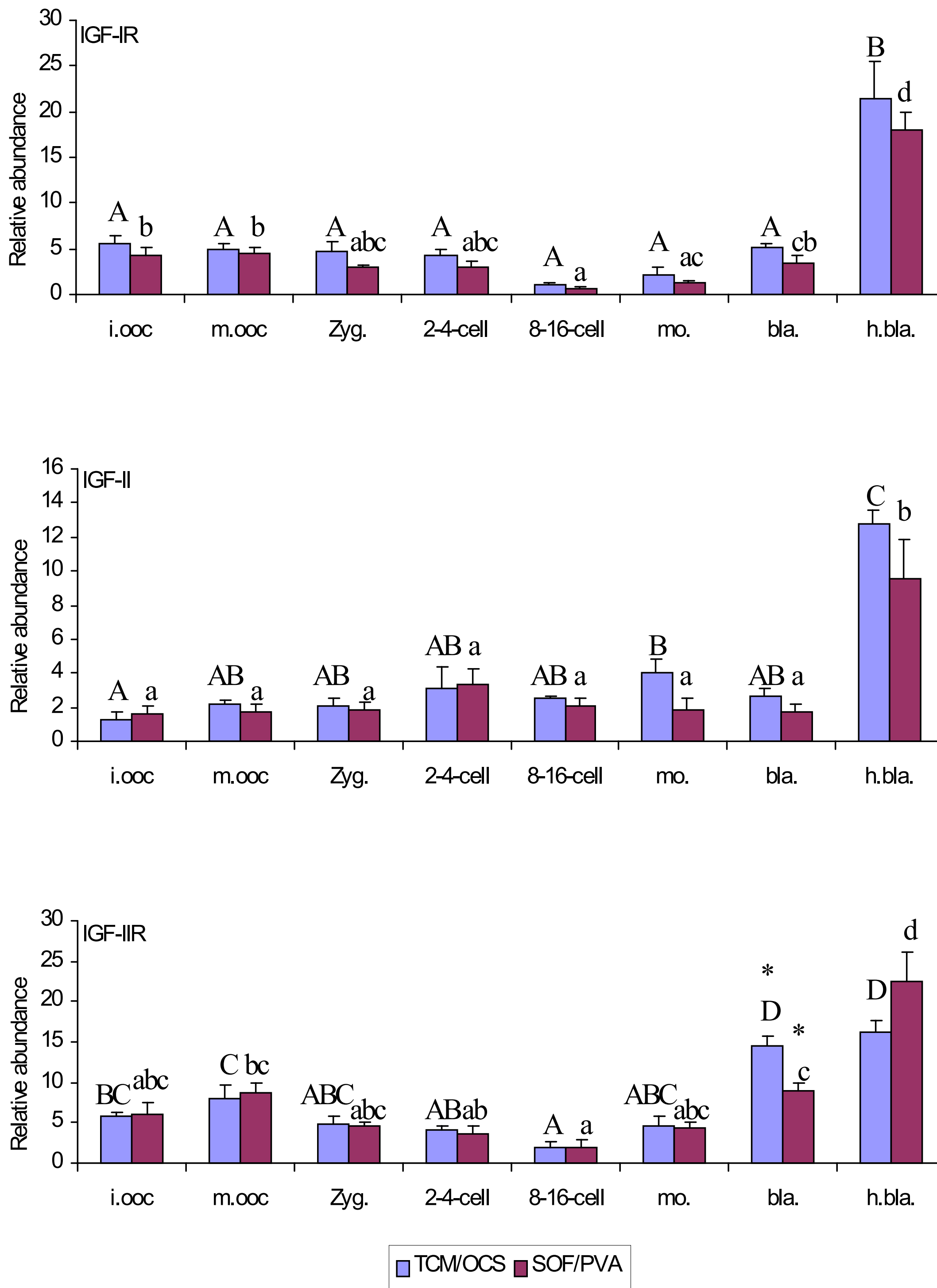


Fig. 32 Relative abundance of IGF-IR, IGF-II and IGF-IIR gene transcripts (values shown as mean ± S.E.M.) throughout bovine preimplantation development (i.ooc = immature oocyte, m.ooc = matured oocyte, Zyg. = zygot, 2-4-cell = 2-4-cell embryo, 8-16-cell = 8-16-cell embryo, mo. = morula, bla. = blastocyst, h.bla. = hatched blastocyst) employing TCM/ECS or SOF/PVA as culture systems. Significant differences throughout preimplantation development are indicated by different superscripts (TCM/ECS A:B:C:D = $P < 0.05$; SOF/PVA a:b:c:d = $P < 0.05$), whereas values sharing the same symbol (*) indicate significant differences ($P < 0.05$) between embryos at the same development stage in the two culture systems.

4.5. Relative abundance of IGFs and their receptors transcripts in bovine embryos in relation to the IVP systems

The relative abundances for IGF-IR, IGF-II, and IGF-IIR transcripts found in the two culture systems are summarized in Table 11. In the TCM-system, IGF-IR was expressed from the immature oocyte onwards with gradually decrease to the 8-16-cell embryo, then increased again from the morula stage up to the hatched blastocyst. No significant differences were observed between the immature oocyte up to the morula, but significant differences were found between hatched blastocysts and all embryonic stages from the immature oocytes to the expanded blastocysts. The same trend was seen in the SOF-system for IGF-IR. The amount of mRNA for IGF-IR decreased (up to the maternal/embryonic transition) at the 8-16-cell stage and then increased during further embryonic development up to the blastocyst stage. The differences between zygotes, 2-4-cells, 8-16-cells and morulae were not significant as way those between immature and mature oocytes, zygotes, 2-4-cells and expanded blastocysts. However, significant differences ($P < 0.05$) were found between hatched blastocysts and the other embryonic stages. Interestingly, the amount of mRNA for IGF-IR was consistently lower in embryos cultured in the SOF-system than in those cultured in the TCM-system.

In the TCM-system, IGF-II was detected from the immature oocyte to the hatched blastocyst. It increased from the immature oocyte to the 2-4-cell stage, then increased at the morula stage when it decreased again at the expanded blastocyst and then sharply increased at the hatched blastocyst. The differences between immature oocytes and expanded blastocysts except for the morula were not significant. Significant differences were found between immature oocytes and morulae and between hatched blastocysts and all the embryonic stages. In the SOF-system, IGF-II was expressed from immature oocytes to the hatched blastocysts but the differences from immature oocytes up to the expanded blastocysts were not significant whereas significant differences ($P < 0.05$) where found between the hatched blastocysts and the other embryonic stages.

In the TCM-system, IGF-IIR was expressed from the immature oocytes up to the hatched blastocysts. It was detected in immature and mature oocytes, decreased to the 8-16-cell and increased again up to the hatched blastocyst. The differences from the zygote to the morula were not significant and also between immature oocytes, mature oocytes, zygotes and morula.

Significant differences ($P < 0.05$) were found between expanded and hatched blastocysts and all embryonic stages. In the SOF-system, IGF-IIR was expressed from the immature oocyte to the hatched blastocyst in a similar fashion. It was decreased (up to the maternal/embryonic transition) at the 8-16-cell stage and then increased during further embryonic development up to the blastocyst stage. No significant differences were found between immature oocytes, zygotes, 2-4-cells, 8-16-cells and morulae and also between immature and mature oocytes, zygotes, and expanded blastocysts. Significant differences ($P < 0.05$) were found between hatched blastocysts and the other embryonic stages (Fig. 32).

The individual differences in IGF-IR, IGF-II and IGF-IIR gene expression of embryos generated in the two culture systems were not significant except at the expanded blastocyst stage when IGF-IIR transcripts were significantly higher in the TCM-system than in the SOF-system.

Table 11: Differences of the relative abundance of IGF-IR, IGF-II, and IGF-IIR transcripts (Means ± SEM) in preimplantation bovine embryos

Developmental stages	Relative Abundance of Transcripts					
	IGF-IR		IGF-II		IGF-IIR	
	TCM-system	SOF-system	TCM-system	SOF-system	TCM-system	SOF-system
Immature oocytes	5.5 ± 0.9^{a}	4.2 ± 1.0^{b}	1.3 ± 0.4^{a}	1.6 ± 0.5^{a}	5.7 ± 0.6^{bc}	6.1 ± 1.3^{abc}
Mature oocytes	4.9 ± 0.7^{a}	4.5 ± 0.6^{b}	2.2 ± 0.2^{ab}	1.7 ± 0.5^{a}	7.9 ± 1.7^{c}	8.7 ± 1.2^{bc}
Zygotes	4.8 ± 0.9^{a}	3.0 ± 0.3^{abc}	2.1 ± 0.4^{ab}	1.8 ± 0.5^{a}	4.9 ± 0.9^{abc}	4.7 ± 0.3^{abc}
2-4-cell embryos	4.3 ± 0.7^{a}	2.9 ± 0.7^{abc}	3.1 ± 1.3^{ab}	3.3 ± 1.0^{a}	4.1 ± 0.4^{ab}	3.7 ± 0.8^{ab}
8-16-cell embryos	1.0 ± 0.3^{a}	0.6 ± 0.2^{a}	2.5 ± 0.2^{ab}	2.1 ± 0.4^{a}	1.9 ± 0.8^{a}	1.0 ± 0.8^{a}
Morulae	2.2 ± 0.9^{a}	1.2 ± 0.4^{ac}	4.0 ± 0.8^{b}	1.8 ± 0.7^{a}	4.5 ± 1.4^{abc}	4.4 ± 0.8^{abc}
Expanded blastocysts	5.2 ± 0.3^{a}	3.4 ± 0.8^{cb}	2.6 ± 0.5^{ab}	1.7 ± 0.5^{a}	14.6 ± 1.1^{d}	8.9 ± 1.0^{c}
Hatched blastocysts	21.4 ± 4.2^{b}	18.0 ± 1.9^{d}	12.8 ± 0.8^{c}	9.6 ± 2.3^{b}	16.1 ± 1.6^{d}	22.4 ± 3.7^{d}

∗No. of replictes: 4 times

∗Values with different superscripts within one column are significantly (a:b $p \leq 0.05$).

5. DISCUSSION

The earliest stages of embryogenesis in mammals and other animal species are regulated by maternally inherited RNAs and proteins stored within the oocyte. Following fertilization, the embryonic genome becomes transcriptionally active and embryonic transcripts and proteins replace some of the maternal components required for early development (Telford et al., 1990). Reproductive biotechnologies have made great progress during the past 15 years.
IVP of bovine embryos has emerged as a reliable alternative method to conventional ovulation induction techniques and as important tool to study preimplantation embryo development. Most culture media are supplemented with serum or serum albumin as protein sources, frequently together with somatic cells in co-culture (Bavister, 1995). Serum and BSA are a complex undefined mixtures containing hormones, growth factors, vitamins, and numerous other unknown factors (Gardner and Lane, 1993). When chemically defined conditions are desired, these commonly used components of media are replaced by synthetic macromolecules, such as polyvinyl alcohol (PVA). As reported by many studies, in vitro environment during the early days of life can influence embryo morphology, fetal gene expression and fetal and placental development in ruminants (for review see Niemann and Wrenzycki, 2000). An important phenomenon seen after in vitro embryo production on development of fetuses and placentas is “Large Offspring Syndrome” (LOS).
Nearly all available information on mRNA expression of growth factor genes during bovine preimplantation development is at the qualitative level (Lonergan et al., 2000; Schultz et al., 1992; Watson et al., 1992). This study is the first to report changes in the relative abundance of mRNAs for IGF-I, IGF-II and their receptors throughout bovine preimplantation development in vitro. Bovine embryos were cultured in two different culture systems (a TCM system vs SOF system) and a semi-quantitative RT–PCR assay was used with the addition of exogenous globin as internal standard (Wrenzycki et al., 1999) to assess the relative abundances of the IGFs. The TCM system is a co-culture with somatic cells. It is a complex and nondefined medium in which 20 IVF-oocytes were cultured in 200 µl droplets of TCM199 + ECS in 5% CO_2 in air in a high humidity atmosphere at 39°C. The SOF system represents a simple, defined medium in which 6-8 IVF-oocytes were cultured in 30 µl droplets of SOF + PVA in 5% CO_2, 7% O_2 and 88% N_2 in a high humidity atmosphere at 39°C. Changes in mRNA expression in the two different culture systems were detected,

providing additional information to the findings reported by Wrenzycki et al. (1999, 2001a) on the effects of culture conditions on mRNA expression.

5.1. Verification of the semi-quantitative RT-PCR assay

As described by Wrenzycki et al. (1999), the changes in mRNA expression detected in this study reflect real differences in the amount of the transcripts present at a certian point of development since similar oocyte or embryo cDNA equivalents had been subjected to PCR. This rules out the possibility that the observed differences were attributed to variation in PCR conditions. For all primer pairs the number of PCR cycles was kept within the linear range of amplification. As the CV levels were only marginally different, the semi-quantitative RT-PCR assay is reliable and possesses a high reproducibility. However, it should be taken into account that due to the possible variation in amplification efficiency of different primer pairs, the results must be viewed as relative and the most of one gene transcript should not be compared with that of a different transcript (Temeles et al., 1994; Wrenzycki et al., 1999). In addition, the absolute number of the transcripts cannot be estimated by this methodological approach. Only transcripts from known genes are readily identified with this assay. Employing subtractive cDNA-libraries (Rothstein et al., 1992; Heyer et al., 1997), or differential display (Zimmermann and Schultz, 1994) enables to overcome the limitation.
Previous studies in our lab (Wrenzycki et al., 1996; 1999) show that after optimization, the RT-PCR assay was sensitive enough to detect bovine mRNA from tongue epithelium at levels from 0.5-5 ng and from 0.5 blastocyst equivalents. This corresponds well with the total RNA levels found in bovine oocytes and blastocysts (2.4-5.3 ng) (Bilodeau-Goeseels and Schultz, 1997b). Thus the RT-PCR assay described in this study was sufficiently sensitive to unravel alterations at the transcriptional level in few embryos.

5.2. Effects of culture systems on embryonic development

IVP of bovine embryos is a useful tool for basic embryological studies as well as commercial applications. Blastocyst developmental rates between 30% and 40% of the matured oocytes can be obtained with optimized IVP systems (Brackett and Zuelke, 1993; Trounson et al., 1994). Usually the basic culture media are supplemented with serum or bovine serum

albumin, which are complex undefined mixtures (Gardner and Lane, 1993) and result in highly variable developmental rates. The protein supplements contain a contamination risk with unknown organic or inorganic factors and therefore further increase non-reproducibility of results (Bavister, 1995; Barnett and Bavister, 1996). This led to the development of more defined protein-free in vitro production systems to standardize culture media. The 'undefined' components are replaced by synthetic macromolecules, such as polyvinyl alcohol (PVA), resulting in chemically defined media (Pinyopummintr and Bavister, 1991; Eckert and Niemann, 1995; Keskintepe and Brackett, 1996).

Results reported in this study provide a significantly higher percentage of two- to four-cell embryos and blastocysts obtained in the TCM system than in the SOF system. Maturation, fertilization, 8-16-cell embryos, unmber of morulae tended to be higher in TCM system than SOF system, but no significant differences were obtained. This is consistent with previous observations that the presence of serum had a biphasic effect, inhibiting the first cleavage and promoting compaction of the morula (for review see Pinyopummintr and Bavister, 1994; Bavister, 1995) Recent studies demonstrated that serum supplementation leads to a higher proportion of blastocyst stages compared to in defined culture systems (Eckert and Niemann, 1995; Eckert et al., 1998; Wrenzycki et al., 1999; 2001a). Wrenzycki et al. (2001a) indicated that despite a similar rate of development in the SOF-system and TCM-system profound differences existed at the transcriptional level. Therefore, the current findings question the commonly used criterion to evaluate the efficiency of a specific IVP system by the 'maximum number of blastocysts' and should encourage implementation of a more suitable parameter to characterize the quality of embryos generated in a specific culture system (Wrenzycki et al. 2001a).

5.3. Expression patterns of IGFs genes and their receptors in bovine IVP embryos produced in the two in vitro culture systems

5.3.1. Temporal patterns

In contrast to previous reports in which IGF-I-mRNA was found in preimplantation bovine and mouse embryos (Schultz et al., 1992; Watson et al., 1992; Doherty et al., 1994; Westhusin et al., 1995; Yoshida et al., 1998a, Lonergan et al., 2000), in the present study,

mRNA for IGF-I was not detected in embryos derived from the two culture systems. This finding is consistent with the observations in preimplantation embryos of rats (Zhang et al., 1994), water buffalo (Daliri et al., 1999), and humans (Lighten et al., 1997a), in which IGF-I mRNA was not detected. Lonergan et al. (2000) used nested PCR to identify IGF-I transcripts in early bovine embryos. Furthermore, in vitro produced bovine embryos did not release IGF-I protein, although large amounts of IGF-II were observed (Winger et al., 1997). The conflicting results with regard to IGF-I expression in bovine embryos may be due to differences in culture systems or interaction of RT-PCR with specific components used in the experiments, particularly the design of primer pairs.

It has been demonstrated that there is a decrease of mRNA levels during early preimplantation development up to the point of activation of the embryonic genome presumably due to degradation of maternal mRNA which is initiated already during oocyte maturation (Paynton et al., 1988; Paynton and Bachvarova, 1994). Differential rates of mRNA degradation can be attributed to differences in mRNA structure and/or the intracellular environment (Ross, 1996). Increased levels of most gene transcripts result from de novo synthesis following embryonic gene activation (Temeles et al., 1994). However, unfortunately the semi-quantitative RT-PCR assay cannot differentiate between an increase in the transcription, or RNA stability or a change in the poly(A) tail length. The concentration of mRNAs for IGF-II, IGF-IIR and IGF-IR seems to follow a pattern similar to most other genes expressed during preimplantation mammalian development. It is consistent with previous observations in which the mRNA content in cultured bovine embryos was declining between the mature oocyte and the 8-16-cell stage and increased again at the blastocyst stage (Bilodeau-Goeseels and Schultz, 1997a; b). This pattern of mRNA loss and re-accumulation is similar to the pattern observed in mice, although the increase occurs by the eight-cell stage in mice, possibly as a result of the earlier onset of embryonic gene activation in this species (Pico and Clegg, 1982). DD-RT-PCR revealed the decline in maternal transcripts and renewal of embryonic transcripts that occur after full activation of the bovine embryonic genome (Natale et al., 2000). The patterns in this study showed that they are in agreement with the timing of the oogenetic-embryonic transition described for bovine embryos by other investigations (Plante et al., 1994; Viuff et al., 1996; 1998; Memili and First, 1999; Memili et al., 1998; Wrenzycki et al., 1999). As reviewed in 2.3.5., in bovine embryos, the full activation of the zygotic genome occurs around the 8-16-cell stage as evidenced by [^{3}H] uridine incorporation into nuclei and nucleoli at the 8-cell stage, and major changes in the ultrastructure of blastomere nucleoli and the pattern of protein synthesis (see Telford et al., 1990; De Sousa et al., 1998b). Studies investigating [^{3}H]

uridine uptake in two- to four-cell embryos revealed that transcriptional activity can be detected earlier than the 8-16-cell stage (Plante et al., 1994; Viuff et al., 1996). Natale et al. (2000) also observed that cDNA banding patterns are largely conserved from the 8-16-cell stage through to the blastocyst stage in bovine embryos. In bovine embryos, most of the blastocyst mRNAs are detectable first at the 8-16-cell stage and only a comparatively small number of unique mRNAs are present in blastocyst cDNA samples (Natale et al., 2000).

During oocyte maturation, IGF-II, IGF-IIR and IGF-IR mRNA transcripts increase, indicating that maturation may depend on increased mRNA levels from these genes. It is consistent with previous reports that IGF-I has stimulatory effects on oocyte maturation in buffalo (Pawshe et al., 1998), cattle (Rieger et al., 1998; Sirotkin et al., 1998) and pigs (Sirotkin et al., 1998).

After maturation, IGFs concentration decreased gradually, as expected, up to maternal-embryonic transition at the 8-16-cell stage. After the maternal-embryonic transition, concentrations of IGF-II, IGF-IIR and IGF-IR mRNA increased up to the hatched blastocyst stage. In human embryos, the relative abundance of mRNAs for IGFs and their receptors increased from the three- to four-cell stage (for example, after maternal-embryonic transition) up to the blastocyst stage (Liu et al., 1997). In mouse embryos, expression of IGF-II increased in a linear fashion at least up to the 8-cell stage of development (Stojanov et al., 1999). In the present study on bovine embryos, the greatest increase in the IGFs mRNA occurred after hatching. The increase in IGF-IR, IGF-II and IGF-IIR mRNA observed when the bovine embryo reached the hatched blastocyst stage may indicate an important role for growth factors of the IGF family when the bovine embryo becomes filamentous in shape. The increase in IGF-IR, IGF-II and IGF-IIR mRNA in the bovine hatched blastocyst may be related to the increase in cell numbers. On the other hand, the temporal variation of the expression of mRNA encoding IGF-I and IGFBP-2 suggests a role for these factors in the uterine environment during early pregnancy in ewes coinciding with rapid development of the embryo and growth of the uterus in preparation for implantation (Cann et al., 1997).

The presence of IGFs and their receptors in bovine preimplantation embryos suggests that the IGF system may indeed play an important role in bovine embryogenesis. Reports from several laboratories support a potential role of growth factors in the intrafollicular control of meiosis. IGF-I is a potent mitogen for granulosa cells (Hernandez et al., 1988) and enhances nuclear maturation in oocytes surrounded by compact cumulus cells both in cattle (Lorenzo et al., 1994) and human (Gomez et al., 1993). Gene expression of IGF-I, IGF-II and their receptors can be a good indicator of the developmental potential for embryos, and finally, the activity and pattern of IGF gene expression can serve as a marker for embryo quality (Liu et al., 1997;

Kowalik et al., 1999). However, Blondin et al. (1999) concluded that at day 222 of gestation there were no differences in levels of IGF-II mRNA in liver of fetuses resulting from transfer of bovine embryos produced in vivo or in vitro. Blondin et al. (2000) reported that the extended in vitro culture period used to generate viable bovine blastocysts from immature oocytes led to significant alterations in IGF-II mRNA steady state concentrations in liver and skeletal muscle of fetuses at day 70 of gestation. These findings indicate that further studies into the role of IGFs in 'Large Offspring Syndrome', are necessary which is frequently observed in calves derived from certain in vitro production systems (Niemann and Wrenzycki, 2000).

As shown by Stojanov et al. (1999), the miotic stimuli in the early mammalian embryo have not been unequivocally identified. One hypothesis is that the embryo releases autocrine growth factors. This hypothesis is supported by: (1) the relative autonomy of preimplantation embryo growth; (2) the observation that the rate of embryo development in vitro is density dependent, with embryos growing in relatively small volumes having faster development rates than embryos developing in larger volumes and those in groups developing better than their individual counterparts (Paria and Dey, 1990; Lane and Gardner, 1992; O'Neill, 1997); (3) the synthesis of a number growth factors and their receptors by the preimplantation embryo, including platelet activating factor (PAF) (O'Neill, 1985; Collier et al., 1988; Roudebush et al., 1997), IGF-I (Rappolee et al., 1990; Doherty et al., 1994), IGF-II (Heyner et al., 1989; Harvey and Kaye, 1991; Rappolee et al., 1992), growth hormone (Pantaleon et al., 1997), TGF-α (Rappolee et al., 1988a); and (4) evidence of trophic actions of a range of growth factors on embryo growth, metabolism and cell progression in vitro (Harvey and Kaye, 1992; O'Neill 1997). Consistent with this prediction is that the stimulatory effects of exogenously added IGF-I on blastocyst metabolism appear to be mediated by insulin receptors (Harvey and Kaye, 1991) and that immunogold electronmicroscopy reveals that the IGF-I receptor is preferentially localized to the basolateral surface of the trophectodermal cells (Smith et al., 1993). Our results indicate that it is preferentially the paracrine rather than the autocrine mechanism which is the mode of action for IGF-I. Pawshe et al. (1998) concluded that IGF-I may act directly through paracrine regulation using receptors present on granulosa cells, cumulus cells, oocytes and preimplantation-stage buffalo embryos and the same results were demonstrated by Daliri et al. (1999). Embryonic expresssion of IGF-I most likely does not begin until post-implantation stages. This is consistent with previous observations that late expression of IGF-I is indicative of paracrine function in early embryo growth and the availability of exogenous IGF-I may be crucial for early embryonic development (Liu et al.,

1997). In contrast to IGF-I, mRNA for IGF-IR, IGF-II, and IGF-IIR were detected in all stages of preimplantation bovine development, which is consistent with previous observations (Watson et al., 1992; Rappolee et al., 1992; Yoshida et al., 1998a; Lonergan et al., 2000). Autocrine and paracrine activities of IGF-II appear to be important as IGF-II mRNA is present in preimplantation embryos and in the reproductive tract (Buhi et al., 1997). In other studies, autocrine and paracrine mechanisms for IGF-II and TGFα-action have been suggested (Eicher et al., 1993; Zhang et al., 1994). Another study on buffalo preimplantation embryos, indicated that IGF-II appears to act through a heterologous autocrine mechanism (Daliri et al., 1999). The presence of transcripts for IGF-IR, IGF-II and IGF-IIR in all preimplantation bovine embryos from the immature oocyte to the hatched blastocyst stage indicates that maternal transcripts occur in the oocyte and that these transcripts persist at cleavage stages before activation of the embryonic genome.

The observed deviation from the normal mRNA expression patterns seen for IVP embryos may reflect epigenetic changes specifically in the pattern of methylation (Niemann and Wrenzycki, 2000). The underlying mechanism is methylation of CpG islands in the gene promotor region which is essential for induction of gene silencing (Jones, 1999). Presently, it is not known which or how many CpG islands have to be methylated to achieve complete silencing of a specific gene. Methylation has long been known to be involved in imprinting, the process by which certain alleles are expressed or silenced depending on the paternal sex from which they are inherited (Sapienza, 1990). More than 30 protein-coding genes, plus a growing number of non-coding RNAs, have now been shown to be imprinted in both mice and humans (Beechey and Cattanach, 2000; Jirtle, 2000). An early perceptive observation on the first two identified imprinted genes has been the basis for a hypothesis that imprinting evolved in mammals because of the conflicting interests of the maternal and paternal genome, in relation to the transfer of nutrients from the mother to her offspring (Haig and Graham, 1991; Moore and Haig, 1999). One prediction from this hypothesis is that paternally expressed imprinted genes would enhance fetal growth at maternal expense while maternally expressed genes would have the opposite action. The majority of imprinted genes do behave as predicted, but some fail to generate a growth phenotype when inactivated in mice, leaving open the possibility of other explantations for the evolution of imprinting in mammals (Hurst and McVean, 1998).

Imprinted genes such as IGF-II, IGF-IIR and H19 are preferentially involved in the control of embryonic, placental, fetal and neonatal growth (Niemann and Wrenzycki, 2000). IGF-II mediates growth in early mouse embryos and forms a pathway in which imprinted genes

affected development during preimplantation stages (Rappolee et al., 1992; O'Dell and Day, 1998; McLaren and Montgomery, 1999; Constância et al., 2000; Kaffer et al., 2000). The IGF-II ligand in mouse is imprinted when inherited maternally and IGF-IIR is imprinted when inherited paternally (De-Chiara et al., 1990; Barlow et al., 1991; Latham et al., 1994; McLaren and Montgomery, 1999; Kaffer et al., 2000; Vu TH et al., 2000), consistent with the hypothesis of Haig and Graham (1991) which predicts that imprinting of growth factors such as IGF-II, IGF-IIR and IGF-IR regulates embryonic growth in the mammalian uterus. The mouse IGF-IIR gene is specifically maternally expressed from the early postimplantation stage (Lerchner and Barlow, 1997). Other studies suggested that IGF-II is parentally imprinted in human preimplantation embryos (Lighten et al., 1997a; b; O'Dell and Day, 1998; McLaren and Montgomery, 1999; Vu TH et al., 2000). However, there is little information available with regard to imprinting of these genes in cattle. In humans, imprinting deviations are responsible for conditions known as Beckwith-Wiedemann syndrome which is characterized by growth aberrations similar for those seen in the large calf syndrome (Hastie, 1997). Imprinted genes have also been found in sheep in which IGF-II gene is paternally expressed (Feil et al., 1998; Hagemann et al., 1998; McLaren and Montgomery, 1999).
Doherty et al. (2000) showed that in vitro culture of preimplantation embryos is associated with epigenetic modifications in the genome. Analysis of multiple growth-related and imprinted genes in murine embryos cultured in a chemically defined medium (M16) with or without fetal calf serum revealed that culture in the presence of serum affected the regulation of imprinted genes (Khosla, 2001). In sheep, methylation and expression of IGF-II receptor gene was reduced in oversized fetuses derived from IVP compared to the control group thereby altering the subtle regulation of expression of the IGFs (Young et al., 2001). Effects of a single imprinted gene were not enough to induce a significant overgrowth in cloned mice (Humpherys et al., 2001).

5.3.2. Medium effects

Despite the recent improvements in bovine IVP, in vitro generated embryos display a number of marked difference compared to their in-vivo counterparts (see Thompson, 1997; Holm and Callesen, 1998; Niemann and Wrenzycki, 2000). Moreover, several reports have clearly demonstrated that an increased proportion of deliveries and calves originating from in vitro production are compromised (Smith et al., 1993; Hasler et al., 1995; Kruip and Dass, 1997).

In addition, extended in vitro culture seems to be associated with the incidence of the 'Large Calf Syndrome' (Kruip and denDaas, 1997). It has been postulated that persistent alterations from the normal expression pattern of developmentally important genes contribute to the Large Offspring Syndrome that is observed in approximately one third of the calves resulting from the transfer of in vitro-produced embryos (Niemann and Wrenzycki, 2000). Indeed, differences in the expression pattern of developmentally important genes have been determined between in vivo and in vitro-derived bovine embryos (Wrenzycki et al., 1996; 1998b; Eckert and Niemann, 1998). For example, transcripts encoding connexin 43 (C×43) were detected in in vivo derived bovine morulae and blastocysts but not in bovine blasocysts produced in vitro and cultured in TCM/ECS (Wrenzycki et al., 1996). In a second study in which the expression of ten marker genes was compared between in vivo derived bovine embryos and in vitro derived embryos cultured in TCM/BSA, only C×43 was differentially expressed, indicating that cultured bovine blastocysts are deficient in this gene product. No other differences in gene expression in these two groups of embryos were reported in this study (Wrenzycki et al., 1998a). The effects of culture on embryonic gene expression patterns were first raised in studies of early mouse embryos (Ho et al., 1994; 1995). Gene transfection studies using a gene construct encoding the heat shock protein (Hsp) 70.1 coupled to sequences encoding a luciferase reporter gene demonstrated that reporter gene activity was up to 15 times higher in embryos derived in vitro than that observed in embryos derived in vivo (Christians et al., 1995). Similar approaches have demonstrated that in vitro derived embryos produced seven times less platelet activating factor (PAF) than in vivo derived embryos (O´Neill, 1997). Additional studies have examined variation in the expression of mRNAs encoding a wide variety of gene products, including actin, glyceraldhyde-3-phosphate dehydrogenase, Na/K-ATPase, Sp1, TATA box binding protein (TBP), IGF-I, IGF-II, IGF-IR and IGF-IIR, in murine preimplantation embryos cultured in KSOMaa or Whitten's medium compared with expression of in vivo derived embryos (Ho et al., 1994; 1995). A single transcript, that encodes TATA box binding protein, was found to vary significantly between the embryos cultured in KSOMaa and those derived in vivo. However, eight of these gene products were observed to be present at significantly different amounts in embryos cultured in Whitten's medium compared with either embryos cultured in KSOMaa or embryos derived in vivo (Ho et al., 1994; 1995). These studies emphasized that culture environments can alter embryonic gene expression patterns which is consistent with recent suggestions (Lequarré et al., 2001; Wrenzycki et al., 1999; 2001a). The timing and magnitude of this alterations vary

among the different transcripts and are affected by the presence of exogenous proteins in a stage-specific manner, predominantly at critical developmental time points.

The widely used IVP system on the basis of TCM199 has been replaced more and more by the SOF system. Culture in the presence of serum can influence the regulation of multiple growth-related imprinted genes in mice embryos, thus leading to aberrant fetal growth and development (Khosla et al., 2001). Presence or absence of serum during in vitro maturation affected changes in gene transcript levels (Rzucidlo et al., 2001; Stojanov and O'Neill, 2001; Watson et al., 2000).

In the present study, the amount of IGF-IR mRNA was slightly higher at all stages of development in embryos derived and cultured in the TCM-system than in embryos that had been cultured in the SOF-system. Although individual stage-specific concentrations were not significantly different, concentrations of IGF-IR were consistently lower in the SOF-system. Another study investigated the expression of ten marker genes (a set of developmentally important genes) between bovine embryos derived in TCM supplemented with either serum or polyvinyl alcohol Hsp 70.1 was higher in the serum supplemented group, whereas the PVA supplemented group displayed increased concentrations of the other gene products (Wrenzycki et al., 1999). The similarity of the patterns (an increase associated with oocyte maturation; a decrease up to maternal-embryonic transition and an increase after maternal-embryonic transition) of growth factor gene expression in the two media indicates an important role of growth factors in preimplantation development. However, in the present study we didn't find significant differences between the relative amounts of IGF-IR, IGF-II and IGF-IIR mRNA in the two in vitro culture medium systems, except IGF-IIR mRNA transcripts were significantly higher in blastocysts derived in TCM/ECS than in those derived in SOF/PVA. It may be attributed to the important role of growth factor genes during the preimplantation development and/or the potential serum content from the growth factors. These differences may not reflect variations in embryo health or developmental potentials but instead may reflect the overall plasticity that the embryo can exhibit to compensate for a suboptimal culture environment. The embryo attempts to respond to a suboptimal environment by adjusting its gene expression pattern, and by investigating these responses it may be possible to improve understanding of the genetic programme controlling the first week of development and also to design improved culture media. For preimplantation mouse embryos it was shown that an optimized culture system is correlated with an mRNA expression pattern that is identical to that found in the in vivo derived counterparts (Ho et al., 1994; 1995). Optimized defined culture media maintain embryonic gene expression patterns

closely resembling those displayed by embryos derived in vivo (Natale et al., 2001; Wrenzycki et al. 2001a).

Wrenzycki et al. (2001a) have investigated the effects of two different culture systems (TCM- and SOF-system) containing either serum, BSA or PVA and compared the results with those for their in vivo derived counterparts on the relative abundance of a set of developmentally important gene transcripts. They showed that embryos generated in the SOF system appear to be more similar to their in vivo counterparts with regard to gene expression patterns than those generated in the TCM system. It means the SOF system provides a better environment for preimplantation development of bovine embryos than the TCM system. This similarity may also be attributed to the reduced oxygen tension of the SOF system via reduction of deleterious effects of reactive oxygen species on early development (Johnson and Nasr-Esfahani, 1994). A similar low oxygen tension has been found in the genital tract of mammalian species (Fischer and Bavister, 1993). Goto et al. (1993) reported an increased generation of reactive oxygen species (ROS) in embryos cultured under increased oxygen tension (20% or 40% O_2). Oxygen free radicals are known to induce various cell lesions, e.g., DNA damage, lipid peroxidation, and oxidative modification of proteins, and may thus contribute to the poorer development of cultured embryos (Johnson and Nasr-Esfahani, 1994; Nakayama et al., 1994). mRNA expression of NADH-ubiquinone oxidoreductase chain 2 (ND2) was increased in blastocysts cultured with the higher oxygen concentration (Koerber et al., 1998). Culture of human oocytes and embryos for the first 2 or 3 days of development under either 20% or 5% O_2 concentrations revealed no significant differences between the two groups in rates of fertilization, pregnancy and implantation. However, culture under 5% O_2 resulted in a significantly higher blastocyst rate and a higher cell number of these blastocysts (Dumoulin et al., 1999). The current results support the findings that environmental stresses, such as free oxygen radicals, induce expression of specific genes (Edwards and Hansen, 1996; 1997; Edwards et al., 1997).

In the present study, the relative amounts of IGF-IR, IGF-II and IGF-IIR transcripts in preimplantation bovine embryos generated in the TCM system tended to be greater than in the SOF system. From these results, it is possible to speculate that IGFs play a role as survival factors in preimplantation bovine embryos. IGF-I has been shown to prevent apoptosis in early rabbit embryos and to act as a survival factor (Herrler et al., 1998). Other studies provide further support for a role of IGF-II as an important growth/survival factor for preimplantation mouse embryos. They show that aberrations in the production of growth factors caused by IVF or culture in vitro may be a significant cause of poor embryo viability

(Stojanov et al., 1999; Stojanov and O'Neill, 2001). Thus, there is evidence to suggest that certain "survival" factors, such as growth factors, produced by the embryo and a maternal reproductive tract play a role in controlling apoptosis level (Brison and Schultz, 1997; 1998). These findings indicate a potentially more versatile role of IGFs in preimplantation development than previously suggested.

IGF transport and function are modulated by interaction with at least six insulin-like growth factor binding proteins (IGFBPs), which are present in many extracellular fluids and in early embryos (Heyner et al., 1993; Hahnel and Schultz, 1994; Kaye and Harvey 1995; Winger et al., 1997; Luciano et al., 2000; Prelle et al., 2001). Moreover, Prelle et al. (2001) demonstrated that exogenous IGF-I regulates also the mRNA levels for components of the IGF system. Clemmons (1998) showed that besides the abundance of IGF-I or its receptors, IGF action is determined by a family of structurally related secreted proteins (IGFBPs) that specifically bind IGFs and modulate IGF bioactivity in different tissue. IGFBP proteolysis may, in turn, contribute to IGF-I biological activity by releasing free IGFs to interact with membrane receptors and there is evidence that IGFBP proteolysis itself may be subject to hormonal regulation (Conover et al., 1993; Smith et al., 2001). With regard to IGFBP modulation of IGF actions, the mechanisms underlying the potentiation of activity by some IGFBPs are still poorly understood. All six IGFBPs have been shown to inhibit IGF action, but stimulatory effects have also been established for IGFBP-1, -3 and -5 (Baxter, 2000; Prelle et al., 2001). Young et al. (1999) suggest that IGFBPs can prolong the half life of IGFs in the circulation and transport them between blood and tissue fluid, modifying local concentrations and access to receptors. Preliminary evidence implicates the IGF axis in the LOS phenotype. Since increased levels of IGF-II have been associated with fetal overgrowth syndromes in both humans and mice, further understanding of the complex IGF system may reveal a related mechanism in Large Offspring Syndrome in cattle and sheep (Young et al., 1998).

In addition to the modulation of IGFs by IGFBPs there is interaction between IGF and some hormones such as gonadotropins (FSH & LH) and growth hormone. Interaction between IGF-I and FSH suggests that they seem to act synergistically as autocrine and paracrine regulators of granulosa cells and therefore together promote mitosis, steroidogenesis and protein synthesis (Pawshe et al., 1998; Zhao et al., 2001). A coordinated expression of the IGF-I gene and GH receptor during development was demonstrated and suggests that GH receptor expression contributes to the tissue specific expression of the IGF-I gene during development (Shoba et al., 1999). Greene and Chen (1999) speculated that the GH-IGF axis may exist

during embryonic development in rainbow trout. Gonadotropins, enhancing IGF peptide and inhibiting IGFBP production, serve to maximize IGF-I or IGF-II availability to receptors in the process of follicular development and during the life time of the corpus luteum (Schams et al., 1999). However, it is concluded that the stimulatory effects of GH on bovine oocyte maturation are dependent on the cumulus cells and are not mediated by IGF-I (Izadyar et al., 1997).

Glucose may be a critical regulator of membrane uptake activity, as transporter recruitment in responce to growth factors appears to be a glucose-requiring event. The action of insulin and IGF-I on glucose availability in vivo may be important for their long-term proliferative effects. Glucose transport in mouse and bovine blastocysts is indeed responsive to IGF-I and insulin, their effects being mediated by the IGF-I receptor. IGF-I is much more important than insulin in the regulation of glucose transport in vivo. The stimulation of glucose transport by IGF-I, requiring up to 80 min to reach maximum, is unlikely to be involved in acute regulation of glucose metabolism, but rather in the long-term coordination of metabolism with cell cycle and embryo development (Pantaleon and Kaye, 1996; 1998; Augustin et al., 2001). Moreover, expression of GLUT1 provides a coordinating link with the proliferative and morphological effects demonstrated by growth factors such as IGF-I and growth hormone. GLUT3 expression required for blastocyst formation in the presence of glucose may be linked to the initiation of events involved in the adaptive response of embryos to their substrate environment (Pantaleon and Kaye, 1998). Expression of the insulin-sensitive GLUT4 was detected in bovine blastocysts (Navarrete-Santos et al., 2000). Two insulin-regulated glucose transporter, GLUT4 and GLUT8, are transcribed during bovine preimplantation development (Augustin et al., 2001). These findings are consistent with our results in which an increase of IGF-IR mRNA expression was detected in bovine blastocysts. Asada et al. (1998) demonstrated that the effects of IGF-I on the expression of GLUTs were almost the same as that of insulin. The increase of the Glut1 mRNA expression in bovine blastocysts (Wrenzycki et al., 1999) and IGF-IR mRNA expression in the present study suggest that glucose transport is mediated by IGF-IR in bovine blastocysts. Zhou et al. (2000) have shown that IGF-I enhances follicular GLUT1 expression, indicating that IGF-I acts through a variety of molecular mechanisms to promote ovarian follicular growth and the aquisition of gonadotropin-responsiveness.

In summary, development to the two- to four-cell stage and blastocyst stage was significantly higher ($P< 0.05$) in embryos cultured in TCM + ECS than in those cultured in SOF + PVA

(61 and 25% vs 55 and 17%, respectively). A semi-quantitative RT–PCR assay did not detect IGF-I transcripts at any stage of preimplantation bovine development, including the hatched blastocyst stage. In the two culture systems, IGF-IR, IGF-II and IGF-IIR were expressed throughout preimplantation development up to the hatched blastocyst stage in a varying pattern. The expression patterns of IGF-IR, IGF-II and IGFIIR in embryos generated in the two culture systems were not significantly different, except at the expanded blastocyst stage, at which significantly ($P< 0.05$) higher amounts of IGF-IIR were observed in the TCM system than in the SOF system. These results indicate that transcripts of IGF-IR and IGF-IIR follow the standard pattern in which maternal stores of mRNA in the oocyte are slowly depleted up to the 8-16-cell stage and then re-established at the onset of embryonic expression of these genes. The lack of detectable IGF-I transcripts in the bovine embryo indicates a predominantly paracrine mode of action. Results indicate an autocrine mechanism for IGF-II and modulation of IGF family expression by culture conditions.

It can be concluded that IGFs have a potentially more versatile role in preimplantation development than previously suggested. Determination of growth factor gene expression in preimplantation bovine development can be used for optimization of in vitro culture systems, with the goal of improving the quality of embryos generated in vitro and ensuring normal offspring. Further studies are underway to investigate growth factor gene expression in embryos produced in vivo in an effort to clarify the effects of culture conditions. In addition, this finding indicates that further studies into the role of IGFs in “Large Offspring Syndrome”, which is frequently observed in calves derived from certain in vitro production systems, might be valuable.

6. SUMMARY

This study aimed to determine the relative abundance of mRNA for the Insulin like growth factors IGFI, IGFII and their receptors in preimplantation bovine embryos produced in vitro using two distinctly different culture systems [TCM199 supplemented with estrous cow serum (ECS) or synthetic oviduct fluid (SOF) supplemented with polyvinyl-alcohol (PVA)]. The TCM-system is a complex, undefined medium and the SOF-system is a simple chemically defined medium.

A total of 6325 oocytes (morphological categories I & II) from 997 ovaries (6.7 ± 1.7 oocytes/ovary) were used for this study. Cumulus-oocyte complexes were isolated from ovaries obtained from a local abattoir and were matured in TCM199 supplemented with hormones (eCG and hCG) and ECS or PVA for 22-24 h. Fertilization in vitro was carried out using frozen-thawed semen of one bull in Fert-TALP containing heparin, hypotaurin, ephinephrine and BSA for 18-19 h.

For in vitro culture, presumptive zygotes were transferred into TCM199 with 10% ECS or SOF supplemented with PVA (6 mg/ml) for 9-10 days. Immature and matured oocytes, zygotes, 2-4-cell and 8-16-cell embryos, morulae, expanded blastocysts and hatched blastocysts were collected at the expected time points and used for this study. Poly(A)$^+$ RNA was isolated from pools of 20-25 oocytes or embryos from each group using a commercial kit (DYNABEADS mRNA DIRECT KIT) and after elution from the beads, the poly(A)$^+$ RNA was transcribed into cDNA using random hexamers. Hotstart PCR was performed with cDNA equivalents corresponding to 4-8 oocytes or embryos using specific primers for IGFI, IGFII, IGFIR and IGFIIR. Rabbit globin mRNA was added to each RT-PCR as an internal standard. Assays were repeated at least four times for each developmental stage. To optimize the amplification reaction for each pair of gene-specific primers, semilog plots of the fragment intensity as a function of cycle number were determined and the range of cycle numbers in which linear fragment production occurred was determined and the number of PCR cycles was kept within this range. A linear range was obtained when the amount of PCR product was approximately doubled upon doubling of the initial RNA input. For the IGF-I, IGF-II, IGF-IR and IGF-IIR genes, a linear increase in the PCR products was observed from 31 to 39 amplification cycles.

The following results are obtained:

1- With regard to the developmental rates of bovine embryos, maturation and fertilization rates tended to be higher in the TCM-system than in the SOF-system (91 ± 4,9 and 70,9 ± 8,3 vs 76,5 ± 2,4 and 64,2 ± 3,4, respectively). A significantly ($P< 0.05$) higher percentage of 2-4-cell and blastocysts was obtained in the TCM-system than in SOF-system (60,7 ± 1,0 and 25,0 ± 1,2 vs 54,8 ± 1,8 and 16,5 ± 0,6, respectively).
2- IGF-I mRNA-transcripts were not detected at any stage of preimplantation bovine development in both culture systems even when the PCR was performed with cDNA equivalents corresponding to 16 oocytes or embryos.
3- IGF-IR, IGF-II and IGF-IIR mRNA transcripts were detected in all stages of preimplantation development derived from the two culture systems.
4- In the TCM-system, IGF-IR was expressed from the immature oocyte, gradually decreased to the 8-16-cell embryo, increased again from the morula stage up to the hatched blastocyst. The same trend was seen in the SOF-system.
5- In the TCM-system, IGF-II was detected from the immature oocyte to the hatched blastocyst. It increased from the immature oocyte to the 2-4-cell stage, then increased at the morula stage, decreased again at the expanded blastocyst and then sharply increased at the hatched blastocyst. In the SOF-system, IGF-II was expressed from immature oocytes to the hatched blastocysts.
6- In the TCM-system, IGF-IIR was detected in immature and matured oocytes, decreased to the 8-16-cells stage and increased again up to the hatched blastocyst. In the SOF-system, IGF-IIR was expressed up to the 8-16-cell stage and then increased during further embryonic development up to the blastocyst stage.
7- In the present study, the amount of IGF-IR mRNA was slightly higher at all stages of development in embryos derived and cultured in the TCM system than in embryos that had been cultured in the SOF-system.
8- The individual differences in IGF-IR, IGF-II and IGF-IIR gene expression of embryos generated in the two culture systems were not significant except at the expanded blastocyst stage where IGF-IIR transcripts were significantly higher in the TCM-system than in the SOF- system.
9- The relative abundance of mRNA for IGF-IR, IGF-II and IGF-IIR seem to follow a pattern similar to most other genes which were expressed during preimplantation mammalian development.

10- Elevated transcript levels of IGF-IR, IGF-II and IGF-IIR mRNA during oocyte maturation in this study, could indicate that maturation depends on an increased mRNA synthesis of these genes.

11- The increase in IGF-IR, IGF-II and IGF-IIR mRNA observed when the bovine embryo reached the hatched blastocyst stage may indicate an important role of growth factors of the IGF family when the bovine embryo becomes filamentous in shape.

12- The results indicate that it is preferentially the paracrine rather than the autocrine mechanism which is the mode of action for IGF-I. Autocrine and paracrine activity appear to be important for IGF-II as IGF-II mRNA is present in preimplantation embryos

The presence of IGFs and their receptors in bovine preimplantation embryos suggests that the IGF system may indeed play an important role in bovine embryogenesis. Gene expression of IGF-II and the IGF-I and IGF-II receptors can be good indicators of the developmental potential for embryos, and finally, activity and pattern of IGF gene expression can serve as markers for embryo quality. Determination of growth factor gene expression in preimplantation bovine development can be used for optimization of in vitro culture systems, with the goal of improving the quality of embryos generated in vitro and ensuring normal offspring. These findings indicate that further studies are needed into the role of IGFs in 'Large Offspring Syndrome', which is frequently observed in calves derived from certain in vitro production systems. Further studies are necessary to investigate growth factor gene expression in embryos produced in vivo in an effort to clarify the effects of culture conditions.

7. ZUSAMMENFASSUNG

Mohamed A. Yaseen

Molekularbiologische Untersuchungen der Expression von Insulin-like Growth Faktor Genen und deren Rezeptoren in in vitro produzierten präimplantatorischen Rinderembryonen.

Diese Untersuchung wurde durchgeführt, um die relativen Transkripthäufigkeiten der Insulin-like-Growth-Faktoren (IGF-I, IGF-II) und ihrer Rezeptoren in präimplantatorischen Rinderembryonen aus zwei verschiedenen Kultursystemen (TCM- und SOF-System) zu bestimmen. Das TCM-System (TCM199 mit OCS supplementiert) ist ein komplexes undefiniertes Medium, dagegen steht das SOF-System (SOF supplementiert mit PVA) ein einfaches chemisch definertes Medium.
Insgesamt 6325 Oozyten (morpholgische Quälität Grad I und II) aus 997 Schlachthausovarien wurden für die IVP eingesetzt. Die isolierten Kumulus-Oozyten-Komplexe wurden in TCM199 mit Hormonzusatz (eCG und hCG) und OCS oder PVA maturiert. Gereifte Metaphase II-Oozyten wurden in vitro fertilisiert. Dazu wurde kryokonserviertes Sperma eines Bullen verwendet. Die IVF wurde in Fert-TALP mit Heparin, Hypotauarin, Ephinephrin und BSA für 18-19 Stunden durchgeführt. Die anschließende In vitro-Kultur wurde in TCM199 mit OCS oder SOF mit PVA-Zusatz für 9-10 Tage durchgeführt. Ungereifte, gereifte Oozyten, Zygoten, 2-4-Zell-, 8-16-Zell-Embryonen, Morulae, expandierte Blastozysten und geschlüpfte Blastozysten wurden dann für die Expressionsanalyse eingesetzt. Dazu wurde die Poly(A)$^+$ RNA aus Gruppen von 20-25 Oozyten oder Embryonen mit Hilfe eines kommerziell erhältlichen Isolationssystems (DYNABEADS mRNA DIRECT KIT) gewonnen. MuLV und Hexamers wurden für die Reverse Transcription der Poly(A)$^+$ RNA in cDNA verwendet. Die spezifische Amplifikation von IGF-I, IGF-II, IGF-IR und IGF-IIR-Amplicons wurde mittels Hotstart-PCR von cDNA aus 4-8 Oozyten oder Embryonenäquivalenten erreicht. Als interner Standard wurde jede RT-PCR-Reaktion mit Kaninchen-Globin-mRNA versetzt. Für jedes Embryonalstadium wurden 4 Wiederholungen durchgeführt. Für jedes genspezifische Primer-Paar wurden die Amplifikationsbedingungen optimiert. Dazu wurden in eine semilogarithmische Graphik die erhaltene Fragmentmenge in Abhängigkeit von der PCR-Zyklenzahl aufgetragen, und die Zyklenzahl, in der eine lineare

Amplifikation erfolgte, bestimmt. Für die untersuchten IGF-I-, IGF-II-, IGF-IR- und IGF-IIR-Transkripte wurde eine lineare Amplifikation zwischen 31-39 PCR-Zyklen ermittelt.

Die folgenden Ergebnisse wurden erzielt:

1- Im TCM-System wurden signifikant höhre Teilungs und Blastozystenraten (60,7 ± 1,0 und 25,0 ± 1,2 vs 54,8 ± 1,8 and 16,5 ± 0,6) als im SOF-System gefunden.
2- IGF-I mRNA konnte in keinem Embryonalstadium, weder im TCM-System noch im SOF-system, nachgewiesen werden.
3- IGF-IR-, IGF-II- und IGF-IIR-Transkripte wurden in allen untersuchten Embryonalstadien detektiert.
4- Im TCM-System wurde ein gradueller Abfall der relativen IGF-IR-Transkripthäufigkeit bis zum 8-16-Zeller, gefolgt von einem erneuten Anstieg bis zur geschlüpften Blastozyste gezeigt. Im SOF-System wurde eine gleiche entwicklungsabhänige Regulation der IGF-IR-Transkripte gefunden.
5- Die IGF-II-Transkripte wurden im TCM-System von der unreifen Oozyte bis zur Blastozyste nachgewisen. Dabei wurde ein Anstieg der relativen Transkripthäufigkeit bis zum 2-4-Zeller, gefolgt von einem Abfall bis zum 8-16-Zell Stadium und einem kontinuierlichen Anstieg bis zur geschlüpften Blastozyste festgestellt. Im SOF-System zeigte IGF-II ein identisches Expressionsmuster.
6- Die IGF-IIR-Transkripte zeigten im TCM- und SOF-system eine Verringerung bis zum 8-16-Zell Stadium und dann einen Anstieg bis zur geschlüpften Blastozyste.
7- Die IGF-IR-mRNA-Häufigkeit war in allen Embryonalstadien, die in TCM kultiviert waren, erhöht gegenüber Embryonen aus dem SOF-System.
8- Die Transkriptehäufigkeit von IGF-IR, IGF-II und IGF-IIR war in der semi-quantitativen RT-PCR nicht signifikant unterschiedlich zwischen dem TCM- und SOF-System. Ausnahme waren signifikant erhöhte IGF-IIR-Transkripte in expandierten Blastozysten, die im TCM-System kultiviert wurden.
9- Die Expressionsmuster von IGF-IR, IGF-II und IGF-IIR entsprechen damit dem Expressionsmuster der meisten anderen Gene, die frühembryonal exprimiert werden.
10- Die höhere Transkripthäufigkeit von IGF-IR, IGF-II und IGF-IIR während der Oozytenreifung deutet auf eine mögliche Rolle der Proteine in der Maturation hin.
11- Der deutliche Anstieg von IGF-IR-, IGF-II- und IGF-IIR-Transkripten in geschlüpften Blastzysten deutet auf eine wesentliche Rolle der IGF-Wachstumsfaktoren in diesem Stadium und der anschließenden Elongation des Embryos hin.

12- Die Ergebnisse weisen auf eine parakrine Regulation des IGF-I-Systems in der frühen Embryogenese hin. Im IGF-II system scheinen sowohl autokrine als auch parakrine steuerungsmechanismen im Embryowirksam zu sein.

Die Expression von IGFs und ihrer Rezeptoren in bovinen präimplantatorischen Embryonen weist auf eine wesentliche Rolle des IGF-Systems in der Embryogenese hin. Die Genexpression von IGF-IR, IGF-II und IGF-IIR kann möglischeweise als Indikator für das Entwicklungspotential und als Marker für die Embryoqualität dienen. Die Bestimmung der Genexpression von IGF-Gene kann möglicherweise für die Optimierung von In vitro Kultur-Systemen mit dem Ziel die Qualität von Rinderembryonen zu erhöhen und gesunden Nachkommen zu garantieren, eingesetzt werden. In den gegenwärtig genutzten In vitro-Systemen kommt es mit einer relative hohen Frequenz zu Kälber, die das 'Large Offspring Syndrome' zeigen. Die Ergebnisse dieses Arbeit liefern die Grundlage, um in weiteren vertiefenden Untersuchungen den Einfluß des Kulturmediums auf die Genexpression des IGF-Systems zu klären.

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9. List of Abbreviations

aa	amino acids
AMV	avian myeloblastosis virus
bla.	Blastocyst
BOEC	bovine oviductal epithelial cells
bp	bases pair
BSA	bovine serum albumin
Cai	internal calcium
cAMP	cyclic adenosin 3' 5'-monophosphate
cDNA	complementary DNA
COC	cumulus oocyte complex
CV	coefficient of variation
DNA	deoxyribonuclic acid
dNTP	deoxynucleotide
dT	Dynabeads oligo
DTT	dithiothreitol
eCG	equine chorionic gonadotropin
ECM	extracellular matrix
ECS	estrous cow serum
EDTA	ethylene-diamine-tetraacetic acid
EGF	epidermal growth factor
EtBr	ethidium bromide
exp.	expanded
FCS	fetal calf serum
fg	femto gram
FGF	fibroblast growth factor
Fig.	Figure
FSH	follicle stimulating hormone
GH	growth hormone
GV	germinal vesicle
GVBD	germinal vesicle breakdown
HB-EGF	heparin binding epidermal growth factor
hbla.	Hatched blastocyst
HCG	human chorionic gonado tropin
Hepes	H-[2-Hydroxyethyl] piperazin-N'-[Ethansulfonic acid]
hnRNA	heterogeneous nuclear RNA
hr	hour
ICM	inner cell mass
IGFBPs	insulin-like growth factor binding proteins
IGF-I	insulin-like growth factor-I
IGF-II	insulin-like growth factor-II
IGF-IR	the type I IGF-I receptor
IGF-IIR	the type II IGF-II receptor
IGFs	the insulin-like growth factor family (IGF-I & IGF-II)
IL-3, –6	interleukin-3, -6
i.ooc.	imature oocyte
IU	international unit
IVC	in vitro culture
IVF	in vitro fertilization
IVM	in vitro maturation
IVP	in vitro production

LH	luteinizing hormone
LIF	leukaemia inhibitory factor
MII	metaphase II
MEM	minimum essential medium
MET	maternal- embryonic transition
min	minute
MM	master mix
MMLV	mouse Moloney leukemia virus
mM/µM	millimol/micromol
mo.	morula
m.ooc.	mature oocyte
MPF	maturation promoting factor
MW	molecular weight
mRNA	messenger RNA
MuLV	murine leukemia virus
MW marker	molecular weight marker
MZT	maternal to zygotic transition
n	numbers
NBCS	newborn calf serum
ng	nano gram
NGF	nerve growth factor
No.	Number
OPU	ovum pick up
PAF	platelet activating factor
PBS	Dulbecco’s phosphate buffered saline
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PH	hydrogen ion concentration
PVA	polyvinyl alcohol
PVP	polyvinyl pyrrolidone
qPCR	quantitative PCR
RNA	ribonucleic acid
ROS	reactive oxygen species
rRNA	ribosomal RNA
RT	reverse transcription
sec	second
SEM	standard error of the mean
sn	small nuclear
snRNPs	small nuclear ribonucleoproteins
SOF	synthetic oviduct fluid
TAE	tris-Acetate-EDTA-buffer
TBE	tris-borate-EDTA-buffer
TE	tris-EDTA-buffer
Taq	Thermus aquaticus
TALP	Tyrode, albumin, lactate, pyruvate
TCM	tissue culture medium
T_m	melting temperature
TGF	transforming growth factor
TRC	transcription requiring complex
tRNA	transfer RNA
UV	ultraviollett
V	volt
VEGF	vascular endothelial cell growth factor
Zyg.	Zygote

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