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Identification and SNP detection for preimplantation active genes and their association with embryo development and male fertility in cattle

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Dedicated to my parents

and

My family

Identifikation und SNP-Detektion von preimplativ aktiven Genen und ihre Assoziation mit der Embryonalentwicklung und Bullenfruchtbarkeit

Vorangegangene Studien legten ihre Schwerpunkte auf die Identifikation und Charakterisierung von Genen die die frühe Entwicklung beeinflussen. Die Erkenntnisse über die Sequenzunterschiede und deren Beziehung zu den Fruchtbarkeitsmerkmalen sind begrenzt. Das Ziel diese Studie war es, SNPs (Einzelbasenaustasch) in den Genen CDH1, DSC2, TJP1, PKP1, COX-2, CD9, GJA1, PLCζ, AKR1B1, N-PAC und EEF1α zu identifizieren und ihre Assoziation mit den männlichen Fruchtbarkeitsmerkmalen Non Return Rate (NRR), Spermaqualität (Volumen je Ejakulation, Konzentration, Motilität und Überlebensfähigkeit) und Spermium-Qualitätsmerkmale (Plasma Membran Integrität, Akrosomen Integrität und DNA Integrität), sowie der frühen Embryoentwicklung zu klären. Zur Detektion der Polymorphismen, wurden die Genotypen von 11 verschiedenen Rinderassen analysiert. Aus dem Sperma von 310 Bullen der Rasse Deutsche Holstein wurde genomische DNA isoliert und für die Auswahl der Kandidatengene genotypisiert. Der Vergleich der Ergebnisse aus der Sequenzierung ermöglichte es, SNPs in den Genen CD9 im Exon 9, bei Pos. 95 (T>C), Diese Polymophismen hatten keinen Einfluss auf die Proteinstrukturen. Weitere SNPs konnten in den Genen PLCζ im Intron 6, Pos. 2749 (G>A), COX-2 im Intron 5, Pos. 185 (T>C) und Intron 7, Pos. 6 (A>T), AKR1B1 im Intron 7, Pos. 159 (A>G), N-PAC im Intron 13, Pos. 5 (C>T), DSC2 im Intron 7, Pos. 281 (T>C), im Intron 9, Pos. 6 (A>G) sowie Pos. 21 (G>A), im Intron 12, Pos. 206 (G>A), Pos. 328 (T>C), Pos. 341 (C>T) sowie Pos. 378 (T>C), CDH1 zwei SNPs im Intron 7, Pos. 43 (G>A) und Pos. 92 (C>G), in TJP1 im Exon 13, bei Pos. 50 (C>T), Diese Polymophismen hatten keinen Einfluss auf die Proteinstrukturen und im Gen PKP1 im Intron 9, Pos. 140 (G>A) detektiert werden. Die Assoziationsanalyse zeigte, dass sich die Gene DSC2 und TJP1 mit dem Volumen der Ejakulation ($P<0.05$), AKR1B1 und CDH1 mit der Überlebensfähigkeit des Spermas ($P<0.05$), CD9, AKR1B1, COX2, DSC2 und TJP1 mit der Motilität ($P<0.05$), sowie CD9, N-PAC, CDH1 und PKP1 mit der Spermiumkonzentration ($P<0.05$) signifikant assoziieren ließen. Die gefundenen SNPs hatten aber keinen Einfluss auf die NRR und auf Merkmale der Spermien. Bei der abschließende Genotypisierung von Embryonen für drei Gene (DSC2, CDH1 und TJP1) konnte ein signifikanter Effekt ($P<0.05$) von TJP1 mit dem Merkmal Entwicklung der Embryonen beobachtet werden.

Identification and SNP detection for preimplantation active genes and their association with embryo development and male fertility in cattle

Previous studies have focused on identification and characterization of genes involved in early development. However, information on the relationship between sequence variance of these genes and fertility traits is limited. Therefore, this study was conducted to identify single nucleotide polymorphisms in CDH1, DSC2, TJP1, PKP1, COX-2, CD9, GJA1, PLCζ, AKR1B1, N-PAC and EEF1α genes and their association with male fertility traits Non Return Rate (NRR), semen quality traits (volume per ejaculation, concentration, motility and survivality after thawing) and sperm flow cytometry parameters (plasma membrane integrity, acrosome integrity and DNA fragmentation index) and early embryo development. To full fill these objectives, 11 different cattle breeds were used for screening polymorphism. Genomic DNA of sperm of 310 German Holstein bulls was genotyped for the selected candidate genes. Comparative sequencing results revealed polymorphisms in CD9 at pos. 95 (T>C) in exon 9 having no change in amino acid, in PLCζ at pos. 2749 (G>A) in intron 6, in COX-2 two SNPs at pos. 185 (T>C) and pos. 6 (A>T) in intron 5 and 7 respectively, in AKR1B1 at pos. 159 (A>G) in intron 7, in N-PAC at pos. 5 (C>T) in intron 13, in DSC2 seven SNPs at pos. 281 (T>G) in intron 7, pos. 6b (A>G) and pos. 21 (G>A) in intron 9, pos. 206 (G>A) and pos. 328 (T>C) and pos. 341 (C>T) and pos. 378 (T>C) in intron 12, in CDH1 three SNPs at pos. 47 (C>T) in exon 8 having no change in amino acid, and two SNPs at pos. 43 (G>A) and pos. 92 (C>G) in intron 7, in TJP1 at pos. 50 (C>T) in exon 13 having no change in amino acid and in PKP1 at pos. 140 (G>A) in intron 9. Association analysis revealed significant association of the following genes with various traits: DSC2 and TJP1 ($P<0.05$) with volume of ejaculation, AKR1B1 and CDH1 ($P<0.05$) with sperm survivality, CD9, AKR1B1, COX-2, DSC2 and TJP1 ($P<0.05$) with sperm motility ($P<0.05$), CD9, N-PAC, CDH1 and PKP1 ($P<0.05$) with sperm concentration. No association was found between identified SNPs and NRR and sperm flow cytometry parameters. Finally, the genotyping results of embryo developed from three genes (DSC2, CDH1 and TJP1) revealed that TJP1 has significant association with embryo development ($P<0.05$).

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List of abbreviations

A	Adenine
APS	Ammonium peroxydisulphate
ATP	Adenosine triphosphate
bp	Base pairs
BLAST	Basic Local Alignment Search Tool
BSA	Bovine serum albumin
C	Cytosine
DFI	DNA fragmentation index
ddH_2O	Distilled & deionized water
DMSO	Dimethyl sulfoxide
DNA	Deoxynucleic acid
dNTP	deoxyribonucleoside triphosphate (usually one of dATP, dTTP, dCTP and dGTP)
DTCS	Dye Terminator Cycle Sequencing
EDTA	Ethylenediaminetetraacetic acid (powder is a disodium salt)
EtBr	Ethidium bromide
ExoSAP	Exonuclease I and Shrimp Alkaline Phosphatase
GLM	General Linear Models
g	Gram
G	Guanine
h	Hour
ICM	Inner cell mass
IPTG	Isopropylthio-ß-D-galactoside
IVC	In vitro culture
IVF	In vitro fertilization
IVM	In vitro maturation
IVP	In vitro production
L	Litre
LSM	Least square means
mg	Milligram
$MgCl_2$	Magnesium chloride

min	Minute
ml	Milliliters
MPM	Modified parker medium
mRNA	Messenger RNA
MW	Molecular weight
NaCl	Sodium chloride
NRR	Non return rate
n.s	Not significant difference
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PLCζ	Phospholipase C zeta
PNA	Peanut (*Arachis hypogea*) agglutinin
PSA	Pisum sativum agglutinin
QTL	Quantitative trait loci
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
rpm	Round per minute
SAS	Statistical Analysis System software
SDS	Sodium dodecyl sulfate
SNP	Single nucleotide polymorphism
SSCP	Single strand conformation polymorphism
T	Thymine
TAE	Tris-acetate buffer
TBE	Tris- borate buffer
TCM	Tissue culture medium
TEMED	N, N, N′, N′-Tetramethylendiamine
UV	Ultra-violet light
X-gal	5-Bromo-4-chloro-3-indolyl-beta-D-galactoside
µl	Microliter

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1 Introduction

The profitability of dairy farms and beef production depends greatly on the reproductive efficiency of dairy cows (Britt 1985, Hamilton 2006, Meadows et al. 2005, Plaizier et al. 1997). Numerous studies have documented that additional days in which cows are not pregnant beyond the optimal time post-calving are costly (Groenendaal et al. 2004, Holmann et al. 1984, Meadows et al. 2005). The value of a new pregnancy has been reported to average approximately $200 (Eicker and Fetrow 2003). The cost of pregnancy loss per abortion has been estimated at $600 to $800 (Eicker and Fetrow 2003, Thurmond and Picanso 1990). Pregnancy loss can have destroying effects on economical success in dairy and beef units. In beef herds, pregnancy loss represents even more important economic factor because most of the income is determined by the number of calves sold. Therefore, pregnancy loss due to, embryonic mortality is a recognized cause of reproductive failure in cattle leading to the loss of a large number of potential calves, delayed genetic progress, and significant loss of money and time in rebreeding cows (Khurana and Niemann 2000, Morris et al. 2001).

Preimplantation development encompasses the period from fertilization to implantation, which occurs in different times in various species (e.g. 4.5 days for mouse, 6–7 days for human, 15–16 days for sheep, and 17–34 days for cow (Ko 2004). Both human and rodent preimplantation embryos express an array of junctional proteins, including components of tight junctions, desmosomes and other cell adhesion molecules (Bloor et al. 2004). An appropriate temporal and spatial pattern of intercellular junctions are needed for successful preimplantation development. Experiments on rodent preimplantation embryos have shown that the onset of E-cadherin expression is essential for compaction (Larue et al. 1994) and expression of the tight junction protein complex is responsible for maintaining cellular polarity of the trophectoderm through positioning the basolateral Na^+/K^+-ATPase (Fleming et al. 2001).

However, in human and also in domestic animals the failure rate for implantation process is high. The reasons for this failure are unclear. Implantation failure may result from failure of particular gene expression or enormously gene expressions at a crucial point in time. In some cases, even a defect in a single but a critical gene is sufficient to cause implantation failure (Copp 1995).

Embryonic mortality results either from intrinsic defects within the embryo, or an inadequate maternal environment, or asynchrony between embryo and mother, or failure of the mother to respond appropriately to embryonic signals (Hansen 2002). With the advent of reproductive technologies this developmental failure becomes more evident. In in vitro production (IVP) of bovine embryos, most of this mortality is sustained within the first 2-3 weeks after fertilization (Diskin and Sreenan 1980, Dunne et al. 2000, Farin et al. 2001). The explanation for this high rate of developmental failure according to the defect within the embryo (intrinsic errors) remains unclear. The extent and regulation of cell death during preimplantation development is likely to be critical for later development of the conceptus (Brison and Schultz 1997), however, the causes, roles and genetic regulation of embryo death and arrest before implantation remain to be elucidated.

Actual fertility levels result from a combination of genetic potential and many environmental factors including nutrition, health and level of management of both cows and the bull. Reproductive traits tend to have a low heritability. This factor and our inability to measure reproductive traits very well mean, we have focused on nutrition and management to improve or at least maintain reproductive performance (Hamilton 2006). Fertility is highly influenced by management and environmental factors, but significant genetic differences exist in both male and female fertility (Weigel 2004).

It has been proposed that candidate gene analysis can be used to identify individual genes responsible for traits of economic importance (Lin 2005, Linville et al. 2001). For selecting fertility traits one can take the genotype into consideration besides the phenotype, which are the externally visible and measurable traits. The accuracy and intensity of selection can be improved by including the genotypic information. The possibility of realizing selection criteria on a molecular genetic level shortens the generation interval. Particularly for fertility traits, this is of greatest interest.

In our study E-cadherin (CDH1), desmocollin2 (DSC2), tight junction protein 1 (ZO1/TJP1), plakophilin1 (PKP1), prostaglandin G/H synthase-2 (PTGS-2/COX-2), CD9, gap junction protein, alpha 1 (Connexin43/ GJA1), phospholipase c zeta (PLCζ), aldo-keto reductase family 1, member b1 (AKR1B1), cytokine-like nuclear factor (N-PAC) and elongation factor 1 alpha (EEF1α) genes were selected because they are indicative of various mechanisms in bovine preimplantation development. E-cad,

DSC2, PKP1 transcripts are sensitive markers for compaction and other genes contributed in embryo development. These genes were investigated for association with Non-Return-Rate (NRR) trait of bulls. In addition, these genes were also tested for association with semen quality traits (sperm concentration, motility, semen volume per ejaculate, survivability after thawing) and sperm flow cytometry parameters (sperm plasma membrane integrity, acrosome integrity and DNA fragmentation index). Finally DSC2, CDH1 and TJP1 genes were tested for embryo development in blastocyst stage.

2 Literature review

2.1 Bull fertility

Reproductive efficiency of dairy cattle depends on the ability of the bull fertility the cow. Since individual bulls service many females, a deficiency in the breeding ability of one bull has a larger impact on herd productivity than fertility problems in a single female. Using a sub-fertile bull may lead to longer calving intervals, a lower number of calves produced and increased costs of wintering open females. All of these results cause serious economic loss to the cow-calf producer. Bull fertility is often measured by the percentage of cycling females exposed to the bull and impregnated during a specific period of time (usually 60–90 days) (Grossman et al. 1995). Reproductive efficiency of bulls is usually measured by non return rate (NRR), which is commonly defined as the proportion of cows that were inseminated and did not return for another service within a specified number of days such as 60 to 90. NRR is the result of two events: conception at the time of insemination and gestation after conception (Grossman et al. 1995, Koops et al. 1995). Conception at insemination depends on the availability of ova and their quality and on the population of spermatozoa inseminated and its characteristics. Factors that influence conception include number or concentration of spermatozoa inseminated, technician, parity, and herd management. Gestation after insemination depends on conception, maternal environment, and maternal and paternal contributions to developmental potential of the conceptus. Factors that influence gestation after insemination include failure of conception, failure of implantation, embryonic or early fetal death, and abortion (Koops et al. 1995). Two types of embryonic death have been distinguished. One type occurs when the conceptus does not survive long enough to provide the signal for maternal recognition of pregnancy, dying before about 15 to 17 d after insemination. The other type occurs when the conceptus survives long enough to provide the signal for maternal recognition of pregnancy, dying after about 15 to 17 d after insemination (Grossman et al. 1995). Male fertility can be regarded as a result of both fertilizing ability of the sperm cells and of viability of the embryo, but under field conditions only the outcome of the inseminations can be recorded, i.e. whether or not the females have returned for repeat insemination (Stalhammar et al. 1994).

NRR overestimate the true rate of conception (Reurink et al. 1990). Measures derived from NRR, such as conception rate and calving rate, might be more reliable for evaluation than NRR itself. Estimated conception rate is a better early measure of efficiency than NRR, because conception rate depends on the population of spermatozoa at insemination and not on developmental potential of the conceptus after insemination (Koops et al. 1995).

2.1.1 Plasma membrane integrity

The maintenance of the sperm fertilizing potential depends on the integrity and functionality of different cellular structures; membranes exert a fundamental role in the maintenance of the sperm fertilizing capability. The plasma membrane is responsible for the mechanism of maintaining the cell osmotic equilibrium, acting as a barrier between intra- and extra cellular mediums. Damages in this structure can conduce to homeostasis loss, leading to cellular death (Flesch and Gadella 2000). Consequently, plasma membrane integrity is crucial to sperm survival inside the female reproductive tract, and in maintaining fertilizing capability (Celeghini et al. 2007). Viable sperm" are defined as cells that possess an intact plasma membrane. This quality is evaluated by staining a sperm sample with propidium iodide (PI), a fluorescent probe that binds to DNA. Cells having an intact plasma membrane will prevent PI from entering into the cell and staining the nucleus. However, cells possessing a damaged plasma membrane will permit PI to enter into the cell and bind to the DNA causing the cells to fluoresce red (Celeghini et al. 2007, Graham 2001). Three kinds of bull sperm populations, green (live), red (dead), and dual-stained (partially damaged) have been detected by flow cytometry and by fluorescence microscopy in fresh and frozen bull semen (Anzar et al. 2002).

Fluorescent staining of spermatozoa to determine viability can be approached in two ways: fluorochromes used to indicate viable cells and those used to indicate non-viable cells. Non-viable cells can be determined using membrane-impermeable nucleic acid stains which positively identify dead spermatozoa by penetrating cells with damaged membranes. An intact plasma membrane will prevent these products from entering the spermatozoa and staining the nucleus. Commonly used examples include phenanthridines, for example propidium iodide (PI) (Gillan et al. 2005). Propidium

iodide has been a probe of choice to assess the extent of cryo-damage of spermatozoa for microscopic, flow cytometric, or fluorometric evaluation (Januskauskas et al. 2003). Their integrity can be assessed using supravital fluorescent stains that depend on the ability of intact membranes to exclude particular stains from the inner compartments of the spermatozoon. Generally, these stains discriminate between dead and live cells, although a third group of cells, damaged or dying spermatozoa, can be recognized when double stains are used (Pena et al. 2003).

2.1.2 Acrosome integrity

The acrosome is a large Golgi/Endoplasmic reticulum derived from acidic secretory organelle. It is filled with hydrolytic enzymes that are organized in a kind of enzyme matrix and most enzymes are heavily glycosylated. Initial sperm-zona binding will trigger the acrosome reaction resulting in the release and activation of acrosomal enzymes. This together with the acquired hyper-activated motility will help the sperm to penetrate the zona pellucida. The acrosome must remain intact before and during the transit of the sperm to the isthmus until zona binding has been accomplished. Early acrosome reactions render sperm infertile (Silva and Gadella 2006), and therefore, acrosomal integrity is a very important functional characteristic which permits evaluation of sperm fertilizing potential (Nikolaeva et al. 1998, Silva and Gadella 2006).

Acrosomal integrity can be measured by a number of methods, but the most commonly used method is with a plant lectin labeled by a fluorescent probe. There are a large number of lectins available for assessing acrosomal integrity. Pisum sativum agglutinin (PSA) is a lectin from the pea plant that binds to α-mannose and α-galactose moieties of the acrosomal matrix. Since PSA cannot penetrate an intact acrosomal membrane, only acrosome-reacted or damaged spermatozoa will stain. Peanut (Arachis hypogea) agglutinin (PNA) is a lectin from the peanut plant that binds to β-galactose moieties associated with the outer acrosomal membrane of fixed spermatozoa, indicating acrosome-intact cells (Gillan et al. 2005). In each case, the lectins, themselves, are not fluorescent, but can be labeled with many different fluorescent probes (Graham 2001). PSA/PNA when bound to Fluorescein Isothiocyanate-conjugated (FITC) marks damaged sperm acrosome in yellow-green (Celeghini et al. 2007). Lectin binding to

sperm samples was examined by fluorescence microscopy to determine the location of sperm-bound fluorescence and the proportion of labelled spermatozoa in the total population (Nikolaeva et al. 1998). Detection can be done on living sperm: the absence of fluorescence is indicative for an intact acrosome, and fluorescence over the anterior sperm head and equatorial regions is indicative for acrosome disruption or acrosome reaction (Marti et al. 2000, Silva and Gadella 2006).

In a regression analysis, it was revealed that sperm motility was positively correlated with sperm membrane integrity ($t = 1.66$, $P = 0.1016$) and sperm acrosome integrity ($t = 3.24$, $P = 0.0019$) (Hua Y 2006). The simple coefficient of correlation among bulls between motility and percentage of sperm with intact acrosomes was only 0.33 (Berndtson et al. 1981).

2.1.3 DNA integrity

The integrity of the sperm DNA is of crucial importance for the accurate transmission of genetic information and further development of an embryo (Agarwal and Said 2003, D'Occhio et al. 2007). The degree of DNA damage is clearly correlated with the impairment of embryo development and severe DNA damage cause male infertility (Agarwal and Said 2003, Silva and Gadella 2006). However, IVF experiments with gamma irradiated sperm showed that sperm with severe DNA damage remained functionally intact at the level of membrane and organelle and motility parameters. In fact, the DNA damaged sperm showed normal ZP binding characteristics and even the fertilization and cleavage rates of the fertilized oocytes remained normal. However, embryo development is blocked after reaching the four to eight cell stages (Silva and Gadella 2006). The sperm chromatin status at the time of fertilization influences the developmental expression of paternally-inherited genes (De Jonge 2000). The contribution of sperm chromatin instability to reproductive wastage in both natural mating and assisted reproduction warrants further investigation as it may prove valuable as a means of decreasing the incidence of embryonic mortality. In this regard, it is possible that 'male factor' infertility may emerge as an even more important component in embryonic development (D'Occhio et al. 2007).

Eid et al. (1994) reported that sperm from bulls possessing either low fertility (69% non-return rate) or high fertility (78% non-return rate), exhibited similar in vitro fertilization rates (77 and 79%, respectively). However, embryos resulting from sperm from the low fertility bulls did not develop as rapidly as embryos resulting from fertilizations by sperm from the high fertility bulls. Since sperm from these low fertility bulls can initiate fertilization, but the resulting embryos can not develop properly, it is likely that relatively high numbers of sperm from some low fertility bulls contain genetic abnormalities which impedes normal embryonic development. Some of these abnormalities affect the overall structure of spermatozoa chromatin, which can be detected using flow cytometry (Graham 2001). The DNA fragmentation (DF) of spermatozoa can be determined using the metachromatic properties of acridine orange (AO) in the sperm chromatin structure assay (SCSA) (Gillan et al. 2005). AO intercalated into double-stranded DNA (native) emits green fluorescence and red fluorescence when associated with single-stranded DNA (Fatehi et al. 2006, Gillan et al. 2005). The ratio of red to total fluorescence can be determined by flow cytometry to provide an index of normality/abnormality. The SCSA has shown good correlation with fertility in a number of species (bull, human and mouse) where spermatozoa from males with low fertility exhibit more chromatin denaturation than spermatozoa from high-fertility males (Gillan et al. 2005).

The multiple regression studies show that parameters (% of intact acrosomes in total population, and percentage of fragmented DNA) present a good predictive value for the sperm fertility ($r = 0.34$, $p < 0.001$) (García-Macías et al. 2007).

2.2 Preimplantation development

Preimplantation development encompasses the period from fertilization to implantation. Fertilization is the union of two haploid gametes to reconstitute a diploid cell ´´zygote``- a cell with the potential to become a new individual. Fertilization is not a single event. Rather, it is a series of steps that might be said to begin when egg and sperm first come into contact and end with the incorporateing of haploid genomes. Prior to fertilization, the two gametes must become fully mature and be transported to a site within the female ``oviduct´´ that will support their interactions with one another. Formation of

zygotes following successful fertilization triggers cohorts of events beginning with repeated cycles of cell division without an increase in cell mass ``cleavage´´, activation of embryonic genome, compaction and differentiation to inner cell mass and trophoblast cells resulting in the formation of blastocyst (Mamo 2004).

In mammals, the preimplantation period is characterized by four major developmental phases that occur after fertilization. these transitions include: 1) cleavage and division, the timing of which is known to be indicator of subsequent developmental potential of the embryo, 2) transition from maternal to zygotic gene activation or embryonic genome activation; 3) Compaction, which results in the formation of communicating polarized epithelium and 4) Differentiation of the morula into the blastocyst, which is composed of totipotent cells of the inner cell mass that will give rise to the embryo proper and the differentiated cells of the trophectoderm, which is a fluid transporting epithelium and that will give rise to extra embryonic tissue (Tesfaye 2003). Preimplantation development is a mammalian-specific event, and is vital for successful implantation and pregnancy (Maekawa et al. 2005). Pre-implantation development occurs with different timing in various species, e.g. 4.5 days for mouse, 6-7 days for human, 15-16 days for sheep, 17-34 days for cow (Ko 2004). These morphological and physiological transitions in preimplantation development of embryos are known to be accompanied and governed by temporal and spatial expression of developmentally important genes (Schultz et al. 1999).

2.3 Embryonic genome activation

Morphological and metabolic transitions in the preimplantation development are regulated by differential expression of developmentally important genes (Schultz et al. 1999). Following fertilization, there is a reprogramming of gene expression as transcription from the zygotic genome begins and maternal messages continue to be degraded (Wang et al. 2004). However, the earliest stages of development are largely dependent on maternally derived messages stored in the oocyte prior to fertilization. As development progresses, maternal RNAs and proteins are depleted and embryo-derived messages become key controlling factors. The shift from dependence on oocyte-derived messages to embryo-produced messages is referred to as the maternal-zygotic transition (Kanka 2003, Telford et al. 1990). In bovine, the activation of the embryonic genome

begins after 8 cells stage (Memili et al. 1998). The transition from maternal to embryonic control of development is characterized by a degradation of maternal RNA and protein and a burst of transcriptional activity from the embryonic genome (Memili and First 2000).

2.4 Compaction and blastocyst formation

Compaction is the first event of morphogenic and cellular differentiation, the process by which an embryo progresses from a collection of rounded, loosely associated blastomeres into a spherical embryo. Compaction includes two interrelated processes; cell flattening and cell surface polarization (Sutherland and Calarco-Gillam 1983). Early indicators of embryonic compaction include slightly uneven blastomere size, vesiculated or grainy cytoplasm, as well as an appearance of increased cell-to-cell adhesion and loss of definition between individual blastomeres (Desai et al. 2000, Wiemer et al. 1996). The process of compaction sets up the initial fate specification of the blastomeres because, after subsequent round of mitosis, an embryo has cell with two distinct lineages. The outside cells will become trophoblastic cells, while the blastomeres on the inside of the compacted embryo will form the inner cell mass (Johnson and Ziomek 1981). Tight junctions provide a permeable seal that allows fluid to move from outside to the inside of the blastocyst without substantial leakage and from the blastocoel. The trophoectoderm ion transport system plays an important role in establishing ion concentration gradients across the epithelium, and thereby providing the force that drive water into the blastocoelic fluid (El-Halawany et al. 2005). The close cell contacts that develop are due to the presence of the cell adhesion molecule E-cadherin, which progressively becomes distributed to areas of cell-cell contact (Johnson et al. 1986). Blastocyst formation is essential for implantation, establishment of pregnancy and is a principal determination of embryo quality prior to embryo transfer (Watson and Barcroft 2001). Compaction appears at the 16-32 cell stage in bovine species (Nikas et al. 1996).

2.5 Genes related to preimplantation development and cell to cell adhesion

2.5.1 Genes related to preimplantation development

2.5.1.1 CD9

In mammals, fertilization is completed by the direct interaction of sperm and egg, a process mediated primarily by gamete surface proteins. To penetrate the substantial cumulus cell barrier surrounding ovulated eggs of most mammalian species, sperm use hyper activated motility and a glycosylphosphatidylinositol (GPI) anchored surface hyaluronidase, (Figure 1). The motility and surface hyaluronidase are necessary, and perhaps sufficient, to digest a path through the extracellular matrix of the cumulus cells (Primakoff and Myles 2002).

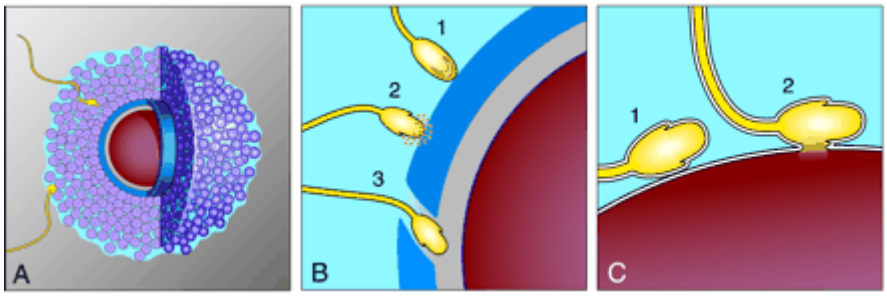

Figure 1: A) Sperm penetration of cumulus cells (purple) to reach zona (navy blue). (B) Egg depicted with cumulus cells removed; sperm 1 binds to the zona (Navy blue); sperm 2 undergoes exocytosis, releasing acrosomal contents (orange-red); sperm 3 penetrates the zona pellucida and begins entry into perivitelline space (gray). (C) Sperm 1 binds to the egg plasma membrane by the side of its head, in a central region (equatorial region); sperm 2 fuses with the egg plasma membrane (Primakoff and Myles 2002)

Fertilization is perhaps the most well-known form of cell to cell fusion. Membrane fusion between sperm and egg occurs after a series of perfusion events, including penetration of the outer layer of the oocyte by sperm, secretion of enzymes by the lysosome-like acrosome in the sperm head, and penetration of the egg's inner layer, the zona pellucida, by the sperm. Only after entry of the sperm into the egg's perivitelline

space does fusion occur between the sperm and egg plasma membranes (Chen and Olson 2005).

To date, the only protein that has been shown to be required for sperm-egg fusion is CD9. The glycoprotein CD9 is a member of the transmembrane-4-superfamily (tetraspanin) and is widely present on cell membranes in animals. It is present on the plasma membrane of oocytes at different developmental stages (Li et al. 2004). CD9 has been implicated in the regulation of cell-biological functions, including cell adhesion, motility, proliferation, differentiation, and signaling (Kaji and Kudo 2004, Le Naour et al. 2000, Liu et al. 2006).

CD9 is closely related to other tetraspanin proteins, integrins, glycoproteins, growth factor and other membrane proteins. Some proteins in this network participate in many different cellular functions, such as adhesion, migration, differentiation, proliferation and signal transduction (Li et al. 2004).

CD9 was significantly increased during the final oocyte maturation, indicating that it is associated with the competence of the oocyte to be fertilized (Li et al. 2004). A study conducted on mice confirmed, CD9 has critical role in fertilization (Kaji et al. 2000), because CD9 null mice showed complete sterility due to a deficiency in egg-sperm fusion (Xiang and MacLaren 2002). Mice lacking CD9 were born healthy and grew normally. However, the litter size from these females was less than 2% of that of the wild type. Results indicated that CD9 is not present on sperm membrane (Miyado et al. 2000).

CD9 has been reported to associate with several integrins in a number of migration, adhesion, and fusion systems, including those containing the α_3, α_5, α_6 or β_1 subunits (Xiang and MacLaren 2002). Integrin $\alpha_6\beta_1$ has been shown to bind to sperm glycoprotein fertilin and could function as a sperm receptor, and there is a possibility that CD9 plays an important role in sperm-egg fusion by modulating $\alpha_6\beta_1$ receptor function. The integrin family provides a physical link between the extra cellular matrix and the cell cytoskeleton and transducers signals, eliciting changes in the intracellular pH, cytoplasmic calcium level, phospholipid metabolism (Le Naour et al. 2000). CD9 associates with integrin $\alpha_6\beta_1$ in eggs (Miyado et al. 2000). Almost in all mutant eggs, intracellular Ca^{2+} oscillations, which signal fertilization, were reported to be absent (Kaji et al. 2000).

The CD9 gene is expressed on blastocysts and endometrium epithelial cells in human and bovine (Liu et al. 2006, Xiang and MacLaren 2002). It was newly found to play a role in inhibiting embryo implantation and involved in embryo invasive behaviors (El-Sayed et al. 2006, Liu et al. 2006).

2.5.1.2 Phospholipase C zeta

Egg activation at fertilization is one of the important Ca^{2+} dependent biological phenomena. In fertilization, the sperm not only provides one half of the genomes to the egg but also awakes the egg that is arrested at a certain stage of meiotic cell division. The release from the meiotic arrest is referred to as “egg activation” characterized by formation of the polar body and male and female pronuclei. Egg activation is caused by a dramatic increase in intracellular Ca^{2+} concentration. The intracellular Ca^{2+} rise is mainly due to Ca^{2+} release from the endoplasmic reticulum forming a “Ca^{2+} wave” that starts from the site of sperm-egg fusion and propagates the Ca^{2+} signal over the whole egg (Miyazaki and Ito 2006). The Ca^{2+} signal is both sufficient and necessary to trigger the events associated with egg activation and embryonic development (Swann et al. 2001).

Microinjection of either single spermatozoa, or soluble sperm extracts into eggs, triggers Ca^{2+} oscillations similar to those at fertilization in mammalian and the consequent cellular processes leading to embryo development (Saunders et al. 2002). Spermatogenic cells contain a specific mRNA encoding the sperm factor protein (Swann et al. 2001). Phospholipase C zeta (PLCζ) which was specifically expressed in mammalian sperm, possesses uniquely all the essential properties of the sperm factor (Saunders et al. 2002, Swann et al. 2004).

PLCζ generates the Ca^{2+} releasing messenger Inositol 1,4,5-trisphosphate (InsP3) by hydrolysis phosphatidylinositol 4-,5-bisphosphate (PIP2) (Swann et al. 2001). Liberated $InsP_3$ then causes Ca^{2+} release by binding to $InsP_3$ receptors located on the endoplasmic reticulum of egg and oocyte (Figure 2) (Nomikos et al. 2005).

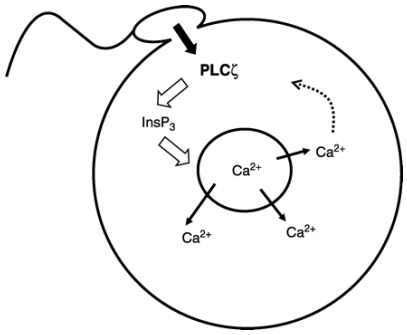

Figure 2: Schematic representation of the basic hypothesis for how PLCζ initiates Ca^{2+} release in mammalian eggs. After fusion of the sperm and egg plasma membranes the sperm-derived PLCζ protein diffuses into the egg cytoplasm. This hydrolyses PIP_2, from an unknown source, to generate $InsP_3$. It may be possible that the subsequent rise in Ca^{2+} leads to the regulation of PLCζ activity (Swann et al. 2004).

There are five subfamilies of PI-PLCs (β, γ, δ, ε, and ζ) classified on the basis of their sequence homology (Nomikos et al. 2005). PLCζ is the smallest PLC isozyme identified to date (Miyazaki and Ito 2006, Saunders et al. 2002).

2.5.1.3 Prostaglandin G/H synthase-2

Prostaglandins (PGs) are arachidonic acid-derived lipids that mediate a wide variety of physiological and pathological processes, including vascular homeostasis, reproductive and gastrointestinal functions, bone metabolism, glomerular filtration, inflammation, and oncogenesis. PGs also contribute to cellular functions such as proliferation and differentiation (Liu et al. 2001, Scherle et al. 2000)

Prostaglandin G/H synthase (PGHS) is the first rate-limiting enzyme in the biosynthetic pathway of PGs from arachidonic acid. Two sequential enzymatic functions are associated with PGHS, a cyclooxygenase reaction responsible for the conversion of arachidonic acid to PGG_2 and a peroxidase reaction involved in the conversion of PGG_2 to PGHS2 and then the most important biologically active end products, PGE_2, $PGF_{2\alpha}$, PGI_2 (prostacyclin), or TXA_2 (thromboxane A_2) (Figure 3). PGHS and phospholipase A_2 (PLA_2) are the key enzymes in the release and conversion of arachidonic acid to PGH_2 (Doualla-Bell et al. 1998, Liu et al. 2001, Smith et al. 1996, Smith et al. 2000).

Two isoforms of PGHS are referred to as PGHS-1 and PGHS-2 (COX-1 and COX-2). They share important similarities at the protein level; their amino acid sequences are 60% identical, and all structural and functional domains are highly conserved. However, the two isoforms are derived from distinct genes located on different chromosomes and encoding different-size mRNAs (2.8 vs. 4.0 kilo bases for COX-1 and-2, respectively). Most importantly, the two isoforms differ markedly in their expression and regulation. For example, COX-1 is present in a variety of tissues and is often referred to as the constitutive isoform involved in the synthesis of PGs necessary for normal biological processes. In contrast, COX-2 is undetectable or present at very low levels in most tissues but can be induced by several agonists and is generally referred to as the inducible form (Liu et al. 2001).

COX-1 is a constitutive enzyme and is associated with the endoplasmic reticulum (ER). PGs are synthesized in the ER by COX-1 and exit cells and function via G protein–coupled cell surface receptors to mediate "housekeeping" functions. In contrast, COX-2 is an inducible enzyme and is primarily associated with the nuclear envelope, suggesting a direct effect on nuclear events (Lim et al. 1997).

Bovine COX-2 consists of 12 exons and 11 introns. The presence of multiple copies of the pentameric sequence 5'-ATTTA-3' in the 3'-untranslated region of the bovine COX-2 cDNA is a structural feature conserved in other species. The position of these motifs within the 3'-untranslated regions could be of functional significance, as revealed by the highly conserved location of five AUUUA elements within 70 nucleotides immediately downstream of the translation stop codon. The 3'-untranslated region plays a key role in the regulation of mRNA stability, and the presence of AUUUA motifs has been described as an instability determinant of rapidly degraded transcripts (Liu et al. 2001). COX-2 mRNA is degraded rapidly or is highly unstable, with a half-life of 1 h in human lung and kidney cells (Guzeloglu et al. 2004).

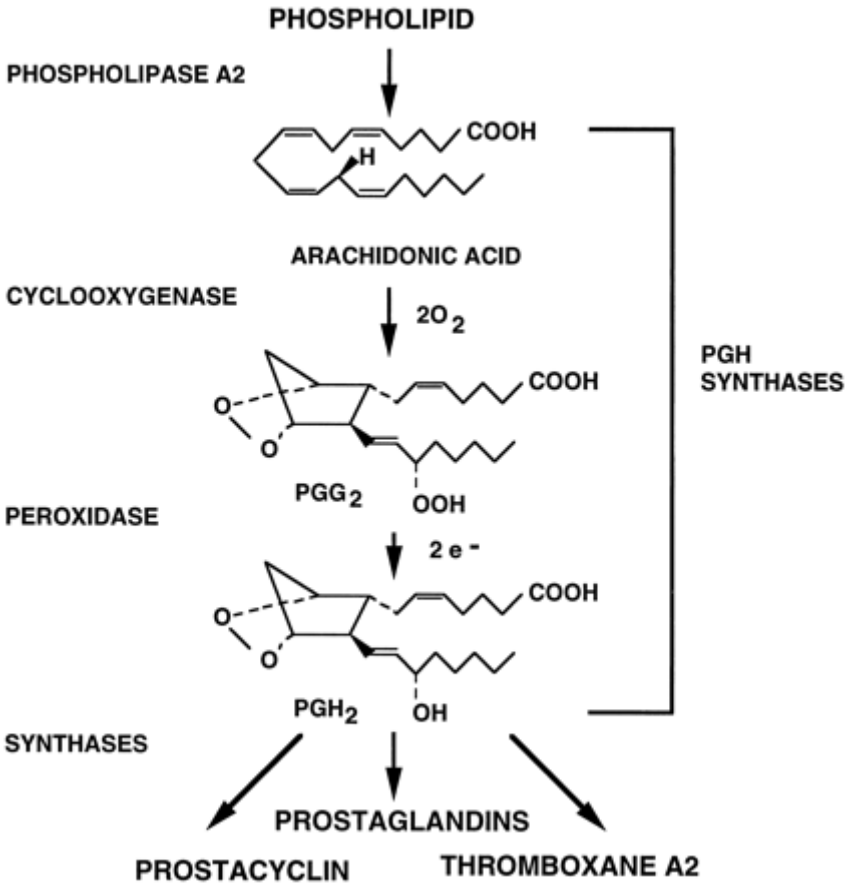

Figure 3: Biosynthetic pathway for the formation of prostaglandins derived from arachidonic acid (Smith et al. 1996).

PG synthesis catalyzed by COX-2 is important for localized increased uterine vascular permeability and attachment reaction. The uterine COX-1 gene is influenced by ovarian steroids, while the COX -2 gene is regulated by the implanting blastocyst during early pregnancy (Chakraborty et al. 1996). COX-2 was expressed during early pregnancy, indicating that PGs are necessary for establishment of pregnancy (Asselin et al. 1997a).
In maintained pregnancies, expression of COX-2 is increased in the bovine endometrium. In pregnant mammals, COX-2 is needed for pregnancy-associated events, such as regulation of localized immune function, angiogenesis and regulation of blood flow, and development of implantation sites (Guzeloglu et al. 2004, Marions and Danielsson 1999). COX-2 expression during the attachment reaction is critical for implantation (Lim et al. 1997). COX-2 is expressed at the time of blastocyst implantation in the rat uterus (Chakraborty et al. 1996, Charpigny et al. 1997). In ruminants, endometrial production of prostaglandins (PGs) plays a central role in the

regulation of the estrous cycle, pregnancy recognition, pregnancy, and parturition (Arosh et al. 2002).

Charpigny et al. (1997) demonstrated that in ovine endometrium, COX-1 protein was expressed at steady levels and COX-2 protein was highly and transiently expressed from Day 12 to Day 15 of the estrous cycle and during pregnancy but disappeared at the end of the cycle. In bovine, COX-2 mRNA and protein were expressed at low levels between Days 1 and 12 and at high levels between Days 13 and 21 of the estrous cycle. COX-2 expression is normally induced immediately before the increase in PG production. Bovine endometrium becomes responsive to produce PGE_2 after Day 13 of the estrous cycle. Days 13–15 may represent optimal uterine receptivity (Arosh et al. 2002).

In early pregnancy, interferon τ (IFNτ) acts as the embryonic signal to inhibit the pulsatile secretory pattern of $PGF_{2\alpha}$ and corpus luteum regression (Roberts et al. 1992). The presence of a viable embryo (IFNτ) may switch on the COX-2 and PGES pathway and PGE_2 production and effect establishment of pregnancy. In the absence of an embryonic signal, other factors may switch on the COX-2 and PGFS pathway, $PGF_{2\alpha}$ production, and luteolysis. The expression of COX-2 mRNA could be modulated by oxytocin, progesterone and estradiol, LH and FSH, and IFNτ under different physiological conditions in bovine uterine tissues and cells (Arosh et al. 2002).

COX-2, rather than COX-1, is responsible for the production of PGE_2 of both embryonic and endometrial origin in ruminants during the establishment of pregnancy (Asselin et al. 1997b, Charpigny et al. 1999). COX-2 deficient females have multiple reproductive failures that include defects in ovulation, fertilization, and implantation (Carson et al. 2000). Gene knockout studies have revealed that, while COX-2 activity was required for early pregnancy, COX-1 activity was required for later pregnancy. Thus, COX-2 gene knockout animals have an infertility phenotype, whereas COX-1 gene knockout animals have limited parturitional defects (Liu and Sirois 1998).

2.5.1.4 Aldo-keto reductase family 1, member b1

Aldose reductase 1 b1 (AKR1B1) is a member of NADPH-dependent aldoketoreductase family and participates in glucose metabolism and osmoregulation. It is believed to play a protective role against toxic aldehydes derived from lipid peroxidation and

steroidogenesis that could affect cell growth/differentiation when accumulated (Lefrancois-Martinez et al. 2004). It is the first enzyme of the polyol pathway (sorbitol-aldose reductase pathway) of sugar metabolism, is most abundantly expressed in adrenal gland, and has been implicated in diabetic complications (Shah et al. 1997).

AKR1B1 is an enzyme in carbohydrate metabolism that converts glucose to its sugar alcohol form, sorbitol, using NADPH as the reducing agent. The reaction equation is glucose + NADPH + $[H^+]$---> sorbitol + $[NADP^+]$ (Chung and LaMendola 1989). This reaction, in particular the sorbitol produced, is important for the function of various organs in the body. Sorbitol is subsequently metabolized to fructose by sorbitol dehydrogenase (Figure 4). Fructose produced from sorbitol is used by the sperm cells, also fructose can be used as an energy source for glycolysis and glyconeogenesis.

AKR1B1 gene which is known for its 20α-Hydroxysteriod dehydrogenase (20α-HSD) was found to be up-regulated in both biopsies derived from blastocysts resulting in no

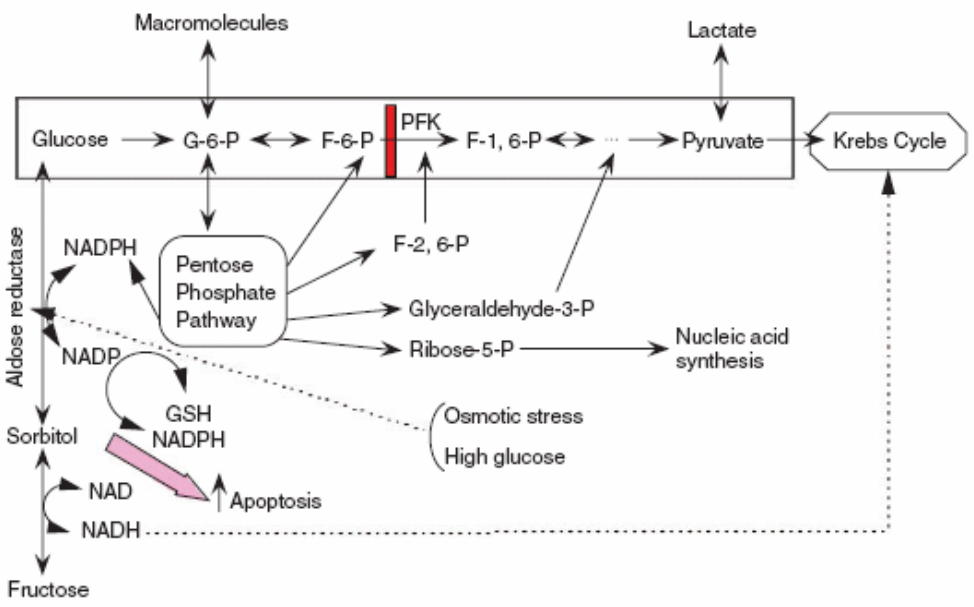

Figure 4: Pathways of glucose metabolism and related factors influencing embryonic development (Wirtu et al. 2004).

pregnancy and resorption (El-Sayed et al. 2006). The AKR1B1 gene was found to be strongly expressed in the endometrium at the time of luteolysis in bovine (Madore et al. 2003), suggesting its potential involvement in pregnancy failure. The enzyme of this gene is known to have two different activities namely; metabolizing progesterone,

which is found to be important to implantation (Jurisicova and Acton 2004) and synthesizing PGF2α and subsequently terminating pregnancy. The concept that catabolism of progesterone to a biologically inactive steroid 20α-dihydroprogesterone (20α-OHP) may constitute an important aspect of the luteolytic process. In rats and rabbits, decreased ovarian venous plasma progesterone levels during luteal regression are associated with a concurrent rise in 20α-OHP levels. Luteal regression in these species is associated with increased ovarian activity of 20α-HSD. Prostaglandins that are elaborated by the uterus are responsible for the increased 20α-HSD activity seen during naturally occurring luteal regression. The relationship between increased uterine $PGF_2α$ content and increased luteal 20α-HSD provides further evidence implicating $PGF_2α$ as the putative factor responsible for inducing luteal 20α-HSD during luteolysis. PGF2α administration results in an increase in serum 20α-OHP and a decline in serum progesterone in pseudopregnant rats (Doebler et al. 1981). AKR1B1 is also known to cause apoptosis in some type of cells like cardiomyocyte being induced by sorbitol as a response to hyperosmotic pressure (Galvez et al. 2003). It may determine the fate of the embryo through its involvement in apoptotic pathways (El-Sayed et al. 2006).

2.5.1.5 Cytokine-like nuclear factor

Embryonic metabolism from the matured oocyte to the 12-cell stage was more or less constant, with pyruvate being the preferred substrate. The first marked increase in oxidation of glucose occurred between the 12- and 16-cell stages. Compaction of morula and blastocyst expansion was accompanied by significant increases in oxidation of all three energy substrates. The incorporation of glucose increased steadily 15-fold from the 1-cell to the blastocyst stage (Khurana and Niemann 2000).

A quantitative calculation was made of the pentose phosphate pathway activity in preimplantation mouse embryos from the 2-cell through the late blastocyst stage. This activity varied with development and showed repeated high and low values. Peak activities occurred at both the 2-cell (15.8%) and compacted morula (13.6%) stages, with lowest activity at the development of the late blastocyst (3.2%) (O'Fallon and Wright 1986).

The pentose phosphate pathway of glucose metabolism plays a very important role in the development of an embryo. It generates both NADPH, which serves as a hydrogen

and electron donor in reductive biosynthesis, and ribose-5-phosphate, which, along with its derivatives, is a constituent of ATP, coenzyme A, NAD^+, FAD^+, RNA, and DNA (O'Fallon and Wright 1986).

Cytokine-like nuclear factor (N-PAC) has Phosphogluconate dehydrogenase (decarboxylating) activity, the process by which glucose is oxidized, coupled to NADPH synthesis. Glucose 6-P is oxidized with the formation of carbon dioxide (CO_2), ribulose 5-phosphate and reduced NADP; ribulose 5-P then enters a series of reactions inter- converting sugar phosphates. The pentose phosphate pathway is a major source of reducing equivalents for biosynthesis reactions and is also important for the conversion of hexoses to pentoses (Robert et al. 2003).

The NADPH is essential for steroidogenesis. It is an essential co-factor for converting progesterone to 17-B-OH-progesterone and the conversion of androstenedione to estrone. The produced estrogen appears to be the embryonic signal for: 1) maternal recognition of pregnancy; 2) activation of the sodium-potassium pump of the allantois; 3) affecting water, electrolyte and glucose transport and 4) increasing uterine blood flow (Bazer 2005, Robert et al. 2003).

The N-PAC transcript was detected at relatively lower levels throughout the preimplantation stages of in vitro-produced embryos compared with those produced in vivo. The transcript was abundant at significantly higher level at two, four and eight-cell stages of in vivo embryos than their in vitro counterparts. This transcript was highly abundant between two and 16-cell stages among in vivo embryos and expression in down regulated during the later developmental period (Tesfaye et al. 2004).

2.5.1.6 Eukaryotic elongation factor 1

Protein biosynthesis requires a large number of components and steps and is thus necessarily highly organized and efficient in cells. Three phases of protein synthesis, initiation, elongation and termination, require GTP and distinct soluble factors. In eukaryotes, elongation factor1 alpha (EF1α) binds GTP and aminoacyl-tRNA (aa-tRNA) to form ternary complexes. These complexes bind to the ribosome (Moore and Cyr 2000, Reed et al. 1994).

The G-protein superfamily members are defined by highly conserved sequence motifs and structural features and regulate a variety of critical cellular processes. G-proteins

have ability to transition between active and inactive forms based on whether GTP or GDP is bound, respectively, allowing them to function as a molecular switch (Carr-Schmid et al. 1999).

EF1α is a member of the G-protein family and its activity is stimulated by binding to aa-tRNA, ribosomes. The ribosome acts as a GTPase-activating protein for EF1α. The regulation of EF1α activity by GTPase-activating proteins is critically important in efficient and accurate protein synthesis and consequent cell growth (Carr-Schmid et al. 1999, Condeelis 1995, Kojima et al. 2002, Reed et al. 1994, Talapatra et al. 2002).

EF1α plays a role in transporting aa-tRNA to the ribosome during protein synthesis. Binding of EF1α with the nucleotide exchange factors EF1β leads to the replacement of GDP with GTP, which switches on the ability of EF1α to interact with aa-tRNA. Subsequently, the binding of EF1α: GTP: aa-tRNA ternary complex with the ribosome triggers the GTPase activity on EF1α causing a conformational change in EF1α. This causes EF1α-GDP to detach from the ribosome, leaving the aa-tRNA attached at the A-site, ready for the next cycle (Figure 5) (Liu et al. 1996).

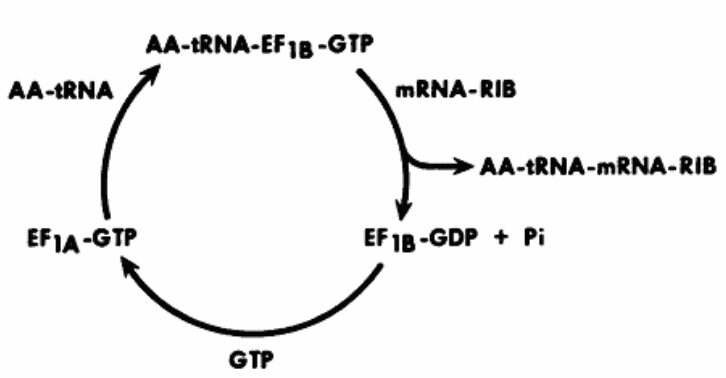

Figure 5: Proposed interconvensions of EF1 during the reactions involved in the binding of aminoacl-tRNA to ribosomes. AA: aminoacyl; RIB: ribosome (Moon et al. 1972)

Evidence suggests that EF1α may have additional functions in cells beyond its conventional role in polypeptide elongation. EF1α binds and cross-link's actin filaments and microtubules (Condeelis 1995). This unique cross-link structure may represent a special property of EF1α that is important in the stability of the cytoskeleton, transport, anchorage, and translation of mRNA (Condeelis 1995, Ermentrout and Edelstein-Keshet

1998 , Liu et al. 1996, Liu et al. 2002, Murray et al. 1996). EF1α may contribute to the coordinate regulation of multiple cellular processes including growth, division, and transformation, are characterized (Negrutskii and El'skaya 1998). Elongation factor EF1α functions to limit the frequency of errors during genetic code translation. Both amino acid mis-incorporation and reading frame errors are controlled by EF1α (Sandbaken and Culbertson 1988).

The ability of the components of translation to influence the assembly, crosslinking and stability of cytoskeletal polymers may be crucial for regulating the transport, anchorage and translation of mRNA. EF1α, in particular, may have a significant impact on the structure of the actin- and tubulin-based polymers in cells. Its abundance relative to and high affinity for actin suggest that EF1α could affect the polymerization and stability of actin filaments (Condeelis 1995, Moore and Cyr 2000).

In mammalian cells, EFlα is present abundantly in almost all kinds of tissues, and the EFlα gene can be regarded as one of the so-called “housekeeping” genes. In eukaryotes, EF1α is a very abundant protein that constitutes about 1-2 % of the total protein in normal growing cells. Large increases in mRNA levels for EFlα are observed in rapidly proliferating cultured cells, in a variety of human tumors and embryos (Condeelis 1995). These results indicate that the level of expression of EFlα correlates with the rate of cell growth and proliferation (Condeelis 1995, Liu et al. 1996, Liu et al. 2002, Uetsuki et al. 1989).

2.5.2 Genes related for cell to cell adhesion and compaction

2.5.2.1 Desmocollin 2

Cell to cell adhesion is thought to play important roles in development, in tissue morphogenesis, in regulation of cell migration and proliferation (Illingworth et al. 2000, Kowalczyk et al. 1999a). Desmosomes are major adhesive intercellular junctions at basolateral membranes of epithelial cells and in other tissues (Bloor et al. 2002). They mediate direct cell-cell contacts and provide anchorage sites for intermediate filaments important for the maintenance of tissue architecture (Syed et al. 2002). Indirect attachment of desmosomal cadherins to intermediate filaments is facilitated by plaque and armadillo proteins including desmoplakin, plakoglobin and plakophilins (Huber

2003). There are increasing evidence that desmosomes in addition to a simple structural function have new roles in tissue morphogenesis and differentiation (Bloor et al. 2002, Legan et al. 1994, South et al. 2003).

Desmosomal cell-cell attachment is mediated through two types of transmembrane glycoproteins of the cadherin super family, desmoglein and desmocollin (Collins et al. 1991). There are currently three known isoforms of both these molecules and each display differentiation-specific and tissue-specific expression patterns. The type 2 desmosomal cadherins are the most widely expressed forms, being present in all desmosome-bearing tissues and cells, whereas expression of types 1 and 3 isoforms is restricted to certain tissues such as tongue, epidermis and stratifying epithelia (Syed et al. 2002).

The molecular constituents of desmosomes and other adhesive complexes are known to function not only in cell adhesion, but also in the transduction of intracellular signals that regulate cell behavior, proliferation and gene expression (Kowalczyk et al. 1999a, Legan et al. 1994). Evidence is beginning to emerge that desmosome respond to both intracellular and extra cellular signals (Legan et al. 1994). Desmosomes are calcium dependent as their formation and internalization is influenced by increasing, or decreasing, Ca^{2+} concentration (Illingworth et al. 2000).

Desmocollin isoform expression has important functional consequences in epithelial proliferation, stratification, and differentiation. Desmocollin expression has a primary role in adhesion at desmosomes. E-cadherin intercellular adhesion is a prerequisite for normal development of tight and desmosomal junctions (Collins et al. 1995). Desmocollins are formed later than adherens junctions during embryogenesis (South et al. 2003). Desmosomes appear very early during murine development, at the early blastocyst stage, suggesting a critical role in developmental processes (Fleming et al. 1991).

2.5.2.2 E-cadherin

Preimplantation development includes the cleavage stages with compaction followed by blastocyst formation (Reuss et al. 1997). An appropriate temporal and spatial pattern of intercellular junctions is needed for successful preimplantation development and implantation of embryos (Bloor et al. 2004). During preimplantation development,

loosely attached blastomeres maximize their cell-cell contacts and form a compact morula as a prerequisite for the generation of two distinct cell types, those of the trophectoderm epithelium and those of the inner cell mass (Larue et al. 1994). Experiments on rodent preimplantation embryos have shown that the onset of E-cadherin (uvomorulin / CDH1) expression is essential for compaction (Bloor et al. 2004). Compaction was shown to be mediated by accumulation and clustering of CDH1 in the cell contact areas of eight-cell stage embryos (Pey et al. 1998).

Cadherins are transmembrane glycoproteins that play an important role in defining cellular shape and degree of contact with neighboring cells (Lu et al. 2002, Wheelock and Johnson 2003). Cadherin expression patterns are critical to the preservation of tissue integrity and developing embryo, to forming cell to cell junctions, promoting cell sorting and cell-signaling events that regulate normal development (Mak et al. 2000, Wheelock and Johnson 2003).

CDH1 is a Ca^{2+} dependent cell-cell adhesion molecule found mainly in epithelial tissues (Kim et al. 2000). It plays an important role in the adhesion of the blastomers, and functionally interfering anti-E-cadherin antibodies cause the early embryo to decompact. Early cell polarization during compaction of the morula is dependent on CDH1. In the homozygous state, the mutation affects preimplantation development severely (Riethmacher et al. 1995). Loss of CDH1 leads to embryonic lethality (Larue et al. 1994). CDH1 null embryos fail to form an intact trophectoderm cell layer (De Vries et al. 2004).

The presence of CDH1 is important in cell differentiation. Down regulation or a complete shut-down of CDH1 expression, mutation of the CDH1 gene, or other mechanisms interfere with the integrity of the adherens junctions (Berx et al. 1998, Riethmacher et al. 1995). Down-regulation of CDH1 expression is associated with local invasion of tumor cells (Tsujii and DuBois 1995). In mouse, inhibition of translation of maternal CDH1 mRNA, causes a developmental arrest at the two-cell stage (Kanzler et al. 2003).

2.5.2.3 Tight junction protein 1

The first tissue to differentiate during mammalian development is the trophectoderm epithelium (TE), which forms the wall of the blastocyst and encloses the ICM (Sheth et

al. 2000). One critical process in TE differentiation is the construction of tight junction protein 1 (TJP1) at the apicolateral border between cells (Miller et al. 2003). Once formed, the trophectoderm engages in vectorial transport driven by a basal membrane Na^+/K^+-ATPase, thereby generating the blastocoelic cavity and regulating exchange of ions, amino acids, sugars and other metabolites between the maternal uterine environment and the ICM. The TJP1 is responsible both for the adhesive permeability seal of epithelial sheets which controls paracellular solute diffusion and transepithelial resistance and for the maintenance of compositionally distinct apical and basolateral membrane domains (Sheth et al. 2000). E-cadherin interact directly or indirectly with this protein (Itoh et al. 1993). The TJP1 engage in intercellular sealing, and link the membrane constituents to the actin cytoskeleton and participate in signaling events that coordinate assembly and functions of the TJP1 seal. During early mouse development, the TJP1 is constructed in a step-wise assembly process once embryo compaction has occurred (Miller et al. 2003). TJP1 is also referred to zonula occludens (ZO-1).
TJP1 appears to perform two fundamental roles in epithelial function. First, it restricts the paracellular passage of ions and small molecules between mucosal and serosal compartments, thereby contributing in large part to the transepithelial electrical resistance. Second, the TJP1 appears to act as a barrier to the lateral diffusion of integral proteins and exoplasmic (outer leaflet) lipids between the two membrane domains of the epithelium, thereby helping to preserve the asymmetry in membrane composition that is essential for polarized function (Fleming et al. 1989).

2.5.2.4 Plakophilin 1

The contact between epithelial cells is mediated by several types of cell-cell junctions, including adherens junctions, desmosomes, tight junctions, and gap junctions (Borrmann et al. 2000). Adherens junctions and desmosomes are intercellular adhesive junctions specialized to provide strong but dynamic cell-cell adhesion in a variety of cell types and tissues (Bornslaeger et al. 2001). These junctions are composed of complex arrays of transmembrane and plaque proteins and are typically connected to cytoskeletal components (Hofmann et al. 2000, South 2004). Desmosomes are found primarily in epithelial tissues and particularly in those that frequently undergo mechanical stress. Desmosomal cadherins provide the adhesion for opposing cell

membranes and desmosomal plaque proteins link cytoskeletal intermediate filaments to these cadherins (Kowalczyk et al. 1999b, Rickman et al. 1999, Schmidt et al. 1997, Smith and Fuchs 1998).

Several junctional plaque proteins of both adherens junctions and desmosomes belong to the armadillo family of proteins. Armadillo gene family encodes a group of highly conserved proteins which play important roles in cell adhesion and in signal transduction mechanisms involved in regulating development. Plakophilin (PKP) is originally known as band 6 protein (Wrenzycki et al. 1999).

PKP proteins contain numerous armadillo repeats, localize to cell desmosomes and nuclei. Several types of PKPs have been detected at different cellular locations or at different stages of development (Choi and Weis 2005).They have been shown to be essential for the formation and function of desmosomes because they are involved in the recruitment and normal association of the other desmosomal proteins and thus confer to normal development, stability, and adhesion of cells and tissues (Schmidt and Jager 2005, Schwarz et al. 2006).

PKPs have multiple members displaying tissue-specific expression patterns that vary with epithelial differentiation (Hatzfeld et al. 1994, Heid et al. 1995). Three PKP isoforms (PKP 1-3) are integrated into the desmosomes dependent on differentiation, and only PKP2 has been detected in all desmosome-producing cell types. In contrast, PKP3 is located in almost all desmosome-bearing cells, with the exception of desmosomes between hepatocytes and between cardiomyocytes (Schwarz et al. 2006). PKP1, however is restricted predominantly in the supra basal layers of stratified, complex, transitional epithelia and also is expressed widely in the nuclei of cells devoid of DMs (Bornslaeger et al. 2001, Choi and Weis 2005, Hatzfeld et al. 2000, James 2005). At least 1 isoform of the PKPs is present in every desmosomes of any cell type. PKP1 increases desmosomal protein content within the cell but does not affect the level of adherens junction proteins CDH1 (Schafer et al. 1993, South et al. 2003).

DMs are calcium dependent as their formation and internalization is influenced by increasing, or decreasing, Ca^{2+} concentration. Conversely, cells at confluency possess DMs that are mostly calcium independent since neither depleting the Ca^{2+} concentration of the medium nor adding chelating agents promote DM disruption. PKP1 has a role in the transition of DMs from a calcium-dependent state to a calcium-independent state and that PKP1 also affects the size and number of keratinocyte DMs (South et al. 2003).

PKP1 plays a role in regulating desmosome integrity in response to low calcium concentrations (South 2004).

Junctional proteins play important roles in carcinogenesis, tumor invasion, and metastasis (Schwarz et al. 2006). Findings point to an essential role of PKP1 in establishing stable cell contacts, desmosomal plaque size, and organization (Chen et al. 2002, Hatzfeld et al. 2000). Loss of PKP1 expression results in epidermal fragility/ ectodermal dysplasia syndrome, which is characterized by a reduction in the number and size of desmosomes in the epithelia of affected individuals (James 2005, McGrath et al. 1997, McMillan et al. 1998, Wessagowit and McGrath 2005).

2.5.2.5 Gap junction protein, alpha 1

Preimplantation development includes the cleavage stages with compaction followed by blastocyst formation (Reuss et al. 1997). At the time of compaction, an induction of direct intercellular communication is first detected in correlation with gap junctional plaques (Lee et al. 1987). Direct cell to cell communication via gap junctions is believed to play a crucial role in preimplantation embryo development, tissue function, and cell homeostasis (Dahl et al. 1996, Houghton 2005, Segretaina and Falk 2004). The formation of gap junctions during compaction will ensure their presence at cavitation and in the trophectoderm and inner cell mass (Houghton 2005). According to Lee et al., 1987, intercellular communication between blastomeres is necessary to maintain compaction.

To extracellular synaptic and hormonal communication, intercellular communication through gap junctions plays an important role in cooperative cell activity (Evans and Martin 2002, Sanderson et al. 1994).

Gap junction is composed of connexins forming intercellular channels that directly connect the cytoplasms of two adjacent cells. The channels are permeable for ions and small molecules with a molecular weight up to 1 KD. They allow the exchange of metabolites like glucose or second messengers like cAMP, Ca^{2+} , and inositol trisphosphate (IP_3) , and play an important role in the metabolic and regulatory integration of signal cells into whole tissues (Bloor et al. 2004, Houghton et al. 2002). Because of these properties, gap junction channels are suggested to be involved in

establishing and maintaining cellular differentiation programs in embryonic tissues (Reuss et al. 1997).

In mammals, gap junction channels are composed of two hemi-channels termed as connexons, each of them provided by one of the two neighboring cells. Each hemi channel is composed of six connexin subunits. Although a ‘‘gap’’ is left between the adjacent cell membranes, two connexons interact and dock in the extra-cellular space to form a tightly sealed, double-membrane intercellular channel (Figure 6) (Bloor et al. 2004, Evans and Martin 2002, Lash et al. 1990).

Connexins are encoded by a large number of genes family predicted to comprise at least 20 isoforms in humans, all represent structurally conserved proteins 25 to 62 kDa in size that differ chiefly in the length of their C-terminal domain. Connexins are classified according to their molecular mass. For example, connexin 43 (GJA1) has a predicted molecular mass of 43 kDa and Connexin31 has a predicted molecular mass of 31 kDa (Houghton 2005). Also based on amino acid similarities, connexins have been classified into subgroups, with α and β being the major subgroups (figure 6) (Segretaina and Falk 2004).

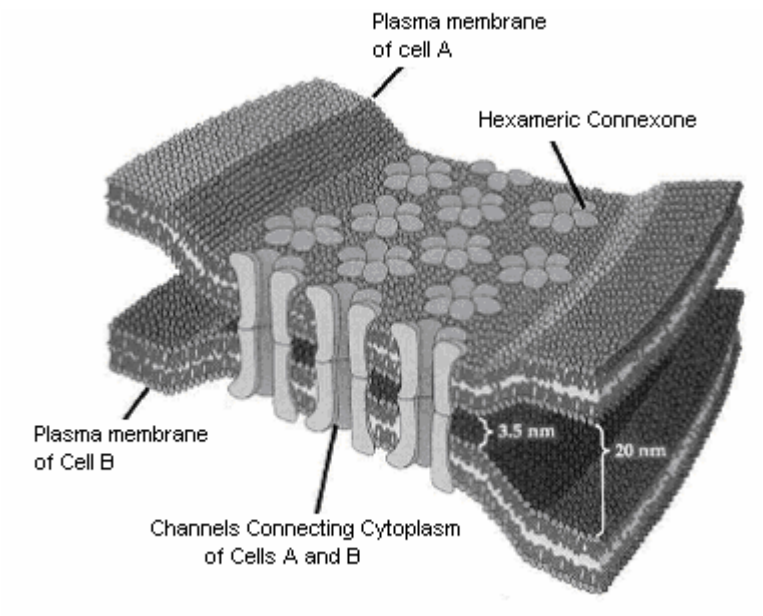

Figure 6: Model of a gap junction. The hexameric connexin subunits in each of the plasma membranes dock to generate the gap junction channel connecting the two cytoplasmic compartments. (Purves et al. 2001)

Expression of multiple connexins in the preimplantation embryo allows the implanting conceptus to undergo rapid diversification of cell types required for establishment of both embryonic (i.e., fetal) and extra-embryonic (i.e., yolk sac and placenta) tissues (Houghton et al. 2002).

GJA1 is widely expressed in several organs and cell types, and is the principal gap junction protein of the heart (Duncan and Fletcher 2002). GJA1 is essential for the development of normal cardiac architecture and ventricular conduction. According to an investigation on all of the connexin isoforms, only GJA1 and Connexin 31 were abundantly expressed and both were identified in the trophectoderm as well as in the inner cell mass (Bloor et al. 2004, Reuss et al. 1997).

GJA1 is the most prominent gap junctional protein expressed during preimplantation and embryonic development (Britz-Cunningham et al. 1995). Evidence suggests that aberrant expression and distribution of the GJA1 channel proteins may affect the survival potential of human embryos (Bloor et al. 2004).

In the bovine embryo, GJA1 expression varies depending whether the embryos are produced in vitro or in vivo. In vitro, GJA1 is expressed in the oocyte and zygote through to the morula stage but is not expressed at the blastocyst stage, whereas GJA1 transcripts are detected in morula and blastocysts produced in vivo (Wrenzycki et al. 1996). A study conducted on mice by (Bloor et al. 2004) revealed that neither GJA1 nor Connexin 31 gene deficiency, result in impaired preimplantation development or inhibited implantation.

2.6 Candidate gene analysis

Candidate genes are loci with a high probability to affect a trait of interest, i.e. these genes are a valuable source for association analyses. In order to investigate the hypothesis that a definite gene may affect a particular trait, analyses are performed where allelic variation detected at the candidate gene locus is used as independent variable enabling to prove statistically the effect of the candidate genes on specific phenotypes (Wimmers et al. 2002). Candidate genes can be identified based on the knowledge of the physiology and biochemistry of a trait (direct candidate gene approach). Other recent approaches to detect candidate genes are based on the analyses

of differences of the expression profile of particular subsets of cells and / or individuals with certain phenotypes.

Gene mutations affect gene expression or enzyme activity, i.e. they might lead to the protein structure alteration and/or protein expression levels. As molecular genetic marker, one is based on sequencing including microsatellite, single nucleotide polymorphisms (SNPs), sequence tagged site (STS) and expressed sequence tag (EST); the other is DNA-finger printing based on PCR technique including random amplified polymorphic DNA (RAPD) or AFLP. Microsatellite markers are multiple tandem repeats of sequence. SNP is mainly a diallelic marker. These markers have become a powerful tool for chromosome mapping and linkage analysis, the study of molecular evolution and population genetics (Lin 2005).

2.7 Single nucleotide polymorphism (SNP)

SNPs are major contributors to genetic variation, comprising some 80% of all known polymorphisms, and their density in the human genome is estimated to be on average 1 per 1,000 base pairs. SNPs are mostly biallelic, more frequent, mutational and more stable, making them suitable for association studies in which linkage disequilibrium (LD) between markers and an unknown variant is used to map disease-causing mutations. In addition, since SNPs have only two alleles, they can be genotyped by a simple plus/minus assay rather than a length measurement, making them more amenable to automation.

SNPs promise considerable advantages over microsatellite markers: (1) lower mutation rates, (2) more robust in laboratory handling and data interpretation (Krawczak 1999), (3) suitability for standardized representation of genotyping results as a digital DNA signature (Fries and Durstewitz 2001) and (4) suitability for various genotyping techniques and high potentiality for automation (Kruglyak 1997). One disadvantage is that SNPs have lower information content, compared with a highly polymorphic microsatellite. But this disadvantage can be compensated for by a higher number of markers.

Individual SNPs are less informative than other marker systems, especially as the widely used microsatellites, but they are more abundant and have a great potential for automation (Wang et al. 1998). Markers used for genome scans should allow the

polymorphisms to be typed quickly, accurately and inexpensively. SNP markers possess the properties that fulfill these requirements. First, DNA sequence variations, based on point mutations are estimated to occur once every 500 to 1000 bp when any two chromosomes are compared. Second, SNPs are diallelic in populations, and their allele frequencies can be estimated easily in any population through a variety of techniques (Kwok 2001). Third, the mutation rate per generation of SNPs is low compared to tandem repeat markers where the high mutation rates can confound genetic analysis in population. Fourth, many of the mutations of the trait result from single nucleotide changes in genes; it is likely that a subset of SNPs is functionally important in complex traits. Last, promising high-throughput genotyping methods are now available for efficient genotyping of SNPs (Wang et al. 1998).

The methods for genotyping of single nucleotide polymorphisms include gel-based and non-gel-based approaches. The gel-based genotyping approaches are for example DNA sequencing, RFLP and single-strand conformation polymorphism (SSCP) analysis (Orita et al. 1989). All non-gel-based genotyping approaches achieve allelic discrimination by one of four mechanisms: allele-specific hybridization, allele-specific primer extension, allele-specific oligonucleotide ligation and allele-specific cleavage of a flap probe. By combining one of these allelic discrimination mechanisms with either a homogeneous or solid-phase reaction format or a detection method such as fluorescence intensity, fluorescence polarization or mass spectrometry fast, reliable, automated and large-scale genotyping is warranted (Kwok 2000).

2.7.1 Single strand conformation polymorphism (SSCP)

Single strand conformation polymorphism (SSCP) has emerged as one of the most popular mutation detection techniques (Masato 1989), which were developed by Orita et al. (1989). The reasons for the popularity of the technique are its technical simplicity and its relatively high sensitivity for the detection of sequence variations (Hayashi 1991). The process of SSCP involves PCR amplification of the fragment of interest, denaturation of the double stranded PCR product with heat and formamide, and electrophoresis on a non-denaturing polyacrylamide gel. During electrophoresis, single stranded DNA fragments fold into a three-dimensional shape according to their primary sequence. The eletrophoretic mobility of separation then becomes a function of the

shape of the folded, single stranded molecules. If the sequence of a wild-type (or reference) sequence differ from that of the fragment being tested, even by only a single nucleotide, at least one of the strands, if not both, will adopt different three- dimensional conformations and exhibit unique eletrophoretic mobility.
Advantages and disadvantages of SSCP analysis were described by (Nataraj et al. 1999) . Advantages: (1) simplicity of usage; (2) no special equipment necessary; (3) mutant bands separated from wild-type can be isolated for analysis and non-radioactive labeling available. Disadvantages: (1) size of fragment analyzed limited; (2) absence of theory; (3) multiple conditions to detect all mutations; (4) gels are sometimes difficult to interpret and less applicable to DNA with unknown sequence.

2.7.2 Restriction fragment length polymorphisms (RFLP)

According (Botstein et al. 1980), restriction fragment length polymorphisms (RFLP) as a powerful new technology is defined to identify variants within a specific region of the genome using digest of total human DNA in order to constitute a genetic linkage map. DNA restriction enzymes recognize specific sequences in DNA and catalyze endonucleolytic cleavages, yielding fragments of defined lengths. PCR-based RFLP marker, restriction fragments may be displayed by electrophoresis in agarose gels, separating the fragments according to their molecular size. Difference among individuals in the lengths of a particular restriction fragment could result from many kinds of genotypic difference: one or more individual bases could differ, resulting in loss of a cleavage site or formation of a new one, alternatively, insertion or deletion of blocks of DNA within a fragment could alter its size. These genotypic changes can all be recognized by the altered mobility of restriction fragments on agarose gel electrophoresis.
On the other hand, fragments encoding specific sequences within a large and complex population of DNA fragments can be detected by hybridization using the method of Southern (Southern 1975). The DNA fragments in agarose gel are transferred onto nitrocellulose paper and hybridized with radioactive probe sequences. The use of probe sequences of very high specific radioactivity has permitted the identification by this method of single-copy sequences in mammalian DNA (Jeffreys and Flavell 1977).

3 Materials and methods

3.1 Materials

3.1.1 Biological samples

For screening SNP, 11 different cattle breeds (Limousin, Gelbvieh, Blond d'Aquitaine, Salers, Vorderwälder, Hinterwalder, Charolais, Red Angus, Piemontese, Pinzgauer, and Galloway) were applied. After finding SNP, 310 sperm samples obtained from Rinder-Union West eG (RUW) station was used for association study. The sperm samples belong to black and red Holstein population. Semen quality of each bull was evaluated with a standard method based on the guidelines of the World Health Organization. Semen quality traits of each sample including sperm volume per ejaculate (ml), sperm concentration (10^9/ml), sperm motility (%), and survivability after thawing (%) underwent an evaluation. From each bull the repeated measurements of sperm quality traits were available. The NRR and other information including inseminator, region, status of cow (heifer, cow and multi calving), insemination date, status of bull (birth date) for each animal were derived from RUW center.
About embryo, the oocytes were collected from ovaries which obtained from local slaughterhouse. After aspiration of 2-8 mm follicles in each ovary, the oocytes in vitro matured and fertilized. The embryos cultured in culture medium up to blastocyst stage.

3.1.2 Materials for molecular analysis

Instruments, software's, chemicals and kits, solution and buffers, primers and biological materials that have been used in this study, will be presented in the following sections.

3.1.3 Instruments

ABI Prism® 7000 Sequence Detection System	Applied Biosystems, Foster City,USA
Centrifuge Hermle	Hermle Z 233MK, Wehingen
Centrifuge Hermle	Hermle Z 323K, Wehingen
CEQ™ 8000 Series Genetic analysis System	Bachman Coulter, Inc, USA
CO_2 incubator (MCO-17AI)	Sanyo, Japan
Electrophoresis chamber	BioRad, München, Germany
Four-well dish	Nunc (Roskilde, Germany)
Gel dryer	BioRad, München
Incubator	Memmert
Memmert CO_2 incubator	Fisher Scientific, Leicestershire, UK
Millipore® Milli Q system	Millipore Corporation, USA (Eschborn)
pH Meter	Kohermann
Power Supply PAC 3000	BioRad, Münich, Germany
PTC-100 Thermal cyclers	Waltham, MA, USA
Savant Speed Vac	GMI, Inc. Minnesota, USA
Spectrophotometer (Ultrospec™ 2100)	Amersham Bioscience, Freiburg, Germany
Stereo microscope (SMZ 2B)	Nikon, Japan
Thermal cycler Bio-Rad iCycler	Bio-Rad, USA
Thermocycler (PTC 100)	MJ Research, USA
Thermoshake Gerhardt	John Morris Scientific, Melbourne, Australia
Tuttnauer autoclave	Connections unlimited, Wettenberg, Germany
UV Transilluminator (Uvi-tec)	Uni Equip, Martinsried, Germany

3.1.4 Softwares

BLAST program	http://www.ncbi.nlm.nih.gov/BLAST
Calculating primer melting temperatures for DNA	http://www.iit-biotech.de/iit-cgi/oligo-tm.pl
Entrez gene	www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene
Gene otology	http://www.geneontology.org
Image analysis program (version 4.10)	Li-Cor Biotechnology, USA
Manipulate and display a DNA sequence	http://www.vivo.colostate.edu/molkit/manip/index.html
Multiple sequence alignment by Florence Corpet	http://prodes.toulouse.inra.fr/multalin/multalin.html
NCBI	http://www.ncbi.nlm.nih.gov/
NEBcutter V2.0	http://tools.neb.com/NEBcutter2/index.php
Oligos parameter calculation	http://proligo2.proligo.com/Calculation/calculation_frame_new.html
Practical molecular biology	http://www.molbiol.ru/eng
Primer3 program	http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi
REBASE^{R}	http://rebase.neb.com/rebase/rebase.html
Resources in animal genetics and breeding	http://resourceagb.50webs.com/index.htm
Microsoft® Exel 2002	Microsoft corporation 1985-2001
Microsoft® word 2002	Microsoft corporation 1985-2001
SAS version 8.0	SAS Institute Inc., Cary, NC, USA
Sorting Intolerant from Tolerant	http://blocks.fhcrc.org/sift/SIFT.html

3.1.5 Chemical and kits

Beckman coulter (Ca, USA): CEQ DTCS-Quick Start Kit

Biomol (Hamburg, Germany): Phenol, Phenol/Chlorophorm/Isoamyl alcohol

GeneCraft (Münster, Germany): Taq polymerase

MBI Fermentas (St. Leon-Rot): Glycogen

Promega (Mannheim, Germany): BSA, pGEM®-T vector, AluI Restriction enzyme;

Roche (Mannheim, Germany): Nonidet P40

Roth (Karlsruhe, Germany): Acetic acid, Agar-Agar, Tris, Ampicillin, Ammonium Peroxydisulphate (APS), Boric acid. Bromophenol blue, Chloroform, Calcium chloride, Dimethyl sulfoxide (DMSO), dNTP, Ethylene diamine tetra acetic acid (EDTA), Ethanol, Ethidium bromide, Formaldehyde, galactopyranoside (X-gal), Hydrochloric acid, Isopropyl β-D-thiogalactoside (IPTG), Nitric acid, Silver nitrate, Proteinase K, N,N,N',N'-Tetramethylethylene-diamine (TEMED), Sodium acetate, Sodium carbonate, Sodium chloride , Sodium hydroxide, Trichloromethane/chlorophorm, 5-bromo-4-chloro-3-indolyl-β-D- Formamide, Glycerin and pertridish plate(Ø=9 mm, and 12 x 12 mm).

RUW (A. G. Münster, Germany): frozen Bull semen

Sigma-Aldrich Chemie GmbH (Munich, Germany): Agarose, Ammonium acetate, Calcium chloride, Calcium chloride dihydrate, Calcium lactate, Formaldehyde, FSH, Dulbecco's phosphate buffered saline (D-PBS), Fetal calf serum Igepal, Glutamine, Heparin, Epinephrine, Hypotaurin, Isopropanol, Magnesium chloride, Magnesium chloride hexahydrate, Taq polymerase,Tween-20, Medium 199, 2-mercaptoethanol , Mineral oil, Penicillin, oligonucleotide primers, Phenol red solution, 10 X PCR reaction buffer, Potassium chloride, Sodium dodecyl sulfate (SDS), Sodium hydrogen carbonate, Sodium hydrogen phosphate, Sodium hydrogen sulfate, Sodium lactate solution (60%), Sodium pyruvate, Streptomycin sulfate, Hepes,

STARLAB GmbH (Ahrensburg): Rigid thin wall 96 X 0.2 ml skirted microplates

Stratagene (Amsterdam, Holland): 5α DH Escherichia coli competent cell

USB Corporation (Staufen): Exo-SAP-IT

3.1.6 Solutions and buffers

All solutions applied in this research were prepared using deionized and demineralized (Millipore) water and pH was adjusted with sodium hydroxide or hydrochloric acid.

Acrylamide (40 %)	Acrylamide	95 g
	Bis-acrylamide	5 g
	Water added to	250 ml
Acrylamide (40%): (49:1)	Acrylamide	39.2 g
	N,N-methylene-bisacrylamide	0.8 g
	Water added to	250 ml
Acrylamide gels (12%)	40% Acrylamide	4.5 ml
	TBE (10X)	750 µl
	Formamide : glycerol(1:3 v/v)	750 µl
	TEMED	10 µl
	APS (100 mg/ml)	150 µl
	Water	9 ml
Agarose loading buffer	Bromophenol Blue	0.0625 g
	Xylencyanol	0.0625 g
	Glycerol	7.5 ml
	Water added to	25 ml
Ampicillin (10 mg/ml)	Ampicillin powder	2 g
	Sterile, distilled water filtrate with 0.45 µl filter	40 ml
APS solution : (10% w/v)	Ammoniumpersulfat	5 g
	Water added to	50 ml
Binding buffer	Tris HCL (1M pH 7.5)	1 ml
	LiCl (5 M)	10 ml
	EDTA (5 mM pH 8.0)	20 ml
	Water added to	50 ml
Blue dextrin buffer	Blue dextrin	1 ml
	EDTA	50 µl
	Formamide	5 ml
Blue dextran loading buffer	Blue dextrin	50 mg
	EDTA (0.5M)	50 µl
	Water	950 µl
	Formamide	5 ml

Capacitation medium	Sodium chloride	0.29 g
	Potassium chloride	0.0115 g
	Sodium hydrogen carbonate	0.105 g
	Sodium dehydrogen sulphate	0.0017 g
	Hepes	0.119 g
	Magnesium chloride $6H_2O$	0.0155 g
	Calcium chloride dehydrate	0.0145 g
	Sodium lactate solution (60%)	184 µl
	Phenol red solution (5% in D-PBS)	100 µl
	Water added to	50 ml
Culture medium (CR-1)	Hemicalcium lactate	0.0273 g
	Streptomycin sulphate	0.0039 g
	Penicillin G	0.0019 g
	Sodium chloride	0.3156 g
	Potassium chloride	0.0112 g
	Sodium hydrogen carbonate	0.105 g
	Sodium pyruvate	0.0022 g
	L-Glutamine	0.0073 g
	Phenol red solution (5% in D-PBS)	100 µl
	Water added to	50 ml
DEPC-treated water (Incubate for 16-20 hr at 37°C and inactivated by autoclaving)	Diethyl pyrocarbonate (DEPC)	1 ml
	Water added to	1 l
Digestion buffer	Sodium chloride	2.5 ml
	Tris	2.5 ml
	EDTA (pH 8)	0.1 ml
	Water added to	50 ml
dNTP solution	dATP (100 mM)	10 µl
	dCTP (100 mM)	10 µl
	dGTP (100 mM)	10 µl
	dTTP (100 mM)	10 µl
	Water added to	400 µl
D-PBS (Sigma)	Magnesium chloride (anhydrous)	0.04683 g/l
Dulbecco's phosphate buffered saline	Potassium chloride	0.2 g/l
	Potassium phosphate monobasic (anhydrous)	0.2 g/l
	Sodium chloride	8 g/l
	Sodium phosphate dibasic (anhydrous)	1.15 g/l
Fertilization medium (FM, TALP)	Sodium chloride	0.33 g
	Potassium chloride	0.0117 g
	Sodium hydrogen carbonate	0.105 g

	Sodium dihydrogen phosphate	0.0021 g
	Penicillin G	0.0032 g
	Magnesium chloride $6H_2O$	0.005 g
	Calcium chloride dehydrate	0.015 g
	Sodium lactate solution (60%)	93 µl
	Phenol red solution (5% in D-PBS)	100 µl
	Water added to	50 ml
IPTG solution	IPTG	1.2 g
	Water added to	10 ml
LB- agar media	Sodium chloride	8 g
	Pepton	8 g
	Yeast extracts	4 g
	Agar-Agar	12 g
	Sodium hydroxide (40.0 mg/ml)	480 µl
	Water added to	800 ml
LB-broth media	Sodium chloride	8 g
	Pepton	8 g
	Yeast extracts	4 g
	Sodium hydroxide (40.0 mg/ml)	480 µl
	Water added to	800 ml
Loading buffer	EDTA	0.1 mM
	Bromophenol blue	0.041 %
	Xylencyanol	0.041 %
	Glycerin	5 ml
	Water added to	2 ml
Lysis buffer	Igepal	0.8 µl
	RNasin	5 µl
	Dithiothreitol (DTT)	5 µl
	Water added to	100 µl
Lysis buffer	SDS (10%)	200 µl
	Tris-HCL (1M) pH 8.0	4 ml
	EDTA (0.5M) pH 8.0	4 ml
	Proteinase K 2% (W/V)	4.44 ml
	Mercaptoethanol	4 ml
	Water added to	200 ml
Millipore® milli Q sterilized water	Millipore® Milli Q water sterilized in an air incubator at 120°C for 1 hr, filtered through a 0.22 µm microcellulose syringe filter (ROTH), aliquoted in 0.2 ml or 1.5ml microcentrifuge tubes and irradiated by UV lamp for 60-90 min	150 µl or 1000 µl per tube

Modified Parker Medium (MPM-110 ml)	Sodium hydrogen carbonate	0.08 g
	Hepes	0.14 g
	Sodium pyruvate	0.025 g
	L-Glutamin	0.01 g
	Gentamycin (10mg/ml)	500 µl
	Tissue culture Medium (TCM-199)	99 ml
	Hemicalcium lactate	0.06 g
	Water added to	10 ml
PBS	Sodium chloride	8.766 g
	Di-Sodium hydrogen phosphate	1.495 g
	Potassium dihydrogen phosphate	0.204 g
	Water added to	1000 ml
PCR buffer (10x)	Tris-HCL	100 mM
	KCL	500 mM
	$MgCL_2$	15 mM
	Gelatin (pH 8.3, Sigma-Aldrich- GmbH, product code P 2192)	0.01%
Sample loading buffer, 4x (50 ml)	Tris-Hcl (1 M pH 6.8)	13 ml
	SDS	6 g
	2-Mercaptoethanol	10 ml
	Glycerin	20 ml
	Bromophenol blue	10 ml
	Water added to	50 ml
PHE medium (30 ml)	Physiological saline (0.9%)	16 ml
	Hypotaurin solution	10 ml
	Epinephrin solution	4 ml
Physiological saline solution (0.9%)	Sodium chloride	9.0 g
	Water added to	1000 ml
Proteinase K buffer	Proteinase K	200 mg
	1 x TE buffer	10 ml
SDS (10%) (w/v)	Sodium dodecyl sulphate (SDS)	10 g
	Water added to	100 ml
Silver staining solution	Sodium carbonate	30.0 g
	Formaldehyde	650 µl
	Water added to	1000 ml
SSCP loading buffer (per 50 ml)	Formamide	47.5 ml
	Sodium hydroxide	200 mg
	Bromophenol blue	125 mg
	Xylenecyanol	125 mg

TAE (50 x) buffer, pH 8	Tris	242 mg
	Acetic acid	57.1 ml
	EDTA 0.5 M (186.1 mg/ml)	100 ml
	Water added to	1000 ml
TBE(10 x) buffer	Tris borate (pH 8.0)	108 g
	Boric acid	55 g
	EDTA (pH8.0) (186.1 mg/ml) (0.5 M)	40 ml
	Water added to	1000 ml
TE (1x) buffer	Tris (1 M) (pH 8.0)	10 ml
	EDTA (186.1 mg/ml) (pH8.0) (0.5M)	2 ml
	Water added to	1000 ml
Washing buffer	Tris-HCL (1M pH 7.5)	0.5 ml
	LiCl (5 M)	1.5 ml
	EDTA (5 mM, pH 8)	1 ml
	Water added to	50 ml
X-gal (Stored at -20°C)	X-gal (5-bromo-4- chloro-3-indolyl- ß- D-galactoside)	50 mg
	N,N'-dimethylformamide	1 ml

3.2 Methods

3.2.1 Experimental design

First the sequence of PCR product for our interest genes was confirmed by sequencing. For screening SNP, genomic DNA of 11 different cattle breeds (Limousin, Gelbvieh, Blond d'Aquitaine, Salers, Vorderwälder, Hinterwalder, Charolais, Red Angus, Piemontese, Pinzgauer, and Galloway) were used for comparative sequencing. The sequence results of these 11 different samples were multi alignment with the sequence of genbank. After finding SNP between these samples, the SNP was confirmed, using RFLP or SSCP methods. After reconfirming SNP, genomic DNA of sperm for 310 Holstein (black or red) were genotyped applying of PCR-RFLP / SSCP for the selected candidate genes.
The SNP's which found in our study were tested for embryos genotyping and identifying their probable association with the early cleavage rate and early blastocyst rate.

3.2.2 In vitro embryo production and sample preparation

3.2.2.1 Oocytes recovery and in vitro maturation

Bovine ovaries were obtained from the local slaughter house and transported to the laboratory within 4 h in a thermo flask (35 ºC) containing physiological saline (0.9% NaCl), and supplemented with 50µl/100 ml Steptocombin®. Before aspiration of cumulus oocyte complex (COCs), the ovaries were washed once with 70% ethanol and two times with 0.9% physiological saline respectively, to eliminate surface organisms. The ovaries were dried with sterile paper to avoid contamination. Subsequently, the content of antral follicles 2-8 mm in diameter was aspirated using a 5 ml syringe attached with 18 G needle. This aspirated fluid was collected in sterilized 50 ml tubes kept at 35 ºC and was allowed to precipitate for 15 min. The competent cumulus oocyte complexes (COCs) which have more than two layers of cumulus cells were selected using glass-pipette and washed three times in drops of modified parker medium (MPM) supplemented with 12% heat inactivated oestrus cow serum (OCS) and 10 µg / ml FSH.

The COCs were transferred in groups of 50 cells in 400 µl maturation medium under mineral oil (Sigma) in four well dishes (Nunc, Roskilde, Denmark) and incubated in a pre-warmed maturation medium covered with mineral oil (Sigma). The MPM was composed of 728 µg / ml of sodium hydrogen bicarbonate 1.28 mg/ml Hepes, 227µg/ml sodium pyruvate, 91 µg L-Glutamine, 55 µg/ml gentamycin and 545 µg/ml hemicalcium lactate already dissolved in 10 ml water. All these chemical were dissolved in 100 ml TCM-199. The solutions are mixed thoroughly, sterile filtered and stored at 4°C. Before use 12 % heat inactivated oestrus cow serum and FSH (10 µg/ml) were added.

Only oocytes with evenly granulated cytoplasm and surrounded by multiple layer of cumulus cells were used for in vitro maturation. During the IVM procedure, these competent COCs were cultured in TCM-199 as basic medium at 39ºC in incubator with humidified atmosphere of 5% CO_2 in air for 22-24 h.

3.2.2.2 Sperm preparation and capacitation

Based on the number of oocytes, 2-4 semen straws prepared from Holstein-Friesian bulls of proven fertility were taken and shortly inserted in a water bath kept at 39°C. After about 8 seconds, the straws were taken out of water, dried with paper towel and disinfected with 70 % ethanol. The motile spermatozoa were then obtained through swim-up procedure (Parrish et al. 1988). During the swim-up procedure frozen thawed sperm cell were incubated in a tube containing 5 ml capacitating medium supplemented with heparin(sodium pyruvate) for 50 min under 39 ºC in an incubator with humidified atmosphere of 5% CO2 in air. The supernatant of the capacitation medium was carefully removed (containing motile sperm cells) and transferred into a 15 ml new falcon tube and was centrifuged at 250g for 5-10 min at room temperature. The sperm cells were pelleted by centrifugation; then the resulting pellet was washed two times however, before resuspending, its concentration was once more determined by diluting 2 µl of sperm preparation in 198 µl of distilled water. The numbers of sperm in each square of a quadratic cell counting plate were counted. Finally pellet resuspended in already prepared 3.5 ml capacitating medium and made ready to use for IVF.

3.2.2.3 In vitro fertilization

Matured oocytes were washed twice in the fertilization medium and transferred into a four-well dish containing 400 µl of fertilization medium supplemented with 6 mg/ml bovine serum albumin (BSA), 2.2 mg/ml sodium pyruvate and 1 mg/ml heparin. Following that, 10 µl of Hypotaurin-Epinephrin-solution (PHE) medium, covered already with mineral oil (Sigma) was added to each well to stimulate sperm motility. Motile spermatozoa selected by the above procedures were finally added to the fertilization medium to have a final concentration of 2 x 10^6 spermatozoa/ml to add to a group of 50 oocytes in each well and co-cultured for 18 h at standard incubation conditions, 39ºC and humidified atmosphere containing 5% CO_2 in air.
PHE is a combination of 3 different solutions. Solution one contains 0.9% NaCl; in solution two, 1.1 mg hypotaurin is dissolved in 10 ml 0.9% NaCl-solution. Solution three contains 40 mg sodium dihydrogen sulphate, 100 µl 60 % sodium lactate solution and 1.8 mg epinephrine, dissolved in 40 ml water. The three solutions are mixed, steriled, filtered and stored at -20°C.

3.2.2.4 In vitro culture

Following IVF, presumptive zygotes were treated with 0.1 % (w/v) hyaluronidase and mechanically denuded by gently vortexed to remove the attached sperm and cumulus cells, and washed three times in the culture medium (CR-1aa) (Rosenkrans and First 1994) supplemented with 10% oestrus cow serum (OCS), 10 µg / ml BME (essential amino acids) and 10 µg / ml MEM (non essential amino acids). The cumulus free zygotes were selected and washed two times with culture medium before being transferred in group of 50-60 cells into four-well dish, each well containing a 400 µl culture medium cover with mineral oil and incubated at the same conditions as used for maturation and fertilization till blastocyst stage. The cleavage rate of the embryos is determined at 28 h, 30 h and 32 h after fertilization followed by incubating for the consecutive days up to blastocyst stage.

3.2.3 Sperm parameters study using flow cytometry

The plasma membrane integrity (PMI), acrosom integrity (PNA / PSA) and DNA fragmentation index of sperms were tested by Hanover University using flow cytometry methods.

3.2.4 General molecular analysis methods

3.2.4.1 DNA isolation from sperm cells

In order to extract genomic DNA, sperm samples were used after defrosting from -20$^{\circ C}$. Sperm samples of 0.5 ml were mixed with 4 ml 0.9% sodium chloride solution, centrifuged at 5000× g for 10 minutes. The supernatant was discarded. The pellets were resuspended in 4 ml of digestion buffer. In order to digest protein in the pellet suspension, 4 ml lysis buffer containing proteinase K, SDS and mercaptoethanol were added and the samples were incubated at 37^{oC} overnight. An equal volume of phenol-chloroform (1:1 v/v) was added and the two phases were mixed until an emulsion was formed. The two phases were separated by centrifugation at 5000× g for 10 minutes. The aqueous supernatant solution was collected in fresh sterilized tubes. Phenol-chloroform extraction was repeated again. Upper parts of liquid was transferred to new tube and add one-tenths volume of 3 M sodium acetate (pH 5.2) and an equal volume of isopropanol were added and the samples were shaken gently until precipitation of DNA. The DNA was washed with 70 % ethanol and dried. Finally the DNA was dissolved with 1 ml TE-buffer and kept at 4^{oC}. The concentration of DNA was measured using spectrophotometer. The working solution of DNA were diluted in 100 µl (50 ng / µl) with deionized and demineralized (Millipore) water and stored at the 4$^{\circ C}$ temperature.

3.2.4.2 DNA isolation from blood samples

For DNA isolation, 10 ml of blood was taken from the jugular vein of the cows, kept in a tube containing 500 µl of 0.5 M EDTA as an anticoagulant and chilled immediately on ice. After centrifugation of blood samples at 1,100 rpm for 30 min, the blood was

separated into: 1) a lower red blood cell phase, 2) a middle phase of white blood cells, and 3) a colorless upper aqueous phase of plasma. The middle layer was transferred to a new sterile 15 ml tube. Nine ml of deionised and demineralised water was added to the sample and the mixture was then mixed by inversion for 20 sec followed immediately by adding 1 ml of 9 % NaOH. The mixture was centrifuged at 1,100 rpm for another 15 min. Supernatant was discarded and the pellet was used for DNA isolation. Genomic DNA from blood samples of cow was isolated using standard phenol/chloroform extraction method. All centrifugation steps were conducted at 12,000x g. To the white blood cell pellet from previous step 0.7 ml of digestion buffer and 20 µl of proteinase K were added. The mixture was incubated at 50°C overnight at 100 rpm in a shaking incubator. Complete pellet digestion resulted in a viscous homogeneous solution. An equal volume of phenol-chloroform was added and mixed by several inversions. The sample was then centrifuged for 10 min at room temperature. After centrifugation, the sample was separated into: 1) a lower phenol-chloroform phase, 2) an interphase of protein and 3) an upper aqueous phase of DNA. The aqueous phase was transferred into a 2.0 ml microcentrifuge tube and washed by mixing with an
equal volume of chloroform to remove residual phenol. After centrifugation for another 10 min at room temperature, the DNA containing the aqueous phase was transferred to a new 2.0 ml tube and precipitated by mixing with 1/10 volume of sodium acetate (3 M, pH 8.5) and a double volume of isopropanol. The mixture was incubated overnight at -20°C or 2 hours at -80°C followed by centrifugation for 20 min at 4°C. Then, the supernatant was drained and the pellet was washed with 0.5 ml of 75% ethanol to remove excess salt. After centrifugation for another 10 min at 4°C, the DNA pellet was briefly air dried and dissolved in 0.3 to 0.5 ml of 1x TE buffer. The DNA concentration was measured by spectrophotometer. A working DNA solution was prepared by diluting stock DNA with 1x TE buffer to the concentration of 50 ng / µl. The stock DNA solution was kept at -20°C and the working solution was kept at 4°C.

3.2.4.3 Polymerase chain reaction

For all gene amplification studies, several pair of primers were designed according to bovine cDNA or genomic DNA sequences found in GenBank (see Table 1 for details) using Primer 3 Software v 2.0 (Rozen and Skaletsky 2000, Wang et al. 1998). These

primers generated a PCR amplicon corresponding to the nucleotides sequence. The identity of the product was confirmed by sequencing. PCR reactions have generally been carried in a 20µl reaction volume containing 2 µl template, 2 µl 10x PCR buffer (Sigma), 0.5 µl dNTP, 50 pmol of each (forward and reverse) primer, 0.5 units of Taq DNA and water has been added to complement the rest volume. Some of PCR reactions were performed in a 'touch down' PCR protocol, for about 2-3 hours. To avoid spurious priming during product amplification, PCR cycles were generally hot start with denaturation at 94°C for 5 minutes. Annealing begins at 5°C above the expected temperature, decreasing 1°C/cycle until it reaches the expected temperature. In all cases, annealing was preceded by 30 seconds of denaturation at 94°C and followed by 60 seconds of extension at 72°C. Then after, following 35-40 cycles of denaturation at 94°C for 30 seconds, annealing at respective temperatures for 30 seconds and extension at 72°C for 60 seconds with final extension at 72°C for 5 minute. Primer sequences, source ESTs and respective PCR conditions are mentioned in Table 1 and 2.

At the end of the PCR reactions, the products were loaded and screened on agarose gel in 1x TAE buffer by staining with ethidium bromide for visualizing and screening products.

Table 1: Detail of primers used for SNP screening and genotyping

Gene Name	Primer Sequence (5' ⟶ 3')	Annaling Temperature(°C)	Product Size (bp)	Accession Number
CDH1	For : GGTCACGGCCACAGATATAGA Rev: TGGGAGCATTATCATTGGTG	56-47	544	AY508164
DSC2-1	For : AAAGTGCAAGACATGGATGG Rev: CCTTCATTGGTTTGGGAATC	59-50	850	M81190
DSC2-2	For : TGACCCTGGAAATTGGTGTA Rev: CCACTTCTGGCTTCTCTATCG	59-50	667	M81190
DSC2-4	For : GGCAGCACGTCTCTCCTATC Rev: GCCAGGAGCTTCAGTGTTTG	57-47	732	M81190
PKP 1	For : GCCTACACCGACAAGTCCTC Rev: CAGTGAGGTTGTGGCTCTCA	63	470	Z37975
TJP	For : CCAGTGGTGCCTGATTCTTT Rev: TAGCGCAGACGATGGTCATA	59	667	NC_007319
N-PAC	For : CCCGGTCTCAGGGAATCAG Rev: TTCTGGTCCAGGAAGATGC	60-50	612	BT021674
DC9	For : GAGGCAAAACTCCAAAACCA Rev: CTCCACTGTCGTTTGTCGTG	61	658	NM_173900
PLCζ	For : TTGTGACCCCATGGACTGTA Rev: CTTTGCATTGCCTCTTCCTC	59	430	NW_001012286
AKR1B1	For : GGTCAATGGGCCTTAGGATT Rev: ACCAGGGCTTACCTGGAAGT	59	796	NW_993718
COX-2	For : TGATCTACCCGCCTCATGTT Rev: CCCTTTGCCTGGTGAATG	58	759	AF031698

Table 2: Primer for SNP genotyping of CDH1

Gene Name	Primer Sequence(5' → 3')	Annaling Temperature(°C)	Product Size (bp)	Accession Number
CDH1	For : CGCACAACAAAATGTTCACC Rev: GGCCTCAAATCTCCAGACAA	56-47	226	AY508164

3.2.4.4 Purification of PCR products

Purification of PCR products from gel

For purification, PCR products were loaded on 0.8 % (w/v) agarose gel and run in 1x TAE buffer containing ethidium bromide. The bands were then visualized under UV light and specific gel slices containing the bands cut for further processing. The bands were put into 1.5 ml tube to be incubated at -20°C for 30 min. 600 µl of 1x TE buffer was added to this gel slice and grounded with the syringe needle until it was dissolved. 600µl of phenol-chloroform (1:1 v/v) were added to homogenized solution and mixed well by vortexing. The mixture was centrifuged at 14,000 rpm for 10 min at 4°C. The supernatant aqueous solution was carefully transferred to new 2.0 ml tube which an equal volume of chloroform was added to it. The mixture was centrifuged by 14,000 rpm for 10 min at 4°C and the supernatant of the solution was transferred to a new 2.0 ml tube. Add 1:10 volume of sodium acetate (3 M, pH 5.2) and two volume of 100% ethanol were then added to the solution which was shaken gently and kept at -20°C overnight. The DNA pellet was precipitated by centrifugation at 14,000 rpm for 30 minutes at 4°C. The pellet was washed two times with 70% ethanol and dried by vacuum drier and then resuspended in 7 µl of Millipore water and stored at -20°C until further use.

Purification of PCR products with using ExoSAP-IT™

ExoSAP-IT™ was used for fast and efficient purification of Polymerase Chain Reaction (PCR) products for sequencing. ExoSAP-IT consists of two hydrolytic enzymes, Exonuclease I and Shrimp Alkaline Phosphates (SAP), in a specially formulated buffer for the removal of unwanted primers and dNTPs from a PCR product mixture with no interference in sequencing.

3.2.4.5 Comparative sequencing for SNP detection

Prior to the sequencing procedure, PCR with specific primer was performed and 5 µl of the PCR product was checked on 2% agarose gel. A sharp band visualized under UV/transilluminator showed primer specifity and the PCR products were ready to be

cleaned up. Sequencing was done by SEQ 8000 Genetic Analysis System (Beckman coulter) using CEQ Dye Terminator Cycle sequencing (DTCS) Quick start kit. For purification of PCR product, a mixture of 1 µl of ExoSAP-IT with 5 µl of PCR product was incubated at 37^{oC} for 30 min followed by ExoSAP-IT inactivation at 80^{oC} for 15 min. The sequencing PCR reaction was done according to the recommendation of the company with minor modification as follows: To the clean PCR product subjected to sequencing PCR, 2 µl of either forward or reverse primer, 4 µl DTCS master mix (DNA polymerase, pyrophosphates, buffer, dNTPs, and dye terminators) and 8 µl of Millipore H_2O were added. The sequencing PCR was done for 30 cycles at 96°C for 20 sec, 50°C for 20 sec and 60°C for 4 minute, followed by holding at 4°C. Precipitation and holding the samples into CEQ sample plate was performed according to the instruction in the kit protocol. Briefly, 2 µl of 3M Sodium Acetate (pH 5.2), 2 µl of 100 mM EDTA (pH 8.0) and 1 µl of glycogen (20 mg / ml) was prepared. To each of the labeled tubes, 5 µl of the stop solution and 60 µl cold 99% ethanol was added, mixed thoroughly and immediately centrifuged at 18,000 rpm at 4^{oC} for 15 min. The supernatant was removed and the pellet was rinsed two times with 200 µl cold 70 % ethanol. For each rinse, a centrifugation was immediately applied at 18,000 rpm at 4^{oC} for 5 min. After that, the supernatant was removed and the pellet was air dried (or vacuum dried) for 10 min. Finally, the pellet was resuspended with 40 µl of sample loading solution and was ready for sequencing. The sample was then resuspended in 40 µl of sample loading solution (SLS) (Beckman Coulter). Samples were transferred to a CEQ sample plate and overlaid with mineral oil at the mean time, the separation buffer was prepared in another plate and both plates were loaded into the instrument and started the desired method in the CEQ™ 8000 Genetic Analysis System.

3.2.4.6 Sequence analysis

The completed sequence of the fragment was utilized to search for homologous sequences in National Center for Biotechnology Information (NCBI) non redundant DNA sequence data base using BLASTN search program (http://www.ncbi.nlm.nih.gov/BLAST/). Sequence similarities were considered to be significant when identity percentage was ≥ 90%.

3.2.4.7 Genotyping methods

3.2.4.7.1 Single strand conformation polymorphism (SSCP)

The SSCP analysis was performed to detect polymorphism in experimented animals. The primers were designed to amplify the specific fragment which contains the SNP of interest for the gene of study. The PCR was performed using designed primers in a touch down condition (Table 2). The PCR products were diluted 1:1 with SSCP loading buffer (95 % formamide, 10 mM NaOH, 0.25 % bromophenol blue and 0.25 % xylene cyanol), then denatured at *95* °C for 5 min., chilled on the ice and loaded on 10 % polyacryamide gel (acrylamide: bisacrylamide =19:1). The gel was run at 12 W for 3.5 hours at room temperature in 1 x TBE buffers ($4^{\circ C}$).

Electrophoresis was performed until bromophenol blue run to the end of gel. The gel was fixed for 10 min in 10 % glacial acetic acid. Then it was moved to 1 % nitric acid for 10 min. The gel was stained for 30 min with 0.2 % silver nitrate, rinsed in ddH2O and developed in 3% sodium carbonate containing 750µl of 37 % formaldehyde and 300 µl of sodium thiosulfate, which were cooled down to 4°C before use. The development time was between 3-5 min. After visualization of the bands, the reaction was stopped by immersion of the gel in 10 % glacial acetic acid for 1 min. Then the gel was washed with ddH2O 3 times and dried for analysis and documentation at room temperature for 3 hours.

3.2.4.7.2 Restriction fragment length polymorphisms (RFLP)

DNA samples from Holstein population were genotyped by using PCR-RFLP (Restriction Fragment Length Polymorphim). The restriction enzymes were selected according to their recognition (http://tools.neb.com/NEBcutter2/index.php) of the polymorphic sites. First, the fragment covers the SNP were amplified with a specific primer (Table 3.2). Next, a restriction digestion of the product was carried out in 10 µl reaction volume containing 1 unit of restriction enzyme, 1 µl of 10x restriction buffer and 3.9 µl Millipore water with 5 µl PCR product was incubated at 37^{oC} for 4 hours. The digested product was checked by electrophoresis on 2% aragose gel, 120 V for 30

min. Different fragment length between non- and digested DNAs reflected the genotype of a specific sample.

3.2.4.8 Embryo genotyping

The genotype of individual blastocysts was determined by PCR technique. Blastocysts were digested with 10 µl of lysis buffer, a solution containing 1.13 mg / ml proteinase K in 50 mM KCl, 50 mM Tris-HCl (pH 8.3), 2.5 mM $MgCl_2$, and 0.5% Tween 20. The digestion was carried out overnight at 56°C. The digest was then diluted 1:3 with water and heated at 95°C for 15 min to inactivate the proteinase K. Two µl of embryo lysate served as the template for the PCR reaction; this was combined with 5.1 µl Millipore water, placed in a PCR reaction tube and heated to 95°C for 15 minutes (to inactivate the proteinase K). The final reaction mixture contained the heat-treated embryo digest plus 0.2 µl of 10 mM dNTPs, 0.3 µl of each primer stock solution (10 µM), 0.1 µl of *Taq* polymerase and 2 µl of 10 × PCR buffer for a total reaction volume of 10 µl. Then PCR was done according special annealing temperature for each primer. Consequently PCR product was used for genotyping using relevant restriction enzyme. The product was visualized on 2 % agarose gels containing ethidium bromide.

3.3 Statistical analysis

3.3.1 Allele and genotype analysis

Allele and genotype frequencies were calculated in order to test for Hardy-Weinberg equilibrium by chi-square analysis.

3.3.2 Association analysis between candidate genes and bull reproductive traits

3.3.2.1 Association analysis for fertility trait

The statistical analyzes were contributed in two ways. In the first part, a linear mixed model ‘’Proc Mixed’’ was used, where the non-return rates were assumed to be normally distributed for each bull. However, as the non-return rate is a binary variable

[the cow either did return (1) or not (0)], a threshold model was used as well. In this threshold model an underlying unobserved liability variable is assumed which is normally distributed. If the threshold is exceeded the observed variable (non-return rate) turns from zero to one. To switch between observed and liability scale a so called link function is used. In our case the logit link ($ex/1+e^x$) was used. All analyzes were conducted with the SAS 9.1 Package (SAS Inc., Cary, NC, USA).

The linear mixed model used was:

$$y_{ijklmn} = \mu + FS_i + CSTAT_j + TEC_k + GEN_l + BSTAT_{m+Bn} + \varepsilon_{ijklmn}$$

Where:

y_{ijklmn}	Non-return rate under investigation per cow i inseminated with bull n,
μ	Overall mean,
FS_i	Fixed effect of farm*season for each cow i,
$CSTAT_j$	Fixed effect for the status of cow j (multi-calving cow, cow and heifer),
TEC_k	Fixed effect for the AI-technician who inseminated cow k,
$BSTAT_l$	Fixed effect for the status of bull l (age),
GEN_m	Fixed effect for the genotype of the gene under investigation for bull m,
B_n	Random effect for the bull n, for which the non-return rate was summarized,
ε_{ijklmn}	Error

For fertility trait NRR, a general linear mixed model with the fixed effects of farm, status of cow (multi-calving cow, cow and heifer), effect of AI-technician, status of bull (age) and genotype and bull as a random effect was used

The threshold model used was:

$$y_{ijk} = \mu + FS_i + GEN_j + c_k + \varepsilon_{ijk}$$

Where:

y_{ijk} Non-return rate under investigation per cow i inseminated with bull j,

μ Overall mean,

FS_i Fixed effect of farm*season for each cow i,

GEN_j Fixed effect of the genotype for the gene under investigation for bull j,

c_k Random effect for the cow k, which could have several inseminations, and

ε_{ijk} Error

3.3.2.2 Association analysis for semen quality traits and sperm flow cytometry parameters

Analyses of variance were performed with the procedures “Proc Mixed” and “GLM’’ of the SAS software package to address effects on semen quality traits, fertility and sperm flow cytometry parameters. For semen quality traits [sperm concentration ($\times 10^9$/ml), sperm motility (%), semen volume per ejaculate (ml), and survivability after thawing sperm (%)] and fertility trait (NRR), statistical analyses were performed using a general linear mixed model with the fixed effects of season and genotype and random effect bull. For sperm flow cytometry parameters [plasma membrane integrity (PMI), acrosome integrity (PSA, PNA) and DNA fragmentation index (DFI)] ‘’General Linear Model’’ were performed. Multiple pair wise comparisons were conducted using the Tukey-Kramer test. Differences of $P \leq 0.05$ were considered significant.

3.3.3 Embryo development

Analysis of variance using "GLM" was performed to investigate the effect of genotypes on embryo development. Multiple pair wise comparisons were down using the Tukey-Kramer test. Differences of $P \leq 0.05$ were considered significant. The following statistical model was used:

$Y = \mu + C_i + \varepsilon_i$

Where;

Y : The investigated embryos

μ : The overall mean

C_i : Effect of genotype

ε_i : Error

4 Results

4.1 Screening SNP and genotyping

The candidate genes sequence was obtained from GenBank (http://www.ncbi.nlm.nih.gov/). Primers were designed from corresponding gene sequence and used to amplify overlapping fragments of about 500-800 bp in size covering exon and intron parts. A competitive-PCR method was used to simultaneously detect the mutated and wild-type alleles. Primers were amplified with genomic DNA of eleven different bovine breeds; Gelbvieh, Blond d'Aquitaine, Salers, Limousin, Vorderwälder, Hinterwalder, Charolais, Red Angus, Piemontese, Pinzgauer, and Galloway. Amplified fragments were analysed for polymorphism. PCR-RFLP and SSCP techniques were established in order to genotype the animals under investigation.

4.2 Polymorphism in genes related preimplantation development

Sequencing result of PLCζ gene revealed one SNP at position 2749 bp in intron 6. The SNP was a transition from Guanine (G) to Adenine (A) and this SNP was confirmed by PCR-RFLP with restriction enzyme HpyCH4III (figure 8). Genotyping results show that among the animals, 23 (0.08) cases were homozygous 'GG', 126 (0.42) were heterozygous 'GA' and 151 (0.50) were homozygous 'AA'. Frequencies of the allele 'G' and 'A' were 0.29 and 0.71 respectively. The detail of the genotypes and allele frequencies in each population (black or red Holstein) are shown in tables 3 and 4.

In CD9 gene, one polymorphism at position 95bp in exon 9 was found. The SNP was a transition from Thymine (T) to Cytosine (C) (figure 7). This SNP was confirmed by PCR-RFLP with restriction enzyme DraI (figure 8). Genotyping was done in 310 Holstein-Frisian bulls using their sperm samples. Among the animals that were genotyped, 247 (0.82) cases were found to be homozygous 'TT', 51 (0.17) were heterozygous 'TC' and 2 (0.01) were homozygous. Frequencies of the allele 'T' and 'C' were 0.91 and 0.09 respectively. The detail of the genotypes and allele frequencies in each population (black or red Holstein) were shown in tables 3 and 4.

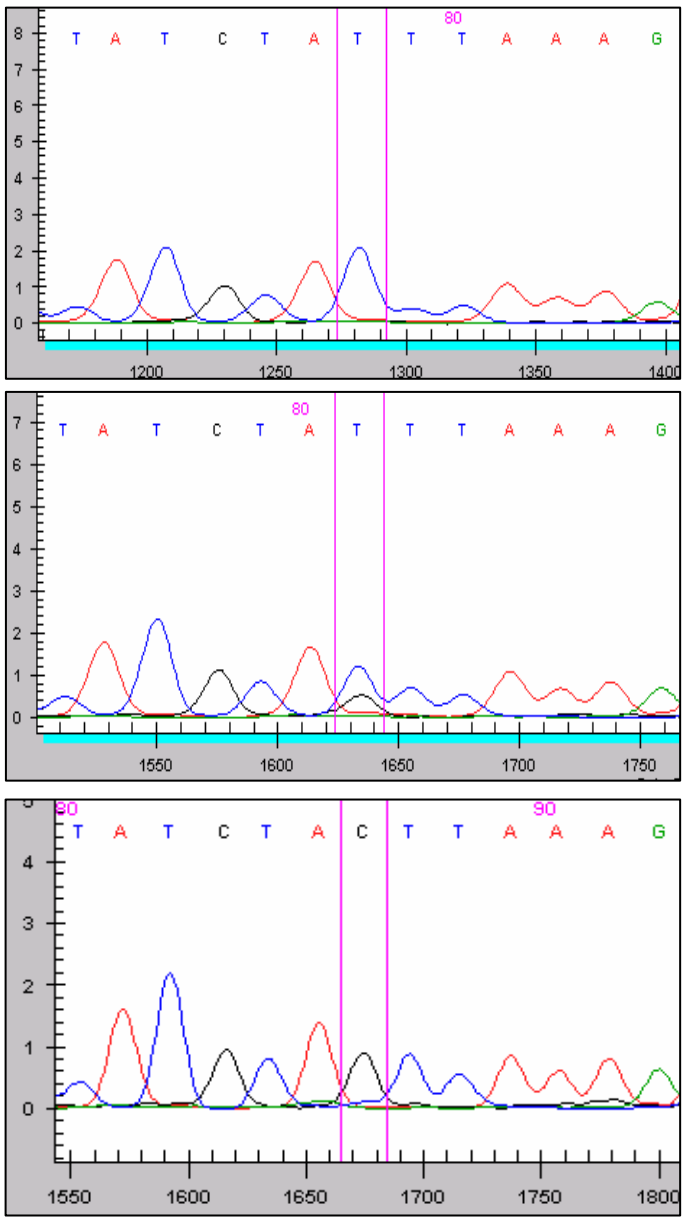

Figure 7: A representative image showing SNP by comparative sequencing. Column show SNP position. A) bull with homozygous T, B) bull with heterozygote TC, and C) bull with homozygous C

For COX-2 gene, two polymorphisms at position 185 bp in intron 5 and 6 bp in intron 7 were found. The former SNP had a transition from Thymine (T) to Cytosine (C) and the later showed a change from Adenine (A) to Thymine (T). These SNPs were confirmed by PCR-RFLP with restriction enzyme AluI (figure 8). Genotyping results show that among the animals 228 (0.76) cases were homozygous 'TT', 14 (0.05) was homozygous

‘CC’ and 58 (0.19) were heterozygous ‘TC’. Frequencies of the allele ‘T’ and ‘C’ were 0.86 and 0.14 respectively. The detail of the genotypes and allele frequencies in each population (black or red Holstein) are shown in tables 3 and 4.

Screening of SNP for AKR1B1 locus showed one SNP in intron 7 at position 159 bp. The SNP was a transition from Adenine (A) to Guanine (G) and this SNP was confirmed by PCR-RFLP with restriction enzyme NdeI (figure 8). Genotyping results shows that among the animals, 192 (0.64) cases were homozygous ‘AA’ and 108 (0.36) were heterozygous ‘AG’. The genotype GG was not detected. Frequencies of the allele ‘A’ and ‘G’ were 0.82 and 0.18 respectively. The detail of the genotypes and allele frequencies in each population (black or red Holstein) were shown in tables 3 and 4.

In the N-PAC gene we found one polymorphism at position 5 bp in intron 13. The SNP was a transition from Cytosine (C) to Thymine (T) and this SNP was confirmed by PCR-RFLP with restriction enzyme Hin1 II (figure 8). Genotyping results show that among the animals, 236 (0.77) cases were homozygous ‘CC’, 70 (0.22) were heterozygous ‘CT’ and 2 (0.01) were homozygous ‘TT’. Frequencies of the allele ‘C’ and ‘T’ were 0.88 and 0.12 respectively. The detail of the genotypes and allele frequencies in each population (black or red Holstein) were shown in tables 3 and 4.
For EEF1α1 we could not found SNP.

4.3 Polymorphism in genes related to cell to cell adhesion and compaction

Comparative sequence study revealed seven polymorphisms for DSC2 gene at positions 281 bp Thymine (T) to Guanine (G) in intron 7; 6 bp Adenine (A) to Guanine (G) and 21 bp Guanine (G) to Adenine (A) in intron 9 and 206 bp Guanine (G) to Adenine (A), 328 pb Thymine (T) to Cytosine (C), 341 bp Cytosine (C) to Thymine (T) and 378 bp Thymine (T) to Cytosine (C) in intron 12. Only the SNP which located in 206 bp Guanine (G) to Adenine (A) in inrton 12 was confirmed by PCR-RFLP with restriction enzyme RsaI (figure 8). Genotyping results show that among the animals, 52 (0.17) cases were homozygous ‘GG’, 144 (0.47) were heterozygous ‘GT’ and 112 (0.36) were homozygous ‘TT’. Frequencies of the allele ‘G’ and ‘A’ were 0.40 and 0.60

respectively. The detail of the genotypes and allele frequencies in each population (black or red Holstein) were shown in tables 3 and 4.

In CDH1 gene three polymorphisms were found, one polymorphism at position 47 bp in exon 8 and two polymorphisms in nucleotide 43 bp and 92 bp in intron 7. The SNPs were transition from Cytosine (C) to Guanine (G), Guanine (G) to Adenine (A) and Cytosine(C) to Guanine (G) respectively. SNP was confirmed by SSCP with using specific primers for the position of 43 bp in intron 7 (figure 9). Genotyping results show that among the animals, 277 (0.90) cases were homozygous 'CC'; 30 (0.10) were heterozygous 'CG' and homozygote 'GG' was not observed. Frequencies of the allele 'C' and 'G' were 0.95 and 0.05 respectively. The detail of the genotypes and allele frequencies in each population (black or red Holstein) were shown in tables 3 and 4.

One SNP was found for TJP1 gene at position 50 bp in coding sequence region of exon 13. The SNP was a transition from Cytocine (C) to Thymidine (T) and this SNP was confirmed by PCR-RFLP with restriction enzyme BmgBI (figure 8). Genotyping results show that among the animals, 187 (0.62) cases were homozygous 'CC', 101 (0.34) were heterozygous 'CT' and 12 (0.04) was homozygous 'CC'. Frequencies of the allele 'C' and 'T' were 0.79 and 0.21 respectively. The detail of the genotypes and allele frequencies in each population (black or red Holstein) were shown in tables 3 and 4.

Screening for polymorphism in PKP1 revealed one SNP at position 140 bp in intron 9. The SNP was a transition from Guanine (G) to adenine (A) and this SNP was confirmed using PCR-RFLP with restriction enzyme DraI (figure 8). Genotyping results show that among the animals, 50 (0.17) cases were homozygous 'GG', 90 (0.30) were heterozygous 'AG' and 160 (0.53) were homozygous 'AA'. Frequencies of the allele 'G' and 'A' were 0.32 and 0.68 respectively. The detail of the genotypes and allele frequencies in each population (black or red Holstein) were shown in tables 3 and 4.
For locus GJA1, no polymorphism was found among eleven different breeds.

So far, the screening for polymorphism in these genes had not been reported.

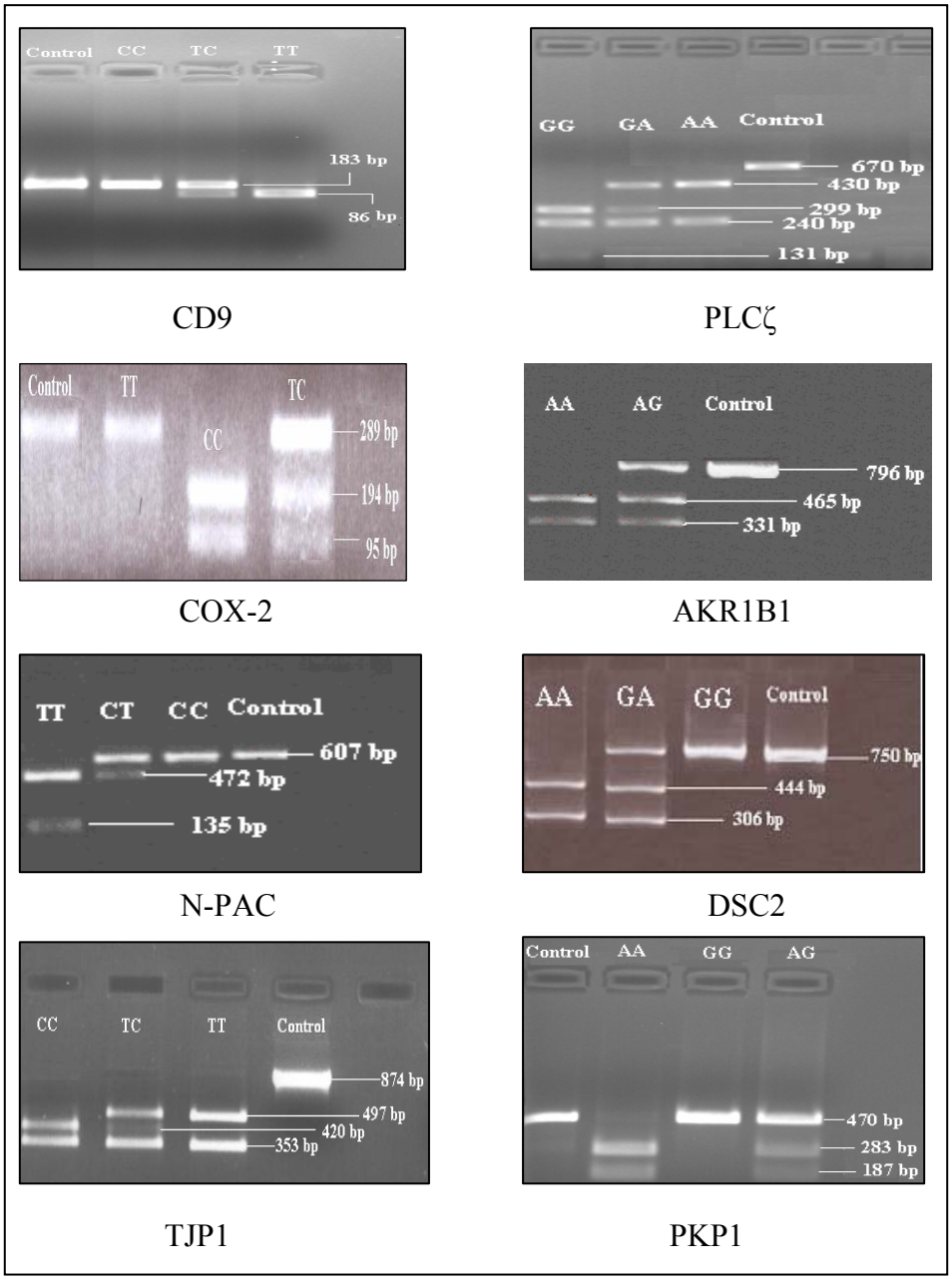

Figure 8: PCR-RFLP for genotyping CD9, PLCζ, COX-2, AKR1B1, N-PAC, DSC2, TJP1 and PKP1 in Holstein population.

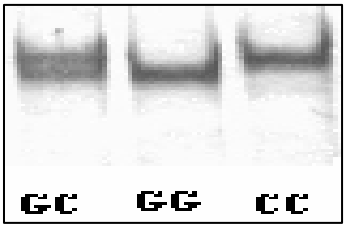

Figure 9: PCR-SSCP for genotyping CDH1 in Holstein population.

Table 3: The genotype frequencies for loci CD9, PLCζ, COX-2, AKR1B1, N-PAC, DSC2, CDH1, TJP1, and PKP1 in black and red Holstein populations

Locus	Genotype	Genotype frequency	
		Black Holstein	Red Holstein
CD9	TT	0.89	0.63
	TC	0.11	0.34
	CC	-	0.03
PLCζ	GG	0.08	0.06
	GA	0.40	0.48
	AA	0.52	0.46
COX-2	TT	0.82	0.49
	TC	0.14	0.37
	CC	0.04	0.14
AKR1B1	AA	0.66	0.59
	AG	0.34	0.41
	GG	-	-
N-PAC	CC	0.78	0.72
	CT	0.21	0.26
	TT	0.01	0.02
DCS2	GG	0.18	0.14
	GA	0.49	0.40
	AA	0.33	0.46
CDH1	CC	0.87	0.99
	CG	0.13	0.01
	GG	-	-
TJP1	TT	0.06	0.32
	CT	0.34	0.68
	CC	0.60	-
PKP1	GG	0.18	0.12
	GA	0.33	0.22
	AA	0.49	0.66

Table 4: Allele frequencies of the loci CD9, PLCζ, COX-2, AKR1B1, N-PAC, DSC2, CDH1, TJP1 and PKP1 for black and red Holstein populations

Locus	Genotype	Genotype frequency	
		Black Holstein	Red Holstein
CD9	T	0.95	0.80
	C	0.05	0.20
PLCζ	G	0.28	0.30
	A	0.72	0.70
COX-2	T	0.89	0.76
	C	0.11	0.24
AKR1B1	A	0.83	0.79
	G	0.17	0.21
N-PAC	C	0.89	0.86
	T	0.11	0.14
DCS2	G	0.43	0.34
	T	0.57	0.66
CDH1	C	0.93	0.99
	G	0.07	0.01
TJP1	T	0.23	0.16
	C	0.77	0.84
PKP1	G	0.35	0.23
	A	0.65	0.77

4.4 Test for Hardy-Weinberg equilibrium

The chi-square test revealed (Table 5) that the loci PLCζ, COX-2, N-Pac and DSC2 were in Hardy-Weinberg equilibrium in both populations. Locus CD9 and TJP1 were in Hardy-Weinberg equilibrium only in red and black Holstein population respectively. For loci AKR1B1, CDH1 (in both population) CD9 and TJP1 (in black and red Holstein population respectively), we could not estimate Hardy-Weinberg equilibrium, because we found 2 genotype in the population and the freedom degree of these gene was zero.

Table 5: Chi-square test revealing genotypes of the loci in Hardy-Weinberg equilibrium

Loucs	Black Holstein		Red Holstein	
	χ^2	P-value	χ^2	P-value
CD9	0.68	-	0.62	0.43
PLCζ	0.05	0.83	1.49	0.22
COX-2	0.58	0.44	2.42	0.12
AKR1B1	9.11	-	5.61	-
N-PAC	3.26	0.07	1.91	0.16
DSC2	0.02	0.89	1.03	0.31
CDH1	1,08	-	0,01	-
TJP1	0.09	0.77	2.91	-
PKP1	16,22	0.0001**	12,06	0.0005**

No significant differences in allele frequencies within population (*P> 0.05*)
** = (*P≤ 0.001*)

4.5 Association analysis

4.5.1 Association analysis between NRR and genotype

The bull fertility trait ‘’Non Return Rate’’ [NRR (%)] were calculated with the insemination records of cows using the statistical Proc. Mixed model. The model comprised the genotype, season, farm, technician effects, cow status (Heifer, calving, multi calving), and bull status (age). The semen quality traits data were analysed for each bull population separately. All genotyped nine marker loci were applied for the statistical analysis. For this study SAS V9.1 software was applied. No association was found between loci and NRR. The association between these candidate genes and NRR are shown in table 6.

Table 6: Estimates of least square means for the loci CD9, PLCζ, COX-2, AKR1B1, N-PAC, DSC2, CDH1, TJP1 and PKP1 genotype effects on NRR (%)

Locus	Genotype	LSM ± SE
CD9	TT	73.94 ± 0.45
	TC	74.45 ± 1.10
PLCζ	GG	74.33 ± 1.48
	GA	73.90 ± 0.63
	AA	74.05 ± 0.57
COX-2	TT	73.85 ± 0.66
	TC	74.66 ± 0.60
	CC	72.74 ± 1.12
AKR1B1	AA	74.17 ± 0.51
	AG	73.84 ± 0.68
N-PAC	CC	74.01 ± 0.47
	CT	74.24 ± 0.87
	TT	74.36 ± 5.49
DCS2	GG	74.08 ± 1.02
	GA	74.01 ± 0.59
	AA	73.89 ± 0.69
CDH1	CC	74.10 ± 0.43
	CG	76.58 ± 2.14
TJP1	TT	74.50 ± 0.95
	CT	74.58 ± 0.70
	CC	73.79 ± 0.52
PKP1	GG	76.57 ± 0.97
	GA	73.57 ± 0.55
	AA	73.53 ± 0.74

4.5.2 Association analysis between semen quality traits and genotypes

Semen quality traits including sperm concentration ($x10^9$/ml), semen ejaculation volume (ml), sperm motility (%) and sperm survival rate after thawing (%) were obtained from each ejaculate. Association analysis of semen quality traits and genotype were performed using Proc Mixed model 3, with the fixed effects of genotype, collected season (year, month), breed and random effect of the bull (repeated measurement). The analysis followed by multiple pair wise comparisons using Tukey-Kramer adjustment. The sperm quality data of black and red Holstein were analysed separately. Results of the statistical analysis are presented as least square means (LSM) and standard errors (SE) of least square means.

4.5.2.1 Semen ejaculate volume

Association analysis results showed that candidate genes DSC2 and TJP1 to be significantly associated with volume of ejaculation ($P<0.05$) in red Holstein population; however no significant effect ($P> 0.05$) was found for these loci in black Holstein population. No significant effects of genotypes in the other loci CD9, COX-2, PLCζ, AKR1B1, N-PAC, CDH1 and PKP1 in both black and red Holstein populations were observed on this specific parameter. Significant effects of different loci on volume of ejaculation were given as means and standard error. In locus DSC2, bulls with homozygote GG had lower ejaculation volume than homozygote AA and heterozygote GA. In locus TJP1, homozygous TT had higher ejaculation volume than heterozygote CT. Due to no observation of one genotype in some genes (CD9, AKR1B1, N-PAC, CDH1 and TJP1) in one or both population; we could not estimate LSM for this genotype in these populations. The association between these candidate genes and semen ejaculate volume are shown in table 7.

4.5.2.2 Sperm survivability

Results of this study showed that candidate genes AKR1B1 and CDH1 were significantly associated with sperm survivability trait ($P<0.05$) in black Holstein population, however no significant association ($P >0.05$) was found for these loci in red

Holstein population. For loci COX-2, CD9, PLCζ, N-PAC, DSC2, TJP1 and PKP1, in both black and red Holstein populations, no significant association effect of genotypes with sperm survivability were observed. Significant effects of different loci on sperm survivability were given as means and standard error. In locus AKR1B1, bulls with homozygote AA had higher survival rate than heterozygote AG. In locus CDH1, homozygous CC had higher survivability rate than heterozygote CG. Due to no observation of one genotype in some genes (CD9, AKR1B1, N-PAC, CDH1 and TJP1) in one or both population, we could not estimate LSM for this genotype in these populations. The association between these candidate genes and sperm survivability are shown in table 8.

4.5.2.3 Sperm motility

Association study between genotypes of candidate genes and sperm motility showed that CD9, COX-2, AKR1B1, DSC2 and TJP1 genes to be associated with sperm motility trait ($P < 0.05$) in both black and red Holstein population. No effects ($P>0.05$) of genotypes in the loci PLCζ, N-PAC, CDH1 and PKP1 were observed in both black and red Holstein populations. Significant effects of different loci on sperm motility were given as means and standard error (table 9). In locus CD9, bulls with homozygote TT had higher sperm motility than heterozygote TC. In locus COX-2, bulls with homozygote TT had higher sperm motility than heterozygote TC. In locus AKR1B1, bulls with homozygote AA had higher sperm motility than heterozygote AG. In locus DSC2, bulls with genotypes GG and GA had higher sperm motility than homozygote AA. In locus TJP1, homozygous TT had lower ejaculation volume than heterozygote CT. Due to no observation of one genotype in some genes (CD9, AKR1B1, N-Pac, CDH1 and TJP1) in one or both population; we could not estimate LSM for this genotype in these populations.

4.5.2.4 Sperm concentration

In this study we found significant association between sperm concentration trait and loci CD9, N-PAC, CDH1 and PKP1 ($P< 0.05$) in red or black Holstein population. No significant effects ($P>0.05$) of genotypes in the loci PLCζ, COX-2, AKR1B1, DSC2 and

TJP1 were observed in both black and red Holstein population. Significant effects of different loci on sperm concentration were given as means and standard error (table 10). In locus CD9, bulls with homozygote CC had higher sperm concentration than homozygote TT and heterozygote TC. In locus N-PAC, bulls with homozygote CC had higher sperm concentration than heterozygote CT. In locus CDH1, bulls with genotypes CC had higher sperm concentration than heterozygote CG. In locus PKP1, bulls with genotype AA and GA had higher sperm concentration than homozygote GG. Due to no observation of one genotype in some genes (CD9, AKR1B1, N-PAC, CDH1 and TJP1) in one or both population; we could not estimate LSM for this genotype in these populations.

Table 7: Estimates of least square means for the loci CD9, PLCζ, COX-2, AKR1B1, N-PAC, DSC2, CDH1, TJP1 and PKP1 genotype effects on ejaculation volume (ml) in black and red Holstein populations

Locus	Genotype	LSM ± SE	
		Black Holstein	Red Holstein
CD9	TT	6.1 ± 0.25	5.5 ± 0.24
	TC	5.4 ± 0.67	5.7 ± 0.37
	CC	-	6.6 ± 1.23
PLCζ	GG	5.7 ± 0.87	5.9 ± 0.54
	GA	6.1 ± 0.35	5.8 ± 0.27
	AA	5.9 ± 0.31	5.2 ± 0.33
COX-2	TT	6.0 ± 0.25	5.3 ± 0.26
	TC	5.9 ± 0.62	5.9 ± 0.38
	CC	6.1 ± 1.07	6.2 ± 0.56
AKR1B1	AA	6.2 ± 0.28	5.6 ± 0.26
	AG	5.5 ± 0.38	5.6 ± 0.32
N-PAC	CC	5.7 ± 0.26	5.4 ± 0.27
	CT	6.1 ± 0.50	5.5 ± 0.46
	TT	-	6.7 ± 1.49
DCS2	GG	5.6 ± 0.54	3.8 ± 0.55 [b]
	GA	5.9 ± 0.32	5.4 ± 0.36 [a]
	AA	5.8 ± 0.41	5.9 ± 0.29 [a]
CDH1	CC	5.9 ± 0.25	5.4 ± 0.24
	CG	5.5 ± 0.71	6.1 ± 0.72
TJP1	TT	5.7 ± 0.95	4.9 ± 0.37 [b]
	CT	5.7 ± 0.38	5.9 ± 0.23 [a]
	CC	6.2 ± 0.29	-
PKP1	GG	6.2 ± 0.60	5.6 ± 0.52
	GA	6.2 ± 0.29	5.6 ± 0.29
	AA	5.6 ± 0.42	5.6 ± 0.32

Table 8: Estimates of least square means for the loci CD9, PLCζ, COX-2, AKR1B1, N-PAC, SC2, CDH1, TJP1 and PKP1 genotype effects on sperm survivability (%) in black Holstein population

Locus	Genotype	LSM ± SE	
		Black Holstein	Red Holstein
CD9	TT	49.1 ± 0.61	48.9 ± 0.52
	TC	49.6 ± 1.03	48.9 ± 0.74
	CC	-	50.5 ± 1.22
PLCζ	GG	48.2 ± 1.24	49.6 ± 0.95
	GA	48.9 ± 0.70	48.7 ± 0.53
	AA	49.2 ± 0.65	49.6 ± 0.66
COX-2	TT	49.0 ± 0.61	48.8 ± 0.54
	TC	49.5 ± 0.69	49.2 ± 0.79
	CC	48.8 ± 1.54	50.0 ± 1.03
AKR1B1	AA	49.4 ± 0.62 [a]	49.5 ± 0.50
	AG	48.2 ± 0.71 [b]	48.5 ± 0.61
N-PAC	CC	49.3 ± 0.62	49.1 ± 0.42
	CT	49.4 ± 0.80	49.1 ± 0.71
	TT	-	51.4 ± 1.19
DCS2	GG	49.4 ± 0.88	48.4 ± 0.82
	GA	49.3 ± 0.66	49.4 ± 0.60
	AA	49.3 ± 0.75	49.3 ± 0.52
CDH1	CC	49.6 ± 0.61 [a]	49.2 ± 0.40
	CG	47.4 ± 0.99 [b]	49.3 ± 1.02
TJP1	TT	47.5 ± 1.27	48.7 ± 0.68
	CT	48.5 ± 0.72	49.3 ± 0.50
	CC	49.5 ± 0.65	-
PKP1	GG	48.6 ± 0.94	49.9 ± 0.81
	GA	49.5 ± 0.65	49.4 ± 0.56
	AA	48.5 ±0.74	48.5 ± 0.58

Table 9: Estimates of least square means for the loci CD9, PLCζ, COX-2, AKR1B1, N-PAC, DSC2, CDH1, TJP1 and PKP1 genotype effects on sperm motility (%) in black Holstein population

Locus	Genotype	LSM ± SE	
		Black Holstein	Red Holstein
CD9	TT	68.9 ± 2.27 [a]	75.1 ± 4.26
	TC	56.7 ± 6.48 [b]	69.6 ± 6.37
	CC	-	57.4 ± 8.15
PLCζ	GG	68.0 ± 3.49	69.2 ± 10.01
	GA	68.5 ± 3.36	66.2 ± 4.89
	AA	68.5 ± 3.03	77.1 ± 5.98
COX-2	TT	68.3 ± 2.39	73.7 ± 4.33 [ab]
	TC	62.4 ±6.04	59.2 ± 6.34 [b]
	CC	67.4 ± 10.43	79.9 ± 9.44 [a]
AKR1B1	AA	71.4 ± 2.56 [a]	75.4 ± 4.50
	AG	59.7 ± 3.54 [b]	65.3 ± 5.49
N-PAC	CC	66.0 ± 2.30	70.9 ± 4.23
	CT	72.8 ± 4.43	73.8 ± 7.44
	TT	-	68.5 ± 13.72
DCS2	GG	65.5 ± 4.69 [a]	62.7 ± 9.16
	GA	71.5 ± 2.79 [a]	71.7 ±5.96
	AA	61.6 ± 3.51 [ab]	67.9 ± 4.77
CDH1	CC	67.5 ± 2.20	71.5 ± 3.78
	CG	66.2 ± 6.39	73.9 ± 11.63
TJP1	TT	58.3 ± 3.25 [b]	67.7 ± 7.17
	CT	61.8 ± 3.60 [a]	72.5 ± 4.12
	CC	71.4 ± 2.71 [a]	-
PKP1	GG	65.9 ± 5.82	63.5 ± 9.43
	GA	69.4 ± 2.79	67.2 ± 5.28
	AA	64.3 ± 4.04	69.7 ± 5.74

Table 10: Estimates of least square means for the loci CD9, PLCζ, COX-2, AKR1B1, N-PAC, DSC2, CDH1, TJP1 and PKP1 genotype effects on sperm concentration (x 10^9/ml) in black and red Holstein populations

Locus	Genotype	LSM ± SE	
		Black Holstein	Red Holstein
CD9	TT	1.4 ± 0.04	1.3 ± 0.05 [b]
	TC	1.4 ± 0.11	1.2 ± 0.07 [b]
	CC	-	1.9 ± 0.23 [a]
PLCζ	GG	1.3 ± 0.13	1.4 ± 0.11
	GA	1.4 ± 0.06	1.2 ± 0.06
	AA	1.4 ± 0.05	1.2 ± 0.07
COX-2	TT	1.4 ± 0.04	1.3 ± 0.06
	TC	1.4 ± 0.10	1.2 ± 0.08
	CC	1.5 ± 0.16	1.4 ± 0.12
AKR1B1	AA	1.4 ± 0.05	1.3 ± 0.06
	AG	1.5 ± 0.06	1.2 ± 0.07
N-PAC	CC	1.4 ± 0.04 [a]	1.3 ± 0.05 [a]
	CT	1.3 ± 0.07 [b]	1.2 ± 0.08 [ab]
	TT	-	0.8 ± 0.24 [b]
DCS2	GG	1.4 ± 0.08	1.2 ± 0.10
	GA	1.4 ± 0.05	1.2 ± 0.07
	AA	1.4 ± 0.06	1.3 ± 0.06
CDH1	CC	1.4 ± 0.04 [a]	1.3 ± 0.04
	CG	1.2 ± 0.10 [b]	1.4 ± 0.12
TJP1	TT	1.3 ± 0.14	1.2 ± 0.01
	CT	1.5 ± 0.02	1.3 ± 0.05
	CC	1.4 ± 0.05	-
PKP1	GG	1.3 ± 0.09	1.1 ± 0.10 [b]
	GA	1.4 ± 0.05	1.3 ± 0.06 [a]
	AA	1.4 ± 0.07	1.3 ± 0.06 [a]

4.5.3 Association analysis between sperm flow cytometry parameters with genotypes

Using flow cytometry technique, four parameters of sperm were measured including: plasma membrane integrity, acrosome integrity (acrosomal matrix and acrosomal membrane) and DNA fragmentation index (DFI) for 56 bull sperm samples. Association study was performed using the procedure "GLM" considering genotype and breed. No significant effect was observed between genotypes and sperm flow cytometry parameters. The association between sperm traits and candidate genes are shown in tables 11.

Table 11: Association between genes and sperm flow cytometry parameters

	AKR1B1	CDH1	CD9	DSC2	N-PAC	PGHS2	PKP1	PLCζ	TJP1
DFI	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
PMI	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
PAS	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
PNA	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s

4.5.4 Genotype dependent development of embryo genes

From the oocyte fertilized in vitro, blastocyst stage embryos at day 7-8 were genotyped for SNP of (DSC2, PKP1 and TJP1) genes which are proven to play an important role in early embryo development. The blastocytes rates are shown in table 12.

Three candidate genes (DSC2, PKP1 and TJP1) related with embryo development were studied. The sperm selected for fertilization of oocytes, was heterozygote in all three genes. Single embryos were genotyped in blastocyst stage. The genotype and allele frequencies of each gene were calculated. The genotyping results were analysed using Chi-square test. The results show that, there is significant difference for TJP1 ($P \leq 0.05$), but for the other two genes no significant effect were observed. The Genotype, allele frequency and Chi-square test are shown in table 13.

Table 12: Embryonic development of in vitro matured and fertilized bovine oocytes

Gene	No. COCs	Cleavage day 2 p.IVF n (percent of total COCs)	Blastocyst day 7-9 p.IVF n (percent of cleaved embryos)
DSC2	700	539(77)	273(51)
CDH1	700	539(77)	273(51)
TJP1	475	355(75)	164(46)

Table 13: Frequency of genotypes and alleles in loci DSC2, CDH1 and TJP1

	No. Blastocyst	GG	GA	AA	Freq. G	Freq. A	χ^2	P-value
DSC2	223	53	105	65	0.53	0.47	0.69	0.71

	No. Blastocyst	CC	CG	GG	Freq. C	Freq. G	χ^2	P-value
CDH1	166	26	66	74	0.36	0.64	2.9	0.24

	No. Blastocyst	TT	TC	CC	Freq. T	Freq. C	χ^2	P-value
TJP1	114	27	72	15	0.55	0.45	8.77	0.01**

No significant differences in allele frequencies within population ($P > 0.05$)
** = ($P \leq 0.05$)

5 Discussion

Reproductive efficiency of dairy cattle depends on the ability of the bull fertilizing the cow. Since individual bull service many females, a deficiency in the breeding ability of one bull has a larger impact on herd productivity than fertility problems in a single female. Using a sub-fertile bull may lead to longer calving intervals, a lower number of calves produced and increased costs of wintering open females. All of these results cause serious economic loss to the cow-calf producer. Male fertility can be regarded as a result both of fertilizing ability of the sperm cells and of viability of the embryo, but under field conditions only the outcome of the inseminations can be recorded, i.e. whether or not the females have returned for repeat insemination (Stalhammar et al. 1994). Heritability for ejaculation volume, concentration and motility were 0.09, 0.16 and 0.22, respectively (Kealey et al. 2006). Correlations between breeding values of semen quality traits and routinely estimated breeding values of male fertility were low and ranged from 0.08 to 0.17 (Gredler et al. 2007). The bull fertility traits are strongly affected by environmental effects. Therefore, effects of single loci are expected to be low and require a higher number of animals to be analyzed (Long and Langley 1999). Previous studies have focused on identification and characterization of preimplantation genes especially in relation to the developmental potential. However information on the relationship between sequence variance and these genes in bull fertility traits is limited. Therefore, this study was conducted to identify single nucleotide polymorphisms in these genes and their association with semen quality, sperm flow cytometry parameters and NRR trait.

5.1 Importance of semen quality traits and sperm flow cytometry parameters on fertility

Sperm concentration, motility and normal sperm rate have usually been used as criteria for semen quality evaluation (Colenbrander et al. 1993). Analysis of semen quality in bull showed that, the ejaculation volume, sperm concentration and motility varied between (0.90-16 ml), [(125-2325)x10^6/ml] and (35-95%) respectively (Hoflack et al. 2006).

The maintenance of the sperm fertilizing potential depends on the integrity and functionality of different cellular structures; membranes exert a fundamental role in the

maintenance of the sperm fertilizing capability. Plasma membrane integrity is crucial to sperm survival inside the female reproductive tract, and in maintaining fertilizing capability (Celeghini et al. 2007).

The acrosome is filled with hydrolytic enzymes which are necessary for penetration sperm to zona pellucida. The acrosome must remain intact before and during the transit of the sperm to the isthmus until zona binding has been accomplished. Early acrosome reactions render sperm infertile (Silva and Gadella 2006), and therefore, acrosomal integrity is a very important functional characteristic which permits evaluation of sperm fertilizing potential (Nikolaeva et al. 1998, Silva and Gadella 2006). In a regression analysis, it was revealed that sperm motility was positively correlated with sperm membrane integrity ($P = 0.1016$) and sperm acrosome integrity ($P = 0.0019$) (Hua Y 2006).

The integrity of the sperm DNA is of crucial importance for the accurate transmission of genetic information and further development of an embryo (Agarwal and Said 2003, D'Occhio et al. 2007). The degree of DNA damage is clearly correlated with the impairment of embryo development and severe DNA damage cause male infertility (Agarwal and Said 2003, Silva and Gadella 2006). The multiple regression studies show that parameters (percentage of intact acrosomes and fragmented DNA) present a good predictive value for the sperm fertility ($r = 0.34$, $p < 0.001$) (García-Macías et al. 2007).

5.2 Detection of single nucleotide polymorphism in CD9, PLCζ, COX-2, AKR1B1, N-PAC, DSC2, CDH1, TJP1, PKP1, GJA1, and EEF1α genes

The polymorphism within a gene can successfully be applied to elaborate on the candidate gene genetically affecting phenotypic variation (Montaldo and Meza-Herrera 1998). Single nucleotide polymorphism within the CD9, PLCζ, COX-2, AKR1B1, N-PAC, DSC2, CDH1, TJP1 and PKP1 genes were detected using genomic DNA by comparative sequencing of eleven breeds (Gelbvieh, Blond d'Aquitaine, Salers, Limousin, Vorderwälder, Hinterwalder, Charolais, Red Angus, Piemontese, Pinzgauer, and Galloway). The polymorphic site of CD9 was detected in the exon 9. In PLCζ one SNP in intron 6 was found. For COX-2, we found two SNP in intron 5 and 7. In AKR1B1 one SNP was found in intron 7. Screening of N-PAC revealed one SNP in intron 13. In locus DSC2, seven SNPs in introns 7, 9 and 12 were found. For CDH1

gene one SNP in exon 8 and two SNP in intron 7 were found. In Locus TJP1, we found one SNP in exon 13. Relating locus PKP1, one SNP was found in intron 9. Finally in screening SNP regarding loci EEF1α1 and GJA1, no SNP could be found. Therefore, these genes seem to be conserved. All the SNP's which we found in exon part, were synonymous SNP, so there were no change in amino acid. These SNP may affect the relevant proteins expression level (Geldermann 1996). In the present study, since the most detected SNPs are located in the intronic region, they could not be the causative polymorphism. The association of such SNPs with observed traits may be explained by the influence of intron on mRNA metabolism including initial transcription, editing and polyadenylation of the pre-mRNA, translation and decay of the mRNA product (Le Hir et al. 2003). Moreover, there is increasing number of reports regarding the role of introns in regulating the expression level of a gene or tissue specific expression pattern (Greenwood and Kelsoe 2003, Jiang et al. 2000, Pagani and Baralle 2004, Van Laere et al. 2003, Virts and Raschke 2001). There is no information available on the SNP or association of this gene with the semen quality and sperm flow cytomerty parameters.

5.3 Hardy-Weinberg equilibrium

Hardy-Weinberg equilibrium is explained as a population with constant allele and genotype frequencies if the population satisfies the following conditions: panmixia and gametes encounter each other randomly; "infinite" population (very large: to minimize differences due to sampling); there must be no selection, mutation, migration (no allele loss/gain); successive generations are discrete (no crosses between different generations) (Falconer and Mackay 1996). Selection and random genetic drift cause allele frequency to change. In this study, candidate gene loci PLCζ, COX-2, N-PAC and DSC2 in both tested bull populations as well as loci CD9 and TJP1 in red and black Holstein population respectively were in Hardy-Weinberg equilibrium. With in loci AKR1B1 and CDH1, due to freedom degree of genotypes, we could not calculate Hardy-Weinberg equilibrium. With in locus PKP1 regardless of loci in Hardy-Weinberg equilibrium present in this study, selection in locus PKP1 might have occurred for a particular trait changing the allele frequency. In absence of selection, the amount of drift depends on allele frequencies and effective population size. Following drift beginning,

an allele frequency will most probably continue changing in the same direction (Falconer and Mackay 1996).

In the past, due to the breeding goals, bulls for milk production trait were selected with low emphasis on fertility and we know these traits have negative correlation with fertility traits (Mackey et al. 2007, Windig et al. 2006). Even so, the selection might lead to changes of allele frequencies of this locus leading to significant expected deviation of the observed genotype frequencies. The deviation from Hardy-Weinberg equilibrium should appear due to inbreeding or hybridization. For a locus, the selection affects normally the deviation from Hardy-Weinberg equilibrium in any population.

5.4 Association analysis in genes related preimplantation development

During fertilization, the motility and surface hyaluronidase are necessary for penetration of sperm to the cumulus cells (Primakoff and Myles 2002). CD9 plays an important and critical role in gamete membrane interactions (Kaji et al. 2000), A study conducted on CD9 null mice, showed their complete sterility due to a deficiency in egg-sperm fusion (Xiang and MacLaren 2002). Studies in mouse and rat testis cells show that, CD9 is commonly expressed on spermatogonial stem cells and membrane of oocytes (Kanatsu-Shinohara et al. 2004, Li et al. 2004). CD9 was significantly increased during the final oocyte maturation, indicating that it is associated with the competence of the oocyte to be fertilized (Li et al. 2004). Another studies show that, CD9 gene which expressed in blastocysts and endometrium epithelial cells in man and in bovines (Le Naour et al. 2000, Xiang and MacLaren 2002), play a role in inhibiting embryo implantation (Liu et al. 2006). This is in agreement with the results that, showing the up-regulation of CD9 in biopsies derived from blastocysts resulting in no pregnancy (El-Sayed et al. 2006). CD9 might be involved in embryo invasive behaviors (Liu et al. 2006). In this study SNP was found in exon 9. There is no change in amino acid sequence. A significant association of this SNP with sperm motility as well as sperm concentration traits has been proved. For sperm concentration animals with genotype CC ($1.9x10^9$/ml) had better performance compare to genotypes TT ($1.3x10^9$/ml) and TC ($1.2x10^9$/ml), but for sperm motility animals with genotype TT (68.9%) had higher motility compare to TC (56.7%). There is no association between this locus and volume of ejaculation, sperm survivability, sperm flow cytometry parameters and NRR.

During fertilization, the sperm not only provides one half of the genomes to the egg but also awakes the egg that is arrested at a certain stage of meiotic cell division. The release from the meiotic arrest is referred to as “egg activation”. PLCζ expressed in mammalian sperm, and possessing uniquely all the essential properties of the sperm factor (Saunders et al. 2002). PLCζ triggers a Ca^{2+} oscillation which is necessary to egg activation and embryonic development (Swann et al. 2001). In this study PLCζ locus screened for SNP and one SNP was found in intron 6. However, no association was found between this SNP and semen quality, sperm flow cytometry parameters and NRR.

Prostaglandin G/H synthesis (COX-1 and COX-2) is the key enzyme in converting arachidonic acid to prostaglandins (PGE_2 and $PGF_{2\alpha}$) (Chakraborty et al. 1996). Prostaglandins are important regulators of sperm motility (Karahan et al. 2006). In man, it has been shown that infertility is often associated with low PGE but high $PGF_2\alpha$ concentrations in seminal plasma (Janett et al. 2005). COX-2 deficient ($PGF_2\alpha$ deficient) have multiple reproductive failures that include defects in ovulation, fertilization, and implantation (Carson et al. 2000). Higher COX-2 mRNA expression was detected in biopsies derived from blastocysts resulting in successful pregnancy and calf delivery. In that study COX-2 gene was found to be 9.6 times more abundant in biopsies resulted in calf delivery compared to those from blastocysts resulted in resorption (El-Sayed et al. 2006). In this study, we found two SNPs in intron 5 and 7. The SNP found in intron 5 has a significant association with sperm motility trait; the animals with genotype CC had higher motility compared to TT and TC (79.9%, 73.9% and 59.2% respectively), but with sperm flow cytometry parameters and NRR no association was found. Study indicate that a SNP in the protamine 1 gene associated with human male infertility (Iguchi et al. 2006).

AKR1B1 is functionally related to the ACTH-responsive gene (Lefrancois-Martinez et al. 2004). Adrenal steroid hormone secretion is regulated by ACTH. ACTH may increase secretion of glucocorticoids, mineralocorticoids, and sex steroids by the adrenal gland. Aldose reductase is an enzyme in carbohydrate metabolism that converts glucose to sorbitol. Sorbitol is used to synthesis fructose (Chung and LaMendola 1989) which will be used by the sperm cells. Also the enzyme of this gene is known to have

another activities namely; metabolising progesterone, which is found to be important to implantation (Jurisicova and Acton 2004) and synthesizing PGF2α and subsequently terminating pregnancy. The microarray study in our house showed that AKR1B1 up-regulated in both biopsies derived from blastocysts resulting in no pregnancy and resorption.

Glucose metabolism has been shown to play an important role in embryo development (Urner and Sakkas 1996). While glucose is not crucial for early preimplantation stages, it becomes the predominant energy substrate after the 8-cell stage in the mouse (Gardner and Leese 1986). Glucose is the major energy substrate utilized by mouse sperm (Hoppe 1976) and that it is necessary to achieve optimum capacitation (Fraser and Herod 1990) and to maintain hyper activated motility (Cooper 1984) and fertilization (Williams and Ford 2001). Glucose plays a crucial metabolic role during gamete fusion. Its metabolism may be needed in a localized region of the male gametes during sperm-oolemma fusion (Urner and Sakkas 1996). The energy for sperm motility and movement characteristics necessary for fertilization is produced by mitochondria in the mid-piece of the spermatozoon (Mahadevan et al. 1997). Mitochondria generate ATP and cAMP (Halangk et al. 1985). In this study one SNP was found in intron 7. The SNP was proved to have a significant association with sperm survivability and motility traits. For sperm survivability and motility animals with genotype AA had higher performance compare to genotype AG (49.4% vs. 48.2% and 71.4% vs. 59.7% respectively). However there is no association between this locus and volume of ejaculation, sperm concentration, sperm flow cytometry parameters and NRR. With considering function of AKR1B1, may be we can say this SNP have the negetive effect on sperm motility and survivability and finally in fertilization.

N-PAC has pentose phosphate pathway activity. It is important for the conversion of hexoses to pentose (Robert et al. 2003). It generates both NADPH and ribose-5-phosphate, which is necessary for the ATP, coenzyme A, NAD^+, FAD^+, RNA, and DNA (O'Fallon and Wright 1986). The NADPH is essential in steroidogenesis for converting progesterone to 17-B-OH-progesterone and the conversion of androstenedione which can be used for producing testosterone and estrogen (De Kretser 2007). It plays a very important role in the development of an embryo. The transcript of N-PAC was detected almost throughout the preimplantation period with maximum level

at 8-cell stage. It has higher relative abundance at 2-cell and 8-cell stages and transcript level was down regulated at 4-cell stage and after 8-cell development stage (El-Halawany et al. 2005). In this study one SNP was detected in the intron 13. We found significant association between this SNP and sperm concentration trait. Animals with genotype CC had higher sperm concentration compare to CT and TT (1.3, 1.2 and $0.8x10^9$ /ml respectively). No association between other semen quality traits, sperm flow cytometry parameters, NRR and SNP was found. Considering metabolic function of the N-PAC, we could perhaps mention that mutation in this gene, would lead to an decrease in ATP and NADPH which are important for steroidogenesis and spermiogenesis (De Kretser 2007). This is the first report for association study of these candidate genes with semen quality traits, sperm flow cytometry parameters and NRR.

5.5 Association analysis for cell to cell adhesion and compaction genes

Desmosomes mediate direct cell-cell contacts and provide anchorage sites for intermediate filaments important for tissue integrity in epithelia, maintenance of tissue architecture and cardiac muscle (Syed et al. 2002). Desmosomes play an important role during murine development (Fleming et al. 1991). In bovine blastocysts, it has significantly higher expression in in vivo-derived embryos compared to in vitro (Knijn et al. 2002, Wrenzycki et al. 2001). It proved that, mutation in intron 5 as a cause of arrhythmogenic right ventricular cardiomyopathy (ARVC) (Heuser et al. 2006). In this study seven SNPs in introns 7, 9 and 12 were found. Significant association was found between SNP, ejaculation volume and motility; for ejaculation volume animals with genotypes AA and GA had beter performance compare to genotype GG (5.9, 5.4 and 3.8 ml respectively) and for sperm motility animals with genotypes GG and GA (65.5% and 71.5%) had higher motility than genotype AA (61.6%). For other traits and sperm flow cytometry parameters and NRR no association were found. Study on succinate dehydrogenase subunits and citrate synthesis genes in human showed one mutation has significant association with impairment of sperm production (Bonache et al. 2007). Other study show that the SNPs in the 5'-flanking region of porcine HSP70.2 are associated with semen quality traits in the hot season (Huang et al. 2002).

Cadherins are calcium-dependent transmembrane glycoproteins. CDH1 plays an important role in mechanisms regulating cell-cell adhesions, mobility and proliferation

of epithelial cells (Lu et al. 2002, Wheelock and Johnson 2003). Cadherin expression patterns are critical to the preservation of tissue integrity and developing embryo, to forming cell to cell junctions, promoting cell sorting and cell-signaling events that regulate normal development (Mak et al. 2000, Wheelock and Johnson 2003). onset of CDH1 expression is essential for preimplantation embryos compaction (Bloor et al. 2004). In bovine blastocysts, CDH1 gene has significantly higher expression (2-fold) in in vivo-derived embryos compared to in vitro (Knijn et al. 2002, Wrenzycki et al. 2001). Defects in CDH1 are involved in dysfunction of the cell-cell adhesion system, triggering cancer invasion (prostate, gastric, stomach, breast, ovary, endometrium and thyroid) and metastasis (Fitzgerald and Caldas 2004, Kamoto et al. 2005, Lei et al. 2002, Lei 2006). Loss of CDH1 leads to embryonic lethality (Larue et al. 1994). CDH1 null embryos fail to form an intact trophectoderm cell layer (De Vries et al. 2004). Our study revealed three SNPs, one in exon 8 and two in intron 7. The SNP in exon part and there is no change in amino acid. In this study, a meaningful association of CDH1 with survivability and concentration traits was proved. The animals with genotype CC had higher performance than animal with genotype CG in these traits; for survivability (49.6% vs. 47.4%) and for sperm concentration (1.4 vs. $1.2x10^9$/ml). No association between this locus, volume of ejaculation, motility, sperm flow cytometry parameters and NRR was found.

TJP1 gene involves in compaction and cavitation of embryo in preimplantation development. Tight junction transcripts dramatically increase during the transition from morulae to blastocyst (Miller et al. 2003). It is evident that the tight junction mRNA level was 1.5-3 fold (P=0.05) higher in in vivo generated morulae and blastocysts than in short-compaction in vitro ones (Miller et al. 2003). In the present study one SNP in exon 13 was found; which does not lead to any change in amino acid sequence. A considerable effect of TJP1 on volume of ejaculation and sperm motility were revealed. Animals with genotype CT had higher ejaculation volume than animal with genotype TT (5.9 vs. $4.9x10^9$/ml) and for motility animals with genotype CC and CT had better performance than animals with genotype TT (71.4%, 61.8% and 58.3%). No association was found between this locus with survivability, concentration, sperm flow cytometry parameters and NRR trait. Study in human, showed that, mutations of the androgen receptor, and Y-micro deletions cause defective sperm production and male infertility

(Yong et al. 2000a). Result of another study showed that, SNPs of the gonadotrophin-regulated testicular helicase (GRTH) gene may be associated with the human spermatogenesis impairment (Zhoucun et al. 2006).

Plakophilin 1 belongs to the armadillo gene family which play important roles in cell adhesion and in signal transduction mechanisms involved in regulating development (Hobmayer et al. 1996). Studies in bovine blastocysts showed that, PKP1 gene has significantly higher relative expression (2-fold) in in vivo-derived embryos compared to in vitro (Knijn et al. 2002, Wrenzycki et al. 2001). Loss of PKP1 expression results in epidermal fragility/ ectodermal dysplasia syndrome, which is characterized by a reduction in the number and size of desmosomes in the epithelia of affected individuals (James 2005, McGrath et al. 1997, McMillan et al. 1998, Wessagowit and McGrath 2005). In human, mutation in the plakophilin 1 gene result in ectodermal dysplasia/skin fragility syndrome (McGrath et al. 1997, Thornhill et al. 2000). In this study we identified one SNP in the intron 9. Association studies revealed significant association between SNP and sperm concentration trait. Animals with genotypes AA and GA had higher sperm concentration compared to animal with genotype GG (1.3 vs. 1.1x 10^9/ml). However there was no association between this locus with volume of ejaculation, survivability, motility, sperm flow cytometry parameters and NRR. The study in human shows that mutations in androgen receptor gene are associated with reduced its function which lead to abnormally low sperm production and male infertility (Yong et al. 2000b). So far, no association study of these genes with semen quality and sperm flow cytometry parameters were reported elsewhere.

5.6 Association study of DSC2, PKP1 and TJP1 genes and embryo development

These candidate genes related with embryo development were studied. All have main role in cell adhesion and embryo compaction and have important function in preimplantation embryo development (Bloor et al. 2004, Mak et al. 2000, Miller et al. 2003, South et al. 2003). The sperm selected for fertilization of oocytes, was heterozygots in all three genes. The embryos were selected from early cleaved embryos and they were cultured up to blastocyst stage. In blastocyst stage each single embryo was genotyped. The genotyping results of TJP1 show that, from 114 blastocyst samples,

the blastocysts with genotype TC had higher frequency comparing other genotypes (0.63 vs. 0.24 and 0.13 respectively for TC, TT and CC). There is significant difference between different genotype in TJP1 ($P \leq 0.05$), but regarding the other two genes no significant effect were observed. Gene expression pattern dependent genotypes are needed to make clear picture for effect of this SNP on preimplantation development.

5.7 The lack of effects

The reproduction traits are quantitative traits. Quantitative traits are regulated by many genes and affected by interactions among them, and thus, a candidate gene associated with a trait in one population may have a different effect, or show no effect at all, in another population due to negative effects of other genes and epistatic interactions of the candidate gene with other genes in the population (Pomp 1997). This theory is supported by many associated studies, in which a polymorphism was significantly associated with performance traits in one family or breed (Casas et al. 1998, Feng et al. 1997, Knorr et al. 1997). Also one SNP may be has different effect in different sex. For example, Hanp1 encodes a histone H1-like protein. Homozygous Hanp1 mutant male mice are infertile, while females are fertile. Hanp1 protein is essential for nuclear formation in functional spermatozoa, and is specifically involved in the replacement of histones with protamines during spermiogenesis (Martianov et al. 2005, Tanaka et al. 2005).

The lack of candidate genes effect could be due to several reasons. First of all, the genes do not affect bull fertility. It is also possible, that the genes do have effect on the traits of interest, but the study failed to elucidate them in both or one of the two groups of bulls. This may be due to the fact that the genes' effects are too small to be detected with the given number and structure of animals.

Reproductive traits are complex traits and an interaction of many genes (epistasis) is responsible for these traits (Geldermann 1996), therefore the effect of the single gene locus can be hidden and not reveal significant association because it may have a very small effect on the phenotype. Background effects of other genes and their interactions with the markers may contribute to estimate the amount of gene effects which in turn would aid to distinguish one bull populations from the other.

Little is known about the magnitude of epistatic variation in the traits studied here. But if epistasis exists, the effect of a particular allele depends on its frequency and the frequency of alleles at other loci. Due to epistasis presence, polymorphisms in a gene could have a small effect in one population, but it may explain a significant portion of the variance across bull populations (Linville et al. 2001).

The other possible reason for the lack of effect in one population, by genes that had positive effects on bull reproductive traits in the other population, is that different linkage disequilibrium existed in these populations. The polymorphism in the studied genes may indirectly affect these traits. These polymorphisms could be markers linked with the causative mutation within the genes or a closely linked gene. Different linkage relationships may be the reason estimates of the effects of genes differ across bull populations (Drogemuller et al. 2001).

Finally, the bull sperms used in this study, have high breeding values including good reproductive performance. However the presence of these animals in the analyses does not cover the negative spectrum of trait inheritance.

5.8 Future prospects

The identification of genes controlling economically important traits provides the basis for new progresses in genetic improvement of livestock species, complementing traditional methods based only on measured performance. Identification of these genes, whether they are major genes or quantitative trait loci (QTL) are an important objective to be pursued; these genes are affecting variability in traits to be improved. Increasing our knowledge will lead to identification of those genes and genetic markers associated with the fertility and preimplantation development traits that subsequently can be incorporated in selection programs to enhance the genetic improvement of breeding stocks through marker-assisted selection.

For understanding more about sperm motility and its function in fertilization and embryo development, study of mitochondrial DNA (mtDNA) will be useful, because mtDNA associated with the process of oxidative phosphorylation , the cells most important ATP generating pathway (Justin et al. 2005).

Besides identification of SNP in our studied genes, gene expression patterns dependent genotypes by accurately quantifying in preimplantation development of embryos help to understanding the molecular mechanisms controlling early embryonic development.

6. Summary

Successful fertilization is dependent on oocyte developmental competence and the ability of the sperm to fuse. Bovine preimplantation embryo development is under constant control of genes activated from either maternal or embryonic genome. Previous studies have focused on identification and characterization of developmentally relevant genes. However information on the relationship between sequence variance of these genes and fertility traits is limited. Therefore, this study was conducted to screen single nucleotide polymorphisms in E-cadherin (CDH1), desmocollin2 (DSC2), tight junction protein 1 (TJP1), plakophilin1 (PKP1), prostaglandin G/H synthase-2 (COX-2), CD9, gap junction protein, alpha 1 (GJA1), phospholipase c zeta (PLCζ), aldo-keto reductase family 1, member b1 (AKR1B1), cytokine-like nuclear factor (N-PAC) and elongation factor 1 alpha (EEF1α) genes and identify their association with the male fertility trait Non Return Rate (NRR), semen quality traits (volume per ejaculation, concentration, motility and survivability after thawing), sperm flow cytometry parameters (plasma membrane integrity, acrosome integrity and DNA fragmentation index) and preimplantation embryo development. These genes were selected based on their biological correlations and/or physiological functions and previous microarray and gene expression analysis studies in bovine preimplantation embryo development. To full fill these objectives, first 11 different cattle breeds (Limousin, Gelbvieh, Blond d'Aquitaine, Salers, Vorderwälder, Hinterwalder, Charolais, Red Angus, Piemontese, Pinzgauer, and Galloway) were used. Blood samples from these animals were used for genomic DNA isolation and subsequent comparative sequencing was done for screening polymorphism. Identified SNPs were confirmed by PCR-RFLP/SSCP methods. Genomic DNA of sperm of 310 German Holstein bulls (black or red) was used for genotyping for identified SNPs.

For locus CD9 one SNP at Pos. 95 (T>C) in exon 9 having no change in amino acid was found. For locus PLCζ one SNP was detected at Pos. 2749 (G>A) in intron 6. In locus COX-2 two SNPs were detected at Pos. 185 (T>C) and Pos. 6 (A>T) in intron 5 and 7 respectively. AKR1B1 gene had one SNP at Pos. 159 (A>G) in intron 7. In locus N-PAC one SNP was detected at Pos. 5 (C>T) in intron 13. For locus DSC2 seven SNPs were detected at Pos. 281 (T>G) in intron 7, Pos. 6 (A>G) and Pos. 21 (G>A) in intron 9, Pos. 206 (G>A) and Pos. 328 (T>C) and Pos. 341 (C>T) and Pos. 378 (T>C)

in intron 12. In locus CDH1 three SNPs were detected at Pos. 47 (C>T) in exon 8 without changing in amino acid, and two SNPs at Pos. 43 (G>A) and Pos. 92 (C>G) in intron 7. In locus TJP1 one SNP was detected at Pos. 50 (C>T) in intron 13 having no change in amino acid. In locus PKP1 one SNP was detected at Pos. 140 (G>A) in intron 9. All SNPs found in CD9, PLCζ, COX-2, AKR1B1, N-PAC, DSC2, TJP1 and PKP1 were confirmed by PCR-RFLP method with corresponding enzymes. The SNP's in CDH1 locus was confirmed by PCR-SSCP method.

The genotyping results show that, in locus CD9 allele T was more frequent than allele C (0.91 vs 0.09). For PLCζ gene, the frequency of allele G was lower than allele A (0.29 vs 0.71). In COX-2 allele T had higher frequency compared to allele C (0.86 vs 0.14). The allele frequency for locus AKR1B1 showed higher frequency for allele A compared to allele G (0.82 vs 0.18). For gene N-PAC, allele C was more frequent than allele T (0.88 vs 0.12). The results show allele G had lower frequency compared to allele A (0.4 vs 0.6). In CDH1 gene, allele G was rare compared to allele C (0.95 vs 0.05). In the candidate gene TJP1, allele T had higher frequency comparing allele T (0.79 vs 0.21) and in locus PKP1 allele A was higher than allele G (0.68 vs 0.32)

Association analysis revealed that loci DSC2 and TJP1 had a meaningful effect on volume of ejaculation ($P<0.05$). For locus TJP1 animal with genotype CT had higher volume per ejaculation (5.9 ml) compared animal with genotype TT (4.9 ml). Significant association was found between loci AKR1B1 and CDH1 and sperm survivability trait ($P<0.05$). For locus CDH1, animals with genotype CC had higher survivability than animal with genotype CG (49.6% vs 47.4%). Loci CD9, AKR1B1, DSC2, TJP1 and COX-2 had considerable effect on sperm motility ($P<0.05$). For locus CD9, animals with genotype TT produced semen with higher sperm motility than animals with genotype TC (68.9% vs 56.7%). Significant association were found between N-PAC, CDH1, CD9, N-PAC and PKP1 genes and sperm concentration ($P<0.05$). In N-PAC, animals with genotype CC had higher sperm concentration compare to genotype TT (1.3 vs 1.2 and 0.8x 10^9/ml).

Finally, three genes (DSC2, CDH1 and TJP1) which have important roles in cell adhesion and embryo compaction were used for association studies with preimplantation embryo development. 475 oocytes were fertilized with the heterozygote bull sperm. From these numbers of fertilized oocytes, 355/475 (75%) embryos were cleaved and finally 164/475 (35%) embryos reached to blastocyst stage. From these

blastocysts, 114 blastocysts were genotyped. Genotyping for locus TJP1 showed that 63% of the blastocysts had TC genotype while the rest of blastocysts found to be TT (24%) and CC (13%). The genotyping results show that TJP1 has a significant effect on embryo development ($P<0.05$). From these results it is possible to see that embryos with genotype TC had more ability to develop and reach to blastocysts stage.

7. Zusammenfassung

Das Entwicklungsstadium der Oozyte und der Fähigkeit des Spermiums mit dieser zu fusionieren, beeinflusst die erfolgreiche Befruchtung. Bovine preimplantative Embryonen reifen unter ständiger Kontrolle von Genen, welche sowohl durch das maternalen als auch embryonalen Genom aktiviert werden. Vorangegangene Studien legten ihre Schwerpunkte auf die Identifikation und Charakterisierung von Genen die die frühe Entwicklung beeinflussen. Jedoch sind die Erkennisse über die Sequenzvariationen in den Genen und deren Beziehung zu den Fruchtbarkeitsmerkmalen begrenzt. Die Aufgabe dieser Studie war es, SNPs (Einzelbasenaustausch) in den Genen E-Cadherin (CDH1), Desmocollin2 (DSC2), Tight Junction Protein (TJP1), Plakophilin1 (PKP1), Prostaglandin G/H Synthase-2 (Cox-2), CD9, Gap Junction Protein Alpha 1 (GJA1), Phospholipase c zeta (PLCζ), Aldo-Keto Reduktase fam. 1 mem. B1 (AKR1B1), Cytokine-like nuklear Faktor (N-PAC) und Elongation Faktor 1 Alpha (EEF1α) zu identifizieren und ihre Assoziation mit den männlichen Fruchtbarkeitsmerkmalen, Non Return Rate (NRR), Spermaqualität (Volumen je Ejakulation, Konzentration, Motilität und Überlebensfähigkeit) und die Morphologie des Spermium [Plasma Membran Integrität, Akrosomen Integrität und DNA Integrität), sowie der frühen Embryonentwicklung zu klären.

Die Gene wurden auf Grundlage ihrer biologischen Korrelationen, ihrer physiologischen Funktionen und aus Ergebnissen vorangegangen Mikroarray-Studien während der bovinen embryonalen Frühentwicklung, ausgewählt. Zur Detektion der Polymorphismen, wurden die Genotypen von 11 verschiedene Rinderrassen (Limousine, Gelbvieh, Blond d'Aquitaine, Salers, Vorderwälder, Hinterwälder, Charolais, Red Angus, Piemonteser, Pnzgauer und Galloway) analysiert. Aus Blutproben wurde genomische DNA isoliert und mit dieser eine vergleichende Sequenzierung vorgenommen. Nach der Bestätigung der SNPs wurden die Methoden RFLP und SSCP zur Genotypisierung verwendet. Dazu wurde genomische DNA aus dem Sperma von 310 Bullen der Rasse Deutsche Holstein gewonnen.

Der Vergleich der Ergebnisse aus der Sequenzierung ermöglichte es, SNPs in den Genen CD9 im Exon 9, Pos. 95 (T>C), CDH1 im Exon 8, Pos. 47 (C>T) und TJP1 im Exon 13, Pos. 50 (C>T) zu identifizieren. Diese Polymophismen hatten keinen Einfluss auf die Proteinstrukturen. Weitere SNPs konnten in den Genen PLCζ im Intron 6, Pos.

2749 (G>A), COX-2 im Intron 5, Pos. 185 (T>C) und Intron 7, Pos. 6 (A>T), AKR1B1 im Intron , Pos. 159 (A>G), N-PAC im Intron 13, Pos. 5 (C>T), DSC2 im Intron 7, Pos. 281 (T>C), im Intron 9, Pos. 6 (A>G) sowie Pos. 21 (G>A), im Intron 12, Pos. 206 (G>A), Pos. 328 (T>C), Pos. 341 (C>T) sowie Pos. 378 (T>C), CDH1 zwei SNPs im Intron 7, Pos. 43 (G>A) und Pos. 92 (C>G), und im Gen PKP1 im Intron 9, Pos. 140 (G>A) detektiert werden. Um die SNPs in den Genen CD9, PLCζ, COX-2, AKR1B1, N-PAC, DSC2, TJP1 und PKP1 nachzuweisen, wurden die Analysemethode PCR-RFLP mit den jeweils spezifischen Enzymen eingesetzt. Die Polymphismen in CDH1 wurden mit einer PCR-SSCP detektiert.

Die Assoziationsanalyse zeigte, dass sich die Gene DSC2 und TJP1 mit dem Volumen der Ejakulation ($P<0.05$) signifikant assoziieren ließen. Mit diesem Merkmal ließen sich keinen weiteren Polymorphismen assoziieren. Dabei wiesen Bullen mit einer Mutation am Genort TJP1 (Genotyp CT) ein höheres Ejakulationsvolumen auf (5.9 ml), als Bullen mit einem homozygoten Genotyp (TT) (4.9 ml). Die Überlebensfähigkeit des Spermas ($P<0.05$) konnte mit den Mutationen in den Genen AKR1B1 und CDH1 in einen signifikanten Zusammenhang gebracht werden. Die Überlebensfähigkeit der Spermien war bei einem Tier mit dem Genotyp CC 2% höher, als bei Tieren mit einem heterozygoten Genotyp CG (49.6% vs 47.4%). Die Mutationen in CD9, AKR1B1, COX2, DSC2 und TJP1 hatten einen signifikanten Einfluss ($P<0.05$) auf die Motilität der Spermien. Am Genort CD9 konnten Tiere mit einem Genotyp TT eine höhere Motilität erzielen, als mit einem Genotyp TC (68.9% vs 56.7%). Mit dem Merkmal Spermiumkonzentration ließen sich die Variationen in den Genen N-PAC, CD9, CDH1 und PKP1 signifikant assoziieren ($P<0.05$). Dabei wiesen Tiere mit einem Genotyp CC für das Gen N-PAC eine höhere Spermienkonzentration auf, als Tiere mit TT (1.3 vs 1.2 und $0.8x10^9$/ml).

Schließlich wurde für die drei Gene DSC2, CDH1 und TJP1, welche eine wichtige Rolle im Zell-Zell Kontakt des Embryos spielen, eine Assoziationsstudie mit Merkmalen der frühembryonalen in vitro Entwicklung Embryonen durchgeführt. 475 Oozyten wurden mit Spermien, die einen heterozygoten Genotyp besaßen, befruchtet. Die Embryonen wurden auf Grundlage ihrer frühen Teilungsrate 355/475 (75%) selektiert und anschließend bis zum Blastozystenstadium 164/475 (35%) kultiviert. Abschließend wurden 114 Blastozysten genotypisiert. Das Ergebnis zeigte einen signifikanten Zusammenhang zwischen TJP1 Genotypen und der embryonalen

Entwicklung. 63% der Blastozysten wiesen einen TC-Genotyp auf, während 24% homozygot TT- bzw. 13% CC-Genotyp waren. Aus diesen Ergebnissen kann geschlossen werden, dass Embryonen mit dem Genotyp TC eine verbesserte Fähigkeit zur Entwicklung und zur Erreichung des Blastozystenstadiums besitzen.

8 References

Agarwal A, Said TM (2003): Role of sperm chromatin abnormalities and DNA damage in male infertility. Hum Reprod Update 9, 331-345

Anzar M, He L, Buhr MM, Kroetsch TG, Pauls KP (2002): Sperm apoptosis in fresh and cryopreserved bull semen detected by flow cytometry and Its relationship with fertility. Biol Reprod 66, 354-360

Arosh JA, Parent J, Chapdelaine P, Sirois J, Fortier MA (2002): Expression of cyclooxygenases 1 and 2 and prostaglandin E synthase in bovine endometrial tissue during the estrous cycle. Biol Reprod. 67, 161-169

Asselin E, Drolet P, Fortier MA (1997a): Cellular mechanisms involved during oxytocin-induced prostaglandin F2{alpha} production in endometrial epithelial cells in vitro: Role of cyclooxygenase-2. Endocrinology 138, 4798-4805

Asselin E, Lacroix D, Fortier MA (1997b): IFN-[tau] increases PGE2 production and COX-2 gene expression in the bovine endometrium in vitro. Mol Cell Endocrinol. 132, 117-126

Bazer FW (2005): Faculty resume. Texas A&M University 7

Berndtson WE, Olar TT, Pickett BW (1981): Correlation between post-thaw motility and acrosomal integrity of bovine sperm. J. Dairy Sci. 64, 346-349

Berx G, Becker KF, Höfler H, van Roy F (1998): Mutations of the human E-cadherin (CDH1) gene. Hum Mutat. 12, 226-237

Bloor DJ, Metcalfe AD, Rutherford A, Brison DR, Kimber SJ (2002): Expression of cell adhesion molecules during human preimplantation embryo development. Mol Hum Reprod. 8, 237-245

Bloor DJ, Wilson Y, Kibschull M, Traub O, Leese HJ, Winterhager E, Kimber SJ (2004): Expression of connexins in human preimplantation embryos in vitro. Reprod Biol Endocrinol. 2, 25

Bonache S, Martinez J, Fernandez M, Bassas L, Larriba S (2007): Single nucleotide polymorphisms in succinate dehydrogenase subunits and citrate synthase genes: association results for impaired spermatogenesis. Int J Androl. 30, 144-152

Bornslaeger EA, Godsel LM, Corcoran CM, Park JK, Hatzfeld M, Kowalczyk AP, Green KJ (2001): Plakophilin 1 interferes with plakoglobin binding to desmoplakin, yet together with plakoglobin promotes clustering of desmosomal plaque complexes at cell-cell borders. J. Cell. Sci. 114, 727-738

Borrmann CM, Mertens C, Schmidt A, Langbein L, Kuhn C, Franke WW (2000): Molecular diversity of plaques of epithelial-adhering junctions. Ann NY Acad Sci 915, 144-150

Botstein D, White R, Skolnick M, Davis R (1980): Construction of a genetic linkage map in man using restriction fragment length polymorphisms. Am J Hum Genet. 32, 314-331

Brison DR, Schultz RM (1997): Apoptosis during mouse blastocyst formation: evidence for a role for survival factors including transforming growth factor alpha. Biol Reprod 56, 1088-1096

Britt JH (1985): Enhanced reproduction and its economic implications. J Dairy Sci. 68(6), 1585-1592

Britz-Cunningham SH, Shah MM, Zuppan CW, Fletcher WH (1995): Mutations of the connexin43 gap-junction gene in patients with heart malformations and defects of laterality. N Engl J Med. 332, 1323-1330

Carr-Schmid A, Durko N, Cavallius J, Merrick WC, Kinzy TG (1999): Mutations in a GTP-binding motif of eukaryotic elongation factor 1A reduce both translational fidelity and the requirement for nucleotide exchange. J Biol Chem, 274, 30297-30302

Carson DD, Bagchi I, Dey SK, Enders AC, Fazleabas AT, Lessey BA, Yoshinaga K (2000): Embryo implantation. Dev Biol. 223, 217-237

Casas E, Keele JW, Shackelford SD, Koohmaraie M, Sonstegard TS, Smith TP, Kappes SM, Stone RT (1998): Association of the muscle hypertrophy locus with carcass traits in beef cattle. J. Anim Sci. 76, 468-473

Celeghini ECC, de Arruda RP, de Andrade AFC, Nascimento J, Raphael CF (2007): Practical techniques for bovine sperm simultaneous fluorimetric assessment of plasma, acrosomal and mitochondrial membranes. Reprod Dom Anim 42, 479-488

Chakraborty I, Das SK, Wang J, Dey SK (1996): Developmental expression of the cyclo-oxygenase-1 and cyclo-oxygenase-2 genes in the peri-implantation mouse uterus and their differential regulation by the blastocyst and ovarian steroids. J Mol Endocrinol. 16, 107-122

Charpigny G, Reinaud P, Creminon C, Tamby JP (1999): Correlation of increased concentration of ovine endometrial cyclooxygenase 2 with the increase in PGE2 and PGD2 in the late luteal phase. J Reprod Fertil 117, 315-324

Charpigny G, Reinaud P, Tamby JP, Creminon C, Martal J, Maclouf J, Guillomot M (1997): Expression of cyclooxygenase-1 and -2 in ovine endometrium during the estrous cycle and early pregnancy. Endocrinology 138, 2163-2171

Chen EH, Olson EN (2005): Unveiling the mechanisms of cell-cell fusion. Science 308, 369-373

Chen X, Bonne S, Hatzfeld M, van Roy F, Green KJ (2002): Protein binding and functional characterization of plakophilin 2. evidence for its diverse roles in desmosomes and beta -catenin signaling. J. Biol. Chem., 277, 10512-10522

Choi HJ, Weis WI (2005): Structure of the armadillo repeat domain of plakophilin 1. J Mol Biol. 346, 367-376

Chung S, LaMendola J (1989): Cloning and sequence determination of human placental aldose reductase gene. J. Biol. Chem. 264, 14775-14777

Colenbrander B, Feitsma H, Grooten HJ (1993): Optimizing semen production for artificial insemination in swine. J. Reprod. Fertil. Suppl. 48, 207-215

Collins JE, Legan PK, Kenny TP, MacGarvie J, Holton JL, Garrod DR (1991): Cloning and sequence analysis of desmosomal glycoproteins 2 and 3 (desmocollins): cadherin-like desmosomal adhesion molecules with heterogeneous cytoplasmic domains. J Cell Biol. 113, 381-391

Collins JE, Lorimer JE, Garrod DR, Pidsley SC, Buxton RS, Fleming TP (1995): Regulation of desmocollin transcription in mouse preimplantation embryos. Development 121, 743-753

Condeelis J (1995): Elongation factor 1[alpha], translation and the cytoskeleton. Trends Biochem Sci. 20, 169-170

Cooper TG (1984): The onset and maintenance of hyperactivated motility of spermatozoa from the mouse. Gamete Res. 9, 55-74

Copp AJ (1995): Death before birth: clues from gene knockouts and mutations. Trends Genet. 11, 87-93

D'Occhio MJ, Hengstberger KJ, Johnston SD (2007): Biology of sperm chromatin structure and relationship to male fertility and embryonic survival. Anim Reprod Sci. 101, 1-17

Dahl E, Winterhager E, Reuss B, Traub O, Butterweck A, Willecke K (1996): Expression of the gap junction proteins connexin31 and connexin43 correlates with

communication compartments in extraembryonic tissues and in the gastrulating mouse embryo, respectively. J Cell Sci 109, 191-197

De Jonge C (2000): Paternal conributions to embryogenesis. Reproductive Medicine Review 8, 203-214

De Kretser DM (2007): Endocrinology of male reproductive In: American association of clinical endocrinologists

De Vries WN, Evsikov AV, Haac BE, Fancher KS, Holbrook AE, Kemler R, Solter D, Knowles BB (2004): Maternal ß-catenin and E-cadherin in mouse development. Development 131, 4435-4445

Desai NN, Goldstein J, Rowland DY, Goldfarb JM (2000): Morphological evaluation of human embryos and derivation of an embryo quality scoring system specific for day 3 embryos: a preliminary study. Hum. reprod. 15, 2190-2196

Diskin MG, Sreenan JM (1980): Fertilization and embryonic mortality rates in beef heifers after artificial insemination. J Reprod Fertil. 59, 463-468

Doebler JA, Wickersham EW, Anthony A (1981): Uterine prostaglandin F2 a content and 20a-hydroxysteroid dehydrogenase activity of individual ovarian compartments during pseudopregnancy in the rat. Biol Reprod 24, 871-878

Doualla-Bell F, Guay JM, Bourgoin S, Fortier MA (1998): Prostaglandin G/H synthase (PGHS)-2 expression in bovine myometrium: Influence of steroid hormones and PGHS inhibitors. Biol Reprod 59, 1433-1438

Drogemuller C, Hamann H, Distl O (2001): Candidate gene markers for litter size in different German pig lines. J. Anim Sci. 79, 2565-2570

Duncan JC, Fletcher WH (2002): Alpha 1 connexin (connexin43) gap junctions and activities of cAMP-dependent protein kinase and protein kinase C in developing mouse heart. Dev Dyn. 223, 96-107

Dunne LD, Diskin MG, Sreenan JM (2000): Embryo and foetal loss in beef heifers between day 14 of gestation and full term. Anim Reprod Sci. 58, 39-44

Eicker S, Fetrow J (2003): New tools for deciding when to replace used dairy cows. Proceedings from Kentucky Dairy conference, Cave City, KY. Univ. Kentucky, Lexington. 33-46

Eid LN, Lorton SP, Parrish JJ (1994): Paternal influence on S-phase in the first cell cycle of the bovine embryo. Biol Reprod 51, 1232-1237

El-Halawany N, Ponsuksili S, Wimmers K, Gilles M, Tesfaye D, Schellander K (2005): Quantitative expression analysis of blastocyst-derived gene transcripts in preimplantation developmental stages of in vitro-produced bovine embryos using real-time polymerase chain reaction technology. Reprod Fertil Dev. 16, 753-762

El-Sayed A, Hoelker M, Rings F, Salilew D, Jennen D, Tholen E, Sirard MA, Schellander K, Tesfaye D (2006): Large-scale transcriptional analysis of bovine embryo biopsies in relation to pregnancy success after transfer to recipients. Physiol. Genomics 28, 84-96

Ermentrout GB, Edelstein-Keshet L (1998): Models for the length distributions of actin filaments: II. polymerization and fragmentation by gelsolin acting together. Bull. Math. Biol 60, 477–503

Evans WH, Martin PEM (2002): Gap junctions: structure and function (Review). Mol Membr Biol. 19, 121-136

Falconer DS, Mackay TFC (1996): Introduction to quantitative genetics. Fourth edition, Published by Addison-Wesley, Longman Press, New York, USA,

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

Fatehi AN, Bevers MM, Schoevers E, Roelen BAJ, Colenbrander B, Gadella BM (2006): DNA Damage in bovine sperm does not block fertilization and early embryonic development but induces apoptosis after the first cleavages. J Androl 27, 176-188

Feng XP, Kuhnlein U, Aggrey SE, Gavora JS, Zadworny D (1997): Trait association of genetic markers in the growth hormone and the growth hormone receptor gene in a White Leghorn strain. Poult Sci 76, 1770-1775

Fitzgerald RC, Caldas C (2004): Clinical implications of E-cadherin associated hereditary diffuse gastric cancer. Gut 53, 775-778

Fleming TP, Garrod DR, Elsmore AJ (1991): Desmosome biogenesis in the mouse preimplantation embryo. Development 112, 527-539

Fleming TP, McConnell J, Johnson MH, Stevenson BR (1989): Development of tight junctions de novo in the mouse early embryo: control of assembly of the tight junction-specific protein, ZO-1. J. Cell Biol. 108, 1407-1418

Fleming TP, Sheth B, Fesenko I (2001): Cell adhesion in the preimplantation mammalian embryo and its role in trophectoderm differentiation and blastocyst morphogenesis. Front Biosci. 1, 1000-1007

Flesch FM, Gadella BM (2000): Dynamics of the mammalian sperm plasma membrane in the process of fertilization. Biochim Biophys Acta 1469, 197-235

Fraser LR, Herod JE (1990): Expression of capacitation-dependent changes in chlortetracycline fluorescence patterns in mouse spermatozoa requires a suitable glycolysable substrate. J Reprod Fertil 88, 611-621

Fries R, Durstewitz G (2001): Digital DNA signatures for animal tagging. Nat. Biotechnol. 19, 508

Galvez AS, Ulloa JA, Chiong M, Criollo A, Eisner V, Barros LF, Lavandero S (2003): Aldose reductase induced by hyperosmotic stress mediates cardiomyocyte apoptosis: differential effects of sorbitol and mannitol. J. Biol. Chem. 278, 38484-38494

García-Macías V, De Paz P, Martinez-Pastor F, Álvarez M, Gomes-Alves S, Bernardo J, Anel E, Anel L (2007): DNA fragmentation assessment by flow cytometry and Sperm-Bos-Halomax (bright-field microscopy and fluorescence microscopy) in bull sperm. Int J Androl. 30, 88-98

Gardner DK, Leese HJ (1986): Non-invasive measurement of nutrient uptake by single cultured pre-implantation mouse embryos. Hum. Reprod. 1, 25-27

Geldermann H (1996): Analyse von Genwirkungen auf Leistungsmerkmale. Dtsch Tierärztl Wschr 103, 369-448

Gillan L, Evans G, Maxwell WMC (2005): Flow cytometric evaluation of sperm parameters in relation to fertility potential. Theriogenology 63, 445-457

Graham JK (2001): Assessment of sperm quality: a flow cytometric approach. Anim Reprod Sci. 68, 239-247

Gredler B, Fuerst C, Fuerst-Waltl B, Schwarzenbacher H, Solkner J (2007): Genetic parameters for semen production traits in austrian dual-purpose Simmental bulls. Reprod Domest Anim. 42, 326-328

Greenwood T, Kelsoe JR (2003): Promoter and intronic variants affect the transcriptional regulation of the human dopamine transporter gene. Genomics 82, 511-520

Groenendaal H, Galligan DT, Mulder HA (2004): An economic spreadsheet model to determine optimal breeding and replacement decisions for dairy cattle. J. Dairy Sci. 87, 2146-2157

Grossman M, Koops WJ, Den Daas JHG (1995): Multiphasic analysis of reproductive efficiency of dairy bulls. J. Dairy Sci. 78, 2871-2876

Guzeloglu A, Subramaniam P, Michel F, Thatcher WW (2004): Interferon-{tau} induces degradation of prostaglandin H synthase-2 messenger RNA in bovine endometrial cells through a transcription-dependent mechanism. Biol Reprod. 71, 170-176

Halangk W, Bohnensack R, Frank K, Kunz W (1985): Effect of various substrates on mitochondrial and cellular energy state of intact spermatozoa. Biomed Biochim Acta. 44, 411-420

Hamilton T (2006): Beef bull fertility factsheet beef bull fertility, Order No. 89-087, Minstry of Agriculture, Food and Rural Affairs-Ontario,

Hansen PJ (2002): Embryonic mortality in cattle from the embryo's perspective. J. Anim. Sci. 80(E. Suppl. 2), E33-E44

Hatzfeld M, Haffner C, Schulze K, Vinzens U (2000): The function of plakophilin 1 in desmosome assembly and actin filament organization. J Cell Biol. 149, 209-222

Hatzfeld M, Kristjansson GI, Plessmann U, Weber K (1994): Band 6 protein, a major constituent of desmosomes from stratified epithelia, is a novel member of the armadillo multigene family. J Cell Sci 107, 2259-2270

Hayashi K (1991): PCR-SSCP: a simple and sensitive method for detection of mutations in the genomic DNA. PCR Methods Appl 1, 34-38

Heid HW, Schmidt A, Zimbelmann R, Schafer S, S W-S, Stumpp S, Keith M, Figge U, Schnolzer M, Franke W (1995): Cell type-specific desmosomal plaque proteins of the plakoglobin family: plakophilin 1 (band 6 protein). Differentiation 58, 113-131

Heuser A, Plovie ER, Ellinor PT, Grossmann KS, Shin JT, Wichter T, Basson CT, Lerman BB, Sasse-Klaassen S, Thierfelder L, MacRae CA, Gerull B (2006): Mutant

desmocollin-2 causes arrhythmogenic right ventricular cardiomyopathy. Am J Hum Genet 79, 1081-1088

Hobmayer E, Hatta M, Fischer R, Fujisawa T, Holstein TW, Sugiyama T (1996): Identification of a Hydra homologue of the [beta]-catenin/plakoglobin/armadillo gene family. Gene 172, 155-159

Hoflack G, Opsomer G, Van Soom A, Maes D, de Kruif A, Duchateau L (2006): Comparison of sperm quality of Belgian Blue and Holstein Friesian bulls. Theriogenology 66, 1834-1846

Hofmann I, Mertens C, Brettel M, Nimmrich V, Schnolzer M, Herrmann H (2000): Interaction of plakophilins with desmoplakin and intermediate filament proteins: an in vitro analysis. J Cell Sci 113, 2471-2483

Holmann FJ, Shumway CR, Blake RW, Schwart RB, Sudweeks EM (1984): Economic value of days open for Holstein cows of alternative milk yields with varying calving intervals. J. Dairy Sci. 67, 636–643

Hoppe PC (1976): Glucose requirement for mouse sperm capacitation in vitro. Biol Reprod 15, 39-45

Houghton FD (2005): Role of gap junctions during early embryo development. Reproduction 129, 129-135

Houghton FD, Barr KJ, Walter G, Gabriel H-D, Grummer R, Traub O, Leese HJ, Winterhager E, Kidder GM (2002): Functional significance of gap junctional coupling in preimplantation development1. Biol Reprod 66, 1403-1412

Hua Y QX, Chen BG, Yang JH, Wu XY, Ma L, Chen CL. (2006): Application of flow cytometry to the evaluation of semen quality. Zhonghua Nan Ke Xue. 12, 608-611

Huang SY, Chen MY, Lin EC, Tsou HL, Kuo YH, Ju CC, Lee WC (2002): Effects of single nucleotide polymorphisms in the 5'-flanking region of heat shock protein 70.2 gene on semen quality in boars. Anim Reprod Sci. 70, 99-109

Huber O (2003): Structure and function of desmosomal proteins and their role in development and disease. Cell. mol. life sci. 60, 1872-1890

Iguchi N, Yang S, Lamb DJ, Hecht NB (2006): An SNP in protamine 1: a possible genetic cause of male infertility? J Med Genet 43, 382-384

Illingworth IM, Kiszka I, Bagley S, Ireland GW, Garrod DR, Kimber SJ (2000): Desmosomes are reduced in the mouse uterine luminal epithelium during the preimplantation period of pregnancy: A mechanism for facilitation of implantation. Biol. reprod. 63, 1764-1773

Itoh M, Nagafuchi A, Yonemura S, Kitani-Yasuda T, Tsukita S, Tsukita S (1993): The 220-kD protein colocalizing with cadherins in non-epithelial cells is identical to ZO-1, a tight junction-associated protein in epithelial cells: cDNA cloning and immunoelectron microscopy. J. Cell Biol. 121, 491-502

James KW, III (2005): A role for plakophilin-1 in the initiation of desmosome assembly. Journal of Cellular Biochemistry 96, 390-403

Janett F, Aebi L, Burger D, Imboden I, Hassig M, Kindahl H, Thun R (2005): Influence of vedaprofen (Quadrisol(R)) on quality and freezability of stallion semen. Theriogenology 64, 1867-1877

Januskauskas A, Johannisson A, Rodriguez-Martinez H (2003): Subtle membrane changes in cryopreserved bull semen in relation with sperm viability, chromatin structure, and field fertility. Theriogenology 60, 743-758

Jeffreys A, Flavell R (1977): A physical map of the DNA regions flanking the rabbit ß-globin gene. Cell 12, 429-439

Jiang Z, Cote J, Kwon JM, Goate AM, Wu JY (2000): Aberrant splicing of tau pre-mRNA caused by intronic mutations associated with the inherited dementia frontotemporal dementia with parkinsonism linked to chromosome 17. Mol. Cell. Biol. 20, 4036-4048

Johnson MH, Maro B, Takeichi M (1986): The role of cell adhesion in the synchronization and orientation of polarization in 8-cell mouse blastomeres. J. embryol. exp. morphol. 93, 239-255.

Johnson MH, Ziomek CA (1981): The foundation of two distinct cell lineages within the mouse morula. Cell 24, 71-80

Jurisicova A, Acton BM (2004): Deadly decisions: the role of genes regulating programmed cell death in human preimplantation embryo development. Reproduction 128, 281-291

Justin CSJ, Roobin PJ, Christopher L, B. (2005): The impact of mitochondrial genetics on male infertility. Int J Androl. 28, 65-73

Kaji K, Kudo A (2004): The mechanism of sperm-oocyte fusion in mammals. Reproduction 127, 423-429

Kaji K, Oda S, Shikano T, Ohnuki T, Uematsu Y, Sakagami J, Tada N, Miyazaki S, Kudo A (2000): The gamete fusion process is defective in eggs of Cd9-deficient mice. Nat Genet. 24, 279-282

Kamoto T, Isogawa Y, Shimizu Y, Minamiguchi S, Kinoshita H, Kakehi Y, Mitsumori K, Yamamoto S, Habuchi T, Kato T, Ogawa O (2005): Association of a genetic polymorphism of the E-cadherin gene with prostate cancer in a Japanese population. Jpn. J. Clin. Oncol. 35, 158-161

Kanatsu-Shinohara M, Toyokuni S, Shinohara T (2004): CD9 is a surface marker on mouse and rat male germline stem cells. Biol Reprod 70, 70-75

Kanka J (2003): Gene expression and chromatin structure in the pre-implantation embryo. Theriogenology 59, 3-19

Kanzler B, Haas-Assenbaum A, Haas I, Morawiec L, Huber E, Boehm T (2003): Morpholino oligonucleotide-triggered knockdown reveals a role for maternal E-cadherin during early mouse development. Mech. dev. 120, 1423-1432

Karahan I, Türk G, Gür S (2006): In vitro effects of prostaglandin F2a and metamizol on the motility of diluted bull semen. Turk J Vet Anim Sci 30, 271-278

Kealey CG, MacNeil MD, Tess MW, Geary TW, Bellows RA (2006): Genetic parameter estimates for scrotal circumference and semen characteristics of Line 1 Hereford bulls. J. Anim Sci. 84, 283-290

Khurana NK, Niemann H (2000): Energy metabolism in preimplantation bovine embryos derived in vitro or in vivo. Biol Reprod 62, 847-856

Kim HC, Wheeler JMD, Kim JC, Ilyas M, Beck NE, Kim BS, Park KC, Bodmer WF (2000): The E-cadherin gene (CDH1) variants T340A and L599V in gastric and colorectal cancer patients in Korea. Gut 47, 262-267

Knijn HM, Wrenzycki C, Hendriksen PJ, Vos PL, Herrmann D, van der Weijden GC, Niemann H, Dieleman SJ (2002): Effects of oocyte maturation regimen on the relative abundance of gene transcripts in bovine blastocysts derived in vitro or in vivo. Reproduction 124, 365-375

Knorr C, Moser G, Muller E, Geldermann H (1997): Associations of GH gene variants with performance traits in F2 generations of European wild boar, Pietrain and Meishan pigs. Anim Genet. 28, 124-128

Ko MSH (2004): Embryogenomics of pre-implantation mammalian development: current status. Reprod Fertil Dev. 16, 79-85

Kojima T, Oshima K, Watanabe H, Komatsu M (2002): Rapid communication: nucleotide sequence of the bovine elongation factor 1 alpha cDNA1. J Anim Sci. 80, 1696-1697

Koops WJ, Grossman M, Den Daas JHG (1995): A model for reproductive efficiency of dairy bulls. J. Dairy Sci. 78, 921-928

Kowalczyk A, Bornslaeger E, Norvell S, Palka H, Green K (1999a): Desmosomes: intercellular adhesive junctions specialized for attachment of intermediate filaments. Int Rev Cytol. 185, 237-302

Kowalczyk AP, Hatzfeld M, Bornslaeger EA, Kopp DS, Borgwardt JE, Corcoran CM, Settler A, Green KJ (1999b): The head domain of plakophilin-1 binds to desmoplakin and enhances its recruitment to desmosomes. Implications for cutaneous disease. J Biol Chem, 274, 18145-18148

Krawczak M (1999): Informativity assessment for biallelic single nucleotide polymorphisms. Electrophoresis 20, 1676-1681

Kruglyak L (1997): The use of a genetic map of biallelic markers in linkage studies. Nat Genet 17, 21-24

Kwok PY (2000): High-throughput genotyping assay approches Pharmacogenomics 1, 95-100

Kwok PY (2001): Methods for genotyping single nucleotide polymorphisms. Annu. Rev. Genomics Hum. Genet. 2, 235-258

Larue L, Ohsugi M, Hirchenhain J, Kemler R (1994): E-Cadherin null mutant embryos fail to form a trophectoderm epithelium. PNAS 91, 8263-8267

Lash JA, Critser ES, Pressler ML (1990): Cloning of a gap junctional protein from vascular smooth muscle and expression in two-cell mouse embryos. J Biol Chem, 265, 13113-13117

Le Hir H, Nott A, Moore M (2003): How introns influence and enhance eukaryotic gene expression. Trends Biochem Sci. 28, 215-220

Le Naour F, Rubinstein E, Jasmin C, Prenant M, Boucheix C (2000): Severely reduced female fertility in CD9-deficient mice. Science 287, 319-321

Lee S, Gilula NB, Warner AE (1987): Gap junctional communication and compaction during preimplantation stages of mouse development. Cell 51, 851-860

Lefrancois-Martinez AM, Bertherat J, Val P, Tournaire C, Gallo-Payet N, Hyndman D, Veyssiere G, Bertagna X, Jean C, Martinez A (2004): Decreased expression of cyclic adenosine monophosphate-regulated aldose reductase (AKR1B1) is associated with malignancy in human sporadic adrenocortical tumors. J Clin Endocrinol Metab 89, 3010-3019

Legan PK, Yue KK, Chidgey MA, Holton JL, Wilkinson RW, Garrod DR (1994): The bovine desmocollin family: a new gene and expression patterns reflecting epithelial cell proliferation and differentiation. J. Cell Biol. 126, 507-518

Lei H, Sjöberg-Margolin S, Salahshor S, Werelius B, Jandáková E, Hemminki K, Lindblom A, Vorcaronechovský I (2002): CDH1 mutations are present in both ductal and lobular breast cancer, but promoter allelic variants show no detectable breast cancer risk. Int J Cancer. 98, 199-204

Lei HH (2006): CDH1 gene mutation for stomach cancer in the Bradfield family.

Li YH, Hou Y, Ma W, Yuan JX, Zhang D, Sun QY, Wang WH (2004): Localization of CD9 in pig oocytes and its effects on sperm-egg interaction. Reproduction 127, 151-157

Lim H, Paria BC, Das SK, Dinchuk JE, Langenbach R, Trzaskos JM, Dey SK (1997): Multiple female reproductive failures in cyclooxygenase 2-deficient mice. Cell 91, 197-208

Lin C (2005): Candidate gene analysis for loci affecting sperm quality and fertility of boar. Dissertation, Bonn University

Linville RC, Pomp D, Johnson RK, Rothschild MF (2001): Candidate gene analysis for loci affecting litter size and ovulation rate in swine. J. Anim Sci. 79, 60-67

Liu G, Grant WM, Persky D, Jr VML, Singer RH, Condeelis J (2002): Interactions of elongation factor 1 alpha with F-actin and beta-actin mRNA: Implications for anchoring mRNA in cell protrusions. Mol. Biol. Cell 13, 579-592

Liu G, Tang J, Edmonds BT, Murray J, Levin S, Condeelis J (1996): F-actin sequesters elongation factor 1alpha from interaction with aminoacyl-tRNA in a pH-dependent reaction. J Cell Biol. 135, 953-963

Liu J, Antaya M, Goff AK, Boerboom D, Silversides DW, Lussier JG, Sirois J (2001): Molecular characterization of bovine prostaglandin G/H synthase-2 and regulation in uterine stromal cells. Biol Reprod 64, 983-991

Liu J, Sirois J (1998): Follicle size-dependent induction of prostaglandin G/H synthase-2 during superovulation in cattle Biol Reprod 58, 1527-1532

Liu WM, Cao YJ, Yang YJ, Li J, Hu Z, Duan EK (2006): Tetraspanin CD9 regulates invasion during mouse embryo implantation. J Mol Endocrinol 36, 121-130

Long AD, Langley CH (1999): The power of association studies to detect the contribution of candidate genetic loci to variation in complex traits. Genome Res. 9, 720-731

Lu DP, Tian L, O' Neill C, King NJ (2002): Regulation of cellular adhesion molecule expression in murine oocytes, peri-implantation and post-implantation embryos. Cell Res. 12, 373-383

Mackey DR, Gordon AW, McCoy MA, Verner M, Mayne CS (2007): Associations between genetic merit for milk production and animal parameters and the fertility performance of dairy cows. Animal 1, 29-43

Madore E, Harvey N, Parent J, Chapdelaine P, Arosh JA, Fortier MA (2003): An aldose reductase with 20 alpha-hydroxysteroid dehydrogenase activity is most likely the enzyme responsible for the production of prostaglandin F2 alpha in the bovine endometrium. J Biol Chem, 278, 11205-11212

Maekawa M, Yamamoto T, Tanoue T, Yuasa Y, Chisaka O, Nishida E (2005): Requirement of the MAP kinase signaling pathways for mouse preimplantation development. Development 132, 1773-1783

Mahadevan MM, Miller MM, Moutos DM (1997): Absence of glucose decreases human fertilization and sperm movement characteristics in vitro. Hum. Reprod. 12, 119-123

Mak A, Blaschuk OW, Jensen GS (2000): The physiological roles of cadherin mediated cell adhesion. Immunology 77, 112-117

Mamo S (2004): Molecular genetics analysis of in vitro produced preimplantation stage bovine embryos for developmental competence. Dissertation, Bonn University

Marions L, Danielsson KG (1999): Expression of cyclo-oxygenase in human endometrium during the implantation period. Mol Hum Reprod. 5, 961-965

Marti JI, Cebrian-Perez JA, Muino-Blanco T (2000): Assessment of the acrosomal status of ram spermatozoa by RCA lectin-binding and partition in an aqueous two-phase system. J Androl 21, 541-548

Martianov I, Brancorsini S, Catena R, Gansmuller A, Kotaja N, Parvinen M, Sassone-Corsi P, Davidson I (2005): Polar nuclear localization of H1T2, a histone H1 variant, required for spermatid elongation and DNA condensation during spermiogenesis. Proc Natl Acad Sci U S A. 102, 2808-2813

McGrath JA, McMillan JR, Shemanko CS, Runswick SK, Leigh IM, Lane EB, Garrod DR, Eady RAJ (1997): Mutations in the plakophilin 1 gene result in ectodermal dysplasia/skin fragility syndrome. Nat Genet 17, 240-244

McMillan JR, McGrath JA, M H, Garrod DR, Hoeger PH, Harper JI, Lake BD, Eady RAJ (1998): Role of plakophilin 1 in epithelial development and desmosome function J Dermat. Sci. 16, 66

Meadows C, Rajala-Schultz PJ, Frazer GS (2005): A spreadsheet-based model demonstrating the nonuniform economic effects of varying reproductive performance in Ohio dairy herds. J. Dairy Sci. 88, 1244-1254

Memili E, Dominko T, First NL (1998): Onset of transcription in bovine oocytes and preimplantation embryos. Mol Reprod Dev. 51, 36-41

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

Miller DJ, Eckert JJ, Lazzari G, Duranthon-Richoux V, Sreenan J, Morris D, Galli C, Renard JP, Fleming TP (2003): Tight junction messenger RNA expression levels in bovine embryos are dependent upon the ability to compact and in vitro culture methods. Biol Reprod 68, 1394-1402

Miyado K, Yamada G, Yamada S, Hasuwa H, Nakamura Y, Ryu F, Suzuki K, Kosai K, Inoue K, Ogura A, Okabe M, Mekada E (2000): Requirement of CD9 on the egg plasma membrane for fertilization. Science 287, 321-324

Miyazaki S, Ito M (2006): Calcium signals for egg activation in mammals. J Pharmacol Sci 100, 545-552

Montaldo HH, Meza-Herrera CA (1998): Use of molecular markers and major genes in the genetic improvement of livestock EJB Electronic Journal of Biotechnology 1, 83-89

Moon HM, Redfield B, Weissbach H (1972): Interaction of eukaryote elongation factor EF 1 with guanosine nucleotides and aminoacyl-tRNA. PNAS 69, 1249-1252

Moore RC, Cyr RJ (2000): Association between elongation factor-1a and microtubules in vivo is domain dependent and conditional. Cell Motil. Cytoskeleton 45, 279-292

Morris DG, Grealy M, Leese HJ, Diskin MG, Sreenan JM (2001): Cattle embryo growth, development and viability.

Murray JW, Edmonds BT, Liu G, Condeelis J (1996): Bundling of actin filaments by elongation factor 1 alpha inhibits polymerization at filament ends. J. Cell Biol. 135, 1309-1321

Nataraj A, Olivos-Glander I, Kusukawa N, Highsmith WJ (1999): Single-strand conformation polymorphism and heteroduplex analysis for gel-based mutation detection. Electrophoresis 20, 1177-1185

Negrutskii BS, El'skaya AV (1998): Eukaryotic translation elongation factor 1 alpha: structure, expression, functions, and possible role in aminoacyl-tRNA channeling. Prog Nucleic Acid Res Mol Biol. 60, 47-78

Nikas G, Ao A, Winston RM, Handyside AH (1996): Compaction and surface polarity in the human embryo in vitro. Biol Reprod. 55, 32-37

Nikolaeva MA, Golubeva EL, Kulakov VI, Sukhikh GT (1998): Evaluation of stimulus-induced acrosome reaction by two-colour flow cytometric analysis. Mol. Hum. Reprod. 4, 243-250

Nomikos M, Blayney LM, Larman MG, Campbell K, Rossbach A, Saunders CM, Swann K, Lai FA (2005): Role of phospholipase C-{zeta} domains in Ca^{2+}-dependent phosphatidylinositol 4,5-bisphosphate hydrolysis and cytoplasmic Ca^{2+} oscillations. J Biol Chem. 280, 31011-31018

O'Fallon JV, Wright RW, Jr (1986): Quantitative determination of the pentose phosphate pathway in preimplantation mouse embryos. Biol Reprod. 34, 58-64

Orita M, Iwahana H, Kanazawa H, Hayashi K, Sekiya T (1989): Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphisms. PNAS 86, 2766-2770

Pagani F, Baralle F (2004): Genomic variants in exons and introns: identifying the splicing spoilers. Nat. rev., Genet. 5, 389-396

Parrish JJ, Susko-Parrish J, Winer MA, First NL (1988): Capacitation of bovine sperm by heparin. Biol Reprod 38, 1171-1180

Pena FJ, Johannisson A, Wallgren M, Rodriguez-Martinez H (2003): Assessment of fresh and frozen-thawed boar semen using an Annexin-V assay: a new method of evaluating sperm membrane integrity. Theriogenology 60, 677-689

Pey R, Vial C, Schatten G, Hafner M (1998): Increase of intracellular Ca2+ and relocation of E-cadherin during experimental decompaction of mouse embryos. PNAS 95, 12977-12982

Plaizier JCB, King GJ, Dekkers JCM, Lissemore K (1997): Estimation of economic values of indices for reproductive performance in dairy herds using computer simulation. J. Dairy. Sci. 80, 2775-2783

Pomp D (1997): Genetic dissection of obesity in polygenic animal models. Behav Genet. 27, 285-306

Primakoff P, Myles DG (2002): Penetration, adhesion, and fusion in mammalian sperm-egg interaction. Science 296, 2183-2185

Purves D, Augustine GJ, Fitzpatrick D, Katz LC, LaMantia JO, McNamara JO, Williams SM (2001): Neuroscience. Sunderland, MA: Sinauer Assoc, p. 681

Reed VS, Wastney ME, Yang DC (1994): Mechanisms of the transfer of aminoacyl-tRNA from aminoacyl-tRNA synthetase to the elongation factor 1 alpha. J. Biol. Chem. 269, 32932-32936

Reurink A, den Daas JHG, Wilmink JBM (1990): Effects of AI sires and technicians on non-return rates in The Netherlands. Livest. Prod. Sci. 26, 107-118

Reuss B, Hellmann P, Traub O, Butterweck A, Winterhager E (1997): Expression of connexin31 and connexin43 genes in early rat embryos. Dev Genet. 21, 82-90

Rickman L, Simrak D, Stevens HP, Hunt DM, King IA, Bryant SP, Eady RA, Leigh IM, Arnemann J, Magee AI, Kelsell DP, Buxton RS (1999): N-terminal deletion in a desmosomal cadherin causes the autosomal dominant skin disease striate palmoplantar keratoderma. Hum. Mol. Genet. 8, 971-976

Riethmacher D, Brinkmann V, Birchmeier C (1995): A targeted mutation in the mouse E-cadherin gene results in defective preimplantation development. PNAS 92, 855-859

Robert M, Peter AM, Victor WR, Daryl KG (2003): Harper's Biochemistry. 26th, McGraw-Hill Companies 688

Roberts RM, Leaman DW, Cross JC (1992): Role of interferons in maternal recognition of pregnancy in ruminants. Proc Soc Exp Biol Med 200, 7-18

Rosenkrans CFJ, First NL (1994): Effect of free amino acids and vitamins on cleavage and developmental rate of bovine zygotes in vitro. J. Anim Sci. 72, 434-437

Rozen S, Skaletsky H (2000): Primer3 on the WWW for general users and for biologist programmers. Methods Mol Biol. 132, 365-386

Sandbaken MG, Culbertson MR (1988): Mutations in elongation factor EF-1{alpha} affect the frequency of frameshifting and amino acid misincorporation in Saccharomyces cerevisiae. Genetics 120, 923-934

Sanderson MJ, Charles AC, Boitano S, Dirksen ER (1994): Mechanisms and function of intercellular calcium signaling. Mol Cell Endocrinol. 98, 173-187

Saunders CM, Larman MG, Parrington J, Cox LJ, Royse J, Blayney LM, Swann K, Lai FA (2002): PLC zeta: a sperm-specific trigger of Ca2+ oscillations in eggs and embryo development. Development 129, 3533-3544

Schafer S, Troyanovsky SM, Heid HW, Eshkind LG, Koch PJ, Franke WW (1993): Cytoskeletal architecture and epithelial differentiation: molecular determinants of cell interaction and cytoskeletal filament anchorage. Acad. Sci. Paris, Ser. IlL 316, 1316-1323

Scherle PA, Ma Wg, Lim H, Key SK, Trzaskos JM (2000): Regulation of cyclooxygenase-2 induction in the mouse uterus during decidualization-an event of early pregnancy. J. Biol. Chem 275, 37086-37092

Schmidt A, Jager S (2005): Plakophilins--hard work in the desmosome, recreation in the nucleus? Eur. j. cell biol. 84, 189-204

Schmidt A, Langbein L, Rode M, Prätzel S, Zimbelmann R, Franke WW (1997): Plakophilins 1a and 1b: widespread nuclear proteins recruited in specific epithelial cells as desmosomal plaque components. Cell Tissue Res. 290, 481-499

Schultz RM, Davis Jr W, Stein P, Svoboda P (1999): Reprogramming of gene expression during preimplantation development. J Exp Zool. 285, 276-282

Schwarz J, Ayim A, Schmidt A, Jager S, Koch S, Baumann R, Dunne AA, Moll R (2006): Differential expression of desmosomal plakophilins in various types of carcinomas: correlation with cell type and differentiation. Hum Pathol. 37, 613-622

Segretaina D, Falk MM (2004): Regulation of connexin biosynthesis, assembly, gap junction formation, and removal. Biochim. biophys. acta, Biomembr. 1662, , 3 – 21

Shah VO, Dorin RI, Sun Y, Braun M, Zager PG (1997): Aldose reductase gene expression is increased in diabetic nephropathy. J Clin Endocrinol Metab 82, 2294-2298

Sheth B, Moran B, Anderson JM, Fleming TP (2000): Post-translational control of occludin membrane assembly in mouse trophectoderm: a mechanism to regulate timing of tight junction biogenesis and blastocyst formation. Development 127, 831-840

Silva PFN, Gadella BM (2006): Detection of damage in mammalian sperm cells. Theriogenology 65, 958-978

Smith EA, Fuchs E (1998): Defining the interactions between intermediate filaments and desmosomes. J Cell Biol. 141, 1229-1241

Smith WL, DeWitt DL, Garavito RM (2000): Cyclooxygenases: structural, cellular, and molecular biology. Annu Rev Biochem. 69, 145-182

Smith WL, Garavito RM, DeWitt DL (1996): Prostaglandin endoperoxide H synthases (cyclooxygenases)-1 and -2. J. Biol. Chem. 271, 33157-33160

South AP (2004): Plakophilin 1: an important stabilizer of desmosomes. Clin Exp Dermatol, 29, 161-167

South AP, Wan H, Stone MG, Dopping-Hepenstal PJC, Purkis PE, Marshall JF, Leigh IM, Eady RAJ, Hart IR, McGrath JA (2003): Lack of plakophilin 1 increases keratinocyte migration and reduces desmosome stability. J Cell Sci 116, 3303-3314

Southern E (1975): Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol 98, 503-517

Stalhammar EM, Janson L, Philipsson J (1994): Genetic studies on fertility in AI bulls. II. Environmental and genetic effects on non-return rates of young bulls. Anim. Reprod. Sci. 34, 193-207

Sutherland AE, Calarco-Gillam PG (1983): Analysis of compaction in the preimplantation mouse embryo. Dev Biol. 100, 328-338

Swann K, Larman MG, Saunders CM, Lai FA (2004): The cytosolic sperm factor that triggers Ca2+ oscillations and egg activation in mammals is a novel phospholipase C: PLC{zeta}. Reproduction 127, 431-439

Swann K, Parrington J, Jones KT (2001): Potential role of a sperm-derived phospholipase C in triggering the egg-activating Ca2+ signal at fertilization. Reproduction 122, 839-846

Syed SEH, Trinnaman B, Martin S, Major S, Hutchinson J, Magee AI (2002): Molecular interactions between desmosomal cadherins. Biochemical Journal 362, 317-327

Talapatra S, Wagner JDO, Thompson CB (2002): Elongation factor-1 alpha is a selective regulator of growth factor withdrawal and ER stress-induced apoptosis. Cell Death Differ. 9, 856-861

Tanaka H, Iguchi N, Isotani A, Kitamura K, Toyama Y, Matsuoka Y, Onishi M, Masai K, Maekawa M, Toshimori K, Okabe M, Nishimune Y (2005): HANP1/H1T2, a novel histone H1-like protein involved in nuclear formation and sperm fertility. Mol. Cell. Biol. 25, 7107-7119

Telford NA, Watson AJ, Schultz GA (1990): Transition from maternal to embryonic control in early mammalian development: a comparison of several species. Mol Reprod Dev. 26, 90-100.

Tesfaye D (2003): Identification and quantitative expression analysis of differentially expressed gene transcripts in preimplantation developmental stages of bovine embryos produced in vitro or in vivo. Dissertation, Bonn University

Tesfaye D, Ponsuksili S, Wimmers K, Gilles M, Schellander K (2004): A comparative expression analysis of gene transcripts in post-fertilization developmental stages of bovine embryos produced in vitro or in vivo. Reprod Domest Anim. 39, 396-404

Thornhill AR, Pickering SJ, Whittock NV, Caller J, Andritsos V, Bickerstaff HE, Handyside AH, Eady RAJ, Braude PR, McGrath JA (2000): Preimplantation genetic diagnosis of compound heterozygous mutations leading to ablation of plakophilin-1 (PKP1) and resulting in skin fragility ectodermal dysplasia syndrome: a case report. Prenat Diagn. 20, 1055-1062

Thurmond MC, Picanso JP (1990): A surveillance system for bovine abortion. Prev Vet Med. 9, 41-53

Tsujii M, DuBois RN (1995): Alterations in cellular adhesion and apoptosis in epithelial cells overexpressing prostaglandin endoperoxide synthase 2. Cell 83, 493-501

Uetsuki T, Naito A, Nagata S, Kaziro Y (1989): Isolation and characterization of the human chromosomal gene for polypeptide chain elongation factor-1 alpha. J Biol Chem. 264, 5791-5798

Urner F, Sakkas D (1996): Glucose participates in sperm-oocyte fusion in the mouse. Biol Reprod 55, 917-922

Van Laere AS, Nguyen M, Braunschweig M, Nezer C, Collette C, Moreau L, Archibald AL, Haley CS, Buys N, Tally M, Andersson G, Georges M, Andersson L (2003): A regulatory mutation in IGF2 causes a major QTL effect on muscle growth in the pig. Nature 425, 832-836

Virts EL, Raschke WC (2001): The role of intron sequences in high level expression from CD45 cDNA constructs. J. Biol. Chem. 276, 19913-19920

Wang DG, Fan JB, Siao CJ, Berno A, Young P, Sapolsky R, Ghandour G, Perkins N, Winchester E, Spencer J, Kruglyak L, Stein L, Hsie L, Topaloglou T, Hubbell E, Robinson E, Mittmann M, Morris MS, Shen N, Kilburn D, Rioux J, Nusbaum C, Rozen

S, Hudson TJ, Lipshutz R, Chee M, Lander ES (1998): Large-scale identification, mapping, and genotyping of single-nucleotide polymorphisms in the human genome. Science 280, 1077-1082

Wang QT, Piotrowska K, Ciemerych MA, Milenkovic L, Scott MP, Davis RW, Zernicka-Goetz M (2004): A genome-wide study of gene activity reveals developmental signaling pathways in the preimplantation mouse embryo. Dev Cell. 6, 133-144

Watson AJ, Barcroft LC (2001): Regulation of blastocyst formation. Front Biosci. 6, D708-730

Weigel KA (2004): Improving the reproductive efficiency of dairy cattle through genetic selection. J. Dairy Sci. 87, E86-92

Wessagowit V, McGrath J (2005): Clinical and molecular significance of splice site mutations in the plakophilin 1 gene in patients with ectodermal dysplasia-skin fragility syndrome. Acta Derm Venereol 85, 386-388

Wheelock MJ, Johnson KR (2003): Cadherins as modulators of cellular phenotype. Annu Rev Cell Dev Biol. 19, 207-235

Wiemer KE, Garrisi J, Steuerwald N, Alikani M, Reing AM, Ferrara TA, Noyes N, Cohen J (1996): Infertility: Beneficial aspects of co-culture with assisted hatching when applied to multiple-failure in-vitro fertilization patients. Hum. Reprod. 11, 2429-2433

Williams AC, Ford WC (2001): The role of glucose in supporting motility and capacitation in human spermatozoa. J Androl 22, 680-695

Wimmers K, Ponsuksili S, Hardge T, Schellander K (2002): Detection of candidate gene for functional traits in pigs, Proceeding of Genomics and biothechnology in livestock breeding Shaker Verlage Achen, Germany, P69-77

Windig JJ, Calus MPL, Beerda B, Veerkamp RF (2006): Genetic correlations between milk production and health and fertility depending on herd environment. J. Dairy Sci. 89, 1765-1775

Wirtu G, Pope CE, Damiani P, Miller F, Dresser BL, Short CR, Godke RA, Bavister BD (2004): Development of in-vitro-derived bovine embryos in protein-free media: effects of amino acids, glucose, pyruvate, lactate, phosphate and osmotic pressure. Reprod Fertil Dev. 15, 439-449

Wrenzycki C, Herrmann D, Carnwath JW, Niemann H (1999): Alterations in the relative abundance of gene transcripts in preimplantation bovine embryos cultured in medium supplemented with either serum or PVA. Mol Reprod Dev. 53, 8-18

Wrenzycki C, Herrmann D, Carnwath JW, Niemann H (1996): Expression of the gap junction gene connexin43 (Cx43) in preimplantation bovine embryos derived in vitro or in vivo. J. reprod. fertil. 108, 17-24

Wrenzycki C, Herrmann D, Keskintepe L, Martins A, Jr., Sirisathien S, Brackett B, Niemann H (2001): Effects of culture system and protein supplementation on mRNA expression in pre-implantation bovine embryos. Hum. Reprod. 16, 893-901

Xiang W, MacLaren LA (2002): Expression of fertilin and CD9 in bovine trophoblast and endometrium during iImplantation. Biol Reprod 66, 1790-1796

Yong E, Lim J, Wang Q, Mifsud A, Ong Y, Sim C (2000a): Genetics of male infertility: role of androgen receptor mutations and Y-microdeletions. Ann Acad Med Singapore. 29, 396-400

Yong E, Lim L, Wang Q, Mifsud A, Lim J, Ong Y, Sim K (2000b): Androgen receptor polymorphisms and mutations in male infertility. J Endocrinol Invest. 23, 573-577

Zhoucun A, Sizhong Z, Yuan Y, Yiongxin M, Li L, Wei Z (2006): Single nucleotide polymorphisms of the gonadotrophin-regulated testicular helicase (GRTH) gene may be associated with the human spermatogenesis impairment. Hum. Reprod. 21, 755-759

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