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Morphological reflectors and molecular predictors of preimplantation developmental competence in bovine oocytes and embryos

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Meinen Eltern
und meiner Oma

Cover Illustration *Reprod Fertil Dev. 2012;24(4)*: Comparative visualization of the same matured bovine oocyte taken by light microscope (left side) and live zona imaging reflecting zona pellucida birefringence using polarized light microscope investigation (right side). Photography by Eva Held, Franca Rings and Michael Hoelker

Morphological reflectors and molecular predictors of preimplantation developmental competence in bovine oocytes and embryos

The efficiency of in vitro production (IVP) of bovine embryos still remains low, reaching blastocyst rates between 25 and 35 %. Accordingly, the necessity to define reliable predictors for developmental competence is indispensible. Up to now most morphological and molecular markers are subjective and partly invasive and thus relatively inaccurately. Therefore, the aim of the first experiment of the present approach was to evaluate the effect of structural changes in zona pellucidas' (ZP) morphology during in maturation on developmental competence using polarized light microscopy. Structural changes in terms of number and size of pores were displayed by the Scanning electron microscopy and determined quantitatively in zona pellucida birefringence (ZPB). Immature oocytes classified as high presumble quality oocytes exhibited highest values for birefringence, and in vivo matured oocytes showed the lowest values for birefringence compared to their in vitro matured counterparts. These results implicate, that decreasing values for ZPB during maturation are typical for high developmental competence. In the second study of the present thesis, the transcriptome profile of one blastomere of a bovine 2-cell stage embryo was directly correlated with the developmental potential of the corresponding sister blastomere. Analyses using microarray technology revealed specific molecular fingerprints for blastomeres whose sister blastomere developed to the blastocyst stage in contrast to those blastomeres whose sister blastomeres stopped cleaving after separation or were blocked before embryonic genome activation (EGA). Pathway analyses uncovered, that differentially regulated genes (DEG) were mostly involved in oxidative phosphorilation, oxidative stress response and antioxidant activity. Five candidate genes, namely, *NDUFS1, MAPK14, CAT, PRDX1* and *PRDX6,* which are known to act as either direct or indirect scavengers of reactive oxygen species (ROS), were selected and their expression levels were further characterized using two independent models for developmental competence. Furthermore, ROS staining revealed high ROS accumulations in late cleaved or rather developmentally incompetent 2-cell stage embryos, compared to low ROS levels in early cleaved and therefore presumable highly competent 2-cell stage embryos. Taken together, noninvasive morphological criteria predicting subsequent developmental competence of matured bovine oocytes were identified in this work. Moreover, a direct correlation between molecular mechanisms and the individual developmental competence, which was found to be already determined in the 2-cell stage, could be detected.

Untersuchungen boviner Eizellen und Embryonen auf morphologischer und molekularer Ebene zur Identifizierung von entwicklungsrelevanten Markern

In Anbetracht der relativ geringen Effizienz der in vitro Produktion (IVP) boviner Embryonen mit Blastozystenraten von 25 bis 35 %, ist es unverzichtbar, zuverlässige Prädiktoren für das Entwicklungspotential von Eizellen und Embryonen zu definieren. Bis heute sind die meisten morphologischen und molekularen Qualitätsparameter subjektiv und von invasiver Natur. Daher war es das Ziel in der ersten Studie der vorliegenden Arbeit, strukturelle Veränderungen in der Zona pellucida (ZP) während der in vitro Maturation (IVM) mit Hilfe der Polarisationslichtmikroskopie quantitativ zu erfassen und mit dem Entwicklungspotential zu korrelieren. Strukturelle Änderungen bezogen auf Anzahl und Größe der Poren in der ZP zeigten sich bei der Analyse mittels eines Rasterelektronenmikroskops und spiegelten sich in der Doppellichtbrechungsintensität der Zona pellucida (ZPB) quantitativ wieder. Immature Eizellen, die als qualitativ sehr gut eingestuft wurden, zeigten die höchsten Werte für die ZPB, wohingegen die in vivo maturierten Eizellen im Vergleich zu den in vitro maturierten die niedrigsten Werte aufwiesen. Diese Ergebnisse implizieren, dass abnehmende Werte für die ZPB während der Maturation als Pärdiktor für hohe Eizellqualität genutzt werden können. Zusammenfassend konnte gezeigt werden, dass die Polarisationslichtmikroskopie als nicht invasisver Prädiktor für das Entwicklungspotential genutzt werden kann. In der zweiten Studie der vorliegenden Arbeit wurde eine direkte Korrelation zwischen dem Transkriptionsprofil einer Blastomere eines bovinen Zweizellers zum Entwicklungspotential der korrespondierenden Schwesterblastomere hergestellt. Beim Vergleich mittels Mikroarraytechnologie wurden spezifische molekulare Fingerabdrücke für Blastomeren ermittelt, deren Schwesterblastomeren sich zur Blastozyste entwickelten im Gegensatz zu denen, deren Schwesterblastomeren sich nicht weiter teilten oder vor der embryonalen Genomaktivierung (EGA) geblockt wurden. Nach Analyse der durch die differentiell regulierten Transkripte betroffenen Stoffwechselwege zeigte sich, dass diese Gene hauptsächlich in oxidative Phosphorilierung, oxidative Stressantwort und antioxidative Aktivität involviert waren. Die Expremierung der daraus selektierten Kandidatengene *NDUFS1, MAPK14, CAT, PRDX1* und *PRDX6*, deren direkte und indirekte Funktionen bei der Eliminierung von freien Radikalen bekannt ist, wurde an Hand von zwei unabhängigen Kompetenzmodellen weiter charakterisiert. Des Weiteren zeigte Färbung, dass spät geteilte Zweizeller mit einem geringeren Entwicklungspotential sehr hohe Anreicherungen an freien Radikalen aufwiesen im Gegensatz zu früh geteilten Zweizellern mit einem hohen Entwicklungspotential, die nur niedrige Anreicherungen zeigten. Schlussfolgernd konnte gezeigt werden, dass spezifische molekulare Mechanismen direkt mit dem Entwicklungspotential korrelieren und es bereits im Zweizeller determinieren.

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List of abbreviations

ART	:	Assisted reproductive technology
BCB	:	Brilliant Cresyl Blue
BME	:	Basal Medium Eagle
BSA	:	Bovine serum albumine
cDNA	:	Complementary Deoxyribonucleic acid
COC	:	Cumulus oocyte complex
CR1aa	:	Charls Rosenkranz 1 amino acid
CV- Mean	:	Cumulated birefringence value
Cy3	:	Cyanine 3
Cy5	:	Cyanine 5
DEG	:	Differentially expressed genes
dNTP	:	Deoxyribonucleoside triphosphate
EGA	:	Embryonic genome activation
ET	:	Embryo transfer
FDR	:	False discovery rate
Fig	:	Figure
FSH	:	Follicle stimulating hormone
G6PD	:	Glucose 6-Phodphat dehydrogease
gDNA	:	Genomic desoxyribonucleic acid
GEO	:	Gene Expression Omnibus
GnRH	:	Gonadotropin releasing hormone
GV	:	Greminal vesicle
GVBD	:	Greminal vesicle breakdown
H_2O_2	:	hydrogen peroxid
hpi	:	hours post insemination
ICM	:	Inner cell mass
IPA	:	Ingenuity Pathway Analyses
IVC	:	In vitro culture
IVF	:	In vitro fertilization
IVM	:	In vitro marturation
IVP	:	In vitro production
LH	:	Luteinizing hormone

MEM	:	Minimum Essential Medium
MII	:	Metaphase II
MPM	:	Modified Parker Media
mRNA	:	messanger ribonucleic acid
mtDNA	:	mitochondrial Desoxyribonucleic acid
NTC	:	No template control
OCS	:	Oestrus cow serum
OPU	:	Ovum pick up
OXPHOS	:	Oxidatve phosphorilation
PCR	:	Polymerase chain reaction
PGD	:	Preimplantation genetic diagnostics
PGF2α	:	Prostaglandin F2α
REDOX	:	Reduction- oxidation
RNA	:	Ribonucleic acid
ROS	:	Reactive oxygen species
RT-PCR	:	Reverse transcriptase polymerase chain reaction
SEM	:	Scanning electron microscop
TCM199	:	Tissue Culture Media 199
TE	:	Trophectoderm
WoW	:	Well of the Well
WT- Mean	:	average thickness of the inner zona pellucida layer
ZP	:	Zona pellucida
ZPB	:	Zona pellucida birefringence

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Chapter 1 General overview

1.1 Introduction

The majority of in vitro produced bovine embryos is used for fundamental research, to understand the whole process of preimplantation embryo development and to examine aberrant developmental processes, related to fertility problems in cattle (reviewed by Lonergan and Fair 2008). Economically considered, in vitro production (IVP) is a promising tool for bypassing infertility in bovine caused by ovulation and fertilization failure, early embryonic death and other causes (Royal et al. 2000). In comparison to in vivo produced embryos in vitro produced embryos are known to exhibit developmental retardation following maturation, fertilization and culture. (Farin et al. 2006; Greve and Callesen 2005; Gutierrez-Adan et al. 2004; Khurana and Niemann 2000; van Soom et al. 1997; Wright and Bondioli 1981). On morphological and molecular levels, several differences between in vivo and in vitro produced embryos have been described so far, which are mainly believed to be induced by maturational and culture environment, but also by breed, oocyte quality and follicular environment. (Holm et al. 1998; Lonergan et al. 2006; Lopes et al. 2007; Thompson 1997; Wrenzycki et al. 2005).

1.1.2 In vitro production and developmental competence of bovine embryos

Although, IVP is an established method for about 35 years, there are two limiting factors in the current in vitro production systems; on the one hand blastocyst rates are still restricted to 25 – 35 % and on the other hand the blastocysts are of poor quality compared to in vivo derived ones. Recently, it was described that in vitro matured and in vitro fertilized embryos, which were transferred to sheep oviducts could improve their quality in terms of cryotolerance, to a level which is similar to that of totally in vivo produced embryos. (Enright et al. 2000; Rizos et al. 2002a; Rizos et al. 2002b). Correspondingly, in vivo matured and fertilized embryos resulted in very low cryotolerance preserved at blastocyst stage, when they were transferred to an in vitro culture system at zygote stage.

In contrast, no influence on blastocyst yield was observed by in vivo culture of presumptive zygotes which were transferred to the oviduct. They resulted in similar blastocyst rates like the in vitro control group, implicating that blastocysts quality is dependent on post-fertilisation culture conditions and blastocyst rates are determined by prefertilization environment (Hendriksen et al. 2000). Therefore, the success of in vitro production depends on the starting material, whose intrinsic quality represents the

basis for the embryonic developmental competence, which seems to be fixed in the oocyte (Lonergan et al. 1999). Although developmental capacity improved during the last decades due to advanced in vitro systems, in vitro produced blastocysts differ significantly from their in vivo derived counterparts on morphological levels like colour of the blastomeres, the extent of compaction, timing of blastocyst formation and expansion and diameter of the embryo at hatching (reviewed by van Soom et al. 2003). They differ on the subcellular level including dissolution of nucleoli and mitochondrial activity (Torner et al. 2008) and on molecular levels (Knijn et al. 2005; McHughes et al. 2009). There is evidence to suggest that there should be a huge repertory of markers to select developmentally competent oocytes and embryos, in detail those vitro embryos with greatest affinity to in vivo derived embryos representing the golden standard embryos. Albeit, most available tools are restricted in their applicability; evaluations of morphological assessment depend mostly on subjective perception and molecular analyses are invasive techniques, avoiding subsequent development. Additionally, many analyses are carried out on pooled groups of oocytes and embryos, raising difficulties in interpretation on an individual basis (reviewed by Lonergan and Fair 2008). As mentioned above, the limiting factor of in vitro development could be the intrinsic quality of the matured oocyte, which is determined by the proportion of oocytes developing to the blastocyst stage (Lonergan et al. 2003). Nevertheless, factors influencing oocytes developmental competence in vivo have been described in the past, like, age of the donors (Salamone et al. 2001), parity, genetic ability for milk and body condition score (Snijders et al. 2000) as well as level of protein feeding (Sinclair et al. 2000) and the season (Al-Katanani et al. 2002).

1.1.3 Effect of maturational environment on developmental competence of bovine oocytes

Investigations during the last years, postulated that in vivo matured oocytes derived by ovum pick up (OPU) are more competent than their in vitro matured counterparts, implicating a higher intrinsic quality of in vivo matured oocytes (Merton et al. 2003). Characterization of oocytes' developmental competence is based on 5 major levels including, the ability to resume meiosis, to cleave following fertilization, to develop to blastocyst stage, to induce pregnancy, to bring it to term and to develop to term in good health (reviewed by Sirard et al. 2006).

The oocytes' quality is acquired during folliculogenesis, growth phase and finally during maturation period. In vitro maturation of immature oocytes does not fully resemble this process, resulting in reduced developmental competence (Krisher 2004).
It is well known, that a greater proportion of in vivo matured oocytes reach the blastocyst stage than those matured in vitro (Greve et al. 1987; McCaffrey et al. 1991; van Soom et al. 1992). Developmental competence is acquired through several mechanisms during folliculogenesis, which are necessary for an accurate maturation. During the acquirement of developmental capacity the oocyte passes several important processes including germinal vesicle breakdown (GVBD) and reaching metaphase I and II (Fair et al. 1995). Within maturational process, which is divided into nuclear and cytoplasmic maturation, the oocytes grow and undergo remodelling on cellular and molecular levels to comply all requirements for subsequent development (Sirard et al. 2006). In our in vitro production systems, the oocytes are collected from ovaries obtained from local slaughterhouses, implicating a wide variety of the oocytes in origin e.g. follicular wave or stage of estrus cycle. These oocytes are usually in different stages of atresia or in different phases of growth (Farin et al. 2006). Generally, the majority of oocytes would never have undergone maturation process in vivo, where the dominant follicle acquires more LH receptors than the subordinates, allowing further growth within low FSH levels and increasing LH levels. As the FSH level is indirectly reduced by the dominant follicle, it deprives essential growth factors for subordinate follicles, leading to their regression (Ginther et al. 2000; Mihm et al. 2008). Furthermore, our in vitro systems cannot fully mimic, in vivo like maturational conditions, which could harm temporal interaction processes during maturation in the cytoplasm and nucleus. De Loos et al. 1992 reported that at the cytoplasmic level some processes were found to be deficient in in vitro maturation e.g., retardation in forming of an organelle free cortex, change of association between endoplasmatic reticulum and lipid is delayed, and small clusters of cortical granules still be present. While evaluation of maturation on the cytoplasmic level still remains difficult, the score of nuclear maturation has turned out as a parameter for success of an in vitro system (de Loos et al. 1992). It was shown, that a higher proportion of oocytes extruding the first polar body between 16 and 20 hours of maturation reach the blastocyst stage than those extruding their first polar body at a later date (van der Westerlaken et al. 1994). Although 90% of in vitro matured oocytes extrude their first polarbody and accomplish

nuclear maturation, the cytoplasmic maturation is considerably affected (de Loos et al. 1992; De Loos et al. 1994; Hyttel et al. 1986), as indicated by the fact, that only one third of oocytes develop to the blastocyst stage in vitro.

1.1.4 Morphological markers for oocytes developmental competence

Several morphological and cellular markers have been introduced, that aimed to select competent and "in vivo like" oocytes. One very common criteria is morphology, with respect to the colour of the cytoplasm and the constitution of cumulus cells, which was defined by Wurth and Kruip 1992, namely I) presence of a clear and compact cumulus and translucent ooplasm, II) dark and compact cumulus and dark ooplasm, and III) dark and expanded cumulus and dark ooplasm. Indeed, the reliability of these morphological, on subjectivity based, quality assessments is complicated by the high heterogeneity of in vitro matured oocytes and in vitro produced embryos, implicating the need for markers, independent of subjective evaluation. Otoi et al. 1997 reported that the developmental competence is related to the oocytes diameter: an increasing diameter goes along with increasing blastocyst rates. Furthermore, growth status, based on Glucose- 6- phosphate dehydrogenase activity (G6PD), being high in growing and nearly absent in fully grown oocytes, has turned out to be a reliable tool for developmental competence. A higher proportion of oocytes, which completed their growth phase, developed to the blastocyst stage compared to their growing counterparts. Brilliant cresyl blue (BCB) staining is a method to visualize the growth status of an oocyte. High G6PD activity in growing oocytes, brakes down the dye and turn the blue colour into colourless (BCB-), whereas fully grown oocytes without G6PD activity stay blue (BCB+) (Pujol et al. 2004; Alm et al. 2005; Bhojwani et al. 2007). This method allows a reliable classification into competent and incompetent oocytes, not depending on subjectivity like the COCs morphology does. However, up to now, the sustainable effect on the oocytes has not been clarified.

Therefore, the introduction of polarized light microscopy in 1997 opened a new window for assessment of oocytes quality. This was the first method, which quantified the developmental competence numerical, by measuring the intensity of birefringence of the zona pellucida (Keefe et al. 1997). These measurements are based on the characteristics of the vibrating light rays created by polarized light. If these light rays pass through orderly arranged filamentous, they are retarded or double refracted,

measured as the so called birefringence intensity, which can be quantified by special software (Montag et al. 2008; Rama Raju et al. 2007). In human it has turned out to be a feasible method to predict pregnancy outcome almost certainly. Oocytes displaying higher birefringence had significantly higher developmental and pregnancy rates than oocytes with low values for birefringence (Montag et al. 2008) Contrasting, in bovine it has been shown, that the developmentally competent zygotes exhibit lower values for birefringence, than incompetent ones (Koester et al. 2011). However, the effect of maturational environment on zona pellucidas birefringence and its implications for developmental competence in the bovine has not been shown yet.

During in vivo maturation the zona pellucida penetrated by cytoplasm appendices of corona radiata cells, which are closely connected to the plasmamembrane. This leads to homogenous porous surface of the zona pellucida. In contrast, after in vitro maturation the surface was shown to be nearly poreless, assuming an insufficient contact between corona radiate cells and zona pellucida (Macchiarelli et al. 1992; Suzuki et al. 1994). Furthermore, it has been reported, that the duration of maturation has crucial impacts on the function and constitution of the zona pelludica. If maturation time is extended to 28 hours and or even longer, the polyspermy rate increases drastically, implicating the loss of zona pellucidas property to harden as thereby block polyspermy after fertilization (Chian et al. 1992). These differences in zona pellucida properties, caused by maturational environment, might be causative for aberrant subsequent in vitro development of bovine IVP embryos. Therefore in chapter 2 the effect of maturational environment on zona pelludia properties was investigated, implicating the hypothesis whether the zona pellucida reflects a suitable maturational environment and in succession can serve as a predictor for developmental competence.

1.1.5 Molecular markers for developmental competence

Beside several morphological criteria for selection of developmentally competent oocytes and embryos, there are also criteria based on the gene expression and transcriptome analyses. It is well accepted, that the ability of an oocyte to develop into a viable embryo depends not only on its morphological appearance but also on the accumulation of specific information and molecules such as RNA, protein or imprinted genes during oogenesis (reviewed by Eichenlaub-Ritter and Peschke 2002). During the last years more and more studies analyzed the molecular level, to discover genes which

are involved in early embryonic development and whose expression is indispensible for preimplantation development (Corcoran et al. 2007; Knijn et al. 2005; Sagirkaya et al. 2006; Tesfaye et al. 2003).

Three major steps within preimplantation development, namely, first cleavage division, embryonic genome activation (EGA) and blastocyst formation, requiring well-orchestrated expression of maternal or embryonic derived genes have been reported (Kidder 1992). Until embryonic genome activation, which occurs at the 8- to 16- cell stage in bovine, early development is regulated by maternal mRNA (Telford et al. 1990). During its growth phase mRNA is transcribed and stored in the oocytes, to support maturation, fertilisation and early development to the point when the genome of the embryo is activated (Donnison and Pfeffer 2004). These stored maternal mRNA sustain the initial cell cycles of the early embryonic development. Bovine embryos, treated with α-amanitin, which acts as an RNA polymerase II inhibitor, develop until the 8- cell stage, indicating, that embryonic development until EGA is mostly dependent on maternal transcripts (Memili and First 2000). Concerning the storage of RNA for further use in later stages, posttranscriptional control like poly- and deadenylation play crucial roles for the establishment of RNA machinery (Richter 1999). Polyadenylation is responsible for the elongation of poly (A) tails which are necessary to induce transcription. Deadenylated transcripts with short poly (A) tails are stabilized and stored until recruitment for translation (Brevini et al. 2007; Eichenlaub-Ritter and Peschke 2002). In fact, not a single gene was found to determine developmental competence, but an interaction between several genes involved in different pathways and biological processes like the regulation of transcription, posttranslational modification of proteins, cell cycle regulation, response to stress and growth factor and cell signalling (reviewed by Wrenzycki et al. 2005). Thus, identification of differentially regulated genes is essential to understand the critical events that occur during the period of early preimplantation development (Khurana and Niemann 2000). It has been shown during the last years that mRNA expression patterns correlate with other quality markers. For example, follicle size, stage of follicular growth and meiotic maturation, maturational environment, COCs quality and time of first cleavage are phenotypic measurable parameters of proven value for indication of developmental competence which are strongly correlated to differences in the

expression of developmentally important transcripts (reviewed by Wrenzycki et al. 2007).

1.1.6 Limiting factors for molecular analyses

Although, numerous studies deal with gene expression pattern during early preimplantation development, describing explicitly how IVP embryos differ from in vivo derived embryos, the interpretation of such data remains difficult, due to the invasive nature of gene expression analyses (reviewed by Lonergan and Fair 2008). This is the main disadvantage of the gene expression studies. The lineage between gene expression profile and given developmental capacity of the embryo to a subsequent stage is missing.

Usually, oocytes and embryos are classified based on competence models, like cumulus morphology or time of first cleavage, to detect transcripts related to developmental competence. However, these studies only display a group effect avoiding the analyses of individual embryos. For instance, early cleaving embryos are more competent to develop to the blastocyst then their later cleaving counterparts and show different expression levels of developmentally important genes than their later cleaving counterparts (Dode et al. 2006; Fair et al. 2004; Mourot et al. 2006), but not all early cleaving embryos develop to the blastocyst stage. Furthermore, it is mostly accepted that in vivo derived blastocyst, differ phenotypically and genetically from in vitro derived blastocyst (Corcoran et al. 2006; Corcoran et al. 2007; McHughes et al. 2009; Mohan et al. 2004; Tesfaye et al. 2004), but not all in vivo blastocysts induce pregnancy and result in calf delivery. Therefore, there is basic necessity to establish study designs for analyses of gene expression profiles directly related to the individual developmental competence of preimplantation embryos.

A method offering a solution to avoid measurements of group effects and allow to correlate transcriptome profiles with developmental potential directly, has been conducted in our laboratory, by analysing the gene expression of biopsies of in vitro produced blastocyst prior embryo transfer to recipients (El-Sayed et al. 2006). These analyses revealed several clusters of genes to be differentially regulated between biopsies derived from blastocysts that resulted in no pregnancy, resorption or calf delivery. Biopsies resulting in calf delivery were enriched in transcripts, necessary for growth factor, signal transduction and implantation, whereas, biopsies from embryos,

that were resorbed or resulted in no pregnancy showed a higher abundance for transcripts related to protein phosphorylation, plasma membrane and glucose metabolism, inflammatory cytokines and inhibitors of implantation. A similar experiment, using in vivo derived blastocyst instead of in vitro produced ones, was conducted in 2011 (Ghanem et al. 2011). Interestingly, a similar gene expression pattern was observed between in vivo and in vitro derived blastocyst resulting in calf delivery. Typical trends of expression for 21 genes could be related to developmental incompetence (Ghanem et al. 2011).These studies were the first, which directly correlated transcriptome profiles with pregnancy outcomes on the individual basis avoiding the group effect as a source of error. However, studies performing embryo transfer (ET) are enormously complex, requiring high technical skills and housing recipient herd. To circumvent this and to investigate molecular mechanisms involved in the early preimplantation phase related to developmental competence of individual embryos, the technique of taking embryo biopsies was transferred to the 2- cell stage.

Several studies dealing with the development of individual blastomeres of 2-cell stage embryos in different species have been conducted during the past decade. It has been shown, that both blastomeres of a 2-cell stage embryo are totipotent and develop to the blastocyst stage independently (Dang-Nguyen et al. 2011; Katayama et al. 2010; Tagawa et al. 2008). But there are contradicting opinions and results concerning the cell fate after the first cleavage division, implicating either a determination of inner cell mass (ICM) and trophectoderm (TE) or undifferentiated cells which contribute to ICM and TE at the same proportion. The group of Magdalena Zernicka-Goetz postulated that the polarity of the 2- cell embryos predicts the embryonic and abembryonic axis of the blastocyst and that the fate of blastomeres is already different (Plusa et al. 2005; Zernicka-Goetz 2011; Zernicka-Goetz 2002; Zernicka-Goetz 2004). This is in line with several observations, demonstrating, that cell-cell contact after second cleavage seems to be the key factor in determining whether a cell adopts a TE or ICM fate (Niwa et al. 2005; Piotrowska et al. 2001). Indeed, in absence of cell-cell contacts due to second cleavage we suggest that singled sister blastomeres in our study not yet established polarity and did not show lineage pre-patterning in the separated blastomeres as it has reported recently (Lorthongpanich et al. 2012). Since both separated blastomeres of a 2- cell embryo are still able to develop to term and to produce identical twins (Hancock 1954; Ozil et al. 1982; Seike et al. 1989; Tagawa et al. 2008) evidencing pluripotency,

we believe that separated sister blastomeres establish lineage pre-patterning as a consequence of polarity through subsequent cleavages one cell cycle later. Recently it was published, that both blastomeres of a murine 2-cell stage embryo are genetically identical (Roberts et al. 2011; VerMilyea et al. 2011), implicating that one blastomere is the reflection of its sister blastomere and can be used as a molecular predictor of the individual developmental competence of the corresponding sister blastomere.

1.2 Scope of the thesis

Taken together, the present work proves the hypothesis that developmental capacity is fixed at a high degree after the first cleavage division. To proof this, two different experiments were conducted on morphological as well as on molecular level.

1. Analyses of the correlation between oocytes' morphology and maturational environment as well as subsequent developmental competence.

2. Identification of typical transcriptomic fingerprints predicting individual developmental competence.

1.3 Material and methods objective 1

To achieve the objectives of this thesis several materials and methods were conducted. Detailed descriptions of material and methods as well as specifics of media composition and quantity can be found in Chapter 2. The most important techniques are described here.

1.3.1 Collection of in vivo matured oocytes by Ovum pick up (OPU)

The first ultrasound-guided transvaginal ovum pick up (OPU) in the cow was reported in 1988 (Pieterse et al. 1988) and conducted on non stimulated animals. This technique allows the recovery of in vivo matured oocytes for in vitro fertilisation and culture, to produce several embryos from one individual cow. The introduction of superstimulation prior to OPU delivered the advantages that more follicles could be aspirated and more oocytes retrieve (Pieterse et al. 1991; Stubbings et al. 1990). There are several protocols for superstimulation procedure. In the present thesis a pre-synchronization prior to superstimulation was conducted. Therefore, donor heifers were injected with Prostaglandine (PGF2α) twice within eleven days, to regress the Corpus Luteum, which induces the completion of lutealphase, implicating the beginning of a new follicular phase or oestrus cycle. After each PGF2α injection the administration of Gonadotropin Releasing Hormone (GnRH) was performed, to receive a higher homogeneity of the follicle state. This allows a more precise forecasting of oestrus on the one hand and a higher probability of synchronized LH- peak on the other hand. Twelve days after the last GnRH injection, the treatment with follicle stimulating hormone (FSH) started. All together, eight consecutive FSH injections over four days in decreasing doses were given, to induce the growth of multiple follicles. The regression of the existing Corpus Luteum is induced by two PGF2α injections. Another GnRH treatment triggers endogenous LH secretion, which in turn induces ovulation. Therefore, the OPU of MII- Oocytes has to be conducted 16 – 20 h after the last GnRH treatment.

In the present approach OPU was conducted endoscopically by U. Besenfelder, who developed this technique.

Prior to OPU the donor heifers have to be restrained and an epidural anaesthesia has to be administered. Than a tocar set consisting of a universal metal tube and an atraumatic

mandrin was placed in the caudodorsal area of fornix vaginae. After replacement of the mandrin by a sharp tocar, the tocar set was inserted through the vaginal wall into the peritoneal cavity. Finally, the sharp tocar was removed and a shaft bearing the endoscope and the punctation line, consisting of a single lumen needle connected to 50 ml Falcon tube was inserted. The whole system is connected to a vacuum pump, creating a constant flow rate of 16 to 20 ml/min. The ovary is manually placed in the right position and all follicles of each ovary aspirated by vacuum pressure, stored at body temperature and finally poured into a square grid dish from where the oocytes were taken out under a stereomicroscope. The OPU procedure has turned out be an efficient method to receive in vivo matured, high quality oocytes, which were used as a control group for high developmental competence.

1.3.2 In vitro production of bovine embryos

The in vitro production of bovine embryos is divided into three steps: in vitro maturation (IVM), in vitro fertilization (IVF) and in vitro culture (IVC).

Prior to IVM the Cumulus- Oocyte- Complexes (COCs) are recovered from ovaries obtained from the local slaughterhouse. Therefore, antral follicles (2 to 8mm in diameter) were aspirated using an 18-gauge needle attached to a 5 ml syringe. The follicular fluid containing COCs is collected in a 50ml conical tube. Under stereomicroscope the COCs are collected and transferred to modified tissue culture medium, which is amongst others supplemented with Hepes, acting as a buffer to maintain the pH value under normal CO_2 and O_2 conditions, and with gentamycin to avoid bacterial-and fungal contaminations. After three washing steps groups of 50 COCs are transferred to a four well dish containing 400µl maturation media, modified Parker Media (MPM), supplemented with oestrus cow serum as a protein source and FSH, to promote nuclear and cytoplasmic maturation *(exact composition and quantity can be found in chapter 2 and 3)*. The media is covered with mineral oil and the four well dish with embryos incubated for 22 hours at 38.7°C in 5% CO_2 in air with highest humidity.

Prior to IVF the frozen and thawed sperm has to undergo a swim-up procedure to separate the vital sperms from dead sperm and cryoprotectives. This procedure is a period of preparation that normally occurs in the female genital tract and takes place in vitro in capacitation media, which additionally induces capacitation, an initial sperm

membrane alteration by supplementation of Heparin. After 50 minutes motile sperms are taken from the supernatant and centrifuged, to collect them in a pellet. After resolving the pellet the sperm is transferred to fertilization media in which the oocytes have transferred before. Additionally to Heparin, fertilization media is supplemented with hypotaurin and epinephrin to induce the acrosome reaction, the fusion of the plasma membrane and the acrosomal membrane (*for exact media composition and quantity see material and methods chapter 2 and 3*). During fertilization oocytes and sperm are incubated at 38.7°C in 5% CO_2 in air with highest humidity. Following 18 hours of in vitro fertilization, cumulus cells of consumptive fertilized oocytes are removed by 2 minutes vortexing and groups of 50 oocytes are transferred to 400 µl culture media, namely Charles Rosenkrans (CR1aa) media, supplemented with oestrus cow serum as a protein source as well as with basal medium Eagle (BME) and minimum essential medium (MEM) for maintenance with essential and non- essential amino acids. Additionally, the amino acids are supposed to act as energy substrates, pH regulators and as pool for de novo protein synthesis (Rosekrans and First 1994). The culture media is covered with mineral oil and incubated for eight days at 38.7°C in 5% CO_2 in air with highest humidity.

1.3.3 Zona pellucida live imaging

In detail, individual measuring of immature oocytes, in vivo and in vitro matured oocytes as well as of growing (BCB-) and fully grown (BCB+) oocytes was done non-invasively with a Leica DM IRB inverted microscope, a circular polarization filter and liquid crystal analyzer optics. The birefringence analysis including autocalibration was fully controlled by a polarization imaging software module (OCTAX Polar Aide) implemented in an imaging software system (OCTAX Eyeware).

The OCTAX PolarAIDE system performs polarization microscopy imaging, which produces an image indicating the special distribution of the retardance in the zona pellucida. The birefringence image obtained is dominated by the inner zona layer, whose radial birefringence intensity is of interest for zona scoring. The image processing on the birefringence image extracted several birefringence intensity profiles ($n>20$) across the inner zona layer. For each of the profiles, i.e. along the entire zona layer, the thickness of the inner layer (WT) and a cumulated birefringence value (CV) over the inner zona layer were calculated. These values (which were computed for each

intensity profile, i.e. over the entire cell's circumference) were averaged and resulted in the values CV-Mean (mean intensity of birefringence) and WT-Mean (mean thickness of the inner layer of the zona pellucida).

1.3.4 Experimental design

Altogether, four successive experiments were conducted:

1. To evaluate COCs quality and the effect of maturational environment on zona pellucidas structure, immature oocytes were classified according to their COCs morphology into quality 1 (Q1, presence of a clear and compact cumulus cells with more than three layers), quality 2 (Q2, compact cumulus with two or three layers) and quality 3 (Q3, oocytes with one or fewer layer of cumulus) before cumulus removal as well as in vivo and vitro matured oocytes were analyses by scanning electron microscopy (SEM).
2. The effect of COCs quality and maturational environment on developmental competence after in vitro fertilization was investigated
3. Effect of COCs morphology and maturational environment on zona pellucida birefringence of immature and matured bovine oocytes.
4. Effect of initial growth status on zona pellucida birefringence.

1.4 Material and methods objective 2

To achieve the objectives of this thesis several materials and methods were conducted. Detailed descriptions of material and methods as well as specifics of media composition and quantity can be found in Chapter 3. The most important techniques are described here.

1.4.1 Bisection of 2-cell stage embryos and individual culture

After the standard in vitro maturation and fertilization of COCs derived from ovaries collected at the local slaughterhouse, in vitro culture of presumptive zygotes in groups of 50 was conducted as described for Objective 1. For culture Charles Rosenkrans (CR1aa) media was used as described above. 28 to 30 hours post insemination (hpi) 2-cell stage embryos were collected and immediately placed in phosphate buffered saline supplemented with 0,5% protease, an enzyme that catabolises proteins by hydrolysis of peptide bonds, like those which can be found in the zona pellucida of mammalian oocytes and embryos. After two minutes the zona pelludcida is removed and the zona free 2-cell stage embryos were carefully transferred to tissue culture medium (TCM199), which stops the protease reaction. Three washing steps were conducted and finally single zona free 2-cell stage embryos were transferred to one well with CR1aa and pipetted gently with a narrow glass pipette. Due to the movements the blastomeres separate from each other.

In a preliminary experiment both blastomeres were cultured in an individual culture system based on the Well of the Well (WoW) system described by (Vajta et al. 2000). WoWs are prepared manually by drilling 16 small holes into the bottom of 4 wells of a five well culture dish. The holes are cylindrical in shape (0.7mm depth and 0.7mm in diameter). The wells were washed with CR1aa and after equilibration single blastomeres were cultured individually until day 8. The aim of this experiment was to find out, whether both blastomeres of a 2-cell stage embryos have the same developmental potential and in which proportion they develop to the blastocyst stage. In the main experiment, blastomeres were separated and one blastomere was cultured individually, while the counterpart was snap frozen in liquid nitrogen. In this process the single blastomere is placed in a lockable plastic tube and carefully soaked in liquid nitrogen, which conserves the RNA from degradation. Finally tubes were stored

in -80°C until usage. On day three and day eight of culture, cleavage and blastocyst rates of the individual cultured blastomeres were counted. According to development, the frozen and stored counterparts were pooled into three groups. The first group were those whose counterparts did not cleave any further after separation (2CB), the second group were those whose counterparts stopped cleaving before embryonic genome activation (8CB) and the reference group were those blastomeres whose counterparts developed to the blastocyst stage (BL).

1.4.2 RNA isolation, amplification and microarray hybridization

Prior to RNA isolation the tubes containing single blastomeres where thawed and centrifuged to ensure, that the sample is on the bottom of the tube and finally pooled in groups of fifteen according to the development of the corresponding sister blastomere as described above.

For RNA isolation the PicoPure™ RNA Isolation Kit (Arcturs, Munich, Germany) was used. Genomic DNA contamination was removed by performing on column DNA digestion using RNase-free DNase (Qiagen GmbH, Hilden, Germany). Quantity and quality of the extracted total RNA was verified on a 2100 Bioanalyzer (Agilent Technologies Inc, CA, USA). Due to the relatively low amounts of isolated RNA, two rounds of amplification were considered to be necessary to receive an adequate amount of RNA for microarray analysis. The RiboAmp® HS kit incorporates a T7 polymerase promoter into a double-stranded complementary DNA (cDNA), from which antisense RNA (aRNA) is transcribed. Two rounds of amplification enables to produce 10^6 – fold the amount of input RNA content. After amplification procedure 2µg of aRNA from each sample were conjugated with either Cy-3 or Cy-5 dyes using ULS Fluorescent labelling kit for Agilent arrays (Kreatech Diagnostics, Amsterdam, Netherlands). Samples from the three pools (biological replicates) of each group were hybridized on EmbryoGENE bovine microarray (Made by Agilent 4 x 44k) using a dye-swap design (technical replicates) and placed in a hybridisation chamber at 65°C for 17 hours. In the next step the microarray slides were washed for 1 min in gene expression wash buffer 1 (room temperature), 3 min in gene expression wash buffer 2 (42°C), 10 sec in 100% acetonitrile (room temperature) and 30 sec in stabilization and drying solution (Agilent), to remove the non-specific bindings of the spots.

Thereafter, the slides were scanned using the GenePix Pro Scanner. Raw data were corrected by background subtraction, and then normalized within and between each array (Loess and quantile, respectively). Statistical comparison between the groups was done with the Limma algorithm. Genes were considered to be differentially expressed at a foldchange >1.5 with adjusted P-value of < 0.05 using the Benjamini and Hochberg false discovery rate method (FDR < 0.1).

1.4.3 Quantitative real time PCR

During the last years real-time PCR has turned out as the method of choice for accurate and sensitive quantification of messenger RNA (mRNA). For array validation three independent biological replicates of the three groups (2CB, 4CB and BL) were used. For verification with the independent model of early and late cleaved 2-cell stage embryos, cDNA was synthesized using oligo dT (23) primers to detect the polyadenylated or rather active forms of the transcripts with a poly A tail of 23 or more nucleotides and random oligomers, which bind to all transcripts irrespectively of the poly A tail length.

For the analyses in the present study, absolute quantification, the so called standard curve method was used. The quantity of the unknown sample is interpolated from a range of standards of known quantity. For standard curve construction, a serial dilution of a template with a known concentration is produced, serving as a standard for the unknown test samples. A serial dilution of 10^1 – 10^9 copy numbers of molecules for each of the selected genes was prepared from plasmid DNA. As an internal control and housekeeping gene GAPDH was used.

In each run the cDNA sample, the serial dilution of plasmid standards and a non-template control (NTC) was loaded to the 96 well plates. The real time PCR analyses were performed in a 20 µl reaction volume containing iTaq SYBR Green Super mix in ABI PRISM® 7000 instrument (Applied Biosystems). Melting curve analyses were constructed to verify the presence of gene specific peak and the absence of primer dimer. Final quantitative analysis was carried out using the relative standard curve method and results were reported as the relative expression after normalization of the transcript amount relative to GAPDH.

1.4.4 Experimental design

Several sub experiments were conducted, to investigate:

1. whether both sister blastomeres of 2-cell stage embryos have the same developmental competence.
2. whether the gene expression profile of 2-stage blastomeres is directly related to the individual developmental competence of the corresponding sister blastomeres.
3. the transcript abundance of selected candidate genes in terms of random and polyadenylization in an independent competence model.
4. the accumulation of reactive oxygen species (ROS) in competent and incompetent bovine 2-cell stage embryos.
5. the effect of different environmental oxygen tensions on developmental competence and transcript abundance of selected candidate genes.
6. transcript abundance of candidate genes in single sister blastomeres.
7. the localization of selected proteins in competent and incompetent bovine 2-cell stage embryos.

1.5 Results

The most important results are summarized here briefly. Detailed explanations can be found in the respective chapters.

1.5.1 Influence of COCs morphology, maturational environment and growth status on Zona pellucida birefringence

In the first experiment (Chapter 2) oocytes with different maturational origin and growth status were quantitatively analysed by polarized light microscopy with respect to developmental competence.

Prior to polarized light microscopic measurements blastocyst rates were counted to evaluate developmental competence. Briefly, in vivo matured oocytes had significantly higher blastocyst rates than their in vitro derived counterparts (39.1% vs. 21.6%) and quality 1 COCs reached significantly higher blastocyst rates than quality 3 COCs (27.7% vs. 16.9%).

The first parameter measured by polarized light microscopy is the mean birefringence of zona pellucida (CV-Mean). In vivo matured (MII) oocytes had significantly lower values for birefringence then in vitro matured oocytes (16.54 ± 2.41 vs. 20.76 ± 2.69). A similar proportion was observed according to the COCs quality of in vitro matured oocytes; good quality oocytes showed a significant lower birefringence than those classified as bad quality oocytes (20.23 ± 2.69 vs. 21.75 ± 3.56). Regarding the second parameter measured by polarized light microscope, the thickness of the inner layer (WT- Mean), the same decreasing trend from in vitro matured to in vivo matured and from quality 1 to quality 3 was observed. In vivo matured oocytes had a WT- Mean of 10.72 ± 1.48 whereas the overall in vitro matured oocytes had a WT-Mean of 12.39 ± 2.19. Shortly summarized, high quality matured oocytes have significantly lower values for birefringence and thickness of the inner layer than low quality oocytes. Concerning immature oocytes (GV), the lowest values for birefringence and thickness of the inner layer were observed for quality 3 oocytes, implicating a high birefringence value (20.95 ± 3.79 vs. 18.03 ± 2.85) and a thicker inner layer (11.44 value 1.92 vs. 10.55 value 1.73) in quality 1 oocytes. In a next step, the results of immature and matured oocytes were correlated, showing that the trend from immature to matured oocytes in terms of birefringence and thickness of the inner layer were significantly

different regarding maturational environment and COCs quality. In vivo maturation leads to a decrease in birefringence and thickness, whereas the in vitro maturation shows an increasing trend for birefringence and thickness. Regarding the COCs quality, the decreasing trend of in vivo maturation could be observed for good quality oocytes (shown in Figure 3 of Chapter 1). Finally, the influence of the oocytes growth status, namely G6PDH activity, on zona pellucida properties was evaluated. Briefly, fully grown oocytes had significantly lower values for birefringence (18.83 ± 3.5 vs. 20.37 ± 4.49) and thickness of the inner layer (10.84 ± 1.95 vs. 11.77 ± 2.35) than their still growing counterparts as well as significantly higher blastocyst rates (32% vs. 11.5%).

1.5.2 Association of bovine 2-cell stage blastomere transcriptome profile with the individual developmental potential of the sister blastomere

The aim of the second experiment (Chapter 3) was to find a significant transcriptome profile and to detect candidate genes which are directly related to the developmental competence.

Prior to gene expression analyses, developmental synchrony was confirmed, by separating both blastomeres of a 2-cell stage embryo followed by an individual culture. The results show, that there is a high synchrony in development, implicating, when the given blastomere develops to the blastocyst, 94% of corresponding sister blastomeres develop to the blastocyst as well. Accordingly, if one blastomere did not cleave any further after separation a proportion of 70% of the sister blastomeres did also not cleave any more. For gene expression analyses a unique custom microarray (Agilent) containing 42,242 oligo probes was used.

The differences in gene expression were uncovered between blastomeres whose counterparts developed to the blastocyst stage, as the control group and those whose sister blastomere either stopped cleaving after separation or cleaved until embryonic genome activation. In the first comparison (BL vs. 2CB) 632 genes were differentially regulated of which 298 were upregulated in blastomeres whose counterparts developed to the blastocyst stage and 334 downregulated. Considering the second comparison (BL vs. 8CB), we found 150 genes to be differentially expressed. 61 genes were upregulated in blastomeres whose sister blastomeres developed to the blastocyst stage and 89 were downregulated.

Considering, transcriptome analyses, we found 632 genes to be differentially regulated between BL and 2CB, 150 between BL and 8CB and 77 were found to be commonly differentially regulated between both incompetent groups and the competent group, 28 of them were upregulated and 39 downregulated. These gene lists were uploaded to two different gene ontology softwares. For biological processes and molecular functions the DAVID software was used and for pathway analyses Ingenuity Pathway Analyses (IPA) software was used.

Genes which were differentially regulated between BL and 2CB were mainly involved in biological processes like: cellular protein and macromolecule process, translation, transcription, protein localization and transport, cell cycle and cellular response to stress as well as in pathways like: protein ubiquitination pathway, estrogen receptor signalling, protein kinase A signalling, molecular mechanism of cancer, oxidative phosphorylation and NRF2- mediated oxidative stress response. Focusing on the commonly differentially regulated genes, molecular functions like peroxidase activity, oxidoreductase activity acting on peroxide as acceptor, antioxidant activity, structural constituent of ribosome, translation initiation factor activity and protein dimerization activity, were manly affected by DEGs. Concerning the most affected biological processes were, ribosome biogenesis, transcription, cell cycle, translation, regulation of oxidative stress and cellular respiration and oxidative phosphorylation. Finally, IPA analyses revealed, nucleotide excision repair pathway, methane metabolism, mitochondrial dysfunction, p38 MAPK signalling, oxidative phosphorylation and NRF2- mediated oxidative stress response as mainly affected. Hence, homogenous results of these cluster analyses for all groups were detected, implicating a strong relation to molecular functions and pathways related to oxidative stress response and oxidative phosphorylation. Therefore, out of these clusters five candidate genes, namely *NDUFS1, MAPK14, CAT, PRDX1* and *PRDX6* were selected for further validation and verification. Real time PCR results showed the expression of eight genes to be in agreement with the array results ($p < 0.05$). Additionally, two genes *(PRDX1, PRDX6)* showed the correct expression pattern (without statistical significance) and one gene *(KRT8)* did not show differences with real time PCR validation. (Figure 4, Chapter 3). For further verification an independent competence model was chosen as described in detail in chapter 3.

Briefly, 2-cell stage embryos were collected according to the time of first cleavage after fertilization (hpi, hours post insemination), which has been demonstrated to be a reliable marker for developmental potential. A significantly higher proportion of zygotes cleaving before 30 hpi develop to the blastocyst stage compared to those cleaving 36 hpi or later.

The expression levels of our five selected candidate genes showed a significant higher abundance in early cleaving embryos compared to their later cleaving counterparts. Furthermore, the amount of reactive oxygen species (ROS) were lower in early cleaving embryos, which is in line with the transcript abundance of the candidate genes, acting as direct and indirect ROS scavengers.

Chapter 2

Zona pellucida birefringence correlates with developmental capacity of bovine oocytes classified by maturational environment, COC-morphology and G6PDH-activity.

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Abstract

In the present study, we aimed to analyse structural changes during in vitro maturation of the bovine zona pellucida (ZP) by scanning electron microscopy (SEM) as well as whether these changes are reflected by zona pellucida birefringence (ZPB). Here we show that alterations during in vitro maturation invasively analysed by SEM are reflected in ZPB. In vivo matured oocytes displayed significantly lower birefringence parameters and significantly higher blastocyst rates compared to in vitro derived oocytes (39.1% vs. 21.6%). The same was observed for in vitro matured oocytes with cumulus oocytes complex (COC) quality grade 1 (Q1) compared to Q3-COC's with respect to zona birefringence and developmental capacity. Immature oocytes with Q1-COC displayed higher ZPB values and a higher developmental capacity to the blastocyst stage (27.7% vs. 16.9%) compared to immature Q3-COC's. Considering in vitro matured oocytes, only those with Q1 COC grade showed a trend for ZPB similar to in vivo matured oocytes. Therefore, a decreasing trend for ZPB during in vitro maturation seems to be typical for high quality oocytes and successful cytoplasmic maturation. In accordance, fully grown immature oocytes reached significant higher blastocystrates (32.0% vs. 11.5%) and lower ZPB values compared to still growing ones.

In conclusion, we successfully evaluated the applicability of zona imaging to bovine oocytes: Alterations during in vitro maturation invasively analysed by scanning electron microscopy were reflected in the birefringence of the zona pellucida of bovine oocytes affecting developmental capacity at the same value. Therefore, ZPB measurement by live zona imaging has got potential to become a new tool to assess correctness of in vitro maturation and to predict developmental competence.

Key words: bovine oocyte, in vitro maturation, environment, zona pellucida birefringence

Introduction

Developmental competence of bovine in vitro produced embryos remains significantly lower compared to their in vivo derived counterparts. Despite intensive research a large proportion of bovine oocytes fail to develop to the blastocyst stage following maturation, fertilisation and in vitro culture (Farin and Farin 1995). It is generally accepted that oocyte quality is a key factor for optimizing the efficiency of reproductive techniques in farm animals as well as for human assisted reproductive technologies (Coticchio et al. 2004; Sirard et al. 2006; Telfer and McLaughlin 2007; van Soom et al. 2007; Wang and Sun 2007). Therefore, identification of credible predictors for developmental competence of bovine oocytes for in vitro production is indispensible. A variety of cellular and subcellular parameters have been investigated whether they are related to developmental competence, such as gene expression pattern (Wrenzycki et al. 2007), mitochondrial status (Stojkovic et al. 2001), calcium stores and calcium current activity (Boni et al. 2002), apoptotic index (Yuan et al. 2005), gene expression profiles in cumulus cells (Assidi et al. 2008; Tesfaye et al. 2009) and factors present in the follicular fluid (Nicholas et al. 2005; Sinclair et al. 2008; van Soom et al. 2007). Unfortunately, these techniques are often complex, time-consuming and most importantly invasive, which excludes further development of oocyte. Therefore, non-invasive criteria to evaluate potent oocytes based on morphology were evaluated during the last decade including homogeneity of ooplasm, thickness and diameter of oocytes as well as compactness of surrounding cumulus layers (Blondin and Sirard 1995; Nagano et al. 2006; Santos et al. 2008). However, the predictive value of these morphological characteristics evaluated by light microscopy is discussed controversial, due to subjectivity and inaccuracy (Lonergan 2007; Nagy 2008; Wang and Sun 2007).

The intrinsic quality of an oocyte, which is acquired during folliculogenesis, relying on vascularisation, oxygen content and cumulus cell characteristics, is a great factor affecting subsequent development of an embryo (Corn et al. 2005; Van Blerkom et al. 1997). Thereby, any negative effect during folliculogenesis will harm the oocyte, resulting in substantial morphological alterations like discoloration (Esfandiari et al. 2006), shape anomaly (Ebner et al. 2008), zona splitting (Shen et al. 2008) or changes of its three-dimensional structure.

It has been reported that the mean difference in thickness between zona pellucida from human conception cycles and failed ones was approximately 1 μm (Shen et al. 2005), a

value, however, which is beyond the limit of verifiability of most systems designed for measuring cells. In contrast to light microscopic analysis of bovine oocytes, scanning electron microscopic (SEM) analysis of bovine oocytes has already identified clear relationships between oocyte morphology and developmental competence: Using SEM, the structure of the ZP in human oocytes matured in vitro is seen as a large multilayered network resembling a sponge, whereas in immature and atretic oocytes the ZP has a compact and smooth surface (Familiari et al. 2006). Santos et al. (2008) reported that the number of pores on the ZP surface varies with the quality of the oocyte. Furthermore, significant differences in the thickness of the ZP were ascertained between species, varying from 5µm in mouse to 27µm in bovine (Dunbar et al. 1994) as well as between in vitro and in vivo embryogenesis (Michelmann et al. 2007). Recent investigations revealed that matured bovine oocytes with high quality cumulus oocyte complex (COC), classified by stereomicroscope, had significantly smaller zona pore diameters compared to those of low quality (Santos et al. 2008). However, application of SEM does not allow further development of the analysed oocytes because of its invasive nature.

In contrast, the introduction of polarization light microscopy opens a new window for non-invasive assessment of morphological zona pellucida properties, recently. Zona pellucida imaging at the metaphase-II stage of oocyte's was established successfully as a predictive marker for human oocyte quality in several studies (Ebner et al 2010.; Madaschi et al. 2009; Montag et al. 2008; Rama Raju et al. 2007).

Although oocyte maturation remains a poorly understood process, the follicle can be considered as the reproductive unit of the ovary. Initiation of germinal vesicle breakdown and completion of the nuclear changes are leading to extrusion of the first polarbody and arranging the second metaphase plate (Lin and Hwang 2006). The formation of a metaphase-II stage oocytes is closely linked to completion of nuclear changes, but neither to the oocytes molecular and structural maturity nor to the developmental competence (Trounson et al. 2001). Any factor affecting follicular recruitment and growth, may influence the secretion of cumulus cells and oocytes (Qi et al. 2002). Hence, physiology of folliculogenesis (Pelletier et al. 2004) and quality of in vitro culture might affect the texture of the zona pellucida. Coincidentally, characteristics and appearance of the ZP may reflect the history of folliculogenesis

(Qi et al. 2002) as marker of correct folliculogenesis and normal oocyte maturation and predictor for subsequent developmental competence.

Previous studies speculated that properties of the zona layers might reflect the history of human oocyte cytoplasmic maturation (Liu et al. 2003) whereupon, the ZPB from germinal vesicle (GV) stage oocytes showed a significantly decreasing trend to MII-stage oocytes (Cheng et al. 2010). In accordance, a higher percentage of high birefringence oocytes was observed in human oocytes being in prophase I compared to metaphase I stage. Interestingly, the percentages of high birefringence oocytes did not change when comparing oocytes before and after in vitro maturation for both prophase I and metaphase I oocytes (de Almeida Ferreira Braga et al. 2010). If zona pellucida birefringence indeed correlates with developmental competence that would imply that developmental competence is already fixed before in vitro maturation. That in turn would suggest that a better selection of oocytes rather than improving in vitro maturation conditions is necessary to improve overall in vitro developmental rates. However, all published studies focused on human ARTs so far and it remains an open question if these results could be transferred to the bovine.

Taking into account, that oocytes are usually of unknown origin with inhomogeneous developmental competence in bovine IVP, any correlation between zona pellucida characteristics with age of donor, follicular origin, maturational stage as well as maturation environment could be fruitful to select more homogenous groups of oocytes bearing higher developmental competence. However, the relationship between oocyte cytoplasmic maturation and ZP birefringence in the bovine is still inexplicit.

The aim of this study was therefore to evaluate the applicability of zona imaging to bovine oocytes. Therefore we analyzed subpopulations of bovine oocytes bearing variable prospective developmental competence, classified according to their environment of maturation (in vivo vs. in vitro), their cumulus cell investment and their G6PDH-activity with respect to zona pellucida properties and their developmental competence, simultaneously. As an invasive technique, we analysed the zona pellucida of in vivo and in vitro derived oocytes by scanning electron microscopy to visualize alterations of the morphological structure before and after maturation related to developmental competence. Using polarisation light microscopy as non-invasive technique, we consequently aimed to find out whether these structural changes are also reflected in the zona birefringence.

Material and Methods

Oocyte collection

Bovine ovaries were obtained from a local slaughterhouse and transported in warm (30-35°C) physiological saline solution within 1-3 hours. Antral follicles (2 to 8 mm in diameter) were aspirated using an 18-gauge needle attached to a 10 ml syringe and collected into a 50 mL conical tube. COCs with evenly granulated oocyte cytoplasm surrounded by more than three compact layers of cumulus cells, were selected and transferred to modified tissue culture medium (TCM199, Sigma, Taufkirchen, Germany) supplemented with 4.4 mM Hepes, 33.9 mM $NaCHO_3$, 2 mM pyruvate, 2.9 mM calciumlactate, 55 $\mu g ml^{-1}$ gentamycin and 12% heat-inactivated oestrus cow serum (OCS).

Assessment of the Cumulus Oocyte Complex (COC) quality

The assessment of the COC morphology was performed as following: Briefly, oocytes with presence of clear and compact cumulus cells with more than three layers were allocated to quality 1 (Q1), oocytes with a compacted cumulus with two or three layers were classified as quality 2 (Q2) and oocytes with one ore less layers of cumulus cells were judged as quality 3 (Q3).

Assessment of Glucos-6-Phosphate-Dehydrogenase (G6PDH) -activity

We performed the brilliant cresyl blue (BCB) stain to separate oocytes undergoing growth and those that have completed their growth phase as described in our previous study (Ghanem et al. 2007). Briefly, immature oocytes with high quality COC's (Q1) were subjected to 26 µM BCB (B-5388, Sigma–Alderich) diluted in mDPBS for 90 min at 38.5 °C in humidified air atmosphere. After washing, stained COCs were examined under a stereomicroscope and categorised into two groups according to their cytoplasma staining: oocytes with any degree of blue colouration in the cytoplasm (BCB+) were classified into low Glucose-6-Phosphate-Dehygrogenase activity group representing fully grown immature oocytes, and oocytes without visual blue colouration (BCB-) were classified into high Glucose-6-Phosphate-Dehygrogenase activity group representing still growing immature oocytes.

In vitro maturation

COCs washed and incubated (in groups of 50) in 400 ml of maturation medium that consisted of TCM-199 (M-2154; Sigma) with Earle salts buffered with 4.43 mM HEPES (H-9136; Sigma) and 33.9 mM sodium bicarbonate (S-5761; Sigma)

supplemented with 12% oestrous calf serum (OCS), 0.5 mM L-glutamine, 0.2 mM pyruvate, 50 mg/ml gentamycin sulphate and 10 µl/ml FSH (Folltropin, Vetrepharm, Canada) in four well dishes (Nunc, Roskilde, Denmark). The maturation medium was covered with mineral oil (Sigma-Aldrich) and was pre-incubated under the maturation conditions for a minimum of 1 h (38.7 °C, 5% CO_2 in air with maximum humidity) and then incubated for 22 h after oocytes were added.

In vitro fertilization and in vitro culture

After maturation COCs were co-incubated with sperm (2×10^6 spermatozoa/ml) in a fertilization medium consisting of Fert-TALP medium supplemented with 10 mM sodium lactate, 1 mM sodium pyruvate, 6 mg/ml BSA, 1 µg/mL heparin, 10 µM hypotaurine, 20 µM penicillamine, and 2 µM epinephrine) at 38.7°C in 5% CO_2 in air. Eighteen hours after insemination (18 hpi), the presumable zygotes were denuded from cumulus cells. Nearly fifty cumulus-free presumptive zygotes were washed three times in CR1aa supplemented with 12% heat inactivated oestrous cow serum (OCS) and then cultured in 400 µl of the same medium in four well dishes (Nunc, Roskilde, Denmark) under mineral oil at 38.7°C in 5% CO_2 in humidified air.

Collection of in vivo matured Oocytes

Pre-synchronization was performed by i.m. administration of 500 µg cloprostenol (PGF2α, Estrumate®; Essex Tierarznei, Munich, Germany) twice within 11 days. Two days after each of the PGF2α treatments animals received 10 µg GnRH (Receptal®; Intervet, Boxmeer, the Netherlands). Twelve days after the last GnRH injection heifers received the first of eight consecutive FSH-injections over 4 days in decreasing doses (in total 300–400 mg FSH equivalent according to body weight; Stimufol, University of Liege, Belgium). Two PGF2α treatments were performed 58 and 72 h after the initial FSH. Finally, 40 h after the first PGF2α application, 10 µg GnRH were given to each animal. Endoscopic guided follicle aspiration to collect in vivo matured MII-Oocytes was performed 16-20 h after GnRH administration. After restraining the recipients, administering 5 ml of a 2% lidocaine-solution (Xylanest purum1, Richter Pharma,Wels, Austria) for epidural anesthesia and disinfecting the vulva (Octenisept1, Schülke/Mayer, Vienna, Austria), a trocar set consisting of an universal metal tube (12.5 mm x 52 cm, Storz, Vienna, Austria) and an atraumatic mandrin was placed caudodorsal of the fornix vaginae. The mandrin was replaced by a sharp trocar and the trocar set was inserted through the vaginal wall into the peritoneal cavity. The trocar

was replaced by a shaft bearing the endoscope (5.5 mm 08 forward Hopkins endoscope, Storz, Vienna, Austria) and the punctation line. The site was illuminated by a fiberoptic cold light (250 W, Storz, Austria) and visualized with a camera (Telecam PAL-Endovision, Storz, Vienna, Austria) connected to a monitor. The aspiration line consisted of a single lumen needle (diameter: 17 G; length: 70 cm; William Cook Europe GmbH, Mönchengladbach, Germany) connected to a 50-ml Falcon tube by 100-cm Teflon tubing. Vacuum pressure was provided by a regulated vacuum pump (V-MAR-5000; Cook) and adjusted to create a flow rate of 16 to 20 ml per min. The collection medium consisted of Hepes buffered TCM supplemented with 50 mg/l gentamicin (Sigma, St. Louis, MO), 60 mg/l heparin (Sigma) and 1% fetal calf serum (FCS; Biochrom, Berlin, Germany).

After bringing the ovary into the right position by transrectal manipulation the aspiration line was advanced to puncture the ovarian follicle. The follicular contents of all follicles of each heifer were aspirated individually and kept at 39°C in thermos. Finally, the follicular fluid contents were poured into a square grid dish to facilitate finding of oocytes under a stereomicroscope.

Preparation of oocytes for scanning electron microscopy (SEM)

For scanning electron microscopy analysis oocytes were placed in fixation medium composed by 2,5% glutaraldehyde for one hour and washed afterwards three times in 0,1M sodium cacodylate in each case 10 minutes, before a two hours fixation in 2% osmiumtetroxide solution. After repeating the washing step, the samples were dehydrated by plunging them into ethyl alcohol at different concentrations (50-100%) and acetone. Following dehydration the samples were dried in a critical-point-dryer (Polaron, Watford; Great Britain). After drying they were coated with 30-nm gold by a Blazers sputtering device (Blazer, Liechtenstein). SEM observations were conducted with ESEM XL 30 FEG, FEI (Philips, Eindhoven, Netherlands) in the institute for pathology RWTH Aachen.

Live zona imaging

Live zona imaging of individual oocytes was performed non-invasively on a Leica DM IRB inverted microscope equipped with ×10, ×20 and ×40 Hoffmann interference optics, ×20 and ×40 stain-free objectives, a circular polarization filter and liquid crystal analyser optics. The birefringence analysis including autocalibration was fully

controlled by a polarization imaging software module (OCTAX ICSI Guard™, OCTAX Microscience GmbH, Altdorf, Germany) implemented in an imaging software system (OCTAX Eyeware™). In detail, the image processing on the birefringence image (Figure 1B&D) extracted several birefringence intensity profiles (n>20) across the inner zona layer. For each of the profiles, i.e. along the entire zona layer, a cumulated birefringence value (CV-Mean) and the average thickness of the inner zona layer (WT-Mean) was calculated. These values (which were computed for each intensity profile, i.e. over the entire cell's circumference) were averaged and resulted in the values CV-Mean and WT-Mean. As plastic dishes interfere with polarized light, glass bottom dishes (WillCo, Amsterdam, Netherlands) were used for examination. Denuded oocytes were separated in 4 µl drops of HEPES-modified tissue culture medium and imaged at 200x magnification. Each drop was covered with mineral oil; screening in groups of 10 did not last longer than two minutes.

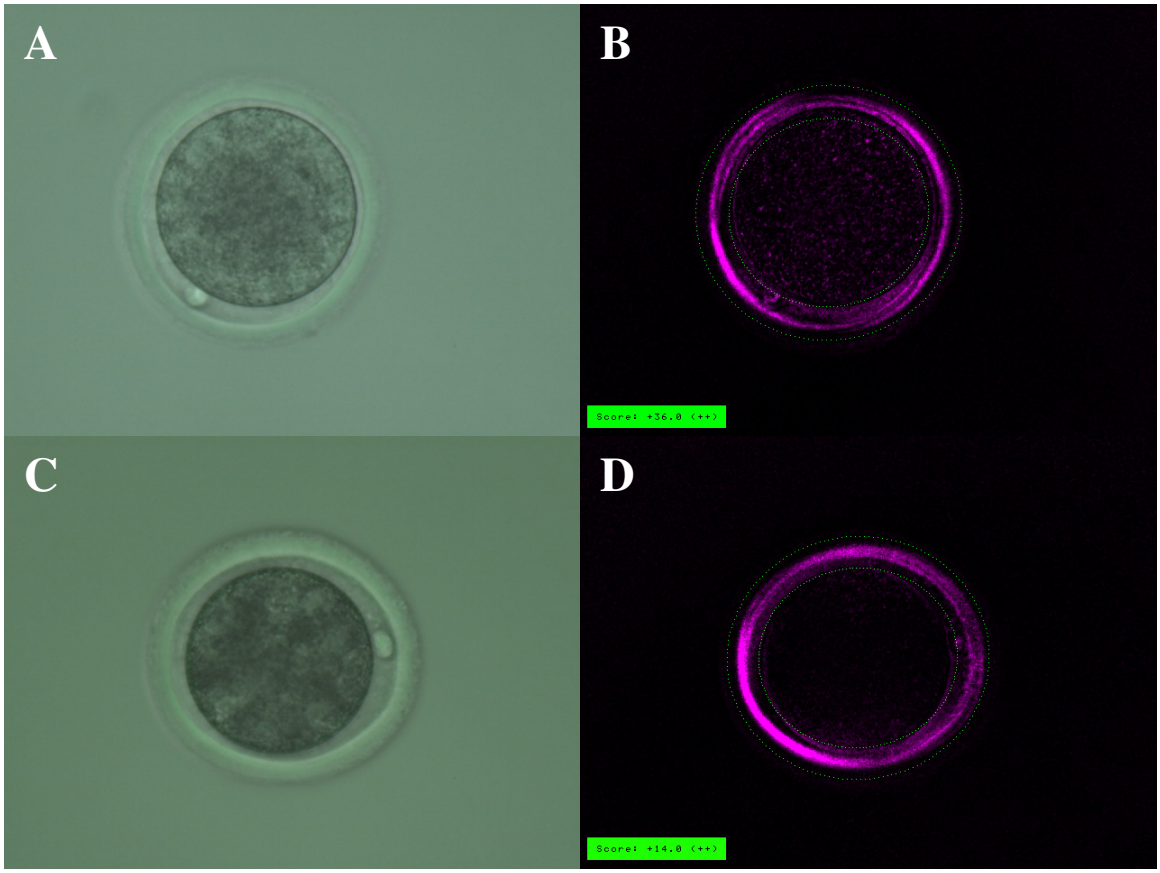

Figure 1: Imaging of matured in vivo derived metaphase II stage oocyte by conventional lightmicroscopy (A) and by polarized light microscopy (B). Similarly, images C and D show in vitro derived metaphase II oocytes.

Statistical analysis

Allocation of oocytes into different morphological groups according to their zona characteristics analyzed by scanning electron microscopy and developmental rates of embryos generated by in vitro fertilization were analyses by χ^2-test. Analysis of variance (ANOVA, two tailed t-test) was performed for comparison of mean values of zona pellucida evaluation. Differences of $p < 0.05$ were considered statistically significant.

Experimental Design

In the present work we performed 4 subsequent experiments: In the first experiment we recapitulated the correlation of the maturational environment (in vivo vs. in vitro) and the COC's quality (Quality 1-3) on the bovine zona pellucida structure measured by scanning electron microscopy (SEM). In the second experiment, we analyzed the effect of the maturational environment and the COC quality on the subsequent in vitro developmental competence after in vitro fertilization. In experiment 3, we aimed to check whether maturational environment and COC quality are reflected in the bovine zona pellucida structure of GV- and metaphase II stage oocytes analyzed by zona pellucida birefringence (ZPB). Finally, in the 4th experiment we compared zona pellucida characteristics measured by ZPB and subsequent developmental competence of immature bovine oocytes classified according to their G6PDH-activity as proven indicator for subsequent developmental competence.

Results

Experiment 1: Effect of maturational environment and COC quality on bovine zona pellucida structure measured by SEM

A total of 21 bovine GV-stage oocytes were analyzed by scanning electron microscopy (SEM). All GV-stage oocytes from Q1+Q2 (n=15) and Q3 (n=6) COC's showed a porous zona pellucida. Considering oocytes of COC quality grade 1+2, a total of 10 (66.6%) displayed a typical fine meshed reticular pore structure (Figure 2A) whereas the zona of 5 oocytes (33.3%) showed an irregular appearing pore structure (Figure 2B). Oocytes of COC quality grade 3 showed a tendency for a higher proportion of irregular appearing pore structure (66.7%) as shown in TABLE 1. A significantly ($p<0.05$) lower proportion of metaphase-II oocytes showed a porous zona structure

compared to GV-stage oocytes (31.3% vs. 100%) irrespective of COC quality with 100% of them showing typical fine meshed reticular pores. A significantly higher proportion of in vivo matured oocytes displayed a porous zona structure with typical fine meshed reticular pores (Figure 2C) compared to in vitro matured oocytes (31.3% vs. 100%) as presented in TABLE 1. Likewise, significantly more in vitro derived MII oocytes showed an imporous structure (68.8% vs. 0%, Figure 2C). Pores with irregular structure were only observed in immature oocytes.

Table 1: Effect of maturational environment and COC quality grade on bovine zona pellucida structure measured by scanning electron microscopy

							Structure of pores			
			imporous		porous		typical		irregular	
Oocyte group	quality	n	n	(%)	n	(%)	n	(%)	n	(%)
GV	Q1+Q2	15	0	0.0%	15	100.0%	10	66.7%	5	33.3%
GV	Q3	6	0	0.0%	6	100.0%	2	33.3%	4	66.7%
		21	0	0.0% [a]	21	100.0% [a]	12	57.1%	9	42.9%
Vitro MII	Q1+Q2	8	5	62.5%	3	37.5%	3	100.0%	0	0.0%
Vitro MII	Q3	8	6	75.0%	2	25.0%	2	100.0%	0	0.0%
		16	11	68.8% [b]	5	31.3% [b]	5	100.0%	0	0.0%
Vivo MII	total	6	0	0.0% [a]	6	100.0% [a]	6	100.0%	0	0.0%

Values with different superscripts within columns differ significantly ($p < 0.05$)

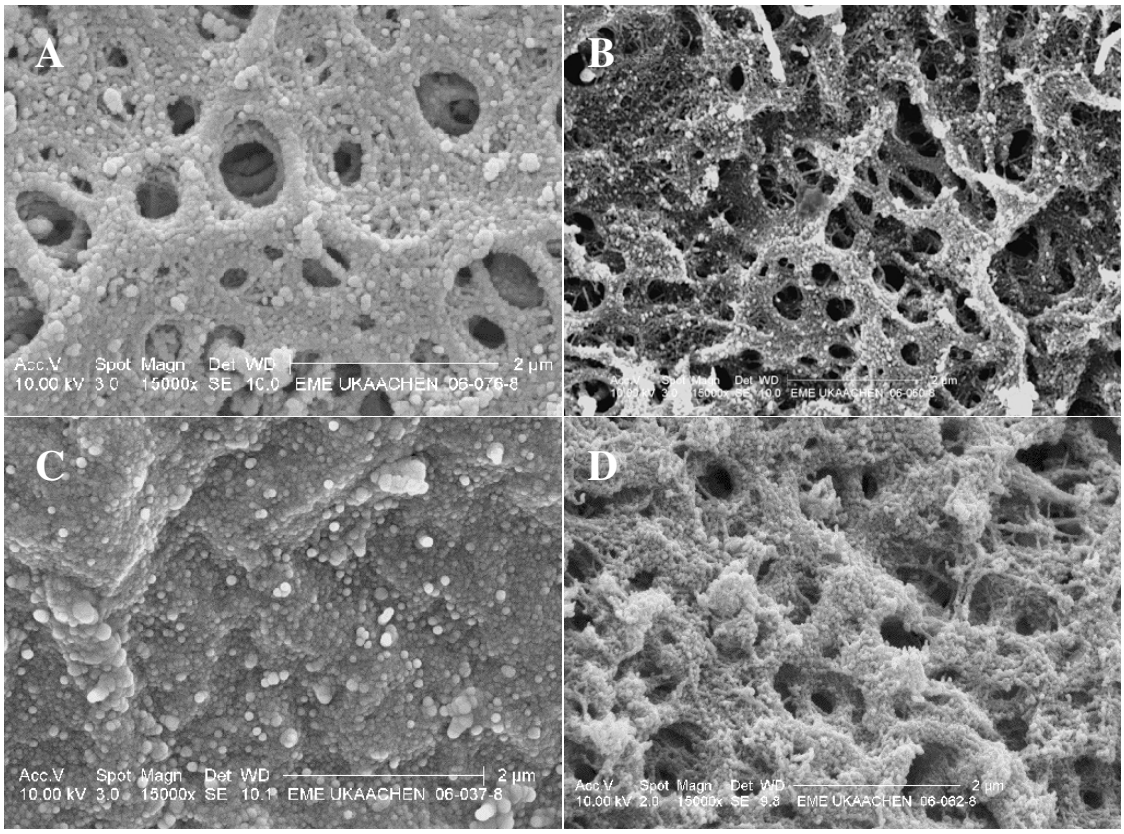

Figure 2: Representative zona pellucida SEM microphotographs of oocytes at different stages of maturity. (A) porous surface with a typical pore structure of an immature GV stage oocyte, (B) porous surface with an irregular degraded emerging pore structure of an immature GV stage oocytes, (C) poreless surface of an in vitro matured metaphase II oocyte and (D) porous surface with a reticular fine-meshed pore structure of an in vivo matured metaphase II oocyte. All pictures are 15000x magnification.

Experiment 2: Effect of maturational environment and COC quality on in vitro developmental competence of bovine oocytes

When 438 oocytes were fertilized in vitro, in vivo matured bovine oocytes (n=68) reached a similar cleavage rate compared to in vitro matured oocytes (n=370, 76.5% vs. 74.3%). Significantly, more in vivo matured oocytes reached the blastocyst stage compared to in vitro matured oocytes (39.1% vs. 21.6%) as shown in Table 2. Considering in vitro matured oocytes of different COC qualities, quality 1 grade COC's reached a significant higher cleavage rate compared to quality 2 and 3 grade COC's (82.5% vs. 71.6% vs. 68.5%), respectively, as well as higher subsequent blastocyst rates (27.7% vs. 20.0% vs. 16.9%, respectively, Table 2).

Table 2: Effect of maturational environment and COC quality grade on in vitro developmental competence of bovine oocytes

Oocyte group	quality	n	Cleavagerate		Blastocystrate	
			n	(%)	n	(%)
Vitro MII	Q1	126	104	(82.5) [a]	35	(27.7) [b]
Vitro MII	Q2	120	86	(71.6) [b]	24	(20.0) [a]
Vitro MII	Q3	124	85	(68.5) [b]	21	(16.9) [a]
		370	275	(74.3) [ab]	80	(21.6) [a]
Vivo MII		68	52	(76.5) [ab]	27	(39.1) [c]

Values with different superscripts within columns differ significantly ($p < 0.05$)

Experiment 3: Effect of maturational environment and COC quality on bovine zona pellucida structure of immature and matured oocytes measured by ZPB

The mean birefringence (CV-Mean) of in vivo matured oocytes (n= 21) was significantly lower ($p<0.05$) compared to their in vitro counterparts (n=247; 16.54 ± 2.41 vs. 20.76 ± 2.69). Within different COC qualities of vitro matured oocytes a significant lower birefringence was observed for COC quality 1 (n=79) compared to quality 3 (n=77; 20.23 ± 2.69 vs. 21.75 ± 3.56) as presented in Table 3. Accordingly, the thickness of the inner layer (WT-Mean) of in vivo matured oocytes was significantly thinner compared to in vitro derived oocytes (10.72 ± 1.48 vs. 12.39 ± 2.19). With respect to COC's quality, oocytes from quality 1 COC's displayed a significantly thinner inner zona layer compared to oocytes of quality 3 COC's (12.3 ± 1.50 vs. 12.95 ± 2.19) as shown in Table 3.

Table 3: Effect of maturational environment and COC quality grade on zona pellucida birefringence of bovine MII stage oocytes measured by polarised light microscopy.

			CV-Mean	WT-Mean
Oocyte group	**quality**	**n**	Mean ± SD	Mean ± SD
Vitro MII	Q1	79	20.23 ± 2.69 ^{A}	12.03 ± 1.50 ^{A}
Vitro MII	Q2	91	20.39 ± 3.54 ^{AB}	12.22 ± 1.98 ^{A}
Vitro MII	Q3	77	21.75 ± 3.56 ^{B}	12.95 ± 2.19 ^{B}
Vitro MII	**total**	**247**	**20.76 ± 3.02** ^{a}	**12.39 ± 1.91** ^{a}
Vivo MII	**total**	**21**	**16.54 ± 2,41** ^{b}	**10.72 ± 1.48** ^{b}

Values with different superscripts within columns differ significantly (a:b; A:B:C; $p < 0.05$)

When we analyzed the zona birefringence and thickness of the inner zona layer of immature oocytes, significant differences for both parameters were observed between oocytes of different COC quality (Q1-Q3). The mean birefringence (CV-Mean) significantly decreased from COC quality 1 to COC quality 3 (20.95 ± 3.79 vs. 19.44 ± 2.91 vs. 18.03 ± 2.85), respectively (Table 4). The thickness of the inner zona layer (WT-Mean) showed the same trend considering COC quality 1 to COC quality 3 (11.44 ± 1.92 vs. 10.79 ± 1.54 vs. 10.55 ± 1.73; $p<0.01$, respectively, Table 4).

Table 4: Effect of COC quality grade of bovine GV stage oocytes on zona pellucida birefringence measured with polarised light microscopy.

			CV-Mean	WT-Mean
Oocyte group	**quality**	**n**	Mean ± SD	Mean ± SD
GV	Q1	79	20.95 ± 3.79 ^{a}	11.44 ± 1.92 ^{a}
GV	Q2	85	19.44 ± 2.91 ^{b}	10.97 ± 1.54 ^{ab}
GV	Q3	77	18.03 ± 2.85 ^{c}	10.55 ± 1.73 ^{b}

Values with different superscripts within columns differ significantly ($p < 0.05$)

Putting the results of the immature (Table 4) and the matured oocytes (Table 3) into relation, the trend from GV- to MII-stage in terms of zona birefringence (CV-Mean) and thickness of the inner zona layer (WT-Mean) were significantly different ($p<0.05$) between different maturational environments and individual COC qualities as presented in Figure 3. In vivo maturation leads to a decrease in ZPB whereas in vitro maturation overall increases birefringence (Figure 3A). With respect to COC quality, only oocytes of high quality COC's (Q1) follow the trend of in vivo matured oocytes whereas oocytes of quality 2 and 3 COC's increased in birefringence (CV-Mean, Figure 3B). In vivo maturation also lead to a decrease in the thickness of the inner zona layer in contrast to the average of in vitro matured oocytes (Figure 3C). More in detail, oocytes of all individual COC qualities increased in thickness of the inner zona layer, however, quality 3 COC's increased strongest and quality 1 COC's most moderate (Figure 3D).

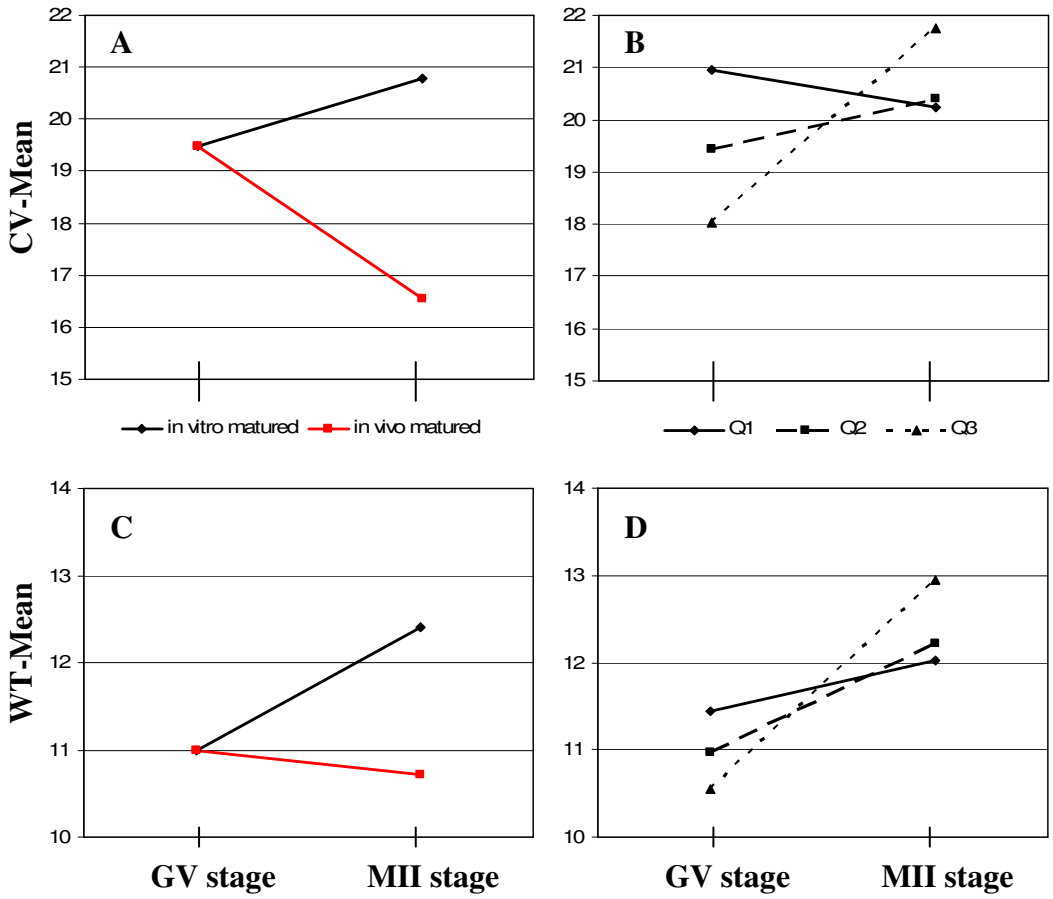

Figure 3: Dynamics from GV stage to MII in terms of zona birefringence (CV-Mean) and thickness of the inner zona layer (WT-Mean) with respect to maturational environment and COC's quality. (A) In vivo maturation leads to a decrease in ZPB whereas in vitro maturation overall increases birefringence. (B) With respect to COC quality, only oocytes of high quality COC's (Q1) follow the trend of in vivo matured oocytes whereas oocytes of Q1 and Q2 COC's increase in birefringence. (C) In vitro maturation also leads to a decrease in the thickness of the inner zona layer in contrast to the average of in vitro matured oocytes. (D) Oocytes of all individual COC qualities increased in thickness of the inner zona layer, however, quality 3 COC's increased the most and quality 1 COC's the least.

Experiment 4: Effect of G6PDH-activity of immature bovine oocytes on in vitro developmental competence and zona pellucida structure measured by ZPB

Immature Oocytes with low G6PDH-activity (BCB+) reached a significant higher cleavage rate compared to oocytes with low G6PDH-activity (BCB-, 75.5% vs. 65.1%) as well as a significantly higher blastocyst rates (32.0% vs. 11.5%) as presented in Table 5.

Table 5: Effect of G6PDH-activity of immature bovine oocytes on subsequent in vitro developmental competence

			Cleavagerate		Blastocystrate	
G6PDH - activity	**Growth status**	**n**	**n**	**(%)**	**n**	**(%)**
low	(fully grown)	416	314	(75.5) [a]	133	(32.0) [a]
high	(still growing)	358	233	(65.1) [b]	41	(11.5) [c]
average	(ordinary)	197	145	(73.6) [a]	47	(23.9) [b]

Values with different superscripts within columns differ significantly ($p < 0.05$)

Concurrently, the mean birefringence of oocytes with high G6PDH-activity and the thickness of the inner layer was significantly lower ($p<0.05$) compared to oocytes with low G6PDH-activity (18.83 ± 3.50 vs. 20.37 ± 4.49 and (10.84 ± 1.95 vs. 11.77 ± 2.35, respectively, Table 6).

Table 6: Effect of G6PDH-activity of immature bovine oocytes on zona pellucida birefringence measured with polarised light microscopy.

			CV-Mean	WT-Mean
G6PDH - activity	**Growth status**	**n**	Mean ± SD	Mean ± SD
low	(fully grown)	105	18.83 ± 3.50 [a]	10.84 ± 1.95 [a]
high	(still growing)	98	20.37 ± 4.49 [b]	11.77 ± 2.35 [b]

Values with different superscripts within columns differ significantly ($p < 0.05$)

Discussion

The characteristics of the ZP have been proposed to reflect the developmental competence of follicles and oocytes in human (Qi et al. 2002). Moreover, it is known that during follicular development in secondary follicles, the developing oocyte and follicular cells secrete the zona pellucida surrounding the plasma membrane of mammalian eggs (Dunbar et al. 1994).

This implies that any harm to the oocyte or to the surrounding cumulus cells caused by suboptimal conditions within the follicle at time of growth or maturation could alter the secretion and patterning of the extracellular coat (Shen et al. 2005). In other words, ZP properties could function as a marker of correct folliculogenesis and oocyte maturation. Studies performing scanning electron microscopy (SEM) reported that the number of ZP pores on the surface varies with oocyte quality (Assidi et al. 2008), with high quality oocytes having a greater number of pores than low quality oocytes in the bovine. With the aim to recapitulate the results of Santos et al. (2008) and the more to analyze whether zona characteristics are affected by maturational environment we compared the zona characteristics of in vivo and in vitro derived MII-stage oocytes. In the present study, we observed differences in zona characteristics analysed by scanning electron microscopy between immature oocytes with cumulus cell investments of different qualities, oocytes of different maturational stages and MII- stage oocytes matured either in vivo or in vitro environments. The COC's quality of immature oocytes correlates with morphological structures of the Zona pellucida. A higher proportion of good quality COC's (Q1-2) had a typical fine meshed pore structure whereas COC's of lower quality (Q3) were found to exhibit an irregular degraded emerging structure at higher proportions. That is inline with recent findings showing that COC quality affects zona characteristics (Santos et al. 2008). That study reported a strong and negative correlation between COC's quality and pores' diameter, including a high number of pores with a significantly smaller size for quality 1 oocytes compared to those of low quality (Santos et al. 2008). Additionally, that study showed that the amount of pores and the meshed structure of the outer surface alter drastically during in vitro maturation. In accordance to Suzuki et al. (1994) we observed alterations in the surface structure as a result of in vitro maturation to such an extent that the zona of all immature oocytes was found to be porous whereas only 31% of in vitro matured oocytes were porous. However, all in vitro matured porous oocytes exhibited a typical

wide meshed, fibrous network with deep pores whereas 43% of all immature porous oocytes showed an irregular pore structure. In contrast, all in vivo matured oocytes showed a homogenous structure with a typical reticular, fine-meshed surface with a very high amount of small pores in our study. This is inline with observations of Santos et al. (2008) who reported that in vitro maturation affects zona characteristics. Moreover, strong differences between the morphology of in vivo and in vitro matured oocytes were observed in porcine oocytes recently. In vivo matured porcine oocytes showed a fine-meshed and rough surface with a high amount of pores, whereas the in vitro matured oocytes had a smooth and tight surface (Funahashi et al. 2000). Taken together, all immature oocytes were found to be porous with only one third of them showing a regular pore structure. After in vitro maturation, the zona of only one third of all oocytes were found to be porous, however, all of them are being of typical structure. Thus, we suggest that irregular pores of immature oocytes disappear during maturation. Macchiarelli et al. (1992) suspected that the pores are generated through penetration of cytoplasm appendages of corona radiata cells, which are in contact with the plasma membrane. Hence, explanations for the alterations during in vitro maturation could be due to an inadequate contact with corona radiata cells, less distinct penetration or an early retraction of cytoplasm appendages in a proportion of oocytes, which may lead to a nearly pore less surface. Only one third of all oocytes entering maturation resulted in matured oocytes with regular pores. Interestingly that is very similar to the rate of development to the blastocyst stage usually obtained after in vitro fertilisation (Rizos et al. 2002b). Having that in mind, we speculate that a certain proportion of oocytes are entering our in vitro maturation system, which are not suitable for maturation because of insufficient contact to surrounding cumulus cells. Our results also confirm that the maturational environments as well as morphological features of the COC's are usefull as predictors for subsequent developmental competence. In vivo matured oocytes reached significant higher blastocyst rates compared to in vitro matured oocytes and high quality COC's reached higher rates compared to lower quality COC's. Whereas high quality COC's reached significant higher cleavage rates compared to low quality COC's no differences in term of cleavage rate were obtained between in vivo and in vitro matured oocytes.

These outcomes are completely comparable to those of an other study in which also no difference in cleavage rate between in vivo and in vitro matured groups but significant

differences in the blastocyst yield were reported (Rizos et al. 2002b). However, although zona characteristics correlate with developmental competence, it has not been possible to introduce this invasive technique to identify oocytes of superior developmental competence into lab routine because it completely damages the oocytes. To circumvent this problem we therefore aimed to test whether zona properties predictive for developmental competence could be evaluated through zona pellucida birefringence (ZPB) measurement, a new technique which has recently been introduced into human ART (Montag et al. 2008; Rama Raju et al. 2007; Shen et al. 2005). Likewise, our study shows that during maturation the structural changes visualized by SEM are reflected in the zona pellucida birefringence (ZPB). To our knowledge, the present study is the first, which analyses the environmental influence on Zona properties of bovine oocytes by polarized light microscopy. In addition, a direct comparison between SEM and ZPB has not been reported so far.

When comparing the ZPB of immature oocytes of different COC quality grades, COC's of high quality grade reached significant higher values compared to oocytes from COC's of lower quality. The higher birefringence is correlated to the thicker internal layer of the Zona pellucida (Kilani et al. 2006). Therefore, immature oocytes from high quality COC's have resulted in higher birefringence values and higher developmental competence at the same value. These results are inline with recent studies performed in human reproduction in which a positive correlation between thicker inner layers and high birefringence scores on the one side and higher developmental potential and a higher development to term on the other side were reported (Ebner et al 2010.; Madaschi et al. 2009; Montag et al. 2008; Rama Raju et al. 2007). In contrast, we observed a lower birefringence for in vivo matured vs. in vitro matured oocytes and matured oocytes of high quality COC's had significant lower values compared to their lower quality counterparts. Due to the higher developmental competence of in vivo matured oocytes, a thinner inner layer could be interpreted as being predictive for better developmental potential. This is in accordance with a recent study which reported that low mean values for zona birefringence parameters were related to superior zygote quality and subsequently led to better preimplantation development following artificial activation or IVF (Koester et al. 2011).

However, these results are not in agreement with a recent study performed in human in which a positive correlation between a thicker inner layer and better developmental

potential in human oocytes was reported (Rama Raju et al. 2007). Analysing the ZPB measurements of immature oocytes compared to matured ones, we found a strong decreasing trend from GV stage oocytes to in vivo matured oocytes. Within the groups of all in vitro matured oocytes only oocytes with high quality COC (Q1) showed a comparable trend for birefringence whereas oocytes from COC's of lower quality as well as in vitro matured oocytes showed an increasing trend. An explanation considering both observations could be that high ZPB values in immature oocytes are an indicator for high quality of immature oocytes and a decrease in ZPB during the process of maturation could be an indicator for successful maturation. Likewise a resent study in human reproduction cycles showed MI oocytes yielded a higher percentage of high-birefringence oocytes compared to MII stage oocytes, indicating that zona birefringence decreases as oocyte nuclear maturation takes place (de Almeida Ferreira Braga et al. 2010). These outcomes are comparable with those of Cheng et al. (2010) who analysed the birefringence of human oocytes during IVF cycles. Similar to our results a decreasing trend regarding zona parameters from GV stage to MII was reported (Bhojwani et al. 2007). The positive correlation between decreasing zona thickness and mean birefringence demonstrated for the in vivo matured oocytes is corresponding with results reported previously (Shen et al. 2005).

Thus, our results clearly show that maturational environment as well as quality of the cumulus cell investment of immature oocytes affects developmental competence as well as zona properties at the same value. However, although classification of oocyte quality by morphological characteristics could provide valuable information for the preselection of oocytes with higher developmental competence, this kind of method is not pretty precise. Therefore, we aimed to investigate if ZPB parameters as indicator for subsequent developmental competence of immature oocytes could be related to Glucose-6-Phosphate-Dehydrogenase (G6PDH) - activity, representing a molecular and subcellular predictor for oocyte quality of proven value (Pujol et al. 2004); (Alm et al. 2005; Bhojwani et al. 2007). In the present study, we observed significant differences between immature oocytes with contrasting G6PDH-activities. Our experiments confirmed a higher developmental competence of oocytes with low G6PDH-activity compared to oocytes with low activity in terms of cleavage rate and blastocyst rate. This is inline with observations in various species reporting different molecular and subcellular characteristics of oocytes due to contrasting G6PDH-

activities (Ghanem et al. 2007; Torner et al. 2008) as well as different developmental capacities including pig (El Shourbagy et al. 2006; Roca et al. 1998; Wongsrikeao et al. 2006), goat (Rodriguez-Gonzalez et al. 2003; Urdaneta et al. 2003), mouse (Mangia and Epstein 1975; Wu et al. 2007), buffalo (Manjunatha et al. 2007) and cattle (Alm et al. 2005; Bhojwani et al. 2007; Pujol et al. 2004). At the same value, immature oocytes with low G6PDH-activity exhibited a lower birefringence and a thinner inner layer compared to their counterparts with high G6PDH-activity. That result seems to conflict with the results of experiments 1-3, which brought us to the suggestion that higher ZPB values for immature oocytes are predictive for high developmental capacity. Indeed, immature oocytes showing low G6PDH-activity are correlated with higher developmental competence and lower ZPB values compared to immature oocytes with low G6PDH-activity. However, we have to keep in mind that immature oocytes with low G6PDH-activity are presumed to have completed their growth phase whereas oocytes with high activity are presumed to undergo still growth (Cheng et al. 2010). Torner et al. (2008) reported a higher proportion of oocytes with low G6PDH- activity being in progressed diakinesis stage whereas immature oocytes with high G6PDH-activity are with higher probability retarded in diplonte stage. Thus, on a hypothetic time line, growing oocytes (high G6PDH-activity) are developmentally retarded compared to ordinary immature oocytes and thereby might be less suitable for in vitro maturation as analyzed in experiment 3. Lower development capacity and higher ZPB scores approved the trend for ZPB during maturation by time, as observed in experiment 1-3, nicely. Concurrently, oocytes having completed their growth phase (lower G6PDH-activity) are developmentally progressed compared to ordinary immature oocytes. Therefore, results of experiment 4 are fitting well rather than being conflictive. Collectively, classifications of immature oocytes by COC morphology and G6PDH-activity are not comparable since oocytes are at different growth stages.

Taken together, in vivo matured M-II oocytes reached higher subsequent developmental competence and lower ZPB values compared to in vitro derived M-II oocytes. Considering in vitro matured oocytes, MII oocytes derived from high quality COC's classified according to cumulus investment, reached a higher developmental competence as well as lower ZPB values compared to those of lower quality COC's. Likewise, immature oocytes derived from high quality COC's reached higher developmental competence as well as higher ZPB values compared to immature

oocytes of lower quality COC's. In accordance, fully-grown immature oocytes reached higher developmental competence and lower ZPB scores compared to growing oocytes. In summary, we suggest a decreasing trend for ZPB during in vitro maturation to be typical for high quality oocytes. To our knowledge, this is the first work correlating zona pellucida birefringence of bovine oocytes with different environmental backgrounds as well as developmental competence. Moreover, by correlating ZPB with G6PDH-activity representing a molecular predictor of oocyte quality, we broke a limitation of similar studies about ZPB in human oocytes enlightening a link between molecular characteristics, zona pellucida properties and developmental capacity.

In conclusion, we successfully evaluated the applicability of Zona imaging to bovine oocytes: Alterations during in vitro maturation invasively analysed by scanning electron microscopy were reflected in the birefringence of the zona pellucida. Our results show that maturational environment and quality of immature oocytes classified by COC's investment and G6PDH-activity correlate with developmental competence and ZPB at the same value. Therefore, the polarized light microscopy is a useful tool offering some opportunities to improve selection of competent oocytes in assisted reproduction. However, further studies are necessary to improve the power of this promising new technique.

Declaration of interest

We declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Chapter 3

Transcriptome fingerprint of bovine 2-cell stage blastomeres is directly correlated with the individual developmental competence of the corresponding sister blastomere

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Abstract

To date, gene expression profiles of bovine preimplantation embryos have only been indirectly related to developmental potential due to the invasive nature of such procedures. This study sought to find a direct correlation between transcriptome fingerprint of blastomeres of bovine 2-cell stage embryos with developmental competence of the corresponding sister blastomeres. Isolated blastomeres were classified according to the sister blastomeres development into three groups: two groups displayed developmental incompetency, including those blastomeres whose corresponding sister blastomeres either stopped cleaving after separation (2CB) or blocked after two additional cleavages before embryonic genome activation (8CB). As a third group, competent blastomeres were defined as those whose sister blastomeres developed to the blastocyst stage (BL). As a result, developmental capacity of corresponding sister blastomeres was highly similar. Microarray analysis revealed 77 genes to be commonly differentially regulated between competent and incompetent as well as blocked blastomeres. Clustering of differentially expressed genes according to molecular functions and pathways revealed antioxidant activity, NRF2- mediated oxidative stress response and oxidative phosphorylation to be the main ontologies affected. Expression levels of selected candidate genes were further characterized in an independent model for developmental competence based on the time of first cleavage post fertilization (hpi). Moreover, overall results of this study were confirmed by higher developmental rates and more beneficial expression of *CAT* and *PRDX1* when cultured in a rather anti-oxidative environment. These results will help to understand molecular mechanisms defining developmental destination of individual bovine preimplantation embryos.

Key words: blastomere separation, gene expression profile, oxidative stress

Introduction

Deviant expression of developmentally important genes has been implicated as a causative factor of embryonic death during preimplantation development (reviewed by Lonergan et al. 2006). Several studies have been published during the last decade (Brevini et al. 2002; Dode et al. 2006; Lonergan et al. 1999) dealing with transcriptomic changes during the first cell cycles with the aim of detecting candidate genes related to developmental competence. However, transcriptome analysis by its nature is an invasive technique and precludes further embryonic development. Therefore, indirect correlation of gene expression between groups of embryos with contrasting developmental rates rather than directly with individual embryo development limits the value of gene expression studies. To circumvent this problem and to enable analysis of developmental competence and gene expression profile of the same embryo in parallel, recently we have performed an experiment in which blastocysts were biopsied before transfer to recipients to correlate transcriptome profile of the biopsy with pregnancy establishment (El-Sayed et al. 2006; Ghanem et al. 2011). A typical genetic signature for in vivo and in vitro derived preimplantation embryos developing to term compared to those lacking the ability to establish pregnancy was detected. In these studies we raised the question whether developmental competence at the blastocyst stage is a consequence of post fertilization culture environment or is due to the intrinsic quality of oocytes and thereby preimplantation embryo competence is predefined much earlier than the blastocyst stage. Since it has already been demonstrated that early cleaving embryos are more likely to develop to the blastocyst than their later cleaving counterparts, accompanied by higher abundance of developmentally related genes (Brevini et al. 2002; Dode et al. 2006; Fair et al. 2004; Lonergan et al. 1999), we assume that determination of developmental competence could be defined already in 2-cell stage embryos. However, direct evidence for that hypothesis is lacking.

To fill this research gap, we modified the technique of taking an embryo biopsy from the very early preimplantation embryo by separating sister blastomeres of bovine 2-cell stage embryos, allowing the correlation of the transcriptome in one blastomere with the developmental capacity of the sister blastomere. This approach should be accomplishable since early blastomeres are assumed to be totipotent and thereby undifferentiated allowing the embryo to regulate its development in order to overcome

failures in its organization such as cell loss. Direct evidence for totipotency is provided by the fact that an isolated blastomere has the ability to develop to term (reviewed by Edwards and Beard 1997). In addition, both blastomeres derived from a bisected 2-cell stage embryo retained a considerable degree of similarity, both in terms of cell number and rate of progression to blastocyst during their development (Katayama et al. 2010). Actually, single bovine blastomeres reach the blastocyst stage at about the same speed as control embryos, despite having two-thirds of the normal number of cells (unpublished from the authors' lab). Similarly, a recent study in bovine describes high synchrony in development of sister blastomeres to the blastocyst stage, to induce pregnancy and to develop to term (Tagawa et al. 2008). In addition to developmental fate, murine 2-cell stage blastomeres have been shown to differ very marginally in their transcriptome fingerprint. Although transcriptome asymmetry within mouse zygotes has been shown, no difference could be detected between early embryonic sister blastomeres, suggesting that any transcriptomic pre-patterning might not be detectable or even non-existing (Roberts et al. 2011; VerMilyea et al.2011). The more, sister blastomeres of bovine 2-cell stage embryos should display even higher similarities than in mice since major genome activation starts at a later developmental stage (Memili and First 2000).

Therefore, the aim of the present study was to evaluate the transcriptomic signatures of blastomeres of bovine 2-cell stage embryos and to relate this to subsequent developmental competence of sister blastomeres to the blastocyst stage. Moreover, we aimed to identify molecular functions and pathways to explain developmental competence enabling us to select developmentally important candidate genes as markers of developmental capacity in early bovine 2-cell stage embryos.

Materials and Methods

Oocyte collection

Bovine ovaries were obtained from a local slaughterhouse and transported to the laboratory in warm (30-35°C) physiological saline solution within 1-3 hours of slaughter. Antral follicles (2 to 8 mm in diameter) were aspirated using an 18-gauge needle attached to a 10 ml syringe and collected into a 50 ml conical tube. Cumulus oocyte complexes (COCs) with evenly granulated oocyte cytoplasm surrounded by more than three compact layers of cumulus cells, were selected and incubated (in

groups of 50) in 400 ml of maturation medium that consisted of TCM-199 (M-2154; Sigma) with Earle salts buffered with 4.43 mM HEPES (H-9136; Sigma) and 33.9 mM sodium bicarbonate (S-5761; Sigma) supplemented with 12% oestrus cow serum (OCS), 0.5 mM L-glutamine, 0.2 mM pyruvate, 50 mg/ml gentamycin sulphate and 10 µl/ml FSH (Folltropin, Vetrepharm, Canada) in four well dishes (Nunc, Roskilde, Denmark). Maturation medium was covered with mineral oil (Sigma-Aldrich) and was pre-incubated under maturation conditions for a minimum of 1 h (38.7 °C, 5% CO2 in air with maximum humidity) and then incubated for 22 h after oocytes were added.

In vitro embryo production until 2-cell stage

Following maturation, COCs were co-incubated with sperm (2×10^6 spermatozoa/ml) in fertilization medium consisting of Fert-TALP medium supplemented with 10 mM sodium lactate, 1 mM sodium pyruvate, 6 mg/ml BSA, 1 µg/mL heparin, 10 µM hypotaurine, 20 µM penicillamine, and 2 µM epinephrine at 38.7°C in 5% CO_2 in air. Eighteen hours after insemination (18 hpi), presumable zygotes were denuded of cumulus cells and washed three times in CR1aa (Rosenkrans et al. 1993) supplemented with 12% heat inactivated oestrus cow serum (OCS). Thereafter, embryos were cultured in 400 µl of CR1aa medium in four well dishes (Nunc, Roskilde, Denmark) in groups of 50 under mineral oil at 38.7°C in 5% CO_2 in humidified air until the 2-cell stage.

Bisection of 2-cell stage embryos

Separation of sister blastomeres in 2-cell stage embryos was conducted 28 to 30 hours after onset of IVF. First, the zona pellucida was removed by exposure of embryos to 0.25% Pronase (Sigma) in Dulbecco's phosphate-buffered saline (DPBS, GIBCO BRL) for 1–2 min, followed by gentle pipetting with a tapered Pasteur pipette. Secondly, sister blastomeres were separated by gentle pipetting in CR1aa medium.

In vitro embryo culture beyond the 2-cell stage

Bisected embryos were cultured individually in small wells (WoW's) until day 8 as described previously (Hoelker et al. 2009). Briefly, we prepared WoW's into the bottom of 5-well culture dishes (Fa. Minitüb, Germany) by using an industrial borer (ULTRA HSSE/Co Bohrer, DIN 1899, Nr. 186, Fa Baer Ultra Präzisionswerkzeuge GmbH, Weinheim). A total of 16 small holes were bored in a 4 × 4 cluster in each well. The holes were cylindrical in shape (0.7 mm depth and 0.7 mm diameter) with a slightly rounded bottom. Bored WoW's were cleaned by washing three times with

CR1aa culture medium. Then each well was overlaid with 400 µl CR1aa medium under mineral oil. After equilibration, one single blastomere and/ or embryo was placed in each WoW and were cultured at 38.7°C in 5% CO_2 in humidified air until day 8.

Comparing the developmental competence of corresponding sister blastomeres

Corresponding blastomeres derived from bisected 2-cell stage embryos were placed individually into the WoW culture system. Subsequent cleavage and development to blastocyst stage were evaluated at day 3 and 8, respectively. Subsequent development of separated blastomeres was classified as I.) no further cleavage (2CB), II.) one additional cleavage (4CB), III.) those which additionally cleaved two times to the 4-cell embryo but did not reach blastocyst stage (8CB) and IV.) those which reached blastocyst stage (BL).

Assessment of individual developmental competence by blastomere biopsy

Single blastomeres derived from bisected 2-cell stage embryos were placed individually into the WoW culture system while the corresponding sister blastomere was frozen stored individually. Subsequent cleavage and development to blastocyst stage were evaluated at day 3 and 8, respectively. Frozen stored blastomeres were pooled according to subsequent development of their corresponding sister blastomeres into one of three groups: I.) corresponding sister blastomeres did not cleave further after bisection (2-cell block, 2CB), II.) corresponding sister blastomeres stopped development after 2 additional rounds of cell cleavage at the 4-cell stage following bisection (8-cell block, 8CB) and III.) corresponding sister blastomeres developed to blastocyst stage (BL). An overview of this main experiment is presented in Figure 1. Embryos that cleaved once after bisection (4-cell block, 4CB) or 3 times (16-cell block, 16-CB) or those which stopped development at morula stage were not included in this experiment.

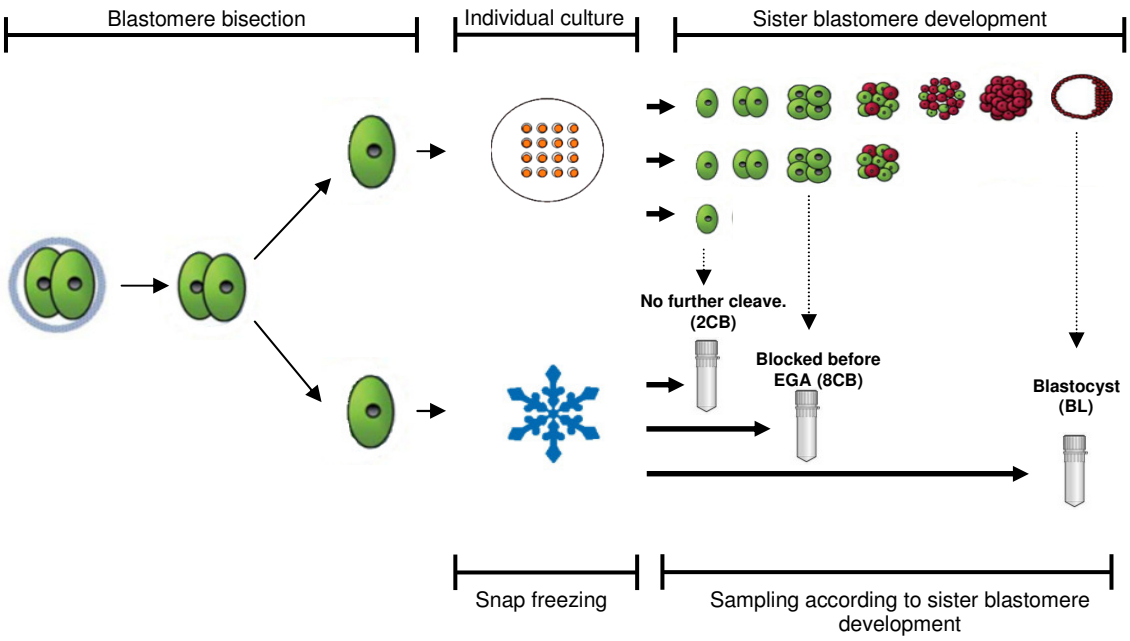


Figure 1: Experimental design: Single blastomeres derived from bisected 2-cell stage embryos were placed individually into the WoW culture system while the corresponding sister blastomere was frozen stored individually. Frozen stored blastomeres were pooled according to subsequent development of their corresponding sister blastomeres into three groups: I.) the corresponding sister blastomeres did not cleave anymore after bisection (2-cell block, 2CB), II.) the corresponding sister blastomeres stopped development after 2 additional rounds of cell-cleavage as 4-cell embryo following bisection (8-cell block, 8CB) and III.) the corresponding sister blastomeres developed to blastocyst stage (BL)

Further characterization of candidate genes using an independent model

Presumptive zygotes were examined at 27, 30, 32, 34, 36, 40 and 42 hours post insemination (hpi) for cleavage. Embryos, which cleaved before 32 hpi were considered to be developmentally competent whereas embryos cleaving later than 36 hpi were considered to be less competent as shown before (Brevini et al. 2002; Dode et al. 2006; Fair et al. 2004; Lonergan et al. 1999). In 4 replicates, freshly cleaved embryos were collected, washed 3 times in DPBS and placed in screw cap tubes (Axygen, California USA) followed by snap freezing in liquid nitrogen. Subsequently, frozen 2-cell stage embryos were pooled according to their time of first cleavage into two groups: I.) early cleavage, cleavage < 30 hpi and II.) late cleavage, cleavage >36 hpi.

Embryo culture under different oxygen tensions

Presumptive zygotes were transferred to CR1aa culture media and were cultured in groups of approximately 50 under 5% oxygen tension compared to 20% oxygen tension. Blastocyst rates were counted on day 7, 8 and 9. Secondly, 2-cell stage

embryos (3 pools each containing 20 embryos) derived from the contrasting oxygen tensions were collected 30 hours after fertilization for measuring the transcript abundance of *CAT* and *PRDX1* using RT real time PCR.

Staining of Reactive Oxygen Species

Intracellular ROS was visualized using the fluorescent probe 6-carboxy- 2',7'-dichlorodihydrofluorescin diacetate (H_2DCFDA). The stock solution was diluted in PBS to a working concentration of 5µM. 2-cell stage embryos were selected according to the time of first cleavage (hpi) as described above, washed twice in PBS and placed in a 4 Well dish containing 400µl of 5 µM H_2DCFDA. After 20 minutes incubation in the dark at 37°C, the embryos were washed twice in PBS and imaged immediately under Leica DM IRB inverted microscope with fluorescence filter.

RNA Isolation and Amplification

For further global gene expression analysis by array technology samples were prepared from 2CB, 8CB and BL pools (3 pools each containing 15 blastomeres). Total RNA isolation was performed using the PicoPure™ RNA Isolation Kit (Arcturs, Munich, Germany) according to the manufacturer's instruction. RNA integrity was evaluated using the Agilent 2100 Bioanalyzer with RNA 6000 Nano LabChip® Kit (Agilent Technologies Inc., CA, USA). Briefly, two rounds of RNA amplified transcription were carried out using Arcturus RiboAmp HS reagents (Molecular Devices, Sunnyvale, CA. USA) as described by (Somers et al. 2006). Likewise, pools of 2-cell stage embryos cultured under high and low oxygen tensions by RT real time PCR for quantification of CAT and PRDX1 were amplified in the same way.

For evaluation of transcript abundance of selected candidate genes in early and late cleaved bovine embryos, 8 pools each containing 60 embryos were sampled both for early and late cleaved embryos. Of these, 4 replicates were amplified using random hexamers (Promega) for reverse transcription to assess abundance of all transcripts whereas 4 replicates were amplified using oligo(dT)23 primers (Promega) for reverse transcription to assess abundance of poly-A-tailed transcripts. Amplified RNA were assessed using the Nanodrop 8000 spectrophotometer (Biotechnology GMBH, Erlangen, Germany).

Reverse Transcription on AmpliGrid Chips for comparative Single-blastomere qPCR

Corresponding blastomeres of 2-cell stage embryos were transferred in 1µl PBS onto an AmpliGrid AG480F slide (Advalytix, Munich, Germany), a glass chip with a surface structure for specific positioning of 48 discrete reaction sites. The blastomeres were air-dried followed by genomic DNA digestions using 0.1µl gDNA Wipeout Buffer (Qiagen, Hilden, Germany) (7-fold) diluted in 0.6 µl RNAse free water. Each reaction site was overlaid with 5µl sealing solution (Advalytix) and incubated at 42°C for 2 minutes using AmpliSpeed ASD100D cycler (Advalytix). For reverse transcription 2.3µl master mix solution containing 0.15µl Quantiscript reverse transcriptase, 0.6µl Quantiscript RT buffer (5-fold) and 0.27µl gene-specific RT-primer mix (3-plex: *GAPDH, CAT, PRDX,* 0.09µl of 10µM primer each) and 1.28 µl RNAse free water. The RT reaction was performed for 60 minutes at 42°C followed by 3 min at 95°C. Prior to quantitative PCR, cDNA was diluted 1:7 with PCR grade water. Real time PCR was performed as described above using 2µl cDNA per well.

Global Gene Expression Analysis

Amplified RNA samples of three pools per group (technical replicates) each consisting of 15 blastomeres (biological replicates) group were hybridized on EmbryoGENE's bovine microarray (made by Agilent 4 x 44k) using a two colour-dye-swap design Cy3 and Cy5 as described previously (Robert et al. 2011). After 17 hours of hybridization at 65°C, the microarray slides were washed first for 3 minutes in gene expression wash buffer (42°C), secondly for 10 seconds in 100% acetonitrile (RT) and 30 seconds in stabilization and drying solution (Agilent). The slides were scanned using the GenePix pro scanner. Data were submitted to a background correction, a Loess within array normalization, a quantile between array normalization, and statistically analyzed using linear models for microarray data analysis (LIMMA). Genes were considered differentially expressed at a fold-change >1.5 with adjusted P-value of <0.05 using the Benjamini and Hochberg false discovery rate method (FDR < 0.1). All analyses were performed using R and limma package. Normalized data were submitted to Gene Expression Omnibus (GEO) under series (GSE37986). A list of differential expressed genes (DEG's) was uploaded to DAVID software to group molecular functions into clusters. Ingenuity Pathway Analysis (IPA) software was used to identify relationships between the genes of interest and to uncover common pathways.

Validation of array results and further characterization of candidates by real time PCR

To validate microarray results, eleven genes were selected for further analysis by real-time PCR (*ATF1, BSG, MAPK14, CAT, NDUFS1, TEAD1, SYCP3, PRDX1, PRDX6, KRT8, SFRS12*) as presented in Table 1. Moreover, for further characterization, 5 validated candidate genes were selected to test their expression in an independent model for developmental competence by real-time PCR (*NDUFS1, MAPK14, CAT, PRDX1, and PRDX6*). Quantitative analysis of cDNA samples was performed using StepOnePlus™ real time PCR system (Applied Biosystems, Foster City, CA, USA). The cDNA synthesized of samples were subjected to real-time PCR using GAPDH primer to test for any variation in the expression of this internal control gene. Standard curves were generated for both target and internal control genes using serial dilution of plasmid DNA (10^1–10^9 molecules). The PCR was performed in a 20 µl reaction volume containing iTaq SYBR Green Supermix with ROX (Bio-Rad Laboratories, Munich, Germany), the cDNA samples and the specific forward and reverse primer in StepOnePlus™ real time PCR system (Applied Biosystem). The thermal cycling parameter was set as 95°C for 3 min, 40 cycles of 95°C for 15 s and 60°C for 1 min. After the end of the last cycle, dissociation curve was generated by starting the fluorescence acquisition at 60°C and taking measurements every 7 s interval until the temperature reached 95°C. Final quantitative analysis was done using the relative standard curve method and results were reported as the relative expression or n-fold difference to the calibrator after normalization of the transcript amount relative to the endogenous control (Tesfaye et al. 2004). In case of singe Cell PCR quantitative analysis was done using the comparative cycle threshold method (CT) and results were reported as the relative expression to the endogenous control. Statistical analysis of the expression values was carried out using the student's t-test.

Table 1: Details of primers used for real-time PCR quantitative analysis

Gene	Accession number	Primer sequences	Annealing temperature (°C)	Product size (bp)
GAPDH	NM_001034034	F:acccagaagactgtggatgg R:acgcctgcttcaccacctt	57	247
ATF1	NM_001075289	F: gacagcataggctcctcaca R: gcaatggcaatgtactgtcc	50	200
MAPK14	NM_001102174	F: cagccgacataattcacagg R: cattatgcatcccactgacc	50	212
CAT	NM_001035386	F: agccagaagagaaaccctca R: ctgcctctccatttgcatta	53	190
NDUFS1	NM_174820	F: ttagcaaatcacccactgga R: tgcctgtagttcccaaatca	55	204
TEAD1	XM_002693050	F: tctggccaggaatgatacaa R: gaagtttggttgtgccaatg	55	183
SYCP3	NM_001040588	F: gttcagaggaggatgccatt R: ggttttgagagaagccttgg	53	194
PRDX1	NM_174431	F: atgccagatggtcagttcaa R: gccaggtgacagaagtgaga	53	197
PRDX6	NM_174643	F: actcatggggcattctcttc R: gcaaggtcccgattcttatc	53	241
KRT8	NM_001033610	F: aatttgcctccttcatcgac R: ttccagcttcagcttctcct	53	185
BSG	NM_001075371	F: aggccagtactcctgcatct R: tcgcttccttgtaccacag	55	186

Protein Localization by Immunofluorescence

After collection of early-cleaving, late-cleaving and uncleaved 2-cell stage embryos, samples were fixed in 4% paraformaldehyde in PBS (pH 7.4) for one hour. The fixed samples were permeabilized in 0.5% (vol/vol) Triton-X100 (Sigma) in PBS and blocked in 3% (wt/vol) BSA (Roche Diagnostics) in PBS. This was followed by a 1 hour incubation in primary antibody Catalase Rabbit anti- Bovine Polyclonal Antibody (LifeSpan BioScience Eching, Germany), Rabbit Polyclonal antibody to Peroxredoxin 1 (Acris Antibodys, Herford Germany) (2–10mg/l) at 39°C and by exposure to secondary antiserum Alexa Flour 568 goat anti-rabbit IgG-FITC (Santa Cruz Biotechnology, Santa Cruz, CA) for 1hour at 39°C . Samples were placed in PBS in a Micro Slide (Lakeside Microscope Accessoirs, Monee, IL) and covered with a glass slide before being visualized on an ApoTome microscope (Carl Zeiss MicroImaging).

Results

Comparing the developmental competence of corresponding sister blastomeres

When corresponding blastomeres derived from bisection of a total of 128 bovine 2-Cell stage embryos were cultured individually, with highest probability none of the separated blastomeres reached the blastocyst stage (84.3%). Considering development to the blastocyst stage as bimorphic phenotype, development of corresponding sister blastomeres were different in only 3.1% of all bisected 2-Cell stage embryos with only one blastomere reaching the blastocyst stage. In contrast, the proportion of observations in which both blastomeres reached the blastocyst stage was 12.5 % resulting in a cumulative probability of 15.6 % (12.5% + 3.1%) that at least one blastomere developed to the blastocyst stage as shown in

More in detail, when a given blastomere derived from a bisected 2-Cell stage embryos did not cleave after separation a proportion of 70% of the corresponding sister blastomeres also did not cleave anymore (2-cell block, 2CB) and no sister blastomere reached the blastocyst stage (Figure 2). In case the given blastomere cleaved exactly once again to a 2-cell embryo (4-cell block, 4CB), 64% of the corresponding sister blastomeres stopped development exactly at the same stage whereas 19 % even did not cleave and 17 % progressed to the 4-cell embryo (8-cell block, 8CB) without reaching the blastocyst stage. When the given blastomere developed beyond the 4-cell embryo (8-cell block, 8CB) without reaching the blastocyst stage (BL), a majority of 80 % of corresponding sister blastomeres developed equally. Likewise, when the given blastomere reached the blastocyst stage (BL) nearly all corresponding sister blastomeres developed in parallel (94%) as presented in Figure 2. Overall, correlation of sister blastomeres developmental capacity was high ($y = 0.87 x + 0.38$, $R^2 = 0.73$).

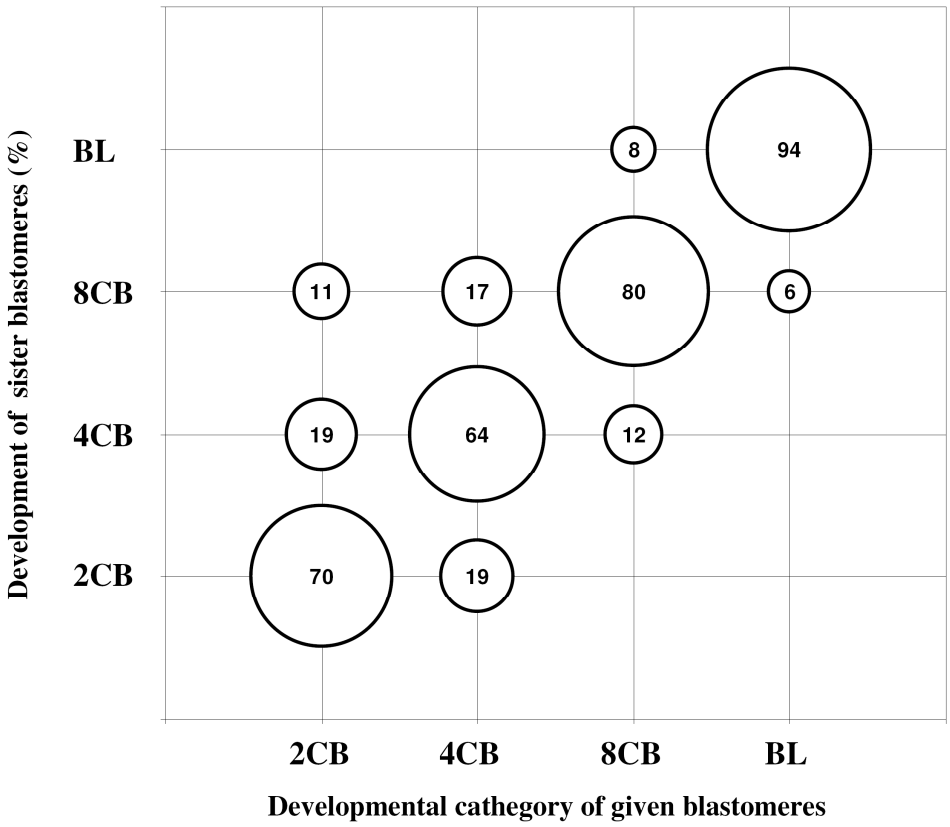


Figure 2: Developmental synchrony of corresponding bovine sister blastomeres
Proportion of sister blastomeres that developed to certain developmental stages (y-axis, 2CB = 2-cell block, 4CB = 4-cell block, 8CB = 8-cell block, BL = Blastocyst) based on developmental classification of the given blastomere (x-axis). When the given blastomere developed to 2-cell stage, 70 % of the corresponding sister blastomeres also stopped development at 2-cell stage. Likewise, when the given blastomere developed to the blastocyst stage 94 % of sister blastomeres also reached the blastocyst stage.

Microarray Results

The EmbryoGENE Microarray analysis revealed a total of 632 genes to be differentially regulated between those blastomeres whose sister blastomeres reached the blastocyst stage (BL) and those which did not cleave at all following separation (fold change ≥ 1.5, P≤ 0.05, FDR≤ 0.1). Of these, 298 genes were up regulated and 334 down regulated in BL group compared to 2CB group. Similarly, 150 genes were differentially regulated between BL group and those whose sister blastomeres stopped cleaving before embryonic genome activation (8CB) group of which 61 genes were up regulated and 89 genes were down regulated (fold change ≥ 1.5, P≤ 0.05, FDR≤ 0.1). Taken together, we found 77 genes (including 20 novel transcripts) to be commonly

differentially expressed in 2CB and 8CB groups compared to BL group as summarized in Figure 3A. Of these, 29 genes were up regulated and 48 genes down regulated (Supplemental Table 1). Moreover, we found 73 genes to be differential expressed exclusively in 8CB vs. BL group. Of these, 32 genes were upregulated and 41 genes were down regulated (Supplemental Table 2).

Molecular functions and pathway analysis

DAVID software clustered most affected molecular functions in BL vs. 8CB (n=150), namely transition metal ion binding, oxidoreductase activity, acting on peroxide as acceptor, peroxidase activity, antioxidant activity. Additionally differentially regulated genes were involved in the following biological processes: oxygen and reactive oxygen species metabolic process, negative regulation of molecular function, hydrogen peroxide catabolic process, cellular response to hydrogen peroxide, hydrogen peroxide metabolic process, response to hydrogen peroxide and cellular response to reactive oxygen species. Clustering of commonly differentially regulated genes (2CB vs. BL and 8CB vs. BL, n=77) uncovered oxidoreductase, peroxidase and antioxidant activity as most important biological functions affected by these genes (Figure 3C) whereas genes exclusively differentially expressed in 8CB vs. BL groups (n=73) uncovered cellular response to stress, regulation of cell proliferation and translation as most important biological functions affected by these genes (Figure 3D).

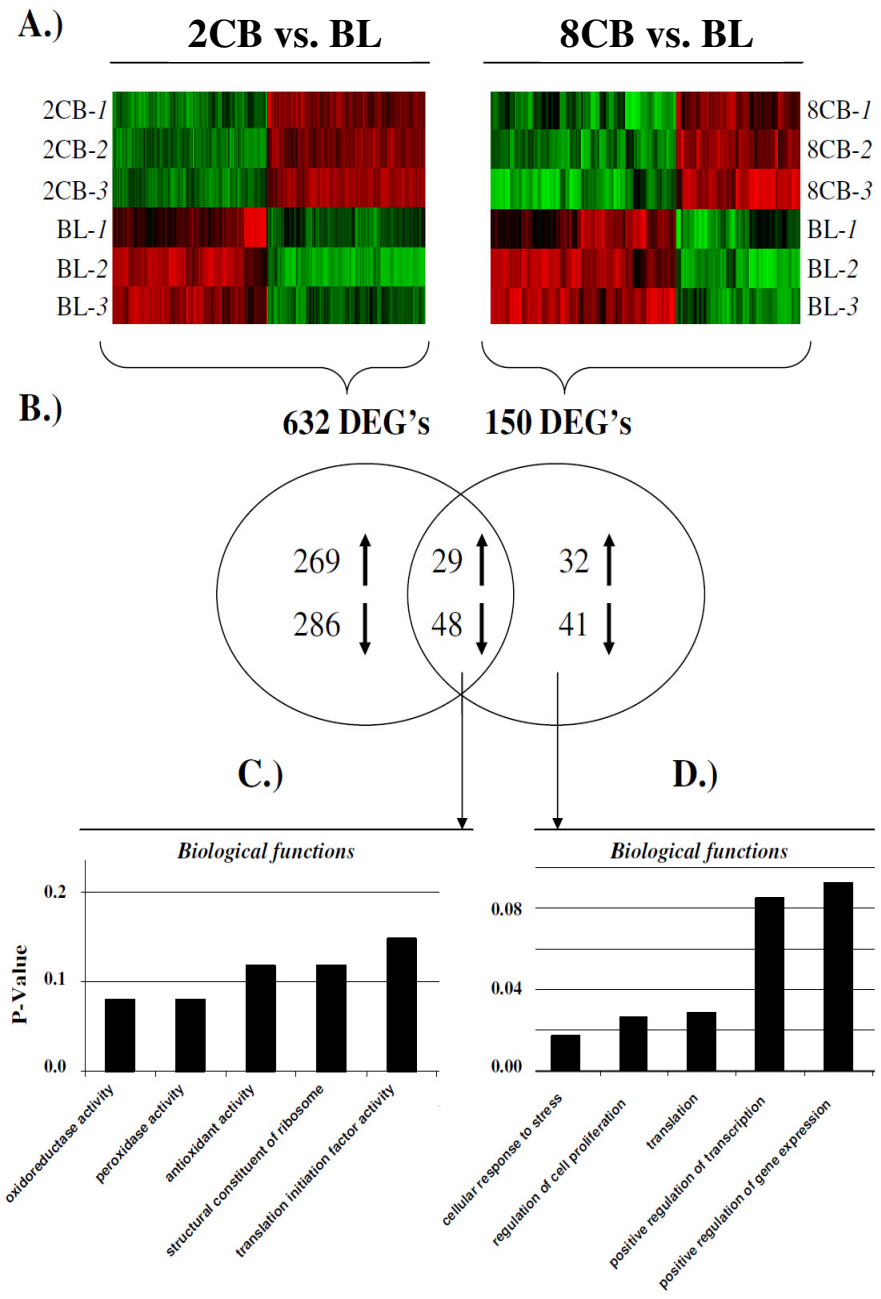


Figure 3: Differential regulated genes between blastomeres with contrasting developmental capacity

(A) Heatmaps of differentially regulated genes between 2CB and BL groups as well as 8CB and BL groups indicate 632 and 150 differential expressed genes. (B) The Venn-Diagram presents 77 genes to be differentially expressed in common for 2CB vs. BL groups and 8CB vs. BL groups. In contrast, 555 genes were differentially expressed exclusively in 2CB vs. BL groups and 73 genes are exclusively differentially expressed in 8CB vs. BL groups. (C.) Biological functions like oxidoreductase activity, peroxidase activity and antioxidant activity were affected with highest relevance (P-Value) by commonly differentially regulated genes (D.) Biological functions like cellular response to stress and regulation of cell proliferation were affected with highest relevance (P-Value) by genes exclusively differentially regulated in 8CB vs. BL groups.

Moreover, differentially expressed genes were uploaded to Ingenuity Pathway analysis (IPA) uncovering 15 pathways in 2CB vs. BL including Protein ubiquitination pathway, Protein kinase A signalling and Molecular mechanisms of cancer, to be affected by the highest numbers of molecules (18, 16 and 16). Additionally, oxidative phosphorylation and NRF2 mediated stress response were significantly affected by differentially expressed genes (Supplemental figure 1A). IPA of genes differentially regulated in 8CB vs. BL groups (Table 2) uncovered 8 pathways including NRF2 mediated stress response (Supplemental figure 1B).

Table 2: List of differentially regulated genes involved in pathways related to oxidative stress

Probe name	Gene Symbol	Description	Fold Change
Oxidative Phosphorylation in BL vs. 2CB			
EMBV3_14488	*NDUFS1*	NADH dehydrogenase (ubiquinone) Fe-S protein 1, 75kDa (NADH-coenzyme Q reductase)	2,05
EMBV3_35406	*SDHD*	succinate dehydrogenase complex, subunit D, integral membrane protein	1,60
EMBV3_12008	*NDUFC1*	NADH dehydrogenase (ubiquinone) 1, subcomplex unknown, 1, 6kDa	1,58
EMBV3_07268	*NDUFB9*	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 9, 22kDa	-1,58
EMBV3_36497	*ATP6V1B2*	ATPase, H+ transporting, lysosomal 56/58kDa, V1 subunit B2	-1,58
EMBV3_37271	*ATP6V0D1*	ATP6DV ATPase, H+ transporting, lysosomal 38kDa, V0 subunit d1	-1,61
EMBV3_34010	*ATP6V0C*	PLP ATPase, H+ transporting, lysosomal 16kDa, V0 subunit c	-1,62
EMBV3_17878	*COX6A1*	cytochrome c oxidase subunit VIa polypeptide 1	-1,62
EMBV3_33704	*NDUFS8*	NADH dehydrogenase (ubiquinone) Fe-S protein 8, 23kDa (NADH-coenzyme Q reductase)	-1,63
NRF2 mediated oxidative stress response in BL vs. 2CB			
EMBV3_36537	*MAP3K7*	mitogen-activated protein kinase kinase kinase 7	2,01
EMBV3_03149	*MAPK14*	mitogen-activated protein kinase 14	1,95
EMBV3_30827	*UBE2E3*	ubiquitin-conjugating enzyme E2E 3 (UBC4/5 homolog, yeast)	1,57
EMBV3_24218	*CAT*	catalase	1,53
EMBV3_20364	*GSTM3*	glutathione S-transferase mu 3 (brain)	-1,52
EMBV3_21587	*JUND*	jun D proto-oncogene	-1,59
EMBV3_39906	*AKR1A1*	aldo-keto reductase family 1, member A1 (aldehyde reductase)	-1,69
EMBV3_10622	*MRAS*	muscle RAS oncogene homolog	-1,85
EMBV3_00987	*DNAJB5*	DnaJ (Hsp40) homolog, subfamily B, member 5	-1,99
EMBV3_28741	*PRDX6*	KIAA0106 peroxiredoxin 6	-2,24
NRF2 mediated oxidative stress response in BL vs. 8CB			
EMBV3_24218	*CAT*	catalase	1,73
EMBV3_40942	*PRDX1*	peroxiredoxin 1	1,57
EMBV3_03401	*PRDX6*	KIAA0106 peroxiredoxin 6	-1,59
EMBV3_00664	*DNAJB12*	DnaJ (Hsp40) homolog, subfamily B, member 12	-1,69
EMBV3_00987	*DNAJB5*	DnaJ (Hsp40) homolog, subfamily B, member 5	-1,95

(Fold Change ≥ 1.5, p≤ 0.05, FDR≤ 0.1)

Array data validation by real time PCR

Eleven genes (*ATF1, BSG, CAT, KRT8, MAPK14, NDUFS1, PRDX1, PRDX6, SFRS12, SYCP3, and TEAD1*) were selected and quantified in three independent samples of 2CB, 8CB and BL groups. PCR results showed the expression of eight genes to be in agreement with the array results ($p < 0.05$). Additionally, two genes *(PRDX1, PRDX6)* showed the correct expression pattern (without statistical significance) and one gene (KRT8) did not show differences with real time PCR validation (Figure 4).

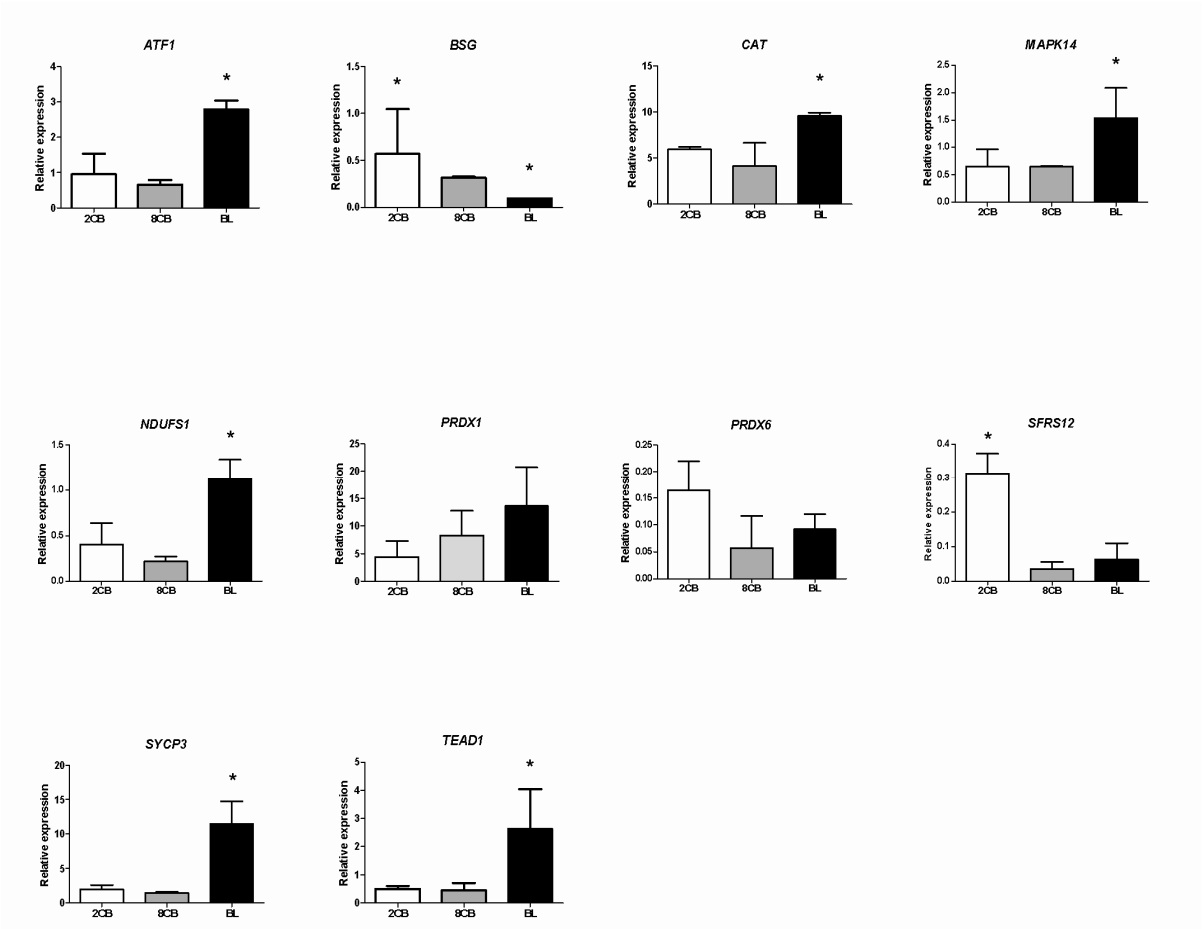


Figure 4: Confirmation of genes related to developmental capacity by RT real-time PCR
Three replicates with pools of 15 blastomeres each were analyzed. The amount of mRNA represents the Mean ± SD of each transcript, corrected with GAPDH as a housekeeping gene. (Columns with * differ significantly $p<0.05$). Overall, RT real time PCR results confirmed

Further characterization of selected candidates in an independent competence model

In order to investigate whether the expression of some of the candidate genes is associated with embryo competency, we used an independent competence model considering the time of first cleavage of 2-cell stage embryos, i.e. early cleaved (competent) vs. late cleaved (incompetent).

For this, we selected 5 candidate genes (*NDUFS1, MAPK14, CAT, PRDX1* and *PRDX6*) involved in oxidoreductase, peroxidase and antioxidant activity as well as in oxidative stress response - and oxidative phosphorylation pathways. In order to differentiate between the active and dormant forms of these transcripts, candidates were analyzed using cDNA synthesized from either random or oligo (dt) primers. Using random primed cDNA all genes were found to be more abundant in early cleaved embryos compared to the late cleaved ones except *NDUFS1* and *CAT*. However using oligo (dt) primed cDNA all genes showed significantly higher abundance in early cleaved embryos compared to the late cleaved ones. (Figure 5)

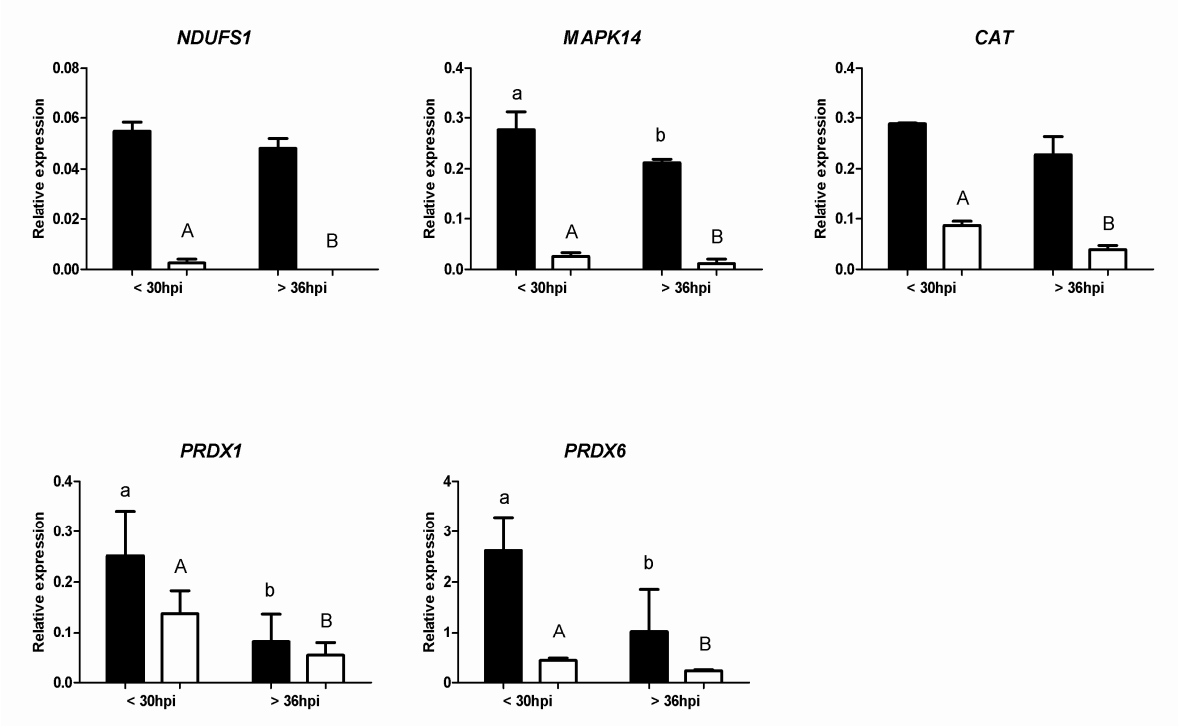


Figure 5: Effect of time of first cleavage post insemination on abundance of selected candidate genes: Abundance of selected candidate genes with respect to random forms (black bars) and polyadenylated forms (white bars) comparing early and late cleaved embryos (< 30 vs. >36 hours post insemination. Columns with different superscripts differ significantly $p<0.05$)

Detection of Reactive Oxygen Species (ROS)

The detection of Reactive Oxygen Species (ROS) after staining with H_2DCFDA revealed relatively low levels of ROS in early cleaving embryos (<30 hpi), higher amounts of ROS in late cleaving embryos (>36 hpi) and highest ROS levels in embryos which reached cleavage very late i.e. 42 hpi as indicated by Figure 6.

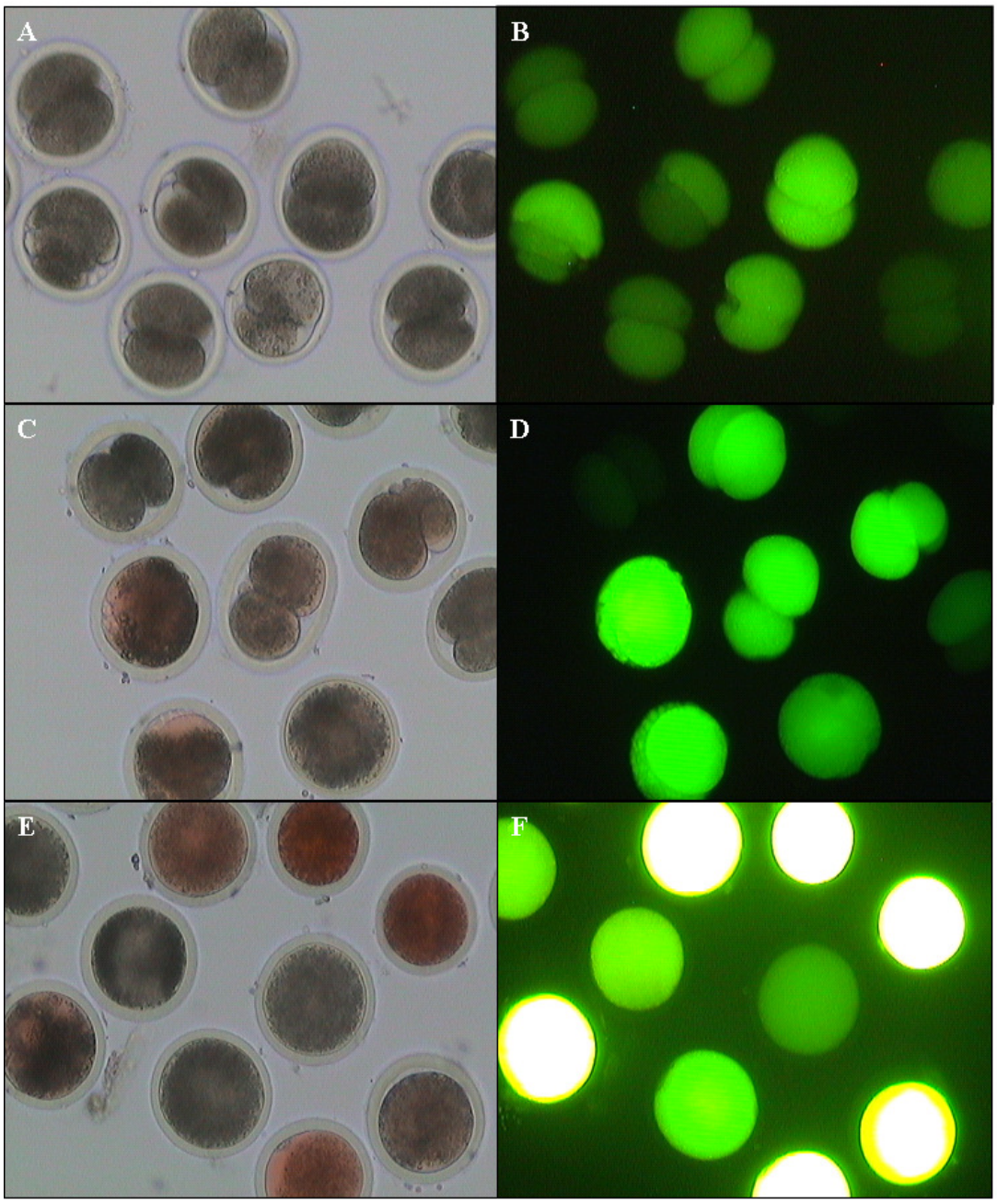


Figure 6: Effect of time of first cleavage post insemination on levels of Reactive oxygen species
Representative pictures revealing lower levels of Reactive oxygen species in early cleaved embryos (B) compared to late cleaved embryos (D) with highest levels observed in uncleaved embryos (F). The left side presents the same groups of early cleaved embryos (A), late cleaved embryos (B) and uncleaved embryos (E) without fluorescence in light field.

Developmental competence and expression levels of *CAT* and *PRDX1* according to environmental oxygen tension

Culturing of embryos in an oxygen tension of 5% representing a rather anti-oxidative environment resulted in significantly higher blastocyst rates compared to those embryos which were cultured under 20% oxygen (37.72% vs. 21.01%) representing a rather pro-oxidative environment (Table 3).

Table 3: Developmental rates of presumptive zygotes cultured under two different oxygen tensions, 5% O_2 and 20% O_2

		Cleavagerate		Blastocystrate d7		Blastocystrate d8		Blastocystrate d9	
Oxygen Tension	**n**	**n**	**(%)**	**n**	**(%)**	**n**	**(%)**	**n**	**(%)**
5 % O_2	517	409	79.11	123	23.79 [a]	174	33.65 [a]	195	37.72 [a]
20 % O_2	533	423	79.36	56	10.51 [b]	84	15.76 [b]	112	21.01 [b]

Values with different superscripts within columns differ significantly $p<0.05$

Considering the transcript abundance in 2-cell stage embryos of *CAT* we found a significantly higher abundance in those embryos cultured under 5% oxygen tension compared to 20%, the same trend was observed for *PRDX1* (Figure 7).

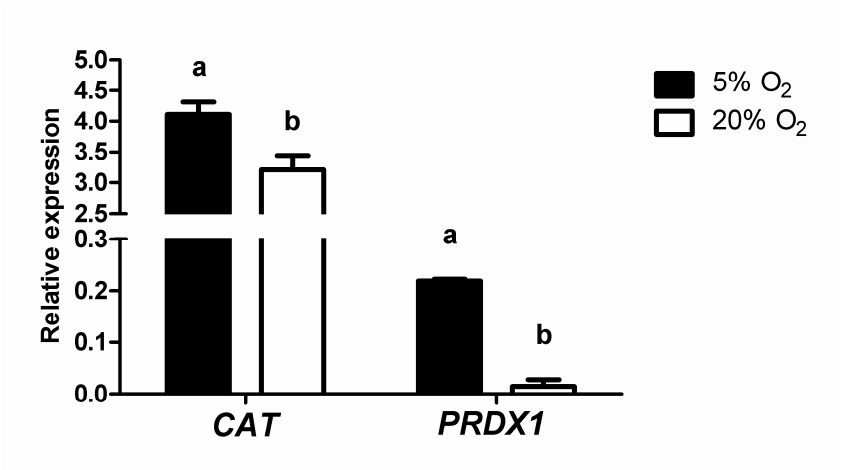


Figure 7: Effect of oxygen tension at culture on relative expression of *CAT* and *PRDX1* in 2-Cell embryos

Embryos cultured under rather anti-oxidative conditions (5% O_2, black bars) showed significantly higher expression of *CAT* and *PRDX1* compared to embryos cultured under rather pro-oxidative conditions (20% O_2, white bars). Columns with different superscripts differ high significantly $p<0.01$

Comparative expression of candidate Genes in single sister blastomeres

To find out whether the selected candidate genes *CAT* and *PRDX1* are expressed at comparable levels in both sister blastomeres of a 2-cell stage embryo a single blastomere RT real time PCR was conducted. When analyzing the corresponding sister blastomeres of 12 bisected bovine 2-Cell stage embryos, the results revealed that there were no significant differences in expression levels of *CAT* and *PRDX1* between the corresponding blastomeres (Figure 8).

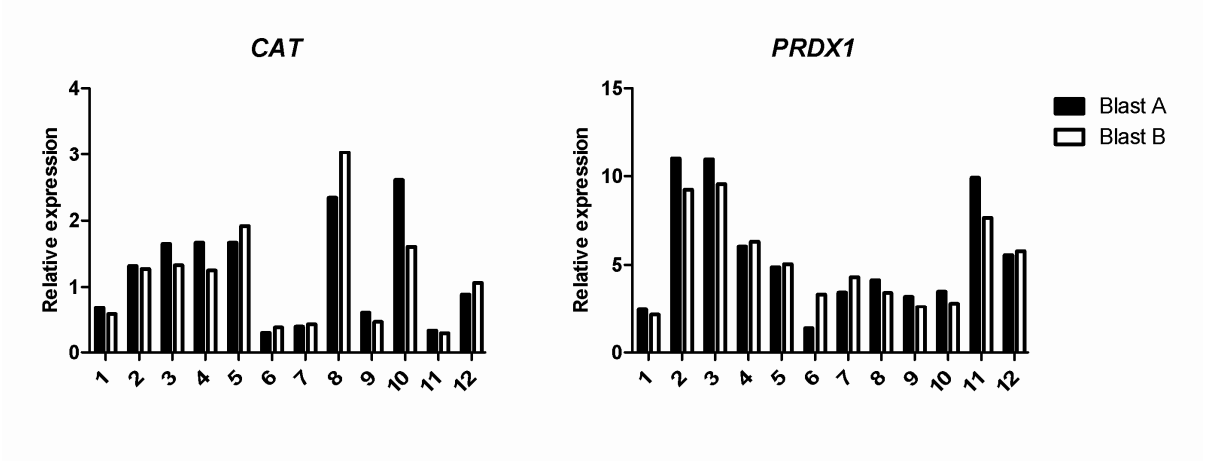


Figure 8: Comparative expression of *CAT* and *PRDX1* in bovine sister blastomeres. Analysis of a total of 12 pairs of sister blastomeres derived by separation of bovine 2-Cell embryos (No 1-12) analyzed by single blastomere RT real time PCR revealed high similarity without any significant difference in gene expression abundance.

Protein Localization by Immunofluorescence

Considering early and late cleaved bovine embryos, higher amounts of CAT protein was observed in early cleaved bovine embryos compared to late cleaved embryos. Similarly, late cleaved embryos showed relatively higher amounts of CAT protein compared to embryos that did not cleave until 42 hpi. No clear difference could be observed between early and late cleaved embryos in protein expression of PRDX1 gene. In contrast, expression of PRDX1 protein was much higher in uncleaved embryos compared to early and late cleaved embryos as presented in Figure 9.

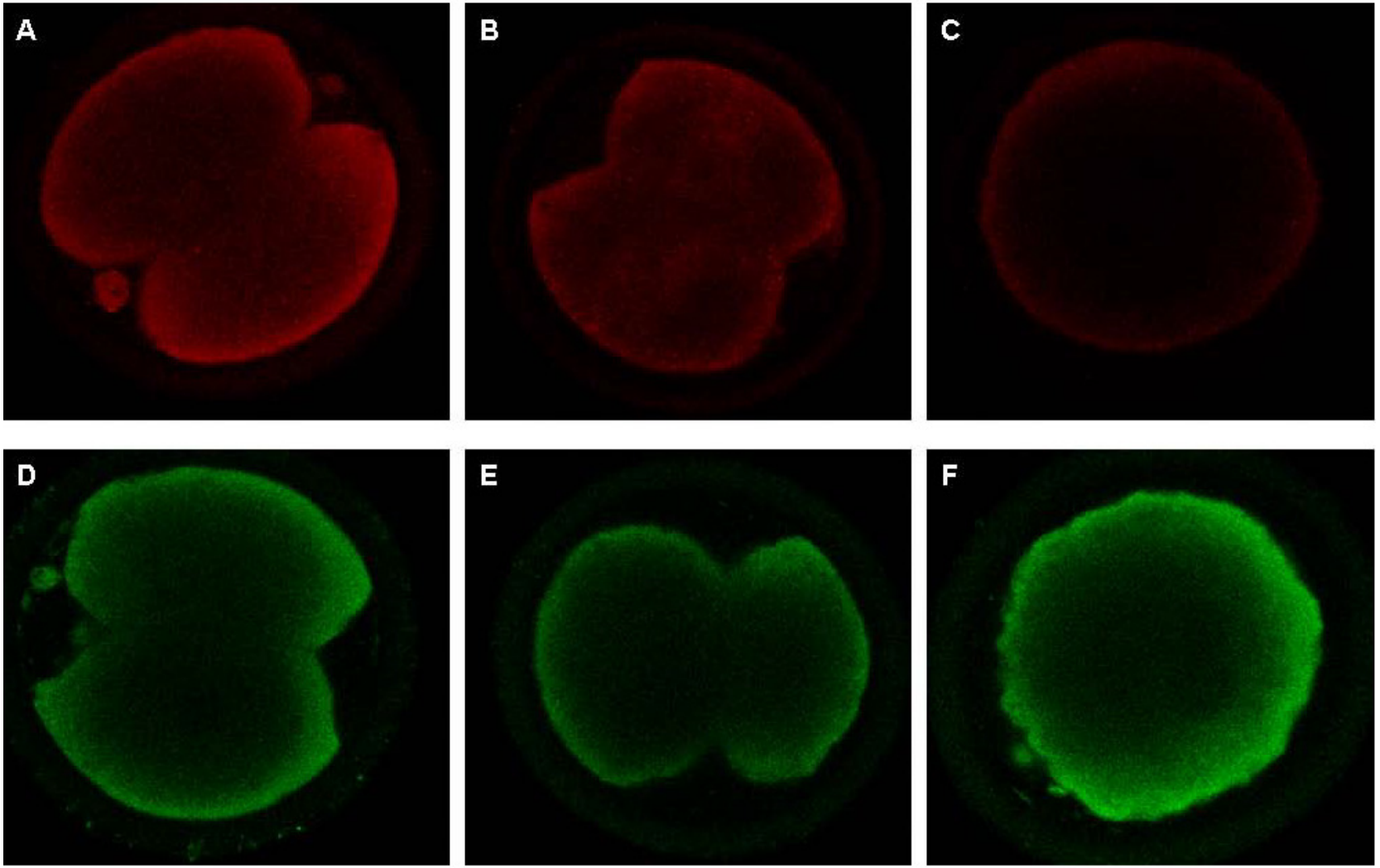


Figure 9: Protein localization of CAT and PRDX1 in bovine 2-Cell embryos
Representative immunoflourescence pictures of CAT (A-C) and PRDX1 (D-F) in early cleaved embryos (A+D), late cleaved embryos (B+E) and uncleaved embryos (C+F)

Discussion

During the last decade, several approaches analyzing transcriptome signatures related to developmental competence of bovine oocytes and embryos have been conducted (Corcoran et al. 2007; Donnison and Pfeffer 2004; Lonergan et al. 2003; Pfeffer et al. 2007; Sagirkaya et al. 2006). The biggest disadvantage of traditional transcriptome analyses is that only average expression characteristics of pools of embryos classified in groups with higher and lower developmental rates due to environmental (McHughes et al. 2009; Mohan et al. 2004; Niemann and Wrenzycki 2000), morphological (van Soom et al. 2003) or sub cellular characteristics (Torner et al. 2008) could be correlated. Efforts have been made to establish a correlation between developmental competence of a single preimplantation embryo and its gene expression characteristics by taking a biopsy of a blastocyst, subsequently transferring the blastocyst to a recipient animal and recording pregnancy rate and retrospectively pooling biopsy samples for transcriptome analysis on the basis of pregnancy outcome (El-Sayed et al. 2006; Salilew-Wondim et al 2010.; Gahnem et al. 2011). These studies clearly indicated that developmental competence of bovine blastocysts is associated with distinct gene expression signatures. However, these studies also raised the question of whether developmental competence is modulated by post fertilization culture environment or is due to intrinsic quality of oocytes used for fertilization and subsequent culture. To answer that question, the present work went some steps backwards on the temporal axis of preimplantation development to determine if transcriptome characteristics of bovine 2-cell stage embryos were related to subsequent in vitro developmental competence. The main strategy of our approach was to bisect 2-cell stage embryos which allows both to follow the developmental capacity of one blastomere and to analyze the transcriptome profile of the corresponding sister blastomere. In a preliminary experiment, we evaluated the developmental potential of intact (control) 2-cell embryos, zona-free 2-cell embryos and individual blastomeres derived from 2-cell embryos (Supplemental Table 3). No significant difference was observed in blastocyst rate between 2-cell stage embryos cultured with or without the zona pellucida. Although separated blastomeres developed at lower rates to the blastocyst stage compared to the other two groups, developmental rates were still sufficient for our strategy. Indeed, in our study we discovered an overall correlation coefficient of 73 % in terms of in vitro development of bisected bovine sister

blastomeres. Considering development to the blastocyst stage as bimorphic phenotype, corresponding blastomeres derived from a bisected 2-cell stage embryo developed differently with a probability of only 3.1%. Moreover, when a given blastomere developed to the blastocyst stage, the corresponding counterpart reached also the blastocyst stage with a probability of 94 %. These results indicate that developmental capacity of both blastomeres of a bovine 2-cell stage embryo is highly similar which is in accordance with previous studies in mouse (Chroscicka et al. 2004; Katayama et al.2010) and pig (Dang-Nguyen et al. 2011). However, it has been reported that murine 2-cell-stage blastomeres show biased differences in their fate (Piotrowska et al. 2001; Zernicka-Goetz 2005) whereas a study of Roberts et al. 2011 showed that both blastomeres of murine 2-cell stage embryos are similar in their individual transcriptomic fingerprint. That could be explained by the finding that the orientation of the first cleavage is generally thought to be meridional, along the animal-vegal axis, with the animal pole marked by the second polar body (Gardner 1997). A result of that is transcriptome asymmetry within blastomeres, but not between the first embryonic sister blastomeres both showing the same gradient similar to that of the former zygote (VerMilyea et al. 2011). In line with that, it has been demonstrated that developmental bias depends on the pattern of the second equatorial cleavage divisions (Piotrowska-Nitsche and Zernicka-Goetz 2005) resulting in molecular heterogeneities between blastomeres evident as early as at the 4- and 8-cell stages (Torres-Padilla et al. 2007) especially with respect to Oct4 kinetics (Plachta et al. 2011). These reports are supported by studies that demonstrated that one of the 2-cell blastomeres (the earlier to divide) contributes significantly more cells to the ICM (Piotrowska et al. 2001) because earlier dividing blastomeres generate more cellular contacts (Niwa et al. 2005) which are required to maintain Oct4 expression levels in embryonic blastomeres (Lorthongpanich et al. 2012). Consequently, cell-cell contact after second cleavage seems to be the key factor in determining whether a cell adopts a TE or ICM fate. Vice versa, in absence of cell-cell contacts due to second cleavage we suggest that singled sister blastomeres in our study not yet established polarity and did not show lineage pre-patterning in the separated blastomeres as it has reported recently (Lorthongpanich et al. 2012). Since both separated blastomeres of a 2-Cell embryo are still able to develop to term and to produce identical twins (Hancock 1954; Ozil et al. 1982; Seike et al. 1989; Tagawa et al. 2008) evidencing pluripotency, we believe that separated

sister blastomeres establish lineage pre-patterning as a consequence of polarity through subsequent cleavages one cell cycle later.

According to our main experimental design, we thus defined pools of blastomeres with high developmental competence which developed to the blastocyst stage (BL) and blastomeres with low developmental competence which stopped cleavage before embryonic genome activation (8-Cell stage, 8CB). Although, these embryos appear phenotypically as 4-cell embryos, they are determined as 8- cell stage embryos, taking into account that they are rather in the fourth cell cycle. Moreover we classified one group of blastomeres with very poor developmental competence which failed to cleave after separation and thereby blocked development in 2-cell stage (2CB). This developmentally high incompetent group was set up to enable identification of genes commonly differential expressed in blastomeres of very poor developmental competence (2CB) as well as in blastomeres which were able to cleave some round without reaching blastocyst stage (8CB). That approached allowed us to exclude genes which are differentially expressed exclusively in the 2CB group since we suggested that failure of second cleavage after separation could have been induced through mechanical damage in that case.

Our results revealed differences at the molecular level for those blastomeres whose counterparts developed to the blastocyst stage (BL group) compared to those with lower developmental competence (2CB and 8CB groups). This is in line with our previous results where biopsies from in vitro derived (El-Sayed et al. 2006) and in vivo derived (Gahnem et al. 2011) blastocysts were taken. Global transcriptome analysis revealed 632 differentially regulated genes between BL group and 2CB group and 150 differentially regulated genes between BL group and 8CB group. Cluster analyses of DEGs between BL and 8CB showed a clear trend towards molecular functions and biological processes related to the cells' response to oxidative stress, including oxidoreductase activity, acting on peroxide as acceptor, peroxidase activity, antioxidant activity and oxygen and reactive oxygen species metabolic process, negative regulation of molecular function, hydrogen peroxide catabolic process, cellular response to hydrogen peroxide, hydrogen peroxide metabolic process, response to hydrogen peroxide and cellular response to reactive oxygen species. A total of 77 transcripts were commonly differentially regulated. Interestingly, all 77 transcripts showed the same expression trend. Functional analysis of those genes again unraveled their

predominant involvement in NRF2- mediated oxidative stress response and oxidative phosphorylation (OXPHOS) pathways and biological functions including oxidoreductase-, peroxidise- and antioxidant- activity. This led us suggest, that those functions and processes described above are mainly responsible for developmental competence. Accordingly, we selected several candidate genes based on these findings as well as some genes by random and successfully validated array results using quantitative real time PCR.

Several studies have evidenced the negative impact of oxidative stress and Reactive Oxygen Species (ROS) on preimplantation embryonic development (Bain et al. 2011; Guerin et al. 2001; Johnson and Nasr-Esfahani 1994). Even marginal elevated levels of ROS can interrupt maturation and early embryonic development (Harvey et al. 2002; Liu et al. 2000; Van Blerkom 2011) and certain amounts of ROS are considered to be important to regulate various cell functions including programmed cell death (Harvey et al. 2002; Rhee 2006). Elevated levels of ROS have been reported to be a consequence of weak culture environments (Goto et al. 1993), but ROS is also produced by the embryo itself within the respiratory chain (reviewed by Guerin et al. 2001). Balance of ROS production and ROS scavenging, described as reduction-oxidation (REDOX) state is suggested to play an important role for optimal growth response (Burdon 1996). Taken together, we hypothesized that expression of genes involved in ROS generation as well as in ROS scavenging correlated with subsequent in vitro developmental competence. To proof our hypothesis, we therefore aimed to further characterize candidate gene expression in an independent model.

Bovine 2-cell stage embryos were classified based on the time of first cleavage post insemination (hpi) which has been shown to be a reliable predictor for developmental competence in a variety of mammalian species. It is widely accepted that a greater proportion of early cleaving bovine embryos develop to the blastocyst stage compared to those cleaving later, which was also accompanied by differences in relative abundance of developmentally related genes (Brevini et al. 2002; Dode et al. 2006; Fair et al. 2004; Lonergan et al. 2000). Consequently, we evaluated gene expression of our candidates related to OXPHOS (*NDUFS1*) and oxidative stress response (*MAPK14, CAT, PRDX1, PRDX6*) in competent (early cleaved) embryos as a model for high developmental competence as well as in incompetent (late cleaved) embryos as a model for low developmental competence in terms of general expression of candidate

genes as well as polyadenylated forms. It is generally accepted that the early embryo is dependent on maternally-inherited transcripts accumulated during oogenesis (Betts and Madan 2008) with variable translational activity of mRNAs due to the proportion of polyadenylated forms. Post-transcriptional regulation mechanisms regulate the activity of maternal inherited transcripts, by shortening the polyA tail for further storage and to protect the transcripts from degradation. With respect to detect active forms of transcripts, real time PCR using oligo dt (23) primers was conducted in the present approach. Considering polyadenylated forms, a significantly higher expression was observed in early cleaving embryos compared to late cleaving embryos with respect to all characterized genes. Accordingly, *NDUFS1* expression was significantly upregulated in blastomeres with the ability to develop to the blastocyst stage compared to those that did not cleave at all after blastomere separation. Previous studies reported NDUFS1 as one of the most important subunits of the NADH:ubiquinone oxidoreductase mitochondrial complex I, which is described as the largest complex of the OXPHOS system (Brandt 2006) regulating energy metabolism (Finel 1998) and producing superoxide (Koopman et al. 2010). Similarly, our results confirmed that *MAPK14,* also known as *p38*, to be significantly up-regulated in early cleaved embryos compared to late cleaved ones both in terms of random and polyadenylated forms. MAPK14 itself was detected to be activated by ROS (Matsuzawa and Ichijo 2008), activates antioxidant enzymes like CAT (Gutierrez-Uzquiza et al.2012) and indirectly regulates oxidative stress response by modulating the expression of antioxidant enzymes including NRF2 (Limon-Pacheco et al. 2007; Niture et al. 2010; Pi et al. 2008; Zipper and Mulcahy 2000). In parallel to elevated *MAPK14*, we found active ROS scavengers *CAT* and *PRDX1* to be up-regulated in early cleaved 2-cell embryos compared to their later cleaved counterparts. Again, this is inline with our results obtained in developmentally competent bisected blastomeres. CAT and PRDX1 eliminate destructive hydrogen peroxide (H_2O_2) from the embryo to assure viability of embryos (Leyens et al. 2003; Orsi and Leese 2001) and thereby acting in sequence in order to reduce H_2O_2. Under low H_2O_2 concentrations, PRDX1 scavenges more efficiently due to its higher affinity towards H_2O_2 whereas under high H_2O_2 concentrations CAT scavenges more efficiently (Neumann et al. 2009). Like NDUFS1, PRDX1 and CAT are located in the mitochondrial membrane and considerable higher expression of all three in developmentally more competent early cleaved embryos may

be correlated with higher numbers of mitochondria reported to be symmetrically segregated in both blastomeres of the 2-cell stage embryo (Tarazona et al. 2006). It is well known that aberrant levels of maternally-inherited mitochondrial DNA (mtDNA) are associated with lower developmental competence (Cummins 2002; Spikings et al. 2007). Likewise, in insulin-resistant mice, a low number of mtDNA in MII oocytes accompanied by oxidative stress causes disruption of ATP biosynthesis resulting in poor oocyte quality and adverse embryonic development (Thouas et al. 2004). Finally, *PRDX6* was found to be significantly upregulated in analysis of random and oligo (dt) primed cDNAs in early cleaving embryos compared to late cleaving ones, being in conflict with a slightly lower expression (without significance) in blastomeres with high developmental competence. However, it has been noticed that *PRDX6* is expressed with decreasing abundance from immature oocytes to 8-cell stage embryos without expression from 16-cell stage to morula and an increase again in blastocyst stage (Leyens et al. 2004). Taking that in mind, lower expression in competent blastomeres could indicate that these embryos are one step ahead on the temporal axis of embryonic development compared to developmentally less competent blastomeres. Likewise, lower expression of *PRDX6* in late cleaving embryos could be explained by being farther from the time point of fertilization than their early cleaving counterparts. In this regard, abundance of *PRDX6* could be interpreted as reflector for developmental speed rather than for direct viability.

Consequently, we aimed to confirm whether expression of genes involved in ROS generation as well as in ROS scavenging fits with levels of ROS in bovine 2-cell stage embryos. ROS staining revealed considerable lower ROS levels in early cleaved embryos compared to late cleaved embryos with highest levels in those embryos which did not cleave. Additionally, analysis of mitochondrial activity during preimplantation development of bovine embryos revealed a significantly higher activity in early cleaved 2-Cell stage embryos compared to their later cleaving counterparts (Tarazona et al. 2006). The amount of mitochondria regulate ROS levels, whereas impaired mitochondria have been reported to produce more ROS (Ou et al. 2012) implicating low ROS levels being a reliable marker for developmental competence (Bain et al. 2011) which is inline with our results in terms of NDUFS1 and PRDX1 gene expression in competent embryos. Taken together, we were able to confirm that expression of genes involved in ROS generation as well as in ROS scavenging

coincidentally correlates with ROS levels and developmental capacity in bovine 2-cell stage embryos.

Thus, culture in presence of anti-oxidants may give rise to and a better gene profile resulting in better developmental competence whereas culture in pro-oxidants may induce a worse gene profile and lower development. To confirm that, we investigated the transcript abundance of the active ROS scavengers *CAT* and *PRDX1* in embryos cultured under high oxygen tension (20% O_2) representing a rather pro-oxidant environment compared to culture in low oxygen tension (5% O_2) representing a rather anti-oxidant environment. As a result, blastocyst rate was significantly higher when embryos were cultured in 5% O_2 compared to 20% O_2 being inline with recent publications (Guerin et al. 2001; Takahashi et al. 2000). Additionally, we identified higher levels of *CAT* and *PRDX1* in 2- cell stage embryos cultured in rather anti-oxidant environments (5% O_2). These results are also in accordance with findings of Correa et al. who detected higher levels of CAT and three other transcripts related to oxidative stress response, in blastocysts cultured under 5% O_2 compared to those cultured under 20% O_2 (Correa et al. 2008). Thus, the more anti-oxidant environment resulted in a better gene expression profile of the active ROS scavengers *CAT* and *PRDX1* as well as in higher developmental competence supporting the overall findings of this study.

To confirm the validity of our experimental approach as well as our conclusions, prior to protein localization we checked at which degree corresponding sister blastomeres are actually representative for each other in terms of *CAT* and *PRDX1* expression. In agreement with studies mentioned above which reported that both sister blastomeres have similar transcriptome profiles (Roberts et al. 2011; VerMilyea et al. 2011), we confirmed by single blastomere PCR that both sister blastomeres of a bisected bovine 2-Cell stage embryo show indeed highly similar expression of *CAT* and *PRDX1*. That is in great accordance to a very recent study reporting that singled sister blastomeres have not established polarity and do not show lineage pre-patterning (Lorthongpanich et al. 2012).

Finally, results of the localization of CAT and PRDX1 protein in early and late cleaving bovine embryos showed that the amount of CAT protein was in line with its relative expression levels in early -, late - a non cleaved embryos. Accordingly, we found marginally higher amounts of PRDX1 protein in early cleaved embryos

compared to late cleaved ones being in agreement with *PRDX1* gene expression results. Surprisingly, higher levels were detected in those embryos, which did not cleave until 42 hpi which did not reflect gene expression abundance in non cleaved embryos. However, that may be explained by the fact that extreme levels of H_2O_2 leads to oligomerization of PRDX1 (Jang et al. 2004) and activation of Mammalian Ste20-like kinase 1 (MST1) inducing apoptosis (Morinaka et al. 2012).

In conclusion, this is the first study highlighting the potential of using blastomeres at the 2-cell stage for transcriptome analysis to correlate with the developmental fate of sister blastomeres. We were able to show that the developmental capacity of the sister blastomeres derived from a bisected bovine 2-cell embryo are highly similar. Consequently, results established distinct molecular fingerprints, which could be related to developmental potential and arrest. In particular, pathways and molecular functions including oxidative phosphorylation and oxidative stress response which are applicable involved preimplantation development were identified. Based on this, candidate genes were selected and further confirmed by characterization in an independent model for developmental competence being in agreement with ROS levels of bovine 2-cell stage embryos supporting their value, implicating that the balance of ROS production and scavenging is decisive for preimplantation development in vitro. Moreover, these results were confirmed by culture of bovine embryos in different oxygen tensions resulting in higher developmental rates and more beneficial expression of *CAT* and *PRDX1* when cultured in rather anti-oxidative environments. Altogether, we suggest, that this method could be adapted as a model for genomic selection of embryos in future breeding programs as well for preimplantation genetic diagnostics (PGD) in human reproductive medicine.

Supplemental data

Table S1: Genes commonly differentially expressed in BL vs. 2CB and BL vs. 8CB

Probe Name	*Gene Name*	*Description*	*foldchange*
EMBV3_40561	DIRC2	disrupted in renal carcinoma 2	2,07
EMBV3_33958	MGC133632	hypothetical protein LOC614279	1,75
EMBV3_17950	BRP44L	brain protein 44-like	1,73
EMBV3_24218	CAT	Catalase	1,73
EMBV3_09660	KDR	flk-1 kinase insert domain receptor (a type III receptor tyrosine kinase)	1,71
EMBV3_40207	NPTN	Neuroplastin	1,70
EMBV3_25638	EPCAM	TACSTD1 epithelial cell adhesion molecule	1,68
EMBV3_11919	PSD2	pleckstrin and Sec7 domain containing 2	1,66
EMBV3_43666	HBXIP	hepatitis B virus x interacting protein	1,64
EMBV3_25866	DCLRE1A	DNA cross-link repair 1A (PSO2 homolog, S. cerevisiae)	1,63
EMBV3_30040	LOC100138864	similar to Eukaryotic initiation factor 4A-II (ATP-dependent RNA helicase eIF4A-2) (eIF4A-II) (eIF-4A-II)	1,61
EMBV3_14488	NDUFS1	NADH dehydrogenase (ubiquinone) Fe-S protein 1, 75kDa (NADH-coenzyme Q reductase)	1,61
EMBV3_14611	ATF1	activating transcription factor 1	1,60
EMBV3_12725	WDR11	PHIP WD repeat domain 11	1,59
EMBV3_35924	LEPROT	leptin receptor overlapping transcript	1,58
EMBV3_34731	SYCP3	synaptonemal complex protein 3	1,58
EMBV3_17165	ANGEL2	angel homolog 2 (Drosophila)	1,57
EMBV3_34635	CCT5	chaperonin containing TCP1, subunit 5 (epsilon)	1,57
EMBV3_28109	COX16	MGC137677 COX16 cytochrome c oxidase assembly homolog (S. cerevisiae)	1,57
EMBV3_14240	DONSON	downstream neighbor of SON	1,55
EMBV3_28693	LOC100336519	FIP1L1 protein-like	1,55
EMBV3_15239	ZNF280B	zinc finger protein 280B	1,55
EMBV3_29105	LOC534360	similar to poliovirus receptor-related 3	1,54
EMBV3_36580	RWDD4A	RWD domain containing 4A	1,54
EMBV3_09584	COMMD8	COMM domain containing 8	1,53
EMBV3_23296	KLRAQ1	MGC151568 KLRAQ motif containing 1	1,53
EMBV3_24891	NDFIP2	Nedd4 family interacting protein 2	1,53
EMBV3_13360	TMEM209	transmembrane protein 209	1,53

EMBV3_33765	BANP	BTG3 associated nuclear protein	1,52
EMBV3_34010	ATP6V0C	PLP ATPase, H+ transporting, lysosomal 16kDa, V0 subunit c	-1,52
EMBV3_30424	LOC100316905	nucleoplasmin 2	-1,52
EMBV3_30676	PSENEN	presenilin enhancer 2 homolog (C. elegans)	-1,52
EMBV3_02632	WIBG	within bgcn homolog (Drosophila)	-1,53
EMBV3_40039	AURKB	STK12 aurora kinase B	-1,54
EMBV3_22837	MAD2L2	MAD2 mitotic arrest deficient-like 2 (yeast)	-1,55
EMBV3_36642	C5H12orf45	MGC133846 chromosome 12 open reading frame 45 ortholog	-1,56
EMBV3_25759	DDOST	dolichyl-diphosphooligosaccharide-protein glycosyltransferase	-1,56
EMBV3_29618	RTF1	Rtf1, Paf1/RNA polymerase II complex component, homolog (S. cerevisiae)	-1,56
EMBV3_13440	SRRM2	serine/arginine repetitive matrix 2	-1,56
EMBV3_17375	C1orf35	chromosome 1 open reading frame 35	-1,57
EMBV3_19544	GTF2H5	general transcription factor IIH, polypeptide 5	-1,57
EMBV3_21021	RTF1	Rtf1, Paf1/RNA polymerase II complex component, homolog (S. cerevisiae)	-1,57
EMBV3_43620	LOC534065	similar to erythroid differentiation-related factor 1	-1,58
EMBV3_07103	NOP56	NOL5A NOP56 ribonucleoprotein homolog (yeast)	-1,58
EMBV3_27964	EIF4E	eukaryotic translation initiation factor 4E	-1,59
EMBV3_03401	PRDX6	KIAA0106 peroxiredoxin 6	-1,59
EMBV3_17388	SRRM2	serine/arginine repetitive matrix 2	-1,59
EMBV3_20195	LOC100337332	splicing factor, arginine/serine-rich 12-like	-1,60
EMBV3_19931	ADAMTSL5	ADAMTS-like 5	-1,61
EMBV3_43173	CCDC106	coiled-coil domain containing 106	-1,61
EMBV3_41957	ZNF804B	zinc finger protein 804B	-1,61
EMBV3_40631	EIF4E	eukaryotic translation initiation factor 4E	-1,62
EMBV3_09407	POLR2J	POLR2J2 polymerase (RNA) II (DNA directed) polypeptide J, 13.3kDa	-1,64
EMBV3_11515	TLCD1	TLC domain containing 1	-1,64
EMBV3_05759	MED27	MGC134262 mediator complex subunit 27	-1,66
EMBV3_38854	POLDIP2	polymerase (DNA-directed), delta interacting protein 2	-1,68
EMBV3_03477	SEPW1	selenoprotein W, 1	-1,68
EMBV3_13532	RBM42	RNA binding motif protein 42	-1,70
EMBV3_42379	NFYC	nuclear transcription factor Y, gamma	-1,71
EMBV3_22673	AP1S1	adaptor-related protein complex 1, sigma 1 subunit	-1,72
EMBV3_16114	RPS15	ribosomal protein S15	-1,72
EMBV3_25823	TRPC4AP	transient receptor potential cation channel, subfamily C, member 4 associated protein	-1,72

EMBV3_08783	LOC616199	similar to Aldose reductase (AR) (Aldehyde reductase) (20-alpha-hydroxysteroid dehydrogenase) (20-alpha-HSD)	-1,73
EMBV3_23913	ACCSL	1-aminocyclopropane-1-carboxylate synthase homolog (Arabidopsis)(non-functional)-like	-1,75
EMBV3_40947	LOC281370	Polyubiquitin	-1,80
EMBV3_09887	SENP3	SUMO1/sentrin/SMT3 specific peptidase 3	-1,83
EMBV3_27192	INTS4	integrator complex subunit 4	-1,84
EMBV3_22215	TPK1	thiamin pyrophosphokinase 1	-1,85
EMBV3_40572	DUSP10	dual specificity phosphatase 10	-1,89
EMBV3_27422	EIF4B	eukaryotic translation initiation factor 4B	-1,89
EMBV3_02194	UBE2L3	ubiquitin-conjugating enzyme E2L 3	-1,89
EMBV3_10127	CDK2AP2	cyclin-dependent kinase 2 associated protein 2	-1,93
EMBV3_00987	DNAJB5	DnaJ (Hsp40) homolog, subfamily B, member 5	-1,95
EMBV3_03600	RPL11	ribosomal protein L11	-1,97
EMBV3_01798	BSG	Basigin	-1,98
EMBV3_18490	NUDT3	nudix (nucleoside diphosphate linked moiety X)-type motif 3	-2,00
EMBV3_40727	SFRS12	splicing factor, arginine/serine-rich 12	-2,21

Table S2: Genes exclusively differentially expressed in BL vs. 8CB

Probe Name	*Gene Name*	*Description*	*foldchange*
EMBV3_16729	CXHXORF15	chromosome X open reading frame 15 ortholog	1.75
EMBV3_31985	COPS7A	COP9 constitutive photomorphogenic homolog subunit 7A (Arabidopsis)	1.73
EMBV3_02417	CT47B1	cancer/testis antigen family 147, member B1	1.66
EMBV3_35780	COMMD6	COMM domain containing 6	1.65
EMBV3_19910	SETD1A	SET domain containing 1A	1.65
EMBV3_10175	ZNF292	zinc finger protein 292	1.62
EMBV3_33763	LOC100300875	hypothetical LOC100300875	1.59
EMBV3_40151	ZNF518B	zinc finger protein 518B	1.59
EMBV3_22539	LOC100301462	similar to cytochrome c oxidase subunit VIIb	1.58
EMBV3_05966	OIP5	Opa interacting protein 5	1.58
EMBV3_34923	RPS13	ribosomal protein S13	1.58
EMBV3_13173	LOC513662	hypothetical LOC513662	1.57
EMBV3_40942	PRDX1	peroxiredoxin 1	1.57
EMBV3_36918	UBAC1	UBA domain containing 1	1.57
EMBV3_30902	LOC100297586	similar to cytochrome c oxidase subunit VIIb	1.56
EMBV3_37190	LOC100336710	N-acylneuraminate cytidylyltransferase-like	1.56
EMBV3_37648	LOC541014	hypothetical protein LOC541014	1.55
EMBV3_27614	RNF14	ring finger protein 14	1.55
EMBV3_14638	NUDCD1	NudC domain containing 1	1.54
EMBV3_36580	RWDD4A	RWD domain containing 4A	1.54
EMBV3_42254	OSBPL8	oxysterol binding protein-like 8	1.53
EMBV3_34782	ZNF235	zinc finger protein 235	1.53
EMBV3_25900	CCDC127	coiled-coil domain containing 127	1.52
EMBV3_20853	CHPF	chondroitin polymerizing factor	1.52
EMBV3_11551	ES1	es1 protein	1.52
EMBV3_39107	ID3	inhibitor of DNA binding 3, dominant negative helix-loop-helix protein	1.52
EMBV3_26552	SYNM	synemin, intermediate filament protein	1.52
EMBV3_00633	CDO1	cysteine dioxygenase, type I	-1.52
EMBV3_10888	LOC509875	similar to Coiled-coil domain-containing protein 3	-1.52
EMBV3_03407	FEM1B	fem-1 homolog b (C. elegans)	-1.53
EMBV3_28774	PTMA	prothymosin, alpha (gene sequence 28)	-1.53

EMBV3_04805	SUMO1	SMT3 suppressor of mif two 3 homolog 1 (S. cerevisiae)	-1.53
EMBV3_24977	ANKRD17	ankyrin repeat domain 17	-1.54
EMBV3_24417	RAVER1	ribonucleoprotein, PTB-binding 1	-1.54
EMBV3_24753	USE1	unconventional SNARE in the ER 1 homolog (S. cerevisiae)	-1.54
EMBV3_14836	GLS2	glutaminase 2 (liver, mitochondrial)	-1.55
EMBV3_11094	ABCC8	ATP-binding cassette, sub-family C (CFTR/MRP), member 8	-1.56
EMBV3_19900	KLF5	Kruppel-like factor 5 (intestinal)	-1.56
EMBV3_31871	RSL1D1	ribosomal L1 domain containing 1	-1.56
EMBV3_12064	SPINT2	serine peptidase inhibitor, Kunitz type, 2	-1.56
EMBV3_12062	CCDC92	coiled-coil domain containing 92	-1.57
EMBV3_11558	ERLEC1	endoplasmic reticulum lectin 1	-1.57
EMBV3_24103	FBXO8	F-box protein 8	-1.57
EMBV3_15424	RPL18A	ribosomal protein L18a	-1.57
EMBV3_17751	LOC784935	similar to Histone acetyltransferase p300 (E1A-associated protein p300)	-1.58
EMBV3_32330	Rpl3	ribosomal protein L3	-1.58
EMBV3_17480	TFG	TRK-fused gene	-1.58
EMBV3_07477	UBAP2	ubiquitin associated protein 2	-1.58
EMBV3_10239	UBL5	ubiquitin-like 5	-1.59
EMBV3_43253	MYL12B	MRLC2 myosin, light chain 12B, regulatory	-1.6
EMBV3_28392	CDKN2D	cyclin-dependent kinase inhibitor 2D (p19, inhibits CDK4)	-1.63
EMBV3_17719	ANKRD40	ankyrin repeat domain 40	-1.64
EMBV3_18860	MTPN	myotrophin	-1.65
EMBV3_43695	PITPNA	phosphatidylinositol transfer protein, alpha	-1.66
EMBV3_38166	HDAC1	histone deacetylase 1	-1.67
EMBV3_02430	MEIS2	Meis homeobox 2	-1.67
EMBV3_29816	SCIN	scinderin	-1.67
EMBV3_38854	POLDIP2	polymerase (DNA-directed), delta interacting protein 2	-1.68
EMBV3_00953	ALDH2	aldehyde dehydrogenase 2 family (mitochondrial)	-1.69
EMBV3_00664	DNAJB12	DnaJ (Hsp40) homolog, subfamily B, member 12	-1.69
EMBV3_00523	PDXDC1	pyridoxal-dependent decarboxylase domain containing 1	-1.7
EMBV3_08338	CDH7	cadherin 7, type 2	-1.72
EMBV3_33525	TMEM57	transmembrane protein 57	-1.72
EMBV3_25905	CASR	calcium-sensing receptor	-1.75
EMBV3_09138	LOC100300550	similar to cytochrome c oxidase subunit VIIb	-1.77

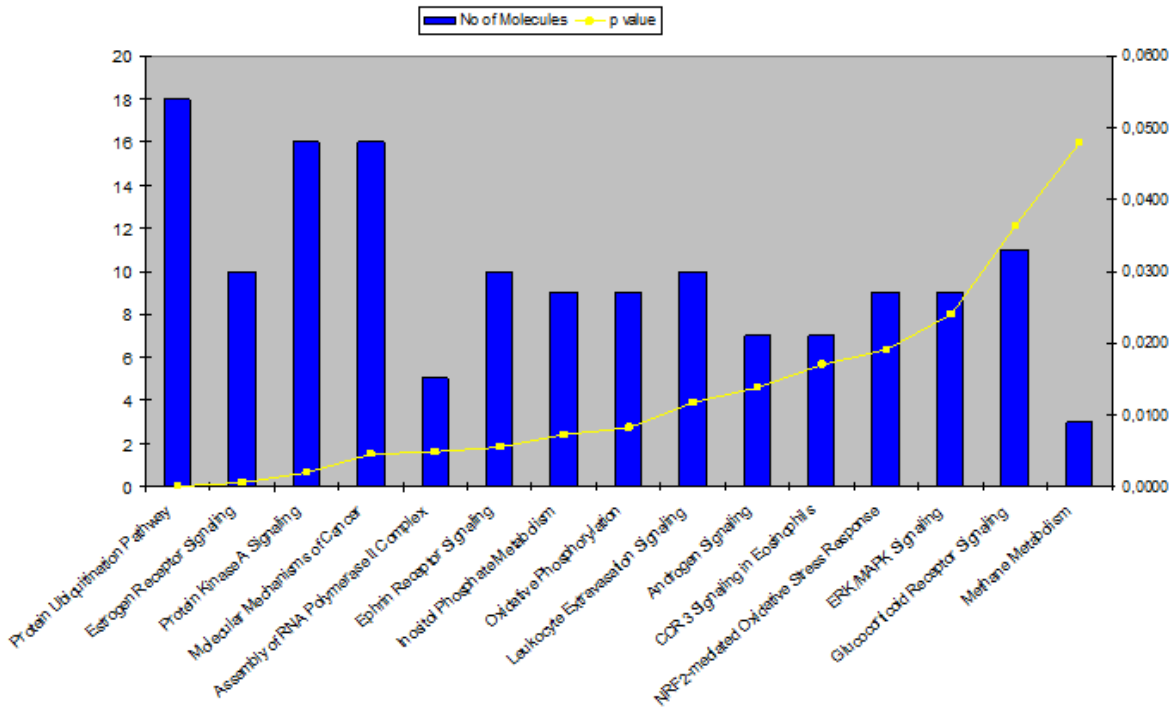
EMBV3_08487	TLK2	tousled-like kinase 2	-1.77
EMBV3_01985	ZYX	zyxin	-1.78
EMBV3_32736	ATP5S	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit s (factor B)	-1.91
EMBV3_31733	TXNIP	thioredoxin interacting protein	-1.91
EMBV3_25709	ZNF24	zinc finger protein 24	-1.91
EMBV3_26294	LOC100294761	hypothetical LOC100294761	-1.96
EMBV3_05230	LOC786299	similar to UL16 binding protein 3	-1.97
EMBV3_00816	NAV1	neuron navigator 1	-2.06

Table S3: Developmental rates according to treatment of 2-cell stage embryos

	2-cell stage	4-cell stage		Blastocyst stage	
Group	**n**	**n**	**(%)**	**n**	**(%)**
2-cell stage embryo (control)	160	146	(91.3)	49	(30.6) [a]
2-cell stage embryo (zona free)	176	167	(94.8)	58	(32.4) [a]
2-cell stage blastomere (bisected)	208	187	(89.9)	45	(21.6) [b]

Different superscripts within raws differ significantly $p < 0.05$

a) 2CB vs. BL



b) 8CB vs. BL

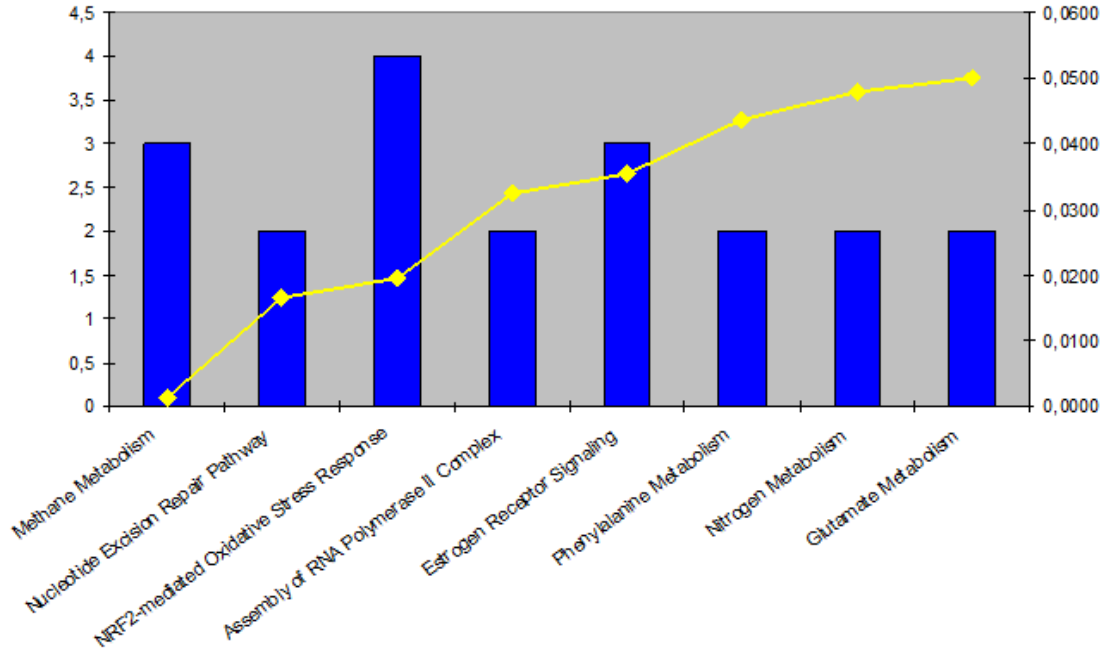


Figure S1: Pathways affected by differentially regulated genes

Chapter 4 General discussion

The present studies aimed to detect direct and non-invasive markers for the developmental competence of bovine oocytes and 2-cell stage embryos on the morphological and molecular level. In the first set of experiments the zona pellucidas' structure and morphology of in vivo and vitro matured oocytes was quantified and set into relation with the developmental competence, implicating measurable effects of maturational environment on developmental competence as well as on zona pellucida properties.

The results show very clearly, that the maturational environment has strong effects on the further development and on the zona pellucidas' morphology, visualized by scanning electron microscopy in a first sub experiment and quantified by polarized light microscopy in a second experiment. This is in line with Rizos et al. 2002b postulating that the developmental fate of the oocyte is highly related to its origin, implicating a significantly higher developmental potential as well as higher blastocysts quality for in vivo matured oocytes compared to their in vitro matured counterparts. Accordingly, one of the weakest points in the field of assisted reproductive technology are high numbers of low quality oocytes used for in vitro production. Therefore, the necessity to select effectively high quality oocytes, which are 'in vivo- like', is indispensible. Indeed, morphological markers for developmental competence are mostly invasive and rather dependent on subjective evaluation. The introduction of polarized light microscopy, however, opened a new window to determine the oocytes quality quantitatively and non- invasively. This technique takes the advantage of double refracting characteristics, called birefringence, of the zona pellucida (Keefe et al. 1997). In human several studies have shown, a strong correlation between birefringence and pregnancy outcome, implicating that alterations of the structure of the zona pellucida are reflected in the parameters measured by polarized light microscopy (de Almeida Ferreira Braga et al. 2010; Ebner et al. 2010; Madaschi et al. 2009; Montag et al. 2008; Rama Raju et al. 2007). The zona pellucida is secreted by the developing oocyte and follicular cells during oogenesis (Dunbar et al. 1994). Shen et al. showed that any harm to the oocytes during its growth phase and/or maturation can alter the secretion or patterning of the extracellular coat (Shen et al. 2005). Thus, polarized light microscopy enables to quantify alterations established during maturation period and therefore to select in vitro matured oocytes which are similar to in vivo matured oocytes, precisely, as shown in the present thesis.

The most interesting finding in the present study was the dynamics in terms of zona properties from immature (GV) oocytes to either in vivo or in vitro matured oocytes (MII) (Figure 3, Chapter 2). In vivo maturation leads to decrease in birefringence, whereas the overall in vitro matured oocytes displayed an increasing trend for birefringence. Decrease in birefringence may result from the porous structure of the zona pellucida after in vivo maturation. Only those in vitro matured oocytes classified by COC morphology as quality 1 followed the decreasing trend. Likewise low quality in vitro matured oocytes had the lowest amount of pores after maturation, implicating a tight structure reflected in higher birefringence. Similarly, a decrease in thickness of the inner layer was observed during in vivo maturation, in contrast to a strong increase during in vitro maturation. These results were in line with those in human reproduction, also reporting a decreasing trend in zona parameters from GV to MII stage (Cheng et al. 2012). Recently, it was reported, that zona pellucida birefringence of bovine presumptive zygotes predicts further development (Koester et al. 2011). The results were in line with our measurements, correlating low zona parameters with high developmental competence and in the opposite to human oocytes, showing a positive correlation between birefringence and developmental competence (Montag et al. 2008), implicating species specific differences within oocytes morphology, which argues with the necessity to find objective and non-invasive tools as predictors for developmental competence and to refrain from subjective evaluation of oocytes. However, this is the first study, showing the differences of in vitro and in vivo matured bovine oocytes, related to their developmental competence in a quantitative way. Thus, we could show that the maturational environment affects zona pellucida properties and developmental competence at the same value.

In accordance to morphological changes of zona pellucida properties taking place during maturation, maternally derived transcripts are synthesized and stored in the growing oocyte to support oocyte maturation and early embryonic development (Wassarman and Kinloch 1992). Therefore, a second set of experiments in this thesis focused on the molecular signatures, predicting high developmental competence. This is based on the fact that the acquisition of high developmental capacity in mammalian oocytes is largely dependent on RNA and protein synthesis and its storage, as well as an expression programme that uses the stored information and proteins in a highly

orchestrated manner during maturation an early development (reviewed by Eichenlaub-Ritter and Peschke 2002).

In contrast to most studies, using pools of embryos selected by competence models, in the present approach a direct correlation between the transcriptome profile and developmental competence could be established. Based on two recent studies, where biopsies of blastocysts were taken to correlate pregnancy outcome with gene expression profiles (El-Sayed et al. 2006; Ghanem et al. 2011), 2-cell stage embryos were biopsied. Preliminary, it was shown, that both blastomeres of 2-cell stage embryo have the ability to develop to the blastocyst stage, with an overall correlation coefficient of 73%, which is in line with studies in mouse (Katayama et al. 2010), bovine (Tagawa et al. 2008), porcine (Dang-Nguyen et al. 2011) and rhesus monkey (Mitalipov et al. 2002). That implicates, that one blastomere is developmentally representative of the other. Molecularly considered, it was shown, that both blastomeres of a murine 2-cell stage embryo are similar in their individual gene expression profile (Roberts et al. 2011), which argues for even higher similarity of bovine sister blastomeres, since bovine embryonic genome activation (EGA) occurs at the late 8-cell stage, whereas in mice EGA takes place at the 2-cell stage (Wang and Latham 1997). As described above, it is well accepted that the developmental competence is highly determined in the matured oocyte. Therefore, 2-cell stage embryos were used for biopsies in order to receive a representative biopsy, which would not have been given by taking a part of an oocytes or zygote, since an irregular mRNA distribution was observed within zygotes but not in blastomeres of 2-cell stage embryos in murine (VerMilyea et al. 2011).

Gene expression analyses revealed a typical transcriptome profile which is strongly related to the developmental competence of bovine 2-cell stage embryos. In detail, analyses of molecular functions, biological processes and pathways showed a specific pattern, implicating genes related to oxidative stress response and oxidative phosphorylation (OXPHOS) as manly affected. This let us suggest, that those functions and pathways, play crucial roles for the early embryonic development. It has already been shown, that oxidative stress, including accumulations of reactive oxygen species (ROS) have negative impact on preimplantation development. Minor changes in ROS balance can interrupt the embryonic development and result in cell death (Bain et al. 2011; Rhee 2006). In contrast, a certain amount of ROS is necessary for different cell

functions, like apoptosis, therefore the balance of ROS production and scavenging seems to be essential for optimal development (Burdon 1996).

The selected candidate genes in the present approach, *CAT* (Orsi and Leese 2001), *PRDX1* (Neumann et al. 2009), *PRDX6* (Leyens et al. 2004) and *MAPK14* (Matsuzawa and Ichijo 2008), are known to act as direct and indirect scavengers of ROS and likewise *NDUFS1*, is a main control element for OXPHOS (Brandt 2006) (*Detailed discussion about individual genes can be found in Chapter 3*). In further sub experiments the expression levels of these genes were verified, for example in an independent competence model, where higher abundances of the candidate genes were detected in early cleaving 2-cell stage embryos, which are assumed to be developmentally more competent compared to their later cleaving counterparts (Dode et al. 2006; Fair et al. 2004; Lonergan et al. 1999). Additionally, we were able to show, that accumulation of ROS is negatively correlated to the expression levels of our candidate genes, in detail, early cleaving embryos exhibit higher levels of ROS scavengers and consequently low ROS accumulation within the cells. For late cleaving embryos lower transcript abundances and accordingly higher levels of intracellular ROS were detected (Figure 6, Chapter 3). This implicates, that further developmental competence is also predicted by ROS accumulations, which is in line with studies postulating negative impacts of elevated ROS levels for development (Bain et al. 2011; Johnson and Nasr-Esfahani 1994). To verify finally at which degree corresponding sister blastomeres are actually representative for each other in terms of *CAT* and *PRDX1* expression, single cell PCR of sister blastomeres was conducted. The results were in agreement with studies mentioned above reporting that both sister blastomeres have similar transcriptome profiles (Roberts et al. 2011; VerMilyea et al. 2011). That is in great accordance with a very recent study demonstrating that singled sister blastomeres have not established polarity and do not show lineage pre-patterning (Lorthongpanich et al. 2012).

Taken together, this is the first experimental design establishing a new method to obtain direct correlation between distinct molecular fingerprints and the developmental competence of bovine 2-cell stage embryos. Moreover, this technique could enables to improve breeding programs on the level of genomic selection as well for preimplantation genetic diagnostics (PGD) in human reproductive medicine.

Both studies in the present thesis were able to provide the basis for new methods to evaluate the developmental competence of oocytes and early embryos non-invasively on the morphological and molecular level. In detail, for the first time the difference in quality of in vivo and in vitro matured oocytes could be measured quantitatively and individual developmental competence of early preimplantation embryos could be directly correlated to the expression of developmentally important candidate genes.

Chapter 5 General conclusion

The results reveal that subsequent developmental competence is morphologically indicated in the matured oocyte and largely genetically determined in the bovine 2- cell stage embryo, as shown in the present studies of this thesis. Therefore, it suggests itself, that the subsequent development is highly affected by the follicular environment.
The follicular environment depends amongst others for example on the mothers' energy status. Disturbances in energy metabolism are known as one of the chief causes for infertility in high yielding dairy cows. Concerning energy metabolism, it has been shown that serum glucose levels go in parallel with follicular glucose levels, which in turns affects follicular growth and development (Leroy et al. 2008).
Likewise, it is well accepted that glucose concentration during maturation determines the subsequent metabolism of the embryo, implicating that an adequate supply with glucose during maturation leads to an improved nuclear maturation and to higher developmental capacity (Krisher and Bavister 1998; Zheng et al. 2001). Therefore, one can suggest, that follicular environment affects the gene expression of the oocyte, which is deciding early embryonic development until the 8-cell stage, when the embryos genome is activated and therefore also gene expression in the first cleavage stages.
In accordance, the oocyte itself has low capacity for glucose uptake (Dan-Goor et al. 1997) (Augustin et al. 2001) hence, it is dependent on the cumulus cells to convert glucose to pyruvate and lactate, which can be metabolized by the oocyte (Harris et al. 2007). High glucose levels during maturation, as existent in the in vitro maturation systems, were detected to increase ROS in the early embryo by affecting the concentrations of anti-oxidant enzymes negatively (Hashimoto et al. 2000). As discussed in the present thesis, oxidative phosphorylation is the predominant ATP production pathway, however, producing ROS as by-products, implicating that high energy metabolic activities during maturation increase ROS levels and decrease developmental competence. (Krisher and Bavister 1999; Steeves and Gardner 1999).
Additionally, the interaction between cumulus cells and oocyte, within the growing follicle, is responsible for the patterning of the zona pellucida (Dunbar et al. 1994), implicating that zona pellucida properties are dependent on high quality oocytes as indicated by gene expression due to an adequate follicular environment.
The maturational environment affects metabolic activities in the cumulus cells and oocytes. Marginal deviations in glucose concentration have detrimental effects on the

glycolysis in cumulus cells, which in turn could affect zona pellucida properties and might define the energy metabolism within the oocyte, which determines the later concentrations of ROS in the early bovine embryo. Thus, aberrant conditions during in vitro maturation and also in in vivo maturation, caused by metabolic diseases in high yielding dairy cows, are suggested to influence zona pellucida properties which reflect successful maturation disturb embryonic development. As shown in the present thesis, early embryonic arrest is closely related to the transcriptome profile of 2-cell stage embryos, in detail genes related to oxidative phosphorylation and oxidative stress seem to decide the developmental fate, which could indicate a major role of the energy metabolism for proper oocyte maturation within the developing embryo.

Chapter 6 References

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Chapter 7 Appendix

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