Transcriptome landscapes of mammalian embryonic cells

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Transcriptome landscapes of mammalian embryonic cells

Transcriptoomlandschappen van embryonale cellen van zoogdieren

(met een samenvatting in het Nederlands)

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Copromotor: Dr. B.A.J. Roelen

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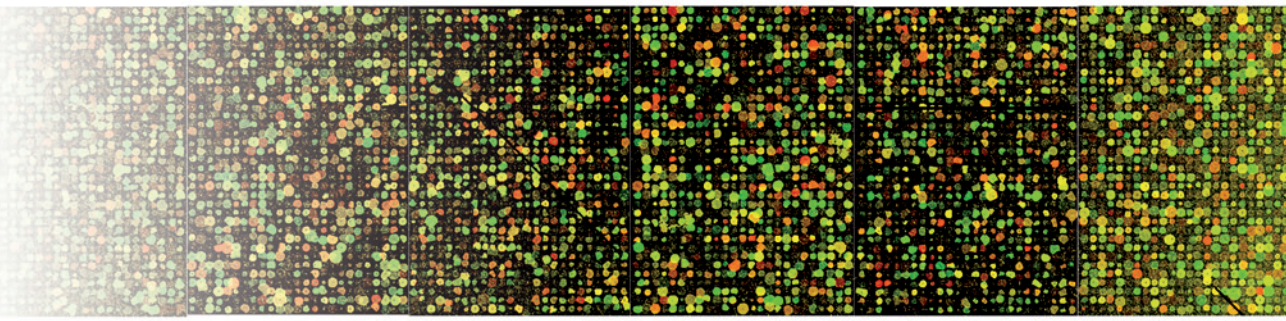

Chapter 1

Introduction[1]

1 Addapted from B.Brinkhof, B.A.J.Roelen and H.P.Haagsman: **Meet the Stem Cells; Production of Cultured Meat from a Stem Cell Biology Perspective** In *Engineering Aspects of Food Biotechnology.* Edited by J.A.Teixeira and A.A.Vicente: CRC Press; 2014:111-142.

DEVELOPMENT

Mammalian life starts with the fertilisation of an oocyte by a sperm cell. These two types of germ cells are some of the most differentiated cells. Yet, in contrast to somatic end stage cells, they can still generate all mammalian tissue after fertilisation (Figure 1). Therefore the resulting cell, the zygote, is considered totipotent (Seydoux & Braun 2006). The zygote starts dividing by a series of cleavage divisions, and a morula stage embryo is formed. The individual cells (blastomeres) start to commit to one of two cell lineages, and the blastocyst stage is attained. The blastocyst is composed of morphologically distinct tissue types; trophectoderm (TE) and inner cell mass (ICM) from which a selection of cells will further differentiate to primitive endoderm (PE). The rest of the ICM becomes the epiblast and will eventually form virtually the entire embryo. During the initial development to the blastocyst stage, cell fate is demarcated by the expression of several proteins. Expression of the transcription factor CDX2 directs TE differentiation, whereas expression of the transcription factor OCT4 (coded by

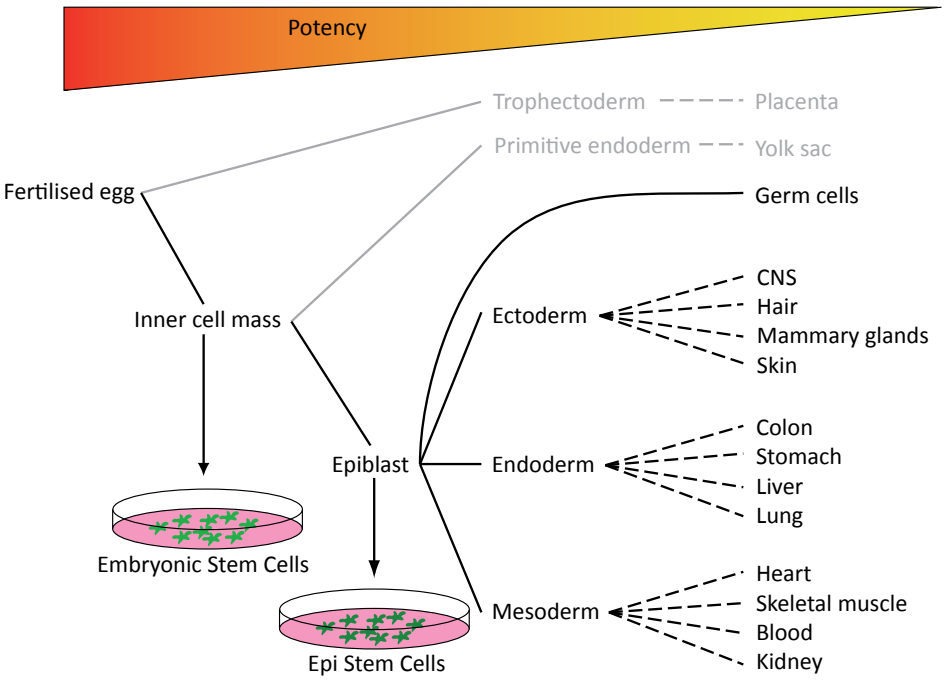

Figure 1.
Developmental potential of cell types during embryogenesis. Cleavage divisions of the fertilised egg lead to the formation of extraembryonic (grey) and embryonic (black) structures. The inner cell mass is composed of pluripotent cells capable of the formation of all cells and tissues of an organism. Position of cell type is relative and not absolute. Dashed line represents a pathway with intermediate cell types.

POU5F1) directs ICM fate. These two proteins mutually inhibit each other's expression, thereby actively promoting lineage segregation. During a second fate decision GATA6 expression in cells antagonizes NANOG expression, and these GATA6-expressing cells segregate from the ICM to form PE, while the opposite mechanism results in formation of the epiblast (Zernicka-Goetz et al. 2009) (Figure 2). *In vivo*, the TE and PE develop to extraembryonic tissues that constitute part of the placenta and yolk sac, whereas the epiblast develops into the foetus and finally a newborn.

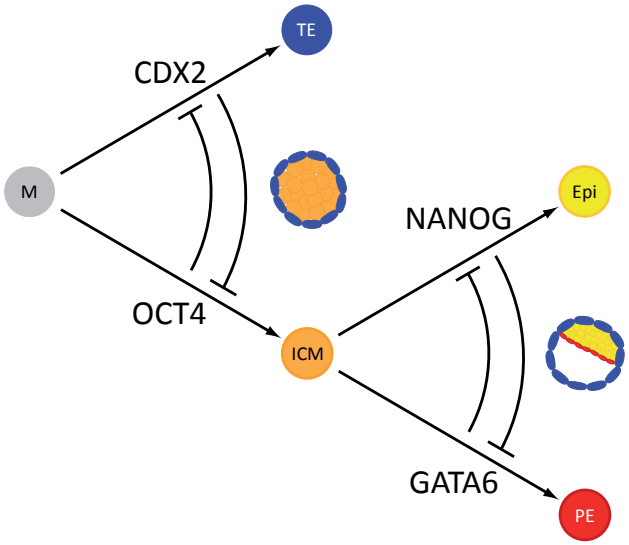

Figure 2
Fate decision during blastocysts formation. During cleavage divisions a zygote develops into a morula. During two fate determining stages first TE and ICM cells are formed depending primarily on CDX2 and OCT4 expression, respectively. During the second stage from ICM cells, PE cells emerge expressing predominantly GATA6, whereas the epiblast cells express NANOG. Epi = Epiblast; ICM = Inner cell mass; M = morula; PE = Primitive endoderm; TE = Trophectoderm.

EMBRYONIC STEM CELLS

Embryonic stem (ES) cells are derived from ICM cells *in vitro* and bear almost indefinite self-renewal potential as well as the ability to generate all cell types within the body. These characteristics distinguish ES cells from the tissue-specific adult stem cells, which have more limited self-renewal and developmental potential (Shenghui et al. 2009). ES cells were generated for the first time in 1981 from mouse blastocyst-stage embryos (Evans & Kaufman 1981, Martin 1981). Remarkably, it only seemed possible to generate ES cells from few mouse strains; efforts to use other strains and species remained unsuccessful. Finally, in 1995, researchers had been able to generate ES cells

from monkey embryos and later in 1998 from human embryos (Thomson et al. 1995, Thomson et al. 1998). Again it took a decade to derive ES cells from another species, the rat (Buehr et al. 2008). In almost 30 years only from two species of rodents and two species of primates ES cell lines have been generated, illustrating the complexity. The conditions to generate and maintain the ES cells from these species are not the same demonstrating the difficulties in generating ES cells and therefore the difficulty in generating ES cell lines from other mammalian species.

To demonstrate the establishment of a bona fide ES cell line, the cells should fulfil a number of criteria. The cells should for instance express several genes and their corresponding proteins that have been associated with pluripotency, such as *Nanog*, *Oct4*, and *Sox2*. To further demonstrate the pluripotent character of ES cells, these cells can be injected into an immune-deficient mouse. If the cells are indeed pluripotent the mouse will develop tumours that originated from the injected cells, and these tumours will consist of tissues from the three germ layers, i.e. endoderm, ectoderm, and mesoderm (Figure 1). To further demonstrate the pluripotent character of the ES cells they can be introduced into a blastocyst-stage embryo that after transfer to a pseudo-pregnant female develops further and is carried to term. If indeed the ES cells are pluripotent the new-born animal will be a chimera with tissues composed of cells originating from both the host blastocyst and the ES cells. If the chimerism is also present in the germ line (i.e. oocytes or sperm cells), breeding with these animals will lead to live offspring completely derived from the ES cells (Kuijk et al. 2011).

The generation of ES cell lines from mammalian species other than rodents and primates, such as farm animals, could benefit traits by gene editing and improve our knowledge of embryonic development. Attempts to generate well-characterised ES cell lines from domestic ungulates such as cattle and pigs have been rather unsuccessful (Keefer et al. 2007). Instead, the generation of cell lines with ES-like characteristics from livestock animals has been reported. In contrast to bona fide ES cells, these ES-like cell lines all showed limited self-renewal potential and undefined developmental potency. The generation of germ line chimeras from these cells has, for instance, not been demonstrated. Moreover, the expression patterns of key genes and proteins that represent pluripotency in the ES-like cells were different from expression patterns of existing ES cell lines. These ES-like cells could not be cultured *in vitro* for a long period, which suggests that these are not suitable to produce a continuous supply of cells which would be needed for tissue engineering, for instance. Therefore it is important to learn more about the mechanisms involved in embryonic development, and which factors are important for maintaining pluripotency in species other than rodents and primates.

EPIBLAST STEM CELLS

Cells with almost the same self-renewal and developmental potential as ES cells are stem cells derived from the epiblast of a mouse postimplantation embryo, so-called epiblast stem cells (EpiSCs) (Brons et al. 2007, Tesar et al. 2007). These cells, when generated from female embryos, exhibit inactivation of one X-chromosome in contrast to mouse ES cells where both X-chromosomes are active leaving EpiSCs in a more "primed" state of pluripotency compared to the "naïve" state of mouse ES cells (Nichols & Smith 2009, Tilo 2011). Furthermore, although expressing the core pluripotency factors *Oct4*, *Sox2*, and *Nanog*, EpiSCs differ from ES cells in their expression of several other transcripts such as reduced *Esrrb*, *Zfp42*, and *Klf4* expression and elevated expression of differentiation markers including *Otx2*, *T,* and *Fgf5* (Tesar et al. 2007, Nichols & Smith 2009). EpiSCs are also different from ES cells in their developmental and functional potential since they are less competent to contribute to chimeras (Tesar et al. 2007, Brons et al. 2007, Guo et al. 2009). It has been postulated that human ES cells, although derived from preimplantation embryos, are in certain aspects more similar to mouse EpiSCs than to mouse ES cells (Tesar et al. 2007, Nichols & Smith 2009). For instance, EpiSCs and human ES cells grow as a monolayer in contrast to the small, compact, and domed appearance of mouse ES cell colonies and for the EpiSC lack the ability to efficiently contribute to chimera formation, which for ethical reasons has not been established for human ES cells. Furthermore, the culture conditions and gene expression profiles of EpiSCs and human ES cells are similar and differ from mouse ES cell conditions (Tesar et al. 2007, Brons et al. 2007). Recently, several reports described the generation of naïve human ES cells rewiring from the primed state of ES cell lines (Hanna et al. 2010, Chan et al. 2013, Gafni et al. 2013, Ware et al. 2014, Takashima et al. 2014, Theunissen et al. 2014) and directly from human blastocysts (Gafni et al. 2013).

ES CELL SELF-RENEWAL AND SIGNALLING PATHWAYS

The core of the regulatory circuit that promotes expression of genes maintaining pluripotency while repressing genes inducing differentiation is formed by three transcription factors: OCT4, SOX2, and NANOG (Figure 3; yellow). The expression of these proteins needs to be carefully regulated as increased OCT4 expression in human ES cells causes differentiation into PE and mesoderm whereas repression leads to differentiation of TE and a loss of pluripotency (Niwa et al. 2000). Furthermore, down-regulation of SOX2 decreases OCT4 expression eventually leading to TE differentiation (Masui et al. 2007). Enhanced expression of the protein NANOG inhibits differentiation to PE both in ES cells as in ICM where it promotes self-renewal and epiblast formation, respectively (Mitsui et

al. 2003). Taken together these results suggest that maintaining the self-renewal potential of ES cells is accomplished by inhibition of differentiation (Katsumoto et al. 2010).

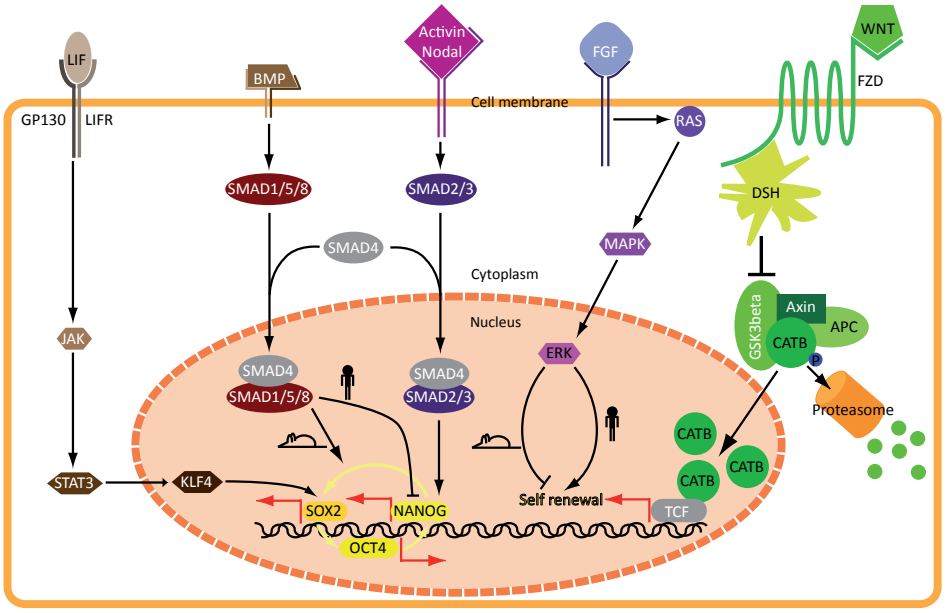

Figure 3
Pathways involved in embryonic stem cell self-renewal and pluripotency. In ES cells derived from human and rodent preimplantation embryos the WNT pathway (shades of green) has comparable effects on the self-renewing machinery. Factors important in maintaining pluripotency and self-renewal in human ES cells (shades of blue) have no or opposing effects in mouse ES cell maintenance, where mouse ES cell specific pathways (shades of brown) may result in differentiation or have no effect in human ES cells. All signals regulate expression of the core pluripotency genes; Sox2, Nanog, and Pou5f1 (coding OCT4), which are also self-regulatory (yellow). The red arrows represent transcription. (Further explanation in text.).

LIF SIGNALLING PATHWAY

The first mouse ES cells were cultured on a layer of non-dividing feeder cells, mouse embryonic fibroblasts, producing the factors required for maintaining self-renewal capacity and pluripotency. Leukemia Inhibitory Factor (LIF) was found to be the factor secreted by mouse embryonic fibroblasts mainly responsible for keeping mouse ES cells in a pluripotent state when cultured in the presence of serum (Williams et al. 1988, Smith et al. 1988). LIF functions through the JAK/STAT pathway (Nemetz & Hocke 1998) (Figure 3; LIF path) antagonising TGFβ signalling (Ulloa et al. 1999) and thereby inhibits ES cell differentiation (Williams et al. 1988).

WNT SIGNALLING PATHWAY

A cellular signalling pathway that likewise appears to be involved in the maintenance of pluripotency in various mammalian species is the WNT signalling pathway (Singh & Brivanlou 2011). Through this pathway the secreted protein WNT is bound by the transmembrane receptor Frizzled (FZD) which leads to the sequestering of the cytoplasmic protein GSK3β from a destruction complex (Figure 3; shades of green). In the absence of WNT ligand this destruction complex is comprised of the proteins GSK3β, APC, and Axin and is responsible for the degradation via the proteasome pathway of phosphorylated and subsequently ubiquitinated β-catenin. However, when degradation of β-catenin is prevented it can accumulate and translocate to the nucleus of the cell activating transcription of WNT target genes. Inhibition of GSK3β also results in elevated nuclear β-catenin levels in ES cells (Bennett et al. 2002). Together with a MAP kinase (MAPK) inhibitor it has been demonstrated this so-called 2i technique permits *de novo* derivation of ES cells from mouse blastocysts as well as the generation of ES cells from rat embryos (Ying et al. 2008, Buehr et al. 2008, Blair et al. 2011). Surprisingly, although human ES cells resemble EpiSCs, it has been reported that activation of the WNT pathway in human ES cells also inhibits differentiation (Sato et al. 2004).

FGF SIGNALLING PATHWAY

Another signalling pathway important for ES cells is mediated through a secreted protein growth factor termed fibroblast growth factor (FGF) (Bellot et al. 1991, Schlessinger 2000) (Figure 3; shades of blue). Activation of FGF receptors can lead to multiple signalling events; one of those is mediated via intracellular MAPK signalling (Ong et al. 2000, Browaeys-Poly et al. 2001). In mouse ES cells, activation of this cascade reduces the self-renewal capacity while inhibition of MAPK signalling enhances self-renewal (Burdon et al. 1999, Kunath et al. 2007) (Figure 3; FGF path & mouse pictogram). Although MAPK inhibition by itself is not sufficient to maintain mouse ES cells in a pluripotent state, MAPK inhibition in combination with GSK3β inhibition (2i) and LIF has resulted in the successful generation of ES cells from previously recalcitrant mouse strains and from the rat (Chen et al. 2006, Ying et al. 2008, Blair et al. 2011).

That early embryonic development is not driven by exactly the same molecular pathways in different mammalian species has been demonstrated by Kuijk and co-workers (Kuijk et al. 2012). When they stimulated FGF signalling in bovine preimplantation embryos, all cells of the ICM expressed GATA6 similar to results obtained with mouse embryos (Yamanaka et al. 2010). However, when MAPK signalling was inhibited a considerable amount of ICM-cells retained GATA6 expression, although a larger percentage

of the ICM cells expressed NANOG compared to control embryos (Kuijk et al. 2012). In mouse all ICM cells expressed NANOG without GATA6 expression (Yamanaka et al. 2010). This suggests that other pathways than those active in mouse embryos are involved and can be important for lineage segregation and possibly the derivation of ES cells from livestock. Interestingly, in human ES cells it seems that FGF signalling is indispensable (Figure 3; FGF path & man pictogram), but inhibition of MAPK in human embryos did not alter the ratio of GATA6 : NANOG expressing cells in the ICM (Kuijk et al. 2012).

BOVINE ES-LIKE CELLS

The generation of various ES-like cell lines from different embryonic developmental stages in cattle has been described (Van Stekelenburg-Hamers et al. 1995, Saito et al. 2003, Roach et al. 2006, Muñoz et al. 2008, Cao et al. 2009, Gong et al. 2010). Some of these cell lines could be expanded and could be maintained in an undifferentiated state for several passages (Muñoz et al. 2008, Cao et al. 2009). Indeed, when some of the ES-like cell lines were cultured in the absence of feeder cells or other differentiation inhibiting factors they formed embryo-like structures known as embryoid bodies made up of a wide variety of differentiated cell types which suggests that the cells were pluripotent (Stice et al. 1996, Roach et al. 2006, Gong et al. 2010). For several ES-like cells the formation of chimeric animals has been reported, but this chimerism was only found in one or a few tissues indicating that the developmental potential of these cells was limited (Cibelli et al. 1998, Iwasaki et al. 2000). Long term culture of bovine ES-like cells has not been demonstrated. These unsuccessful attempts to generate genuine bovine ES cells suggest that in maintaining pluripotency other genes and pathways are involved in cattle.

For farm animals, most embryos that have been used in attempts to generate ES cell lines were so-called *in vitro*-derived embryos. Usually, oocytes are collected from ovaries obtained as left-over tissue from slaughterhouses. Oocytes within ovarian follicles at final stages before ovulation, antral follicles, can be aspirated from the ovaries and cultured *in vitro*. These oocytes are not yet ready to be fertilised as they still need to undergo a substantial part of meiosis. After maturation *in vitro*, the oocytes can be fertilised *in vitro* and developing embryos cultured *in vitro* until the blastocyst stage. The *in vitro* culture steps are suboptimal when compared to the steps as they occur *in vivo,* and indeed the quality in terms of numbers of viable cells of *in vitro* blastocysts is reduced in comparison with the quality of *in vivo* blastocysts (Rubio Pomar et al. 2005). Possibly therefore, *in vivo* obtained embryos are a better source for the generation

of ES cells, but as a disadvantage these embryos can only be obtained from timely inseminated and slaughtered animals.

PORCINE ES-LIKE CELLS

Pigs have many physiological as well as anatomical similarities with humans, for instance the size of their organs. Therefore, apart from being an important livestock species, the pig is a much appreciated model in translational research, tissue engineering, and even xeno-transplantation (Matsunari & Nagashima 2009). Indeed, efforts have been made to establish porcine ES cell lines particularly for tissue regeneration purposes but with limited success. Culture and maintenance for prolonged periods of undifferentiated porcine ICM-derived cells has been reported using human recombinant LIF and other heterologous cytokines (Piedrahita et al. 1990a, Hochereau-de Reviers & Perreau 1993, Moore & Piedrahita 1997, Puy et al. 2010, du Puy et al. 2011). As in mouse ES cells most of these cell lines could differentiate *in vitro* and several were capable of the formation of carcinomas consisting of cell types from all germ layers upon transplantation to immunocompromised mice (Hochereau-de Reviers & Perreau 1993, Gerfen & Wheeler 1995). Although some genetic markers were expressed similar to the expression in mouse ES cells a putative porcine ES cell line was documented to have an epithelial appearance (phenotype) as indicated by the expression of cytokeratin (Piedrahita et al. 1990b). Interestingly, ICM cells of pig early blastocysts do not express NANOG yet suggesting that later stage pig embryos would be more suitable for the derivation of pluripotent cell lines (Kuijk et al. 2008).

Culture of porcine ICM in EpiSC conditions resulted in the appearance of colonies with an ES-like morphology that expressed NANOG, but none of these colonies could be maintained in their undifferentiated state (du Puy et al. 2011). In addition, although pig chimeras have been described when porcine ES-cell like cells had been introduced into blastocyst stage embryos, the level of chimerism, and therefore developmental potency of these cells, has not been documented (Wheeler 1994).

GENERATING PLURIPOTENT CELL LINES FROM LIVESTOCK SPECIES

Mouse ES cells differ from human ES cells in several aspects such as the signalling pathways important for maintenance of pluripotency and the expression of marker proteins. It is therefore not unlikely that in different mammalian species, different signalling pathways need to be activated or repressed in order to generate ES cell lines from embryos. In addition, there are important differences in pre- and peri-implantation

development of the different mammalian species, particularly in the timing of developmental processes. Most preimplantation embryos of livestock species are generated by *in vitro* fertilisation, and it has been suggested that it would be difficult to establish cell lines from pig and cattle embryos because of the low number of cells present in the ICM of *in vitro* produced embryos (Anderson et al. 1994, Van Soom et al. 1996). Unfortunately, a well-characterised cell line from an embryo of livestock that fulfils the criteria of a bona fide ES cell line has not been established yet.

The identification of pluripotency-related candidate genes or gene products and their pathways by comparative studies (including microarray studies) might aid in the establishment of embryo-derived livestock stem cell lines (Keefer et al. 2007). Cytokines and growth factors involved in inhibition of differentiation in mouse and primate ES cell lines such as LIF and FGF apparently have different functions in ungulate ICM or epiblast cultures indicating other pathways need to be activated or repressed to induce self-renewing capacity in these species (Talbot et al. 1995, Moore & Piedrahita 1997). Although bovine ICM outgrowths express LIF receptor and the transmembrane signal transducer GP130, the stimulation of this pathway has not succeeded in the establishment of bovine ES cells (Pant & Keefer 2006, Keefer et al. 2007). Therefore, pathways demonstrated to be involved in ES cell line establishment and maintenance can have different effects in livestock embryo-derived cells or require different concentrations of the proteins involved in these pathways. The difficulty to formulate culture conditions (most importantly media components) is presumably the reason for the absence of ES cell lines from livestock.

TISSUE SPECIFIC STEM CELLS

It is generally thought that stem cells reside in all tissue types. The function of these tissue specific stem cells is to help regenerate tissue that is damaged by for instance physical trauma or disease. Although these cells have self-renewal capacity it does not mean that they actually self-renew extensively under physiological conditions.

Hematopoietic stem cells were the first adult cells identified that can self-renew and also give rise to differentiated cells (Becker et al. 1963). These hematopoietic stem cells are exemplary as adult stem cells; they reside in the bone marrow and can give rise to all types of blood cells *in vivo*. This makes them extremely suitable in regenerative medicine such as replenishing blood cells after treatment of diseases like leukaemia and autoimmune disorders (Bryder et al. 2006). To no surprise muscle contains stem cells as well and as it seems more than one type (Asakura 2003). Still, in comparison to ES cells the proliferative capacity of adult stem cells is limited, and adult stem cells have the tendency to differentiate spontaneously *in vitro* (Langelaan et al. 2010).

DIFFERENTIATION TO MUSCLE CELLS

In order to understand the differentiation to muscle cells studies have been conducted in developing embryos but also in adult organisms investigating proliferation and differentiation of muscle stem cells after injury (Grefte et al. 2007, Tedesco et al. 2010, Ten Broek et al. 2010, Bentzinger et al. 2012). Different from most mammalian tissue, skeletal muscle is generated by fusion of cells, more specifically myoblasts, instead of cell division only (Mintz & Baker 1967). Myoblasts can fuse with other myoblasts, resulting in myotubes and, subsequently, myofibres or they can fuse with pre-existing myofibres. These myofibres therefore contain multiple cell nuclei.

MYOGENESIS

During embryogenesis the mesoderm is separated into paraxial, intermediate, and lateral mesoderm (Aulehla & Pourquié 2006). From the paraxial mesoderm, marked by *Pax3* expression (Williams & Ordahl 1994), somites are formed (Figure 4). During subsequent stages of development the dermamyotome is formed (Gros et al. 2005, Relaix et al. 2005). Relatively few cells that originate from the dermamyotome localize between the formed myofibres and surrounding basal lamina (Schultz et al. 1978) (Figure 5A) and become a distinct cell type known as the satellite cell (Gros et al. 2005, Ciemerych et al. 2011). Satellite cells, already identified in 1961 by Katz and Mauro (Katz 1961, Mauro 1961), are located within muscle tissue and provide new myonuclei to growing muscle fibres during postnatal development (Moss & Leblond 1971) similar to what myoblasts do during embryonic development. The satellite cells are mitotically quiescent in normal adult muscle (Schultz et al. 1978) but retain the ability to proliferate and differentiate in response to muscle injury (Grounds et al. 1992, Collins et al. 2005, Montarras et al. 2005).

It appears that *Pax7*-expressing satellite cells are indispensable in muscle repair (Oustanina et al. 2004, Relaix et al. 2006, Lepper et al. 2011). It has therefore been suggested that PAX7 acts as a key regulator in satellite cell biogenesis (Bryson-Richardson & Currie 2008). Commitment to muscle-progenitor fate begins with cellular co-expression of both *Pax7* and *Myf5* (Figure 5A). *Pax7*-expressing satellite cells that do not express *Myf5* are not yet committed to muscle cells. These cells can divide either asymmetrically, where only one daughter cell becomes *Pax7+/Myf5+* and the other remains *Pax7+/Myf5-* and therefore uncommitted, or symmetrically giving rise to either two committed *Pax7+/Myf5+* satellite cells or two uncommitted *Pax7+/Myf5-* satellite cells (Kuang et al. 2007, Bryson-Richardson & Currie 2008). Porcine satellite cells expressing α6 integrin (*α6ITG*) represent a highly myogenic cell population (Wilschut et al. 2011). Furthermore, this receptor is involved in activation of the *Myf5* promoter

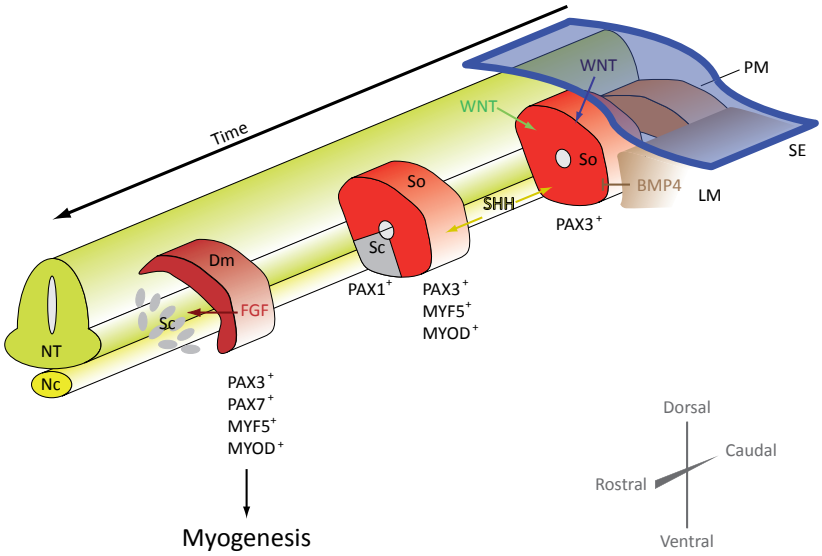

Figure 4

Embryonic myogenesis. Several extrinsic factors determine the differentiation of the somite into dermamyotome and subsequent commitment to myogenesis. Each stage and structure is identified by their expression of stage (and location) -specific genes (details in text). Dm: dermamyotome; LM: lateral mesoderm; Nc: notochord; NT: neural tube; PM: paraxial mesoderm; Sc: sclerotome; SE: surface ectoderm; So: somite.

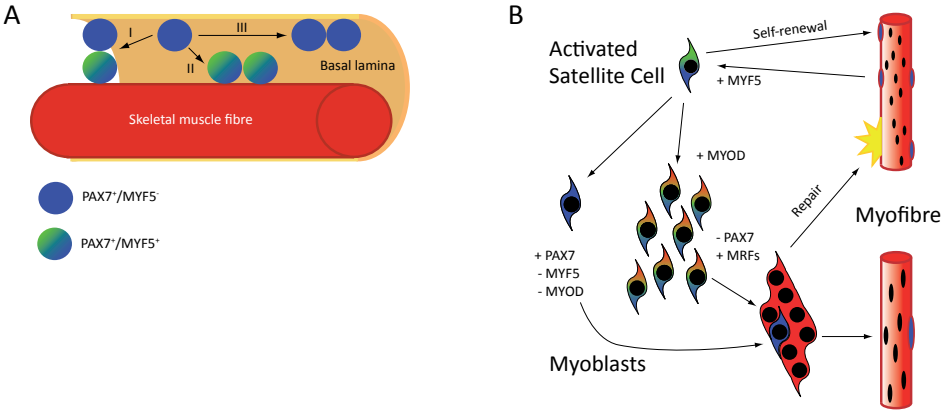

Figure 5

Satellite cell commitment (A) and post-natal myogenesis (B). Satellite cells expressing *Pax7* are quiescent and reside between the myofibre and the basel lamina of the muscle. A: Cell division can result in muscle lineage commitment of the daughter cell when co-expressing *Pax7* and *Myf5*. This coincides with an apical location of the cell. Cells retaining their basal location remain uncommitted and do not express *Myf5*. A satellite cell division can result in I) one committed and one uncommitted satellite cell (asymmetric division) or via symmetric division in II) two committed satellite cells or III) two uncommitted satellite cells. B: In *de novo* myogenesis or upon injury, *Pax7* expressing satellite cells are activated and express *Myf5*. *MyoD* and other MRFs positive myoblasts and deactivated *Pax7* expressing myoblasts without *Myf5* and *MyoD* expression can fuse resulting either in repair or formation of new myofibres.

region upon Dickkopf 3 (Dkk3) protein binding in zebrafish (Hsu et al. 2010, Hsu et al. 2011, Fu et al. 2012).

MyoD expression is up-regulated in *in vitro* cultured mouse muscle progenitor cells and isolated mouse muscle myofibres correlating with satellite cell activation and induction of differentiation (Yablonka-Reuveni et al. 1999, Zammit et al. 2004, Ciemerych et al. 2011) (Figure 5B). Activated satellite cells proliferate and become committed to the myogenic lineage, thereby expressing myogenic regulatory factors (MRFs) and lineage markers, such as *Myf5*, *MyoD*, *α7itg*, and desmin (*Des*) (George-Weinstein et al. 1993, Creuzet et al. 1998). Further development during myogenesis is associated with up-regulation of *Des* responsible for induction of myoblast fusion (Li et al. 1994). Recently, a muscle specific marker, called myomaker (Tmem8c), expressed during and sufficient to promote mammalian myoblast fusion, was identified (Millay et al. 2013, Landemaine et al. 2014). *Tmem8c* was found to be essential during muscle regeneration in adult satellite cells (Millay et al. 2013, Millay et al. 2014). The information about the extrinsic and intrinsic pathways in muscle development will therefore be helpful in regenerative medicine as well as in tissue engineering.

CULTURING MEAT

Tissue engineered muscle from livestock species is not only useful as a model for mammalian myogenesis but could also reduce the rather dramatic impact on the environment by the meat industry. Livestock currently uses 26% of ice free land for grazing, and the area used for feed crop production is about 33% of all cropland, totalling to 30% of the planet's land surface (Steinfeld et al. 2006). A further increase in land use is bound to come at the cost of natural habitats, including rainforests, and therefore alternatives for conventional meat production are demanded. Possible alternatives are: (1) make current meat production more sustainable; (2) increase product quality (and reduce meat consumption); (3) replace animal protein by plant or fungal proteins; (4) make animal protein without animals. To improve meat quality and produce a protein source without the use of animals, stem cells and tissue engineering could be employed.

The idea of culturing animal tissue *in vitro* for human consumption is not new. Already in the early twentieth century Winston Churchill proposed the idea to grow animal tissue instead of whole animals (Churchill 1932). Unfortunately, this idea was at that time never experimentally explored. Supported by NASA, in 2002 the possibilities were explored to increase goldfish (*Carassius auratus*) muscle explants by *in vitro* culture, and a limited increase in muscle mass was established (Benjaminson et al. 2002). Recently, in August 2013, an *in vitro* cultured hamburger grown from satellite cells isolated from muscle biopsies, was presented and, in contrast to the goldfish

prepared muscle explants (Benjaminson et al. 2002), sampled after preparation by a chef (Post 2014). The culturing of one 85 g hamburger needed 3 months of culturing, and therefore this proof of concept of *in vitro* meat still needs improvement reducing production costs and enhancing muscle development.

THESIS OUTLINE

In this thesis expression profiles of embryonic cells present in the bovine preimplantation embryo or in different bovine or porcine developmental stages are described.

To identify genes involved in early bovine embryonic development the transcriptome of morula stage and blastocyst stage embryos is evaluated in **chapter 2**. Since the blastocyst comprises the TE and the pluripotent ICM the gene expression profiles of these cell types were compared. After elevating the *NANOG* : *GATA6* expressing cell ratio in the ICM by culturing bovine blastocysts in the presence of a MAPK inhibitor (PD0325901), these transcriptomes were used to identify genes associated with pluripotency in the bovine ICM.

The use of additional small molecule inhibitors is reported to rewire human ES cells from a primed signature to a naïve state similar to mouse ES cells. To study the effect of this naïve human stem cell medium (NHSM) in bovine pluripotency bovine blastocysts were generated in NHSM. In **chapter 3**, the mRNA expression profiles of ICMs isolated from these blastocysts were compared with the transcriptome of ICMs cultured under standard (SOF) condition.

Not only NHSM could lead to derivation of allegedly naïve human ES cells hence two additional media were used in **chapter 4** to culture bovine blastocysts to compare gene expression levels between blastocysts cultured in these two media, NHSM and SOF. Genes differentiating between TE, PE, and epiblast were assayed, and expression profiles of genes identified as markers for pluripotency and more particular naïvety were generated. The findings described in these chapters could aid in the generation of bovine embryonic stem cell lines.

Epiblast cells *in vivo* differentiate into three germ layers of which one is mesoderm. Skeletal muscle is amongst others derived from the embryonic mesoderm. For regenerative medicine and tissue engineering purposes, understanding the differentiation of muscle stem cells to become muscle is important. In **chapter 5** the function of Dickkopf 3 protein in the development of myotubes from porcine satellite cells is investigated. The expression levels of gene transcripts marking stages during this process were determined to identify expression levels of *MYF5* and if this could result in improved myotube formation.

The established bovine and porcine transcriptome landscapes are compared with available data from other mammalian species in the discussion chapter of this thesis.

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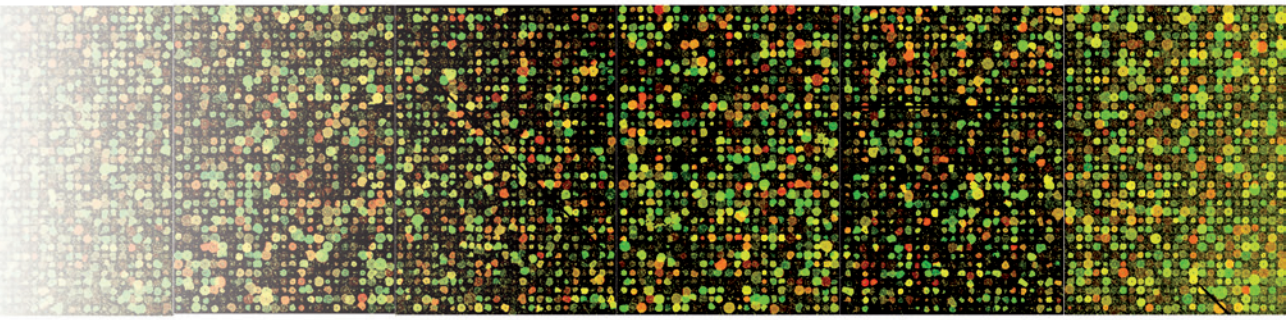

A mRNA landscape of bovine embryos after standard and MAPK inhibited culture conditions: a comparative analysis

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ABSTRACT

Background

Genes and signalling pathways involved in pluripotency have been studied extensively in mouse and human preimplantation embryos and embryonic stem (ES) cells. The unsuccessful attempts to generate ES cell lines from other species including cattle suggest that other genes and pathways are involved in maintaining pluripotency in these species. To investigate which genes are involved in bovine pluripotency, expression profiles were generated from morula, blastocyst, trophectoderm, and inner cell mass (ICM) samples using microarray analysis. As MAPK inhibition can increase the *NANOG : GATA6* ratio in the inner cell mass, additionally blastocysts were cultured in the presence of a MAPK inhibitor, and changes in gene expression in the inner cell mass were analysed.

Results

Between morula and blastocyst 3,774 genes were differentially expressed, and the largest differences were found in blastocyst up-regulated genes. Gene ontology (GO) analysis shows *lipid metabolic process* as the term most enriched with genes expressed at higher levels in blastocysts. Genes with higher expression levels in morulae were enriched in the *RNA processing* GO term. Of the 497 differentially expressed genes comparing ICM and TE, the expression of *NANOG*, *SOX2*, and *POU5F1* was increased in the ICM confirming their evolutionary preserved role in pluripotency. Several genes implicated to be involved in differentiation or fate determination were also expressed at higher levels in the ICM. Genes expressed at higher levels in the ICM were enriched in the *RNA splicing* and *regulation of gene expression* GO term. Although *NANOG* expression was elevated upon MAPK inhibition, *SOX2* and *POU5F1* expression showed little increase. Expression of other genes in the MAPK pathway including *DUSP4* and *SPRY4*, or influenced by MAPK inhibition such as *IFNT*, was down-regulated.

Conclusion

The data obtained from the microarray studies provide further insight in gene expression during bovine embryonic development. They show an expression profile in pluripotent cells that indicates a pluripotent, epiblast-like state. The inability to culture ICM cells as stem cells in the presence of an inhibitor of MAPK activity together with the reported data indicates that MAPK inhibition alone is not sufficient to maintain a pluripotent character in bovine cells.

BACKGROUND

In mammals, early life starts with the formation of a zygote as a result of the fertilisation of an oocyte. Sequential cleavage divisions lead to the formation of a morula stage embryo wherein a fluid-filled cavity emerges called the blastocoel. Two differentiated groups of cells can be distinguished in the embryo that is now called a blastocyst. A group of cells adjacent to the blastocoel, the inner cell mass (ICM), is able to contribute to all cells of the three germ layers and is therefore referred to as being pluripotent. The other group of cells, called the trophectoderm (TE), forms an epithelium surrounding the blastocoel and the ICM and is important for implantation within the uterus and contributes to the non-maternal part of the placenta. In the ICM further differentiation occurs by the formation of the epiblast, that will form the foetus, and the formation of extraembryonic primitive endoderm (PE) contributing to the yolk sac.

Studies with mouse embryos have advanced our understanding of how a pluripotent cell population is established during preimplantation development (Tanaka et al. 1998, Ralston & Rossant 2005, Chazaud et al. 2006, Plusa et al. 2008). During the first differentiation, the transcription factors CDX2 and OCT4 are key regulators for the formation of respectively TE and ICM. CDX2 represses the activity of OCT4 in mouse TE (Strumpf et al. 2005) and is virtually absent in ICM cells (Wallingford et al. 2013). OCT4 in turn can counteract CDX2 activity in the inner cells of the morula. The second differentiation is indicated by the expression of either NANOG or GATA6 in ICM cells fated to become the epiblast or PE, respectively (Zernicka-Goetz et al. 2009). Like for CDX2 and OCT4 in the morula, NANOG and GATA6 inhibit each other's transcription (Ralston & Rossant 2005). Whether the same genes and signalling pathways are also involved in the formation of a pluripotent cell population in other mammals remains to be established. Indeed, in contrast to the mouse, OCT4 protein remains present in the TE of bovine blastocysts even after transcription is down-regulated (Kurosaka et al. 2004), and its expression is not negatively regulated by CDX2 (Berg et al. 2011). In mouse embryos it has been established that GATA6-stimulated fibroblast growth factor (FGF) signalling via the extracellular signal-regulated protein kinase (ERK) is responsible for NANOG repression and thereby the formation of primitive endoderm (Chazaud et al. 2006, Nichols & Smith 2009, Yamanaka et al. 2010, Schrode et al. 2014). In bovine and human embryos however, although GATA6 expression is specific for primitive endoderm, inhibition of ERK signalling had a more moderate (bovine) or no (human) effect on the numbers of NANOG and GATA6 expressing cells suggesting that in these species other pathways are involved in the formation of the pluripotent cell population (Kuijk et al. 2012, Roode et al. 2012, Van Der Jeught et al. 2013). These

findings suggest species-specific mechanisms active in the specification of ICM, TE, epiblast, and PE lineages, and that further insight is needed into the molecular basis of cell sorting during the two first differentiation events.

When mouse ICM cells are cultured under defined conditions, their pluripotent character can be maintained (Evans & Kaufman 1981, Martin 1981). However, the establishment of such embryonic stem (ES) cells has only been successful for mice, non-human primates (Thomson et al. 1995), humans (Thomson et al. 1998), and rats (Buehr et al. 2008). Although pluripotency refers to the capacity to give rise to all embryonic and adult cell types, including the germ line, various states of pluripotency have been described. These states are referred to as "naïve" and "primed", with "primed" being more developmentally restricted (Hackett & Surani 2014). In mammals other than primates and rodents, the correct stages of embryos that contain pluripotent cells and culture conditions that maintain pluripotency have yet to be established (Telugu et al. 2010).

In order to identify genes that may be important for the acquisition and maintenance of pluripotency in bovine embryos a genome-wide gene expression analysis was performed in morulae, intact blastocysts, TE, and ICM. Analyses of gene expression patterns in preimplantation embryos to distinguish between pluripotent cells of the ICM versus those of the TE have previously made use of cell lines because of the technical difficulties of separating ICM from TE (Tanaka et al. 2002, Hamatani et al. 2004). Here we have manually dissected individual ICMs from TE. As the late ICM is composed of both pluripotent epiblast cells and the PE, the pluripotent character of the ICM was enhanced by inhibition of the ERK-pathway resulting in an increased percentage of ICM cells that express *NANOG*.

RESULTS

Gene expression profile of preimplantation embryos

To identify genes involved in bovine pluripotency, gene expression profiles of morula and blastocyst embryos, ICM, and TE were generated using microarray analysis. Bovine cumulus oocyte complexes (COCs) were *in vitro* matured, fertilised, and cultured for 5 or 9 days to obtain morula and blastocyst stage embryos, respectively (Figure 1A,B). In addition, ICM and TE were manually dissected from day 9 blastocysts (Figure 1C,D). From all samples RNA was isolated and only those with an RNA integrity number (RIN) ≥8.0 were used for further analysis. To compensate for biological and technical errors, two biological replicates of each sample were labelled with either Cy3 or Cy5 and hybridized on the arrays with a common reference pool consisting of blastocysts so all samples could be compared (Figure 1E).

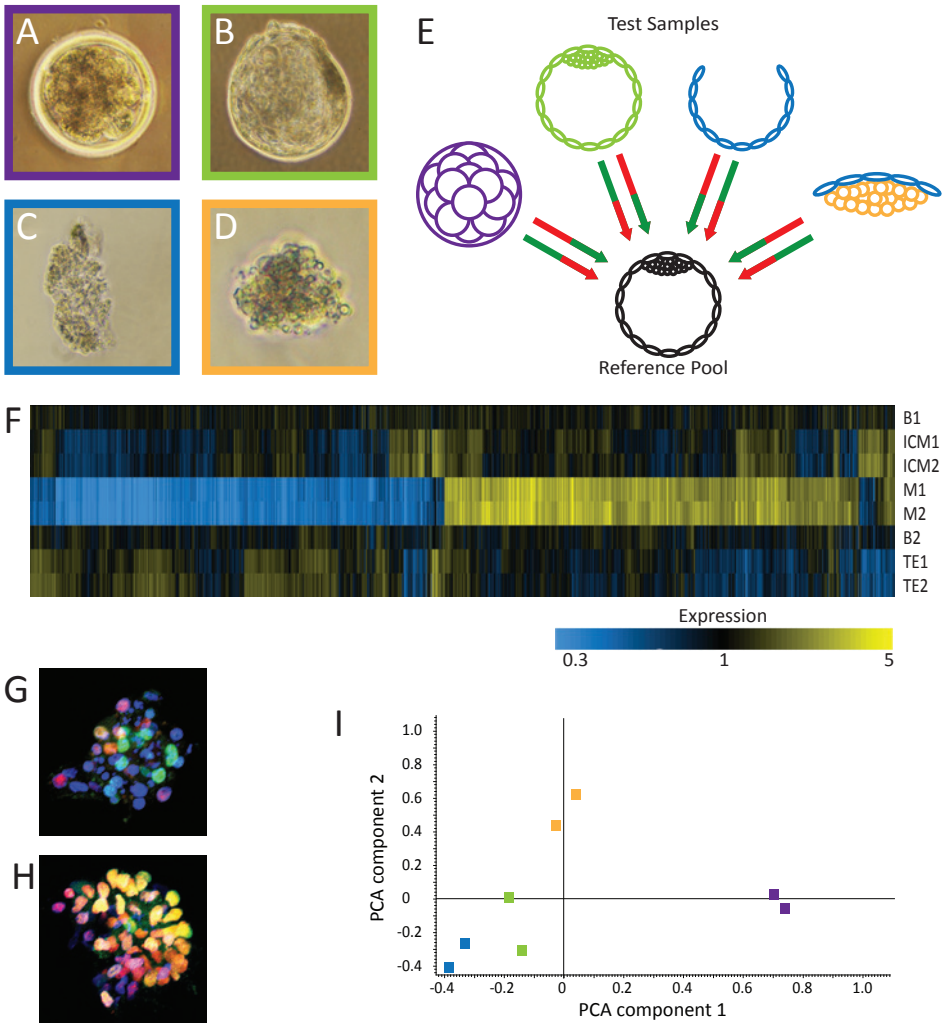

Figure 1.

Microarray set-up and sample validation. RNA from morula (A, purple), blastocyst (B, green), trophectoderm (C, blue), and inner cell mass (D, orange) was hybridized on microarrays. A common reference sample composed of blastocysts was hybridized with each sample in duplicate in balanced dye-swap (E, arrows indicate an array and the used fluorescent label; Cy3: green; Cy5: red). The heat map (F) illustrates pairwise clustering of microarray sample replicates. Yellow colour represents over-expressed probes, and blue colour represents under-expressed probes as indicated by the colour legend (B = blastocyst; ICM = inner cell mass; M = morula; TE = trophectoderm). Representative pictures of immunofluorescent labelling of dissected ICM (G) and TE (H) for GATA6 (green) and CDX2 (red); double GATA6-CDX2 nuclei appear yellow. Nuclear staining by DAPI (blue). A 2D principal component analysis plot (I) with sample position indicates clustering of trophectoderm (blue), blastocyst (green), inner cell mass (orange), and morula (purple) replicates.

Samples were hybridized on a microarray slide containing almost 44,000 probes per array coding for ~14,000 gene transcripts indicating that for a subset of genes more than one probe was present. If the position of the probe is nearer to the 3' end of the corresponding gene, signal intensity is expected to be higher (Auer et al. 2003), and chance of incorrect signal by variations in RNA integrity is smaller (Eklund & Szallasi 2008). Therefore, the expression of the probes corresponding to the most 3' ends of genes was used for the analysis (Shi 2006, Li et al. 2011). Gene expression levels in morula, blastocyst, ICM, and TE were determined, and a hierarchical clustering analysis was performed. The constructed heat map shows clear pairing of the morula, ICM, and TE samples (Figure 1F). This is particularly important for the TE and ICM samples since these were manually dissected and confirms the reproducibility of the dissection. We used mechanical isolation of ICM from TE using tungsten needles. A selection of isolated ICMs was stained for CDX2 and GATA6 to identify the contribution of TE cells to the pooled microarray samples. The isolated ICMs contained only ~20% CDX2 positive TE (Figure 1G). Since however some TE cells remained attached to the ICM, throughout the manuscript "ICM" refers to the ICM containing few TE cells. In the TE samples all cells were CDX2 and GATA6 positive (Figure 1H). Blastocyst samples did not pair since their difference with the reference is minimal indicated by a near black appearance in the heat map. A principal component analysis (PCA) further identified four categories according to cell type or developmental stage. In a 2D plot, morula samples separate the farthest from the other samples. TE and ICM samples are clearly separated from each other with the blastocyst replicates in between (Figure 1I).

To further confirm the specificity of the samples, expression levels of genes that are known to be differentially expressed in bovine embryos were compared. Of the selected genes, *HMGB1*, *SOX2*, and *POU5F1* (coding for OCT4 protein) are known to be expressed at relatively high levels in bovine morula embryos (Xie et al. 2010, Khan et al. 2012, Madeja et al. 2013), *CDX2* and *KRT18* have the highest expression levels in TE (Ozawa et al. 2012, Madeja et al. 2013) while *FN1* and *NANOG* are abundantly expressed in the ICM (Madeja et al. 2013). The relative expression levels of the selected genes as determined by microarray analysis in morula, blastocyst, ICM, and TE were as expected, with highest levels of *HMGB1* and *SOX2* expression in morulae, *POU5F1* expression predominantly in morulae and ICM, highest *FN1* and *NANOG* expression in the ICM and *CDX2* and *KRT18* expression at highest levels in TE (Figure 2A). These selected genes and five additional genes (Supplemental Figure 1) were also analysed for their expression levels by qRT-PCR, revealing a similar expression profile validating sample identity and demonstrating that the microarray data accurately reflect relative expression levels (Figure 2B).

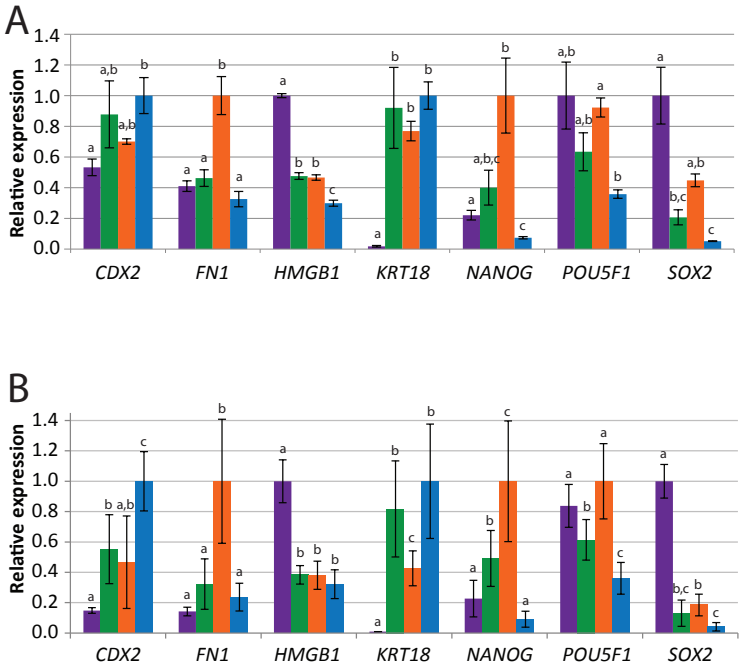

Figure 2.
Relative gene expression. Microarray data were analysed for selected genes known for their expression in morula (purple), blastocyst (green), ICM (orange), and TE (blue) (A). The same genes were analysed by qRT-PCR in 4-6 samples per embryo stage or cell type revealing the same pattern (B). Normalization was performed with the reference genes encoding RPL15, SDHA, and YWHAZ. Y-axis depicts relative mean expression to sample with highest expression set at 1 per gene. Bars with different letters are significantly different ($p<0.05$) and error bars indicate standard deviation.

Genes differentially expressed between morulae and blastocysts

Most differences in gene expression were found between morulae and blastocysts. When gene expression in morulae was compared with that in blastocysts using a >2-fold difference with p-value <0.05 as cut-off, 3,774 genes were differentially expressed. In the blastocyst, 1,960 genes were up-regulated, up to 107-fold, while 1,814 genes were expressed at higher levels in morulae (Figure 3A and Supplemental Table 1). The 25 most differentially expressed genes were expressed with at least a 30-fold difference of which only 1 gene (*ETNPPL*) was expressed at higher levels in the morula (Table 1).

To identify properties of the differentially expressed genes a Parametric Analysis of Gene Set Enrichment (PAGE) was performed using AgriGO (Du et al. 2010), and a list of the five most-enriched gene ontology (GO) terms per categorie was generated (Table 2). A large group of differentially expressed genes was involved in the *lipid metabolic process* (GO:0006629) in the blastocyst. A much smaller group of genes that function in *pepsin A activity* (GO:0004194) was over-represented in blastocysts, and indeed, a

large group of genes up-regulated in the blastocyst located to the *plasma membrane* (GO:0005886). Compared to the blastocyst, in the morula more genes were involved in the *nucleobase-containing compound metabolic process* (GO:0006139) and more specifically in *RNA processing* (GO:0006396). Morula up-regulated genes were found in the enriched molecular function GO terms *nucleic acid binding* (GO:0003676) and *transcription regulator activity* (GO:0030528).

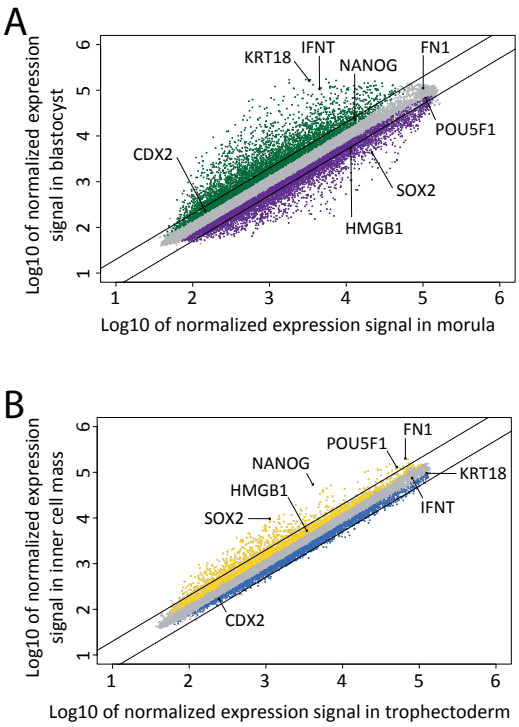

Figure 3.
Relative gene expression in blastocyst versus morula and inner cell mass versus trophectoderm samples. All probes representing genes (grey) were plotted for their expression levels. Black lines represent the 2-fold cut off. Axes are Log10 transformed and depict the normalized expression signal in the indicated sample. Genes analysed in qRT-PCR are indicated. (A) Genes with significant ($p \leq 0.05$) higher expression levels in blastocyst or morula are indicated with green or purple dots, respectively. (B) Genes with significant ($p \leq 0.05$) higher expression levels in inner cell mass or trophectoderm are indicated with orange or blue dots, respectively.

Genes differentially-expressed between ICM and TE

When gene expression in the ICM was compared with that of the TE, 497 genes were differentially expressed. Here, the majority (406) of the differentially expressed genes were expressed at higher levels in the ICM. Of all genes, the difference in expression levels of *NANOG* was the largest (13-fold up-regulated in the ICM, Table 3 and

Table 1. List of the most differentially expressed genes between blastocyst and morula.

Rank	Entrez Gene name	Gene discription	AgriGO ID	FC
1	PLS1	Plastin-1	ENSBTAP00000053019	107.2
2	PAGE4	G antigen family C member 1	ENSBTAP00000047904	103.9
3	PGHS-2	Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase) (PTGS2), mRNA.	ENSBTAP00000018774	94.1
4	ANXA3	Annexin A3	ENSBTAP00000042843	70.5
5	ALDH1A3	Aldehyde dehydrogenase 1 family, member A3	ENSBTAP00000012030	64.9
6	SEPP1	Selenoprotein P, plasma, 1 (SEPP1), mRNA	NP_776884	57.8
7	GCA	Grancalcin, EF-hand calcium binding protein	ENSBTAP00000024550	54.1
8	MYOF	Myoferlin	ENSBTAP00000038769	52.0
9	KRT8	Keratin, type II cytoskeletal 8	ENSBTAP00000001108	51.9
10	KRT18	KRT18 protein, Uncharacterised protein	ENSBTAP00000001988	49.2
11	SLC23A1	Solute carrier family 23 (ascorbic acid transporter), member 1	ENSBTAP00000010823	48.7
12	ZFP37	Uncharacterised protein	ENSBTAP00000024598	45.6
13	PRSS22	Uncharacterised protein	ENSBTAP00000021967	43.2
14	AGRN	Agrin	ENSBTAP00000017563	39.4
15	ETNPPL	Bos taurus alanine-glyoxylate aminotransferase 2-like 1 (AGXT2L1), mRNA.	ENSBTAP00000013587	-38.8
16	CYP51A1	Lanosterol 14-alpha demethylase	ENSBTAP00000002582	36.3
17	LDLR	Low-density lipoprotein receptor	ENSBTAP00000016342	34.4
18	CNN2	Calponin-2	ENSBTAP00000027670	33.8
19	LOC787705	Uncharacterised protein		33.7
20	HMOX1	Heme oxygenase 1	ENSBTAP00000020701	33.2
21	PLAU	Urokinase-type plasminogen activator Urokinase-type plasminogen activator long chain A, Urokinase-type plasminogen activator short chain A, Urokinase-type plasminogen activator chain B	ENSBTAP00000007806	32.9
22	PQLC3	PQ-loop repeat-containing protein 3	ENSBTAP00000027051	31.6
23	PDZK1	Na(+)/H(+) exchange regulatory cofactor NHE-RF3	ENSBTAP00000007638	31.0
24	LOC523509	Uncharacterised protein	ENSBTAP00000050990	30.9
25	TMEM20	Bos taurus solute carrier family 35, member G1 (SLC35G1), mRNA.	ENSBTAP00000045606	30.0

The 25 most differentially expressed genes (≥30-fold; $p \leq 0.05$) between blastocyst and morula are listed. FC = fold change; positive values indicate higher expression in blastocyst, and negative values have higher expression in morula.

Table 2. AgriGO parametric analysis of gene set enrichment (PAGE) analysis (blastocyst vs. morula).

GO_acc	ontology	description	genes #	p-value	FDR
GO:0006629	P	**lipid metabolic process**	311	0	0
GO:0016070	P	RNA metabolic process	840	2.70E-29	2.70E-26
GO:0006396	P	RNA processing	242	1.00E-28	7.00E-26
GO:0010467	P	gene expression	1068	6.50E-22	3.20E-19
GO:0006139	P	nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	1169	4.20E-20	1.70E-17
GO:0003676	F	nucleic acid binding	929	5.70E-35	2.40E-32
GO:0003723	F	RNA binding	252	1.00E-20	2.20E-18
GO:0003677	F	DNA binding	623	7.00E-19	1.00E-16
GO:0030528	F	transcription regulator activity	445	4.40E-11	4.70E-09
GO:0004194	F	**pepsin A activity**	14	1.40E-10	1.20E-08
GO:0016020	C	**membrane**	1629	0	0
GO:0005886	C	**plasma membrane**	907	0	0
GO:0044425	C	**membrane part**	1280	0	0
GO:0044421	C	**extracellular region part**	564	0	0
GO:0016021	C	**integral to membrane**	1012	0	0

Genes differentially expressed between blastocyst and morula were assessed for their properties described by gene ontology (GO) terms in respect to their relative expression. The five most significantly enriched GO-terms (according to p-value) are listed for each GO domain. P=Biological Process, F=Molecular Function, and C=Cellular Component. Bold descriptions indicate enrichment for blastocyst. Regular descriptions indicate enrichment for morula. FDR = False Discovery Rate (Hochberg).

Supplemental Table 2). *SOX2* and *POU5F1* that together with *NANOG* form the core transcriptional regulatory circuitry in pluripotent cells were also over-expressed in the ICM compared with TE (Figure 3B).

For human ES cells, a network of NANOG-, SOX2-, and POU5F1-target genes that encode transcription factors and chromatin modulators has been established (Boyer et al. 2005). From the list of positively regulated genes in human ES cells, apart from the core network only expression of *STAT3* and *ZIC3* was significantly up-regulated in bovine ICM versus TE (Supplemental Table 2). Again, we performed a parametric analysis of gene set enrichment with the genes differentially expressed between ICM and TE in AgriGO (Du et al. 2010). With a false discovery rate (FDR) ≤0.1, *enzyme linked receptor protein signaling pathway* (GO:0007167) and *peptidyl-tyrosine phosphorylation* (GO:0018108) terms were enriched for the ICM whereas for the TE, genes were enriched in the *sterol biosynthetic process* (GO:0016126) (Table 4). Only genes up-regulated in respect to the TE were used for a singular enrichment analysis (SEA) in order to identify characteristics specific for the cells of the ICM (Supplemental Table 3). In particular, terms containing genes involved in *RNA splicing* (GO:0008380) and *regulation of gene*

Table 3. List of the most differentially expressed genes between inner cell mass and trophectoderm.

Rank	Entrez Gene name	Gene Description	AgriGO ID	FC
1	NANOG	Homeobox protein NANOG	ENSBTAP00000027863	13.2
2	UPP1	Uridine phosphorylase 1	ENSBTAP00000011088	8.9
3	SOX2	SRY (sex determining region Y)-box 2	ENSBTAP00000015411	8.6
4	CAV1	Caveolin-1	ENSBTAP00000023751	7.8
5	AK3L1	Adenylate kinase isoenzyme 4, mitochondrial	Q0VCP1	7.5
6	LOC616039	Pancreatic trypsin inhibitor-like	XP_873093	7.4
7	OTX2	Bos taurus orthodenticle homeobox 2 (OTX2), mRNA	ENSBTAP00000019616	7.2
8	GPC4	Bos taurus glypican 4 (GPC4), mRNA	ENSBTAP00000027510	7.1
9	HAS2	Hyaluronan synthase 2	ENSBTAP00000026503	7.1
10	SLC4A7	Solute carrier family 4, sodium bicarbonate cotransporter, member 7	AAI42306.1	7.1
11	HNF4A	Bos taurus hepatocyte nuclear factor 4, alpha (HNF4A), mRNA	ENSBTAP00000016078	7.0
12	CLIC6	Chloride intracellular channel 6	ENSBTAP00000002299	6.9
13	LGALS4	Galectin-4	ENSBTAP00000021701	6.8
14	ID1	DNA-binding protein inhibitor ID-1	ENSBTAP00000021521	6.7
15	FLRT3	Fibronectin leucine rich transmembrane protein 3	ENSBTAP00000004298	6.6
16	PDGFRA	Bos taurus platelet-derived growth factor receptor, alpha polypeptide (PDGFRA), mRNA	ENSBTAP00000009441	6.6
17	NID1	Nidogen 1	ENSBTAP00000009531	6.5
18	KIT	Mast/stem cell growth factor receptor	ENSBTAP00000003498	6.5
19	GRP	Bos taurus gastrin-releasing peptide GRP mRNA, complete cds.	ENSBTAP00000006297	6.5
20	LOC100139916	LOC100139916 interleukin 32-like		6.5
21	TKTL1	Transketolase-like protein 1	ENSBTAP00000036249	6.4
22	A2M	Alpha-2-macroglobulin	ENSBTAP00000006167	6.4
23	PDYN	Proenkephalin-B preproprotein	AAI51344.1	6.1
24	ACTG2	Actin, gamma 2, smooth muscle, enteric	ENSBTAP00000036954	6.0
25	MME	Membrane metallo-endopeptidase	ENSBTAP00000002681	6.0

The 25 most differentially expressed genes (>6-fold; $p \leq 0.05$) between inner cell mass and trophectoderm are listed according to their relative expression. FC = Fold change and positive values indicate higher expression in inner cell mass.

expression (GO:0010468) were enriched even to a more specific level of enriched child terms like *nuclear mRNA splicing* (GO:0000398) and *chromatin silencing* (GO:0006342), respectively (Figure 4, Supplemental Table 3 and Supplemental Figure 2).

Table 4. AgriGO parametric analysis of gene set enrichment (PAGE) analysis (ICM vs. TE).

GO_acc	ontology	description	Genes #	p-value	FDR
GO:0016126	P	sterol biosynthetic process	12	1.40E-11	9.10E-09
GO:0016125	P	sterol metabolic process	20	2.00E-09	6.50E-07
GO:0008202	P	steroid metabolic process	28	1.00E-07	1.70E-05
GO:0008203	P	cholesterol metabolic process	18	8.90E-08	1.70E-05
GO:0006694	P	steroid biosynthetic process	20	2.00E-07	2.60E-05
GO:0006629	P	lipid metabolic process	62	6.40E-07	6.10E-05
GO:0008610	P	lipid biosynthetic process	34	6.40E-07	6.10E-05
GO:0006720	P	isoprenoid metabolic process	13	2.10E-05	1.70E-03
GO:0006066	P	alcohol metabolic process	54	1.50E-04	1.10E-02
GO:0044255	P	cellular lipid metabolic process	39	2.20E-04	1.40E-02
GO:0010038	P	response to metal ion	14	3.70E-04	2.20E-02
GO:0006721	P	terpenoid metabolic process	11	5.20E-04	2.90E-02
GO:0023034	P	**intracellular signaling pathway**	65	1.70E-03	8.70E-02
GO:0018212	P	**peptidyl-tyrosine modification**	12	2.30E-03	9.40E-02
GO:0018108	P	**peptidyl-tyrosine phosphorylation**	12	2.30E-03	9.40E-02
GO:0007167	P	**enzyme linked receptor protein signaling pathway**	39	2.00E-03	9.40E-02
GO:0031090	C	organelle membrane	70	1.90E-04	7.40E-03
GO:0005773	C	vacuole	18	1.40E-04	7.40E-03
GO:0042175	C	nuclear envelope-endoplasmic reticulum network	23	1.40E-04	7.40E-03
GO:0005789	C	endoplasmic reticulum membrane	22	4.70E-04	1.00E-02
GO:0005764	C	lysosome	17	5.10E-04	1.00E-02
GO:0000323	C	lytic vacuole	17	5.10E-04	1.00E-02
GO:0005768	C	endosome	23	1.40E-03	2.20E-02
GO:0012505	C	endomembrane system	62	1.50E-03	2.20E-02
GO:0044432	C	endoplasmic reticulum part	25	2.30E-03	3.00E-02

All genes differentially expressed between ICM and TE were assessed for their properties described by gene ontology (GO) terms in respect to their relative expression. This reveals 25 enriched GO-terms with a FDR ≤ 0.1 in the GO domains Biological Process (P) and Cellular Component (C). Ranked according to z-score. Bold descriptions indicate enrichment for inner cell mass. Regular descriptions indicate enrichment for trophectoderm. FDR = False Discovery Rate (Hochberg).

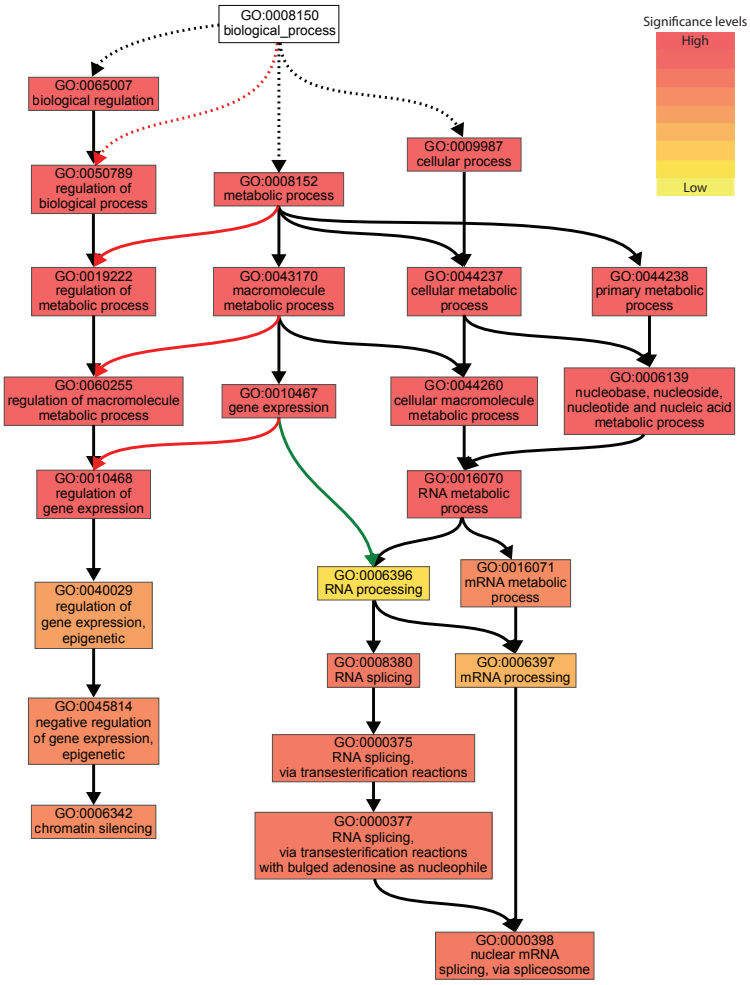

Figure 4.
Biological processes in inner cell mass (partial). All genes expressed at higher levels in inner cell mass compared with trophectoderm were assessed for their enrichment (GO term analysis in AgriGO) in *biological processes* (GO:0008150). The hierarchical tree graph contains a highly enriched group of processes ending in *chromatin silencing* (GO:0006342) and *nuclear mRNA splicing, via spliceosome* (GO:0000398).

Genes differentially expressed between MAPK-inhibited and control ICMs.

It has been described that culture of ICM cells in the presence of an FGFR inhibitor, a MAPK inhibitor, and an inhibitor of GSK3β, the so-called 3i culture system, leads to stable ES cell lines from non-permissive mouse strains (Ying et al. 2008, Nichols & Smith 2009) and rat embryos (Buehr et al. 2008). Culture of mouse embryos in the presence of a MAPK inhibitor resulted in all cells of the ICM expressing *Nanog* while the

expression of *Gata6* was down-regulated (Yamanaka et al. 2010). Similarly, when bovine embryos were cultured in the presence of the MAPK inhibitor PD0325901, a larger percentage, although not all, of the ICM cells expressed NANOG (Kuijk et al. 2012). Therefore, in order to identify NANOG target genes and genes that may be important for pluripotency, ICMs were isolated from bovine embryos cultured in the presence of the MAPK inhibitor PD0325901. Gene expression in these ICMs was compared with that from control ICMs using microarray analysis. In total 94 genes were differentially regulated between control (DMSO) and MAPK-inhibited ICMs, ≥2-fold difference with p-value ≤ 0.05 as cut-off, with the expression of 44 genes up-regulated, and the expression of 50 genes down-regulated (Supplemental Table 4). As expected, *NANOG* expression was up-regulated in the MAPK-inhibited ICM as detected by microarray analysis (Figure 5A,B). Expression differences between control ICM and ICM from embryos exposed to the MAPK inhibitor detected by qRT-PCR verified the microarray data (Figure 5B,C, and Supplemental Figure 3). Furthermore, immunostaining showed an increase in the percentage of NANOG expressing cells in the ICM after MAPK inhibition (Supplemental Figure 4) as we had previously established (Kuijk et al. 2012). To our surprise several interferon coding genes were dramatically down-regulated after MAPK inhibition such as *IFNW1* and *IFNT* (Figure 5A). Gene expression analysis by qRT-PCR confirmed the microarray results and further showed a decreased *IFNT* expression in TE and to a greater extend in ICM upon MAPK inhibition (Figure 5D).

Genes up-regulated in the PD-treated or in the control ICMs were enriched in 189 of the same GO terms after a singular enrichment analysis containing at least two genes and a FDR ≤ 0.1 (Supplemental Table 5 and Figure 5E). When the relative gene expression was taken into account only 6 GO terms were found to be enriched with a FDR < 0.1, of which 4 were under-represented in the PD-treated ICMs (Table 5). The only function-describing term was *receptor binding* (GO:0005102), and this term was under-represented in the PD group.

Cross-comparisons

The 497 genes differentially expressed in the ICM versus TE and the 95 differentially expressed genes in the MAPK inhibited versus control ICMs were compared revealing that 42 genes were shared (Table 6). Of these genes 15 were both up-regulated in ICM and after MAPK inhibition while 27 were contra-regulated (Figure 6A). Only *PRPH* was expressed at lower levels in ICM than in TE, and 26 genes were down-regulated after MAPK inhibition, and the expression of 16 genes was further up-regulated (Table 6). Although it has been described that NANOG activates *POU5F1* and *SOX2* transcription, expression of these genes was not significantly altered after up-regulation of *NANOG* expression by MAPK inhibition.

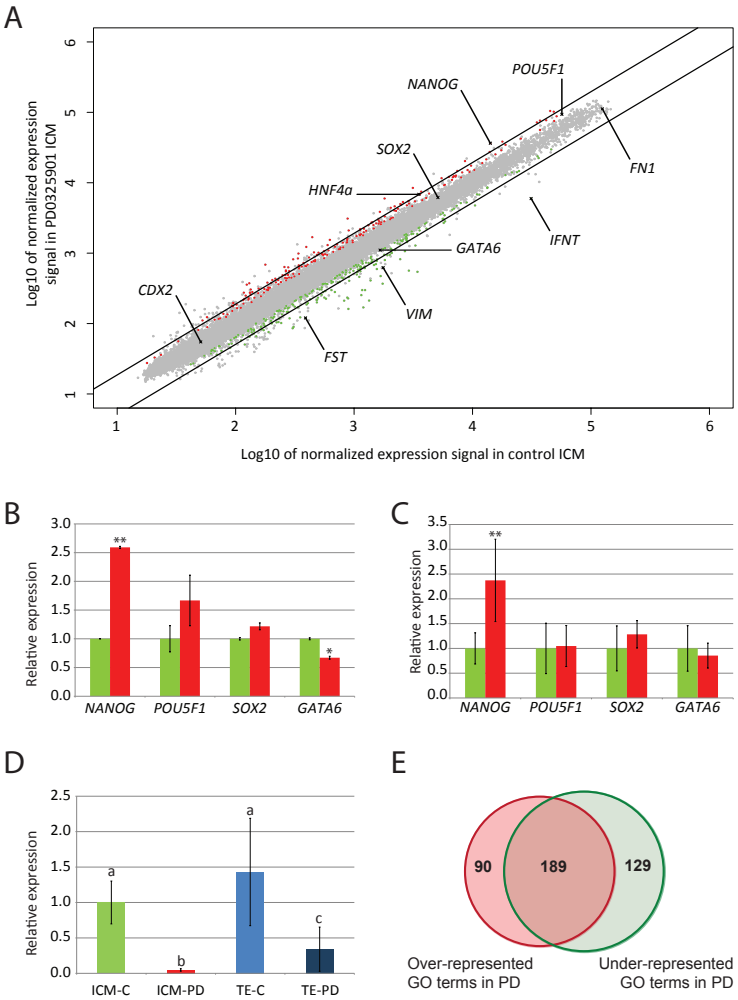

Figure 5.

E**xpression in and GO-analysis of MAPK-inhibited and control inner cell mass.** All probes representing genes (grey) were plotted for their expression levels. Black lines represent the 2-fold cut-off. Axes are Log10 transformed and depict the normalized expression signal in the indicated sample. Genes discussed in text (and analysed by qRT-PCR) are indicated. Genes with significant ($p \leq 0.05$) higher expression levels in MAPK-inhibited and control inner cell masses are indicated with red and green dots, respectively. MAPK-inhibited ICM (red) gene expression of *NANOG*, *POU5F1*, *SOX2*, and *GATA6* was determined by microarray (B) and qRT-PCR (C), presented relative to gene expression in control ICM (green). Significant differences are indicated; * $p<0.05$; ** $p<0.005$. *IFNT* expression (D) was determined by qRT-PCR in control TE (light blue), PD0325901-treated TE (dark blue), and PD0325901-treated ICM (red) and presented relative to control ICM (green). Normalization was performed with the reference genes *RPL15*, *SDHA*, and *YWHAZ*. Error bars indicate standard deviation, and data bars with different letters are significantly different ($p<0.05$). A Venn diagram (E) shows the numbers of over-represented (red; total 279) and under-represented (green; 318 in total) GO-terms in the MAPK-inhibited inner cell mass. 189 GO-terms are in common.

Table 5. AgriGO parametric analysis of gene set enrichment (PAGE) analysis (MAPK inhibited vs. control).

GO_acc	ontology	description	genes #	p-value	FDR
GO:0005102	F	receptor binding	12	0.0016	0.027
GO:0005615	C	extracellular space	26	7.60E-05	0.003
GO:0044421	C	extracellular region part	28	0.00019	0.0036
GO:0005576	C	extracellular region	34	0.0033	0.043
GO:0043232	C	**intracellular non-membrane-bounded organelle**	18	0.0093	0.073
GO:0043228	C	**non-membrane-bounded organelle**	18	0.0093	0.073

All genes differentially expressed between MAPK-inhibited and control ICMs were assessed for their properties described by gene ontology (GO) terms in respect to their relative expression. This revealed 6 enriched GO-terms with a FDR ≤ 0.1 in the GO domains Molecular Function (F) and Cellular Component (C). Bold descriptions indicate enrichment in MAPK-inhibited ICM. Regular descriptions indicate enrichment in control ICM.

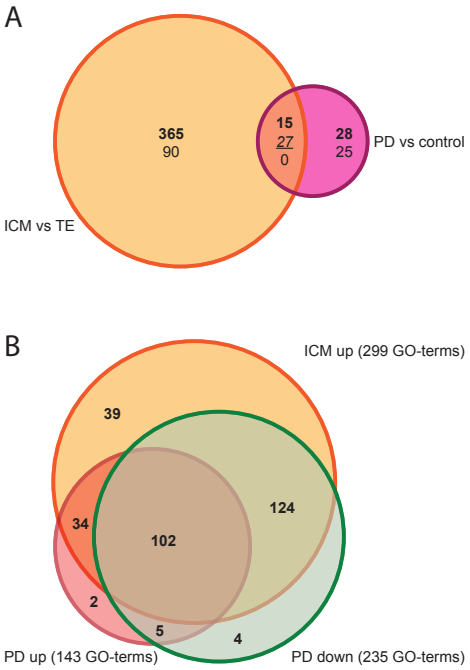

Figure 6.
Gene expression and processes in the inner cell mass. (A) Of the 497 genes differentially expressed (≥2-fold; p≤0.05) between ICM and TE (orange) several are up-regulated (bold) or down-regulated (regular), and 42 of these are also differentially expressed when inner cell masses treated with the MAPK-inhibitor PD0325901 (PD) are compared with control inner cell masses (magenta). This latter comparison retains 95 differentially expressed genes. Of the 42 shared genes 15 are up-regulated in both comparisons and 27 are contra-regulated (italics and underlined). (B) A gene ontology analysis with 41 ICM up-regulated genes (orange), 16 PD up-regulated (red) and 26 PD down-regulated (green) genes indicates several GO-terms of which 34 are enriched in both up-regulated gene analyses.

Table 6. List of genes differentially expressed in ICM vs. TE and PD treated vs. control ICMs.

Gene name	FC (ICMvsTE)	FC (PDvsControl)
ADH6	3.336	-2.993
AHCYL2	5.379	2.068
C8A	2.558	2.009
CD8B	3.392	-2.010
CKB	3.660	2.062
CTSC	3.064	-2.517
CYP1A1	4.041	-2.563
DHRS7	3.717	2.170
DUSP4	3.685	-6.320
EMILIN2	5.463	2.697
FBLN1	2.215	-2.160
FST	2.468	-3.235
GKN2	2.615	-2.426
HAS2	7.117	-2.237
JAM2	2.205	-2.186
LOC100139049	2.661	2.276
LOC100139916	6.461	-2.002
LOC100140174	3.096	2.880
LOC616039	7.437	-2.203
MAP1B	3.657	2.032
MEIS2	3.638	-2.508
MFAP5	4.520	-2.403
MYL9	2.257	3.305
NANOG	13.215	2.575
NID1	6.536	-2.072
P4HA3	3.279	2.083
PDGFRA	6.583	-2.116
PDYN	6.053	-2.398
PHLDA1	2.679	-2.133
PRPH	-2.552	2.124
PRSS12	4.640	2.334
RSPO3	2.572	-2.619
S100B	3.875	3.516
SELP	2.758	2.466
SERPINA5	5.908	-7.057
SERPINH1	2.673	-2.110
SLC1A3	3.230	-2.249
SPRY4	4.314	-2.065
TGM2	3.363	-2.231
TIFA	3.394	-2.569
unknown	2.868	2.076
VIM	4.636	-2.827

Alphabetical ranking of differentially expressed genes ($p<0.05$) in inner cell mass versus trophectoderm (ICMvsTE) and MAPK-inhibited versus control inner cell masses (PDvsControl). FC = Fold change in respect to ICM or PD.

The expression of *GATA6* was significantly down-regulated in the ICM after MAPK inhibition, although the difference was less than 2-fold and was not significant in the qRT-PCR analysis. The expression of 26 other ICM-specific genes was significantly down-regulated upon MAPK inhibition suggesting that these genes are involved in primitive endoderm formation (Figure 5 and Table 6). Expression of a number of the genes was also analysed by qRT-PCR showing a similar pattern and validating the microarray data (Figure 5 and Supplemental Figure 3). Indeed mouse follistatin, coded by *Fst*, has been implicated as a marker for primitive endoderm derivatives (Veltmaat et al. 2000). Other genes down-regulated in the MAPK inhibited bovine ICMs themselves code for negative regulators of MAPK activity such as *DUSP4* and *SPRY4*.

The 42 shared genes were analyzed for their properties by gene ontology analysis. A PAGE analysis in AgriGO revealed 59 enriched GO-terms of which none had a FDR<0.1. When the SEA was performed using either ICM up-regulated, PD up-regulated, or PD down-regulated genes (Supplemental Table 6), a Venn diagram with the enriched terms (FDR<0.1) revealed 34 enriched GO-terms shared between the up-regulated gene comparisons (Figure 6B, Supplemental Table 6).

DISCUSSION

During bovine preimplantation development several cell types display a pluripotent character. The failure in generating true pluripotent ES cell lines from *Bos taurus* embryos however indicates that, compared with murine and human embryos, other genes are involved in maintenance of pluripotency, that the correct embryonic stage with pluripotent cells has not been used, or that the culture conditions employed did not sufficiently inhibit differentiation. Interestingly, induced pluripotent stem (iPS) cells generated from bovine cells also behave differently than mouse iPS cells. Similar to porcine iPS cells, the introduced transgenes are not silenced in the currently used culture conditions, but remain expressed in these cells suggesting that other factors are needed for maintenance of pluripotency (Sumer et al. 2011). We performed a microarray analysis comparing morula, blastocyst, ICM, and TE gene expression profiles to identify genes possibly involved in pluripotency. Further enrichment of the pluripotent character of the ICM was achieved by inhibiting the ERK pathway through exposure to the MAPK inhibitor PD0325901 during *in vitro* culture thereby increasing the percentage of *NANOG* expressing cells in the ICM/epiblast (Kuijk et al. 2012, Van Der Jeught et al. 2013).

To obtain samples for the microarray analysis embryos were cultured up to the morula stage or blastocyst stage. Blastocysts were dissected manually to separate ICM and TE. The advantage of this technique is that ICM and TE are isolated from the

same embryo, in contrast to for example a technique like immunosurgery. Manually dissecting blastocysts is challenging however, and it is unavoidable that few TE cells remain attached to the ICM. We therefore verified that the separation of the two cell types was successful. For separation of ICM from TE, Nagatomo et al have used either a micromanipulator or mild trypsin treatment to separate ICM (Nagatomo et al. 2013). ICMs isolated using the micromanipulator still contained 43.3% TE cells (Nagatomo et al. 2013). In our hands the percentage of TE cells remaining in the ICM isolates was ~20% as determined by CDX2 expression, indicating a low contribution of TE cells to the ICM transcriptome. The disadvantage of mild trypsin treatment to isolate 'pure' ICM cells, is that TE and ICM cannot be compared from the same embryo and that the trypsin treatment by itself may cause a difference in gene expression. The observation that duplicate samples paired together and that TE and ICM clustered apart from each other together with the expected expression patterns of known TE- and ICM-specific genes in the microarray as well as by qRT-PCR indicates that indeed the separation was specific and reproducible.

Several genes were represented on the array by multiple probes, and in those cases we only used the expression data of the most 3'-located probe. Unfortunately, the *Bos taurus* genome is not completely annotated (Zimin et al. 2009), and indeed approximately 5% of the probes representing genes differentially expressed between blastocyst and morula could not be identified. The other comparisons could be made with all probes linked to a known differentially expressed gene. For the Gene Ontology analysis genes need to be associated with a GO-term. Not all genes are associated with a GO-term, and therefore 3.7% – 5.3% of the genes could not be analysed in the AgriGO gene ontology analysis.

In vitro derived embryos were used as this enabled us to generate the numbers needed for RNA extraction. Particularly for the ICM and TE samples large numbers of embryos were needed to obtain sufficient amounts of RNA for hybridization (Supplemental Table 7). Although a significant difference in gene expression between *in vitro* and *in vivo* derived embryos has been demonstrated (Kues et al. 2008) the birth of healthy animals from *in vitro* derived embryos indicates that the pathways for pluripotency are functional in these embryos. When gene expression was compared between different stages of *in vivo* derived bovine embryos most genes were found to be differentially expressed between early development (oocyte-4 cell stage) and later stages (8-cell stage-blastocyst) (Kues et al. 2008). Most likely these differences in gene expression are caused by embryonic genome activation around the 8-cell stage (Kues et al. 2008, Sirard 2012, Graf et al. 2014). A larger number of genes (~1800) was expressed in *in vivo* derived oocytes compared with *in vitro* matured oocytes (Kues et al. 2008), indicating that in our study with *in vitro* derived embryos important genes may not have been detected. However, since *in vitro* derived embryos are commonly

used for embryo transfer and give rise to healthy animals, it can be expected that genes important for pluripotency are sufficiently expressed, and the pathways for pluripotency are functional in *in vitro* derived embryos.

We started our analysis by comparing gene expression in blastocysts with that in morulae. This indicated that most differentially expressed genes are expressed at higher levels in the blastocyst, but a GO-analysis revealed that most genes expressed at higher levels in morulae are involved in gene transcription. This might be a result of the embryonic genome activation initiated during the 8-16 cell stage in cattle embryos preceding the morula stage (Kues et al. 2008, Sirard 2012, Graf et al. 2014). Next we tried to identify genes involved in pluripotency by comparing gene expressions in ICM and TE. Mouse and human ES cell pluripotency is regulated by NANOG, SOX2, and OCT4, and these factors enhance each other's transcription (Jaenisch & Young 2008, Loh & Lim 2011, Wang et al. 2012). Indeed, their gene expression levels were found higher in the bovine ICM samples compared with TE. Remarkably, in the comparison of the ICM with the TE, expression of genes in the GO-category *RNA splicing* was specifically up-regulated in the ICM. This indicates a higher transcriptional activity in ICM cells than in TE cells. This is further reflected in the >400 genes up-regulated in ICM compared with TE. Using deep sequencing, Ozawa et al. examined genes differentially expressed between ICM and TE of day 8 *in vitro* derived embryos (Ozawa et al. 2012). All of 8 ICM-characteristic genes that Ozawa et al. found were also up-regulated in our study, except for *ZC3HAV1* and *Il6R*. Expression of *Il6R* was indeed significantly up-regulated in the ICM, but the difference was below the cut-off used (2-fold). These results confirm the specificity and reliability of the ICM isolation and microarray analysis. Compared with our results Ozawa et al. found more genes (870 versus 497 in our study) to be differentially expressed between ICM and TE, most likely because of the less stringent cut-off value used (1.5- versus 2.0-fold difference in our study) (Ozawa et al. 2012).

By enhancing the overall *NANOG* expression in the ICM we had anticipated that *SOX2* and *POU5F1* expressions were similarly enhanced. Surprisingly however, in ICMs from embryos cultured in the presence of a MAPK inhibitor, gene expression levels of *NANOG* were up-regulated while those of *POU5F1* and *SOX2* remained relatively unchanged. These results suggest that in bovine cells *NANOG* by itself is not sufficient in maintaining the core pluripotency network.

An unexpected result was the expression of several interferon-coding genes in the ICM. Various reports have described exclusive *IFNT* expression in trophectoderm or TE derived cell lines (Ezashi et al. 2001, Johnson et al. 2006, Sakurai et al. 2009). We detected *IFNT* expression in the isolated ICMs at similar levels as in TE however, and the expression in the ICM was down-regulated upon MAPK inhibition even to a greater extent than in TE. In ungulates, interferon tau (coded by *IFNT*) expression by TE is important for maternal pregnancy recognition (Roberts 2007). In bovine day 7 blastocysts

interferon tau has been detected at varying intensity in the TE and was concentrated at the border of the ICM and TE (Johnson et al. 2006). By dissecting the ICM, part if not all of the interferon tau-positive adjacent cells have been included in the ICM samples accounting for the observed *IFNT* expression in the ICM samples. Together with *CDX2* predominantly expressed in TE cells and capable of increasing *IFNT* transcription (Imakawa et al. 2006, Sakurai et al. 2009), this might explain the greater expression reduction found in ICM (20-fold) than in TE (4-fold). Nevertheless, our results and the previously reported location of interferon tau expression (Johnson et al. 2006) do not exclude *IFNT* expression in the ICM even though its function in the ICM is unknown.

After exposure to PD0325901 expression of *NANOG* in the ICM was enhanced as compared to control ICMs. In the mouse more than 3,000 genes have been identified containing NANOG binding sites (Loh et al. 2006). Of the 42 genes identified to be differentially expressed in bovine ICMs and after MAPK inhibition, only five were homologous to murine genes containing NANOG binding sites. Of those genes only expression of *NANOG* was up-regulated after MAPK inhibition. Of the remaining four, *CD8B*, *DUSP4*, *JAM2,* and *SPRY4*, the expression was enhanced in the ICM, but their expression was down-regulated after MAPK inhibition. The role of the glycoprotein CD8B in early embryonic development, and more specifically in the ICM, is unclear. Its expression can be regulated however by MAPK signalling (Wilkinson & Kaye 2001, D'Souza et al. 2008) possibly accounting for the observed down-regulation after PD0325901 treatment. DUSP4 is suggested to function in the negative feedback control of MAPK signalling specifically dephosphorylating ERK1/2 (Chu et al. 1996, Kondoh & Nishida 2007). Also SPRY4 is known for its involvement in the MAPK pathway by interacting with GRB2 and GAP1 and as such inhibiting RAS activation (Casci et al. 1999) and antagonizing FGF activity (Fürthauer et al. 2001). Therefore, the down-regulation of *DUSP4* and *SPRY4* expression by MAPK inhibition is most likely a direct result of the MAPK inhibition rather than result from the up-regulation of *NANOG* expression. *JAM2* is expressed in both embryonic and adult stem cell lines (Ivanova et al. 2002), and its expression is enhanced in undifferentiated mouse ES cells compared to early stages of differentiation. Since mouse ES cells that genetically lack *Jam2* maintain pluripotency however, the function of JAM2 in stem cells remains unknown (Sakaguchi et al. 2006). In mouse Sertoli cells inhibition of ERK activity did not affect *Jam2* transcription (Wang & Lui 2009), suggesting that the observed reduced *JAM2* expression resulted from increased *NANOG* levels. Interestingly, *JAM2* expression was also down-regulated after OCT4 had been exogenously introduced into human cells, suggesting that low levels of JAM2 induce or indicate differentiation (Kim et al. 2009). In our bovine ICMs *POU5F1* expression was however not significantly up-regulated after enhanced *NANOG* expression. Surprisingly, no other genes that had been identified as overlapping NANOG putative

targets in mouse and human ES cells (Loh et al. 2006) appeared to be up- or down-regulated in bovine ICMs with enhanced *NANOG* expression.

Apart from the core pluripotency markers NANOG, SOX2, and OCT4, other transcription factors are reported to be involved in mouse or human pluripotency. Of all transcription factors differentially expressed between ICM and TE, *OTX2* ranked third and was 7.2-fold higher expressed in ICM. In mouse ES cells OTX2 was reported to be required for the transition to a stable epiblast stem cell condition (Acampora et al. 2013). Recently, it was shown that OTX2 is one of the earliest transcription factors to be activated during exit from a naïve ground state in mES cells (Yang et al. 2014). Although the MAPK pathway is important in cell differentiation (Ying et al. 2008) and therefore might influence *OTX2* expression, we did not detect a difference in *OTX2* expression in the PD treated bovine ICMs. Together, these findings suggest that the ICMs under investigation were already in a "primed" state.

Transcription factors involved in the LIF or BMP pathway were also amongst the genes with up-regulated expression levels in the ICM. Although *BMP4* was not differentially expressed, *STAT3* (2.5-fold), *ID3* (2.7-fold), and *ID1* (6.7-fold) were expressed at higher levels in ICM than in TE. STAT3 is capable of suppressing mesoderm and endoderm commitment whereas ID genes suppress neuroectoderm commitment in mES cells. Fibronectin, with expression levels almost 4-fold higher in ICM, can induce *Id* expression, and also NANOG is capable of activating STAT3 and inducing ID genes (Ying et al. 2003). Up-regulated *NANOG* expression did however not induce *STAT3* or *ID* expression in MAPK inhibited ICMs. Although the level of expression might not be high enough, the increased expression of *STAT3*, *ID1*, and *ID3* suggests that, although in a primed state, differentiation is not initiated yet in the bovine day 9 ICMs.

The transcription factor PRDM14 is implicated to act as a safeguard for maintaining pluripotency (Ma et al. 2011) and is uniquely expressed in mouse compacted morula, ICM, the early epiblast, primordial germ cells, and ES cells (Kurimoto et al. 2006, Yamaji et al. 2008, Grabole et al. 2013). Indeed, the expression of *PRDM14* was found to be up-regulated in bovine morulae compared to blastocysts (2.4-fold; p=0.00097) and ICM versus TE (2.5-fold; p=0.0015). It has been reported that in mouse ES cells PRDM14 attenuates FGF-induced differentiation (Grabole et al. 2013). We did however not observe a difference in levels of *PRDM14* expression in MAPK-inhibited ICMs (1.07-fold difference; p=0.87), suggesting that the FGF- or MAPK-signalling pathways do not repress *PRDM14* expression in bovine pluripotent cells. Expression of the ZIC gene family members *ZIC2* and *ZIC3* was also up-regulated in the ICM (4.3-fold and 2.2-fold, respectively). *Zic2* and its orthologues are expressed in frog (Kuo et al. 1998) and zebrafish (Grinblat & Sive 2001) pregastrulation embryos, and in mouse E0.5 and ICM of E4.5 embryos (Brown & Brown 2009). *Zic3* is implicated to play an important role in maintaining pluripotency in mouse ES cells (Lim et al. 2007) and contains OCT4,

NANOG, and SOX2 binding sites (Boyer et al. 2005, Loh et al. 2006). Indeed, after increased *NANOG* expression by MAPK inhibition *ZIC3* expression increases 1.5-fold ($p=0.038$), but *ZIC2* expression decreased 2.2-fold ($p>0.05$). Other reported genes to safeguard pluripotency such as *PARP1* and *PARP7* (Roper et al. 2014) were expressed at higher levels in morula than in blastocyst (*PARP7*), did not show a differential expression between ICM and TE and were not differentially expressed after MAPK inhibition. All together, these findings indicate that, despite the increased *ZIC3* and *NANOG* expression, MAPK inhibition by PD0325901 is insufficient to maintain a pluripotent state in bovine ICM cells.

CONCLUSION

We have identified whole genome expression profiles of different stages of bovine embryos and TE and the pluripotent ICM of blastocysts. In addition, the transcriptome of ICMs with enhanced *NANOG* expression after inhibition of MAPK activity was established. Unfortunately, these expression profiles did not lead to (new) pathways or indications how to maintain pluripotency and possibly generate genuine bovine ES cells. Furthermore, it became apparent that although MAPK inhibition increased *NANOG* and *ZIC3* expression, this is insufficient to maintain pluripotency. Comparing transcription factor expression in the bovine ICMs used in the microarray with known expressions in mouse pluripotent cells indicates a "primed" or epiblast state. Therefore, the data presented in this paper can act as a starting point for further research on bovine pluripotency.

METHODS

Bovine in vitro embryo culture and mechanical separation

Bovine embryo culture was performed at 39°C in a humidified atmosphere with 5% CO_2, unless stated otherwise. Three to eight mm follicles of ovaries, obtained from a local slaughterhouse, were aspirated to retrieve COCs. Groups of 35-60 COCs were incubated for 23 h in 500 µl M199 (Life Technologies, Bleiswijk, The Netherlands) supplemented with 0.05 IU/ml recombinant hFSH (Organon, Oss, The Netherlands) and with 1% penicillin-streptomycin (Life Technologies). Fertilisation was performed as described previously (Parrish et al. 1988) with modifications as described (Izadyar et al. 1996). In short, matured COCs were transferred to fertilisation medium (Fert-TALP) supplemented with heparin at a final concentration of 10 µg/ml (Sigma Aldrich, Zwijndrecht, The Netherlands), 20 µM D-penicillamine (Sigma Aldrich), 10 µM hypotau-

rine (Sigma Aldrich), and 1 μM epinephrine (Sigma Aldrich). Frozen-thawed sperm from a bull with proven fertility was centrifuged over a Percoll-gradient (GE Healthcare Europe GmbH, Eindhoven, The Netherlands) and added to the COCs at a final concentration of 1.0×10^6 spermatozoa/ml. Fertilisation day was considered as day 0. After incubation for 20 h the COCs were denuded by vortexing for 3 min, and the cumulus-free oocytes were placed in synthetic oviduct fluid (SOF) medium. The presumptive zygotes were incubated at 39°C in a humidified atmosphere with 7% O_2 and 5% CO_2. At day 5 either morulae were collected or embryos were transferred to fresh SOF medium, cultured to blastocyst stage embryos and collected on day 9. Embryos were cultured until day 9 of development as this resulted in a higher percentage of hatching and hatched blastocysts (Rizos et al. 2003, Nicacio et al. 2012, Madeja et al. 2013), which facilitated ICM from TE separation. To ensure good quality embryos only stage codes 7-9 blastocysts with quality code 1 or 2, according to the IETS manual, were collected (Stringfellow & Givens 2009, Bó & Mapletoft 2013). To obtain ICMs containing a higher percentage of NANOG-expressing cells, SOF medium was supplemented with a final concentration of 0.5 μM PD0325901 (Stemgent, Cambridge, MA, USA) at day 5. Embryo culture for control ICM samples was performed with equal concentrations of the solvent DMSO.

Blastocysts collected to obtain inner cell mass and trophectoderm were placed in wash buffer containing 6.67 mg/ml NaCl (Merck, Schiphol-Rijk, The Netherlands), 0.24 mg/ml KCl (Merck), 0.168 mg/ml $NaHCO_3$ (Sigma Aldrich), 0.047 mg/ml NaH_2PO_4 (Merck), 0.217% (v/v) of a 60% sodium lactate solution (Sigma Aldrich), 2.38 mg/ml HEPES (Sigma Aldrich), 0.2% (v/v) phenolred (Sigma Aldrich), 0.39 mg/ml $CaCl_2 \cdot 2H_2O$ (Sigma Aldrich), 0.10 mg/ml $MgCl_2 \cdot 6H_2O$ (Merck), 0.11 mg/ml sodium pyruvate, 100 U/ml penicillin-streptomycin (Life Technologies), and 6.0 mg/ml bovine serum albumin fraction 5 (MP Biomedicals, Santa Ana, CA, USA), set at an osmolality of 280 osmol/kg and adjusted to pH 7.3.

Sharpened tungsten needles were used to manually separate the trophectoderm from the ICM. This procedure was performed in wash medium under a stereo microscope.

RNA isolation

Collected cells and embryos were harvested per tissue type or treatment and stored in 100 μl extraction buffer (Life Technologies) at -80°C until RNA isolation. RNA isolation and on column DNA digestion (Qiagen, Venlo, The Netherlands) was performed using the PicoPure® RNA isolation kit (Life Technologies) according to the manufacturer's protocol. Total RNA quality and quantity assessment was performed by microelectrophoresis on a Bioanalyzer 2100 using the RNA 6000 Pico LabChip kit (Agilent Technologies, Amstelveen, The Netherlands) according to manufacturer's instructions. RNA was stored at -80°C until further use.

Microarray gene expression analysis

Selected total RNA samples were compared in a common reference experiment design using 12 dual channel microarrays (8 for the stage-/cell-specific microarray and 4 for the MAPK-inhibition microarray) with each sample hybridized against an identical common reference total RNA sample consisting of a pool of blastocysts total RNA. Within each group of two microarrays for each stage/tissue type/treatment, sample versus common reference hybridizations were performed in balanced dye-swap.

Microarrays used were bovine whole genome gene expression microarrays V2 (Agilent Technologies) representing 43,653 *Bos taurus* 60-mer oligos in a 4x44K layout. cDNA synthesis, cRNA double amplification, labelling, quantification, quality control, and fragmentation were performed with an automated system (Caliper Life Sciences NV/SA, Teralfene, Belgium), starting with 10-20 ng total RNA from each sample, all as previously described in detail (Roepman et al. 2006, van Wageningen et al. 2010). Microarray hybridization and washing was with an HS4800PRO system with QuadChambers (Tecan, Mechelen, Belgie) using 700 ng, 1-2% Cy5/Cy3 labelled cRNA per channel as described (van Wageningen et al. 2010). Slides were scanned on an Agilent G2565BA scanner at 100% laser power, 30% PMT. After automated data extraction using Imagene 8.0 (BioDiscovery, Hawthorne, CA, USA), Loess normalization was performed (Yang et al. 2002) on mean spot-intensities. Gene-specific dye bias was corrected by a within-set estimate (Margaritis et al. 2009). Data were further analysed by MAANOVA (Wu et al. 2003), modelling sample, array, and dye effects in a fixed effect analysis. P-values were determined by a permutation F2-test, in which residuals were shuffled 10,000 times globally. Gene probes with $p < 0.05$ after false discovery rate determination (FDR by Benjamini-Hochberg) were considered significantly changed. In cases of multiple probes per gene, the values from the most 3' probe were used (Shi 2006, Li et al. 2011). To determine differentially expressed genes a fold change cut-off of 2-fold was used. All microarray gene expression data have been deposited in NCBI's Gene Expression Omnibus (Edgar et al. 2002) and are accessible through GEO Series accession number GSE63054 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=cxytiuworbupdsf&acc=GSE63054).

Quantitative reverse transcription-PCR

RNA was converted to cDNA using the iScript™ cDNA Synthesis Kit (BioRad, Veenendaal, The Netherlands) according to manufacturer's instructions. Primers (Life Technologies; Supplemental table 8) for specific *Bos taurus* mRNA templates (http://www.ncbi.nlm.nih.gov/nucleotide/) were designed using a Primer3 based platform (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Further *in silico* validation was performed by predicting PCR product folding structures using the Mfold web server http://mfold.rit.albany.edu/?q=mfold/DNA-Folding-Form (SantaLucia 1998, Zuker 2003). For quantitative

reverse transcription PCR (qRT-PCR) we used iQ™ SYBR® Green supermix on a MyiQ detection system (Biorad) in a 25 µl reaction volume with a final primer concentration of 400 nM according to manufacturer's instructions. To confirm specificity of primer pairs and establish melting temperatures (Tm) a temperature gradient was performed ranging from 57.0°C – 65.3°C using a 4 times dilution series of cDNA from blastocyst samples. Reactions started with a 5 min enzyme activation cycle at 95°C continued with 45 cycles in which the first step was 20 s denaturing at 95°C, followed by 30 s at Tm (Supplemental table 8) for annealing, and the third step for 30 s at 72°C for elongation. To generate a dissociation curve the reaction continued by increasing the temperature from 60°C to 98°C per 0.5°C for 15 s each step. For expression analysis of the individual samples the primer specific optimal Tm was chosen (Supplemental table 8), and the dissociation curve was generated with 1°C temperature increments per step until 98°C.

Immunostaining

ICM, TE, and blastocyst samples were collected and fixed in 4% paraformaldehyde (PFA) for 15 min and stored in 1% PFA at 4°C until further use. Samples were permeabilised in PBS + 10% FCS + 0.5% Triton X100 (Sigma Aldrich) for 30 min. Next, aspecific binding was blocked by incubating the samples in PBS + 10% FCS + 0.1% Triton X100 (PBST) for 1 h before overnight incubation with primary antibodies rabbit anti-GATA6 (Santa Cruz; sc-9055; 1:100), mouse anti-CDX2 (Biogenex; CDX2-88; 1:200) or mouse anti-NANOG (eBiosciences; 14-5768-82; 1:250) at 4°C. Secondary antibody incubation for 1 h with appropriate goat anti mouse Alexa647 or goat anti rabbit Alexa 488 dye (Invitrogen, Venlo, The Netherlands) and subsequent nuclear staining using DAPI (Sigma Aldrich) for 5 min preceded Vectashield (Brunschwig Chemie, Amsterdam, The Netherlands) mounting in Grace Bio-Labs SecureSeal™ imaging spacers (Sigma Aldrich). All incubations were performed at room temperature unless stated otherwise.

Fluorescent images were obtained using an inverted semi-automated confocal microscope (SPE-II – DMI4000; Leica, Son, The Netherlands) and further analysed with Fiji software (Schindelin et al. 2012).

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

BB participated in study design, carried out embryo culture, RNA isolation, microarray analysis, qRT-PCR, Immunostaining, and co-wrote the manuscript. HTAT assisted in embryo culture and sample collection. MGK carried out microarray and performed statistical analysis. FMR performed statistical analysis. SIJ carried out qRT-PCR. KM performed statistical analysis. HH participated in the design and data analysis and co-wrote the manuscript. BAJR designed and coordinated the study, assisted in sample collection and co-wrote the manuscript.

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SUPPLEMENTALS

Supplemental figures and tables in this chapter can be found in the electronic version of the paper as published in BMC Genomics (http://www.biomedcentral.com/1471-2164/16/277/).

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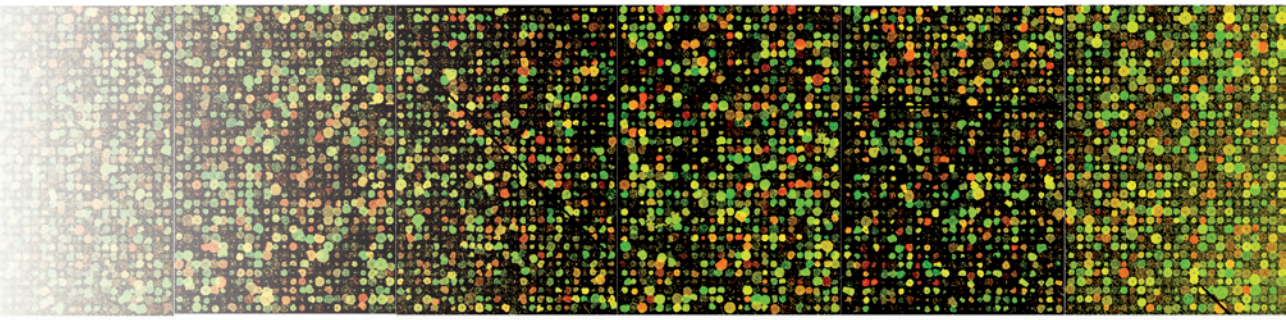

Chapter 3

Gene expression profile of bovine embryos cultured in naïve human stem cell medium

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ABSTRACT

A culture medium sustaining a naïve state in cells cultured from human ICMs has been reported recently. This naïve human stem cell medium (NHSM) could also maintain the naïve state of mouse ES cells. Therefore we investigated the effect of this NHSM on the transcriptome of bovine ICM cells. Microarray analysis was performed to identify gene expression differences in ICMs dissected from blastocysts cultured either in control conditions or in NHSM. Bovine NHSM embryos developed faster and contained more lipid droplets than control embryos. Neither the pluripotency markers *NANOG*, *POU5F1* and *SOX2* were differentially expressed nor were markers for naïvety or differentiation, whereas the expression of *XIST*, involved in X-chromosome inactivation, was down-regulated and *HPRT1* up-regulated in NHSM. The results hint at a more naïve character in the ICM of embryos cultured in NHSM.

INTRODUCTION

Embryonic stem (ES) cells form a valuable type of cells to study gene function and to understand embryonic development. These cells are considered to be pluripotent, are amenable to gene targeting by homologous recombination (Smithies et al. 1985, Thomas & Capecchi 1987, Brandon et al. 1995), and can contribute to a chimeric animal when introduced into a blastocyst (Bradley et al. 1984). Generally, ES cells are derived from the inner cell mass (ICM) of a blastocyst stage embryo (Rossant 2001, Keller 2005). Where the ICM is a transient population of cells that differentiates to form a fetus, ES cells can be expanded clonally and therefore retain their pluripotency when cultured under appropriate conditions (Rossant 2001). Pluripotent stem cells have also been derived from the epiblast of early postimplantation mouse embryos (Brons et al. 2007, Tesar et al. 2007), but these so-called EpiSCs have a more limited state of pluripotency referred to as the primed state, opposed to the naïve state of ES cells. Differences between naïve and primed pluripotency are displayed in the cells' culture requirements, in the epigenome, and the X-chromosome status in female cells. Importantly, naïve stem cells are capable of generating high-grade chimeras after introduction into embryos, including germ-line chimerism while primed stem cells rarely contribute to chimeras (Nichols & Smith 2009).

Although not derived from postimplantation embryos, but from blastocyst stage embryos, human ES cells are suggested to be analogous to mouse EpiSCs (Brons et al. 2007, Tesar et al. 2007, Rossant 2008) and are at a primed state of pluripotency (Nichols & Smith 2009). Recently however, a culture medium, termed naïve human stem cell medium (NHSM) has been described that facilitated the derivation of naïve ES cells from human blastocyst stage embryos. Importantly, this culture medium also facilitated the conversion of mouse EpiSCs to ES cells that efficiently contributed to germ line chimerism (Gafni et al. 2013). It has been suggested that already established primed human ES cells cultured under these conditions also adapt a more naïve state, although distinct proof by analysis of contribution to chimeras is not possible.

One of the hallmarks of naïve pluripotency is the presence of two active X-chromosomes in female cells. Random inactivation of one of the X-chromosomes in female cells is a mechanism for dosage compensation and is thought to be set around the blastocyst stage. The long non-coding RNA molecule *XIST* is the master regulator of X-chromosome inactivation in placental mammals (Escamilla-Del-Arenal et al. 2011).

Remarkably, the derivation of true embryonic stem cell lines, either naïve or primed, from other mammals apart from rodents and primates has not been successful, although pluripotent stem cells from large domestic animals such as cattle would be of great interest for biomedical research (Nowak-Imialek & Niemann 2013). Possibly, the

correct stage of embryos has not been used, or the culture conditions used have not been able to capture pluripotency and elicit self-renewal in the cells.

Here we cultured bovine embryos from the morula to the blastocyst stage in NHSM and compared the gene expression in the inner cell mass with the gene expression profile of embryos cultured in 'regular' medium using microarray analysis. The results show a slightly more naïve gene palette in bovine ICMs under NHSM conditions, including a down-regulation of *XIST* expression, but self-renewal was not stimulated.

RESULTS

Embryo culture in NHSM causes morphological and biochemical differences

Since embryonic stem cell lines with characteristics of naïve pluripotency have been derived from human ICMs cultured under NHSM conditions we investigated how gene expression responded in ICMs of bovine blastocysts cultured in NHSM. *In vitro* produced bovine embryos that were cultured from day 5 until day 9 in NHSM appeared to develop faster compared to embryos cultured in standard SOF medium. At day 9 post fertilisation most SOF embryos were still contained within their zona pellucidae whereas most NHSM embryos had already hatched. The NHSM blastocysts contained more cells (1.96-fold; p=0.0007) of both ICM and TE than SOF embryos (Figure 1A – D). NHSM-embryos at this stage had ascended in the culture medium to just below the liquid surface, possibly as a result of lipid accumulation, while embryos cultured in SOF medium remained at the bottom of the culture dishes. Indeed, the NHSM cultured embryos contained numerous perinuclear lipid droplets in both TE cells and ICM whereas lipid droplets in SOF embryos were predominantly detected in the ICM and only in few TE cells (Figure 1E). The accumulation of lipid droplets in NHSM was not a result of the more developed state of the blastocysts since enhanced (hatched or stage 8/9) blastocysts in SOF medium only had few lipid droplets, and stage 7 NHSM blastocysts clearly contained many lipid droplets in both TE and ICM (Figure 1E).

Microarray analysis

We next used microarray analysis to examine gene expression patterns of blastocysts cultured in NHSM in comparison to those cultured in SOF. Embryos were cultured until day 9 after which the ICMs were manually isolated and subjected to RNA extraction. Only those samples with good RNA quality, indicated by RNA integrity number (RIN) values above 8, were used. Because of the manual isolation of ICM from TE, it cannot be excluded that ICM samples contained TE cells. Indeed, we previously determined that about 20% of the cells of the samples had TE characteristics indicated by *CDX2* expression (Brinkhof et al. 2015). We however think that the presence of a small percentage

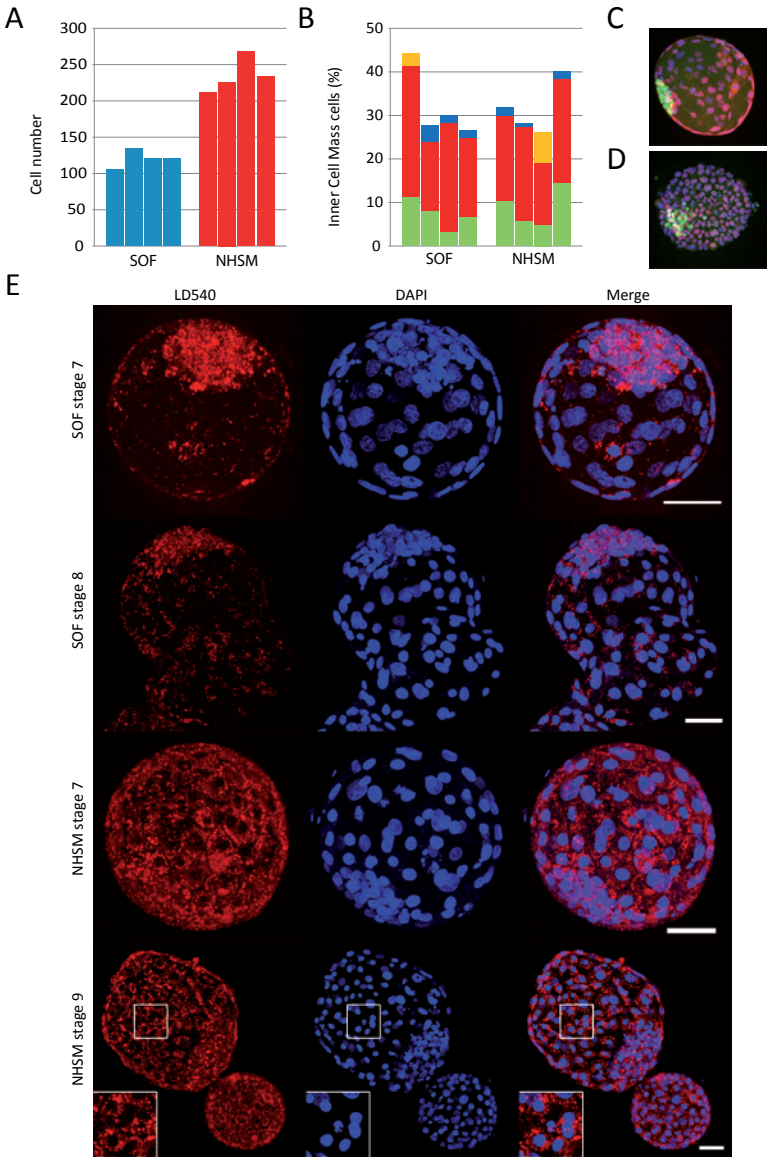

Figure 1.

Embryo composition. Bovine embryos were stained for GATA6 as a primitive endoderm marker, for NANOG as an epiblast marker and DAPI for nuclear staining. A) Cell numbers were determined in four embryos cultured in SOF (blue) and in four embryos cultured in NHSM (red) by counting DAPI positive nuclei. B) Of these embryos the ICM was counted for cells positive for NANOG (green), GATA6 (red), double positive (orange) or only DAPI (blue). Representative pictures for SOF (C) and NHSM (D) blastocysts stained for GATA6 (red) and NANOG (green). Cells positive for both GATA6 and NANOG appear orange, and cells not expressing GATA6 and NANOG only show DAPI (blue) staining. E) Early (stage 7) and late (stage 8/9) blastocysts cultured in SOF and NHSM were stained by LD540 (red) for lipid droplets and counterstained with DAPI (blue). The inset shows a higher magnification of trophectoderm cells with perinuclear lipid droplets.

of TE cells had little effect on the results and the conclusions. For simplicity we will refer to manually separated samples as ICM, rather than ICM + TE. From each culture condition four separate pools of ICMs were generated and compared as such that one NHSM pool was hybridized on an array together with one SOF pool labelled with either Cy5 or Cy3. This resulted in a total of four microarrays analysed.

Microarray expression profiles revealed 769 probes to be differentially expressed (cut-off 1.5-fold; $p<0.05$) between NHSM- and SOF-cultured ICMs, from a total of 43,653 probes (Figure 2A). Comparing the four array results by generating a heatmap with these probes showed similar expression profiles indicating the reproducibility (Figure 2B). Expression levels of 368 probes were higher in ICMs from NHSM-cultured embryos whereas 401 were more abundantly expressed in ICMs from SOF-cultured embryos (Figure 2A, C). The distribution of the differentially expressed probes on the *Bos taurus* genome was comparable for all chromosomes (average = 1.52% ± 0.58) apart from chromosome 8 (3.0%) and the X-chromosome (3.5%) that had a higher percentage of differentially expressed genes (Figure 3A). On chromosome 8 the largest expression difference was detected in a confined region between 22.6 Mb and 23.2 Mb containing interferon (IFN) coding genes including *IFNW*, *IFNA* and *IFNT* (Figure 3B). The X-chromosome exhibited a more diverse pattern of differentially expressed probes of which 44 were >1.5-fold up-regulated and 22 down-regulated in NHSM (Figure 3C). Several genes were represented on the microarray by more than one probe, and for consistency we used the probes representing the most 3-primed end of the genes. Accordingly, a remaining 641 genes were differentially expressed (Supplemental Table 1). A list of the 25 most differentially expressed genes also indicates an over representation of genes located on the chromosomes X and 8 (Table 1). We validated the expression of 16 genes by qRT-PCR, and indeed differences in gene expression between SOF and NHSM cultured ICMs were similar for these 16 genes (Figure 4).

Since human embryonic cells cultured with NHSM acquire a more naïve signature, we were surprised that in the bovine ICMs the expression of core pluripotency genes *NANOG*, *SOX2*, and *POU5F1* was at similar levels (*SOX2*, *POU5F1*) or down-regulated (*NANOG*) in NHSM, although the down-regulation was below the 1.5-fold cut-off (Figure 2C and 4A) and could not be confirmed by qRT-PCR (Figure 4B). Genes of which, at least in the mouse, the products direct OCT4 to enhancer sites related to a primed state (Buecker et al. 2014) were inconclusively expressed. Whereas *OTX2* showed no differential expression (-1.01-fold, $p=0.987$), *ZIC3* was mildly down-regulated (1.20-fold, $p=0.064$), and *ZIC2* exhibited marked down-regulation (2.01-fold, $p<0.001$, Supplemental Table 1) in NHSM. Expression of other transcription factor genes associated with self-renewal and pluripotency (Boyer et al. 2005, Loh et al. 2006, Jiang et al. 2008, Zhao et al. 2012, Takashima et al. 2014, Theunissen et al. 2014, Hackett & Surani 2014) was either unchanged (FC<1.1-fold; *DPPA2*, *TFCP2L1* and *ESRRB*), not signifi-

cantly changed (p>0.05; *KLF2* and *KLF4*) or moderately down-regulated (1.5>FC>1.25, p<0.05; *DPPA3*, *KLF5* and *TBX3*) in NHSM. Results from qRT-PCR data showed similar expression dynamics although most expression levels were not significantly different between NHSM and SOF (Figure 4B).

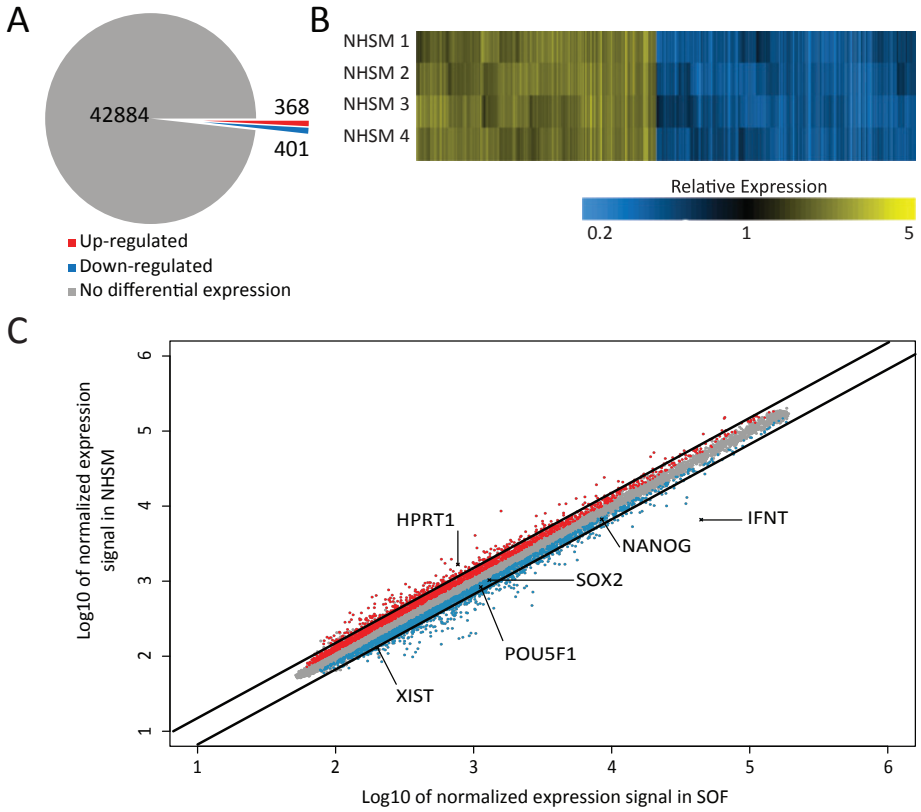

Figure 2.

Microarray data. A) Pie diagram showing the numbers of genes examined by microarray analysis. Number of genes up-regulated in NHSM medium is indicated in red, and down-regulated gene number in blue. A total of 769 probes out of 43,653 were differentially expressed (1.5-fold change; p<0.05) between embryos cultured in SOF and in NHSM. B)Heatmap comparing four different comparisons between NHSM and SOF ICMs. These results were obtained from four separate microarrays showing a similar expression in NHSM cultured embryos relative to SOF cultured embryo expression. C) Scatter plot indicating differences in gene expression between ICM cells from NHSM-cultured and SOF-cultured embryos. Probes expressed at significantly higher levels in NHSM ICMs (red), in SOF ICMs (blue) or with no significantly different expression levels (grey) are depicted in this scatter plot. Diagonal lines indicate the 1.5-fold cut-off used for analysis. Several genes discussed in the manuscript are indicated.

Gene ontology analysis

Gene Ontology (GO) can be used to describe gene products in relation to their known cellular component (C), molecular function (F) or biological process (P). A Parametric Analysis of Gene Set Enrichment (PAGE) was used to determine the most significantly enriched GO terms (Du et al. 2010). Of the 641 genes found to be differentially expressed, 560 could be related to at least one GO term resulting in 927 enriched GO terms after PAGE (Supplemental Table 2). The most significantly enriched GO terms

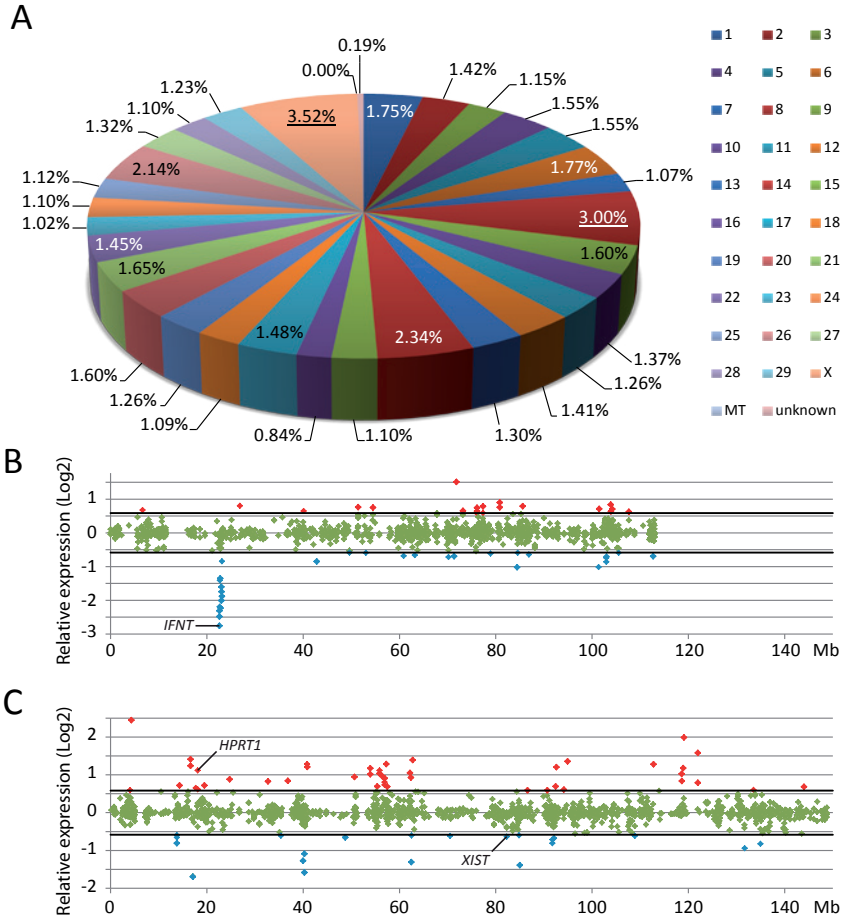

Figure 3.

Chromosome distributions. A) Proportional distribution of probes per chromosome, mitochondrion (MT) or of unknown location. Chromosome 8 (red; 3.00%) and the X-chromosome (pink; 3.52%) were over-represented (as indicated with the underlined percentages) by differentially expressed probes. The relative expression (Log2) of probes (green) with higher (cut-off 1.5-fold change indicated with thick black lines) expression levels in NHSM (red) or in SOF (blue) developed ICMs are plotted with respect to the location on chromosome 8 (B) and the X-chromosome (C).

describing biological processes (P) contained genes generally down-regulated in NHSM and were involved in early differentiation, in particular neural differentiation. These results suggest that in bovine embryos cultured in NHSM the differentiation from ICM to epiblast and beyond was inhibited. Processes up-regulated by NHSM were enriched in GO-terms describing cell death or mitosis (Figure 5). The observed enhanced proliferation rate (Figure 1A, B) is also reflected in the up-regulated expression of genes involved in M-phase and M-phase of mitotic cell cycle (Figure 5).

Table 1. List of most differentially expressed genes.

Gene Symbol (NCBI)	Description	Chr.	NHSM vs SOF (FC)
IFN-tau-c1	Interferon-tau 3g precursor	8	-6.75
CT47B1	Cancer/testis antigen family 147, member B1	X	5.45
DUSP4	Bos taurus dual specificity phosphatase 4 (DUSP4), mRNA.	27	-5.15
LOC781778	Uncharacterised protein	8	-4.71
ENSBTAG00000046119	Uncharacterised protein	8	-4.67
LOC100847720	Uncharacterised protein	8	-4.56
ENSBTAG00000034320	Novel pseudogene	8	-4.03
LOC101905249	Melanoma-associated antigen B1-like	X	3.97
ABCC2	Uncharacterised protein	26	-3.76
LOC523509	Uncharacterised protein	8	-3.69
DAZL	Deleted in azoospermia-like	1	3.36
LOC618985	Uncharacterised protein	8	-3.35
LOC618806	Melanoma-associated antigen 10-like	10	3.34
GPC4	Glypican-4 precursor	X	-3.21
ENSBTAG00000045727	Novel pseudogene	14	-3.19
ACTA1	Actin alpha skeletal muscle	28	3.17
PDYN	Proenkephalin-B preproprotein	13	-3.13
LOC100336885	Uncharacterised protein	8	-3.03
TKTL1	Transketolase-like protein 1	X	-3.00
LOC520085	Bos taurus similar to melanoma antigen (LOC520085).	X	2.99
QPRT	Nicotinate-nucleotide pyrophosphorylase	25	-2.99
PLAU	Urokinase-type plasminogen activator Urokinase-type plasminogen activator long chain A Urokinase-type plasminogen activator short chain A Urokinase-type plasminogen activator chain B	28	-2.94
SPRY4	Sprouty homolog 4 (Drosophila)	7	-2.93
SLC13A4	Solute carrier family 13 member 4	4	-2.87
STC1	Stanniocalcin-1	8	2.85

The 25 genes with the largest expression difference between NHSM- and SOF-cultured ICMs are presented. Negative values indicate fold change (FC) down-regulated in NHSM, and positive values are up-regulated.

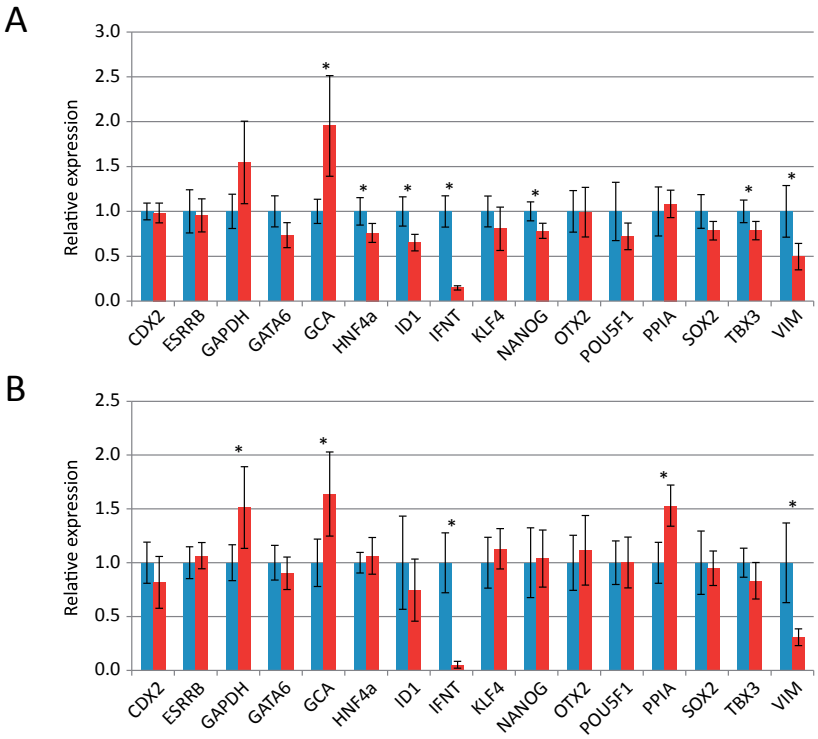

Figure 4.
Relative gene expression levels. The expression of selected genes in SOF-cultured ICMs (blue) is set at 1 to show the relative gene expression in NHSM-cultured ICMs (red) after microarray analysis (A) and qRT-PCR (B). Asterisks indicate levels significantly ($p<0.05$) different between SOF and NHSM.

XIST expression is down-regulated in NHSM

One of the hallmarks of naïve pluripotency is the presence of two active X-chromosomes in female ES cells. In contrast, primed female ES cells are characterised by one inactivated X-chromosome for dosage compensation. This is in parallel with random X-chromosome inactivation in early embryos that in the mouse starts at the blastocyst/epiblast stage (Gardner & Lyon 1971, Takagi 1974, Mukherjee 1976). The long non-coding RNA *XIST* is driving X-chromosome inactivation (Payer & Lee 2008, Dupont & Gribnau 2013). Although in cattle *XIST* transcripts have been detected at low levels from day 2 post fertilisation (De La Fuente et al. 1999), strong expression coincides with major zygote genome activation (Ruddock et al. 2004). *XIST* transcripts were detectable in female as well as in male bovine embryos up to the morula stage, becoming exclusively expressed in female blastocysts (Peippo et al. 2002, Dupont & Gribnau 2013). Although little is known about the start of X-chromosome inactivation in bovine embryos, we anticipated that *XIST* expression had

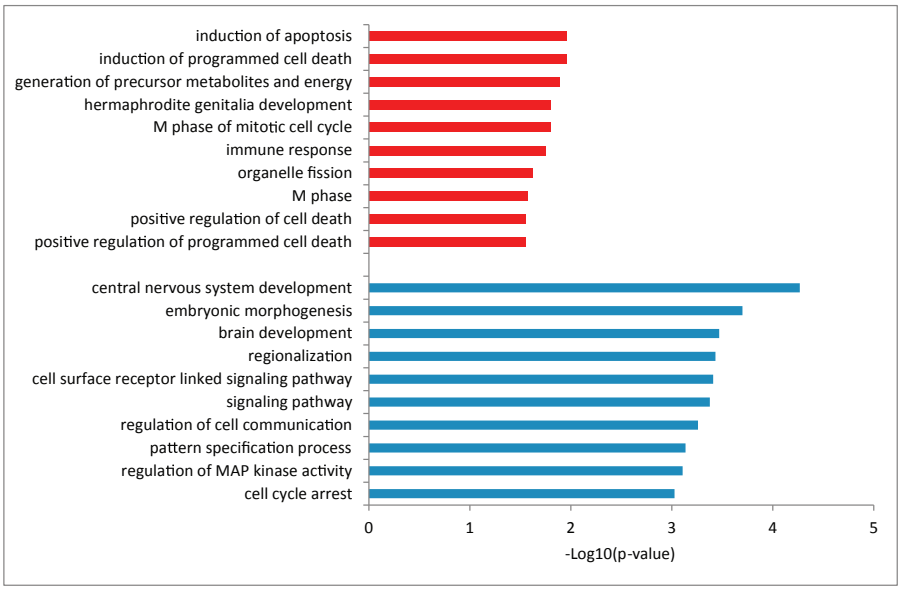

Figure 5.
Gene ontology analyses. Top 10 of GO terms enriched with genes showing a higher expression in NHSM (red) or a higher expression in SOF (blue).

already started in the day 9 blastocysts and was discriminating between male and female embryos (De La Fuente et al. 1999, Peippo et al. 2002, Wrenzycki et al. 2002, Ruddock et al. 2004). The expression of *XIST* was significantly reduced in the ICMs from NHSM embryos (Figure 2C, 3 and 6B). With embryos of mixed sex, a reduction of *XIST* expression could be due to a higher percentage of male embryos that are expected to have much lower *XIST* levels. Therefore, we examined whether culture in NHSM skewed the sex ratio with a higher percentage of male embryos reaching the blastocyst stage. The presence of the Y-chromosome specific *SRY* gene in the genome was analysed by qPCR. In embryos from parthenogenetically activated oocytes, the *SRY* gene was not present, and the genomic *SRY* copy numbers increased when we mixed these samples with those from mixed sex IVF embryos (Figure 6A). In SOF and NHSM blastocysts the copy numbers of *SRY* were similar indicating that there was no difference in the sex ratio of embryos cultured in SOF and NHSM (Figure 6A). The results suggest that NHSM indeed delayed *XIST* expression and therefore X-chromosome inactivation. We further investigated expression of X-linked genes in the NHSM embryos. Expression of *HPRT1*, known to be stochastically inactivated in female cells (Basrur et al. 2004, Wu et al. 2014), was significantly up-regulated in NHSM ICMs which could have resulted from reduced *XIST* RNA coating of the X-chromosome (Figure 2C, 3 and 6C). In total 41 genes on the X-chromosome were significantly up-regulated while 16 were down-regulated (cut-off 1.5-fold) (Figure 2C; Table 2) indicating reduced X-chromosome inactivation under NHSM conditions.

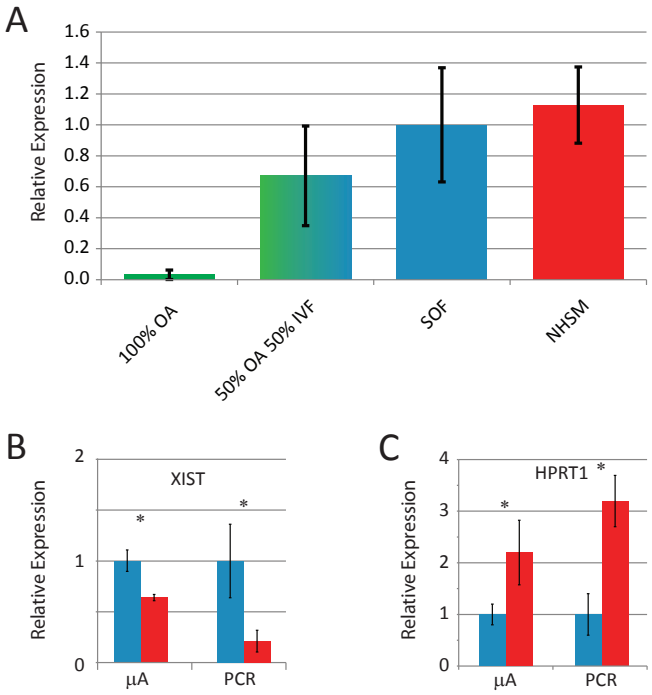

Figure 6.
***SRY*, *XIST* and *HPRT1* expression.** A) Parthenogenetically derived embryos (OA) and IVF SOF- or NHSM-cultured embryos were analysed for the presence of genomic *SRY*. Analysis of relative expression of *XIST* (B) and *HPRT1* (C) from microarray (µA) and qRT-PCR (PCR) data showed significant ($p<0.05$) differences as indicated with asterisks.

NHSM does not stimulate bovine self-renewal

Since the gene expression data hinted at a more naïve level of pluripotency in the embryos cultured in NHSM, we analysed whether cell lines could be derived from these embryos. Embryos were cultured from day 5-9 in NHSM, and at day 9 hatched blastocysts or dissected ICMs were plated on wells coated with vitronectin or gelatin with vitronectin and cultured in NHSM. Initially, the blastocysts attached and the TE proliferated (Figure 7). The ICMs on top of the TE did not proliferate and isolated ICMs did not attach. After 7 days in culture, TE cells started to detach and like the ICM cells they died. Isolated ICMs did not attach to the wells despite coating and did not proliferate.

The results suggest that in contrast to what has been described for mouse and human embryos, culture of bovine ICM cells in NHSM does not lead to generation of stem cell lines.

Table 2. Differentially expressed genes on the X-chromosome.

Gene Symbol (NCBI)	Description	NHSM vs SOF (FC)
CT47B1	Cancer/testis antigen family 147. member B1	5.45
LOC101905249	Melanoma-associated antigen B1-like	3.97
GPC4	Glypican-4 precursor	-3.21
TKTL1	Transketolase-like protein 1	-3.00
LOC520085	Bos taurus similar to melanoma antigen (LOC520085).	2.99
LOC786942	Uncharacterised LOC786942	2.66
BC108185.1	Bos taurus cDNA clone IMAGE:8059386.	2.62
SLC7A3	Cationic amino acid transporter 3	-2.62
LOC100847577	P antigen family member 3-like	2.56
ACSL4	Acyl-CoA synthetase long-chain family member 4	-2.48
BC149677.1	Bos taurus cDNA clone IMAGE:8057572.	2.43
NGFRAP1	Protein BEX3	2.43
MAGEB16	Melanoma antigen family B. 16	2.43
L1CAM	Neural cell adhesion molecule L1 precursor	-2.42
LOC786718	Uncharacterised LOC786718	2.36
LOC618023	Cancer/testis antigen 1-like	2.31
PAGE4	G antigen family C member	2.30
ENSBTAG00000012835	Novel pseudogene	2.26
LOC783543	Eso3 protein-like	2.26
HPRT1	Hypoxanthine-guanine phosphoribosyltransferase	2.17
BT.50520	Uncharacterised protein	2.07
BEX5	Protein BEX5	2.05
MGC133764	Melanoma antigen family B-like	2.03
LOC100126053	LOC100126053 protein	2.02
NXF3	Nuclear RNA export factor 3	1.94
LOC101902201	Cancer/testis antigen 1-like	1.92
GPR64	Uncharacterised protein	-1.92
NXT2	NTF2-related export protein 2	1.90
CXORF57	Uncharacterised protein	1.88
ENSBTAG00000018098	Known pseudogene	1.84
MAGEB4	Melanoma antigen family B. 4	1.78
LOC508820	Melanoma-associated antigen 9-like	1.77
AP1S2	AP-1 complex subunit sigma-2	-1.77
UTP14A	U3 small nucleolar RNA-associated protein 14 homolog A	-1.75
RNF128	E3 ubiquitin-protein ligase RNF128 precursor	1.75
MAGEB10	Uncharacterised protein	1.73
LOC787476	Uncharacterised protein	1.65
LOC540312	Protein DDX26B-like	1.65
RBM3	Uncharacterised protein	-1.64

Table 2. Differentially expressed genes on the X-chromosome. (continued)

Gene Symbol (NCBI)	Description	NHSM vs SOF (FC)
LOC100848555	X antigen family member 5-like	1.62
ARMCX6	Protein ARMCX6	1.62
MGC140151	Protein BEX4	1.61
EIF1AY	Eukaryotic translation initiation factor 1A, Y-linked	1.60
PIM2	Serine/threonine-protein kinase pim-2	-1.60
Unknown	Unknown	-1.58
ENSBTAG00000030507	Ferritin	1.57
XIST	X (inactive)-specific transcript	-1.56
BCOR	BCL-6 corepressor	-1.53
SH3BGRL	SH3 domain-binding glutamic acid-rich-like protein	-1.53
NSDHL	Sterol-4-alpha-carboxylate 3-dehydrogenase, decarboxylating	-1.53
NUDT11	Diphosphoinositol polyphosphate phosphohydrolase 3-alpha	1.53
NUDT10	Nudix (nucleoside diphosphate linked moiety X)-type motif 10	1.52
FOXO4	Forkhead box protein O4	-1.52
ENSBTAG00000031256	Uncharacterised protein	1.52
RNF113A	RING finger protein 113A	1.51
RGN	Regucalcin	1.51
ENSBTAG00000021026	Known pseudogene	1.50

All genes differentially expressed (1.5-fold; $p<0.05$) located on the X chromosome are listed. Negative fold change (FC) values indicate lower expression levels in NHSM ICMs, and positive values are at higher expression levels in NHSM ICMs compared with SOF.

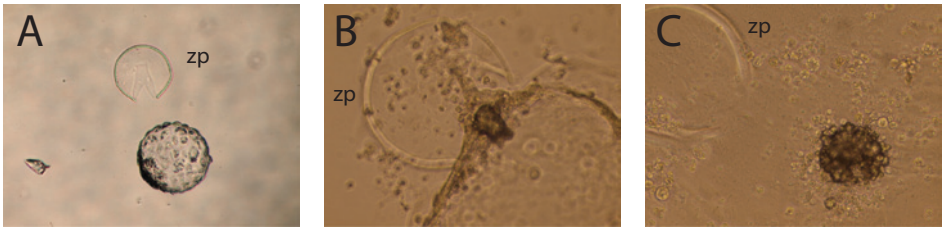

Figure 7.
Culture of plated blastocysts. Intact NHSM blastocysts were plated and cultured in NHSM on vitronectin-coated wells. After one day (A) trophectoderm cells started to attach and after another 3 days had spread (B). This continued up to day 6 (C) whereafter detachment occurred. zp = zona pellucida.

DISCUSSION

The embryonic stage and culture conditions that allow the derivation of bovine ES cell lines are not known. Since it has been described that culture of ICMs from mouse and human embryos in NHSM facilitates the derivation of naïve ES cells, we studied the effect of NHSM on the development of bovine embryos and the gene expression

profile in the ICM. *In vitro* matured and fertilised oocytes were cultured until the morula stage in SOF. From this point, embryos were either cultured in SOF or in NHSM until day 9. In most studies bovine embryos are cultured until day 8, but an extra day of culture facilitated the collection of more embryos that were no longer enclosed by the zona pellucida (stage 8 and 9) and improved ICM isolation. During culture from day 8 post fertilisation hatched blastocysts in NHSM started to float near the surface of the medium while embryos cultured in SOF remained at the bottom of the dishes, even after hatching. The NHSM embryos also appeared to be darker than their SOF counterparts, most likely due to increased numbers of lipid droplets. Indeed, lipid droplets could be detected in ICM and TE cells of the NHSM embryos whereas almost no lipid droplets could be detected in TE from SOF embryos in early or in late blastocysts. Preimplantation embryos of ungulates such as cattle contain a relatively high number of lipid droplets, which is most likely used as an energy source (Ferguson & Leese 2006). The ratio of saturated and unsaturated fatty acid species in the lipid droplet could be important for the quality of the developing embryo (Aardema et al. 2011), but at this stage it is not known whether the lipid composition of the droplets changed as a result of NHSM. Furthermore, the GO-analysis indicated an under-representation of genes involved in *lipid metabolism* (GO:0006629), whereas the *generation of precursor metabolites and energy* (GO:0006091) was enriched in NHSM.

In cattle, high numbers of lipid droplets have been associated with low quality embryos (Abe et al. 2002a), and the number seems to be dependent on culture conditions. In particular, the presence of foetal bovine serum induced the formation of lipid droplets in bovine embryos (Abe et al. 2002b), but neither SOF nor NHSM contained foetal bovine serum. The higher proliferation rate of NHSM embryos, which might not be related to pluripotency, suggests good quality embryos, and the proportional increment in cell number of ICM as well as TE (~2-fold compared to SOF) indicates fast, though normal, development.

Large numbers of embryos are needed in order to detect expression differences by microarray and qRT-PCR. Therefore, we used *in vitro* derived embryos that have demonstrated to be fit for embryo transfer and can give rise to healthy animals indicating functional pluripotency pathways. The isolation of ICMs was performed manually, and although inevitably some TE cells remained attached to the ICM their contribution to the transcriptome is expected to be minimal (Brinkhof et al. 2015). Although the *Bos taurus* microarrays contained 520 probes of unknown location only one was (up-) regulated (>1.5-fold; $p<0.05$) in our NHSM versus SOF comparison. Another 768 probes were differentially expressed, and after correction for multiple probes 640 genes or genomic locations could be identified. Not all these genes and locations could be associated with a GO term resulting in a reduced number of 560 genes suitable for GO-analysis. This analysis reveals over-representation of genes up-regulated in NHSM in the GO-

terms associated with *cell division* (GO:0051301), including *M phase* (GO:0000279), *mitosis* (GO:0007067), and *M phase of mitotic cell cycle* (GO:0000087), indicating an increased proliferation rate. On the other hand, genes up-regulated in NHSM were also over-represented in GO-terms associated with *programmed cell death* (GO:0012501), including *positive regulation of cell death* (GO:0010942), *induction* (GO:0012502) and *positive regulation* (GO:0043068) *of programmed cell death,* and *induction of apoptosis* (GO:0006917).

This could imply reduced quality of embryos after NHSM culture, but NHSM embryos developed normally, and, furthermore, genes involved in inducing cell death may also be involved in cell cycling (Wu et al. 2006). Genes differentially expressed could be mapped evenly along the bovine genome apart from chromosome 8 and the X-chromosome, both harbouring a higher percentage of differentially expressed genes than the other chromosomes. For chromosome 8, most genes were located at a small area coding for interferon proteins. Although *IFNT* expression has been detected in the ICM part of bovine blastocysts (Degrelle et al. 2005, Brinkhof et al. 2015), its protein expression is considered to be restricted to the TE (Johnson et al. 2006, Sakurai et al. 2009, Ezashi et al. 2009). As we used dissected ICM, few TE cells might still have been attached accounting for the detected *IFNT* expression. However, the *IFNT* expression was down-regulated more than 6-fold in NHSM, possibly resulting from reduced c-JUN phosphorylation by SP600125 (Bennett et al. 2001).

The X-chromosome harboured several differentially expressed genes distributed over the entire chromosome, representing twice the average amount of differentially expressed genes found on the autosomes. One of these genes was *XIST*. This long non-coding RNA is involved in X-chromosome inactivation (XCI) during early development of female embryos. XCI is important to compensate for the reduced expression in male embryos of genes located on this chromosome. In this study the expression of *XIST* was found to be 1.5-fold reduced in NHSM suggesting a reduced or delayed XCI. The possibility that a lower *XIST* expression level was attributed to a skewed sex ratio was dismissed by the equal expression of genomic *SRY* in SOF-cultured embryos and NHSM-cultured embryos. The presence of two active X-chromosomes in female ESC lines is considered a marker for naïvety as opposed to the primed state with random X-chromosome inactivation. One of the genes regulated by XCI is *HPRT1* (Basrur et al. 2004, Okamoto et al. 2011). *HPRT1* expression was 2-fold up-regulated in NHSM indicating that full X-chromosome inactivation was not completed. *G6PD* expression levels, also known to be XCI-regulated (Basrur et al. 2004), were comparable between SOF and NHSM cultured ICMs (Oliveira et al. 2013) whereas *SMCX* was slightly up-regulated (Basrur et al. 2004). Genes known to escape XCI in bovine like *ZFX* (Basrur et al. 2004), *UTX*, *MED14* (*CRSP2*) and *UBA1* (Yen et al. 2007) were either not differentially expressed or slightly down-regulated in NHSM. *XIST* transcripts have already been

detected in bovine 8-cell-stage female embryos whereas the timing of onset of XCI in bovine embryos is unknown (De La Fuente et al. 1999). However, in elongated day 14 female bovine embryos, XCI is present (Kimura et al. 2004). Taken together, these results indicate that besides one active X-chromosome the other X-chromosome is also still active, although probably partially. This indicates that after NHSM culture these bovine ICM cells display a status between naïve and primed. Since the naïve or primed state is not fixed, but a continuum (Hough et al. 2014) the cell population in the ICMs could very well be in a naïve as well as in a primed state with more naïve cells in NHSM ICMs. In addition, it has been reported that the transcriptomes of mouse and human naïve ES cells are quite distinct and that they more resemble the transcriptomes of the respective blastocyst embryos (Huang et al. 2014).

Genes known to be important for pluripotency (*NANOG*, *POU5F1,* and *SOX2*) were not up-regulated in ICMs collected from NHSM cultured blastocysts, whereas *NANOG* expression is elevated in naïve human ESCs compared to their primed counterparts (Gafni et al. 2013). Elevated *NANOG* expression was expected since MAPK inhibition has been shown to increase NANOG expression levels in bovine embryos (Kuijk et al. 2012, McLean et al. 2014, Brinkhof et al. 2015). Possibly this effect has been counteracted by FGF in the NHSM. Neither genes associated with naïvety (*ESRRB* and *TBX3*) nor genes indicative for epiblast formation (KLF4) or germ layer differentiation (*OTX2*) were differentially regulated. Of the DUSP genes, only *DUSP4* and *DUSP6* were significantly regulated. These genes showed a reduced expression whereas human naïve ES cells show an increased expression of DUSP genes (*DUSP3*, *DUSP5,* and *DUSP10*) (Gafni et al. 2013). *SPRY2* and *SPRY4* were both down-regulated as well and function, like DUSP proteins, in a feedback-loop negatively regulating ERK (Hanafusa et al. 2002, Eblaghie et al. 2003). This might explain the down-regulated expression levels of these genes in NHSM, since ERK is inhibited via p38, JNK, and MAPK inhibitors.

Unfortunately, attempts to culture these blastocysts in NHSM in gelatin/vitronectin coated dishes were unsuccessful. Possibly, the ICM could not migrate over the TE (Roelen et al. 1998) and was therefore inhibited in its proliferation potential. In conclusion, NHSM is partially capable of generating or maintaining a more naïve character of the bovine ICM although for propagation of cells capable of self-renewal other factors are required which are so far unknown.

MATERIALS AND METHODS

Culture of bovine embryos

Embryo culture was performed in a humidified atmosphere with 5% CO_2 at 39°C, unless stated otherwise. Ovaries were obtained from a local slaughterhouse, and fol-

licles of 3 – 8 mm in diameter were aspirated to retrieve cumulus oocyte complexes (COCs). COCs were matured for 23 h in groups of 35 – 60 per 500 ml M199 (Life Technologies, Bleiswijk, The Netherlands) supplemented with 0.05 IU/ml recombinant hFSH (Organon, Oss, The Netherlands) and 1% penicillin-streptomycin (Life Technologies). Subsequently, fertilisation was performed as described previously (Parrish et al. 1988) with modifications (Izadyar et al. 1996). In short, matured COCs were transferred to fertilisation medium (Fert-TALP) supplemented with 10 mg/ml heparin (Sigma Aldrich, Zwijndrecht, The Netherlands), 20 µM D-penicillamine (Sigma Aldrich), 10 µM hypotaurine (Sigma Aldrich), and 1 µM epinephrine (Sigma Aldrich). Frozen-thawed sperm from a bull with proven fertility was centrifuged over a Percoll-gradient (GE Healthcare Europe GmbH, Eindhoven, The Netherlands) and added to the COCs at a final concentration of 1.0×10^6spermatozoa/ml. This was considered as day 0. After 20 h oocytes were freed of cumulus cells by vortexing for 3 min and placed in synthetic oviductal fluid (SOF). After 4 days culturing in a humidified atmosphere with 5% CO_2 and 7% O_2 at 39°C embryos were transferred to fresh SOF or to naïve human stem cell medium (NHSM) (Gafni et al. 2013) containing KO DMEM (Life Technologies) supplemented with 20% knock-out serum replacement (Life Technologies), 12.5 µg/ml bovine insulin (Sigma Aldrich), 1 mM glutamine (Life Technologies), 1% non-essential amino acids (Sigma Aldrich), 0.1 mM beta mercaptoethanol (Life Technologies), 1% penicillin-streptomycin (Life Technologies), 20 ng/ml recombinant human LIF (Prospec, Rehovot, Israel), 8 ng/ml recombinant bFGF (Peprotech, Rocky Hill, NJ, USA), 1 ng/ml recombinant TGFbeta1 (Peprotech) and the small molecule inhibitors: 1 µM PD0325901 (Erk1/2; Stemgent, Cambridge, MA, USA), 3 µM CHIR99021 (GSK3beta, Bio-connect, Huissen, The Netherlands), 5 µM Go6983 (PKC, Tocris, Bristol, UK), 10 µM SP600125 (JNK, Tocris) and 10 µM SB203580 (p38, Tocris). Subsequent culture continued until day 9 to obtain a higher percentage of stage 9 (Stringfellow & Givens 2009) blastocysts facilitating ICM isolation (Rizos et al. 2003, Nicacio et al. 2012, Madeja et al. 2013). To ensure good quality embryos only blastocysts scored as stage 7 – 9 with quality code 1 or 2 (Stringfellow & Givens 2009) were collected.

Generation of bovine parthenogenetically activated embryos

Matured bovine oocytes were stripped from cumulus cells and placed in fertilisation medium as described above. The fertilisation medium was further supplemented with 5 mM Ionomycin (Sigma Aldrich), and oocytes were incubated for 5 min. After three times washing in SOF the oocytes were transferred to SOF supplemented with 1.9 mM 6-DMAP (Sigma Aldrich) and incubated for 3.5 h. Hereafter, the oocytes were washed three times in SOF and cultured in SOF. At day 5 of culture, cleaved embryos were transferred to fresh SOF and cultured for an additional 4 days when blastocysts were collected for DNA extraction.

DNA extraction and PCR

DNA from IVF blastocysts and parthenogenetically activated blastocysts was extracted using the prepGem kit (ZyGem, Hamilton, New Zealand) according to manufacturer's instructions. For quantitative PCR DNA was mixed with iQ™ SYBR© Green supermix (BioRad, Veenendaal, The Netherlands) in a 25 µl reaction volume with final primer concentrations of 400 nM, and quantity measured on a CFX detection system (Biorad). Primer sequences for detection of *SRY* (forward; 5'-ACAGTCATAGCGCAAATGATCAGTG-3' and reverse; 5'-GGGTTGCATAGTATTGAAGAGTCTGC-3') were used with an annealing temperature (Ta) of 67°C. For normalization primers detecting *Gremlin* (forward; 5'-CATCAACCGCTTCTGCTACG-3', reverse; 5'-TGGCTGGAGTTCAGGACAGT-3'; Ta = 64.5°C) and *CDC42* (forward; 5'-GTGCCTGAGATAACTCACCA-3', reverse; 5'-GGAGTGATAGGCTTCTGCTT-3'; Ta = 61°C) were used. PCR reactions started with a 5 min enzyme activation step at 95°C. This step was followed by 40 cycles, each cycle containing a denaturing step of 10 s at 95°C, and an annealing and elongation step for 30 s at the appropriate Ta. To confirm product purity a dissociation curve was generated by increasing the temperature from 60°C to 95°C with 1°C temperature increments each step. Results were further analysed with Biorad's CFX3.1 software.

ICM collection and RNA isolation

ICMs were separated from the TE as described before (Brinkhof et al. 2015). In short, blastocysts were placed in wash buffer, and sharpened tungsten needles were used to separate the ICM from the TE. The procedure was performed under a stereo microscope. Isolated ICMs were collected and stored in RLT (Qiagen, Venlo, The Netherlands) in groups of 11 – 24 at -80°C until RNA isolation. RNA isolation and on column DNA digestion was performed using an RNA micro kit (Qiagen) according to manufacturer's protocol. A Bioanalyzer 2100 and the RNA 6000 Pico LabChip kit (Agilent Technologies, Amstelveen, The Netherlands) were used for assessment of total RNA quality and quantity according to manufacturer's instructions. RNA was stored at -80°C until further use.

Microarray analysis

A selection of total RNA samples isolated from dissected ICMs cultured in SOF or NHSM were compared using four dual channel microarrays. On each array one pool of NHSM ICM total RNA was hybridized together with one pool of SOF ICM total RNA. This was performed twice with independent biological replicates. This comparison was again performed with two other biological replicates for each culture condition in a balanced dye-swap. Bovine whole genome gene expression microarrays V2 (Agilent Technologies) representing 43,653 *Bos taurus* 60-mer oligos in a 4x44K layout were used. From each sample 10 ng total RNA was used for cDNA synthesis, cRNA double amplification, labelling, quantification, quality control and fragmentation performed on an automated

system (Caliper Life Sciences NV/SA, Teralfene, Belgium), all as previously described in detail (Roepman et al. 2006, van Wageningen et al. 2010). Microarray hybridization and washing was performed with an HS4800PRO system and Quad-Chambers (Tecan, Mechelen, Belgium) using 500 ng, 1-2% Cy5/Cy3 labelled cRNA per channel as described (van Wageningen et al. 2010). Slides were scanned on an Agilent G2565BA scanner at 100% laser power, 30% PMT. After automated data extraction using Imagene 8.0 (BioDiscovery, Hawthorne, CA, USA), Loess normalization was performed (Yang et al. 2002) on mean spot-intensities. Correction of gene-specific dye bias was performed by a within-set estimate (Margaritis et al. 2009), and data were further analysed by MAANOVA (Wu et al. 2003), modelling sample, array, and dye effects in a fixed effect analysis. By a permutation F2-test, in which residuals were shuffled 10,000 times globally, p-values were determined. After false discovery rate determination (FDR by Benjamini-Hochberg), gene probes with $p<0.05$ were considered significantly changed when the difference was at least 1.5-fold. The values from the most 3' probe were used in case of multiple probes per gene (Shi 2006, Li et al. 2011). All microarray gene expression data have been deposited in NCBI's Gene Expression Omnibus (Edgar et al. 2002) and are accessible through GEO Series accession number GSE69399.

Quantitative reverse transcription PCR

To generate cDNA, RNA was converted using the iScript™ cDNA Synthesis Kit (BioRad) according to manufacturer's protocol. For primers (Supplemental table 3) to be designed a primer3 based platform (Primer-Blast; http://www.ncbi.nlm.nih.gov/tools/primer-blast) was used with specific *Bos taurus* mRNA templates (Genbank; http://www.ncbi.nlm.nih.gov/nucleotide). A four-times dilution series of cDNA from blastocysts was used for confirmation of primer pair specificity and establishing annealing temperatures (Ta) by performing a temperature gradient ranging from 57°C - 65°C. All quantitative reverse transcription PCR (qRT-PCR) reactions were performed in 25 µl iQ SYBR Green supermix (Biorad) with a final primer concentration of 400 nM on a CFX detection system (Biorad) according to manufacturer's protocol. Reactions started with 5 min at 95°C for enzyme activation followed by 40 cycles of first a denaturing step at 95°C for 10 s followed by an annealing step at appropriate Ta (Supplemental table 3) for 10 s and finally a 30 s elongation step at 72°C. The reaction continued with the generation of a dissociation curve by increasing the temperature by 1°C for 15 s each step from 60°C to 98°C.

Immunostaining

Collected blastocysts were fixed in 4% paraformaldehyde (PFA) for 15 min and stored in 1% PFA at 4°C until further use. Blastocysts were washed briefly in PBS containing 0.1% Triton X100 (Sigma Aldrich) and 10% FCS (PBST) and permeabilised in PBS +

0.5% Triton X100 + 10% FCS for 30 min. For detection of GATA6 and NANOG, blastocysts were blocked for aspecific binding by 1 h incubation in PBST and subsequently incubated overnight with the primary antibodies rabbit anti-GATA6 (Santa Cruz, Dallas TX, USA; sc-9055; 1:100) and mouse anti-NANOG (eBiosciences, San Diego CA, USA; 14-5768-82; 1:250) at 4°C. After 1 h incubation with secondary antibodies goat anti-mouse Alexa647 and goat anti-rabbit Alexa488 (both Life Technologies; 1:200) and 5 min incubation with DAPI (0.1 μg/ml) (Sigma Aldrich), embryos were mounted in Vectashield (Brunschwig Chemie, Amsterdam, The Netherlands) in Grace Bio-Labs SecureSeal™ imaging spacers (Sigma Aldrich). Nuclear and lipid droplet staining (Spandl et al. 2009) was performed after permeabilisation with DAPI (0.1 μg/ml) and LD540 (0.05 μg/ml), respectively. After 15 min embryos were mounted in Vectashield (Brunschwig Chemie) in Grace Bio-Labs SecureSeal™ imaging spacers (Sigma Aldrich). For image acquisition an inverted semi-automated confocal microscope (SPE-II – DMI4000; Leica, Son, The Netherlands) was used. Images were further analysed using Fiji software (Schindelin et al. 2012).

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AUTHOR DISCLOSURE STATEMENT

All authors state no competing financial interests exist.

SUPPLEMENTALS

Supplemental tables from this chapter can be found in the electronic (ePub) version of this thesis.

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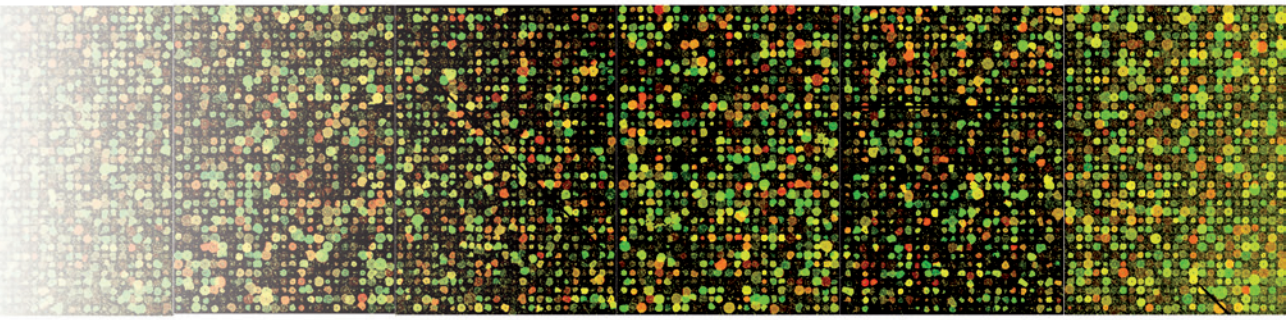

Delayed differentiation in bovine blastocysts cultured under naïve (human) conditions

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To be submitted

ABSTRACT

Recently, several different culture protocols have been described capable of generating human naïve pluripotent stem cells. To investigate the behaviour of bovine embryos cultured under naïve conditions we cultured bovine blastocysts in a selection of these reported culture conditions. Gene expression was compared with that of blastocysts cultured under standard conditions. The morphology of day 8 blastocysts was similar after culture in the different media with slightly different cell numbers. Transcript levels identifying trophectoderm, primitive endoderm, or epiblast only showed reduced expression of some primitive endoderm markers. Of the key pluripotency markers (*NANOG*, *SOX2*, and *POU5F1*), *POU5F1* expression was reduced, whereas levels of *NANOG* and *SOX2* were not changed. The naïve culture media resulted in a down-regulated *XIST* expression compared to blastocysts cultured under standard conditions, but no significant differential expression of *HPRT1* could be detected. Genes involved in differentiation showed reduced expression levels compared to blastocysts cultured under standard conditions. These data indicate that culture conditions for the generation of human naïve pluripotent stem cells can reduce differentiation in bovine embryos and may aid in the generation of bovine embryonic cell lines.

INTRODUCTION

Embryonic stem (ES) cells are a valuable type of cells to study gene function and embryonic development. These cells have the potency to develop into any cell of the foetus and adult, a trade called pluripotency, and can be propagated without losing this capacity (self-renewal). Generally, ES cells are derived from the inner cell mass (ICM) of a preimplantation embryo. While the ICM develops into a foetus *in vivo*, when isolated and cultured under appropriate conditions cells from the ICM can retain their pluripotency and be expanded clonally. Ultimately, their pluripotency can be verified by introducing the ES cells into an embryo to be carried to term and able to successfully reproduce, generating progeny originating from the ES cells (Rossant 2001, Jaenisch & Young 2008).

To compensate the double dosage of X-linked genes in female mammalian embryos either the maternally or paternally inherited X-chromosome is randomly inactivated. The timing of X-chromosome inactivation (XCI), initiated by the expression of the long noncoding RNA *XIST*, is not exactly known in human and bovine embryos, despite *XIST* expression in the female blastocysts of these species (De La Fuente et al. 1999, Dupont & Gribnau 2013, Sado & Sakaguchi 2013). This is in contrast to the situation in mice, the standard XCI model, where both X-chromosomes are active (XaXa) at the early blastocyst stage, but XCI is complete (XaXi) at the late blastocyst stage (Bermejo-Alvarez et al. 2012). Murine ES cell lines are generated from early preimplantation embryos and therefore exhibit an XaXa status in female cells which is considered a hallmark of true (naïve) pluripotency (Lengner et al. 2010, Payer et al. 2011).

Early postimplantation mouse embryos have also been used to derive pluripotent stem cells (Brons et al. 2007, Tesar et al. 2007). These so-called EpiSCs rarely, if at all, contribute to the generation of chimeras when introduced into embryos (Huang et al. 2012), and in female EpiSC lines XCI has been established. Although ES cells and EpiSCs are both considered pluripotent stem cells they are referred to as being naïve (ES) opposed to primed (EpiSCs), and they require different culture conditions.

Human ES cells have also been derived from preimplantation embryos, but their epigenome, X-chromosome status, and culture conditions more resemble those of mouse EpiSCs. Human ES cells are therefore considered to be at a primed state of pluripotency. Recently however, several studies described different culture media capable of deriving naïve human ES cells (Gafni et al. 2013, Chan et al. 2013, Theunissen et al. 2014, Takashima et al. 2014, Ware et al. 2014, Valamehr et al. 2014). These culture media also facilitate the conversion of primed cells towards a naïve state independent of the origin (human or mouse). Obviously however, distinct proof of a naïve character of human ES cells by the contribution to the formation of germ-line chimeras is not possible. Human-mouse chimeras have been generated, but with mixed success (Gafni

et al. 2013, Theunissen et al. 2014). Transcriptome analysis of the naïve-like human ES cells derived with different culture media revealed some differences between the cell types, and all were rather similar to the transcriptome of the human preimplantation blastocyst (Huang et al. 2014).

The recent establishment of naïve human ES cell lines led us to investigate the influence of these culture conditions on bovine blastocysts. Gene expression profiles of blastocysts cultured in naïve human stem cell medium (NHSM) (Gafni et al. 2013), 3iL (Chan et al. 2013) and 5i/L/A (Theunissen et al. 2014) were compared to gene expression profiles of embryos cultured in synthetic oviductal fluid (SOF), the standard culture medium for bovine *in vitro* embryo production. Transcripts characteristic for primitive endoderm formation were reduced in all three culture media compared to SOF. Trophectoderm-expressed *CDX2* levels were similar between all culture conditions, and only 5i/L/A culture medium increased epiblast characteristic gene transcript levels. *XIST* expression levels were decreased by all culture conditions compared with standard culture conditions.

RESULTS

Bovine embryo production in naïve media

To determine the effect of culture conditions allowing for the generation of naïve human ES cells on expression levels of certain genes in bovine blastocysts, *in vitro* culture of embryos was performed in three of different "naïve" media and compared with the expression levels in standard SOF cultured embryos. All cultures started with the *in vitro* maturation and fertilisation of oocytes followed by embryo culture in SOF for four days (5 days post fertilisation; 5dpf). Hereafter, morula stage embryos were cultured until 8dpf in either SOF, NHSM (Gafni et al. 2013), 3iL (Chan et al. 2013) or 5i/L/A (Theunissen et al. 2014) (Table 1). The percentage of embryos that had developed to the blastocyst stage at 8dpf was similar in each group with no difference in the percentage of embryos hatched from their zona pellucidae (Figure 1A). The embryos in the different groups had a similar morphology except for NHSM-cultured embryos that appeared darker than the other embryos (Figure 1B-E), and hatched embryos ascended to just below the surface in NHSM as has been described before (Chapter 3). The average embryo diameter was not significantly different between embryos from the different culture conditions (Figure 1F). Counting DAPI positive cell nuclei (Figure 1G – J) showed different cell numbers depending on the culture condition. The NHSM day 8 embryos were composed of the smallest number of cells (95 ± 49) whereas day 8 embryos cultured in 5i/L/A contained the largest number of cells (219 ± 74).

Table 1. Components of culture media used.

SOF	NHSM	3iL	5i/L/A	Component	Function
*	**KO-DMEM**				
		TeSR1			
			1:1 DMEM/F12 & Neurobasal		
	20%			KOSR	
			1%	N2 supplement	
			2%	B27 supplement	
4 mg/ml			50 µg/ml	BSA	
	12.5 µg/ml			Rec. Hum. Insulin	
1%	1%		1%	NEAA	
2 mM	1 mM		1 mM	glutamine	
2%	0.1 mM		0.1 mM	β-Mercaptoethanol	
0.1%	1%		1%	penicillin-streptomycin	
	20 ng/ml	10 ng/ml	20 ng/ml	Rec. Hum. LIF	
	8 ng/ml			Rec. bFGF	
	1 µM	1 µM	1 µM	PD0325901	MAPK-inh
	10 µM			SP600125	JNK-inh
	10 µM			SB203580	p38-MAPK-inh
	5 µM			Go6983	PKC-inh
			0.5 µM	SB590885	B-Raf-inh
	3 µM			CHIR99021	GSK3a/b-inh
			1 µM	IM-12	GSK3b-inh
		2 µM		BIO	GSK3a/b-inh & JAK-inh
		2 µM		Dorsomorphin	AMP kinase-inh & BMP-inh
	1 ng/ml			TGFb1	TGFb - SMAD pathway
			20 ng/ml	Activin A	TGFb - SMAD pathway
			1 µM	WH-4-023	Lck/Src-inh
			10 µM	Y27632	ROCK1-inh

Of each media 5 ml was prepared and sterilised over a 0.22 µm filter. Main component in bold.
* details of SOF can be found in methods section.

Gene expression and normalization

From each culture group consisting of 15 – 26 pooled embryos RNA was extracted resulting in comparable amounts per embryo. After RNA conversion to cDNA qRT-PCR was performed to determine gene expression levels. To accurately normalize gene expression data four genes (*SDHA*, *YWHAZ*, *RPL15*, and *UBB*) were selected as candidate reference genes described as suitable for normalization of gene expression (Bogaert et al. 2006) in bovine embryos (Brinkhof et al. 2015). The Genorm-based script ranked

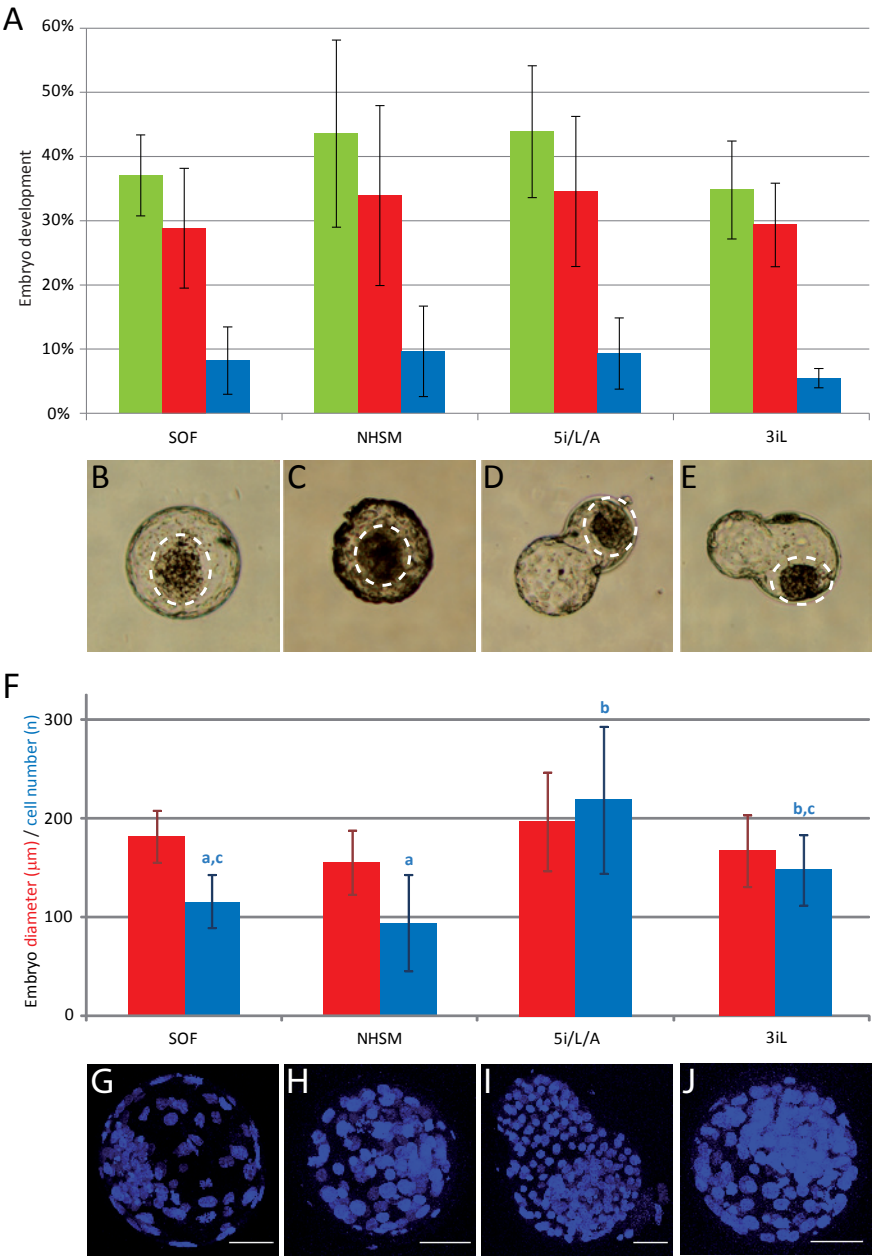

Figure 1.

Embryo characteristics. On day 8 of embryo culture, percentage of blastocysts (green) was determined and scored as enclosed by zona pellucida (red) or hatched (blue) (A). Representative blastocysts from SOF (B), NHSM (C), 5i/L/A (D) and 3iL cultures are shown with the ICM indicated by a white dashed oval. Average blastocyst diameter (red) and total cell number (blue) per embryo was determined (F) after nuclear DAPI (blue) staining of SOF (G), NHSM (H), 5i/L/A (I) and 3iL (J) blastocysts. Scale bar represents 50 µm.

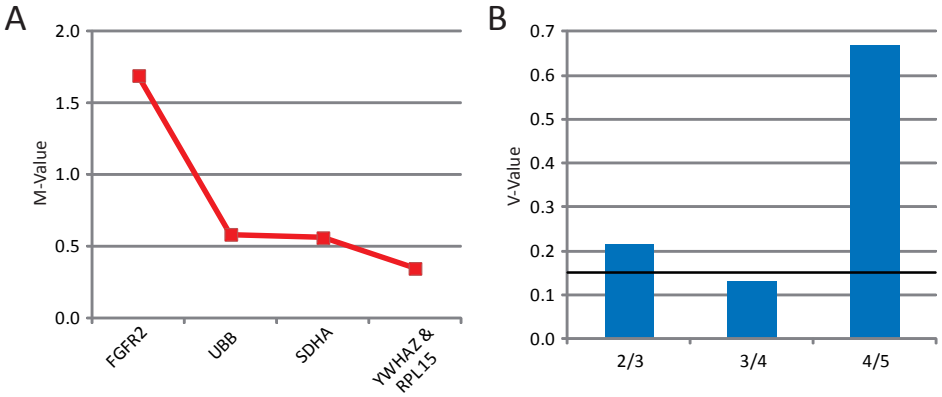

Figure 2.
GeNorm analysis. By calculating M-values the least and most stable genes can be plotted (A). After plotting the pairwise variation between NFs of a given number of genes the minimum amount of reference genes can be determined (B). Cut-off value of 0.15 is indicated by black horizontal line.

YWHAZ and *RPL15* as most stably expressed, followed by *SDHA* and *UBB* (Figure 2A). The result of *FGFR2* expression was included and indicated a high M-value, hence we could expect large expression differences between samples for *FGFR2*. By calculating the pairwise variation (V-value) between the candidate reference genes, the minimal amount of reference genes needed for accurate normalization could be determined. Gene expression data from our candidate reference genes indicate the use of three reference genes was sufficient. Again the inclusion of *FGFR2* resulted in an increased V-value, indicating large expression differences (Figure 2B). Therefore, *YWHAZ*, *RPL15*, and *SDHA* were used for normalization.

To determine differences in blastocyst composition, gene expression was determined with regard to trophectoderm (TE) specific transcript *CDX2* (Strumpf et al. 2005), primitive endoderm (PE) characteristic markers *GATA6* (Koutsourakis et al. 1999), *HNF4α* (Morrisey et al. 1998), *FGFR2* and *PDGFRα* (Niakan et al. 2010), and epiblast markers *FGF4* (Rappolee et al. 1994) and *KLF4* (Chan et al. 2013). None of the culture conditions induced significant differences in TE- and epiblast-specific gene expression. PE-specific gene expression was down-regulated in the naïve culture conditions although only significant for *FGFR2* and *GATA6* (Figure 3A). *POU5F1* expression levels were lower in NHSM and 3iL, whereas the other core pluripotency genes *NANOG* and *SOX2* showed no significant expression difference. Gene transcripts related to naïvety were either not differentially expressed (*PRDM14* (Yamaji et al. 2013), *TBX3* (Niwa et al. 2009), and *ZFP42* (Toyooka et al. 2008)) or expressed at a higher level (*ESRRB* (Martello et al. 2012)) in SOF cultured embryos (Figure 3B). Combined, these data suggest comparable blastocyst composition and pluripotent state in any of the media under investigation.

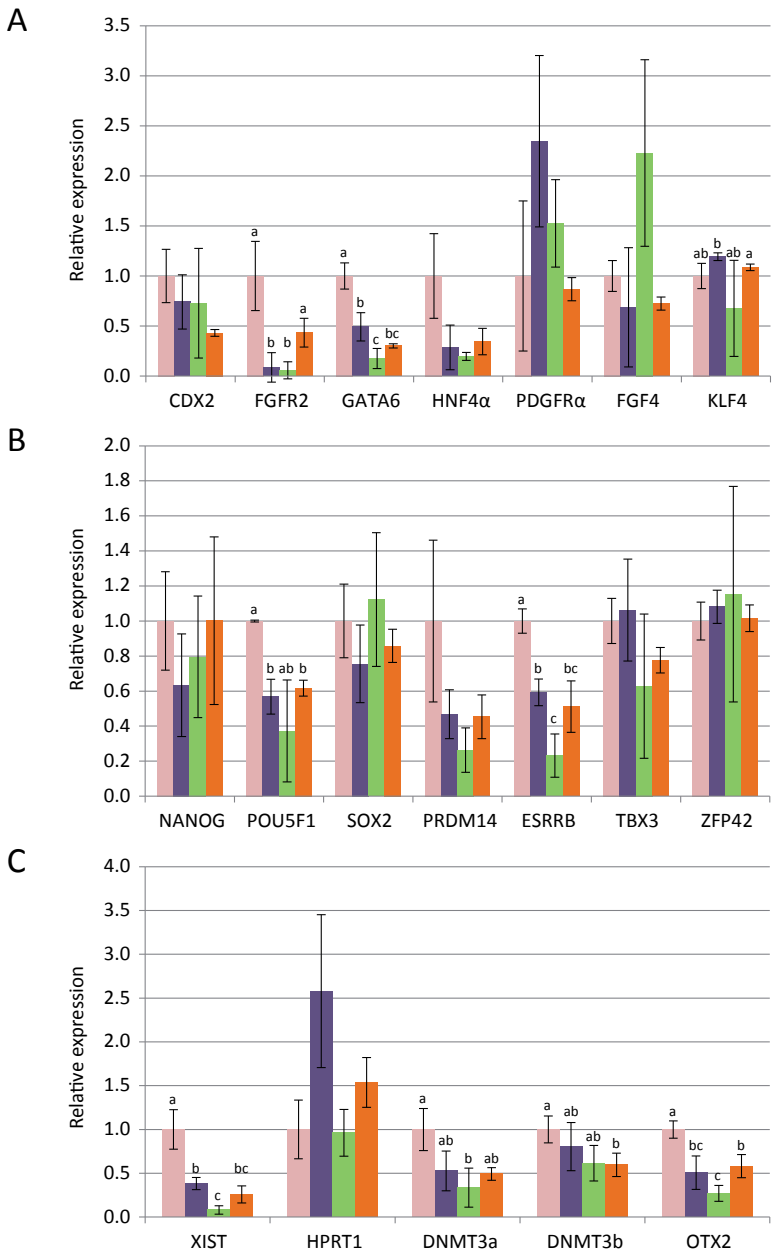

Figure 3.
qRT-PCR results. Expression of genes marking TE, PE, and epiblast (A), involved in pluripotency (B) and implicated in XCI, methylation, or differentiation (C) is plotted with respect to expression in SOF cultured embryos (pink) for NHSM (purple), 5i/L/A (green) and 3iL (orange). Significant differences ($p<0.05$) are indicated by different letters.

X-chromosome inactivation is not completed indicated by reduced *XIST* expression

In contrast to naïve ES cells that, at least in the mouse, have two active copies of the X-chromosome in female cells, primed ES cells are characterised by having one active and one inactive X-chromosome. XCI is established by covering of the candidate chromosome with *XIST* transcripts. Interestingly, all naïve culture media caused a reduction in *XIST* expression of at least 2.5-fold (Figure 3C). *HPRT1,* located on the X-chromosome, is one of the genes of which the expression is down-regulated after XCI and is frequently used as a marker for XCI (Farazmand et al. 2001, Peippo et al. 2002, Basrur et al. 2004, Jeon et al. 2008, Merighe et al. 2009, Jeon et al. 2012). Despite decreased *XIST* expression the expression levels of *HPRT1* in ICMs from embryos cultured under the "naïve" conditions did however not significantly differ from those of the control group (Figure 3C).

It has been reported that ES cells cultured in 2i conditions exhibit hypomethylation and reduced expression of the *de novo* methyltransferases DNMT3a and DNMT3b capable of modifying the epigenome to a primed state (Yamaji et al. 2013, Leitch et al. 2013). Genes for both proteins showed a reduced expression in bovine embryos cultured in "naïve" conditions although only the expression of *DNMT3a* was significantly reduced in 5i/L/A, and expression of *DNMT3b* was only significantly reduced in 3iL (Figure 3C). When pluripotent cells transit from a naïve state to a primed character one of the first genes to be expressed is *OTX2;* a gene involved in neuronal differentiation and brain development. It has been proposed that *OTX2* expression keeps cells in a primed state by blocking a return to naïvety (Acampora et al. 2013). Blastocysts cultured in any of the naïve culturing media showed a reduced *OTX2* expression compared to SOF cultured blastocysts (Figure 3C).

DISCUSSION

ES cell lines have only been developed from rodent and primate embryos, but differ substantially in developmental potential, transcriptome and their culture requirements (De Los Angeles et al. 2012). ES cells from mouse early preimplantation embryos are in a naïve state whereas cells derived from mouse postimplantation and human blastocysts can generate primed ES cells (Nichols & Smith 2009). Recently, several studies described the use of culture media capable of generating naïve human ES cell lines (Gafni et al. 2013, Chan et al. 2013, Theunissen et al. 2014, Takashima et al. 2014, Ware et al. 2014, Valamehr et al. 2014). Remarkably, ES cell lines from mammalian species other than rodents or primates, whether naïve or primed, have not been derived yet. Whether the culture media used were not sufficient to capture and maintain pluripo-

tency of the cells or whether for instance the developmental stage of embryos used was not correct is not known. Since the recently described culture media were able to re-direct human and mouse ES cells from a primed to a naïve state we anticipated that these media could be helpful in capturing pluripotency in bovine cells, less dependent on the developmental stage of the embryo. Bovine embryos were cultured in three different media that had been successful for the generation of mouse and human naïve pluripotent cell lines, and we examined the gene expression profiles of these embryos. The results were compared with expression data from SOF cultured bovine embryos.

In the four different media used in this study (SOF, NHSM, 5i/L/A and 3iL) the successful development of blastocysts from morula stage embryos was similar. Also, no differences were determined in diameter of the embryos and the percentage of hatched blastocysts. Nevertheless, average cell number in the embryos was different depending on culture condition. In particular blastocysts from the 5i/L/A medium contained twice as many cells as blastocysts cultured in NHSM. These findings indicate that proliferation rate is higher in 5i/L/A than in NHSM. The behaviour of complete embryos in selected media is most likely different compared with the behaviour of isolated and plated ICM cells. It has not been described however if the expression profiles change when whole human or mouse embryos are cultured in the different naïve media. Previous attempts to generate cell lines by culturing plated ICMs in NHSM medium were unsuccessful (Chapter 3) indicating that apparently for bovine cells to inhibit differentiation and stimulate self-renewal other stimuli are needed. The embryos used in this study were generated by *in vitro* maturation and fertilisation of oocytes derived from slaughterhouse ovaries. It has been established that the quality of *in vitro* embryos is compromised compared with the quality of *in vivo* embryos (Corcoran et al. 2006, Kues et al. 2008). *In vitro* embryos can give rise to healthy calves however after embryo transfer indicating that *in vitro* embryos are of good quality (Massip et al. 1996, Van Wagtendonk-de Leeuw et al. 2000, Fischer-Brown et al. 2005).

A hallmark of naïve pluripotency is the presence of two active X-chromosomes in female ES cell lines (Nichols & Smith 2009, Payer et al. 2011, De Los Angeles et al. 2012). XCI is initiated by *XIST* expression covering the X-chromosome in *cis* upon completion. A reduced *XIST* expression is therefore associated with delayed or reversed XCI. Indeed, *XIST* transcripts were less abundant in the naïve media than in SOF cultured embryos. In contrast to human ES cells cultured in 5i/L/A that exhibited XaXi (Theunissen et al. 2014), *XIST* expression in bovine blastocysts in 5i/L/A showed even the largest reduction in *XIST* expression. To our surprise, *HPRT1* expression, influenced by XCI (Lock et al. 1987), remained at similar levels in the different culture media. Previously, we reported an increased *HPRT1* expression and *XIST* down-regulation in NHSM cultured day 9 blastocysts (Chapter 3). Although *XIST* transcripts are present, covering of the X-chromosome and thereby reducing the *HPRT1* expression in female

cells, might not have been achieved in any or all day 8 blastocysts in any of the culture media, although the naïve culture conditions are capable of delaying *XIST* expression. Moreover differences in X-chromosome activity might be obscured in whole embryos since in the TE XCI is most likely imprinted (Xue et al. 2002, Dindot et al. 2004) and not affected by culture media. It should also be emphasized that the cultured embryos can be considered a mixture of female and male genotypes. A possible increase in *HPRT1* expression in the female embryos may be obscured by the presence of male embryos. In future experiments, when studying X-chromosome inactivation it might be worthwhile to use sexed semen to enhance for female or male embryos specifically (Seidel Jr. 1999, Johnson 2000). Alternatively, individual embryos could be sexed by for instance blastomere biopsy (Handyside et al. 1989) or removal of part of the TE (Monk et al. 1988, Summers et al. 1988). This could shed more light on how different culture media affect XCI.

The use of 2i in bovine embryo culture has been reported to improve blastocyst development and elevate the expression of epiblast markers *NANOG* and *SOX2* (Harris et al. 2013), but failed to accomplish the generation of bovine ES cells (Verma et al. 2013). MAPK inhibition results in elevated expression levels of *NANOG* in the ICM of bovine embryos (Kuijk et al. 2012, Brinkhof et al. 2015), but blocking GSK3β activity with CHIR99021 had minimal effect on *NANOG* expression during embryonic development (Kuijk et al. 2012). Despite these previous findings, *NANOG* expression was not changed when we cultured bovine embryos in any of the naïve human stem cell culture conditions compared to the standard SOF culture, neither was the expression of *SOX2*. We have previously established enhanced cell proliferation and unchanged *NANOG* or *SOX2* expression levels in bovine embryos cultured in NHSM (Chapter 3). Genes involved in pluripotency (*PRDM14* (Yamaji et al. 2013), *ESRRB* (Martello et al. 2012), *TBX3* (Niwa et al. 2009) and *ZFP42* (Toyooka et al. 2008)) showed no expression difference when compared to SOF embryos except for *ESRRB*, which was more than 1.6-fold down-regulated in all three naïve media. Although this suggests a reduced pluripotent state, *OTX2* expression, implicated in ES cell progression from naïve to primed (Buecker et al. 2014), was down-regulated in the three media tested. These data suggest that more pluripotent cells from bovine blastocysts cultured in "naïve" media are delayed in their developmental progression and in an intermediate state opposed to being naïve or primed than from SOF cultured blastocysts.

Bovine ES cell lines have not been established yet; therefore a comparison of gene expression profiles from human and bovine whole embryos cultured in the "naïve" media could be helpful in interpreting these results and identify genes important for bovine pluripotency and self-renewal.

METHODS

Bovine in vitro embryo production

Embryo production was performed in a humidified atmosphere with 5% CO_2 at 39°C, unless stated otherwise. From ovaries, obtained from a local slaughterhouse, follicles of 3 – 8 mm in diameter were aspirated to retrieve cumulus oocyte complexes (COCs). COCs in groups of 35 – 60 per 500 µl M199 (Life Technologies, Bleiswijk, The Netherlands) supplemented with 0.05 IU/ml recombinant hFSH (Organon, Oss, The Netherlands) and 1% penicillin-streptomycin (Life Technologies) were matured for 23 h. Matured COCs were fertilised as described previously (Parrish et al. 1988) with modifications (Izadyar et al. 1996). In short, COCs were transferred to fertilisation medium (Fert-TALP) supplemented with 10 mg/ml heparin (Sigma Aldrich, Zwijndrecht, The Netherlands), 20 µM D-penicillamine (Sigma Aldrich), 10 µM hypotaurine (Sigma Aldrich), and 1 µM epinephrine (Sigma Aldrich). Frozen-thawed sperm from a bull with proven fertility was centrifuged over a Percoll-gradient (GE Healthcare Europe GmbH, Eindhoven, The Netherlands) and added to the COCs resulting in a final concentration of 1.0×10^6 spermatozoa/ml. This was considered as day 0. After 20 h, vortexing for 3 min resulted in oocytes freed of cumulus cells which were placed in SOF. SOF medium consisted of 107.63 mmol/l NaCl (Sigma Aldrich), 7.16 mmol/l KCl (Sigma Aldrich), 1.19 mmol/l KH_2PO_4 (Sigma Aldrich), 3.20 mmol/l Sodium DL-lactate (60% syrup; Sigma Aldrich), 0.74 mmol/l $MgSO_4 \cdot 7H_2O$ (Merck Millipore, Billerica, MA, USA), 25 mmol/l $NaHCO_3$ (Sigma Aldrich), 1.78 mmol/l $CaCl_2 \cdot 2H_2O$ (Sigma Aldrich), 0.33 mmol/l Sodium pyruvate (Sigma Aldrich), 2.05 mmol/l L-Glutamine (Sigma Aldrich), 4 mg/ml BSA (Merck Millipore), 100 U/ml penicillin-streptomycin (Life Technologies), 1% MEM NEAA (Sigma Aldrich), 2% BME Amino Acids (Sigma Aldrich) and 0.5 µl/ml Phenolred 0.5% (Sigma Aldrich) in LAL water (Lonza, Basel, Switzerland). After 4 days culturing in a humidified atmosphere with 5% CO_2 and 7% O_2 at 39°C, embryos were transferred to fresh SOF or to one of the media used for human naïve stem cell generation; NHSM (Gafni et al. 2013), 3iL (Chan et al. 2013) or 5i/L/A (Theunissen et al. 2014). Concentrations of media components; recombinant human LIF (Prospec, Rehovot, Israel); recombinant human bFGF (Peprotech; Rocky Hill, NJ, USA); recombinant human TGFβ1 (Peprotch); recombinant human Activin A (Peprotech); PD0325901 (Stemgent, Cambridge, MA, USA); CHIR99021 (Bio-connect, Huissen, The Netherlands); Go6983 (Tocris, Bristol, UK); SP600125 (Tocris); SB203580 (Tocris); WH-4-023 (Selleckchem.com; Houston, TX, USA); Dorsomorphin (Sigma Aldrich); BIO (Sigma Aldrich); IM-12 (Sigma Aldrich); Y27632 (Sigma Aldrich); SB590885 (Sigma Aldrich); bovine Insulin (Sigma Aldrich); NEAA (Sigma Aldrich); glutamine (Life Technologies); BSA (Life Technologies); N2 supplement (Life Technologies); B27 supplement (Life Technologies); β-mercaptoethanol (Life Technologies); KOSR (Life Technologies); KO-DMEM (Life Technologies); TeSR1 (Stem

Cell Technologies, Grenoble, France); Neurobasal (Life Technologies); DMEM/F12 (Life Technologies) and penicillin-streptomycin (Life Technologies) are listed in Table 1. At day 8, blastocysts of stage 7–9 with quality code 1 and 2 (Stringfellow & Givens 2009) were collected to determine embryo properties or for RNA isolation.

Embryo properties

Blastocysts for embryo properties determination were fixed in 4% paraformaldehyde (PFA) for 15 min and stored in 1% PFA until further use. Fixed embryos were washed in PBS containing 0.1% Triton X100 and 10% FCS (PBST) and subsequently permeabilised in PBS + 0.5% Triton X100 + 10% FCS for 30 min. Next, after washing in PBST, embryos were incubated with DAPI (0.1 µg/ml) (Sigma Aldrich) for 5 min and mounted in Vectashield (Brunschwig Chemie) in Grace Bio-Labs SecureSeal™ imaging spacers (Sigma Aldrich). For image acquisition an inverted semi-automated confocal microscope (SPE-II – DMI4000; Leica, Son, The Netherlands) was used. Images were further analysed using LASAF software (Leica) for diameter acquisition and Fiji software (Schindelin et al. 2012) for cell counting.

RNA isolation and cDNA generation

For RNA isolation 3 pools of 15–26 blastocysts were collected per culture condition and lysed and stored in RLT buffer (Qiagen, Venlo, The Netherlands) at -80°C until further use. An RNA micro kit (Qiagen) was used for RNA isolation and on column DNA digestion according to manufacturer's protocol. Total RNA quality and quantity was assessed using a Bioanalyzer 2100 and the RNA 6000 Pico LabChip kit (Agilent Technologies, Amstelveen, The Netherlands) according to manufacturer's instructions. RNA was stored at -80°C until further use. RNA was converted to cDNA using the iScript™ cDNA Synthesis Kit (Biorad, Veenendaal, The Netherlands) according to manufacturer's protocol. cDNA was stored at -20°C until qRT-PCR performance.

Quantitative reverse transcription PCR

New primer sets were designed using Primer-Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast) (Ye et al. 2012) a primer3 based platform (Untergasser et al. 2012). Specific *Bos taurus* nucleotide sequences (Genbank; http://www.ncbi.nlm.nih.gov/nucleotide) were used as a template. Further *in silico* validation was performed by predicting PCR product folding structures using the Mfold web server (SantaLucia 1998, Zuker 2003). To confirm primer specificity and establish annealing temperature, a temperature gradient ranging from 57°C - 65°C was performed, using a four-time dilution series of cDNA from blastocysts. All quantitative reverse transcription PCRs (qRT-PCRs) were performed with a final primer concentration of 400 nM in 25 µl iQ SYBR Green supermix (Biorad) on a CFX detection system (Biorad) according to manufacturer's protocol.

Reactions started with 5 min at 95°C for enzyme activation followed by 40 cycles of first a denaturing step at 95°C for 10 s followed by an annealing step at appropriate annealing temperature (Table 2) for 10 s and finally a 30 s elongation step at 72°C. Hereafter, a dissociation curve was generated by increasing the temperature each step by 1°C for 15 s from 60°C - 98°C.

Statistics

Expression data from candidate reference genes (cRG) were analysed using GeNorm (Vandesompele et al. 2002). GeNorm calculates a stability of expression (M) value of a cRG based on the average pair-wise variation between all studied cRGs. By stepwise elimination of the least stable cRG, the two most stable genes are identified. In addition, GeNorm determines a normalization factor (NF) for a given number (n) of cRGs, and the pairwise variation (V-value) is defined between consecutive normalization factors NFn and NFn+1. When this variation is below the cut-off value of 0.15 there is no need to include more genes for accurate normalization (Vandesompele et al. 2002). GeNorm analysis was performed using the Bioconductor software package in the statistical programming language R and the GeNorm script from the SLqPCR package (De Spiegelaere et al. 2015). All other statistical analyses were performed using Excel and performing a student t-test after determining variances.

AUTHOR DISCLOSURE STATEMENT

All authors state no competing financial interests exist.

Table 2. Primer details

Gene [1]	Discription	NCBI # [2]	Forward Primer	Reverse Primer	Ta (°C)
CDX2	Caudal type homeobox 2	(Brinkhof et al. 2015)	AACCTGTGCGAGTGGA	GCGACTGTAGTGAAACTCC	60
DNMT3a	DNA (cytosine-5-)-methyltransferase 3 alpha	(McLean et al. 2014)	CTGGTGCTGAAGGACTTGGGC	CAGAAGAAGGGGCGGTCATC	57
DNMT3b	DNA (cytosine-5-)-methyltransferase 3 beta	(McLean et al. 2014)	CCGCAGATCAAGCTCAC	GTTATTTCGGGTTCGGAC	60
ESRRB	Estrogen-related receptor beta	XM_606860	ATGAAATGCCTCAAAGTGG	TCAGAGCCTTGATGTCG	61
FGF4	Fibroblast growth factor 4	(McLean et al. 2014)	TACGGCTCGCCTTTCTTCAC	TTCTTGGCCTTGCCGTTCTT	60
FGFR2	Fibroblast growth factor receptor 2	(McLean et al. 2014)	TGTAAGAGAAAAGGAGATCCCA	GTTGAAGAGAGGCGAGTTG	56
GATA6	GATA binding protein 6	XM_002697727	GTGAACTGCGGCTCCATCCA	AGGCATTGCACACAGGCTCG	67
HNF4α	Hepatocyte nuclear factor 4, alpha	(Nagatomo et al. 2013)	CGGGCCACGGGCAAACACTA	CACCACGCACTGCCGGCTAA	58
HPRT1	Hypoxanthine phosphoribosyltransferase 1	(Goossens et al. 2005)	TGCTGAGGATTTGGAGAAGG	CAACAGGTCGGCAAAGAACT	58
KLF4	Kruppel-like factor 4	NM_001105385	TCTCATCTCAAGGCACACCTGCG	GCGGGCAAACTTCCACCCAC	61
NANOG	Nanoghomeobox	(Brinkhof et al. 2015)	GAGAGCACAGAGAAGGAAGA	CTGGTGGTAGGAATAGAAGC	60
OTX2	Orthodenticle homeobox 2	XM_005211840	GGGCTGAGTCTGACCACTTC	AACCATACCTGTACCCTGGACT	58
PDGFRα	Platelet-derived growth factor receptor, alpha polypeptide	(McLean et al. 2014)	GCCAACCAGATGTGAGGTGA	AAAGACCACGCTGGCAGTAA	60
POU5F1	POU class 5 homeobox 1	(Brinkhof et al. 2015)	TAGCCACATCGCCCAGCAGC	GAAAGGAGACCCAGCAGCCTCA	60
PRDM14	PR domain containing 14	(McLean et al. 2014)	ATTTTCGTTCCGCCGCCCCC	TCAGCCCCGGCGCTATCGGT	60
RPL15 [r]	Bos taurus ribosomal protein L15 mRNA	(Brinkhof et al. 2015)	CACAAGTTCCACCACACTATTGG	TGGAGAGTATTGCGCCTTCTC	61
SDHA [r]	Bos taurus succinate dehydrogenase complex, subunit A, flavoprotein (Fp)	(Brinkhof et al. 2015)	GCAGAACCTGATGCTTTGTG	CGTAGGAGAGCGTGTGCTT	64
SOX2	SRY (sex determining region Y)-box 2	(Brinkhof et al. 2015)	CCATGCAGGTTGACATCGT	ACACAACTACGGAAACTAAAAGTGG	60
TBX3	T-box 3	(McLean et al. 2014)	CGGATTTACTTTGGCCTTCCC	CTGGTATGCAGTCACAGCGA	60
UBB [r]	Bos taurus polyubiquitin	NM_174133	CTGACCGGCAAGACCATCAC	AGATGAGCCTCTGCTGGTCG	61
XIST	X (inactive)-specific transcript	NR_001464	AGCATTGCTTAGCATGGCTC	TGGCTGTGACCGATTCTACC	60
YWHAZ [r]	Bos taurus tyrosine-3-monooxygenase/tryptophan 5-monooxygenase Activation protein zeta polypeptide	(Goossens et al. 2005)	GCATCCCACAGACTATTTCC	GCAAAGACAATGACAGACCA	56
ZFP42	ZFP42 zinc finger protein	(Huang et al. 2011)	TGCCTGTCCTCACAACGGATGC	AGTGTGGGTGCGCACGTGTG	60

Details of primer sequences and annealing temperatures (Ta) to detect specific *Bos taurus* gene transcripts. 1) Gene [r] indicates candidate genes for normalization. 2) Newly developed primer sets are designed according to listed Genbank accession number. Primer sets described in previous reports are referred to as appropriate.

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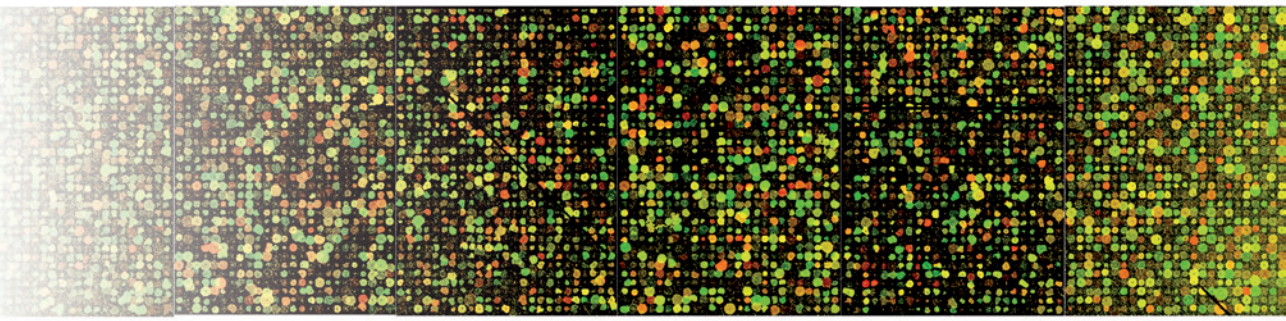

DKK3 enhances *MYF5* expression in cultured porcine satellite cells

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ABSTRACT

The myogenic regulatory factor MYF5 plays an important role during skeletal muscle development and regeneration. In zebrafish, Dkk3 protein can bind to α6 integrin and stimulate *Myf5* promoter activity during myogenesis. To examine the function of DKK3 in porcine satellite cells, these cells were cultured in the presence of different concentrations of DKK3 protein. We observed fusion of cells after induction of differentiation, indicative of myogenic development. Gene expression analysis showed that *MYF5* expression was elevated after 48 h of DKK3 exposure compared to control differentiation medium. Markers expressed later in myogenic differentiation and involved in the fusion of myoblasts also showed differential expression. Nevertheless, these expression levels already decreased 24 h after initiating DKK3 exposure. Taken together we conclude that *MYF5* expression is stimulated by DKK3 in porcine satellite cells and accelerates myoblast fusion.

INTRODUCTION

Pigs have been used for several decades as model species in human medicine and even for xeno-transplantation because of their comparable weight, size and physiology (Matsunari & Nagashima 2009, Flisikowska et al. 2014). Myogenesis has been studied in pigs to provide basic knowledge for the development of therapies to treat human muscle disease, such as Duchenne muscular dystrophy (Perry & Rudnick 2000, Muráni et al. 2007, Klymiuk et al. 2013). Additionally, myogenesis in agricultural species has been studied to improve meat quality. Meat quality is in particular defined by muscle fibre characteristics (Rehfeldt et al. 2000, Picard et al. 2002, Wimmers et al. 2007). Manipulation of these characteristics can improve meat quality and has a profound impact on the profitability of the meat industry (Joo et al. 2013).

During embryogenesis, somites are formed from paraxial mesoderm. The somites subsequently differentiate into dermamyotome and sclerotome, and it is the dermamyotome that harbours the first muscle progenitor cells (MPCs). The MPCs, identified by PAX3 and PAX7 expression (reviewed in (Christ & Ordahl 1995, Buckingham et al. 2003, Buckingham & Relaix 2007)) migrate to the trunk and limb areas to further differentiate into skeletal muscle cells (Gros et al. 2005). A small percentage of the cells residing in the muscle tissue, known as satellite cells, maintains the capacity to self-renew and is available for post-natal muscle growth and regeneration (Katz 1961, Mauro 1961, Seale et al. 2001). Upon activation, for instance due to injury, satellite cells can differentiate into myoblasts and also repopulate the satellite stem cell pool (Church et al. 1966, Tajbakhsh 2003, Kuang et al. 2007, Bryson-Richardson & Currie 2008). Although satellite cells form a heterogenic cell population (Kang & Krauss 2010), most are characterised by the expression of PAX7 (Seale et al. 2000). Highly myogenic stem cells are part of the heterogeneous satellite cell population and can be identified by their α6 integrin (*α6ITG*) expression in pigs (Wilschut et al. 2011). Differentiation of satellite cells results in progenitor cells that express *MYF5* and *MYOD* (Rudnicki et al. 1993). In zebrafish (*Danio rerio*), Dickkopf 3 (DKK3) is associated with positive regulation of *MYF5* expression (Hsu et al. 2010, Hsu et al. 2011). The family of Dickkopf proteins consists of four members (DKK1–4); DKK1, 2, and 4 can bind LRP5/6, thereby inhibiting WNT signalling (Semënov et al. 2001, Mao et al. 2002), but DKK2 is also known to promote WNT signalling (Wu et al. 2000, Brott & Sokol 2002, Li et al. 2002). DKK3, by contrast, is reported either not to interfere with WNT signalling (Mao et al. 2001, Mao & Niehrs 2003, Du et al. 2011, Fujii et al. 2014) or to block WNT signalling (reviewed with references therein in (Veeck & Dahl 2012)). Importantly, DKK3 has been described to bind α6ITG and to activate *MYF5* transcription (Fu et al. 2012). Expression of *MYF5* can lead to Desmin (DES) expressing myoblasts (Kaufman & Foster 1988, Tajbakhsh 2003). Eventually these myoblasts fuse to form myotubes and muscle fibres (Chen &

Olson 2005). Recently, TMEM8c, also known as myomaker, was identified as a muscle specific protein regulating myoblast fusion in mouse and zebrafish (Millay et al. 2013, Landemaine et al. 2014).

To examine a function of DKK3 in mammalian skeletal muscle formation, freshly isolated pig satellite cells were cultured in the presence of human DKK3. Expression levels of genes involved in satellite cell differentiation and myoblast fusion were determined. Indeed, satellite cells differentiated and fused. Although *MYF5* expression was down-regulated during differentiation, exposure to DKK3 resulted in increased *MYF5* expression.

RESULTS

To examine a function of DKK3 in the differentiation of mammalian satellite cells towards skeletal muscle, a muscle biopsy was obtained from the hind leg of a neonatal piglet after removal of fat and connective tissue. To eliminate fastly adhering fibroblasts, isolated cell fractions were preplated before culturing on Matrigel coated-well plates. Cells were allowed to proliferate to confluency in growth medium (GM), and subsequently differentiation was induced for 24 h in differentiation medium (DM) (Figure 1). Hereafter, at day 0, medium was replaced every 24 h with DM alone or supplemented with two different concentrations of DKK3 for a subsequent 5 days. Myoblast fusion was visible already after 24 h of differentiation even in the absence of DKK3 (Figure 1). In the cultures containing DKK3, myotubes seemed to form earlier than in cultures without DKK3 (Figure 2).

Muscle stem cells including satellite cells are characterised by the expression of *PAX7*. Indeed, the presumptive porcine satellite cells expressed high levels of *PAX7* after proliferation in GM. The expression decreased after culture in DM, indicating differentiation (Figure 3A). In the presence of DKK3 the expression of *PAX7* was further reduced, but only at a low concentration of DKK3. After 5 days of culture in the presence of both high and low concentrations DKK3, no difference in *PAX7* expression was observed when compared with culture in regular DM. It has been reported that satellite cells express ***α6ITG*** (Wilschut et al. 2011), and this expression could possibly be enhanced by exposure to DKK3 protein. Indeed, expression of ***α6ITG*** was detected in the satellite cells after culture in GM (Figure 3B). The levels of ***α6ITG*** expression reduced significantly already after 24 h of differentiation. In the presence of low concentrations of DKK3 the expression of ***α6ITG*** remained at the same level whereas a high concentration of DKK3, but also the absence of DKK3, resulted in increased ***α6ITG*** expression levels (Figure 3B).

DKK3 interacts with the α6ITG receptor enhancing *MYF5* expression (Hsu et al. 2011). Upon differentiation in DM, *MYF5* expression decreased almost 5-fold (Figure 4A). Nevertheless, after 48 h incubation in the presence of DKK3 a significant *MYF5* expression

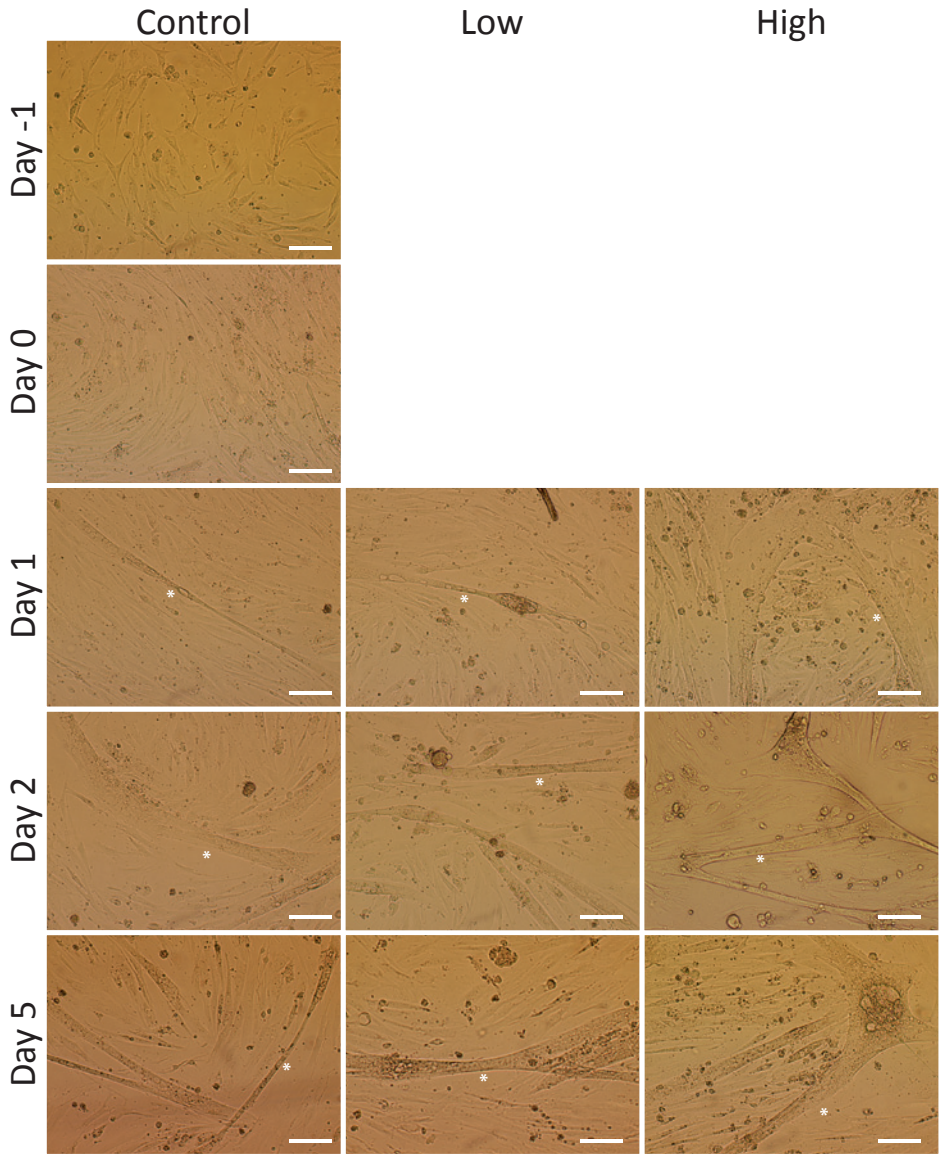

Figure 1.

Morphological representation of differentiating satellite cells. Porcine satellite cells were cultured in growth medium until confluency (day -1) when medium was replaced with differentiation medium and cell cultured for another 24 h. At day 0, medium was replaced with differentiation medium (control) or supplemented with a low (50 ng/ml) or high (500 ng/ml) concentration of DKK3. Pictures were taken on the indicated days. Examples of fused myoblasts are indicated by an asterisk. Scale bars indicate 100 µm

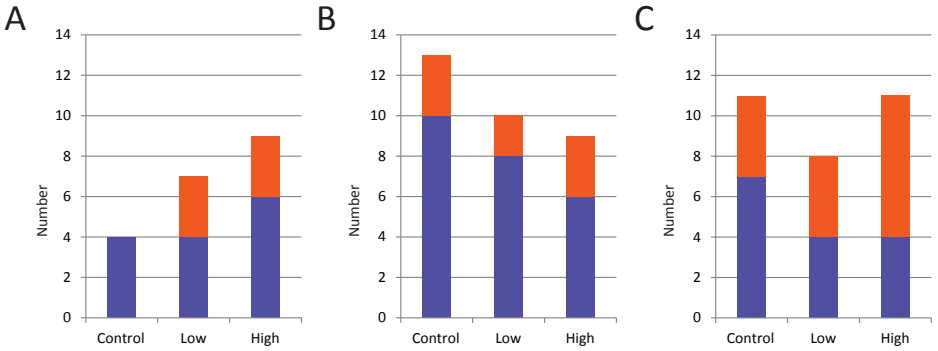

Figure 2.
Myotube formation. Diameter of myotubes was measured in 4 separate sections at day 1 (A), day 2 (B) and day 5 (C). Cells were cultured in differentiation medium (Control) or supplemented with a low (50 ng/ml) or high (500 ng/ml) concentration of DKK3. Total number of myotubes in these sections were further subdivided in number of myotubes with a diameter smaller than 30 μm (purple) or larger than 30 μm (orange).

increment was observed. This enhanced expression was not correlated with the concentration of DKK3. After 5 days of differentiation, *MYF5* transcript levels had equalled to that of regular DM expression levels (Figure 4A). *MYOD* is expressed approximately simultaneously with *MYF5* in satellite cells (Buckingham 1992). In contrast to *MYF5*, *MYOD* expression increased dramatically when differentiation was induced to subsequently decrease during further differentiation to initial expression levels (Figure 4B). After 48 h this reduction was significantly lower in DKK3-exposed cells compared to cells in regular DM again irrespective of protein concentrations (Figure 4B).

When muscle stem cells start to differentiate, *DES* expression increases (Smythe et al. 2001, Shi & Garry 2006) as was indeed observed (Figure 5A). After initial differentiation, a low concentration of DKK3 after 24 h further increased *DES* expression, whereas no protein or a high concentration of DKK3 reduced *DES* expression. Cells cultured in both high and low concentrations of DKK3 continued or started to express lower *DES* levels whereas the regular DM increased *DES* expression. Eventually, at day 5 all culture conditions showed lower *DES* expression levels, although the levels at 5 days of differentiation were significantly higher compared with the levels in proliferating stem cells. Furthermore, *DES* levels at day 5 in DKK3-cultured cells were significantly lower than in regular DM (Figure 5A). To identify the formation of myoblasts, we analysed cells for expression of *TMEM8c*. Initial differentiation in DM had no significant effect on *TMEM8c* transcript levels (Figure 5B). Only after an extra 24 h and 48 h culture in DM, *TMEM8c* levels were first up-regulated to subsequently reduce to initial GM levels after 5 days of differentiation. The *TMEM8c* expression profile of cells cultured in the presence of DKK3 was very similar to the observed *DES* expression profile, albeit less pronounced (Figure 5B).

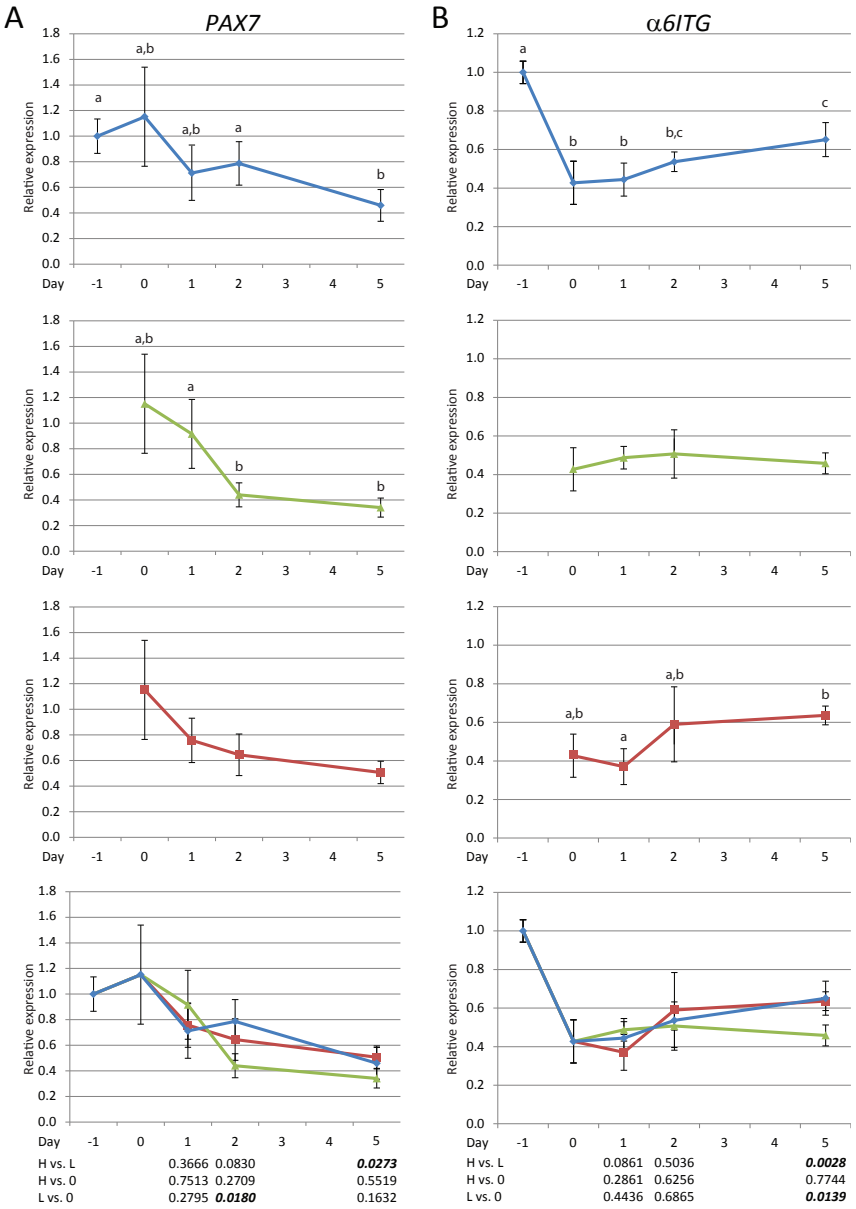

Figure 3.

Expression levels of *PAX7* and *α6ITG* during myogenesis. The expression levels of *PAX7* (A) and *α6ITG* (B) were determined at the indicated time points (days; x-axis) in porcine satellite cells during myogenesis in growth medium until day -1 (set at 1) followed by differentiation medium (blue line) or exposed to a low (green line) or a high (red line) concentration of DKK3 from day 0 onwards. Expression levels are mean values ± standard deviation. Different letters represent statistically different expression values ($p<0.05$). Significance values (p) of expression differences between culture conditions per time point (figures at the bottom are combined from the three figures above) are given below each graph, and significant ($p<0.05$) differences are indicated in bold and italic.

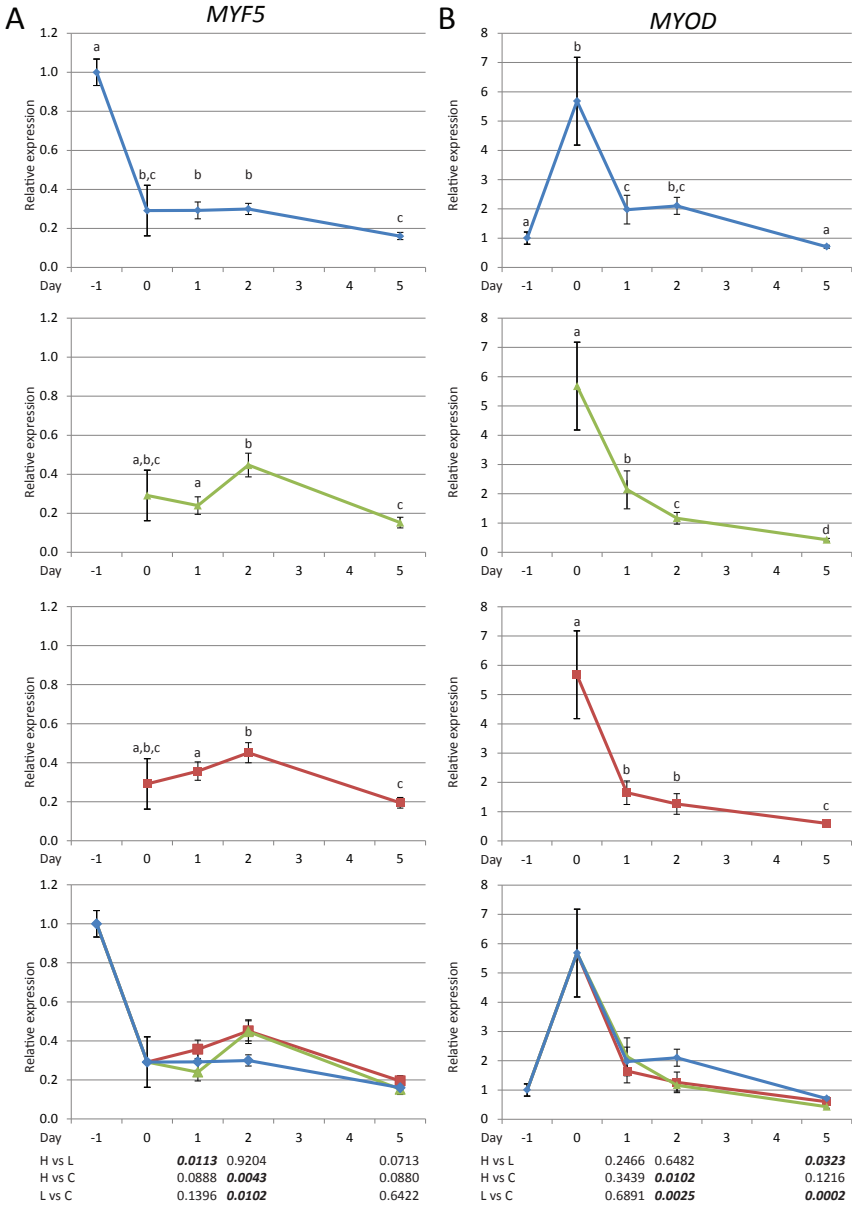

Figure 4.

Expression levels of *MYF5* and *MYOD* during myogenesis. The expression levels of *MYF5* (A) and *MYOD* (B) were determined at the indicated time points (days; x-axis) in porcine satellite cells during myogenesis in growth medium until day -1 (set at 1) followed by differentiation medium (blue line) or exposed to a low (green line) or a high (red line) concentration of DKK3 from day 0 onwards. Expression levels are mean values ± standard deviation. Different letters represent statistically different expression values ($p<0.05$). Significance values (p) of expression differences between culture conditions per time point (figures at the bottom are combined from the three figures above) are given below each graph, and significant ($p<0.05$) differences are indicated in bold and italic

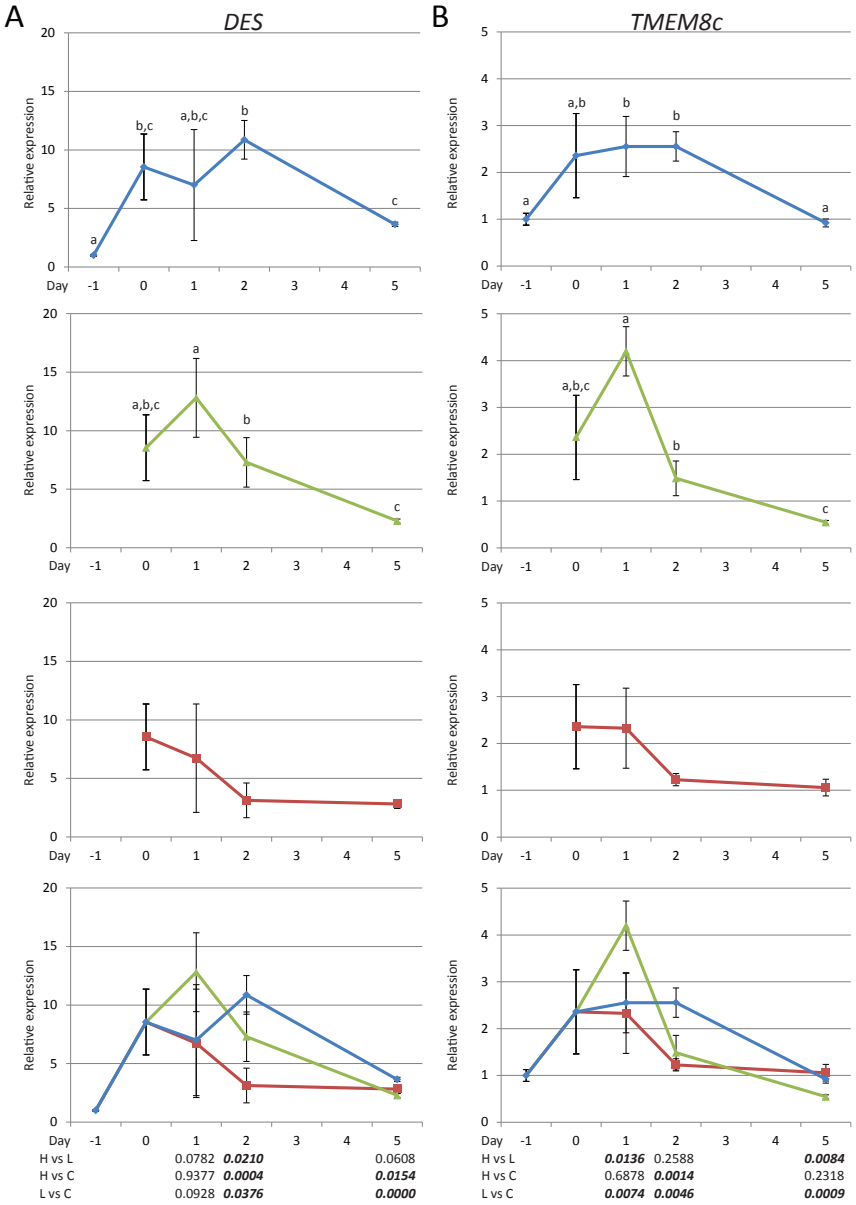

Figure 5.

Expression levels of *DES* and *TMEM8c* during myogenesis. The expression levels of *DES* (A) and *TMEM8c* (B) were determined at the indicated time points (days; x-axis) in porcine satellite cells during myogenesis in growth medium until day -1 (set at 1) followed by differentiation medium (blue line) or exposed to a low (green line) or a high (red line) concentration of DKK3 from day 0 onwards. Expression levels are mean values ± standard deviation. Different letters represent statistically different expression values ($p<0.05$). Significance values (p) of expression differences between culture conditions per time point (figures at the bottom are combined from the three figures above) are given below each graph, and significant ($p<0.05$) differences are indicated in bold and italic.

DISCUSSION

Muscle satellite cells can be kept in a proliferating state in 10% FBS, whereas differentiation is induced by reduced serum concentrations of bovine, horse or sheep origin (Doumit & Merkel 1992), or by the absence of serum (Mau et al. 2008). Blood serum contains undefined components including growth factors, and it has been reported that serum enhances adherence to ECM (Lawson & Purslow 2000). Not only can muscle satellite cells differentiate towards the myogenic lineage, differentiation towards adipogenic (Csete et al. 2001, Yue et al. 2010), osteogenic (Asakura et al. 2001, Huang et al. 2012) and neuronal (Redshaw & Loughna 2012) lineages have been reported. To accomplish non-myogenic differentiation additional factors are needed such as a combination of methyl-isobutylxanthine, dexamethasone, indomethacin and insulin, bone morphogenetic protein, low O_2 concentrations and γ-linolenic acid (Asakura et al. 2001, Wada et al. 2002). We did not include any of these factors, but serum contains unknown factors that could possibly contribute to differentiation into non-myogenic lineages. The morphology of the differentiating satellite cells presented here indicated myogenic differentiation by the fusion of myoblasts under all circumstances. Furthermore, the expression of *TMEM8c* was up-regulated in line with the observed myoblast fusion.

Postnatal satellite cells express *PAX7,* and this is essential since *PAX7* mutants lose their satellite cell pool, and, in contrast to what has been observed in embryonic muscle cells, this cannot be rescued by *PAX3* expression (Soleimani et al. 2012, Buckingham & Rigby 2014). *PAX7*-expressing quiescent satellite cells express *MYF5* and *MYOD* upon activation (Montarras et al. 2013), and *MYOD* expression is preceded by *MYF5* expression (Buckingham & Rigby 2014). *PAX7* expression is then down-regulated during differentiation (Seale et al. 2000) as observed in our cultures. This reduction was found in all cultures and was not related to DKK3 exposure. After 24 h in DM, *MYOD* expression increased indicating that differentiation towards myoblasts had been initiated. The rapid decrease in *MYOD* expression after another 24 h would suggest that *MYF5* and *MYOD* expression are only needed at the initiation of the differentiation process and not during the fusion process. Indeed, *MYOD* expression is reduced in adult muscle fibres (Voytik et al. 1993, Hughes et al. 1993).

Integrins interact with the ECM, and their expression depends on the type of ECM (Hynes 1992, Danen & Sonnenberg 2003, Barczyk et al. 2010). Integrins form heterodimers composed of an alpha and a beta unit and during *in vivo* myogenesis eight different alpha chains and two beta chains have been reported to be involved (Thorsteinsdóttir et al. 2011). The expression of α6ITG was found to be important for myogenic stem cell differentiation, although a possible beta chain associated with this alpha chain was not reported (Wilschut et al. 2011). Furthermore, the expression of

α6ITG could be increased by culturing porcine muscle stem cells on Matrigel (Wilschut et al. 2010). In the results presented here, the expression of *α6ITG* was reduced after initial differentiation, despite continued culture on Matrigel. Hereafter, *α6ITG* expression increased irrespective of the presence of DKK3. Therefore, this effect could be a belated effect of the Matrigel coating and is most likely not related to DKK3 exposure. Nevertheless, at day two, *MYF5* was found to be up-regulated 1.5-fold when DKK3 was present. Furthermore, the presence of DKK3 accelerated myotube formation.

The more than 3-fold reduction in *MYF5* expression within the initial differentiation and subsequent 3-fold reduction of *MYOD* immediately after this initial induction of differentiation suggest that the muscle stem cells were already forming myotubes when they were exposed to DKK3. Consequently, the effect of DKK3 might have been less pronounced, illustrated by the marginal up-regulation of *MYF5* after 48 h and no observable effect on myoblast fusion. It would therefore be interesting to differentiate porcine satellite cells in the immediate presence of DKK3 protein, and investigate its effects on gene expression and differentiation as a function of time from the start of differentiation.

MATERIALS AND METHODS

Satellite cell isolation

A post mortem muscle biopsy was taken from the hind leg of a three days old, female piglet and kept in DMEM (Life Technologies, Bleiswijk, The Netherlands) supplemented with 30% FBS (Life Technologies) and 1% penicillin-streptomycin (Life Technologies) during transport. All procedures were approved by the institutional animal ethics committee. Tissue was grinded and digested in pre-warmed 1 mg/ml pronase (Sigma Aldrich, Zwijndrecht, The Netherlands) in PBS (Braun, Melsungen, Germany) with 1% HEPES (Life Technologies) for 1 h at 37°C while shaking, followed by thorough trituration and centrifugation (2 min at 150*g*) to separate dissociated cells and undigested tissue. Dissociated cells were washed (2 min centrifugation at 150*g* at 4°C), and the supernatant, containing cells, filtered through a 70 μm cell strainer followed by a 40 μm cell strainer. This cell fraction was centrifuged (5 min at 1000*g* at 4°C), and the pellet kept on ice until further use. Undigested tissue was incubated in 0.10% w/v Collagenase XI (Sigma Aldrich) for 30 min at 37°C while shaking, followed by thorough trituration at 4°C. After centrifugation (2 min at 150*g* at 4°C) the supernatant containing dissociated cells was filtered through a 70 μm cell strainer followed by a 40 μm cell strainer. After centrifugation for a second time (5 min at 1000*g* at 4°C) and filtration through a 40 μm cell strainer, these cells were pooled with the pronase dissociated cell fraction. The isolated cells were resuspended in hypotonic buffer (0.2 M NH_4Cl,

13mM $KHCO_3$, pH 7.4) for 8 min on ice to shock the erythrocytes (Qu et al. 1998). After centrifugation (5 min at 1000*g* at 4°C), the cell pellet was resuspended at 1.0 x 10^6 cells/ml in DMEM-HG (Life Technologies) + 10% DMSO (Sigma Aldrich) + 40% FBS (Life Technologies), kept overnight in a Freezing container (Nalgene® Mr. Frosty; Sigma Aldrich) filled with 2-propanol (Sigma Aldrich) at -80°C before storage in liquid nitrogen.

Cell culture

Frozen cells were quick-thawed in a water bath at 37°C and transferred to DMEM-HG (Life Technologies) containing 40% FBS (Life Technologies) before centrifugation (5 min at 700*g*). The cell pellet was resuspended in growth medium [GM; DMEM-HG supplemented with 5 ng/ml recombinant human bFGF (Peprotech; Rocky Hill, NJ, USA), 10% FBS and 1% penicillin-streptomycin] and after plating were cultured for 1 h at 37°C and 5% CO_2 to remove fast-adhering fibroblasts, a technique known as preplating (Qu et al. 1998). Cells that had not adhered were transferred to 12-well plates coated with 1 mg/ml Matrigel (BD Bioscience, Bedford, MA, USA) in GM and allowed to proliferate to confluency. Subsequently, the GM was replaced by differentiation medium [DM; DMEM-HG, 2% horse serum (Invitrogen) and 1% penicillin-streptomycin]. After 24 h, DM was replaced by DM supplemented with 50 ng/ml (low concentration), 500 ng/ml (high concentration), or no (control) recombinant human DKK3 protein (R&D Systems, Minneapolis, MN, USA). This was considered as day 0. Then, media were refreshed every 24 h until day 5.

RNA isolation and cDNA synthesis

Cells were washed in PBS, lysed in RLT buffer (Qiagen, Venlo, The Netherlands), and stored at -80°C until further use. For RNA isolation and on column digestion an RNA micro kit (Qiagen) was used according to the manufacturer's protocol. Quality and quantity of total RNA was assessed using a Bioanalyzer 2100 and the RNA 6000 Pico LabChip kit (Agilent Technologies, Amstelveen, The Netherlands) according to manufacturer's instructions. RNA was stored at -80°C until further use. The iScript™ cDNA Synthesis Kit (Biorad, Veenendaal, The Netherlands) was used to convert RNA into cDNA according to manufacturer's protocol. cDNA was stored at -20°C until further use.

Quantitative reverse transcription PCR

New primer sets were designed using the primer3 based platform (Untergasser et al. 2012) Primer-Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast) (Ye et al. 2012). Specific *Sus scrofa* nucleotide sequences (Genbank; http://www.ncbi.nlm.nih.gov/nucleotide) were used as a template (Table 1). Further *in silico* validation by predicting amplicon folding structures was performed using the Mfold web server (SantaLucia 1998, Zuker 2003). Confirmation of primer specificity and establishment of an anneal-

Table 1. Details of primers used for quantitative gene expression measurement.

Gene	Discription	NCBI [1)]	Forward primer	Reverse primer	Ta (°C)
GAPDH [r]	glyceraldehyde-3-phosphate dehydrogenase	(Kuijk et al. 2007)	TCGGAGTGAACGGATTTG	CCTGGAAGATGGTGATGG	54
ACTB [r]	actin, beta	(Kuijk et al. 2007)	CATCACCATCGGCAACGAGC	TAGAGGTCCTTGCGGATGTC	56
UBC [r]	ubiquitin C	(Kuijk et al. 2007)	TTCGTGAAGACCTTGACTG	GGACTCCTTCTGGATGTTG	54
PGK1 [r]	phosphoglycerate kinase 1	(Kuijk et al. 2007)	AGATAACGAACAACCAGAGG	TGTCAGGCATAGGGATACC	56
PAX7	transcription factor pax-7	(Wilschut et al. 2008)	GGTGGGGTTTTCATCAATGG	GTCTCTTGGTAGCGGCAGAG	55
MYF5	myogenic factor 5	NM_001278775	ACGACTAACCCCAACCAGAG	GGCAACTGGAGAGAGAGAGGC	55
α6ITG	integrin, alpha 6	(Wilschut et al. 2010)	AAACGAGAAATTGCTGAAAGAC	CACTAGAATGATCCACCAAGG	54
DES	desmin	(Wilschut et al. 2010)	CCGAGATCTACGAGGAGGAG	TCTCGGATTCTTCTTTCAGC	56
MYOD	myogenic differentiation 1	(Blanton Jr. et al. 2000)	TCCGCGACGTAGATTTG	ACGGGAAGTGCGAGTGTT	54
TMEM8c	transmembrane protein 8C	XM_003353702	GACTTTCGTGATGTTTGGC	TTGGTCCTTCATCTGCTG	60

Gene [r] indicates a reference gene used for normalization. 1) Newly developed primers are designed using indicated NCBI accession number, otherwise original paper is referred to. Ta = annealing temperature.

ing temperature (Ta) was done by a temperature gradient ranging from 57°C - 65°C, using a four-time dilution series of cDNA from GM lysates. Quantitative reverse transcription PCR (qRT-PCR) was performed on a CFX detection system (Biorad) with a final primer concentration of 400 nM in 25 µl iQ SYBR Green supermix (Biorad) according to the manufacturer's protocol. Reactions were initiated by activating the enzyme 5 min at 95°C. Reactions proceeded with 40 cycles consisting of first a denaturing step at 95°C for 10 s followed by an annealing step at appropriate Ta for 10 s and finally a 30 s elongation step at 72°C. Hereafter, a dissociation curve was generated by increasing the temperature each step by 1°C for 15 s from 60°C - 98°C.

AUTHOR DISCLOSURE STATEMENT

All authors state no competing financial interests exist.

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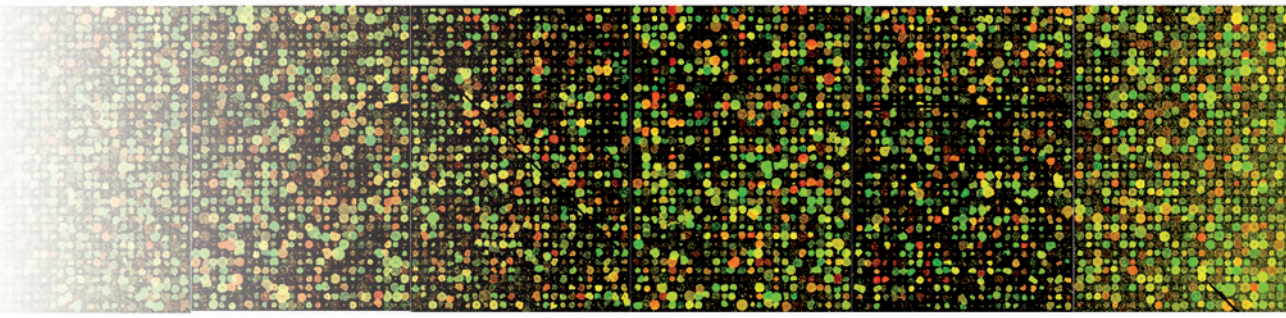

Chapter 6

General Discussion

During embryonic development a totipotent fertilised egg, the zygote, divides becoming a compacted morula stage embryo. In this stage the first lineage segregation is initiated resulting in the formation of a blastocyst stage embryo composed of an outer epithelial layer called the trophectoderm (TE) enclosing a fluid filled cavity, the blastocoel, and a polar group of cells, the inner cell mass (ICM). In the late blastocyst, cells in the ICM have undergone the second lineage segregation distinguishing the primitive endoderm (PE) cells from the pluripotent epiblast cells. The epiblast cells can subsequently differentiate into the three germ layers; ectoderm, endoderm and mesoderm, and germ cells. Of these different layers all cells found in the body are formed. The ICM cells can be cultured in a Petri-dish and kept pluripotent while proliferating when the appropriate culture medium is used. The ability to sustain a high self-renewing capacity without losing pluripotency is distinctive for these embryonic stem (ES) cells.

The generation of such cell lines from mouse (Evans & Kaufman 1981, Martin 1981) and human (Thomson et al. 1998) origin has advanced our knowledge of embryonic development. Most importantly, the pluripotency of these cells in combination with their susceptibility to homologous recombination has led to the possibility of targeted gene manipulation in mice. Human pluripotent stem cells can be useful for regenerative medicine, or in the near future more likely drug discovery and screening methods. After the generation of the first ES cell line from mouse embryos it was expected that similar cell lines would be generated from many mammalian species including livestock animals. The availability of ES cells from livestock species could aid in modelling human disease representing the human more accurate than with mouse ES cells (Whyte 2011), enhance pharmaceutical or drug production and pharmacokinetic studies and serve as a genetic engineering tool improving the generation of livestock harbouring genes more advantageous for economic and disease-resistant traits (Blomberg & Telugu 2012). Furthermore, theoretically they could serve as a more or less inexhaustible source for the production of organs such as liver and muscle tissue. This could serve in biomedicine and tissue engineering purposes like testing pharmaceuticals and an addition to existing edible protein sources; *in vitro* meat. Surprisingly however no bona fide ES cell lines from livestock species have been generated to date (Gandolfi et al. 2012).

There are several possibilities for the failure of generating stable ES cell lines from farm animal species. Possibly, blastocyst stage embryos from cattle or pigs do not contain pluripotent cells. As the totipotent zygote develops to a blastocyst stage embryo, epigenetic changes such as demethylation followed by *de novo* methylation take place preparing distinct cells to form the foetus. It is not known whether cells in blastocyst stage embryos of all mammalian species have already progressed to this stage and indeed are pluripotent, or that cells of some mammalian species acquire this characteristic only later during development. Clearly, at least in the mouse, epiblast

cells of late blastocyst stage embryos are pluripotent, and is it possible to 'capture' and maintain this pluripotency when cells are cultured *in vitro,* but whether the same would apply to animals with slightly different preimplantation embryological timing, such as developmental stage of implantation, is unknown.

Therefore, in **chapter 2** the transcriptomes of bovine morula and blastocyst-stage whole embryos were compared. In addition the expression profile of the ICM from which the foetus will develop was compared with that of the TE that will give rise to the embryonic part of the placenta. Hereto, the ICM was mechanically separated from the TE. Indeed, gene expression levels of key transcription factors (NANOG, SOX2, and OCT4) that are central in the pluripotency network in mouse and human were found to be expressed at higher levels in the ICM than in the TE from bovine blastocysts. Of these genes *SOX2* was expressed at higher levels in morulae compared to blastocysts and to ICM, indicating a decreasing expression during embryonic development. *POU5F1* coding for OCT4 was also expressed at higher levels in morula than in blastocyst and TE and equal to expression levels in the ICM. The expression level of *NANOG* was higher in blastocyst than in morula and predominantly found in the ICM. These results reflect the SOX2/OCT4-regulated *NANOG* expression in the ICM as has been established for mouse (Rodda et al. 2005). Importantly, these data demonstrate that the key genes involved in pluripotency are expressed in the bovine ICM. For the derivation of bovine ES cells the blastocyst stage is therefore suggested to be the appropriate stage, and it is inhibition of differentiation rather than induction of pluripotency that is currently lacking in cultured bovine ICM cultures for the derivation of stem cell lines.

By exposing embryos to the MAPK-inhibitor PD0325901 and increasing the percentage of cells expressing *NANOG* at the expense of *GATA6*-expressing cells, we expected to identify genes influenced by the shift in the *NANOG* : *GATA6* ratio. As described in **chapter 2**, *NANOG* levels increased upon MAPK inhibition, and so were *ZIC3* expression levels. The PE-specific genes *GATA6* and *PDGFRα* were down-regulated after PD0325901 treatment.

Another type of pluripotent cells can be generated from somatic tissues. By introducing four factors; OCT4, SOX2, c-MYC and KLF4 (also known as the Yamanaka factors), adult cells can be dedifferentiated to a pluripotent state exhibiting gene expression profiles, morphology and growth properties of ES cells (Takahashi & Yamanaka 2006, Takahashi et al. 2007). A drawback of the technique generating these so-called induced pluripotent stem (iPS) cells, is the use of viral vectors potentially causing insertional mutagenesis (Wu & Dunbar 2011), and moreover, c-MYC and KLF4 have oncogenic potential (Pelengaris et al. 2002, Vangapandu & Ai 2009) making iPS cells less suitable for human medicine, regenerative medicine or as a food source. Methods other than viral integration have been reported in the generation of iPS cells; however, with reduced reprogramming efficiency (reviewed in (O'Malley et al. 2009, Saha & Jaenisch 2009,

Stadtfeld & Hochedlinger 2010)). Somatic cells from farm animals have also been used to generate iPS cells, but these cell lines exhibited incomplete reprogramming illustrated by the necessity of maintaining expression of the exogenous factors and limited or absence of chimera contribution (reviewed in (Nowak-Imialek & Niemann 2013) and (Chronowska 2013)). Possibly other techniques using zinc finger nucleases (Beerli et al. 2000) combined with Cre recombinase treatment (Ramalingam et al. 2013) or using TALENs (Hockemeyer et al. 2011) could stably incorporate autologous Yamanaka factors in farm animal derived somatic cells establishing iPS cells from these species. Also the CRISPR/Cas9 technique is able to knock-in genes and helped establishing bovine iPS cells (Heo et al. 2015) and could also be used in embryonic gene targeting (Liang et al. 2015). In addition, the bovine or porcine iPS cells could be extremely useful in composing culture media that enable full reprogramming and maintenance of the iPS cells in absence of the exogenous factors. With a factorial design this could be done in a high throughput manner. The same media or media components that would allow culture of bovine or porcine iPS cells in the absence of exogenous factors could be used to maintain bovine or porcine ICM cells in a pluripotent state during culture after plating.

The difference between mouse and human ES cells is not only reflected in their origin, but, originally, also in their culture conditions, transcriptome and, in female cell lines, X-chromosome status. The original key for deriving mouse ES cells was culturing in the presence of leukaemia inhibitory factor (LIF). For the generation of mouse ES cells from formerly recalcitrant mouse strains, LIF is a prerequisite activating STAT signalling thereby inhibiting ES cell differentiation and promoting viability (Nichols & Smith 2009). In bovine embryogenesis both receptors GP130 and LIFR are expressed (Eckert & Niemann 1998), although *in vitro* no effect is observed after media supplementation with bovine LIF (Yamanaka et al. 1999, Yamanaka et al. 2001), and the use of human or mouse LIF has no or adverse effects on the ICM (Rodríguez et al. 2007). For the successful generation of bovine ES cells other signals activating STAT3, such as the interleukin oncostatin M (Kakutani et al. 2015), or inhibiting pathways reducing *STAT3* expression might be needed. SOCS3 and PIAS3 are known to inhibit JAKs and STAT3, respectively. Peptide or antibody mediated inhibition of these genes could possibly enhance STAT3 activation, and Genistein is capable of enhancing BMP and STAT3 signalling in HepG2 cells (Zhen et al. 2013).

To generate and maintain mouse ES cells inhibition of the FGF – MAPK pathway and GSK3β in the WNT pathway is sufficient (Ying et al. 2008). This dual inhibition (2i) is insufficient for human ES cell generation and propagation (Van Der Jeught et al. 2013) and in porcine putative iPS cells even reduces the expression of pluripotency-related genes (Petkov et al. 2014) although porcine iPS cells can be generated by 2i and LIF in combination with ectopic expression of Yamanaka factors (Zhang 2014). Bovine ICMs show

increased expression of *NANOG* and *SOX2* after blastocyst culture in 2i-supplemented medium (Harris et al. 2013) although propagation beyond 8 passages and chimera contribution could not be confirmed (Verma et al. 2013). Increased *NANOG* expression in bovine ICM is possibly a result of only MAPK inhibition and not by GSK3β inhibition (Kuijk et al. 2012). For mouse ES cells WNT signalling is essential for self-renewal, it inhibits differentiation into EpiSCs and together with LIF is sufficient to support and derive mouse ES cell lines (Ten Berge et al. 2011). Culturing bovine blastocysts on a MEF feeder layer in the presence of WNT3A and LIF resulted in colony formation, but these colonies could not be propagated beyond 3 passages (Figure 1). These results further indicate WNT signalling in bovine pluripotency might be dispensable.

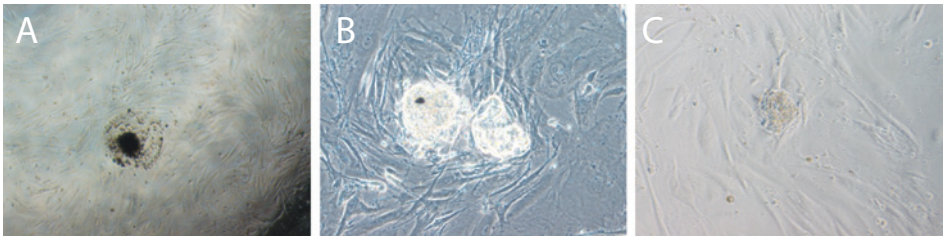

Figure 1.
Bovine embryo culture in WNT3A and LIF. Bovine zona pellucida-free blastocysts were cultured for 6 days (p0, A), and after a 5 min 0.25% trypsin-EDTA treatment at room temperature, the ICM was partially dissociated and cultured for an additional 6 days (p1, B). This procedure was repeated after again 6 days in culture (p2, C). After the third passage no colonies remained. All culturing was performed in the presence of mouse WNT3A and LIF protein on MEF feeder layers. Pictures are representative for each stage.

Culturing human ES cells under 2i conditions, surprisingly, results in differentiation or cell death (Nichols & Smith 2009). The original culture media keeping human ES cells proliferative and self-renewing required activin/nodal and FGF signalling (Vallier et al. 2005). Interestingly, these exact culture conditions could also be applied to epiblast cells isolated from the mouse postimplantation embryo referred to as EpiSCs (Tesar et al. 2007, Brons et al. 2007). EpiSCs further resemble human ES cells in morphology, transcriptome and X-chromosome status in female cells. A failure to contribute to chimera formation defines the EpiSCs, and thereby also human ES cells, as primed, as opposed to the naïve status of mouse ES cells (Nichols & Smith 2009). Recent reports show the use of small molecule inhibitors in addition to 2i facilitated the generation of naïve human ES cells (Chan et al. 2013, Gafni et al. 2013, Takashima et al. 2014, Ware et al. 2014, Theunissen et al. 2014, Van Der Jeught et al. 2015, Chen et al. 2015).

The use of a culture medium containing 2i and additional growth factors and inhibitors was able to rewire the primed status of human ES cells and mouse EpiSCs towards

a status similar to the naïve mouse ES cells. Moreover, also the direct generation of naïve ES cell lines from human blastocysts was successful using this naïve human stem cell medium (NHSM). In **chapter 3**, we describe the formation of bovine embryos in NHSM and investigated the transcriptome of ICMs dissected from blastocysts either cultured in standard SOF or in NHSM.

The conditions of NHSM were used to culture whole blastocysts and dissected ICMs in an attempt to generate ES cells from bovine *in vitro* embryos and porcine *in vivo* embryos. When porcine *in vivo* day 6 and day 8 whole blastocysts or their dissected ICMs were cultured on MEF feeder layers, they disappointingly did not show any proliferative capacity over a maximum period of 14 days. Bovine ICMs and intact blastocysts were grown on vitronectin and were passaged after 6 days using dispase (Figure 2A). After 2 days only one ICM attached (Figure 2B) and showed no further proliferative capacity at day 5 after passage (Figure 2C). These results indicate that NHSM cannot keep bovine or porcine ICMs pluripotent whether derived from *in vitro* or *in vivo* blastocysts. Nevertheless, according to the transcriptomes, NHSM is able to delay bovine differentiation.

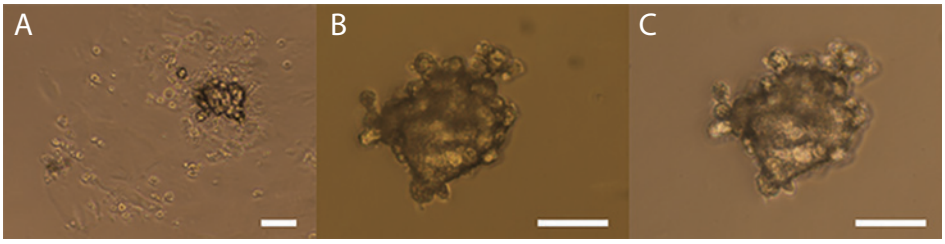

Figure 2.
Bovine ICM culture in NHSM. Bovine blastocyst was cultured on vitronectin in NHSM for 6 days (A) when ICM was passaged by dispase dissociation (=p1). Although the ICM had attached after 2 days (B), no proliferation occurred at day 6 after p1 (C). White bars indicate relative size.

To generate the transcriptomes in **chapters 2 and 3**, microarray analysis has been performed. Since the first publication describing the use of microarrays (Schena et al. 1995), this technique has become the technology of choice in large scale transcriptome studies (Zhao et al. 2014). For microarray analysis, a reference transcriptome is a prerequisite (Raz et al. 2011), and high amounts of input mRNA are needed. When mRNA is converted to cDNA and not hybridized to immobilized oligos as in microarray studies, but directly sequenced, less starting material is needed and no prior knowledge about probe selection is needed. This technique known as RNA-Seq (Nagalakshmi et al. 2008) is becoming more widely used and has strong potential to replace microarrays for whole-genome gene expression studies (Zhao et al. 2014) (Figure 3). Comparative studies indicate high correlation between these techniques

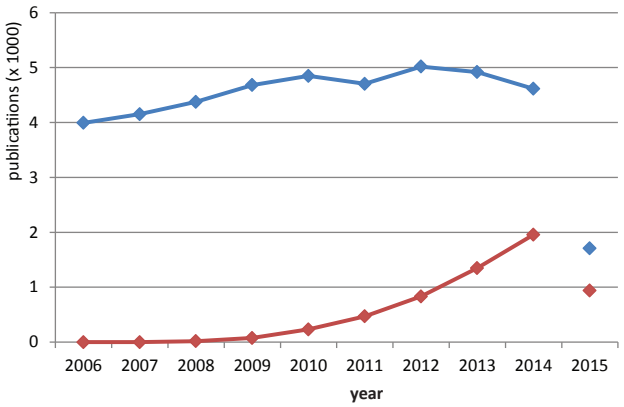

Figure 3.
Microarray and RNA-Seq reports. A literature survey using either "microarray" (blue) or "RNA-Seq" (red) as query from January 2006 until June 2015.

although RNA-Seq becomes more favourable because of high reproducibility, accuracy and dynamic range (Kogenaru et al. 2012). Still, microarray technology remains more popular, possibly because it is readily affordable by many laboratories (Zhao et al. 2014). A disadvantage of microarray analysis and RNA-Seq was the quantity of RNA needed for adequate measurements. Very recently however, RNA sequencing at the single cell level has become available (Yan et al. 2013, Streets et al. 2014). It would be very interesting to make a detailed comparison of the transcriptomes of individual mouse, human and cattle embryonic cells during early embryonic development. These techniques are now becoming available.

The obtained transcriptomes described in chapters 2 and 3 together with literature identified several genes discriminating embryonic cell types or progression through pluripotency. In **chapter 4** it is described if other reported "naïve" conditions could establish a pluripotent character in bovine embryos. Therefore, a selection of these marker-genes has been used to determine expression levels in blastocysts cultured in some of these media. According to a down-regulation of selected differentiation markers these "naïve" media could delay lineage commitment in bovine embryos. Nevertheless, markers for naïve pluripotency were also down-regulated. The expression levels of these genes in human blastocysts after culture in any of these "naïve" media are unknown, making a good comparison difficult. Cell culturing of dissected ICMs from bovine blastocysts generated by culture in any of these naïve media would make a comparison with human naïve stem cells possible. Deep sequencing and epigenome characterisation should be able to reveal how to maintain pluripotency in these cells.

For the generation of the transcriptome landscapes of bovine origin, described in **chapters 2, 3 and 4**, blastocysts were *in vitro* cultured from *in vitro* matured and fer-

tilised oocytes. Several differences are reported between *in vivo* and *in vitro* derived embryos, not only in bovine. Sex ratio differences are reported between *in vivo* and *in vitro* derived mouse preimplantation embryos (Peippo & Bredbacka 1995), *in vitro* produced bovine blastocysts have demonstrated reduced quality indicated by lower survival rates after vitrification than their *in vivo* counterparts (Rizos et al. 2002), and gene expression profiles were found to differ (Wrenzycki et al. 2001, Rizos et al. 2003, Kues et al. 2008, Bauer et al. 2010). Gene expression differences have also been reported in early passages of mouse ES cells derived from *in vivo* or *in vitro* developed blastocysts (Horii et al. 2010). Despite the differences, both *in vivo* and *in vitro* derived embryos are developmentally competent (Otoi et al. 1996, Macháty et al. 1998) indicating their pluripotent capacity. Furthermore, human ES cell lines are exclusively derived from *in vitro* cultured embryos indicating that the *in vitro* culture per se does not exclude ES cell derivation. Culturing human embryos in any of the naïve media (Gafni et al. 2013, Chan et al. 2013, Ware et al. 2014, Takashima et al. 2014, Theunissen et al. 2014) and comparing their genome-wide transcriptomes with those of embryos cultured without inhibitors could be very informative about pathways influenced by the inhibitors. This might provide new insights in pluripotency, which could be helpful for the generation of ES cells from other species. The transcriptomes generated and described in this thesis further indicate that differentiation can be delayed in bovine preimplantation embryos and their cell types by media containing small molecules promoting self-renewal and a naïve pluripotent character in human ES cell derivation. Each transcriptome defines the mRNA levels present in a population of cells, averaging the transcriptomes of individual cells. RNA sequencing enables the genome-wide analysis of transcripts isolated from one individual cell (scRNA-seq) (Nawy 2014). This technique could provide information about onset of differentiation events in preimplantation embryos (Ohnishi et al. 2014) or discriminate between cells susceptible for inhibiting or enhancing molecules. Together, this might indicate the appropriate cell (or cells) to select for in ES cell derivation. More information can be obtained from epigenomics and proteomics, since they influence or are influenced by the transcriptome.

The efficient engineering of muscle tissue could benefit regenerative medicine and, if in farm animals, establish a completely novel method of meat production, known as *in vitro* meat. In **chapter 5** we describe the function of Dickkopf 3 (DKK3) protein in myotube formation from porcine satellite cells. We observed improved myotube formation by earlier myoblast fusion and larger diameter of myotubes. It has been reported that DKK3 activates the *MYF5* promoter after binding to the α6 integrin (α6ITG) receptor, activating phosphorylation of p38, mediating the formation of a SMAD2/3/4-complex (Hsu et al. 2010, Hsu et al. 2011, Fu et al. 2012). The small increase in *MYF5* expression could be related to the exposure of the activated cells to DKK3 after 24 h in differentiation medium. Possibly the cells were already beyond initiation of activation, and when

cells are exposed to DKK3 from the moment differentiation is induced the satellite cells are more susceptible for activation of *MYF5*. Furthermore, muscle tissue contains a heterogeneous population of stem cells of which not all express *α6ITG*. Since DKK3 is thought to act through binding this receptor for activation of *MYF5* transcription, selection or enrichment of α6ITG expressing satellite cells, e.g. by FACS sorting, could help establish DKK3 function in myogenesis. The satellite cells were grown in wells coated with Matrigel, demonstrated to induce *α6ITG* (Wilschut et al. 2010). More extra cellular matrix (ECM) types bind integrins such as fibronectin, laminin, vitronectin and collagens (Hynes 1992), but they control cell growth differently exemplified by fibronectin keeping myoblasts proliferating while laminin initiates the formation of myotubes (Adams & Watt 1993). Culturing with different ECMs would probably obtain different *MYF5* expression levels and result in differences in myotube formation.

The success of *in vitro* meat depends on feasibility, cost, quality and possibilities of scaling-up. First and foremost, to obtain such a product that could add to existing meat sources the use of cells able to proliferate infinitely and with the potential to differentiate into muscle is a prerequisite. Adult stem cells such as muscle satellite cells can readily differentiate into muscle, but only have a limited proliferation potential, making harvesting new muscle biopsies at a regular frequency a necessity. Probably developmental progression and efficiency need improvement, and also quality is inextricably linked to the success of *in vitro* meat. Embryonic stem cells from farm animals such as cattle and pigs could be the source of cells capable of infinite proliferation and the potency to participate in myogenesis. Mouse and human pluripotent cells have been reported to be able to contribute to myogenic development (reviewed in (Salani et al. 2012)). Establishment of ES cell lines from farm animal species also efficiently differentiating into muscle with good quality and in high quantities could have *in vitro* meat ending up in supermarkets as a sustainable product.

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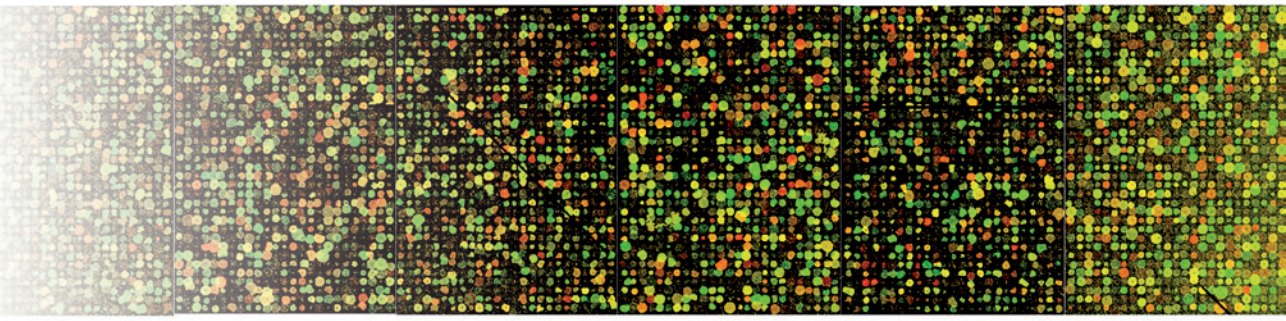


Chapter 7

Summary in English

In mammals, successful fertilisation of an oocyte by a sperm cell results in the formation of a totipotent zygote. This single cell embryo has the potency to develop into any of the tissues, both embryonic and extraembryonic, that compose the species. To accomplish this, a zygote undergoes cleavage divisions until a morula stage embryo is formed, and the morula will further develop into a blastocyst stage embryo. The blastocyst is composed of an outer layer of cells, the trophectoderm (TE) enclosing a fluid filled cavity, the blastocoel, and a group of cells referred to as the inner cell mass (ICM). This ICM further differentiates into primitive endoderm (PE or hypoblast) and epiblast. Because of their capacity to contribute to the formation of all tissues found in an organism, the ICM and epiblast are referred to as being pluripotent, as opposed to the TE and PE forming the extraembryonic tissues like placenta and yolk sac. When the pluripotent cells are cultured in a Petri-dish in the appropriate medium, differentiation can be inhibited, and the cells can proliferate without losing their pluripotency (self-renewal). The derivation of such embryonic stem (ES) cells was successful for the first time in 1981 using ICM cells from mouse blastocysts. Later, ES cells have been derived from monkey, human and rat blastocysts. Interestingly, the culture conditions for the generation of ES cell lines are different for the cells of different species. Even more surprisingly, the generation of ES cell lines from other mammals such as cattle and pig has not been accomplished to date.

ES cells are characterised by expression of the core transcription factors for pluripotency NANOG, SOX2 and OCT4. Two distinct states of pluripotency can be recognised in ES cells; naïve and primed. Naïvety is characterised by the efficient contribution to chimera formation and the expression of specific early embryonic markers. Primed ES cells have very low capacity of chimera contribution and express markers of lineage specification. Furthermore, to retain the specific pluripotent state, culture conditions are different. Cells from male embryos contain an X-chromosome and a Y-chromosome whereas female embryonic cells have two X-chromosomes. To avoid double transcription of X-linked genes, the paternally inherited X-chromosome is inactivated during early cell divisions in female embryos. In ICM cells from female embryos this is reversed, and random X-chromosome inactivation will take place. Naïve female ES cells show no X-chromosome inactivation whereas primed female ES cells do.

The availability of ES cells from pigs and cattle could help improving traits by genetic modifications and could improve general knowledge of mammalian embryogenesis. Despite several attempts, no authentic ES cells of these species have been derived showing similar gene expression profiles or developmental potency like in ES cells from mouse or human origin. This thesis describes the research of gene expression profiles from different embryonic stages and cell types to identify genes involved in pluripotency or differentiation in bovine or porcine cells. The results are compared with data from other mammals.

At about the morula stage, the first differentiation is initiated resulting in the formation of a blastocyst. To identify genes involved in this process, gene expression profiles from morula and blastocyst stages were generated and compared by means of microarray analysis (**chapter 2**). The blastocyst is composed of the TE and pluripotent ICM. By comparing the transcriptomes of the TE and ICM, genes were detected that were expressed at higher levels in the ICM than in the TE and vice versa. In particular, genes involved in RNA splicing and gene expression regulation showed higher expression levels in the ICM than in TE. NANOG, SOX2 and OCT4, coded by *NANOG*, *SOX2* and *POU5F1* respectively, are transcription factors that are central in the pluripotency network. Expression levels of these genes were higher in the ICM than in the TE of bovine embryos. Genes involved in human ES cell maintenance like *STAT3* and *ZIC3* were also expressed at higher levels in the bovine ICM. Previously, it has been described that in bovine embryos inhibition of MAPK activation results in a larger percentage of ICM cells expressing NANOG. Therefore, embryos were cultured in the presence of a MAPK inhibitor (PD0325901), and indeed the embryos showed elevated *NANOG* levels compared with ICM cells from control embryos. Surprisingly, expression levels of interferon coding genes were dramatically down-regulated in the ICM from MAPK inhibitor treated blastocysts. A function of interferons in the ICM and a possible relation to pluripotency remains unknown. Interestingly, expression levels of *SOX2* and *POU5F1* were not increased in the ICM of PD0325901 treated blastocysts. Other genes, known to be expressed in naïve ESC, like *PRDM14* and *ZIC3* showed higher expression levels in ICM than in TE, and these levels could only be increased for *ZIC3* after enhancing *NANOG* transcription levels. A gene related to primed pluripotent cells is *OTX2*, and its transcripts are present in the ICM, but the transcription levels were not reduced after MAPK inhibition. It is concluded that the transcriptome of the ICM from bovine embryos indicates a pluripotent cell population which cannot be maintained by MAPK inhibition alone.

It is not known whether the successful generation of bovine ES cells depends on the pluripotent state (naïve or primed) of the cells present in preimplantation embryos. In contrast to mouse ES cells, human ES cells exhibit a primed character and culture conditions are different from the conditions used for mouse ES cells. In 2013, a method was published using a medium capable of reversing primed human ES cells to a naïve state. Furthermore, this medium allowed the derivation of naïve ES cells from human blastocysts. This naïve human stem cell medium (NHSM) has also been successful for the culture of mouse naïve ES cell lines. To further determine which genes are involved in bovine pluripotency, bovine embryos were cultured in NHSM, and the transcription profile of the ICM was compared with the gene expression profile of ICM cells from blastocysts cultured in standard medium (SOF). As described in **chapter 3**, most NHSM cultured bovine blastocysts were no longer enclosed by their zona pellucidae and

ascended to just below the liquid level. Also, these embryos appeared darker than their SOF cultured counterparts possibly caused by the presence of lipid droplets in both the ICM and the TE, whereas in the SOF cultured embryos lipid droplets were only present in the ICM. Microarray analysis resulted in a list of 641 unique gene entrees differentially expressed between ICM cells isolated from NHSM- and SOF-cultured embryos. Remarkably, there was an over-representation of differentially expressed genes located on chromosomes 8 and X. Most genes differentially expressed on chromosome 8 encode for interferons as has been described in chapter 2. The X-chromosome is of particular interest since in female cells two active copies of the X-chromosome indicate a naïve state while X-chromosome inactivation indicates a primed state. Interestingly, expression levels of *XIST*, a long noncoding RNA directing X-chromosome inactivation, were reduced in ICM cells from bovine blastocysts cultured in NHSM. Furthermore, the expression level of *HPRT1*, also located on the X-chromosome, was elevated in these ICM cells. These data suggest that one of the X-chromosomes in these cells had not been (completely) inactivated in contrast to the inactivated state in ICM cells from SOF-cultured embryos. The expression levels of *NANOG*, *SOX2* and *POU5F1* were not changed in ICM cells from bovine blastocysts cultured in NHSM. This was surprising since *NANOG* expression levels increased when human ES cells were cultured in NHSM. This medium also contains the MAPK-inhibitor PD0325901, and therefore an enhanced *NANOG* expression level was expected in bovine ICM cells from NHSM cultured blastocysts. Other genes, associated with naïvety (*ESRRB* and *TBX3*), epiblast specification (*KLF4*) or differentiation (*OTX2*) showed no differential gene expression. Cell culture of isolated bovine ICM cells in NHSM did not result in establishment of a bovine embryonic cell line. Combined, the results suggest that NHSM is capable of delaying X-chromosome inactivation, but that other factors are needed to capture and maintain pluripotency of bovine cells.

Shortly after the publication of using NHSM to generate human naïve pluripotent stem cells, similar culture media with different composition have been published. All these media contained inhibitors of several differentiation pathways. By culturing bovine embryos in these various media and analysing gene expression patterns, pathways important in bovine pluripotency might be indicated. Expression levels of selected genes specific for embryonic cell type (TE, PE or epiblast), pluripotency and the pluripotent state (naïve or primed) were determined (**chapter 4**) using qRT-PCR. NHSM cultured bovine embryos were darker and ascended to the liquid level as described in chapter 3. Bovine embryos obtained from a culture medium with five inhibitors, LIF and Activin (5i/L/A) contained more cells than the NHSM-cultured embryos. Embryos from a medium with three inhibitors and LIF (3iL) showed no morphological differences compared with embryos cultured in the standard medium (SOF). Expression levels of genes indicative for TE (*CDX2*) and epiblast (*FGF4* and *KLF4*) were similar between

all blastocysts whereas expression levels of genes indicating PE fate (*GATA6*, *HNF4a*, *FGFR2*, and *PDGFRa*) were all down-regulated in the "naïve" media compared to the embryos cultured in SOF. The expression levels of pluripotency network genes showed a reduced expression for *POU5F1* in embryos cultured in NHSM and 3iL whereas *SOX2* and *NANOG* expression levels were similar in all the media. Gene expression levels of genes involved in naïve pluripotency were not differentially expressed (*PRDM14*, *TBX3*, and *ZFP42*) or were down-regulated (*ESRRB*) in the blastocysts from the "naïve" media. Also, the early differentiation marker *OTX2* showed a reduced expression level in the "naïve" media compared to SOF-cultured bovine blastocysts. In general, in embryos cultured in the different "naïve" media, the expression levels of *XIST* were reduced, but the expression levels of *HPRT1* were not significantly different from embryos cultured in SOF. These data suggest that bovine embryos cultured in any of the "naïve" media contain more pluripotent cells that are in an intermediate state between naïve and primed.

For the characterisation of the gene expression profiles large quantities of RNA are needed. To obtain sufficient amounts of RNA, *in vitro* produced bovine embryos were used for the transcriptome analysis described in chapters 2, 3, and 4. It is known that the transcriptome from *in vitro* derived bovine blastocysts differs from that of *in vivo* blastocysts. Nevertheless, *in vitro* embryos can still develop into healthy calves when transferred to a pseudopregnant cow. Therefore, these *in vitro* bovine embryos contain sufficient pluripotent cells that enable the determination of genes important for bovine pluripotency.

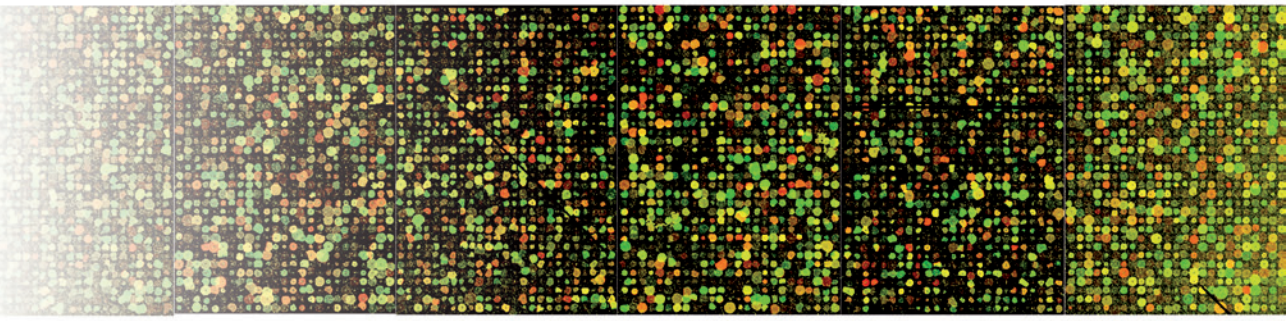
To gain as much information as possible about expression levels in the various embryonic cell types we used microarray analysis. This technique has been widely used since the '90s. In the last decade other techniques have however been described facilitating more detailed and complete transcriptome analysis, even single cell sequencing.

Obviously, for the generation of ES cell lines pluripotent cells need to be inhibited to differentiate. MAPK-inhibition was insufficient, and also exposure to several other inhibitors present in NHSM, 5i/L/A or 3iL could not maintain pluripotency of bovine embryonic cells. The lack of authentic bovine ES cells merits the investigation of the transcriptome of embryonic cells from mouse or human embryos produced in any of the "naïve" media, and, together with the gene expression profiles from mouse and human ES cells, compare them with the transcription profiles described in this thesis.

ES cells from livestock species could also serve as a source for edible proteins such as cultured meat (*in vitro* meat). The growing world population and the demand for meat come at the cost of living area and the environment caused by (an increasing) livestock. This merits the investigation of alternatives for regular meat sources. Apart for the development of *in vitro* meat, also for the improvement of meat quality from farm animal species, knowledge about muscle development (myogenesis) needs to

be improved. It has been established that almost all adult tissues contain stem cells. At injury these cells can develop new tissue cells to regenerate damaged organs and tissues. In muscle a specific groups of cells called satellite cells can function as muscle stem cells. In **chapter 5** the differentiation of porcine satellite cells in the presence of a Dickkopf protein (DKK3) is described. Research with zebrafish suggested that an interaction of DKK3 with α6 integrin (α6ITG) enhances *MYF5* expression. Together with MYOD, MYF5 initiates myogenic differentiation. When porcine satellite cells were cultured in differentiation medium with DKK3 indeed *MYF5* transcription levels initially increased, only to decrease again after 5 days. During myogenic differentiation individual cells will fuse and thereby form myotubes. These myotubes appeared during culturing in differentiation medium and were observed earlier and had a larger diameter in the presence of DKK3 compared to the culture conditions without DKK3. During these differentiation and fusion processes several genes were expressed including respectively desmin (*DES*) and *TMEM8c* (also known as myomaker). Expression levels of these genes were highest shortly after combined expression of *MYF5* and *MYOD* followed by the initiation of fusion. The expression levels of *DES* and *TMEM8c* were very similar when compared between the culture conditions. Possibly, the expression levels of these genes only peak at a short time span, not to be detected within the 24 h interval used here. Alternatively, the fusion process had already started before DKK3 exposure. Future experiments should include culture of porcine satellite cells in the presence of DKK3 protein from the start of differentiation.

Mouse and human pluripotent stem cells can efficiently differentiate to muscle cells. Nevertheless, meat from these species as a food source will not be a commercially successful product for obvious reasons. Therefore, the derivation of pluripotent stem cells from farm animal species such as cattle and pigs capable of self-renewal and efficient differentiation to skeletal muscle tissue could lead to a sustainable additive to existing meat products in supermarkets. The results described in this thesis can possibly aid to that goal.



Chapter 8

Summary in Dutch

Het resultaat van een succesvolle eicelbevruchting bij zoogdieren is de vorming van een totipotente zygote. Vanuit dit eencellig embryo worden alle weefsels gevormd die nodig zijn om een organisme te vormen: zowel de embryonale weefsels als de extra-embryonale weefsels. Hiervoor klieft een zygote enkele malen tot een morula stadium en zal er vervolgens een blastocyst gevormd worden. Dit embryo bestaat uit een buitenste laag cellen, het trophectoderm (TE), met daarin een met vloeistof gevulde holte, de blastocoel en een groep cellen die de *inner cell mass* wordt genoemd. Deze inner cell mass (ICM) specialiseert (differentieert) zich weer verder in primitief endoderm (PE of hypoblast) en epiblast. De cellen van de epiblast kunnen nog bijdragen aan alle celtypen en weefsels in een organisme, terwijl het TE en het PE alle extra-embryonale cellen en weefsels vormen, zoals een deel van de placenta en de dooierzak. Vanwege hun vele mogelijkheden in de ontwikkeling worden de ICM en de epiblastcellen pluripotent genoemd. Als deze cellen in een kweekschaaltje met de juiste voedingsmiddelen worden gekweekt, kan de differentiatie geremd worden en kunnen deze cellen blijven delen zonder dat ze pluripotentie verliezen (*self-renewal*). Deze cellen worden embryonale stamcellen (ESC) genoemd en de eerste ESC werden van de muis gegenereerd in 1981. Later is dit ook gelukt met ICM-cellen uit blastocysten van aap, mens en rat. De kweekomstandigheden voor deze verschillende ESC zijn echter verschillend en ook is het tot op heden niet gelukt om zulke cellijnen van andere zoogdieren, zoals runderen en varkens, te verkrijgen, wat de complexiteit hiervan aangeeft.

ESC moeten onder andere de transcriptiefactoren NANOG, SOX2 en OCT4 tot expressie brengen. Twee verschillende stadia van pluripotency kunnen onderscheiden worden bij ESC; naïef en *primed*. Naiviteit kenmerkt zich door de efficiënte bijdrage aan de vorming van chimeren en de expressie van specifieke vroeg embryonale markers. Primed ESC zijn nauwelijks in staat bij te dragen aan de vorming van chimeren en expresseren markers voor cellijn differentiatie. Daarnaast zijn ook de kweekomstandigheden om de pluripotentie te behouden verschillend. Cellen van mannelijke embryo's hebben een Y-chromosoom en een X-chromosoom, terwijl vrouwelijke embryocellen twee X-chromosomen hebben. Om te voorkomen dat de genetische informatie op het X-chromosoom in vrouwelijke cellen dubbel afgeschreven wordt, zal het paternale X-chromosoom geïnactiveerd worden tijdens de vroege celdelingen. In de ICM-cellen van de blastocyst wordt dit weer teniet gedaan, waarna random inactivatie van een X-chromosoom zal plaats vinden. In naïeve vrouwelijke ESC heeft deze random inactivatie nog niet plaats gevonden en in primed cellen wel.

ESC van varkens en runderen zouden kunnen helpen om soorten te verbeteren door genetische modificatie en onze kennis over embryonale ontwikkeling bij zoogdieren kunnen vergroten. Ondanks meerdere pogingen zijn er geen authentieke ESC van deze soorten die een genexpressiepatroon hebben vergelijkbaar met ESC van muis of mens

of dezelfde ontwikkelingspotentie hebben als ESC van muis of mens. In dit proefschrift staat het onderzoek beschreven naar de genexpressieprofielen in verschillende embryonale stadia en celtypen met als doel genen te identificeren welke betrokken zijn bij pluripotentie dan wel differentiatie in het rund en het varken. De resultaten worden vergeleken met gegevens van ander zoogdieren.

Rond het morula-stadium vindt de eerste differentiatie plaats wat resulteert in de vorming van een blastocyst. Om te bepalen welke genen hier een rol bij spelen zijn de genexpressieprofielen van deze verschillende embryonale stadia van het rund met elkaar vergeleken d.m.v. *microarray* analyse (**hoofdstuk 2**). De blastocyst bestaat uit het TE en de pluripotente ICM. Door het transcriptoom van de ICM met dat van het TE te vergelijken hebben we genen kunnen identificeren welke meer tot expressie komen in de ICM dan in het TE en andersom. Deze genen spelen dus een rol bij specifieke processen in deze celtypen. Zo zijn er veel genen betrokken bij RNA *splicing* en bij het reguleren van genexpressie in de ICM. De transcriptiefactoren NANOG, SOX2 en OCT4 hebben een sleutelrol in pluripotentie bij de muis en de mens. De genen die hiervoor coderen (respectievelijk *NANOG*, *SOX2* en *POU5F1*) kwamen op een hoger niveau tot expressie in de ICM dan in het TE van runderblastocysten. Ook de genen *STAT3* en *ZIC3*, welke een rol spelen in het behoud van pluripotentie in humane ESC, kwamen hoger tot expressie in de runder-ICM. Er is eerder beschreven dat door de kinase-activiteit van MAPK te remmen het aantal cellen dat in de ICM NANOG tot expressie brengt, verhoogd kan worden. Hierdoor zullen er ook meer cellen zijn waarin het effect van NANOG op de genexpressie te meten is. Het transcriptiepatroon van ICM-cellen die geïsoleerd waren uit runderblastocysten gekweekt met een remmer van MAPK (PD0325901) vertoonde inderdaad een hogere expressie van *NANOG* dan van de ICM uit blastocysten gekweekt zonder deze remmer. Verrassend was de verlaagde expressie van interferon-coderende genen in de ICM. Van deze genen is geen functie bekend in de ICM en dus ook niet in relatie tot pluripotentie. Zeer interessant was dat de expressieniveaus van *SOX2* en *POU5F1* niet verhoogd waren in de ICM van MAPK-geremde blastocysten. Bepaalde genen die tot expressie komen in naïeve ESC, zoals *PRDM14* en *ZIC3* hadden hogere expressieniveaus in de ICM dan in het TE, maar deze expressie kon alleen verder verhoogd worden voor *ZIC3* als gevolg van een verhoging van *NANOG* transcriptieniveaus. Een gen dat als een van de eersten tot expressie komt in primed cellen is *OTX2*. Dit gen komt ook hoger tot expressie in de ICM, maar dit expressieniveau kon niet worden verlaagd door remming van MAPK activatie. Er is geconcludeerd dat het transcriptoom van de ICM duidt op een pluripotente cel populatie, maar de remming van MAPK activatie is onvoldoende om een pluripotent karakter te behouden in de ICM van runderembryo's.

Het is onduidelijk of het succesvol kweken van runder-ESC afhankelijk is van het stadium (naïef of primed) waarin de pluripotente cellen zijn als deze uit een preimplantatie embryo worden gehaald. In 2013 werd een methode gepubliceerd om humane

ESC die in een primed staat waren weer in een naïeve staat te krijgen. Het hiervoor gebruikte medium was ook succesvol voor de derivatie van ESC van humane embryo's en hield deze in een naïeve staat. Tot dan waren alle humane pluripotente cellijnen in een primed staat. Ook was het standaard medium dat voor de humane ESC werd gebruikt anders dan wat voor de kweek van naïeve muizen-ESC werd gebruikt. Dit naïeve humane stamcel medium (NHSM) kon echter ook worden gebruikt om muizen-ESC in een naïeve staat te kweken en behouden. Dit suggereert dat voor het kweken van naïeve ESC het embryonale stadium en de naïeve of primed pluripotente staat niet van belang is.

Om te bepalen welke genen van belang zijn bij het behoud van pluripotentie en of deze methode kan leiden tot ESC van runderen, hebben we runderblastocysten gekweekt in NHSM en het transcriptieprofiel van deze ICM-cellen vergeleken met het transcriptieprofiel van ICM-cellen uit blastocysten gekweekt onder standaard (SOF) omstandigheden (**hoofdstuk 3**). Aan het einde van de kweek bleek dat veel van de NHSM-embryo's niet meer omhuld waren door de zona pellucida en bovendien richting het oppervlak van het kweekmedium waren gedreven. Ook waren deze embryo's een stuk donkerder dan de SOF-embryo's. Dit werd mogelijk veroorzaakt door de aanwezigheid van lipide druppels in zowel de ICM- als de TE-cellen; dit in tegenstelling tot de SOF-embryo's waar lipide druppels alleen in de ICM cellen konden worden aangetoond. De microarray studie resulteerde in een lijst met 641 unieke genen die verschillend tot expressie kwamen tussen in NHSM en in SOF gekweekte ICM-cellen. Hierbij viel het op dat chromosoom 8 en het X-chromosoom relatief meer genen bevatten die verschillend tot expressie kwamen dan de andere chromosomen. Wederom hadden de interferon genen op chromosoom 8 een gereduceerd expressieniveau, zoals beschreven in hoofdstuk 2. Erg interessant was het expressieprofiel van het X-chromosoom, want hier ligt o.a. *XIST*. Dit gen is verantwoordelijk voor de inactivatie van het X-chromosoom in vrouwelijke cellen. Het expressieniveau van *XIST* was verlaagd in ICM-cellen van in NHSM gekweekte embryo's. Verder was het expressieniveau van *HPRT1* dat ook op het X-chromosoom ligt verhoogd. Dit suggereert dat het X-chromosoom in deze cellen nog niet (volledig) was geïnactiveerd in tegenstelling tot ICM-cellen van in SOF gekweekte blastocysten. In tegenstelling tot de verwachting waren de expressieniveaus van *NANOG*, *SOX2* en *POU5F1* niet anders in de ICM van in NHSM gekweekte runderembryo's. Het expressieniveau van *NANOG* is wel verhoogd in humane ESC als deze in NHSM worden gekweekt en aangezien dit medium ook de MAPK activatie remmer PD0325901 bevat was een verhoogd *NANOG* expressieniveau in de ICM van runderblastocysten gekweekt met dit medium wel verwacht. Andere genen geassocieerd met naïviteit (*ESRRB* en *TBX3*), epiblast vorming (*KLF4*) of differentiatie (*OTX2*), waren niet differentieel geëxpresseerd. Het kweken van ICM-cellen in NHSM resulteerde niet in het uitgroeien

van deze cellen. NHSM kan dus wel X-chromosoom inactivatie vertragen, maar er zijn andere factoren nodig om het pluripotente karakter te behouden.

Nadat de publicatie van de methode om naïeve humane pluripotente stamcellen te kweken in NHSM was verschenen, volgden er meer publicaties met vergelijkbare resultaten, maar verschillende medium samenstellingen. Alle media bevatten remmers van verschillende differentiatiepaden. Door runderembryo's in deze verschillende media te produceren zouden er aanwijzingen kunnen worden gevonden welke van deze paden een rol spelen in het behoud van pluripotentie in runderembryo's. Hiervoor zijn d.m.v. kwantitatieve *reverse transcription* (RT) PCR, expressieniveaus van geselecteerde genen gemeten in embryo's gekweekt in verschillende media (**hoofdstuk 4**). Deze genen waren specifiek voor embryonaal celtype (TE, PE of epiblast), pluripotentie en de pluripotente staat (naïef of primed). De runderembryo's gekweekt in de verschillende kweekmedia vertoonden enkele verschillen. De embryo's in NHSM waren donkerder en sommigen dreven naar het oppervlak, zoals beschreven in hoofdstuk 3. Embryo's gekweekt in een medium dat vijf remmende stoffen, LIF en Activine bevatte (5i/L/A) bestonden uit meer cellen dan embryo's gekweekt in NHSM. Embryo's gekweekt in medium dat drie remmende stoffen en LIF bevatte (3iL) of het standaard medium voor het produceren van *in vitro* runderembryo's (SOF) waren vergelijkbaar van samenstelling. De expressieniveaus van genen specifiek voor TE (*CDX2*) en epiblast (*FGF4* en *KLF4*) waren niet verschillend tussen de verschillende embryo's terwijl de expressieniveaus van de genen specifiek voor PE (*GATA6*, *HNF4α*, *FGFR2* en *PDGFRα*) allen waren verlaagd t.o.v. expressieniveaus in embryo's gekweekt in SOF. Van de belangrijkste genen voor pluripotentie was *POU5F1* expressie verlaagd in NHSM en 3iL gekweekte embryo's, terwijl de expressieniveaus van *SOX2* en *NANOG* niet verschilden. Ook de genen gerelateerd aan een naïeve staat kwamen niet verschillend tot expressie (*PRDM14*, *TBX3* en *ZFP42*) of hadden een verlaagd expressieniveau (*ESRRB*) t.o.v. in SOF gekweekte blastocysten en ook de vroege differentiatiemarker *OTX2* liet een verlaagd expressieniveau zien. In de "naïeve" embryo's was ook *XIST* expressie verlaagd, maar desondanks waren de *HPRT1*-expressieniveaus vergelijkbaar. Het lijkt er dus op dat de runderembryo's gekweekt in welk "naïeve" medium dan ook pluripotente cellen bevatten die meer in een tussenstadium zitten dan dat deze cellen naïef dan wel primed zijn.

Voor het karakteriseren van genexpressieprofielen is een relatief grote hoeveelheid RNA nodig. Om voldoende RNA te kunnen isoleren is gebruik gemaakt van *in vitro* (in het laboratorium) geproduceerde embryo's in de hoofdstukken 2 t/m 4. Het is wel bekend dat er een verschil is in transcriptoom tussen *in vivo* (in het lichaam) en *in vitro* geproduceerde embryo's, maar ook *in vitro* embryo's kunnen na terugplaatsing in een koe verder uitgroeien tot gezonde kalveren. Deze *in vitro* embryo's bevatten dus ook voldoende pluripotente cellen en zouden dus informatie moeten kunnen geven over de regulatie van pluripotentie bij runderen.

Om zoveel mogelijk informatie te krijgen over de expressieniveaus in de verschillende celtypen en als gevolg van de embryoproductie onder verschillende omstandigheden, hebben we gebruik gemaakt van microarray analyse. Deze techniek wordt sinds de jaren '90 veelvuldig gebruikt. Het laatste decennium zijn er echter ook andere technieken beschreven, die zelfs het transcriptoom van een enkele cel kunnen bepalen.

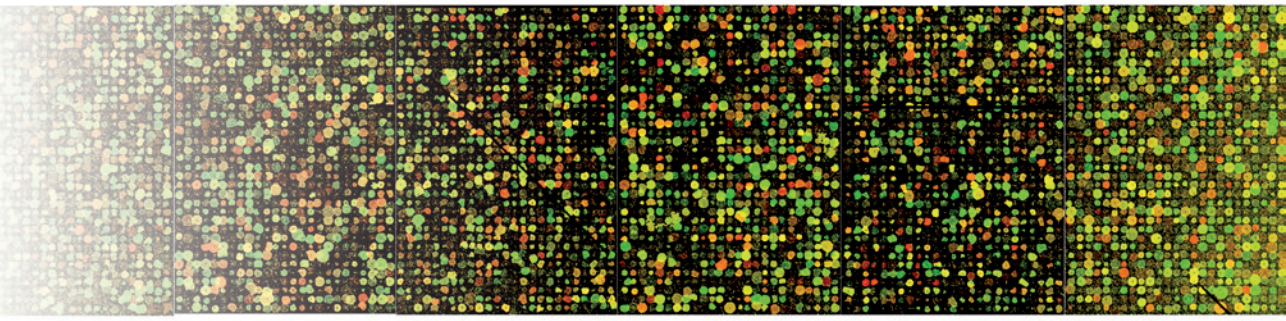
Het is duidelijk dat pluripotente cellen geremd moeten worden in hun differentiatie drang. Alleen remming van MAPK is onvoldoende, maar ook het gebruik van media met meerdere remmende stoffen zoals NHSM, 5i/L/A en 3iL is niet geschikt om pluripotentie te behouden in embryonale cellen van het rund. Omdat dit niet mogelijk is en er dus nog geen authentieke runder-ESC zijn, zou het wel interessant kunnen zijn om het transcriptoom in humane of muizen- embryo's gekweekt in de "naïeve" media te bepalen en samen met de data van humane en muizen ESC onder deze condities te vergelijken met de in dit proefschrift beschreven transcriptieprofielen van runderembryo's.

ESC van landbouwhuisdieren kunnen ook gebruikt worden als bron voor eetbare eiwitproducten zoals kweekvlees (*in vitro meat*). Vanwege de toenemende wereldbevolking en de vraag naar vlees, maar het gebrek aan ruimte en de zware belasting op het milieu dat een (vergrootte) veestapel heeft, zijn dit soort alternatieven zeer gewenst. Voor een product als kweekvlees, maar ook voor het verbeteren van de vleeskwaliteit van landbouwhuisdieren zelf, is het van belang te begrijpen hoe de ontwikkeling van spiercellen plaats vindt (myogenese). Het is verondersteld dat alle organen van een organisme stamcellen bevatten. Deze cellen zijn in volwassenen aanwezig om in geval van schade weer nieuwe orgaancellen te kunnen produceren. Ook spieren hebben deze stamcellen welke satellietcellen worden genoemd.

In **hoofdstuk 5** wordt de differentiatie van varkens-satellietcellen met toevoeging van een Dickkopf eiwit (DKK3) beschreven. Onderzoek in zebravissen suggereert dat DKK3 kan binden aan α6 integrine (α6ITG) en dan uiteindelijk *MYF5* transcriptie bevordert. MYF5 is samen met MYOD betrokken bij het initiëren van de myogene differentiatie. Vanaf het moment dat de varkens-satellietcellen werden blootgesteld aan DKK3 werd het expressieniveau van *MYF5* inderdaad verhoogd, maar na 5 dagen waren de niveaus weer gedaald. Tijdens de spierdifferentiatie gaan individuele cellen fuseren waardoor er *myotubes* ontstaan. Ook tijdens de differentiatie van de satellietcellen in aanwezigheid van DKK3 werd deze fusie waargenomen en dit proces startte eerder en resulteerde in dikkere myotubes dan bij de kweekomstandigheden zonder DKK3. Tijdens deze differentiatie- en fusie- processen worden ook specifieke genen tot expressie gebracht zoals respectievelijk desmine (*DES*) en *TMEM8c* (ook bekend als *myomaker*). De expressieniveaus van deze genen waren verhoogd op het moment waarop dit verwacht kon worden; kort na de gecombineerde expressie van *MYF5* en *MYOD* en ten tijde van fusie. De expressieniveaus van *DES* en *TMEM8c* verschilden

echter nauwelijks tussen de verschillende kweekomstandigheden. Het zou nog wel zo kunnen zijn dat er een verschil is, maar dat de duur van expressie van deze genen zo kort is, dat deze in de 24-uurs interval waarbij wij hebben gemeten, niet waarneembaar was. Ondanks dat DKK3 het *MYF5* expressieniveau kon verhogen en de vorming van myotubes bevorderde tijdens de differentiatie van varkens-satellietcellen, was verwacht dat de resultaten duidelijker verschillend zouden zijn. Dat dit niet zo was, kan verklaard worden door slechts een beperkte functie van DKK3 in relatie tot spiercel differentiatie, maar mogelijk ook doordat de satellietcellen eerst 24 uur in een differentiatiemedium waren geplaatst en daarna pas een deel van deze cellen werd blootgesteld aan DKK3. Het differentiatieproces en de fusie van cellen was mogelijk al geïnitieerd voordat DKK3 functioneel kon zijn. Door vanaf het begin van de differentiatie al DKK3 aan het medium toe te voegen zou differentiatie tot spiercellen mogelijk meer gestimuleerd kunnen worden.

Van muizen en humane pluripotente cellen is het bekend dat deze efficiënt tot spiercellen gedifferentieerd kunnen worden. Het eten van muizen- of mensen-"vlees" zal echter om verschillende redenen geen succes kunnen zijn. Daarom zou het verkrijgen van pluripotente cellen van landbouwhuisdieren zoals varkens en runderen, welke oneindig kunnen delen zonder differentiatie, maar bij het wisselen van medium zullen differentiëren tot spierweefsel, kunnen leiden tot een milieuvriendelijke toevoeging aan het bestaande vlees in de supermarkt. De resultaten zoals beschreven in dit proefschrift kunnen daar mogelijk aan bijdragen.



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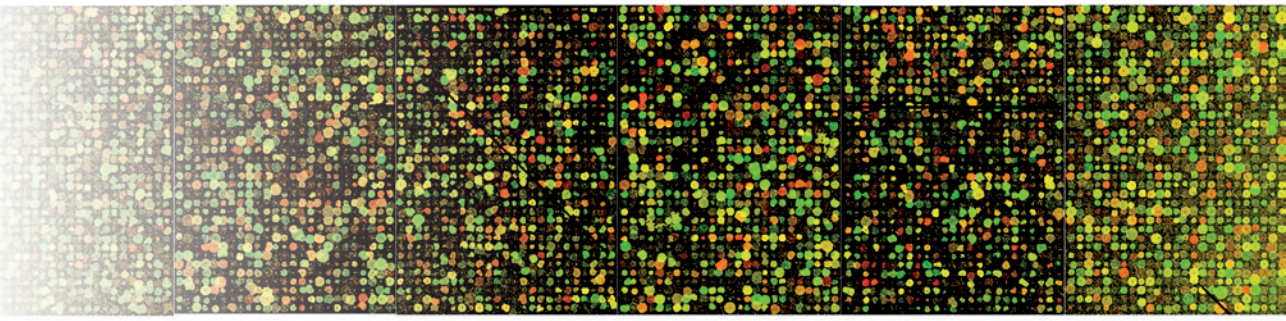
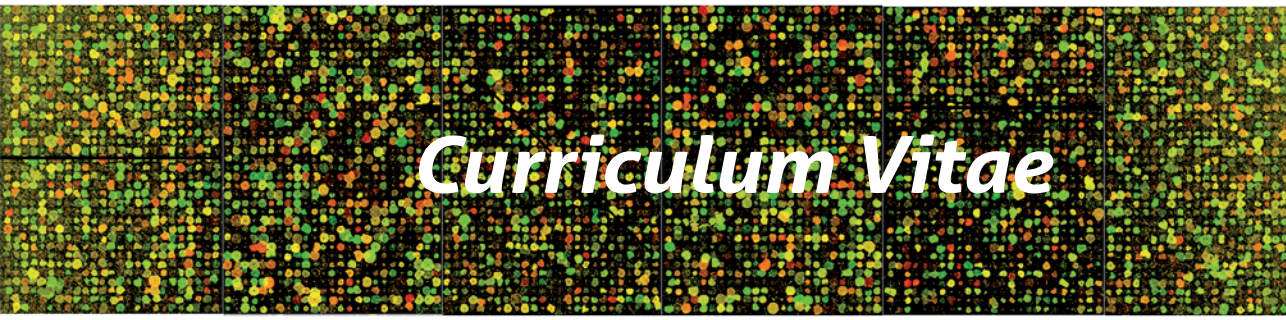
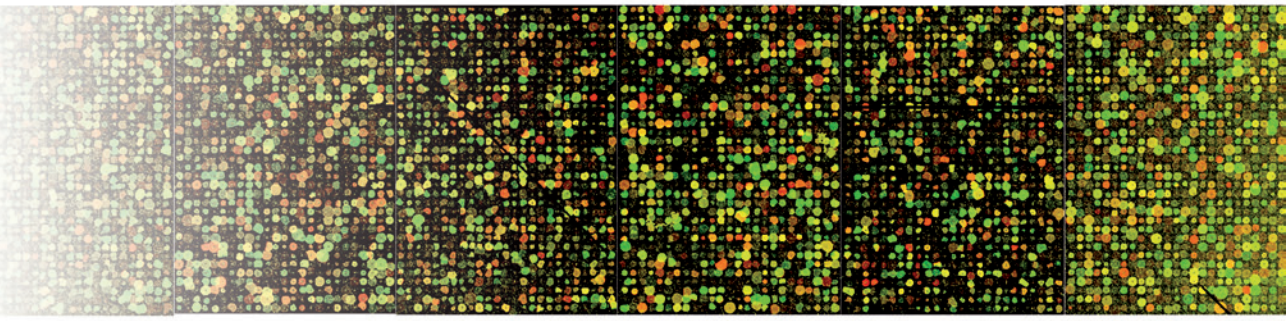




Curriculum Vitae

Bas Brinkhof, geboren op 15 augustus 1975 in Eindhoven, behaalde in 1992 zijn MAVO diploma aan de R.K. MAVO "Willem van Duvenvoorde" te Oosterhout. Na zijn MLO diploma medische microbiologie aan het Spectrum-College te Breda in 1996 en vervolgens in 1999 zijn HLO diploma biologische laboratorium techniek aan de Hogeschool Rotterdam & Omstreken te hebben behaald, startte hij bij de Erasmus Universiteit Rotterdam als research analist bij de afdeling Immunologie. Hier deed hij onderzoek naar de ontwikkeling van FISH technologie ter identificatie van translocaties bij leukemie. Na een onderbreking van een jaar backpacken in Australië, vervolgde Bas zijn carrière bij de afdeling Kindergeneeskunde van de Erasmus Universiteit Rotterdam waar hij assisteerde bij promotieonderzoek naar oorzaken van glucocorticoide resistentie bij kinderen met leukemie. In 2004 startte Bas als research analist bij de faculteit Diergeneeskunde van de Universiteit Utrecht. Bij het departement Geneeskunde van Gezelschapsdieren was hij betrokken bij onderzoeken naar bloed-, nier- en leverafwijkingen bij verschillende hondenrassen. Zijn eigen promotieonderzoek dat resulteerde in de totstandkoming van dit proefschrift begon in december 2010 bij het departement Gezondheidszorg Landbouwhuisdieren bij de faculteit Diergeneeskunde van de Universiteit Utrecht.

Bas Brinkhof, born 15 August 1975 in Eindhoven, the Netherlands completed his secondary education at the R.K. MAVO "Willem van Duvenvoorde" in Oosterhout in 1992. After finishing his medical microbiology study at the MLO of the Spectrum-College in Breda in 1996 and obtaining his BSc-degree for biological laboratory techniques at the HLO of the Hogeschool Rotterdam & Omstreken in 1999, he was employed by the department of Immunology of the Erasmus University Rotterdam as a research technician. Here, he developed FISH-techniques to identify translocations involved in leukaemia. After a break of one year backpacking in Australia, Bas continued his career at the department of Paediatric Medicine of the Erasmus University Rotterdam participating in PhD-research studying the cause of glucocorticoid resistance in paediatric leukaemia. Hereafter, in 2004, Bas went to the University of Utrecht to join the faculty of Veterinary Medicine as a research technician. At the department of Clinical Sciences of Companion Animals he participated in the studies of blood, kidney, and liver diseases in dogs. His own PhD research started in December 2010 at the department of Farm Animal Health of the faculty of Veterinary Medicine and resulted in the present thesis.



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