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Expression profiles of genes regulating dairy cow fertility: recent findings, ongoing activities and future possibilities*

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Subfertility has negative effects for dairy farm profitability, animal welfare and sustainability of animal production. Increasing herd sizes and economic pressures restrict the amount of time that farmers can spend on counteractive management. Genetic improvement will become increasingly important to restore reproductive performance. Complementary to traditional breeding value estimation procedures, genomic selection based on genome-wide information will become more widely applied. Functional genomics, including transcriptomics (gene expression profiling), produces the information to understand the consequences of selection as it helps to unravel physiological mechanisms underlying female fertility traits. Insight into the latter is needed to develop new effective management strategies to combat subfertility. Here, the importance of functional genomics for dairy cow reproduction so far and in the near future is evaluated. Recent gene profiling studies in the field of dairy cow fertility are reviewed and new data are presented on genes that are expressed in the brains of dairy cows and that are involved in dairy cow oestrus (behaviour). Fast-developing new research areas in the field of functional genomics, such as epigenetics, RNA interference, variable copy numbers and nutrigenomics, are discussed including their promising future value for dairy cow fertility.

Keywords: dairy cow, fertility, genomics, microarray, transcriptomics

Introduction

Dairy cattle bred primarily for production, with relatively little attention for functional traits, are subfertile (Royal *et al.*, 2000; Roxstrom *et al.*, 2001), as shown by suboptimal ovarian cyclicity (Opsomer *et al.*, 2002), behavioural expression of oestrus (Van Eerdenburg, 2006), success rate of artificial insemination (Jorritsma and Jorritsma, 2000; Royal *et al.*, 2000) and embryo survival (Sheldon *et al.*, 2006). Subfertility leads to between-calving intervals that are longer than economically optimal (Huirne *et al.*, 2002), it increases replacement rates (Beaudeau *et al.*, 2000) and, thus, affects profitability, animal welfare and sustainability of animal production. Decreasing profit margins per kg milk pushes farmers to reduce cost, increase herd size and, consequently, minimize labour input and cost of getting cows pregnant. This restricts options to repair dairy cow fertility through additional management and so genetic improvement will become increasingly important (Veerkamp and Beerda, 2007). Though variation in dairy cow fertility is in part genetic (Flint, 2006), heritability for fertility traits is typically low (Pryce and Veerkamp, 2001). This reflects the large unexplainable residual variation in statistical models predicting traits like calving interval and pregnancy rate at the individual cow level and does not mean genetic information is unimportant: daughters of different sires may have differences in mean calving interval of up to 30 days and the pregnancy rates of daughters sired by extreme Holstein bulls are as high as 7% (Weigel, 2006).

It is often perceived that improving fertility by genetic selection is incompatible with the dairy industry's aim of achieving ever-higher milk production levels. Focussing on increasing genetic merit primarily for yield reduces genetic merit for fertility (Veerkamp *et al.*, 2003), but conjoint improvement for reproductive performance and milk yield, say maintaining 70% to 80% of the yearly increase in yield (Veerkamp *et al.*, 2000), is possible (Andersen-Ranberg *et al.*, 2005; Jamrozik *et al.*, 2005). In line with this, most leading dairy cattle breeding programmes incorporate fertility, as derived from calving dates and insemination dates, in their selection indices (Miglior *et al.*, 2005).

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Nowadays, detailed information on the Bovine genetic code (i.e. ~3 billion nucleic base pairs packaged in 29 autosomes and two sex chromosomes plus ~16 000 mitochondrial base pairs, (Lewin, 2003)) is available. New high-throughput technologies open up ways to effectively exploit genomic information and accelerate genetic improvement. The aim of the present study is to review and discuss relatively new approaches, like genomic selection and functional genomics, as applied in the field of dairy cow fertility.

Genomic selection

Information on variation between animals in nucleotide sequence, as established by micro-satellites, restriction fragment length polymorphisms (RFLPs) and single nucleotide polymorphisms (SNPs), has been utilized in selection via the candidate gene approach and whole genome scan approach (Veerkamp and Beerda, 2007). Information on the function of a gene in species other than dairy cows may be used for a candidate gene approach: variation in the gene is linked to phenotype, as applied for genes encoding for gonadotropin-releasing hormone (Schneider *et al*., 2006), leptin (Liefers *et al*., 2005) and the bovine luteinizing hormone receptor (Hastings *et al*., 2006). In the past years, attention has shifted from identifying variation in individual (causal) genes towards genomic selection.

The whole genome scan approach builds on a large number of polymorphic markers and involves the identification of QTL that are associated with specific phenotypic traits. Of the 55 studies with dairy cattle reviewed by Khatkar *et al*. (2004), 45 reported on QTL for milk production traits and somatic cell score. The few reporting on reproduction traits were about gestation length, dystocia/stillbirth, success/failure per insemination of daughters and (90 days) non-return rate. In addition, dairy cow genetic markers have been linked to ovulation rate, multiple ovulations (Kappes *et al*., 2000) and twinning (Lien *et al*., 2000). The latter two are associated with QTL on bovine chromosome 7 (BTA7) and may be the same as the one validated for its effect on conception rate (Weller *et al*., 2008). The confidence interval for QTL location typically spans hundreds of genes and the identification of the causal genes involved may require fine mapping of the QTL (Meuwissen *et al*., 2002) by linkage disequilibrium (LD) mapping and the construction of higher density marker maps. Several QTL are suspected to be used by breeding companies, although generally these are not published.

Part of phenotypic variation is linked to SNPs, causally or indirectly by LD, and individual animals can be genotyped on the basis of many tens of thousands of SNPs. Once correlations between SNPs and phenotypes have been established, such information allows detailed genotyping in the absence of further phenotypic records, and genomic selection (Meuwissen *et al*., 2001) has the ability to reduce the cost and increase the effectiveness of breeding programmes. The Dutch breeding company, CRV, announced the implementation of genomic selection in dairy cattle (using 3000 SNPs) in 2006.

Functional genomics: transcriptomics

Functional genomics builds on information on static aspects of the genome (nucleotide sequence) as produced by genome sequencing projects, but focuses on dynamic aspects such as gene transcription (of DNA into mRNA) and translation. Gene expression divergence may result from variation in the sequence of regulatory regions, control of transcriptional initiation, RNA processing and translation (an estimated 60% of genes in humans use alternative splicing), chromatine structure and DNA methylation (Kehrer-Sawatzki and Cooper, 2007).

With the application of high-throughput technologies, such as oligo or cDNA microarray-based transcriptome analysis, gene functions are studied in a genome-wide fashion, bridging the gap between sequence and biological function. Fan *et al*. (2006) reviewed how microarray platforms for parallel genomic analysis were first developed about 15 years ago (Southern *et al*., 1992), resulting some years later in gene expression profiling assays for measuring mRNA abundance (Schena *et al*., 1995). mRNA levels are assumed to serve as a proxy for phenotypic variation, although mRNA levels need not always reflect the level of functional proteins associated with the gene (Preuss *et al*., 2004). Microarray techniques allow quantitative analyses, which is of great relevance as the effects of gene expression levels may be subtle. Niwa *et al*. (2000) showed that transcriptional regulators do not function as an on–off control system and the precise level of expression of the POU transcription factor Oct-3/4 determines the fate of embryonic stem cells. Differential expression of 50% above or below normal expression levels in undifferentiated stem cells triggers differentiation to endoderm/mesoderm or trophectoderm, respectively.

Gene expression profiling can be combined with QTL mapping (genetical genomics) and the usefulness of the approach has been demonstrated in mice (Schadt *et al*., 2003).

Micorarray-based high-throughput technology has great potential in improving dairy cow fertility (Dawson, 2006; Wolf *et al*., 2006) and facilitates progress in a number of fast-developing research areas such as epigenetics and genomic structural variation (see further on).

Recent transcriptomic studies on dairy cow fertility

Microarray-based gene expression profiling in dairy cattle has been discussed recently (Beerda and Veerkamp, 2006) for studies on oocyte maturation (Dalbiès-Tran and Mermillod, 2003; Vallee *et al*., 2005), non-regressed and regressed corpus luteum tissue (Casey *et al*., 2005), oviduct epithelial cell function (Bauersachs *et al*., 2003 and 2004), the endometrium during the oestrous cycle (Bauersachs *et al*., 2005), pre-implantation embryonic development (El-Halawany *et al*., 2004; Ushizawa *et al*., 2004; Sirard *et al*., 2005) and embryo-induced transcriptome changes of the endometrium in the pre- and peri-attachment period (Ishiwata *et al*., 2003; Klein *et al*., 2006). Briefly, the findings indicate that at the ovarian level, processes are typically controlled

by genes involved in steroid biosynthesis, oxygen radical metabolism, apoptosis, regulation of cell cycles (including DNA replication and repair), cell structure and tissue remodelling. In the oviducts, variation (e.g. during the oestrus cycle) involves the expression of genes encoding for signal transduction pathways, cell-surface proteins, cell–cell interaction proteins, immune-related proteins and those that promote proliferation. Changes in the endometrium are associated with expression divergence in genes involved in immune function, regulation of cell cycles, apoptosis, tissue remodelling, intercellular communication, cell–cell interaction, cell adhesion molecules, cell motility, intracellular metabolism, protein secretion and modification, and regulation of gene expression.

Hashizume *et al*. (2007) reviewed bovine gene expression profiles during pregnancy, which involves a cascade of different processes like pre-implantation embryogenesis, maternal recognition of pregnancy, implantation embryogenesis, placentation and foetogenesis. Embryonic mortality is a major contributor to reproductive failure and in about 80% of the cases is caused by suboptimal placentation (Cross *et al*., 1994). Reciprocal signalling and molecular exchange between the mother and the embryo/foetus, at discrete sites that together make up the placentome, is likely to play a critical role. During bovine placentation there is a continuing migration of foetal trophoblast-derived binucleate cells (BNC) and subsequent fusing with maternal uterine epithelial cells into feto-maternal syncytial plaques (Wooding and Flint, 1994). BNC produce an array of compounds, like steroids, prostanoids, bovine placental lactogens (bPLs), pregnancy-associated glycoproteins (PAGs), bovine prolactin-related protein (bPRP)-1, and the gene expression profiles for BNC-specific bPLs, bPRPs and PAGs vary during pregnancy with apparent strategic effects on implantation, placentogenesis, fetogenesis and overall progression of pregnancy (Hashizume *et al*., 2007).

Bovine embryos grown *in vivo* are of superior quality compared to those grown *in vitro*. A subset of expression profiling studies targeting events in early development from oocyte to embryo has been performed in the context of optimizing embryo production and transfer. Mamo *et al*. (2006) investigated gene expression profiles in *in vitro*-produced bovine matured oocytes and blastocysts using an 82 target gene cDNA microarray and quantitative real-time polymerase chain reaction (qPCR). Out of the 82 genes, 25 were confirmed as potential candidates for characterizing developmental competence. Clearly, gene expression studies are not restricted to microarray-based approaches and mRNA abundances in bovine oocytes and blastcystes have been determined for a limited number of selected genes using (semi) quantitative PCR (Knijn *et al*., 2005; Lingenfelter *et al*., 2007; Warzych *et al*., 2007). Wrenzycki *et al*. (2004) reviewed PCR technology-based gene expression patterns in pre-implantation bovine embyos produced *in vitro* (IVP), including those derived from somatic nuclear transfer (NT), compared to *in vivo*-grown embryos. The study evaluated the expression patterns of approximately 100 genes, which constitutes a limited sample as 15 000 to 16 000 genes may be expressed throughout pre-implantation development in mice (Stanton *et al*., 2003), and likely other mammals. Gene expression profiles in IVP and especially NT-derived embryos deviate from *in vivo*-grown embryos and the differences supposedly underlie the abnormalities of which a high birth weight is the predominant feature (large offspring syndrome or LOS). The genes that show expression divergence were suggested as genetic markers for embryo quality and viability.

Whereas IVP-produced embryos may lead to lasting pregnancy in 50% of the cases, for NT this may be 15% or less. Using a 2640 cDNA chip, Pfister-Genskow *et al*. (2005) found 18 genes that were expressed differently in bovine pre-implantation embryos produced by NT or IVP. NT embryos under-expressed three genes that encode for intermediate-filament proteins, possibly foreboding abnormal differentiation and placenta formation, and heat shock protein 27, which may be linked to abnormally high apoptosis (Pfister-Genskow *et al*., 2005). Up-regulated genes in NT embryos were suggested to compromise membrane formation (nidogen 2) and increase maternal immune responses to over-expressed MHC-I genes.

Corcoran *et al*. (2006) identified differences in gene expression profiles between bovine blastocysts derived from *in vivo v. in vitro*. Of the 384 genes and expressed sequence tags that were differentially expressed, 85% were down-regulated in the latter. The inferior quality of *in vitro*-derived embryos was associated with a decreased level of gene expression and, possibly, caused by a deficiency of the machinery associated with transcription and translation (Corcoran *et al*., 2006). Gene expression profiles can be used to define normal and deviant embryos and from here optimize embryo production protocols. Interestingly, NT- and IVP-derived embryos with widespread dysregulation of genes frequently survive the postnatal period, indicating that mammalian development can tolerate a substantial degree of epigenetic abnormality (Wrenzycki *et al*., 2004).

The research summarized typically is descriptive. The description of changed expressions of (many) genes is often indefinite and non-exclusive, which reflects the biological reality but complicates a clear interpretation of the findings. Computational tools are needed to translate gene expression patterns into metabolic pathways and meaningful biological functions and bioinformatics are increasingly needed for understanding processes underlying reproduction. Noteworthy is the absence of research into gene expression patterns in the brain of dairy cows in relation to fertility. A project has been started to fill this gap and the first findings are reported here. The work is part of collaborate research on the genomics of female fertility, targeting segregating QTL affecting conception rate in dairy cows (Agricultural Research Organization, Israel) and gene expression patterns associated with folliculogenesis (INRA, France), embryonic developmental competence (University of Bonn, Germany and MTT Agrifood Research, Finland) and oestrus/oestrus behaviour (Animal Sciences Group of Wageningen UR, The Netherlands). These and other animal breeding research groups and businesses (33 in total) have

joined forces in the EU integrated project SABRE (http://www.sabre-eu.eu/): Cutting Edge Genomics for Sustainable Animal Breeding (6th Framework Programme, Priority 5: Food Quality and Safety).

Genomic regulation of oestrus (behaviour) from the brain

Oestrus behaviour and central mechanisms underlying female sexual behaviour

Oestrus behaviour marks the time of ovulation when cows can be inseminated successfully and oestrus detection is a key factor in the management of fertility: the timing of insemination to the time of ovulation, which ideally is 7 to 18 h before ovulation (Hall *et al.*, 1959), is critical for the tuning of sperm and oocyte ageing (Hunter and Greve, 1997). Roughly about 30 h prior to ovulation, cows start expressing oestrus behaviour (Roelofs *et al.*, 2005). By definition, the standing reflex that cows show when mounted by others identifies heat, but only about 50% or less of the cows may display this (Roelofs *et al.*, 2005). Relatively little is known about the regulation of oestrus behaviour in farm animals and an experiment was performed to identify genes expressed in the brain of dairy cows that are involved in oestrus and the expression of oestrus behaviour. Different brain areas that are known to play a role in female sexual behaviour have been collected and here findings are presented for a part of the brain containing the ventral tegmental area (VTA).

Studies in rodents show that female reproductive behaviour in mammals hinges on oestrogen actions in the hypothalamus (Pfaff, 2005), with a central role for the ventromedial hypothalamus (VMH) and involvement of the arcuate nucleus, pre-optic area, paraventricular nucleus and suprachiasmic area. Extra-hypothalamic sites that modulate reproductive behaviour include the limbic system, i.e. amygdala, hippocampus, septum and cingulate gyrus, the stria terminalis and the VTA. Oestrogen primed female rodents, through actions in the VMH, exhibit both appetitive (soliciting) and consummatory (lordosis) behaviour following progestin actions in the VTA. Progesterone from peripheral origin or central biosynthesis is metabolized to dihydroprogesterone (DHP) by 5α-reductase and, subsequently, into 5α-pregnan-3α-ol-20-one (3α,5α-THP) by 3α-hydroxysteroid oxidoreductase (3αHSOR) (Rupprecht, 2003). 3α,5α-THP facilitates female sexual behaviour via a mechanism involving the receptors dopamine type 1 (D1, stimulatory), dopamine type 2 (D2, inhibitory) (Frye *et al.*, 2004) and γ-aminobutyric acid (GABAA)/benzodiazepine receptor complex (GBR, stimulatory) (Petralia and Frye, 2005). A key role is assumed for the mitochondrial benzodiazepine receptor (MBR)-mediated increase in neurosteroidogenesis, including that of 3α,5α-THP (Petralia and Frye, 2005).

Experimental approach

For the identification of genes that are expressed in the brain of dairy cows and that are involved in oestrus (behaviour), a comparison was made between gene expression patterns in brain samples of Holstein Friesian heifers that were euthanized on estimated day 0 (hereafter indicated by 0) of their oestrus cycle with those of heifers slaughtered on estimated day 12 (12), i.e. during the luteal phase when progesterone levels are maximal and before the onset of luteolyse on day 15. This first comparison identifies genes that are involved in regulating oestrus, including the expression of oestrus behaviour. A second comparison was made to narrow down the number of candidate genes that underlie the variability in oestrus expression and identify genes that may be targeted for improving oestrus behaviour. Cows that in general showed a low expression of oestrus behaviour (L) during lactation were compared to those that showed a high expression of such behaviour (H). A total of four comparisons were made. The comparison of group H0 to H12 and L0 to L12 identifies genes that are differentially expressed around oestrus and the luteal phase, and assesses whether this differs between cows that tend to show oestrus behaviour clearly and those that typically do not. The comparison of group H0 to L0 and H12 to L12 indicates whether the H group differs from the L group due to differential gene expression during day 0 or during d12 or for that matter if differences occur regardless of the phase of the oestrus cycle.

Methods

Holstein Friesian heifers ($n = 28$) were purchased before first calving and group housed in a freestall (research farm the Waiboerhoeve, Lelystad, The Netherlands). During lactation, cows were monitored for oestrus behaviour (continuous observations of half an hour in the morning and afternoon). Behaviours were recorded and scored according to the protocol by Van Eerdenburg (2006): mucous vaginal discharge (3 points), cajoling/flehmen (3), restlessness (5), being mounted but not standing (10), sniffing the vulva of another cow (10), resting with chin on the back of another cow (15), mounting other cows, or attempting to (35), mounting head side of other cows (45), standing heat (100). Points are summed over a rolling 24-h interval and it is assumed that the total score identifies oestrus when accumulating to 50 or more. Milk progesterone concentrations were assessed twice a week and repeated transrectal ultrasonography was performed in the period prior to predicted oestrus. The integrated information was used to determine the moment of euthanasia (by injection of T61), which was done between 77 and 131 days in milk, and calculate mean individual behavioural oestrus expression scores. The latter ranged between 0 and 920 with averages of 375, 414, 87 and 64 for groups H0, H12, L0 and L12, respectively. Efforts were made to euthanize the animals in a stress-free, quick and standardized way, i.e. on the research farm and near the cows' home section. Brain samples were collected within the hour from euthanasia, immediately stored in liquid nitrogen and maintained at -70°C for long-term storage. Different brain areas were sampled, including a 1-cm-thick slice in a coronal plane

taken directly rostral from the colliculus superior in a rostral direction. From the left half of the brain a strip was isolated by cutting from mid-saggital in the lateral direction (1 cm) and from the most inferior in a posterior direction up to the cerebral aquaduct. This means that a relatively large brain sample was taken that included more than only VTA cells (e.g. cells of the substantia nigra and the interpeduncular nucleus). For each experimental group, RNA of seven cows was pooled.

RNA extraction and 20k microarray (Bovine Oligo Microarray Consortium, Jerry Taylor, University of Missouri – Columbia) procedures were performed as described by Niewold *et al*. (2007) with only minor modifications. RNA was isolated without sodium citrate/NaCl precipitation and 5 μg RNA was used per labelling. Following hybridization, and before removal of the coverslip, a 5-min delay at room temperature was followed by three subsequent washings of 15 min each in: 2 × SSC + 0.2% SDS (at 42°C), 2 × SSC (room temperature) and 0.2 × SSC (room temperature), after which the procedures conformed to the Micromax TSA protocol (Niewold *et al*., 2007). Differences in gene expression levels of factors ⩾3 were considered significant.

The study ran from September 2006 to April 2007 and was approved by the Animal Care and Ethics Committee of the ASG-WUR (Lelystad).

Preliminary findings and discussion

Group H0, as compared to H12, showed up-regulation of multiple immunoglobulin superfamily proteins. Immunoglobulins provide the ideal structure for protein–protein interactions and, thus, cell–cell interactions (Rougon and Hobert, 2003). Not surprisingly, they play important roles in brain developmental processes, such as axon pathfinding neuronal migration and synapse formation, and the functioning of neuronal networks in adults. Pfaff (2005) describes how oestrogens promote female sexual receptive behaviour via increased gene transcription in VMH neurons, causing expansion of dendrites and synapses. Such remodelling of synaptic networks requires signalling by cell–cell interactions, which may be facilitated by the up-regulation of immunoglobulin superfamily proteins that mediate this. Interestingly, the assumed mobilization of immunoglobulin superfamily proteins during oestrus was observed for the H0 group relative to H12, but not for the L0 group relative to L12, suggesting that the mechanism could play a role in the degree of oestrus behaviour expression. Against this interpretation is the fact that the difference between the H0 group and the H12 group reflects low gene expression in the H12 group, not high gene expression in the H0 group. This was because genes encoding for immunoglobulin superfamily proteins were down-regulated in the H12 group as compared to the L12 group.

A gene up-regulated in group H0 as compared to H12 was that encoding for protein phosphatase 1 beta (PP1 beta). Protein phosphatases (PP), including PP1 beta, are widely expressed in the mammalian brain. One of the known PP1 functions is activating Wnt/beta-catenin signalling, a critical event in both cellular proliferation and organismal development (Luo *et al*., 2007). Compared to the H12 group, the H0 group showed down-regulation of the gene encoding tyrosine hydroxylase, which converts tyrosine into dopa and mediates the rate-limiting step in the catecholamine biosynthesis. Central catecholamine signalling strongly affects behaviour, including female sexual behaviour (Pfaff, 2005).

Comparing the L0 and L12 groups revealed no differences in gene expression levels.

Relative to the L12 group, the H12 group showed the aforementioned down-regulation of genes encoding immunoglobulin superfamily proteins together with the gene encoding the prohormone, promelanin-concentrating hormone (PMCH). PMHC is proteolytically processed into the orexigenic hormone melanin-concentrating hormone (MCH), as well as into neuropeptide EI (NEI) and neuropeptide GE (NGE). The PMHC gene is involved in the regulation of energy homoeostasis as PMCH-deficient mice have high metabolic rate, are hypophagic and lean whereas mice that over-express the gene are hyperphagic and mildly obese (Marsh *et al*., 2002). The relative high level of PMHC gene expression in cows that tend to poorly express oestrus behaviour may be explained, for example, as a defence mechanism against energy deficit or as a route to establish different energy allocation strategies. The H12 group, relative to the L12 group, showed up-regulation of SCO-spondin. The glycoprotein SCO-spondin, which is an extracellular matrix-like protein and a member of the thrombospondin type 1 repeat (TSR) superfamily (Meiniel and Meiniel, 2007), is expressed early in the CNS and is involved in developmental processes such as commissural axon pathfinding (Lehmann and Naumann, 2005). The subcommissural organ (SCO) is an ependymal gland located in the roof of the third ventricle that secretes glycoproteins (e.g. SCO-spondin) into the cerebrospinal fluid (CSF), leading to a structure known as Reissner's fibre (RF). The SCO–RF complex may participate in the clearance of CSF monoamines like catecholamines (Richter *et al*., 2004).

The comparison of the H0 group to the L0 group showed down-regulation of gamma 2 actin (γ-actin, ACTG2) and desmin (DES). Actins are involved in different cell functions such as cell motility, adhesion and shape (Kaksonen *et al*., 2006). γ-actin influences cell growth and plays a role in the readout of genetic information (Fojo, 2006). Miles *et al*. (2006) reported on plasminogen-binding proteins identified as rat β/γ-actin on cell surfaces that, upon binding, increased prohormone processing, leading to inhibition of catecholamine release. Desmin plays a role in the structural as well as functional interaction of mitochondria and the cytoskeleton (Capetanaki, 2002) and is typically associated with mitochondrial function in muscle cells (Fountoulakis *et al*., 2005).

Several genes that were differentially expressed between the groups encode for proteins that are involved in general functions like intra-cellular signalling, cell shape, motility and proliferation (γ-actin, DES, PP1 beta). Other findings point to divergences in cell–cell interactions (immunoglobulin superfamily proteins) and catecholamine signalling

(tyrosine hydroxylase, SCO-spondin, γ-actin) and energy homeostasis (PMCH). There is no easy way to understand the consistent pattern in gene expression that unambiguously relates to the experimental factors oestrus cycle phase and the cows' trait to (minimally) express oestrus behaviour, although a more detailed evaluation of the genes and possible functions within the specific brain area is yet to be done and interesting candidate mechanisms are identified. This seems rather unsatisfying, but relates to one of the strong points of gene expression profiling in that it identifies novel directions to explore. The fact that PMCH has been described in more detail than DES does not mean that the former has more biological relevance for oestrus (behaviour) than the latter, but simply that it was more easy to integrate in known concepts. Clearly, the interpretation of the data will be much supported by an increasing level of specificity of the measurements. For example, in terms of homogeneous animals, precise predefined moment of oestrus cycle, exact cell group and standardization of sample collection procedures. Also, data will be more powerful when gene expression profiles are determined at an individual level as these can be statistically linked to detailed phenotypic measurement and, subsequently, tested for multiple factors. With the rapidly decreasing costs of microarray platforms, this has become realistic and is what we will do for tissues from the anterior pituitary, hypothalamus, hippocampus and amygdala.

Developing new genomic research areas

Epigenetics

Epigenetics is about alterations in phenotype due to changes in cellular properties that can be inherited, but do not represent alterations in genotype or, in short, alterations in DNA function without alterations in DNA sequence (Jones and Takai, 2001). Epigenetic modulations of DNA and histones determine the pattern of gene expression and silencing (Jaenisch and Bird, 2003). Histone modifications, like de-acetylation of histones by histone deactylases and site-specific methylation of histone H3, may trigger methylation of cytosine residues by methyltransferases (DNMT3a and DNMT3b), which results in gene silencing that is maintained over cell generations by DMNT1 (Li, 2002; Jaenisch and Bird, 2003). The epigenetic regulation of gene expression seems inevitable for multicellular organisms as it underlies the development of cell lineage-specific gene expression and phenotypes from one genotype (Jablonka and Lamb, 1998). Also, DNA methylation allows the silencing of virus-derived and potentially destabilizing repeat sequences that make up large proportions of the mammalian genome (International Human Genome Sequencing Consortium, 2001). Epigenetics determines the formation and specification of germlines, the erasure and re-establishment of methylation patterns in embryonic primordial germ cells and, subsequently, re-establishment of sex-specific patterns during gametogenesis and results in different modifications of the paternal and maternal genome after fertilization (Allegrucci *et al.*, 2005). The differential expression of paternal and maternal alleles based on epigenetic differences stemming from the germlines is known as genomic imprinting. The resulting monoallelic expression of imprinted genes is tissue specific, seems restricted to the prenatal period (Moore, 2001) and functions to limit the production of transcripts (Allegrucci *et al.*, 2005). Imprinted genes may account for only a fraction of the genome (e.g. ~0.3% (Miozzo and Simoni, 2002)), but these affect early development strongly and may have a major impact on fertility (Fowden *et al.*, 2006). In 2006, Gebert *et al.* (2006) compared bovine oocyte to sperm DNA and reported on a differentially methylated region in the imprinted insulin-like growth factor 2 gene and this allows further work on gene-specific methylation patterns during pre-implantation development. This phenomenon has been hypothesized to cause subfertility in offspring from parents under malnutrition.

RNA interference

Validating gene function through knock-out studies in farm animals using conventional methods is possible (Denning and Priddle, 2003; Hofmann *et al.*, 2004), though typically it will be costly and complicated (Wolf *et al.*, 2000). The application of RNA interference (silencing) may, in part, circumvent practical problems. Long double-stranded RNAs and micro-RNA precursors are processed into short (~22 nucleotides) double-stranded RNAs, i.e. micro-RNAs and short interfering RNAs (Meister and Tuschl, 2004). The latter two are processed into micro-RNA containing ribonucleoprotein particles, which repress the translation of target mRNA, and RNA-induced silencing complex, which increases the degradation of target mRNA. Hiendleder *et al.* (2005) discuss how RNA interference can be applied without transgenic approaches. Small organisms and cells are treated effectively by soaking them in double-stranded RNA solutions, but in mice short interfering RNAs have been injected effectively in target tissues. The resulting decrease in gene expression may be in the range of 30% to 60% for over a week, and the approach mediated significant effects on plasma metabolite concentrations (Soutschek *et al.*, 2004). Possible future applications of RNA interference tools are discussed by Lew *et al.* (2005).

Variable copy numbers

Higher-order architectural features of the genome, as compared to primary sequence information, are linked to genomic instability and susceptibility to genomic rearrangements like deletions, duplications, inversions or translocation of genomic segments, marker chromosomes and isochromosomes. The human genome has been estimated to be composed of ~5% duplicated sequences that occur both within and between chromosomes (Sharp *et al.*, 2006). Such DNA segments of 1 kb or larger that are present as variable copy numbers (CNVs) are known to alter gene dosage, interfere with coding sequences and have gene-regulating effects. For example, three instead of two copies of the normal PMP22 gene causes serious neurological

disease (Lee and Lupski, 2006). For a population of humans, Redon *et al*. (2006) found that 12% of the genome consisted of copy number variable regions, and since this encompasses more nucleotide content than SNPs, the contribution of CNVs to phenotypic variation may be substantial. Stranger *et al*. (2007) linked gene expressions (14 072 genes) in human lymphoblastoid cell lines to SNPs and CNVs, with the former being investigated at a relatively high resolution as compared to the latter (meaning that the effect of CNV will have been underestimated). There was little overlap between SNPs and CNVs and each captured 83.6% and 17.7% of the genetic variation in gene expression, respectively. Contemporary CNV maps are far from complete (Redon *et al*., 2006) and increasing power for the detection of the smaller CNVs will likely increase the importance of CNVs in phenotypic variation. DNA microarrays have proved to be a powerful tool in detecting CNVs of 40 to 50 kb and larger.

Nutrigenomics

Nutrients can be seen as dietary signals that influence gene expression and, subsequently, protein and metabolite production (Muller and Kersten, 2003). Nutritional genomics or nutrigenomics deals with the genome-wide influences of nutrition and studies the effects of dietary components on gene expression patterns (transcriptome), chromatin organization (epigenome), protein expression patterns (proteome) and metabolite profiles (metabolome) (Afman and Muller, 2006). Macronutrients (fats, carbohydrates and proteins), micronutrients (vitamins and minerals) and food components like flavonoids all participate in the binding of nuclear receptors to response elements in gene promoter regions (Muller and Kersten, 2003) and, through the resulting co-repressor dissociation and co-activator recruitment, facilitate transcriptional activation. Nutrient-mediated activation of nuclear receptors may affect many cell functions as, for example, fatty acid-sensitive nuclear receptor peroxisome proliferator activator receptor-α (PPAR α) targets 3000 to 4000 (hepatic) genes involved in processes such as acute-phase protein responses, cell proliferation, fatty acid oxidation, gluconeogenesis and amino acid metabolism (Mandard *et al*., 2004). Early work and prospects for nutritional genomics in cattle are discussed by Dawson (2006), including the effects of endophyte-infected forages and poor-quality feed. The author argues on the basis of dietary-induced changes in gene expression how selenium, through triiodothyronine, modulators of oxidative stress and the thioredoxin carrier system, could affect fertility in cattle. Future nutrigenomic work will result in detailed insight in cows' nutritional and (reproductive) physiological status and help tailor feeding strategies to improve fertility. For example, as it may no longer be necessary to apply diets in a relatively extreme form over a prolonged period of time in order to assess its effects (Dawson, 2006). Before nutrigenomics results in the tailoring of rations to the needs of groups of individuals (genotypes), some hurdles must be overcome. Mammals handle high concentrations of different nutrients and associated metabolites, with different substances binding multiple targets with different affinities. This complexity obstructs the interpretation of findings from nutrigenomic studies and would require extreme standardization and control of experimental conditions. In general, individual-specific variation in the degree of gene expression may be considerable (Radich *et al*., 2004) and variation in environmental factors for the different experimental animals may cause false positives. Realistically, in the near future complex problems within the field of nutrigenomics may need to be downsized to small feasible projects that, for example, make use of model systems (Afman and Muller, 2006).

Summarizing conclusions

Increases in genetic merit for female fertility can be combined with high milk yield and nowadays dairy cattle breeding programmes incorporate fertility in their selection indices. Traditional breeding value estimation procedures are combined with gene-assisted selection and, more recently, genomic selection based on genome-wide information. Functional genomics builds on information on static aspects of the genome and is about the implementation of high-throughput technologies like gene expression profiling by microarray platforms. Recent gene expression profiling studies have produced a wealth of data regarding possible genes and physiological processes underlying phenotypic fertility traits. Typically, processes under study involve a large number of genes, which by their transcripts effectuate multiple functions depending on tissue, developmental stage etc. Extracting clear-cut interpretations from the data that reflect the biological reality are complex. Currently, gene expression profiling is already a powerful tool for identifying candidate genes/mechanisms underlying fertility traits, as demonstrated for centrally expressed genes involved in oestrus (behaviour). When aided by advanced computational tools and bioinformatics, it may produce gene expression data that become increasingly self-explanatory. A practical application is the use of gene expression patterns for defining normal and deviant embryos and to optimize embryo production protocols. Studies on alterations in DNA function without alterations in DNA sequence (epigenetics) provide new insights and angles to investigate physiological problems. For example, imprinted genes strongly affect early development and likely have a major impact on fertility. Research into gene-specific methylation patterns during pre-implantation development is underway. RNA interference, as applied without transgenic approaches, may develop into a useful tool to manipulate gene expression and thus validate hypothesized gene functions and physiological mechanisms in farm animals. Nutrigenomics deals with the effects of dietary components on gene expression patterns and such information can help to tailor feeding strategies in order to improve dairy cow fertility. It is recognized that genome-wide influences of nutrition are complex and tackling practical problems by

this route may take considerable effort. Recent findings indicate that CNVs may contribute significantly to phenotypic variation. Microarrays allow high-throughput CNV detection and new insights in the genomic regulation of phenotypic fertility traits are to be expected. In the years to come, large amounts of genomic information will become available to be used for the unravelling of genomic and physiological mechanisms underlying fertility traits and fine-tuning of effective selection strategies. Dealing with the information stress may prove challenging. There is great need for bioinformatic solutions to integrate different types of information on, for example, gene expression, DNA methylation, QTL and SNPs, and aid the processing of such data into biologically meaningful interpretations. The integration of large amounts of new genomic information with that on physiological mechanisms is expected to open novel ways to combat issues like subfertility in dairy cattle.

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