Epigenetics and risk assessment in livestock production

• Genomics and epigenetics in livestock production
• ARTs and epigenetics in livestock production
• Examples for epigenetic studies in early bovine embryos
• Perspectives for risk assessment

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Mariensee, Neustadt, Germany
A new era in biology: Genome sequencing, somatic cloning and embryonic stem cells

1997: A flock of clones

1998: First draft of bovine and chicken genome; 2006: dog, bee;

2004: first draft of bovine and chicken genome; 2006: dog, bee;


Nature 426, 2003
Sequencing and annotation of the bovine genome

- ~22,000 genes
- Size: 2.87 Giga base pairs, 60 chromosomes
- ~5% of the genome are transcriptionally active
- High degree of homology with human, dog, rat, mouse
- Transposons and ruminant-specific repeats
- Bovine-specific variation in genes related to lactation, reproduction, energy efficiency, and the immune system.
The ENCODE project clarifies the role of „junk DNA“

Pennisi, Science 337, 1159-1161, 2012
DNA methylation and histone modification as main factors of epigenetic modulation

Other epigenetic mechanisms:
- Telomere length regulation
- X-Chromosome-inactivation
  - miRNAs

All epigenetic mechanisms:
- Epigenome

Qiu, Nature 441, 143-145, 2006
Germ cell reprogramming during development

Von Meyenn & Reik, 2015
The Callipyge gene: Prominent example for epigenetic effects on phenotype in Merino sheep

Only inheritance of the mutation from the copy of the **paternal** callipyge gene in combination with the „normal“ copy of the **maternal** gene (imprinting) leads to the specific phenotype in skeletal muscle formation.
The ovine DLK1-GTL2 (IG) region with the Callipyge mutation

- The Callipyge mutation (CLPG) refers to the replacement of „G“ by „A“ within the intergenetic region DLK1-GTL2.

- CLPG changes the epigenotype of the DLK1-GTL2 region, incl. hypomethylation and bidirectional long range IG transcription.

Takeda et al. PNAS 2006
Observation
Pregnant Dutch women suffering from big local famine (1944/45) gave birth to low-weight babies. These women than again gave birth to low-weight babies, although no longer suffering from famine.

Conclusion
Epigenetic imprint during pregnancy can be inherited.

Heijman et al., PNAS 2008, 105:17046-9
Genetically identical mice with different DNA methylation patterns causing kinks in the tail of one but not the other.

**Agouti viable yellow (Avy) gene**
The Avy locus is a retrotransposon that is inserted upstream of the agouti gene. Normally, the Avy elements are methylated, thus, shut off. However, in the Agouti mouse, they are unmethylated and active, leading to a yellow coat and very pronounced obesity.
Increasing evidence suggests transgenerational inheritance of epigenetic features

Paternally induced transgenerational inheritance of susceptibility to diabetes in mammals

Yanchang Wei, Cai-Rong Yang, Yan-Ping Wei, Zhen-Ao Zhao, Yi Hou, Heide Schatten, and Qing-Yuan Sun

Low paternal dietary folate alters the mouse sperm epigenome and is associated with negative pregnancy outcomes


Paternally Induced Transgenerational Environmental Reprogramming of Metabolic Gene Expression in Mammals

Benjamin R. Canova, Lucas Fausquier, Naimi Habib, Jeremy M. Shea, Caroline E. Hart, Ruowang Li, Christoph Bock, Chengjian Li, Hongchang Gu, Phillip D. Zamore, Alexander Meissner, Zeping Weng
ART - Assisted reproductive techniques in cattle

- Improvement of genetic quality in herds (AI, ET, IVP)
- Multiplication of identical animals of high genetic merit (MOET and SCNT)
- Conservation of rare breeds
- Experimental model for human reproduction
  - uniparous
  - similarity of preimplantation development (EGT, cleavage patterns, blastocyst features, etc.)
  - gestation period of 9 months
  - minimum sized follicle is necessary to complete maturation in vitro
  - similar with regard to biochemical and intrinsic paternal and maternal regulatory processes
  - bovine genome is sequenced and annotated (~ 80% similarity with human genome)
Methylation reprogramming in preimplantation mammalian (bovine) embryos

Dean et al. (2001), PNAS 98, 13734-13738
Steps involved in *in vitro*-production of embryos (IVP)

1. Collection of oocytes (abattoir oocytes, OPU)
2. In vitro-maturation (IVM)
3. In vitro-fertilisation (IVF)
4. In vitro-culture (IVC)
5. Transfer
6. Cryopreservation
In vivo and in vitro production of bovine embryos

Bovine ET-industry:

575,785 in vivo and 366,854 in vitro produced embryos transferred in 2014 worldwide

- Zygote (Day 1)
- 4-cell-embryo (Day 2-2.5)
- 8-16-cell-embryo (Day 3-4)
- Morula (Day 5-6)
- Expanded blastocyst (Day 7-8)
- Hatched blastocyst (Day 8-9)
No. of transferred bovine IVP-embryos in Europe

Total number of embryos transferred in Europe with the percentage of IVC embryos.
## European countries with >1000 bovine embryos transferred in 2014

<table>
<thead>
<tr>
<th>Country</th>
<th>Transfers 2014</th>
<th>Transfers 2013</th>
</tr>
</thead>
<tbody>
<tr>
<td>The Netherlands</td>
<td>37,923</td>
<td>36,964</td>
</tr>
<tr>
<td>France</td>
<td>37,347</td>
<td>35,205</td>
</tr>
<tr>
<td>Germany</td>
<td>21,897</td>
<td>21,502</td>
</tr>
<tr>
<td>Italy</td>
<td>7,573</td>
<td>5,996</td>
</tr>
<tr>
<td>Belgium</td>
<td>6,751</td>
<td>4,876</td>
</tr>
<tr>
<td>Russian Federation</td>
<td>4,171</td>
<td>2,148</td>
</tr>
<tr>
<td>Denmark</td>
<td>3,712</td>
<td>3,581</td>
</tr>
<tr>
<td>Spain</td>
<td>3,710</td>
<td>3,209</td>
</tr>
<tr>
<td>Finland</td>
<td>3,283</td>
<td>2,973</td>
</tr>
<tr>
<td>Switzerland</td>
<td>2,929</td>
<td>2,210</td>
</tr>
<tr>
<td>Ireland</td>
<td>2,231</td>
<td>no data reported</td>
</tr>
<tr>
<td>Austria</td>
<td>1,456</td>
<td>998</td>
</tr>
<tr>
<td>Luxemburg</td>
<td>1,430</td>
<td>no data reported</td>
</tr>
</tbody>
</table>
Somatic cloning of cattle

Cattle in Mariensee cloned from adult (left) or fetal (right) fibroblasts

2nd generation of cloned cattle:
The cloned calf "Blondie" in Mariensee

* 28.03.2000  66.5 kg birthweight
Cloned cattle and their offspring
Epigenetic reprogramming of gene expression in IVP and SCNT derived early embryos

- Housekeeping Genes
- Developmentally important genes
- Pluri-/Totipotency genes
- Tissue-specific genes

In vivo embryo

IVP embryo

cloned embryo

somatic cell
### Gestation length and birthweights of calves derived from *in vitro* and *in vivo* production

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of calves</th>
<th>Gestation length [days]</th>
<th>Birthweights [kg]</th>
<th>&gt;50 kg [%]</th>
<th>&gt;60 kg [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOF-BSAaa</td>
<td>23</td>
<td>281.1±7.1$^a$</td>
<td>52.8±9.4$^a$</td>
<td>13 (59.1$^a$)</td>
<td>5 (22.7$^a$)</td>
</tr>
<tr>
<td>SOF-Serum</td>
<td>10</td>
<td>280.1±5.9$^a$</td>
<td>56.7±12.1$^a$</td>
<td>5 (50.0$^a$)</td>
<td>5 (50.0$^a$)</td>
</tr>
<tr>
<td>sheep oviduct</td>
<td>34</td>
<td>279.2±5.3$^a$</td>
<td>44.1±5.5$^b$</td>
<td>4 (11.8$^b$)</td>
<td>1 (2.9$^b$)</td>
</tr>
<tr>
<td><em>In vivo</em> (SO)</td>
<td>63</td>
<td>279.4±5.1$^a$</td>
<td>41.1±3.0$^b$</td>
<td>1 (1.6$^c$)</td>
<td>0</td>
</tr>
<tr>
<td><em>In vivo</em> (AI)</td>
<td>24</td>
<td>281.7±4.3$^a$</td>
<td>43.4±4.3$^b$</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Lazzari et al. (2002), Biol. Reprod. 67, 767-775
Epigenetic changes in bovine embryos caused by *in vitro* culture

- Deviating mRNA expression patterns
- Aberrant methylation marks on imprinted and non-imprinted genes
  - *SNRPN, PEG3, PEG10, PEG11, IGF2, IGF2R* show abnormal reprogramming of imprinted genes
- Increased embryo density could induce aberrant expression
- X-linked aberrant expression
### Study into the role of IVM on epigenetic marks in bovine oocytes: Bovine oocyte maturation: Conditions and Media

<table>
<thead>
<tr>
<th>Medium (O₂ atmosphere)</th>
<th>MII oocytes (%)</th>
<th>Blastocysts (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TCM (20%)</strong></td>
<td>403/518 (78 ± 7.5)</td>
<td>45/179 (25 ± 7.3)</td>
</tr>
<tr>
<td><strong>mSOF (5%)</strong></td>
<td>367/481 (76 ± 9.2)</td>
<td>44/142 (31 ± 6.6)</td>
</tr>
</tbody>
</table>

Heinzmann et al., 2011, MRD 78, 188-201
### Genes selected for analysis

<table>
<thead>
<tr>
<th>Imprinted genes</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>IGF2R</em> - Insulin like growth factor 2 receptor; paternal imprint</td>
</tr>
<tr>
<td><em>H19</em> - non-coding RNA; paternal imprint</td>
</tr>
<tr>
<td><em>PEG3</em> - Paternal Expressed 3; maternal imprint</td>
</tr>
<tr>
<td><em>SNRPN</em> - Small Nuclear Ribonucleoprotein N, maternal imprint</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Non-imprinted genes</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>GDF9</em> - Growth differentiation factor 9</td>
</tr>
<tr>
<td><em>Glut8</em> - Glucose Transporter 8</td>
</tr>
<tr>
<td><em>PRDX1</em> - Peroxidoredoxin</td>
</tr>
<tr>
<td><em>Dnmt3a/b</em> - DNA Methyltransferases</td>
</tr>
<tr>
<td><em>Dnmt1a/b</em> - DNA Methyltransferases</td>
</tr>
</tbody>
</table>

Heinzmann et al., 2011, MRD 78, 188-201
Gene Expression Profiles

- RT Real Time analysis based on RNA preparations (n≥4) of single oocytes
- Statistical test: Kruskal-Wallis-Test

Heinzmann et al., 2011, MRD 78, 188-201
Identification and characterization of 3 novel bovine DMRs

*In silico* analysis of evolutionary conserved known human/mouse DMRs in the bovine genome (*H19, SNRPN, PEG3*) using basic local alignment search tool (BLAST), multiVISTA alignment tool (http://gsd.lbl.gov/vista/index.shtml) and Meth-Primer for CpG-island prediction (www.urogene.org/methprimer).

<table>
<thead>
<tr>
<th>Tissue/Cell Type</th>
<th>Methylation level (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>Igf2-H19</em></td>
</tr>
<tr>
<td></td>
<td>CTCF 1</td>
</tr>
<tr>
<td>Sperm</td>
<td>97</td>
</tr>
<tr>
<td>Parthenogenetic embryos</td>
<td>1</td>
</tr>
<tr>
<td>Placenta</td>
<td>47</td>
</tr>
<tr>
<td>Heart</td>
<td>47</td>
</tr>
<tr>
<td>Kidney</td>
<td>45</td>
</tr>
<tr>
<td>Liver</td>
<td>47</td>
</tr>
</tbody>
</table>

Hansmann et al. 2010, Cytogenet Genome Res
Methylation analysis by Limiting Dilution: Outline of methodological strategy

Starting material: 10 oocytes

- bisulfite conversion (EZ DNA Methylation-Direct™ Kit; Zymo Research)

Limiting Dilution (1:10 in H₂O)

Division (into 10 x10µl)

Nested PCR

MP Multiplex-PCR (outer Primers)

SP Singleplex-PCRs for each gene (inner Primers)

Pyro-sequencing/direct sequencing

- Oocyte
- DNA of one oocyte

# Imprinting mutations and single CpG errors

<table>
<thead>
<tr>
<th>DMR</th>
<th>Group</th>
<th>No. of single CpG errors / CpGs analyzed</th>
<th>No. of abnormal alleles / alleles analyzed</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>H19-IGF2</strong> (20)</td>
<td>Immature (IM)</td>
<td>13/1820</td>
<td>2/93</td>
</tr>
<tr>
<td></td>
<td>mSOF</td>
<td>19/2080</td>
<td>0/104</td>
</tr>
<tr>
<td></td>
<td>TCM</td>
<td>22/1835</td>
<td>2/94</td>
</tr>
<tr>
<td></td>
<td>vivo</td>
<td>6/760</td>
<td>1/39</td>
</tr>
<tr>
<td><strong>SNRPN</strong> (30)</td>
<td>Immature (IM)</td>
<td>19/1620</td>
<td>2/56</td>
</tr>
<tr>
<td></td>
<td>mSOF</td>
<td>28/1619</td>
<td>0/54</td>
</tr>
<tr>
<td></td>
<td>TCM</td>
<td>24/1914</td>
<td>0/64</td>
</tr>
<tr>
<td></td>
<td>vivo</td>
<td>3/450</td>
<td>1/16</td>
</tr>
<tr>
<td><strong>PEG3</strong> (18)</td>
<td>Immature (IM)</td>
<td>13/918</td>
<td>3/54</td>
</tr>
<tr>
<td></td>
<td>mSOF</td>
<td>14/1026&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0/57</td>
</tr>
<tr>
<td></td>
<td>TCM</td>
<td>27/990&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0/55</td>
</tr>
<tr>
<td></td>
<td>vivo</td>
<td>7/414</td>
<td>0/23</td>
</tr>
</tbody>
</table>

Heinzmann et al., 2011, MRD 78, 188-201
Studies on reprogramming in bovine cloned embryos

• Selection of 25 developmentally important genes (Gap-junctions, translation, glucose transporter, DNA-methylation, growth factors, pluripotency, Telomeres, differentiation, imprinting, trophoblast function, etc.)

• Screening of 42 amplicons from 25 developmentally important genes located on 15 different chromosomes, including a total of 1069 CpG sites

• Somatic cells (blood, fibroblasts) - Bovine blastocysts (in vivo, in vitro, SCNT)

• Genomic DNA from 80 blastocysts (~70ng DNA)

• Bisulfite sequencing, PCR amplification and sequencing

Niemann et al., Cellular Reprogramming 12, 33-42, 2010
Genes selected for DNA methylation analysis in bovine embryos

- **Gap Junctions:** Cx43
- **DNA-Methylation:** DNMAP 1, DNMT 3a, 3b
- **Translation:** EIF2-AK3, ARFGEF 2
- **Glucose Transporter:** Glut-3, -8
- **Growth factors:** IGFII, IGFIIr
- **Differentiation:** LIF, LIFr, NNAT
- **Pluripotency:** Oct4, Nanog
- **Telomere regulation:** Telomerase rev. transcriptase
- **Trophoblast function:** Interferon tau 1, Mash 2
- **Imprinting:** PEG-3, -10, -11
- **Maternal expression:** ZAR 1, MATER
- **Epigenetic regulation:** SUV39H1, G9A

Niemann et al., Cellular Reprogramming 12, 33-42, 2010
DNA methylation analysis in early bovine embryos: A detailed view into one gene with its various CpGs

Example: PEG 3

Niemann et al., Cellular Reprogramming 12, 33-42, 2010
# Methylation analysis in bovine embryos

Grading of IVP, in vivo and SCNT derived blastocysts related to the extent of DNA methylation

<table>
<thead>
<tr>
<th>Gene</th>
<th>In vitro</th>
<th>In vivo</th>
<th>SCNT</th>
</tr>
</thead>
<tbody>
<tr>
<td>CX43</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DNMT1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DNMT3a</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DNMT3b</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G9a</td>
<td>0</td>
<td>87</td>
<td>NA</td>
</tr>
<tr>
<td>Glu8</td>
<td>29</td>
<td>33</td>
<td>20</td>
</tr>
<tr>
<td>H19</td>
<td>14</td>
<td>40</td>
<td>27</td>
</tr>
<tr>
<td>IGF2</td>
<td>38</td>
<td>40</td>
<td>24</td>
</tr>
<tr>
<td>IGF2R</td>
<td>18</td>
<td>34</td>
<td>18</td>
</tr>
<tr>
<td>Interferon tau</td>
<td>83</td>
<td>32</td>
<td>8</td>
</tr>
<tr>
<td>LIF</td>
<td>17</td>
<td>37</td>
<td>NA</td>
</tr>
<tr>
<td>LIFR</td>
<td>50</td>
<td>45</td>
<td>10</td>
</tr>
<tr>
<td>Mater</td>
<td>87</td>
<td>66</td>
<td>52</td>
</tr>
<tr>
<td>NNAT</td>
<td>2</td>
<td>47</td>
<td>8</td>
</tr>
<tr>
<td>NNAT-1</td>
<td>42</td>
<td>61</td>
<td>20</td>
</tr>
<tr>
<td>PEG3</td>
<td>60</td>
<td>36</td>
<td>79</td>
</tr>
<tr>
<td>PEG10</td>
<td>85</td>
<td>57</td>
<td>46</td>
</tr>
<tr>
<td>PEG11</td>
<td>3</td>
<td>93</td>
<td>64</td>
</tr>
<tr>
<td>Suv39H1</td>
<td>27</td>
<td>52</td>
<td>34</td>
</tr>
<tr>
<td>Suv39H1-1</td>
<td>49</td>
<td>78</td>
<td>10</td>
</tr>
<tr>
<td>Telomerase</td>
<td>77</td>
<td>41</td>
<td>60</td>
</tr>
</tbody>
</table>

Niemann et al., Cellular Reprogramming 12, 33-42, 2010
Methylation analysis in bovine embryos

A: Blood - In vivo blastocysts

B: ♂/♀ Fibroblasts - cloned blastocysts

C: IVP - In vivo - SCNT-blastocysts

Niemann et al., Cellular Reprogramming 12, 33-42, 2010
The bovine IGF2 gene as model for gene specific DNA methylation studies

- Growth factor, important for embryonic and fetal development
- Contains an intragenic DMR within its last exon
- This DMR is methylated on the paternal allele, i.e. is expressed from the maternal allele.

- Bisulfite sequencing for analyses of methylation patterns

Gebert et al., Genomics 88, 222-229, 2006
Sex specific methylation pattern of the intragenic DMR within the bovine IGF2 gene

Cloned expanded blastocysts (day 7) and NT donor cells

Bonferroni t-test (a:b:c; p<0.05)

Gebert et al., Genomics 94, 63-69, 2009
Methylation pattern of the intragenic DMR within the bovine IGF2 gene in blastocysts of different origin

Expanded blastocysts (day 7)

Dunn’s method (a:b:c; p<0.05)

Cloned female and male blastocysts show a different level of methylation at the DMR

Gebert et al., Genomics 94, 63-69, 2009
Sex-specific reprogramming of the IGF 2 DMR in SCNT derived bovine blastocysts

Gebert et al., Genomics 94, 63-69, 2009
## Epigenetic features related to somatic cloning (SCNT) of livestock

<table>
<thead>
<tr>
<th>DNA methylation</th>
<th>mRNA gene expression</th>
<th>Imprinted gene expression</th>
<th>X-chromosome inactivation</th>
<th>Telomere length adjustment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Broad demethylation of the DNA</td>
<td>- Silencing and/or activation of specific genes</td>
<td>Aberrant expression of NDN, XIST, SNRPN, IGF2/H19, IGF2R, PEG 3</td>
<td>- Deviant XIST expression</td>
<td>- Elongation of telomeres at morula-blastocyst transition as in conventionally produced embryos</td>
</tr>
<tr>
<td>Embryo related variability of DNA-methylation profiles</td>
<td>- Up-and-down-regulation of genes related to various biological functions in development</td>
<td>- Premature inactivation of X-chromosome</td>
<td>- Normal telomere length in cloned offspring</td>
<td></td>
</tr>
<tr>
<td>Hyper- or hypomethylation of specific genes and/or satellite DNA</td>
<td>- Random inactivation of X-chromosomes</td>
<td>- Telomere length may be dependent on the type of donor cells</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Niemann, Theriogenology 2016
http://dx.doi.org/10.1016/j.theriogenology.2016.04.02.
Contributions to phenotypic variation

D. Pomp, The future of livestock genomics, EU-Workshop, Brussels, July 2006
Summary and conclusions

• Epigenetic phenomena are essential for physiological embryonic and fetal development in livestock.
• Epigenetic mechanisms play a crucial role in the application of ARTs, specifically for somatic cloning (SCNT), *in vitro* production of embryos and reprograming to induced pluripotent stem cells (iPSCs).
• Epigenetic features may contribute substantially to the phenotype of the offspring.
• Transgenerational inheritance of specific epigenetic phenotypes has been observed.
• Epigenetic mechanisms are involved in certain human diseases; this is likely also the case for livestock species.
• Current evidence suggests that epigenetics do not pose an additional risk on food producing animals that cannot be assessed by current surveillance schemes.
• More research is needed to explore the role of epigenetics in livestock production.
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