# Transcriptomics and reproduction: Revealing molecular signatures of fertility in mammals

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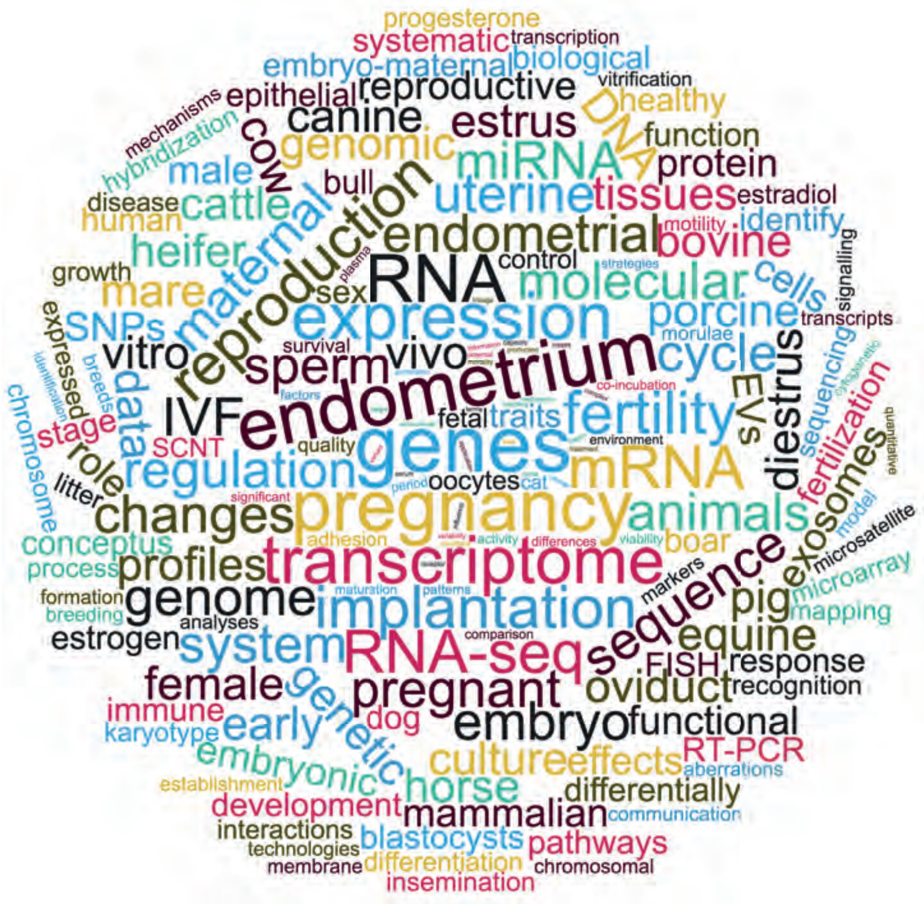
# Group Genetics and Functional Genomics at AgroVet-Strickhof

| | |
|---|---|
| Carmen Almiñana Brines | Research Scientist (SNSF) |
| Siyka Bozukova | Lab manager/technician |
| Shuqin Zeng | PhD student (Chinese government scholarship) |
| Alba Rudolf Vegas | PhD student (ProPferd/Meier-Birkel Foundation) |
| Esin Keles | DVM student (joint supervision with H. Bollwein) |
| Katharina Weber | DVM student (joint supervision with H. Bollwein) |
| Claude Schelling | Molecular Genetics |
| Aldona Pieńkowska-Schelling | Academic guest, Chromosome analysis |

- Molecular biology lab (RNA, DNA, protein)
- Histology (LCM), endocrinology, cell culture
- Sperm analysis, IVF, chromosome analysis
- Animal genomics and bioinformatics
- Animal nutrition labs
- Modern animal housing and trial facilities
- Metabolism center

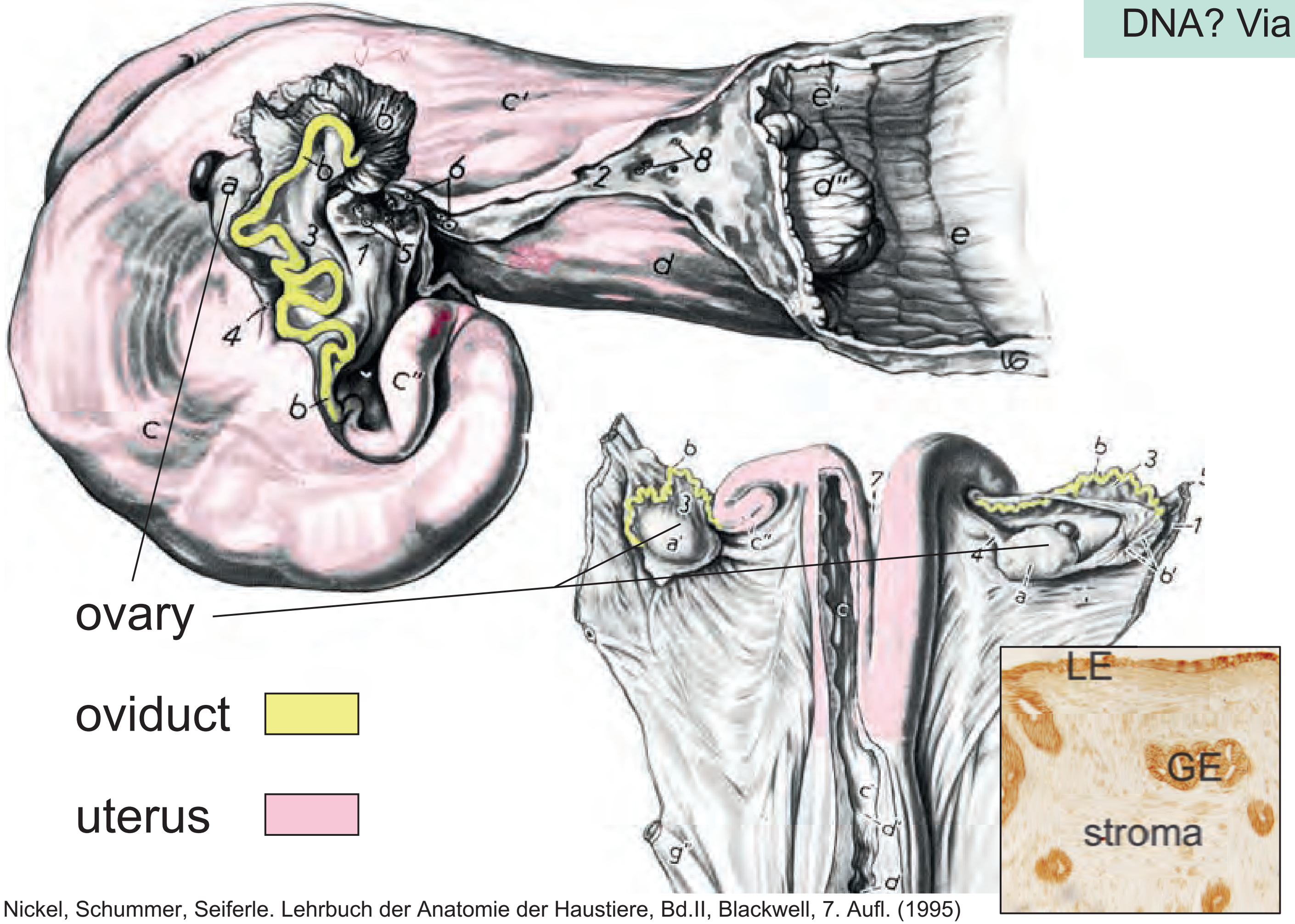
# Current main research topics of the group

- Recognition and establishment of pregnancy – embryo-maternal interactions
- Role of extracellular vesicles (exosomes and microvesicles) in gamete/embryo-maternal interactions
- Molecular mechanisms of endometrial receptivity and fertility
- Mammalian gene annotation and ortholog identification
- Male fertility (small RNAs in sperm)
- Diseases and genetic factors associated with disturbed fertility
- Recognition of factors associated with cryptorchidism, umbilical hernias and cesarean section in dogs



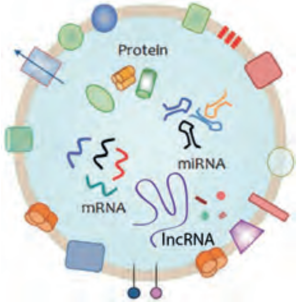
# Embryo-maternal interactions

Reproductive tract



Nickel, Schummer, Seiferle. Lehrbuch der Anatomie der Haustiere, Bd.II, Blackwell, 7. Aufl. (1995)

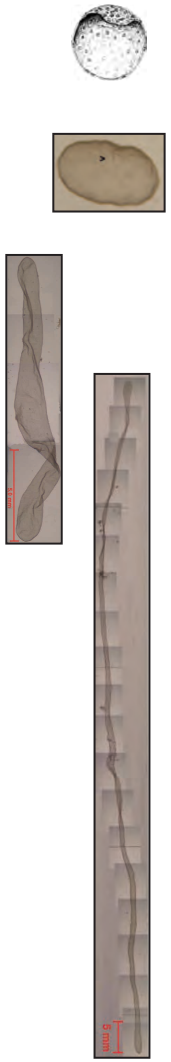
Exchange of proteins, peptides, lipids and other small molecules, small ncRNA, other ncRNA, mRNA, DNA? Via fluid and vesicles



exosomes

Oviductal/ uterine fluid

Embryo/conceptus



# Similarities and differences in reproductive biology of cattle, pig and horse compared to humans

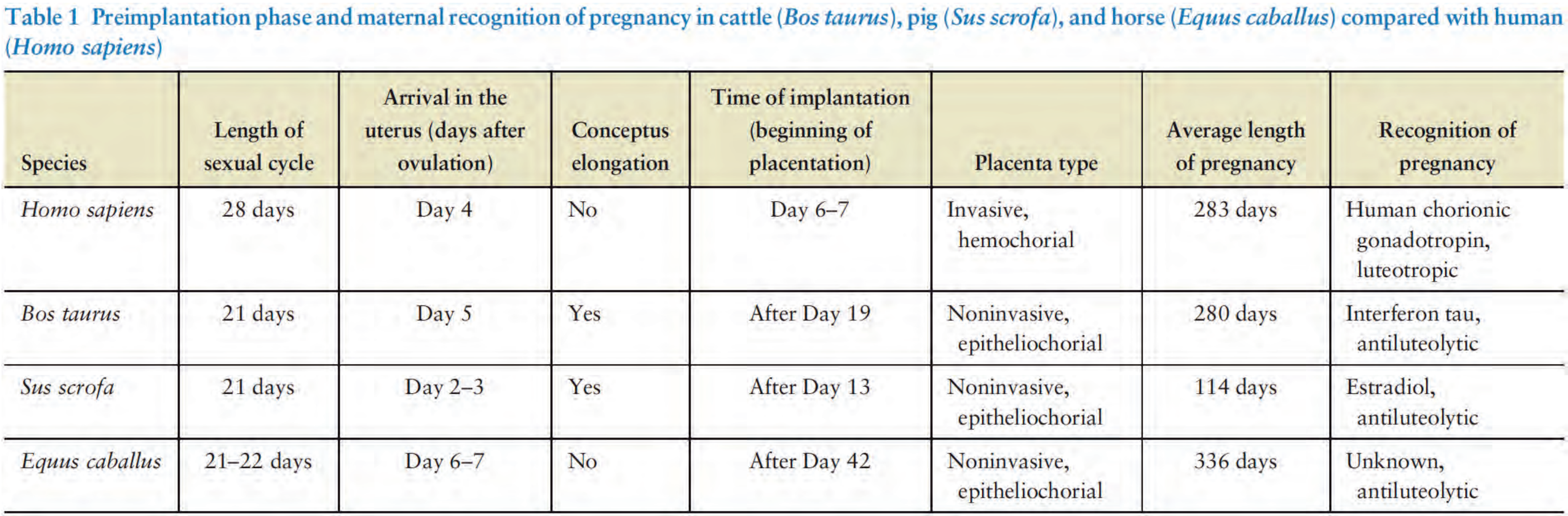
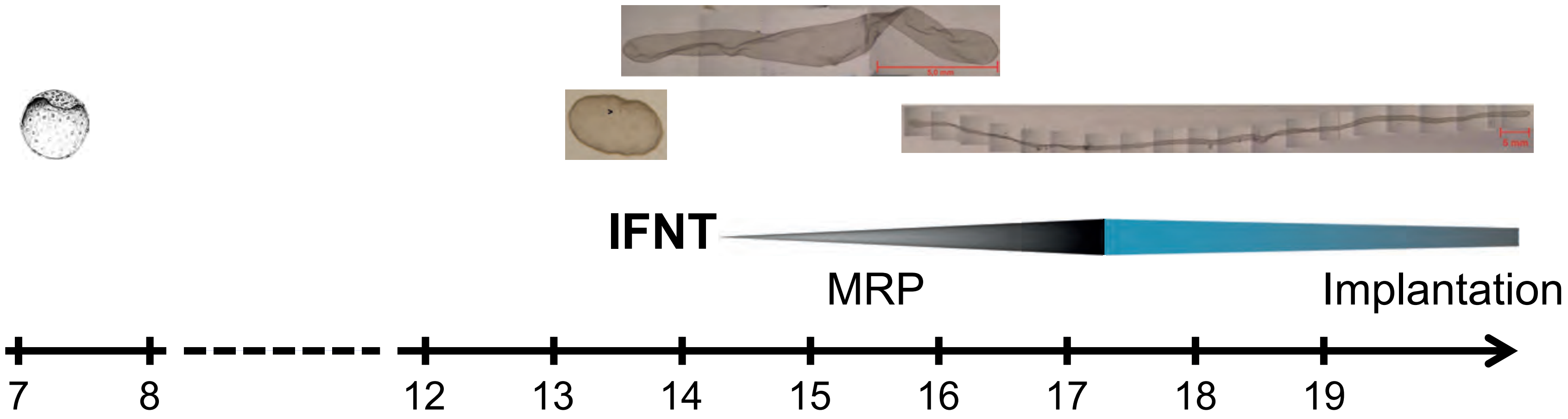


Table 1 Preimplantation phase and maternal recognition of pregnancy in cattle (*Bos taurus*), pig (*Sus scrofa*), and horse (*Equus caballus*) compared with human (*Homo sapiens*)

| Species | Length of sexual cycle | Arrival in the uterus (days after ovulation) | Conceptus elongation | Time of implantation (beginning of placentation) | Placenta type | Average length of pregnancy | Recognition of pregnancy |
|---|---|---|---|---|---|---|---|
| *Homo sapiens* | 28 days | Day 4 | No | Day 6–7 | Invasive, hemochorial | 283 days | Human chorionic gonadotropin, luteotropic |
| *Bos taurus* | 21 days | Day 5 | Yes | After Day 19 | Noninvasive, epitheliochorial | 280 days | Interferon tau, antiluteolytic |
| *Sus scrofa* | 21 days | Day 2–3 | Yes | After Day 13 | Noninvasive, epitheliochorial | 114 days | Estradiol, antiluteolytic |
| *Equus caballus* | 21–22 days | Day 6–7 | No | After Day 42 | Noninvasive, epitheliochorial | 336 days | Unknown, antiluteolytic |

Bauersachs & Wolf Annu Rev Anim Biosci 2015

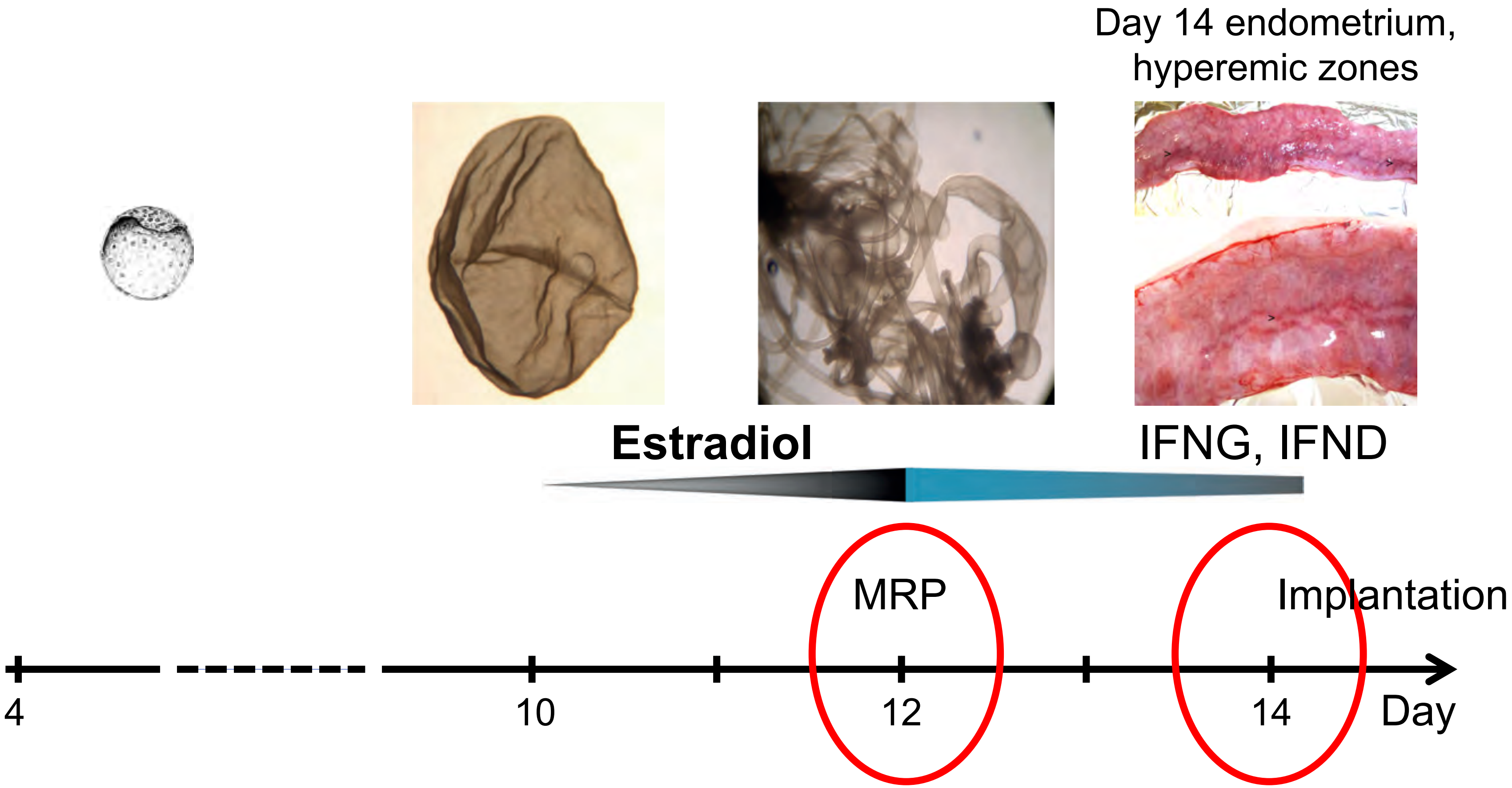
# Pre-implantation phase in cattle



MRP: maternal recognition of pregnancy

- Conceptus interferon tau as pregnancy recognition signal
- Prevention of release of endometrial PGF2a by repression of OXTR
- ➢ Prevention of luteolysis

# Pre-implantation phase of pregnancy in pigs



Day 14 endometrium,
hyperemic zones

**Estradiol**

IFNG, IFND

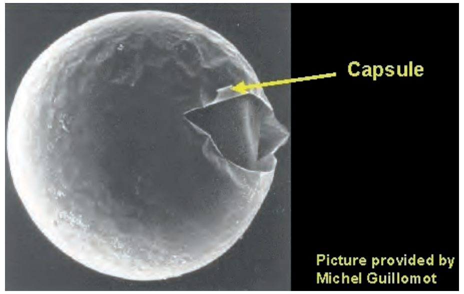
MRP

Implantation

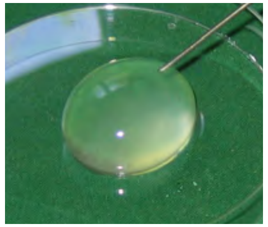
4
10
12
14
Day

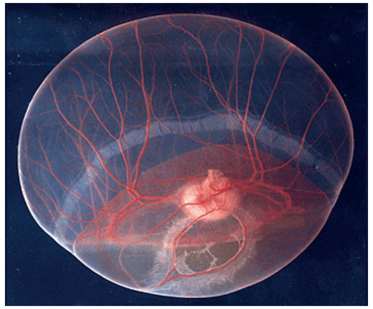
- Day 10-12: transition from spherical blastocyst to filamentous conceptus
- Increase of conceptus estrogen synthesis with conceptus elongation
- Day 14: Conceptus attachment, increased vascularization at implantation zones

# Pre-implantation phase in the horse



Picture provided by Michel Guillomot





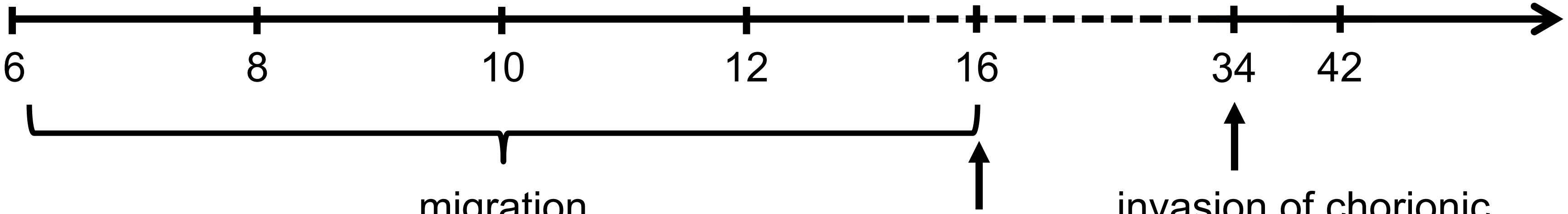
© Doug Antczak

E2, PGE2, etc.

IFND

MRP I

Epitheliochorial implantation



migration

fixation

invasion of chorionic girdle, endometrial cups, eCG

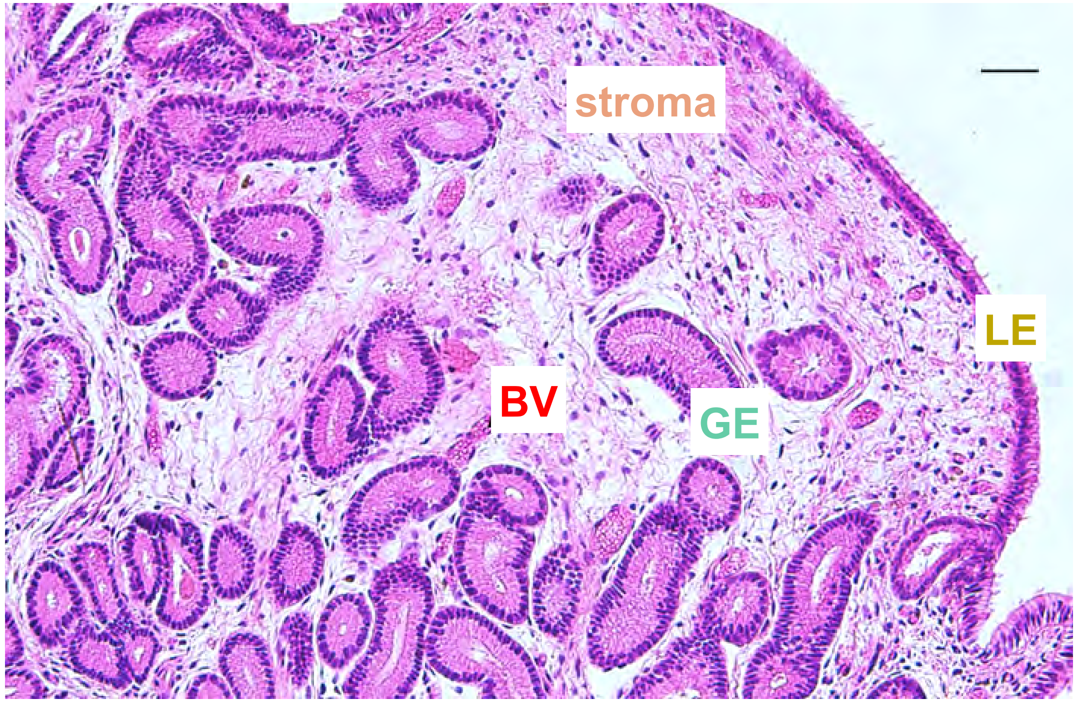
# Functions of the endometrium

- Support of embryonic development
- Recognition of pregnancy (in mammals with late implantation)
- Preparation for implantation and placentation

Image from Merkl et al., *Biol Reprod* 2010

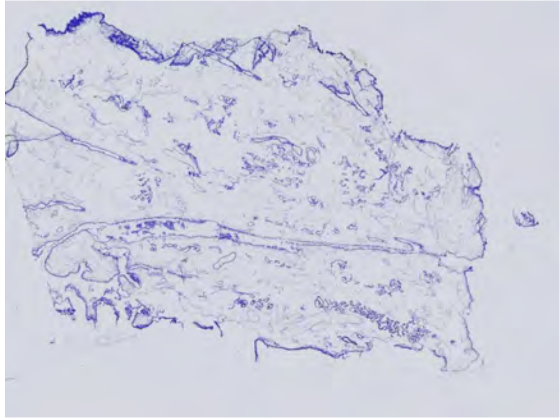
# Cell type-specific analyses of transcriptome changes in porcine endometrium

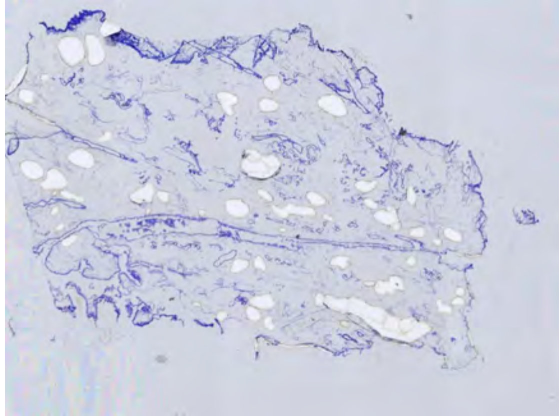
- Days 12 and 14 of pregnancy
- Isolation of endometrial cell types/compartments by laser capture microdissection (LCM)
- Isolation of total RNA and RNA-sequencing using a low-input library protocol

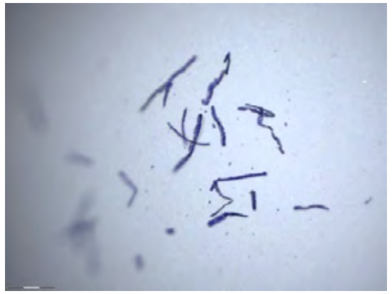


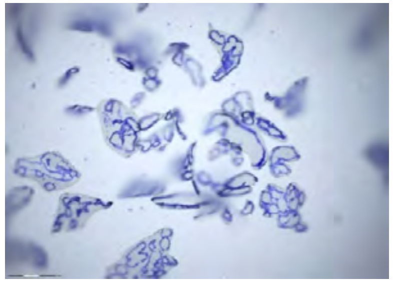
LE luminal epithelium; GE glandular epithelium; BV blood vessels

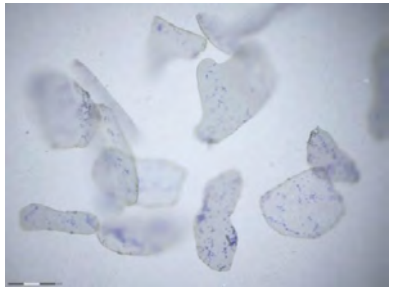
# Laser Capture Microdissection (LCM)


Before LCM


After LCM


Luminal
Epithelium
(LE)

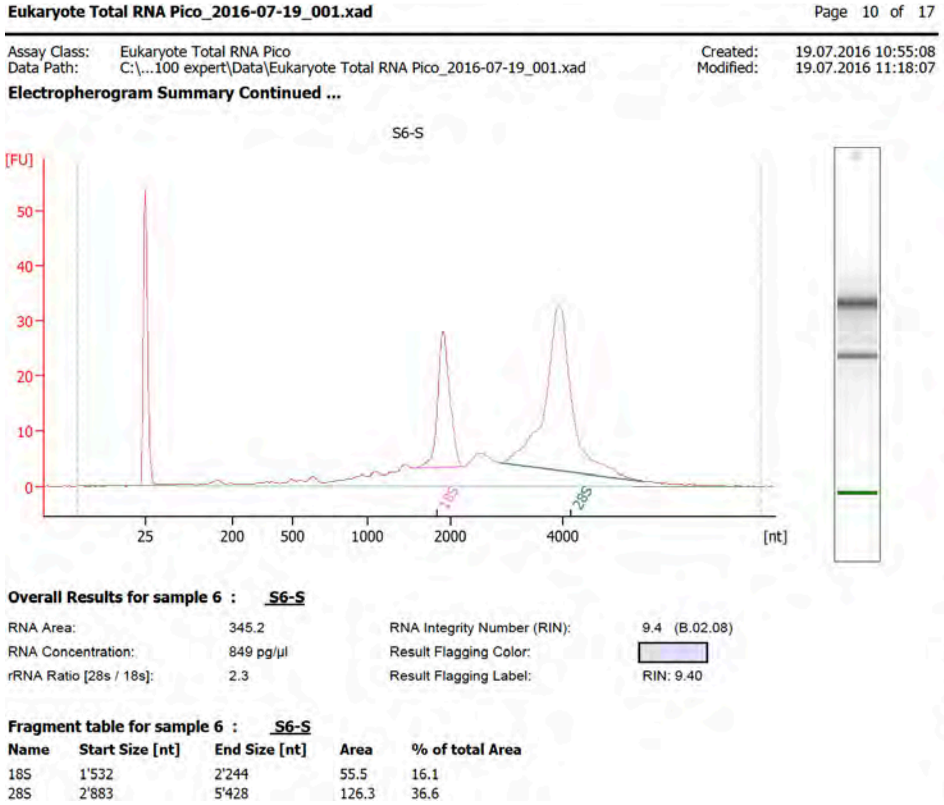

Glandular
Epithelium
(GE)


Stroma
(S)

# RNA isolation and quality control

Arcturus PicoPure
RNA isolation kit

stroma



**Eukaryote Total RNA Pico_2016-07-19_001.xad** Page 10 of 17

Assay Class: Eukaryote Total RNA Pico Created: 19.07.2016 10:55:08
Data Path: C:\...100 expert\Data\Eukaryote Total RNA Pico_2016-07-19_001.xad Modified: 19.07.2016 11:18:07

**Electropherogram Summary Continued ...**

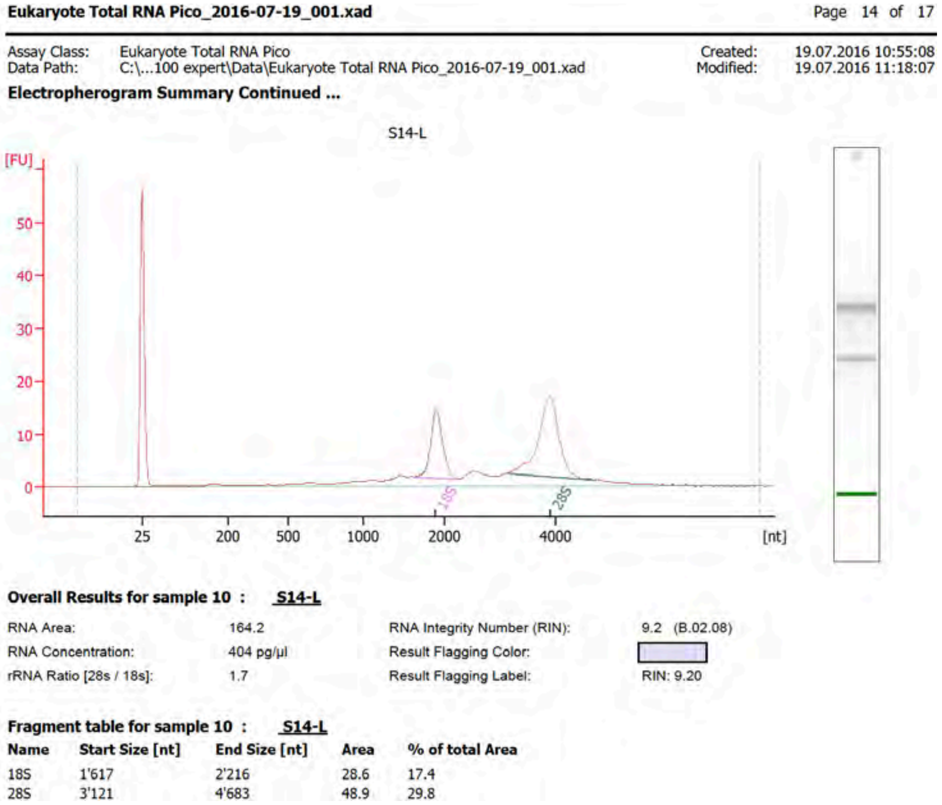
**Overall Results for sample 6 : S6-S**

| | | | |
|---|---|---|---|
| RNA Area: | 345.2 | RNA Integrity Number (RIN): | 9.4 (B.02.08) |
| RNA Concentration: | 849 pg/µl | Result Flagging Color: | |
| rRNA Ratio [28s / 18s]: | 2.3 | Result Flagging Label: | RIN: 9.40 |

**Fragment table for sample 6 : S6-S**

| Name | Start Size [nt] | End Size [nt] | Area | % of total Area |
|---|---|---|---|---|
| 18S | 1'532 | 2'244 | 55.5 | 16.1 |
| 28S | 2'883 | 5'428 | 126.3 | 36.6 |

luminal epithelium



**Eukaryote Total RNA Pico_2016-07-19_001.xad** Page 14 of 17

Assay Class: Eukaryote Total RNA Pico Created: 19.07.2016 10:55:08
Data Path: C:\...100 expert\Data\Eukaryote Total RNA Pico_2016-07-19_001.xad Modified: 19.07.2016 11:18:07

**Electropherogram Summary Continued ...**

**Overall Results for sample 10 : S14-L**

| | | | |
|---|---|---|---|
| RNA Area: | 164.2 | RNA Integrity Number (RIN): | 9.2 (B.02.08) |
| RNA Concentration: | 404 pg/µl | Result Flagging Color: | |
| rRNA Ratio [28s / 18s]: | 1.7 | Result Flagging Label: | RIN: 9.20 |

**Fragment table for sample 10 : S14-L**

| Name | Start Size [nt] | End Size [nt] | Area | % of total Area |
|---|---|---|---|---|
| 18S | 1'617 | 2'216 | 28.6 | 17.4 |
| 28S | 3'121 | 4'683 | 48.9 | 29.8 |

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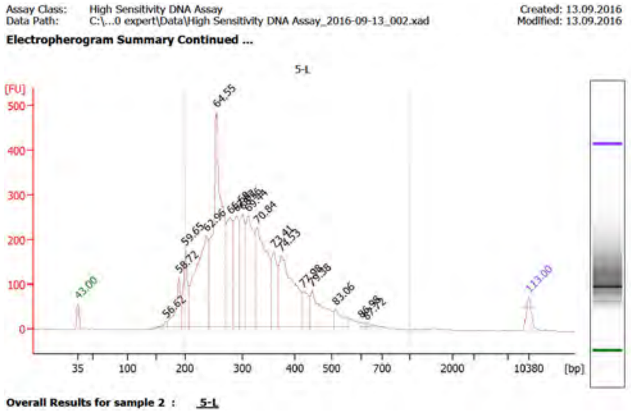
# Preparation of RNA-Seq libraries and sequencing

Ovation SoLo RNA-Seq System
(NuGEN)

Illumina Sequencing



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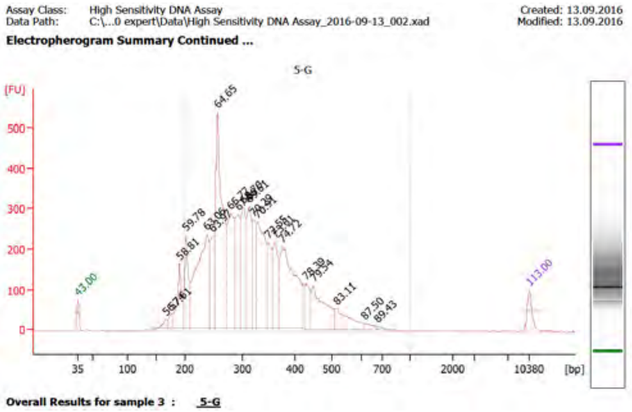
Created: 13.09.2016
Modified: 13.09.2016

**Electropherogram Summary Continued ...**

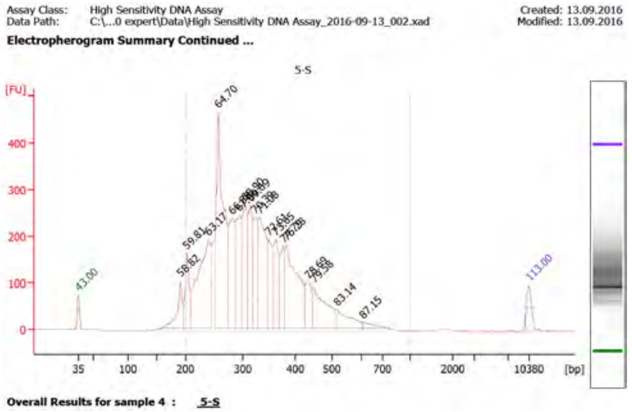
**Overall Results for sample 2 : 5-L**



Assay Class: High Sensitivity DNA Assay
Data Path: C:\...0 expert\Data\High Sensitivity DNA Assay_2016-09-13_002.xad
Created: 13.09.2016
Modified: 13.09.2016

**Electropherogram Summary Continued ...**

**Overall Results for sample 3 : 5-G**



Assay Class: High Sensitivity DNA Assay
Data Path: C:\...0 expert\Data\High Sensitivity DNA Assay_2016-09-13_002.xad
Created: 13.09.2016
Modified: 13.09.2016

**Electropherogram Summary Continued ...**

**Overall Results for sample 4 : 5-S**

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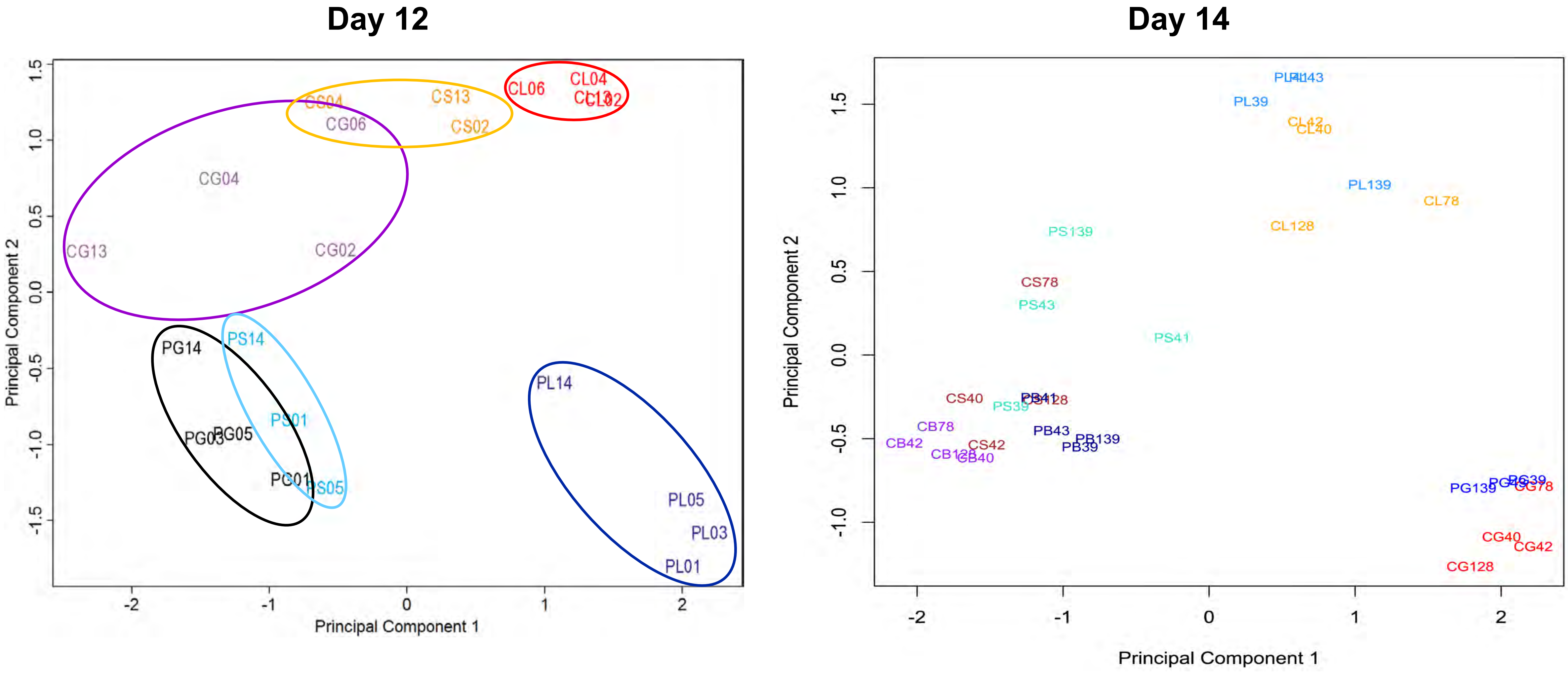
# Multidimensional scaling plots (principal component analysis)



P: pregnant; C: control; L: luminal epithelium; G: glandular epithelium; S: stroma; B: blood vessels

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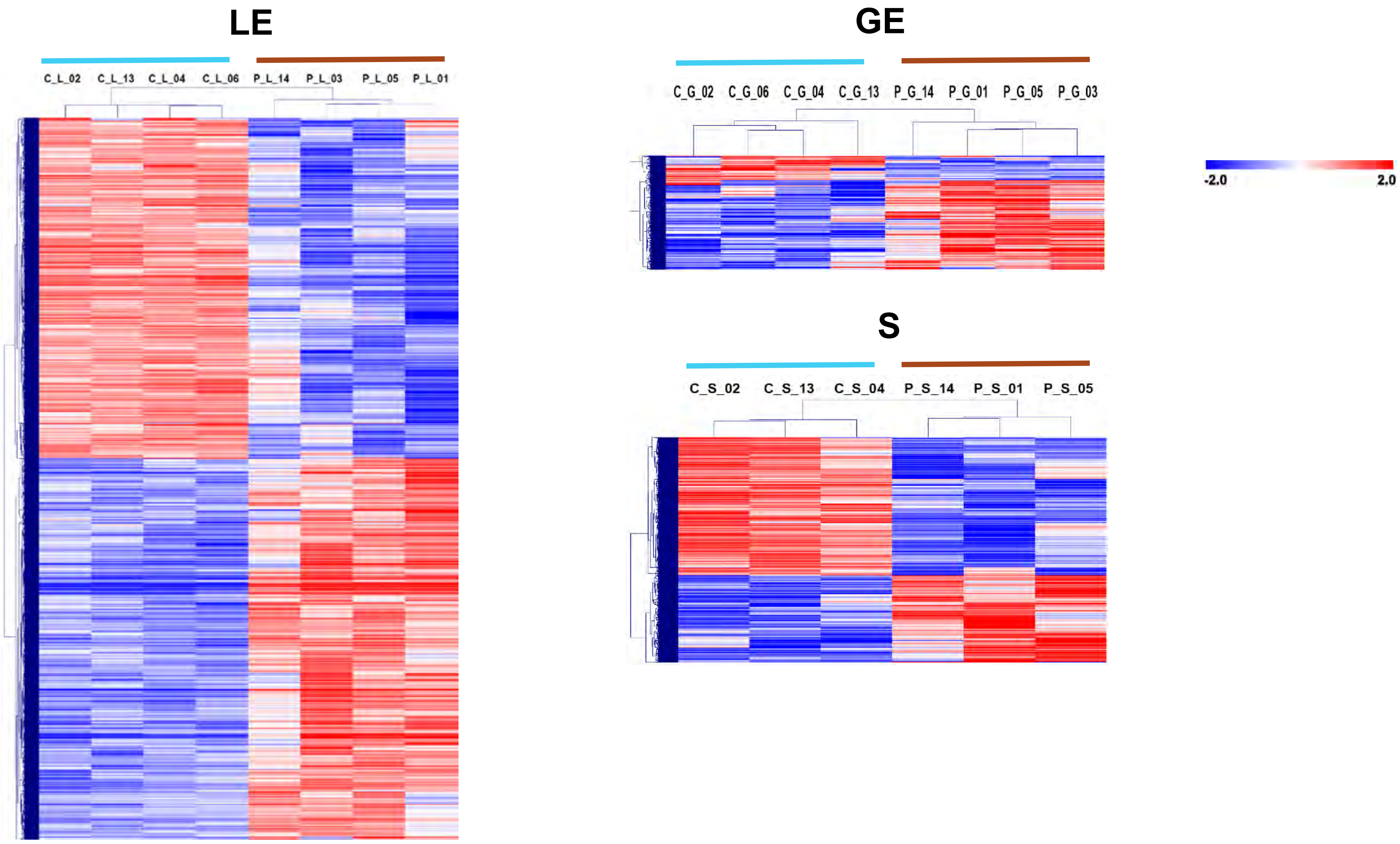
# Hierarchical cluster analysis of DEGs Day 12 of pregnancy

control

pregnant



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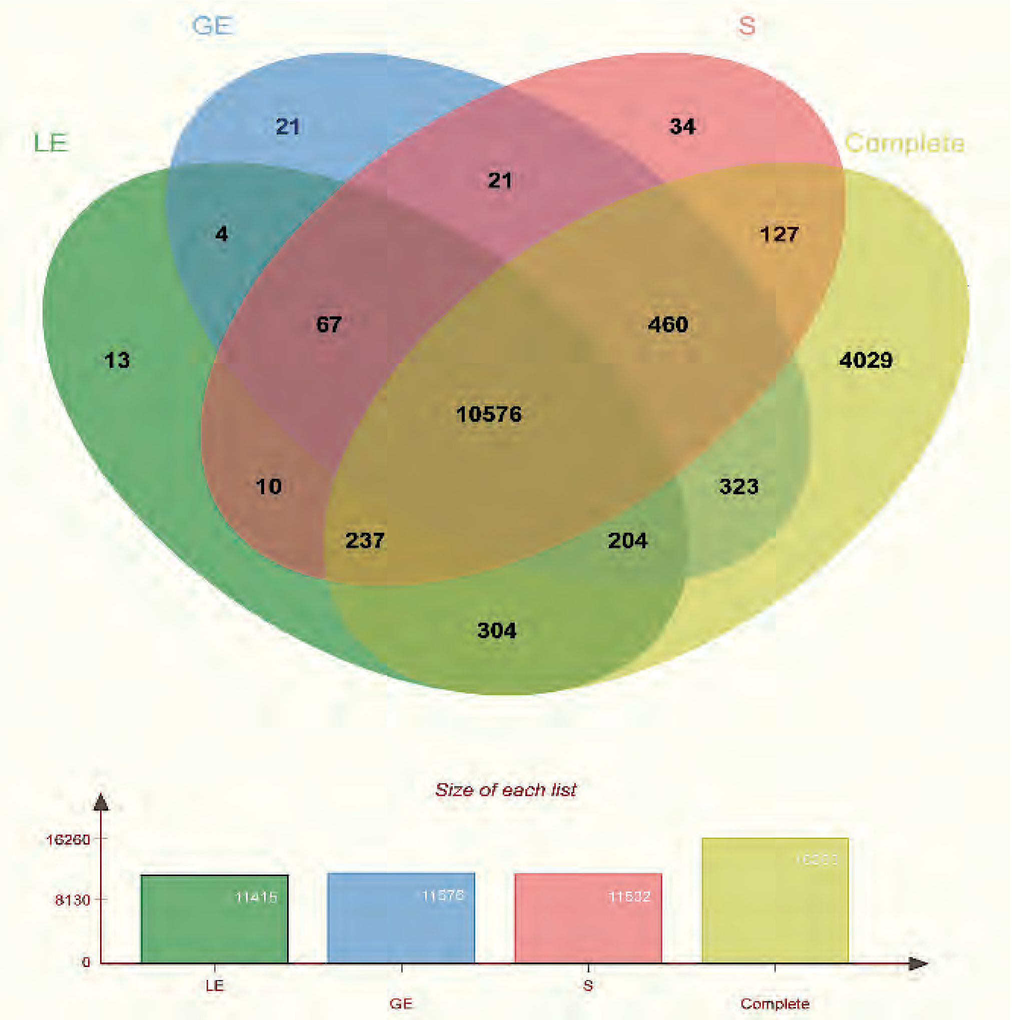
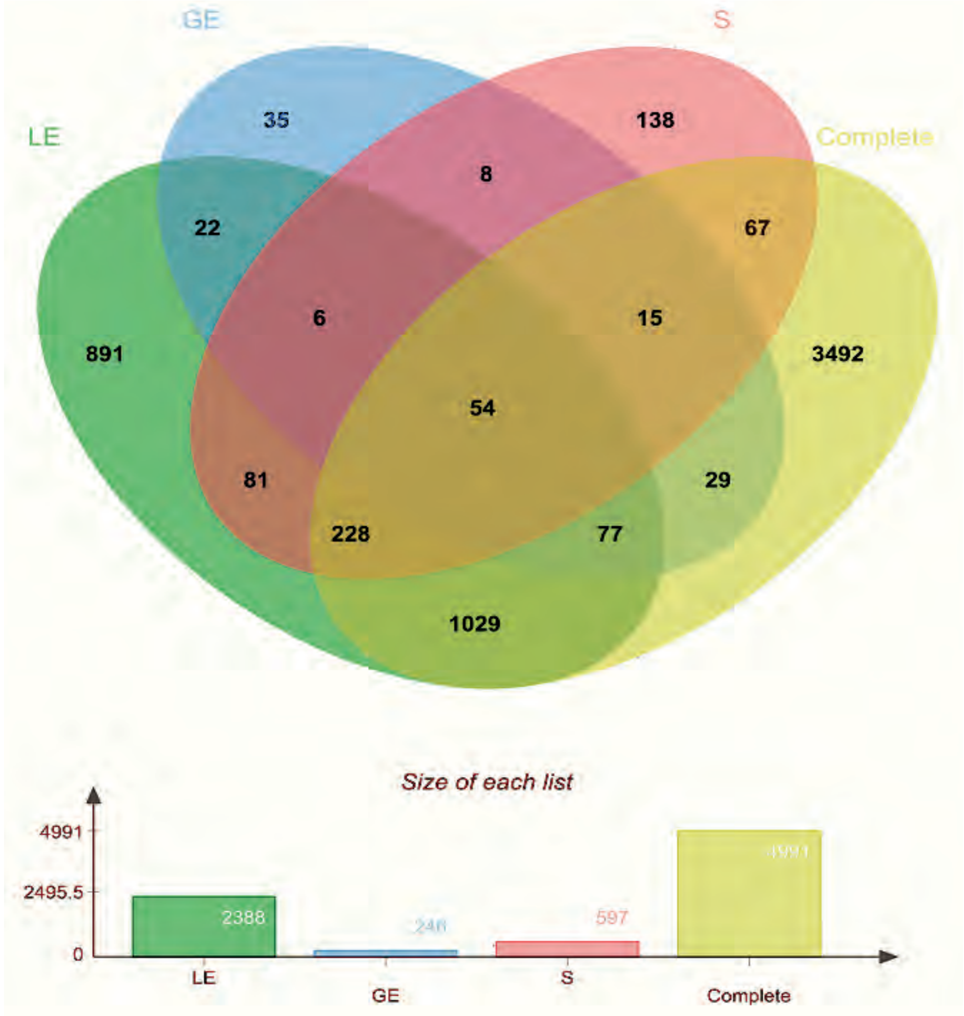
# Overlap of expressed genes and DEGs between LCM samples on Day 12

## Detectable genes



## Differentially expressed genes



LE: luminal epithelium; GE: glandular epithelium; S: stroma

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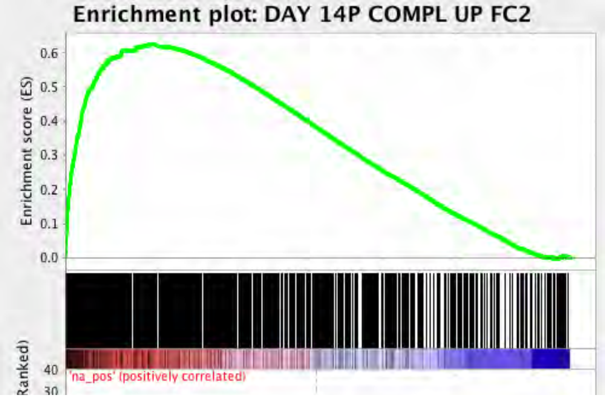
# Comparison of Day 14 DEGs to Day 12 RNA-seq data sets

### Complete tissue

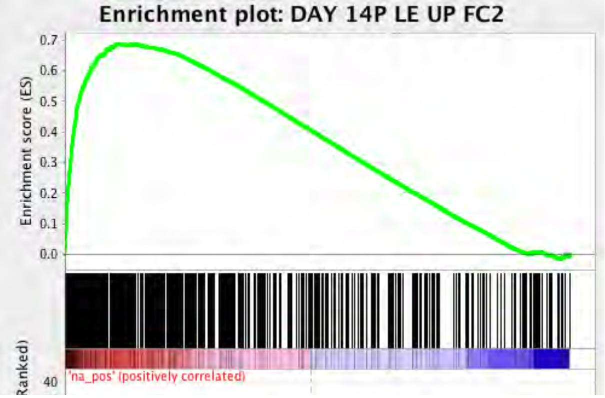
Day 14 complete tissue up



Day 14 complete tissue down

### Luminal epithelium

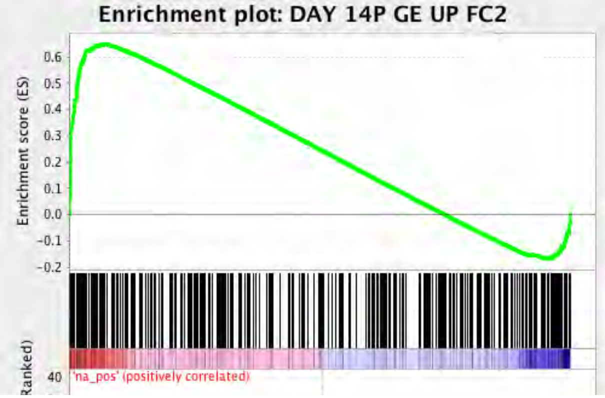
Day 14 LE up



Day 14 LE down

### Glandular epithelium

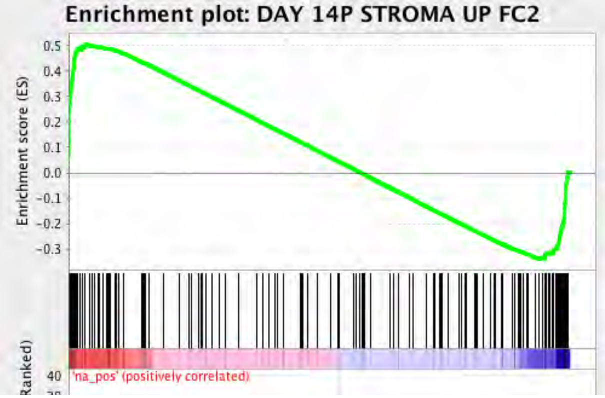
Day 14 GE up



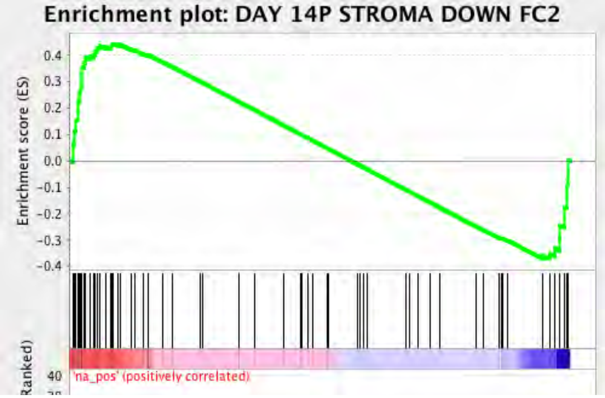
Day 14 GE down

### Stroma

Day 14 stroma up

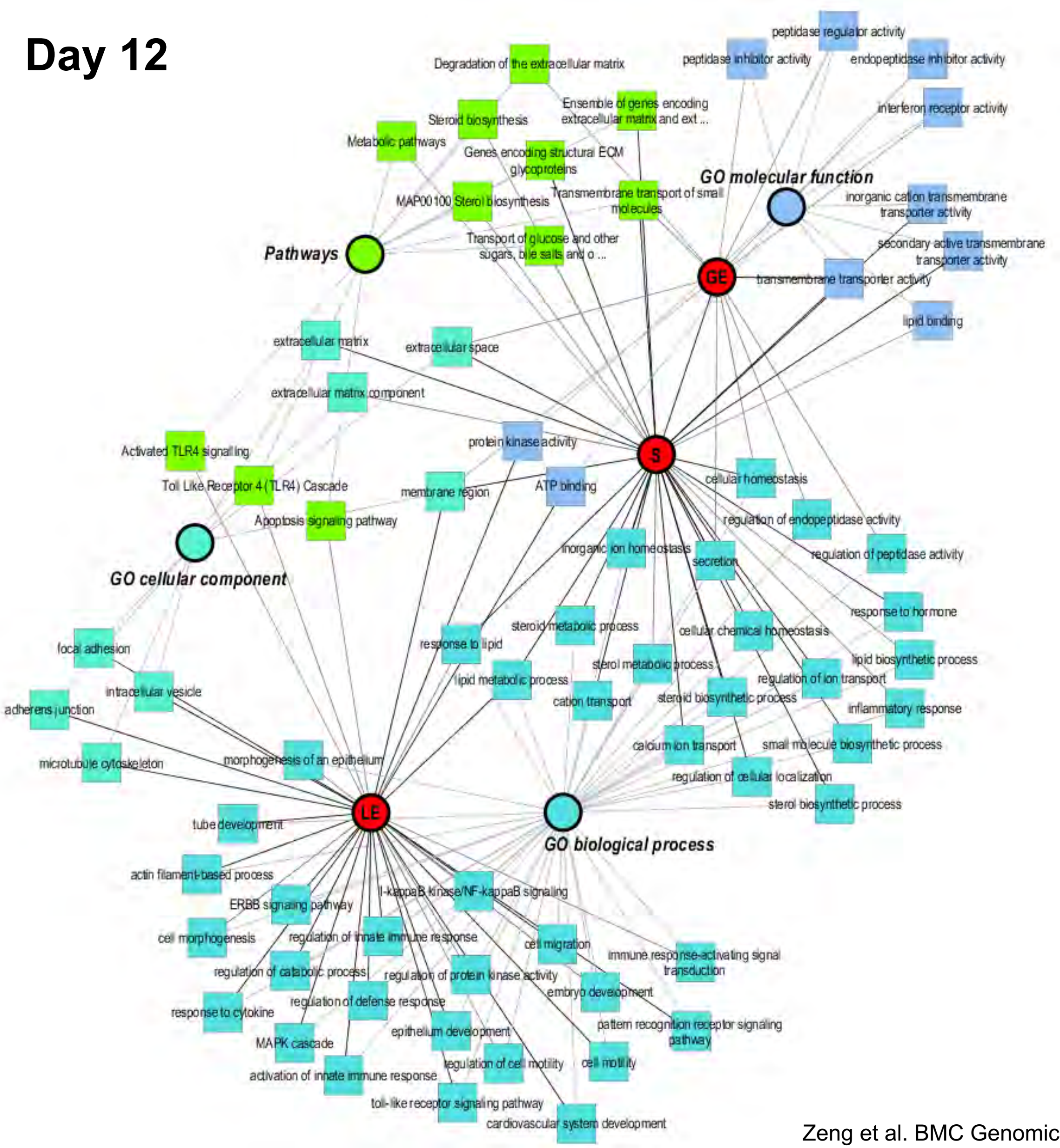


Day 14 stroma down



# Cell type-specific overrepresentation of GO categories and pathways

**Day 12**



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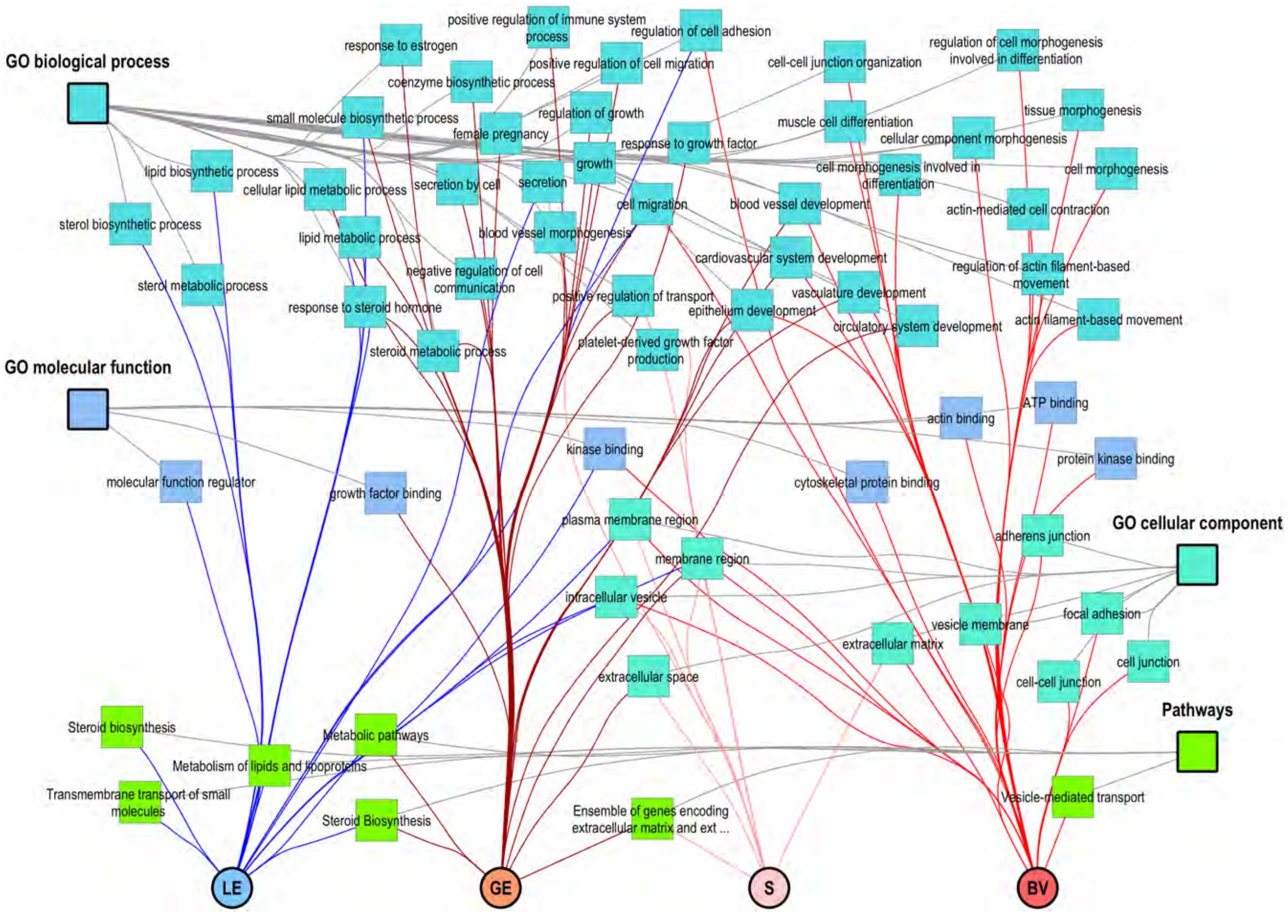
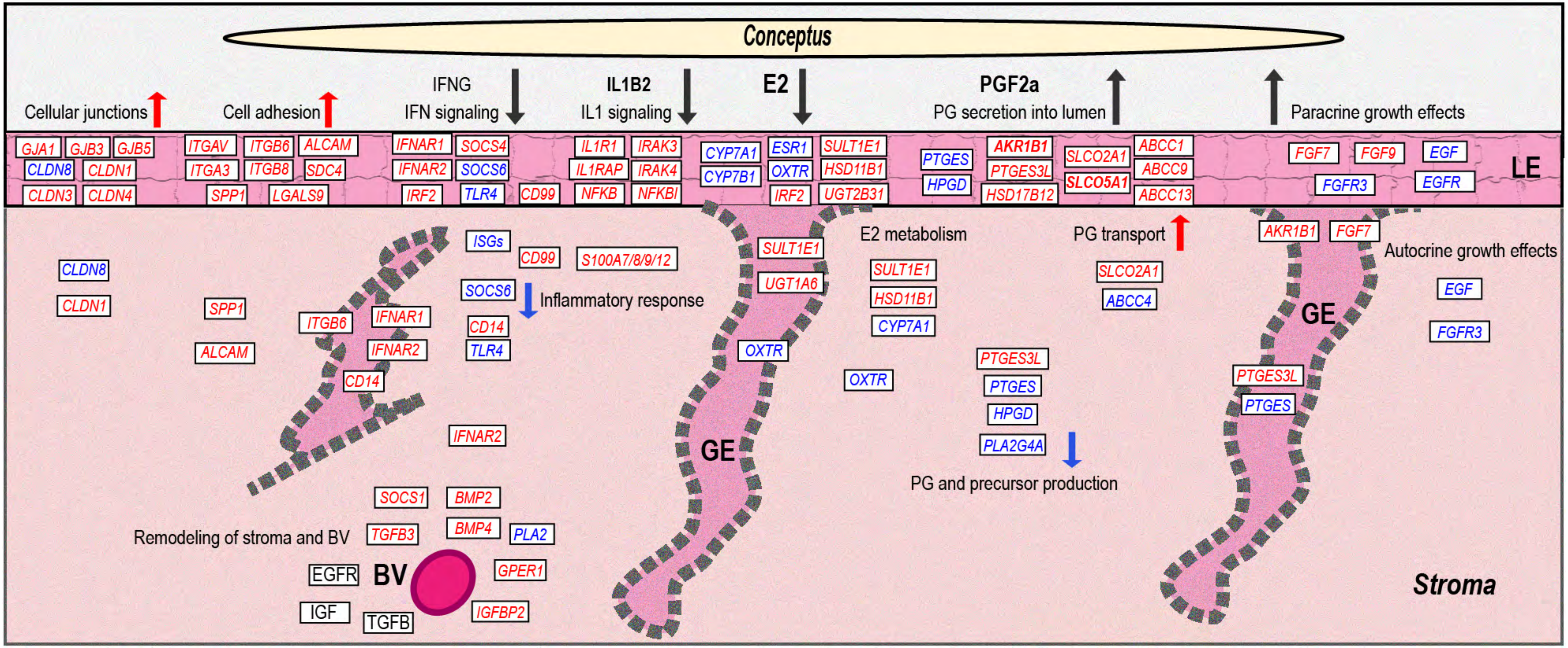
**Day 14**



Zeng et al. submitted

# Data interpretation in consideration of localization of DE on Day 12



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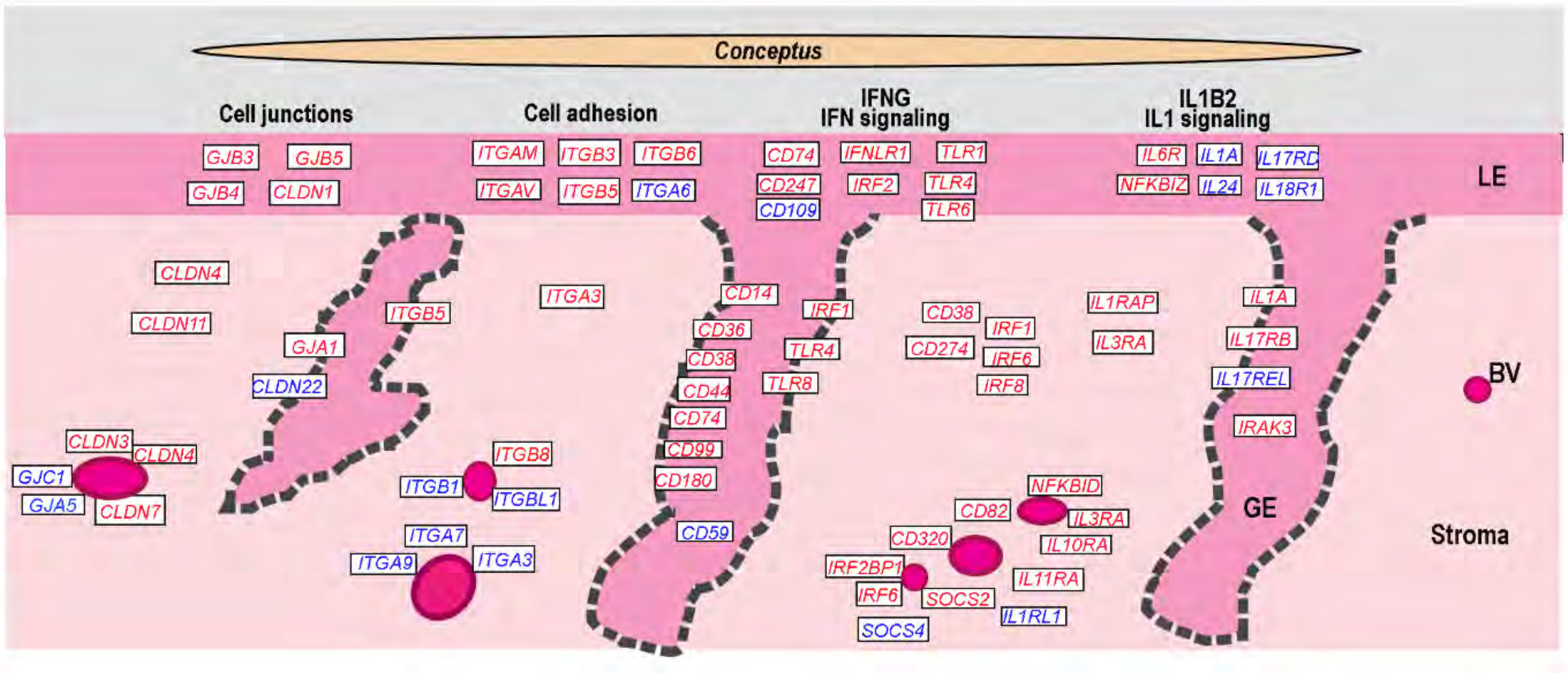
# Data interpretation in consideration of localization of DE on Day 14



Zeng et al. submitted

# Main overrepresented processes for the identified DEGs

**Main processes for DEGs on Day 12:**

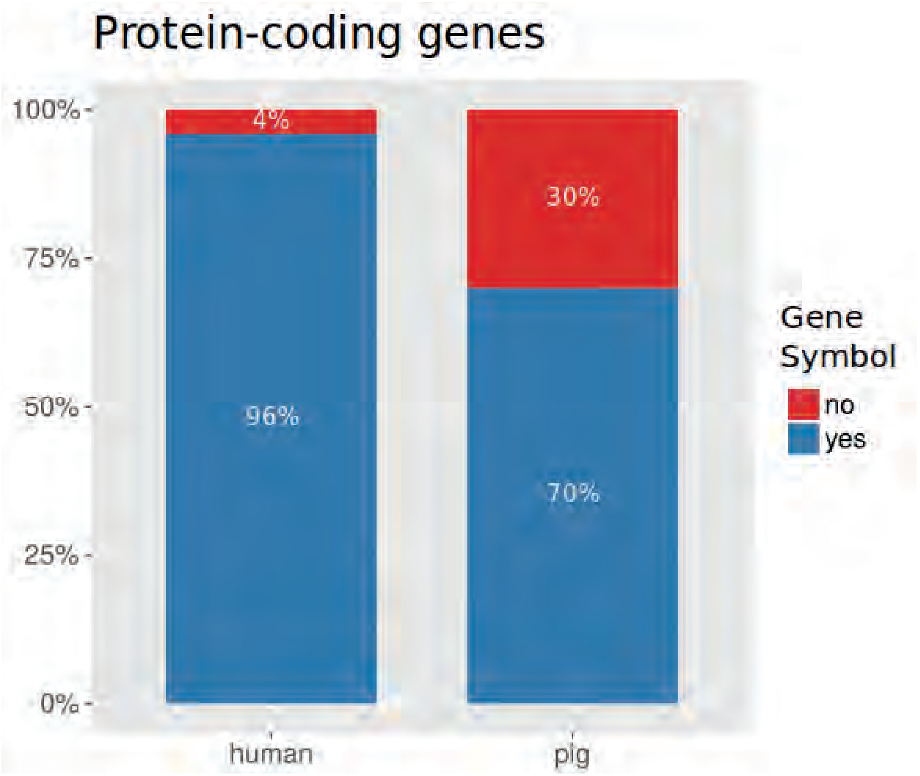
- Potential molecular players for the switch from endocrine to exocrine $PGF_{2a}$ secretion and the regulation of $PGE_2$ production (mechanism of prevention of luteolysis)
- FGF, IGF, EGFR, and TGFB growth factor systems – conceptus growth and endometrial remodeling
- Cell adhesion in preparation for attachment of the conceptus trophectoderm to the LE
- Maternal immune response to support conceptus growth and to avoid negative inflammatory effects
- Estrogen metabolism

**Main processes for DEGs on Day 14:**

- Control of uterine fluid secretion
- Trophoblast to endometrium adhesion
- Growth regulation by Wnt signaling
- Modulation of the maternal immune system
- Endometrial remodeling (mainly blood vessels and stroma)

# Problem of gene annotation in livestock and other species

- Improve functional annotation of DEG lists
- Many genes are not yet assigned to functional categories



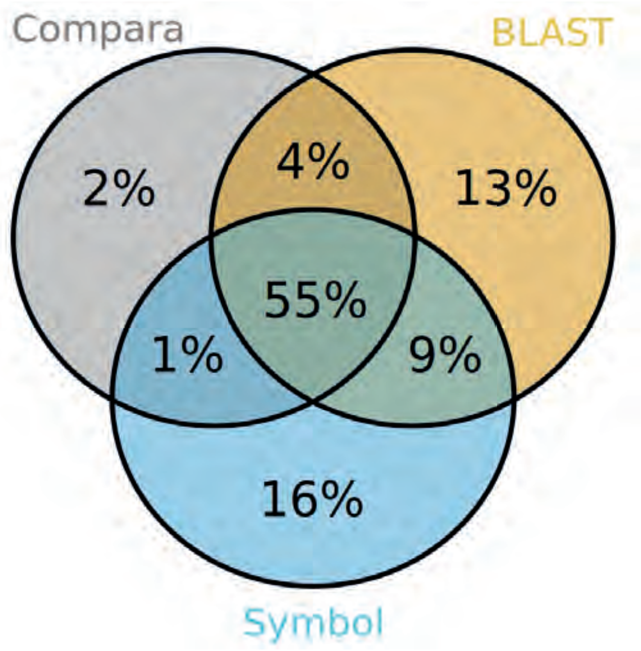
- Databases for microRNAs are incomplete

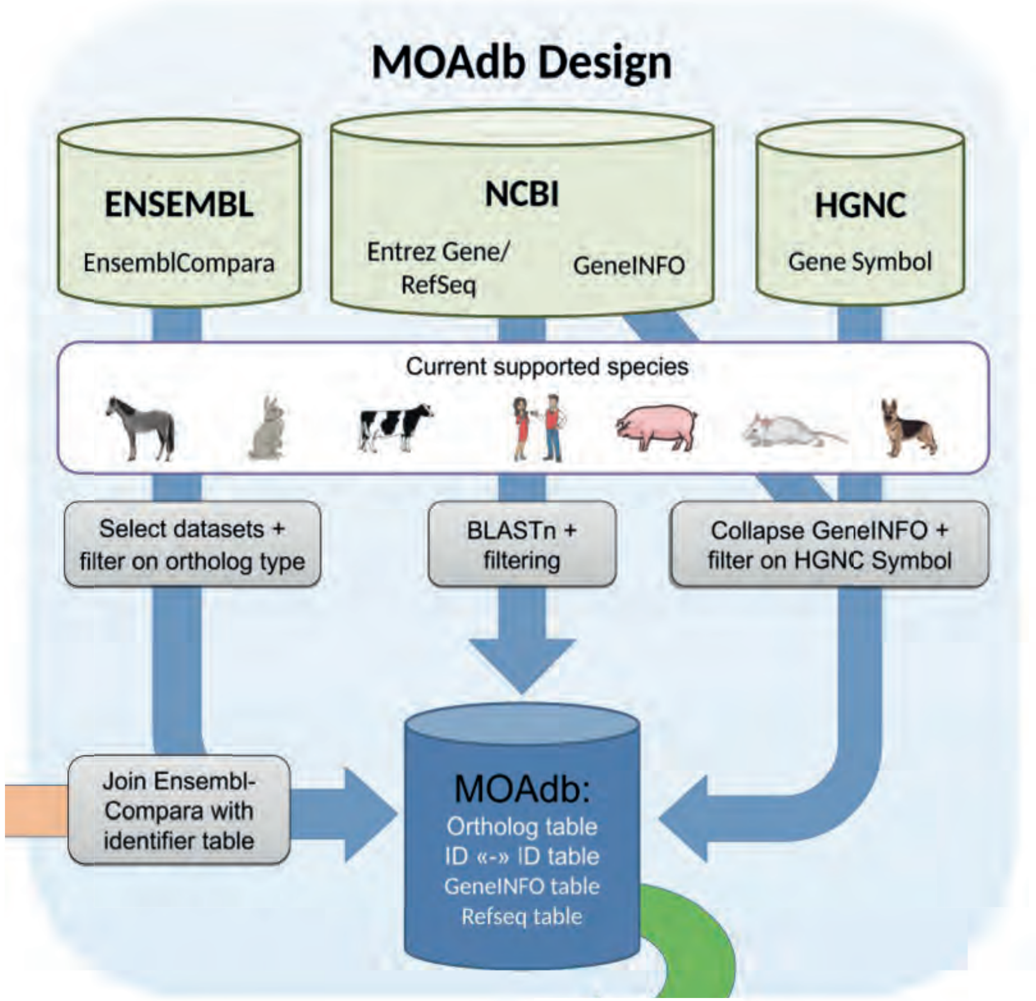
| **Species** | **Known miRNAs** |
|---|---|
| Human | ~ 2,000 |
| Cow | ~ 1,000 |
| Pig | ~ 400 |
| All species | ~ 40,000 |

Bick et al. Database 2019

# Mammalian Annotation database MAdb https://moadb.ethz.ch/

- cross-matching of official gene symbols
- using ortholog information from Ensembl Compara
- pairwise BLAST comparisons

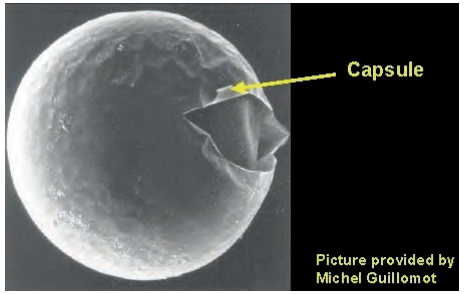




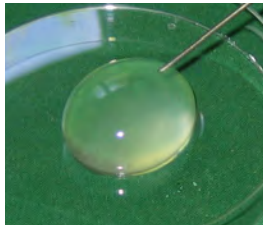
Bick et al. Database 2019

# Holistic analysis of embryo-maternal interactions in the mare

- Endometrial biopsies –> RNA analysis (LCM RNA-seq)
- Conceptus (pregnant mares) –> RNA analysis
- Uterine flush (uterine fluid) –> exosomes, free proteins
- Blood samples –> steroid hormones



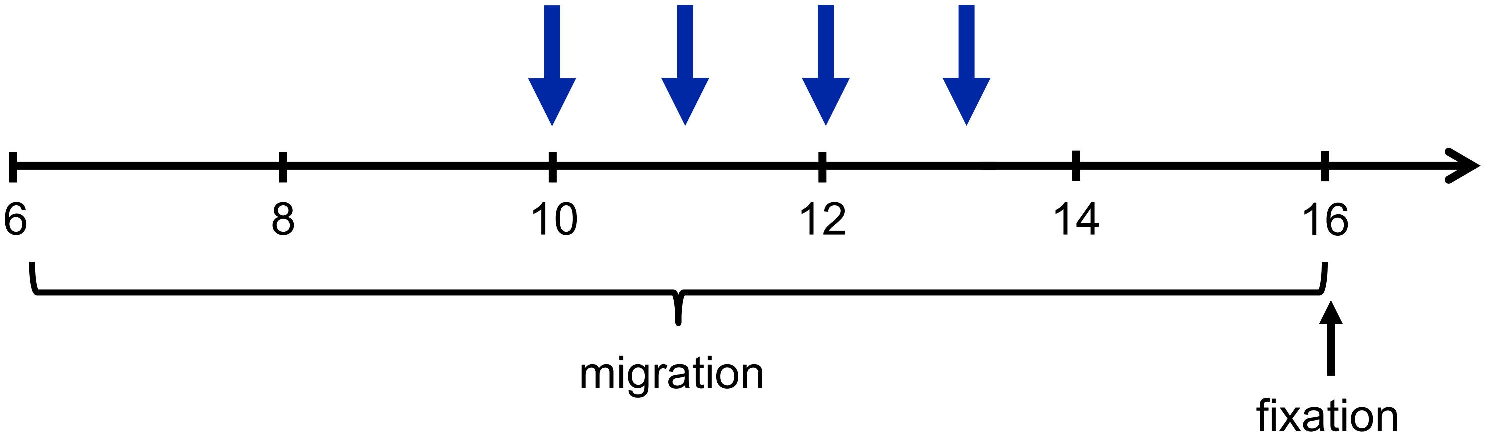
Picture provided by Michel Guillomot



E2, PGE2, etc.

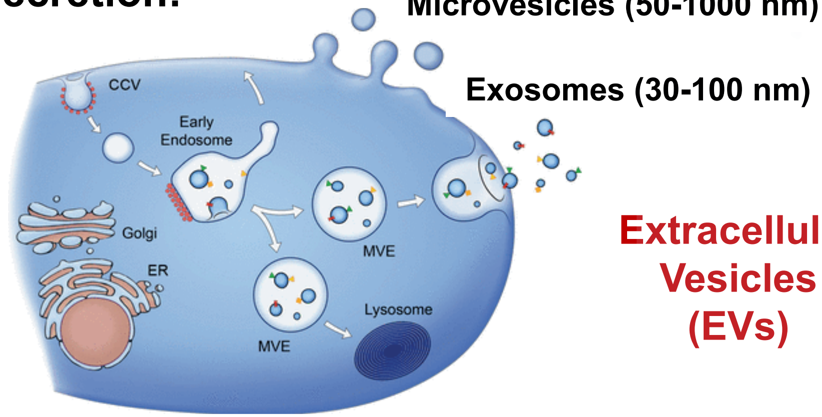
IFND

**M R P**

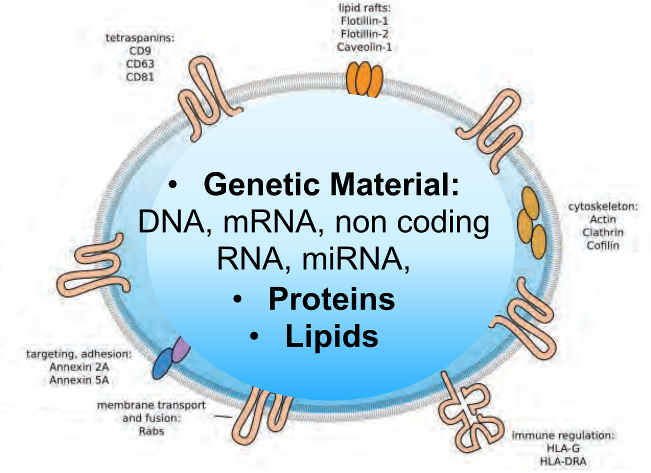


# Uterine extracellular vesicles (EVs): Mediators of embryo-maternal communication

## ❖ Secretion:



## ❖ Content:



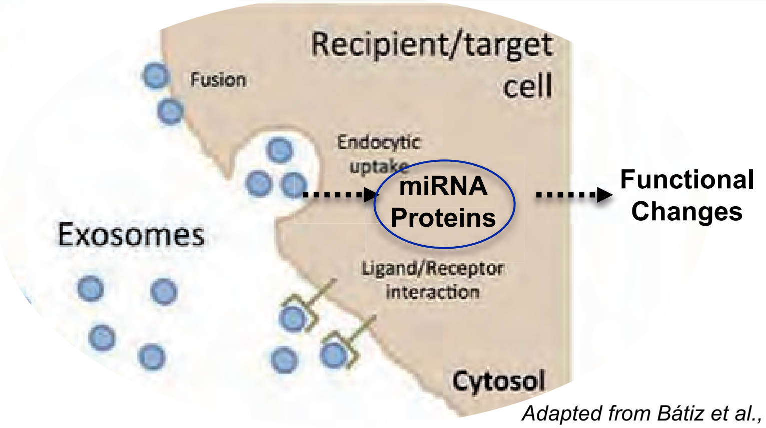
- **Genetic Material:** DNA, mRNA, non coding RNA, miRNA,
- **Proteins**
- **Lipids**

*Adapted from http://www.antibodies-online.com/*

**Extracellular Vesicles (EVs)**

## ❖ Exosomes

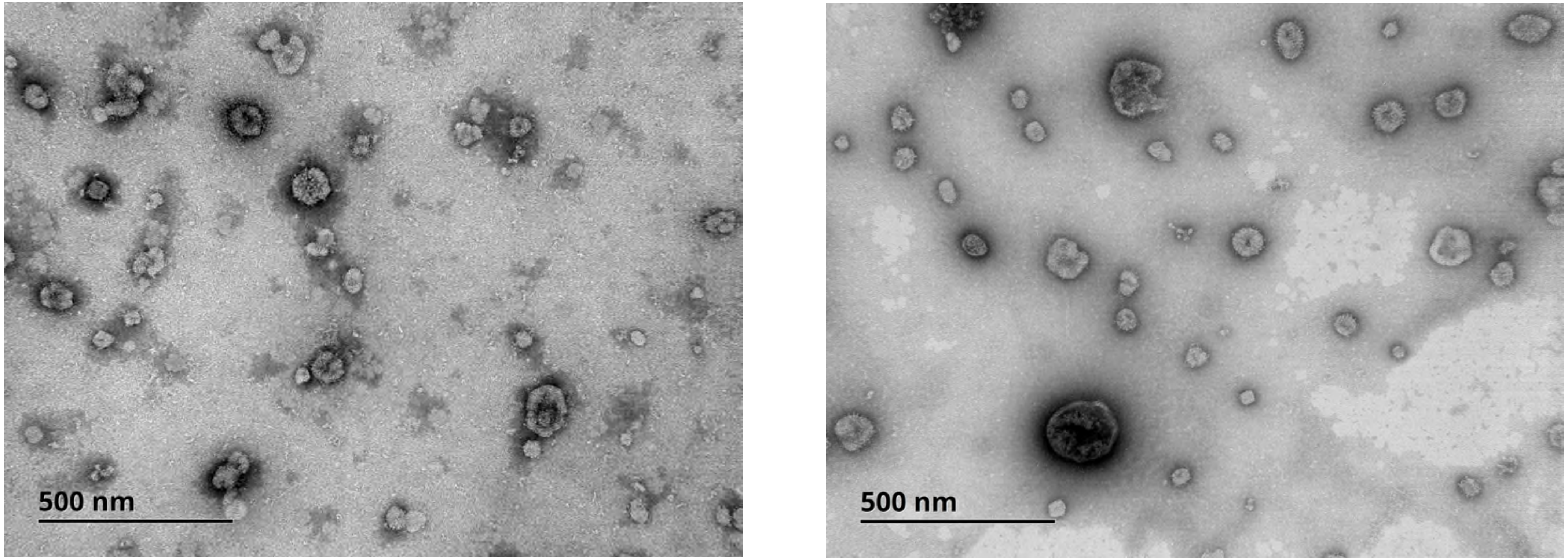
- Small membrane **vesicles** secreted by most cell types.
- Contain **genetic material, proteins and lipids.**
- **Ability to transfer their content** to target cells as a new way of **cell-to-cell communication**.
- Exert **functional effects in the recipient cells.**



*Adapted from Bátiz et al., 2016*

# Collection and characterization of uterine EVs

- Establishment and optimization of EVs isolation from uterine flush (70-80 ml)
- Comparison of different isolation methods and characterization by TEM, Western blot, QExactive HF-X mass spec proteomics, RNA analysis
- Aim: Comparative analysis of the uterine EVs (protein and RNA) in pregnant mares and corresponding cyclic controls



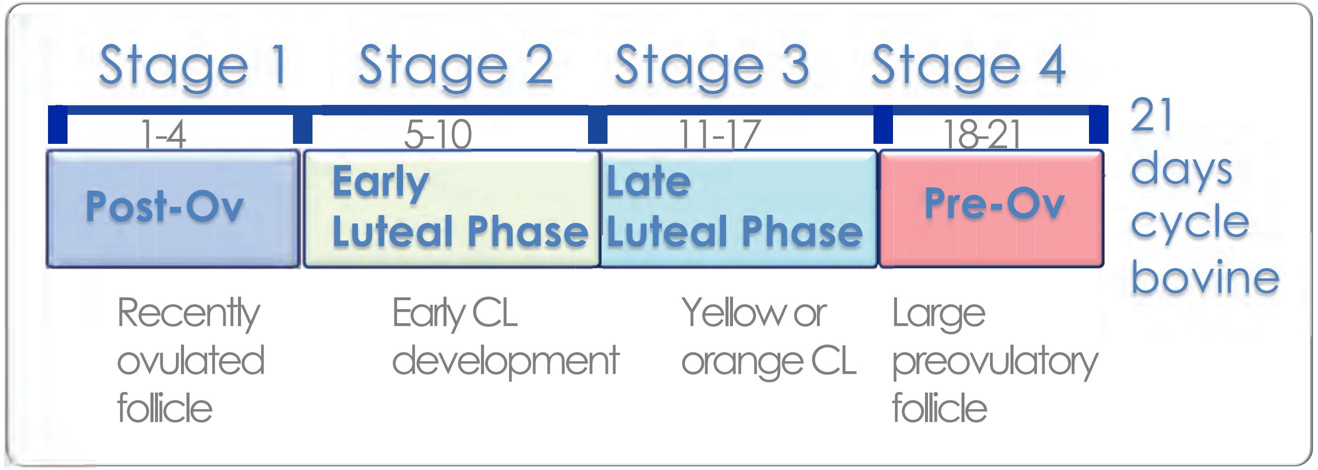
Transmission electron microscope (TEM) images of EVs derived from uterine fluid of the mare

EVs

# ➢ Characterization bovine oviductal EVs content

✓ **Different hormonal regulation (stages estrus cycle)**

**Oviductal fluid**



| Stage 1 | Stage 2 | Stage 3 | Stage 4 |
|---|---|---|---|
| 1-4 | 5-10 | 11-17 | 18-21 |
| **Post-Ov** | **Early Luteal Phase** | **Late Luteal Phase** | **Pre-Ov** |
| Recently ovulated follicle | Early CL development | Yellow or orange CL | Large preovulatory follicle |

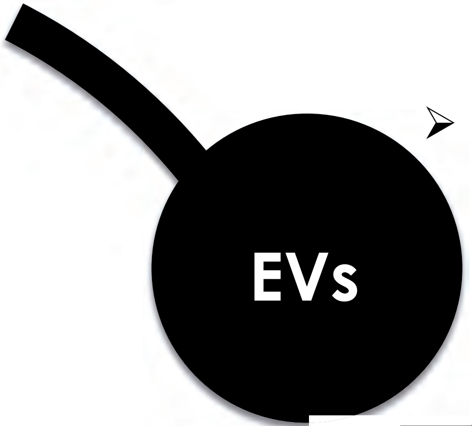
21 days cycle bovine

INRA SCIENCE & IMPACT

ETH Eidgenössische Technische Hochschule Zürich Swiss Federal Institute of Technology Zurich

Universität Zürich

*(Almiñana et al., 2018, BMC Genomics)*

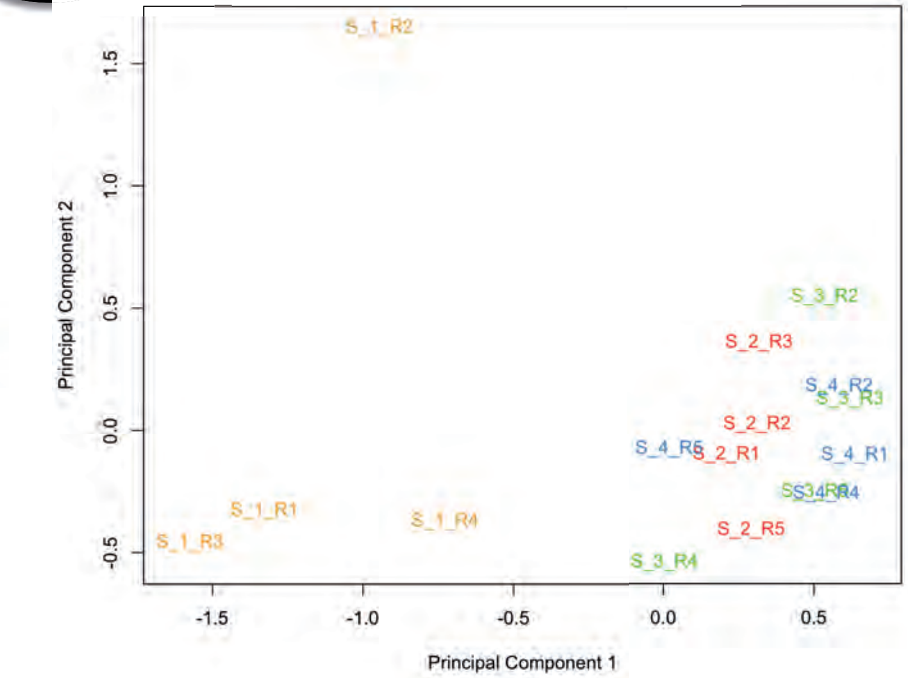


- ➢ Characterization oviductal EVs content

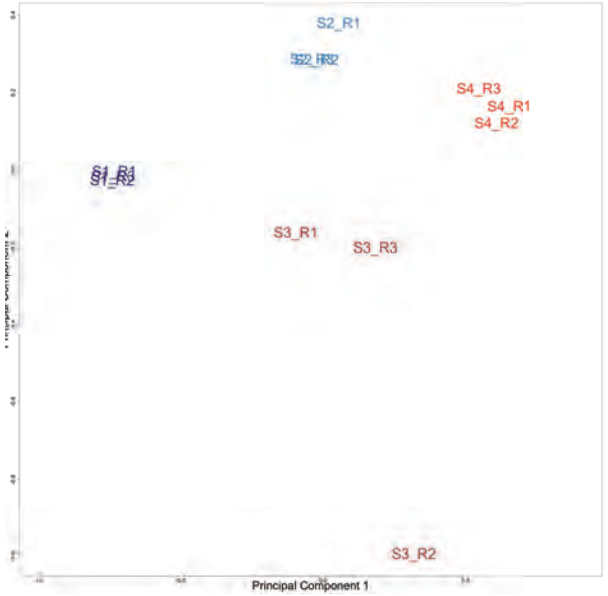
✓ **Different hormonal regulation (stages estrus cycle)**

**Principal Component Analysis (PCA)**

**mRNA content**

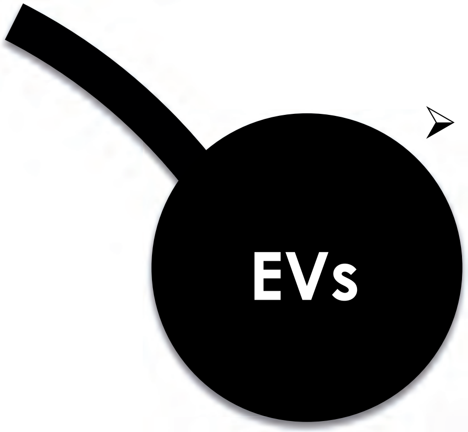


**Protein content**



- ➢ Identification of **mRNAs and proteins** involved in:
- **Sperm-oviduct interactions** (OVGP, HSP70; ANXA2)
- **Embryo development** (OVGP, HSPB1)

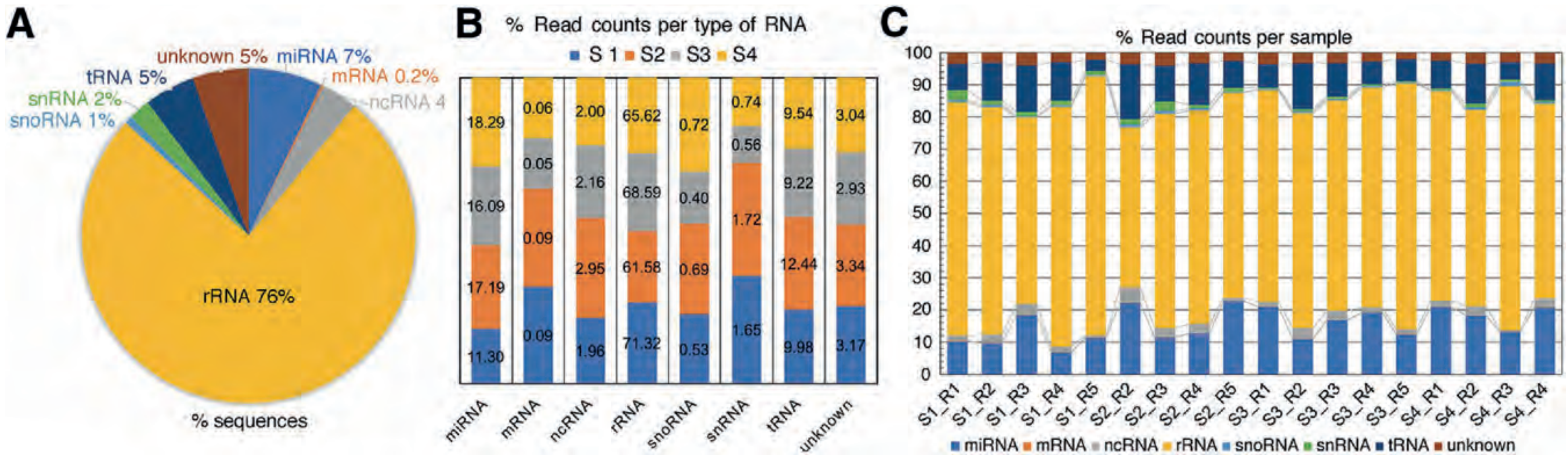
*(Almiñana et al., 2018, BMC Genomics)*



➢ Characterization oviductal EVs content

✓ **Different hormonal regulation (stages estrus cycle)**

**Small RNA content**



➢ Identification of **miRNAs** associated with:

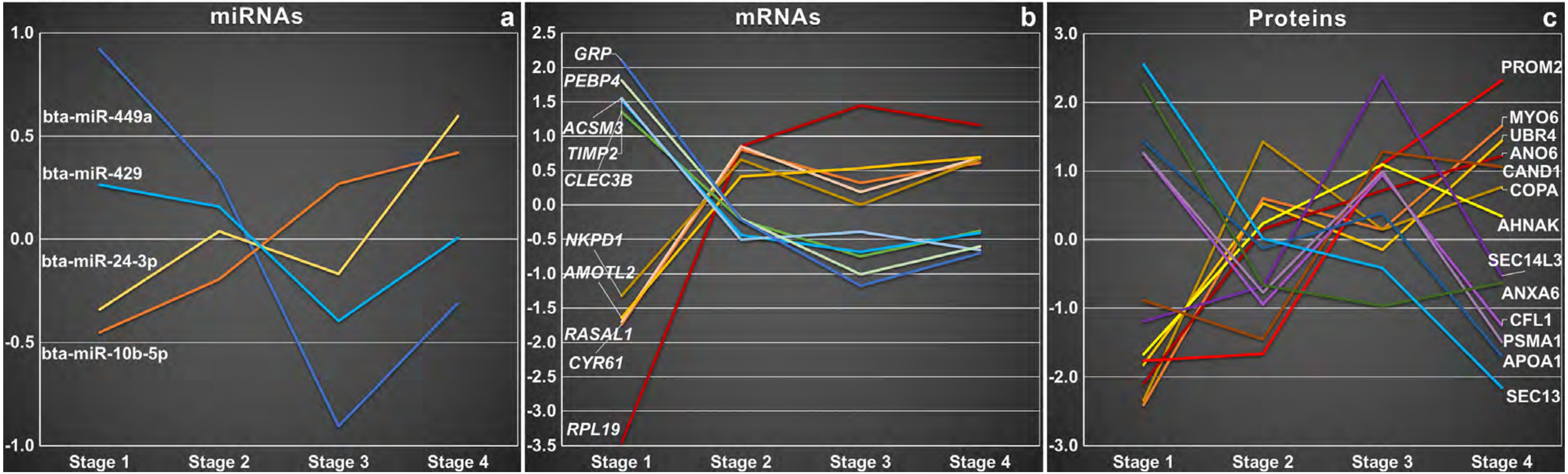
- **dysregulation of cilia, Infertility,** (bta-miR-449a)
- early phases of **embryonic development** (bta-miR-34b)

*(Almiñana et al., 2018, BMC Genomics)*

**EVs**

- Characterization oviductal EVs content
  - ✓ **Different hormonal regulation (stages estrous cycle)**

**Typical expression profiles**



*(Almiñana & Bauersachs., 2019, Bioengineering)*

# Analysis of subfertility in the mare

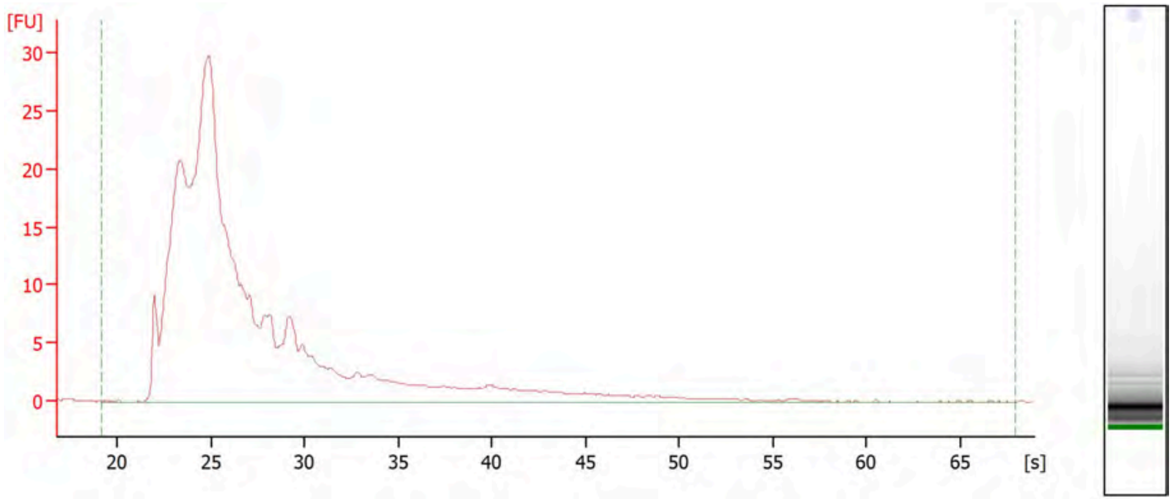
- Collection of cytobrush samples during estrus (1-3 days before ovulation) of 64 mares of the stud Lewitz (D)
- Group of fertile mares (pregnant after 1 AI, n=19)
- Group of mares with reduced fertility (no pregnancy after ≥3 AIs, n=12)
- Bacteriology and cytology to exclude mares with clinical endometritis
- Isolation of RNA and DNA from cytobrush samples
- RNA: RNA sequencing
- DNA: - PCR for selected bacterial and fungus species
  - 16S rRNA gene sequencing for the analysis of the uterine microbiome

Top: Mares of the stud Lewitz
Bottom: Processing of samples in the lab

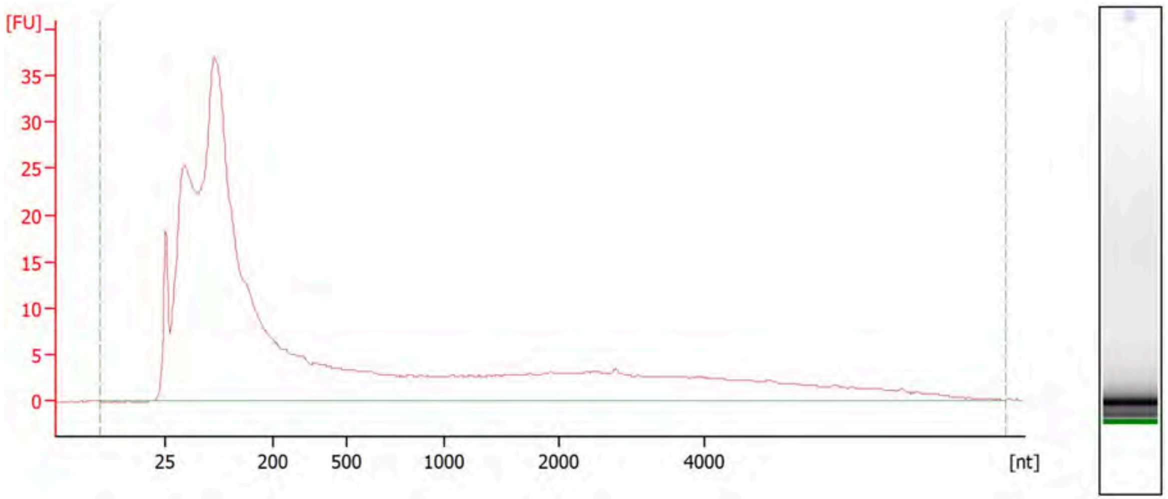
# Male fertility – analysis of small RNA in sperm

- Establishment of a total **RNA extraction protocol** for cryopreserved bovine semen
- Exploration of **sperm small RNA populations**
- Small RNA-seq from low-input samples and data analysis pipeline

Sperm samples from stallions with different fertility



RNA from bulls with different performance for sex-sorted sperm



# Thank you for your attention!!!

## Research station AgroVet-Strickhof

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