EFFECTS OF BOVINE OVIDUCT EPITHELIAL SPHEROIDS DURING *IN VITRO* EMBRYO CULTURE ON QUALITY OF EMBRYO AND TRANSCRIPTOMICS PATTERN

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ผลของเซลล์เยื่อบุท่อนำไข่โคแบบสเฟียรอยด์ในการเลี้ยงตัวอ่อนภายนอกร่างกายต่อคุณภาพของตัวอ่อนและรูปแบบทรานสคริปโตมิกส์

นางสาวธัญญา ประนมพนธ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาปรัชญาดุษฎีบัณฑิต
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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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ธัญญา ประนมพนธ์ : ผลของเซลล์เยื่อบุท่อนำไข่โคแบบสเฟียรอยด์ในการเลี้ยงตัวอ่อนภายนอกร่างกายต่อคุณภาพของตัวอ่อนและรูปแบบทรานสคริปโตมิกส์ (EFFECTS OF BOVINE OVIDUCT EPITHELIAL SPHEROIDS DURING *IN VITRO* EMBRYO CULTURE ON QUALITY OF EMBRYO AND TRANSCRIPTOMICS PATTERN) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร.รังสรรค์ พาลพ่าย, 132 หน้า.

คำสำคัญ: เซลล์เยื่อบุท่อนำไข่โคแบบสเฟียรอยด์/การเลี้ยงตัวอ่อนภายนอกร่างกาย/ทรานสคริปโตมิกส์

แบบจำลองภายนอกร่างกายของเซลล์เยื่อบุท่อนำไข่ ที่ใช้ศึกษาเชิงลึกเกี่ยวกับการสื่อสารระหว่างตัวอ่อนและแม่ ซึ่งเหนี่ยวนำให้เกิดเซลล์ดิฟเฟอเรนทิเอชันหรือมีความท้าทายทางด้านเทคนิคการใช้ ในการศึกษานี้ผู้วิจัยได้เสนอแบบจำลองเซลล์เยื่อบุท่อนำไข่โคแบบสเฟียรอยด์ (OES) ที่มีรูปทรงเฉพาะ ที่ขนาดเส้นผ่านศูนย์กลาง 100–200 ไมโครเมตร อย่างไรก็ตาม ปัจจุบันหนึ่งในการอภิปรายที่สำคัญที่สุดในช่วงการเจริญของตัวอ่อนก่อนระยะการฝังตัวภายนอกร่างกาย คือ ความเครียดจากปฏิกิริยาออกซิเดชันซึ่งส่งผลเสียต่อการพัฒนาและคุณภาพของตัวอ่อนระยะบลาสโตซิสต์ ผู้วิจัยทดสอบ OES เพื่อใช้เป็นแนวทางการเพาะเลี้ยงตัวอ่อนภายนอกร่างกายโดยการเพาะเลี้ยงร่วมแบบใหม่เพื่อสนับสนุนการเจริญของตัวอ่อนภายใต้สภาวะการเพาะเลี้ยงแบบปกติ (5% ออกซิเจน) และสภาวะที่มีความเครียดจากปฏิกิริยาออกซิเดชัน (20% ออกซิเจน)

ในการทดลองแรกมีวัตถุประสงค์เพื่อศึกษา 1) สภาวะการเพาะเลี้ยงที่เหมาะสมของ OES ในการเพาะเลี้ยงแบบแขวนลอย โดยการประเมินสัณฐานวิทยา จำนวนเซลล์ทั้งหมด ความมีชีวิต และกิจกรรมของเซลล์ที่มีขน; 2) ติดตามการแสดงออกของยีนใน OES ณ ช่วงเวลาที่มีการก่อตัว (วันที่ 0) และในช่วง 10 วันของการเพาะเลี้ยง และ 3) ทดสอบว่าตัวอ่อนระยะกำลังเจริญส่งผลต่อเกณฑ์คุณภาพ OES หรือไม่ จากผลการทดลอง ณ วันที่ 10 พบว่ามีสัดส่วนของ OES รูปทรงเวสิเคิล (V-OES) สูงใน M199/500 (เพาะเลี้ยงด้วยน้ำยา HEPES-buffered TCM-199 ปริมาตร 500 ไมโครลิตร) และในน้ำยาเลี้ยงตัวอ่อน synthetic oviduct fluid (SOF)/25 (หยดน้ำยา SOF ขนาด 25 ไมโครลิตร ภายใต้ mineral oil) สัดส่วนของเซลล์มีชีวิตใน V-OES ไม่ได้รับผลกระทบจากการเพาะเลี้ยงและยังคงสูง (>80%) จนถึงวันที่ 10 ของการเพาะเลี้ยง จำนวนเซลล์ทั้งหมดต่อ V-OES ลดลงเมื่อเวลาผ่านไป ยกเว้นใน SOF/25 ในขณะที่สัดส่วนของเซลล์ที่มีขนเพิ่มขึ้นเมื่อเวลาผ่านไปใน M199/500 แต่ลดลงใน M199/25 และ SOF/25 แอมพลิจูดของการเคลื่อนที่ของ OES ในการเพาะเลี้ยงแบบแขวนลอยลดลงเมื่อเวลาผ่านไปภายใต้สภาวะการเพาะเลี้ยงทั้งหมด นอกจากนี้ การแสดงออกของยีน *ANXA1*, *ESR1*, *HSPA8* และ *HSPA1A* ใน OES ยังคงมีเสถียรภาพในระหว่างการเพาะเลี้ยง ในขณะที่การแสดงออกของยีน *PGR* และ *OVGP1* ลดลงจากวันที่ 0 ถึงวันที่ 10 ท้ายสุดนี้ การเพาะเลี้ยงร่วมระหว่างตัวอ่อนระยะกำลังเจริญกับ OES ใน SOF/25 เพิ่มสัดส่วนของ V-OES เมื่อเทียบกับการเพาะเลี้ยง OES เพียงอย่างเดียว

การทดลองที่สองมีวัตถุประสงค์เพื่อตรวจสอบผลของ OES ต่อการเจริญและคุณภาพของตัวอ่อนในแง่ของจำนวนเซลล์บลาสโตซิสต์และทรานสคริปโตมของบลาสโตซิสต์ตามช่วงเวลาการเพาะเลี้ยงร่วม (4 วัน เทียบกับ 7 วัน) และปริมาณของออกซิเจน (5% เทียบกับ 20%) ภายใต้สภาวะ 5% ออกซิเจน OES เพิ่มจำนวนเซลล์ต่อบลาสโตซิสต์เมื่อเพาะเลี้ยง OES ร่วมกับตัวอ่อนเป็นเวลา 7 วัน (137.6 ± 10.8 เทียบกับ 102.6 ± 8.4 เซลล์ในกลุ่มควบคุม; P <0.05) ภายใต้สภาวะ 20% ออกซิเจน OES เพิ่มอัตราตัวอ่อนระยะบลาสโตซิสต์ในวันที่ 7 และ 8 อย่างมีนัยสำคัญทางสถิติ (30.7% และ 31.8% สำหรับ 4 และ 7 วัน ของการเพาะเลี้ยงร่วมเทียบกับ 19.8% ในกลุ่มควบคุม; P<0.05) และเพิ่มจำนวนเซลล์ต่อบลาสโตซิสต์โดยไม่มีความแตกต่างระหว่างการเพาะเลี้ยงร่วม 4 และ 7 วัน (112.7 ± 7.8. และ 138.1 ± 10.5 เซลล์ ตามลำดับ เทียบกับ 82.1 ± 4.5 เซลล์ในกลุ่มควบคุม; P <0.0001) จำนวนและสัดส่วนสูงสุดของยีนที่มีการแสดงออกแตกต่างกัน (DEGs) แบบเฉพาะพบในการเพาะเลี้ยงร่วมกับ OES 7 วัน เทียบกับกลุ่มควบคุมภายใต้ 20% ออกซิเจน ท้ายสุดนี้ คำศัพท์ด้านหน้าที่ ซึ่งพบมากที่สุดของ DEGs ที่ได้รับผลกระทบจากการเพาะเลี้ยงร่วม OES โดยไม่คำนึงถึงระดับออกซิเจนและเวลาของการเพาะเลี้ยงร่วม รวมถึง "การเผาผลาญของไขมัน" "การตอบสนองต่อการกระตุ้นภายนอกเซลล์" "การตอบสนองต่อระดับออกซิเจน"

โดยสรุป ผลลัพธ์เหล่านี้ชี้ว่า OES เป็นแบบจำลองที่ใช้งานง่าย ได้มาตรฐาน และเป็นแบบจำลองทางสรีรวิทยาเพื่อศึกษาปฏิสัมพันธ์ระหว่างตัวอ่อนกับแม่ในโค ซึ่งเป็นการศึกษาแรกที่รายงานผลสนับสนุนของ OES ต่อการเจริญของตัวอ่อนและผลสนับสนุนของตัวอ่อนระยะกำลังเจริญต่อสัณฐานวิทยาของ OES นอกจากนี้ OES ยังเปลี่ยนแปลงรูปแบบทรานสคริปโตมของตัวอ่อนโดยมีผลกระทบสูงสุดภายใต้สภาวะความเครียดจากปฏิกิริยาออกซิเดชัน ซึ่งพิสูจน์ให้เห็นเป็นครั้งแรกถึงการปรับเปลี่ยนการโต้ตอบของตัวอ่อนและ OES ตามสภาพแวดล้อมของตัวอ่อน

สาขาวิชาเทคโนโลยีชีวภาพ
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ลายมือชื่อนักศึกษา _____
ลายมือชื่ออาจารย์ที่ปรึกษา _____
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม _____

THANYA PRANOMPHON : EFFECTS OF BOVINE OVIDUCT EPITHELIAL SPHEROIDS DURING *IN VITRO* EMBRYO CULTURE ON QUALITY OF EMBRYO AND TRANSCRIPTOMICS PATTERN. THESIS ADVISOR : ASSOC. PROF. RANGSUN PARNPAI, Ph.D. 132 PP.

Keyword: Bovine Oviduct Epithelial Spheroids/*In Vitro* Embryo Culture/Transcriptomics

Most *in vitro* models of oviduct epithelial cells (OEC) used thus far to gain insights into embryo–maternal communication induce cell dedifferentiation or are technically challenging. Here, we propose a model based on bovine oviduct epithelial spheroids (OES) with specific shape and diameter (100–200 µm) criteria. So far, however, one of the most significant discussions during pre-implantation embryo development *in vitro* is oxidative stress with deleterious effects on blastocyst development and quality. We tested OES as a new co-culture approach to support embryo development under both usual (5% O_2) and oxidative stress (20% O_2) culture conditions.

The objectives in the first experiment were to 1) determine the appropriate culture conditions of bovine OES cultured in suspension by evaluating their morphology, total cell number, viability, and activity of ciliated cells; 2) monitor gene expression in OES at the time of their formation (day 0) and over the 10 days of culture; and 3) test whether the vicinity of developing embryos affects OES quality criteria. On day 10, the proportions of vesicle-shaped OES (V-OES) were higher in M199/500 (500 µL of HEPES-buffered TCM-199) and synthetic oviduct fluid (SOF)/25 (25-µL droplet of SOF medium under mineral oil) than in M199/25 (25-µL droplet of M199 under mineral oil). The proportion of viable cells in V-OES was not affected by culture conditions and remained high (>80%) through day 10. The total number of cells per V-OES decreased over time except in SOF/25, while the proportions of ciliated cells increased over time in M199/500 but decreased in M199/25 and SOF/25. The movement amplitude of OES in suspension decreased over time under all culture conditions. Moreover, the gene expression of *ANXA1*, *ESR1*, *HSPA8*, and *HSPA1A* in OES remained stable during culture, while that of *PGR* and *OVGP1* decreased from day 0 to day 10. Last, the co-culture of developing embryos with OES in SOF/25 increased the proportion of V-OES compared to OES cultured alone.

The objective in the second experiment was to examine the effect of OES on embryo development and quality in terms of blastocyst cell numbers and blastocyst transcriptome according to the co-culture time (4 vs 7 days) and oxygen tensions (5%

vs. 20%). Under 5% O_2, the presence of OES increased the number of cells per blastocyst when OES were co-cultured for 7 days (137.6 ± 10.8 vs. 102.6 ± 8.4 cells in control; $P<0.05$). Under 20% O_2, the presence of OES significantly increased blastocyst rates on days 7 and 8 (30.7% and 31.8% for 4 and 7 days vs. 19.8% in controls; $P<0.05$) and cell numbers per blastocyst with no difference between 4 and 7 days of co-culture (112.7 ± 7.8. and 138.1 ± 10.5 cells, respectively, vs. 82.1 ± 4.5 cells in controls; $P<0.0001$). The highest number and proportion of specific differentially expressed genes (DEGs) was observed for the 7dOES vs. control under 20% oxygen. Finally, the most enriched functional terms of DEGs impacted by OES co-cultured regardless of the oxygen level and time of co-culture including "metabolism of lipids", "response to extracellular stimulus", "response to oxygen levels".

In conclusion, these results point to OES as an easy-to-use, standardizable, and physiological model to study embryo–maternal interactions in cattle. This is the first study reporting the supportive effect of OES on embryo development and of developing embryos on OES morphology. The OES altered the embryonic transcriptome with the highest impact under oxidative stress conditions, also evidencing for the first time a modulation of the embryo-OES dialog according to the embryonic environment.

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Thanya Pranomphon

TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

Page

TABLE OF CONTENTS (Continued)

Page

TABLE OF CONTENTS (Continued)

LIST OF TABLES

Table **Page**

LIST OF TABLES (Continued)

Table **Page**

LIST OF FIGURES

Figure **Page**

LIST OF FIGURES (Continued)

Figure **Page**

LIST OF ABBREVIATIONS

ALI	air-liquid interphase
ARTs	assisted reproductive technologies
BSA	bovine serum albumin
CAT	catalase
cDNA	complementary DNA
CL	corpus luteum
cm	centimetre
CM-H2DCFDA	chloromethyl-2′-7′-dichlorodihydrofluorescein diacetate
CO_2	carbon dioxide
COCs	cumulus-oocyte complexes
CSH	cysteamine
Cu	copper
Cu,Zn-SOD	copper, zinc superoxide dismutase
DCF	2′,7′-dichlorofluorescein
DEGs	differentially expressed genes
E1	estrone
E2	estradiol
E3	estriol
ECM	extracellular matrix
EGA	embryonic genome activation
EGF	epidermal growth factor
ET	embryo transfer
ETC	electron transport chain
EVs	extracellular vesicles
FBS	fetal bovine serum
FCS	fetal calf serum
FGF	fibroblast growth factor
FSH	follicle stimulating hormone
FTAI	fixed-time artificial insemination
GnRH	gonadotropin-releasing hormone
GPx	glutathione peroxidase
GPx-4	glutathione peroxidase 4
GR	glutathione reductase
GSH	glutathione
GSSG	oxidized glutathione
GST	glutathione S-transferase

LIST OF ABBREVIATIONS (Continued)

H^+	proton
H_2O	water
H_2O_2	hydrogen peroxide
HPLC	high performance liquid chromatography
HPLC-ESI-MS/MS	high-performance liquid chromatography-mass spectrometry
ICM	inner cell mass
ICSI	intracytoplasmic sperm injection
IFNT	interferon tau
IGF	insulin-like growth factor
IGFBP	IGF-binding protein
IMFs	isthmic mucosa fragments
IVC	*in vivo* culture
IVD	*in vivo* development
IVF	*in vivo* fertilization
IVM	*in vivo* maturation
IVP	*in vivo* embryo production
LH	luteinizing hormone
M	molar
M199	HEPES-buffered TCM-199
MET	maternal-to-embryonic transition
min	minute
mL	millilitre
mm	millimeter
MOET	multiple ovulation embryo transfer
mRNA	messenger ribonucleic acid
MVs	microvesicles
N_2	nitrogen
$NADP^+$	oxidized nicotinamide adenine dinucleotide phosphate
NADPH	reduced nicotinamide adenine dinucleotide phosphate
$NO^{\bullet}$	nitric oxide
NO_2	nitrogen dioxide
NOX	NADH oxidase
O_2	oxygen
$O_2^{\bullet-}$	superoxide anion
°C	degree Celsius
OEC	oviduct epithelial cells
OES	bovine oviduct epithelial spheroids
oEVs	*in vivo* EVs

LIST OF ABBREVIATIONS (Continued)

OF	oviductal fluid
$OH^{\bullet}$	hydroxyl
$ONOO^{-}$	peroxynitrite
OPU	ovum pick-up
P_4	progesterone
PBS	phosphate buffered saline
PGF	prostaglandin F2 alpha
PGs	prostaglandins
Prxs	peroxiredoxins
PVA	polyvinyl alcohol
RNA-seq	RNA sequencing
RNS	reactive nitrogen species
ROH	organic alcohol
ROOH	organic hydroperoxides
-ROOH	reduction of organic hydroperoxides
ROS	reactive oxygen species
RT	room temperature
RT-qPCR	reverse transcription-quantitative polymerase chain reaction
SCNT	somatic cell nuclear transfer
Se	selenium
sec	second
SOD	superoxide dismutase
SOF	synthetic oviduct fluid
SS	sexed semen
T°	temperature
TE	trophectoderm
TGF	transforming growth factor
$Trx(SH)_2$	reduced thioredoxin
$TrxS_2$	oxidized thioredoxin
UF	uterine fluid
VOCs	volatile organic compounds
V-OES	vesicle-shaped OES
ZGA	embryonic genome activation
Zn	zinc
µg	microgram
µL	microliter
µm	micrometre
µM	micromolar

CHAPTER 1
INTRODUCTION

1.1 General introduction

Assisted reproductive technologies (ARTs) have been gaining attention to enhance infertility treatment and animal breeding (Fluks et al., 2024). ARTs offer the opportunity to increase the number of embryos consequent to a higher number of healthy offspring with desired sex and genetic trait per unit of time (Ferré et al., 2020a; Besenfelder & Havlicek, 2023). Examples of ARTs are fixed-time artificial insemination (FTAI), sexed semen (SS), *in vitro* embryo production (IVP), multiple ovulation embryo transfer (MOET), somatic cell nuclear transfer (SCNT), intracytoplasmic sperm injection (ICSI) and others (Velazquez, 2008; Menchaca, 2023). Nevertheless, major factors limiting IVP are the quality of embryos produced *in vitro* that is still less efficient than those *in vivo* (Maillo et al., 2016). Therefore, IVP has been extensively researched in many aspects including applying *in vitro* models of oviduct epithelial cells (OEC) in a co-cultured system.

Crucial events for early embryo development take place in the oviduct: the first mitotic cell divisions, the consumption and decrease of maternal mRNA stored in the oocyte, the embryonic genome activation (EGA) at the 8-16 cell stage, the morula compaction (16-32 cells) during which the first cell lineages differentiate (Maillo et al., 2016). Then the embryo reaches the blastocyst stage and enters the uterus for further development. Any dysfunction or interruption of these key steps may lead to early embryo loss or embryos with lower aptitude to development.

Thus, the oviduct endorses major functions to secure the success of pregnancy (Li & Winuthayanon, 2017). Although *in vitro* embryo production has been successfully achieved in different species, it bypasses this oviduct milieu, resulting in low success in terms of blastocyst rate and capacity to give birth to healthy offspring (Ferré et al., 2020a). Therefore, oviduct epithelial cells (OEC) cultured *in vitro* have been widely used to mimic the maternal environment and improve embryo development in different species, including rabbits (Carney et al., 1990), sheep (Gandolfi & Moor, 1987), goats (Yadav et al., 1998), mice, pigs, cattle, and humans (Li

& Winuthayanon, 2017). Co-culturing with OEC has been shown to enhance blastocyst quality in terms of cell numbers, cryotolerance (Schmaltz-Panneau et al., 2015), and expression of target genes in cattle (Cordova et al., 2014), as well as pregnancy rates in humans (Yeung et al., 1992), red deer (Locatelli et al., 2005), and goats (Rodríguez-Dorta et al., 2007).

Nevertheless, most *in vitro* models of oviduct epithelial cells (OEC) have been shown to induce cell dedifferentiation or are technically challenging. OEC monolayers grown on plastic dishes rapidly dedifferentiate, losing morphological criteria of cell differentiation like cilia and secretory granules after 3 days in culture (Walter, 1995). Moreover, after 5 to 10 days in culture, OEC displayed a decrease in gene expression of steroid hormone receptors and oviduct-specific glycoprotein 1 (*OVGP1*) (Schmaltz-Panneau et al., 2015). As an alternative system, OEC monolayers grown on inserts in air-liquid interphase (ALI) allow to maintain a steady differentiation status for several weeks of culture. However, this model requires a long time to grow to confluence (three to four weeks, depending on the species), is technically challenging and so far, did not show any supporting effect on development of co-cultured bovine embryos (Chen et al., 2017; van der Weijden et al., 2017). In the same way, oviduct-on-a-chip platforms are promising but technically challenging. Ferraz et al (2017) succeeded in applying this device for IVF and IVC, however the cleavage rate and 8-16 cells formation were lower than optimised standard *in vitro* embryo production. OEC organoids developed from oviduct epithelial progenitor cells are capable of maintaining stemness, however their production is long and challenging (Schoen & Chen, 2018). In addition, the apical side of OEC is inside the organoid, making it difficult to interact with developing embryos without microdissection. OEC explants or aggregates in suspension culture have been proposed as an alternative. Rottmayer et al. (2006) proposed a short time suspension culture of OEC aggregates for 24 h and evidenced a maintenance of their morphology and stable gene expression of *OVGP1* and steroid hormone receptors after 6 h of culture. However, the possibility of culturing OEC in suspension during the time needed for supporting embryo development, i.e., 7-9 days, has not yet been assessed. Here, we propose a model based on **oviduct epithelial spheroids (OES)**, which form rapidly (within 48–72 h) from isthmic mucosa fragments (IMF), containing an already-differentiated epithelium. We previously reported OESs with

specific shape and size criteria as a good model to study sperm–oviduct interactions (Mahé et al., 2023). Here we proposed to use OES for co-culture with embryos.

In vitro culture and manipulations generate physical and metabolic stress, resulting in oxidative stress that affects early embryo development (Guérin et al., 2001; Hardy et al., 2021; Agarwal et al., 2022; Deluao et al., 2022; Mauchart et al., 2023). Even under low oxygen concentration (5% O_2), reactive oxygen species (ROS) accumulate in the embryo blastomeres up to the morula stage (Dalvit et al., 2005). Here we proposed to use OES to consume the O_2 in excess and produce ROS scavenger proteins, thus overcoming oxidative stress during *in vitro* embryo development. Based on our previous data using oviduct fluid and OECs, the OES are also assumed to secrete soluble proteins, metabolites and extracellular vesicles containing coding and non-coding RNAs, proteins, lipids and metabolites with supporting effects on cell mitosis and gene expression in the developing embryos (Lamy et al., 2016; Banliat et al., 2020; Bauersachs et al., 2020; de Alcântara-Neto et al., 2022). The EGA is a key step occurring around 4-5 days after fertilization (at the 8-16 cell stage) and which highly differs in terms of gene and protein expression pathways between *in vivo* and *in vitro* produced bovine embryos (Gad et al., 2012; Cao et al., 2014; Banliat et al., 2022a; Banliat et al., 2022b). In this thesis, we tested the hypothesis that the exposure to OES up to the EGA (i.e. during 5 days) would be enough to support embryo development and gene expression under usual culture (5% O_2 and 5% CO_2) and oxidative stress culture conditions (5% CO_2 in air).

1.2 Objectives of the thesis

This introduction showed that *in vitro* production of mammalian embryos still requires improvements, particularly regarding the developmental competence of the resulting blastocysts. It has been clearly demonstrated that the maternal environment (the oviduct) plays a major role in orienting embryo development toward high developmental potential. Therefore, a better knowledge of embryo–maternal communication in the oviduct would open the way for the set-up of optimised *in vitro* development systems. This knowledge would highly benefit from relevant *in vitro* systems allowing to mimic and analyse embryo – maternal communication and the factors involved in this dialogue. Here we proposed a new model of oviduct, close to physiological conditions. Consequently, the objectives of this thesis were:

1.2.1 To characterize oviduct epithelial spheroids (OES) as a new *in vitro* model for the study of embryo-maternal dialog.

1.2.2 To study the effect of OES on embryo development and quality according to the time of exposure (4 vs 7 days) and oxygen levels (5% vs. 20%).

1.2.3 To study the effect of OES on embryo gene expression according to the time of exposure (4 vs 7 days) and oxygen levels (5% vs. 20%).

CHAPTER 2
LITERATURE REVIEWS

2.1 The mammalian oviduct, a complex and dynamic environment for early embryo development

2.1.1 Anatomy and histology of the oviduct

The oviduct is a part of the female reproductive tract, a tube, connecting ovary to uterus, and constitutes a crucial organ for early reproductive events (Maillo et al., 2016; Coy et al., 2018; Winuthayanon & Li, 2018). The oviducts are also called uterine tubes or fallopian tubes in humans. They are thin paired and symmetrical tubular organs located between the ovary and the uterus and connected to the pelvic wall by the broad ligament that contains blood vessels, nerves, and lymphatics tissues (Fig. 2.1).

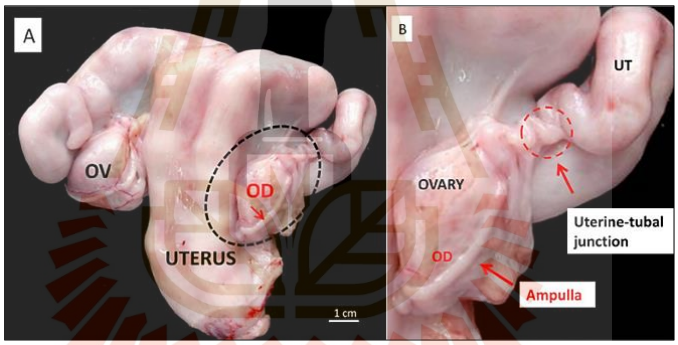

Figure 2.1 Bovine female genital tract. (A, B) The uterus (UT), the ovary (OV), and the oviduct (OD) are shown in detail (Avilés et al., 2015).

The oviducts are composed of three parts: **i) the infundibulum**, is the funnel-shaped section covering the ovary and contains the fimbria that is responsible to pick up the cumulus-oocyte complex at the time of ovulation and drive it into the oviduct by cilia beating; **ii) the ampulla** is the widest part of the oviduct in which takes place the final maturation of the gametes and the fertilization; **and iii) the isthmus** is the narrowest portion and the site for sperm reservoir (spermatozoa bind to the ciliated epithelial cells) and early embryo development during the first 4-5 days after fertilization in cattle. The utero-tubal junction is a selective barrier allowing the passage of viable spermatozoa from the uterus in order to fertilize the egg (Mahé et al., 2021) and the entry of the embryo into the uterus for further differentiation

and implantation (Maillo et al., 2016; Coy et al., 2018; Winuthayanon & Li, 2018) (Fig. 2.2).

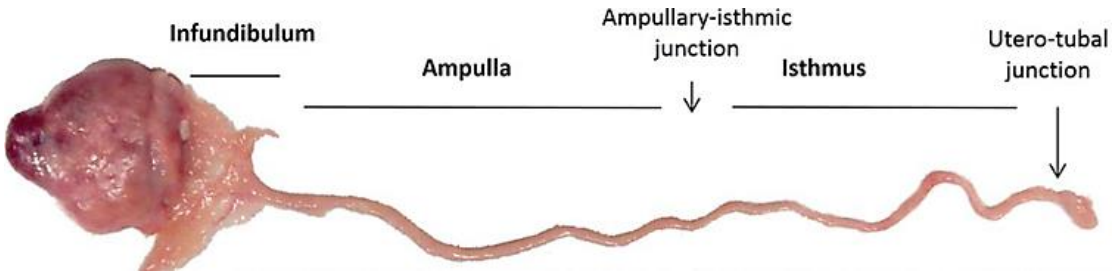

Figure 2.2 Anatomy and regions of the bovine oviduct (Maillo et al., 2016).

The oviduct wall consists in four cell layers: **i) the outer serosa** is formed mainly by connective tissue; **ii) the muscularis** is formed mainly by smooth muscular cells. It consists of an outer longitudinal muscle layer and an inner circular muscle layer that are responsible for peristaltic contractions to convey gametes (egg and sperm), early embryo, and the secretory products; **iii) the submucosa** with blood vessels, nerves, and lymphatics; **and iv) the mucosa** containing an epithelium, which is a simple columnar cell layer that consists of ciliated and non-ciliated (secretory) cells. Underneath the epithelium is the lamina propria which maintains as well as nourishes the lining of mucosa as it consists of microcapillary vessels and connective tissue with fibroblasts (or stromal cells) (Barton et al., 2020), immune cells, and progenitor cells expressing the leucine-rich repeat-containing G-protein-coupled receptor 5 (LGR5) (Ng et al., 2014; Snegovskikh et al., 2014; Winuthayanon & Li, 2018) (Fig. 2.3).

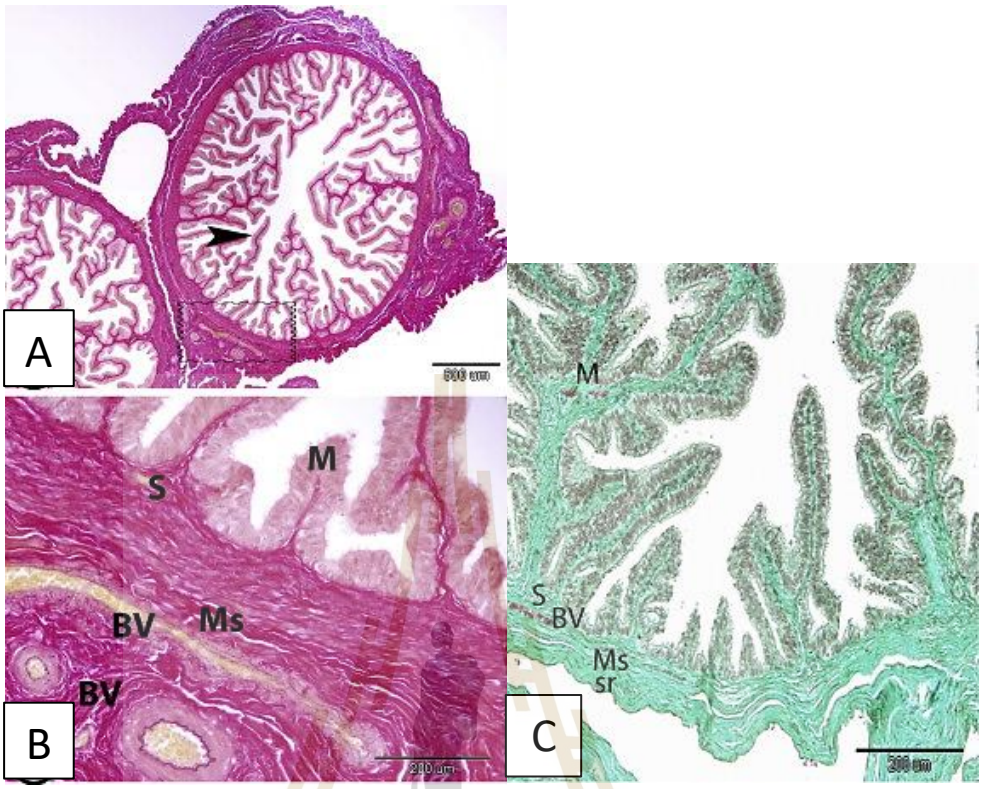

Figure 2.3 Histology of bovine oviduct. (A) The ampulla stained by Van Geison Resorcin Fuchsin (arrowhead; highly folded mucosa with large number of leaf-like folds). (B) Magnified square from (A). (C) The ampulla stained by Goldner's Trichrome. Mucosa (M), submucosa (S), muscularis (Ms), serosa (Sr) and blood vessels (BV) (Mokhtar, 2015).

In the oviduct epithelium, the ciliated cells display motile cilia that are complex structures containing tubulin protein, located at the apical surface of cells (Coy et al., 2018), while the secretory cells lack cilia and present microvilli and secretory granules at the apical membrane (Winuthayanon & Li, 2018) (Fig. 2.4).

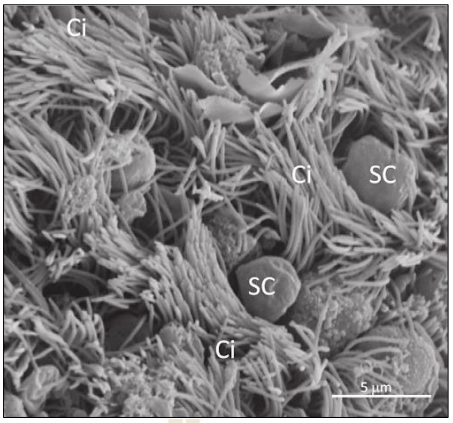

Figure 2.4 Epithelial cells of the bovine oviduct observed by scanning electron microscope. The ciliated cells with numerous cilia (Ci) and the secretory cells (SC) (Avilés et al., 2015).

Moreover, microvilli and primary cilia were also observed at the surface of the secretory cells, the latter associated with the sensory mechanism (Coy et al., 2018). Ciliated cells facilitate the transportation that occurs within the oviduct by ciliary beating; oocyte pick up, embryo transport and tubal fluid flow, while secretory cells participate to the production of the oviductal fluid (OF), mixture between specific secretory components and the transudation (passage of serum components through cell membrane by osmotic pressure) from the blood and the interstitial tissue, and intracytoplasmic content of the dead epithelial cells (Hunter, 1988; Maillo et al., 2016; Winuthayanon & Li, 2018; Saint-Dizier et al., 2019). Considering the anatomical region of the oviduct, the epithelial (mucosal) folds are abundant and complex in infundibulum and ampulla segments while there are fewer, simpler and smaller in the isthmus (Coy et al., 2018).

Among those four cell layers, the composition of muscle cells (muscularis) and epithelial cells (mucosa) are specific to each oviduct segment. In the infundibulum, there are less muscle cells but abundant ciliated cells to catch up and transport the oocyte (Croxatto, 2002; Winuthayanon & Li, 2018). On the other hand, in the isthmus, the muscle wall is thicker and secretory cells are predominant in the epithelium (Pedrero-Badillo et al., 2013; Winuthayanon & Li, 2018). Nevertheless, the proportion of ciliated cells and secretory cells are also regulated by the phases of the reproductive cycle (Coy et al., 2018). Ciliated cells are abundantly found in the infundibulum and ampulla at the follicular phase of the cycle while secretory cells are predominantly observed during the luteal phase. Controversy, the proportion of

both cell types does not differ in the isthmus during follicular or luteal phases (Abe, 1996; Maillo et al., 2016).

2.1.2 Roles of sex steroid hormones across the estrous cycle

The reproductive cycle in cattle can be mainly divided into two phases: follicular phase and luteal phase like most mammals including primates (Coy et al., 2018). The average estrous cycle length is 21 days in cattle (Forde, 2018). The follicular (or pre-ovulatory) phase is the period when ovarian follicles grow. In cattle, the follicles grow in wave-like patterns, showing 2 or 3 waves of follicular recruitment and growth in each estrous cycle by the secretion of follicle stimulating hormone (FSH) from the anterior pituitary gland that is stimulated by the gonadotropin-releasing hormone (GnRH) from the hypothalamus. However, among follicles there is a larger follicle secreting high level of estradiol (E2), which occurs in response to FSH, called the dominant follicle. High E2 level will exert negative feedback effect on FSH secretion while exert positive feedback effect on the luteinizing hormone (LH) secretion, thus resulting in a surge of LH followed by ovulation. The luteal phase is the period that begins after ovulation in which the ovulated follicle transforms into a corpus luteum (CL) causing progesterone (P4) levels rise. In the absence of fertilization and embryo development, the luteal phase ends due to corpus luteum regression (El-Sadi et al., 2013; Coy et al., 2018; Forde, 2018; Nowak, 2018; Pohler et al., 2020). In case of fertilization, the trophectoderm of the blastocyst secretes an increasing quantity of interferon tau (IFNT), which is responsible for maternal recognition of pregnancy. IFNT inhibits endometrial production of prostaglandin F2 alpha (PGF) and therefore inhibits CL regression (Imakawa et al., 1987; Bazer et al., 1997; Rodríguez-Alonso et al., 2020).

Estrogens are produced by the developing ovarian follicles and can be divided into estrone (E1), estradiol (E2) and estriol (E3). However, estradiol is pivotal estrogen secreted by granulosa cells of the ovarian follicle (Nowak, 2018). E2 exerts its functional effect via classical nuclear estrogen receptor alpha (ESR1) and beta (ESR2) or membrane ERs (GPR30, ER-X) (Merchenthaler, 2018). P4 is secreted by the corpus luteum in the ovaries and acts via nuclear progesterone receptors (PGRs), PGR-A and PGR-B, and membrane-bound PGRs (mPRs) (Winuthayanon & Li, 2018). P4 is essential for pregnancy maintenance, embryo development and implantation (Coy et al., 2018).

Estradiol (E2) and progesterone (P4) are ovarian sex steroid hormones that mainly regulate oviduct physiology and functions (Winuthayanon & Li, 2018). E2 causes cell hypertrophy, oviduct fluid secretion (Verhage et al., 1979; Winuthayanon

& Li, 2018), stimulated ciliary beat frequency, and muscle contraction (by inhibiting P4 activity) (Mahmood et al., 1998; Winuthayanon & Li, 2018), while P4 gives rise to cell atrophy, decreasing ciliary beat frequency and muscle contraction after binding to classical progesterone receptors (PGRs) (Wånggren et al., 2008; Bylander et al., 2013; Winuthayanon & Li, 2018). P4 also contributes to the loss of ciliated cells and reduction of the secretory activity in the oviduct epithelium (Donnez et al., 1985; Pedrero-Badillo et al., 2013; Winuthayanon & Li, 2018)

2.1.3 Oviduct secretions

1) Oviduct fluid

The oviductal fluid (OF) is the source of nutrients for early embryo development but also supports sperm transport (Guérin & Menezo, 2011; Winuthayanon & Li, 2018). In cattle, the rate of OF secretion was 0.2 mL per day at luteal phase while 2.0 mL per day during estrus (Małysz-Cymborska et al., 2013; Saint-Dizier et al., 2019). The oviductal fluid (OF) is a mixture of specific secretions from the oviduct epithelium, the transudation (passage of the fluid through cell membrane by osmotic pressure) from the blood and the interstitial tissue, and intracytoplasmic content of the desquamating cells (Hunter, 1988; Maillo et al., 2016; Coy et al., 2018; Saint-Dizier et al., 2019). The OF is made up with carbohydrates, ions, lipids, phospholipids and metabolites, in which lactate, pyruvate, amino acids and glucose are energy substrates for the developing embryos (Hugentobler et al., 2007; Hugentobler et al., 2008; Maillo et al., 2016; Saint-Dizier et al., 2019). The OF also contains prostaglandins (PGs), steroid hormones (E2 and P4) and growth factors; epidermal growth factor (EGF), fibroblast growth factor (FGF), transforming growth factor (TGF), insulin-like growth factor (IGF) and IGF-binding protein (IGFBP) (Aguilar & Reyley, 2005; Barton et al., 2020). Concerning the proteins in the OF, serum albumin, oviductin (OVGP1), heat shock proteins (HSP90B1, HSP90AB1, HSP90AA1, HSPA8, HSPA1B, HSPA5), annexins (ANXA1, ANXA4), tubulin subunits (TUBB5, TUBA4A), complement C3 (C3), and myosins (MYH9, MYH14) are at high abundance (Wijayagunawardane et al., 1998; Smits et al., 2016; Rodríguez-Alonso et al., 2019; Saint-Dizier et al., 2019).

Oviductin, also called oviductal glycoprotein 1 (OVGP1), is an estrogen-dependent oviductal glycoprotein secreted by the oviductal epithelium (Coy & Yanagimachi, 2015). OVGP1 is known to be involved in fertilization and early embryo development (Choudhary et al., 2019). OVGP1 has also been reported to reduce polyspermy by inducing zona hardening in pigs and cattle (Coy et al., 2008; Coy &

Yanagimachi, 2015). Moreover, OVGP1 mRNA expression is regulated by E2 during the oestrous cycle (Buhi, 2002).

Annexin A1 (ANXA1) is located on the apical membrane of oviduct epithelial cells and associated with sperm binding at the oviduct sperm reservoir (Ignotz et al., 2007).

Heat-shock protein family A (Heat Shock Cognate, Hsc70) member 8 (HSPA8), encoded by *HSPA8* gene is involved in sperm viability and fertilizing ability *in vitro* in pigs and cattle (Elliott et al., 2009).

Finally, heat shock protein family A (Hsp70) member 1A encoded by *HSPA1A* gene has been reported to be induced during heat stress in OECs (Rąpała et al., 2018).

2) Extracellular vesicles (EVs)

Extracellular vesicles (EVs) are abundantly found in the OF (Al-Dossary et al., 2013; Lopera-Vásquez et al., 2016; Almiñana et al., 2017; Almiñana & Bauersachs, 2019). Extracellular vesicles are membrane-delineated vesicles (Yáñez-Mó et al., 2015), frequently mentioned to microvesicles and exosomes (Raposo & Stoorvogel, 2013). Exosomes are rounded phospholipid bilayer vesicles belonging to a class of EVs with the size ranges from 40 to 150 nm in diameter (Pavani et al., 2016). Exosomes occur by inward budding of multivesicular bodies membrane and these bodies finally fuse to the plasma membrane from secreting cells and liberate the exosomes in the extracellular space (Kalluri & LeBleu, 2020). Microvesicles (MVs), also called shedding vesicles or ectosome (Gradilla et al., 2018), are another subtype of EVs with the size ranges from 100 to 1,000 nm in diameter (Pavani et al., 2016). The formation of MVs arises from directly outward budding of plasma membrane to extracellular space (Cruz et al., 2018).

EVs have been proposed to play a role in cell-to-cell communication and serve as the biomarker for disease diagnosis. Since, EVs were isolated from most cell types, both eukaryotic and prokaryotic cells as well as from various types of biological fluids, including urine, saliva, breast milk, plasma, serum (Yáñez-Mó et al., 2015), follicular fluids (da Silveira et al., 2012), oviductal fluid (Al-Dossary et al., 2013) and others including conditioned culture medium *in vitro* (Jaiswal & Sedger, 2019).

EVs may convey nucleic acid (mRNAs, non-coding RNA), lipids and proteins (Pavani et al., 2016) from secreting cells to both nearby and long-distance target cells (Jaiswal & Sedger, 2019) in order to provide their functional effects on target cells (van Niel et al., 2018; Almiñana & Bauersachs, 2019). Therefore, EVs can be uptaken by recipient cell through fusion with plasma membrane or binding to a

specific cell surface receptor on recipient cell using EVs cell-surface proteins (Pavani et al., 2016).

2.1.4 Reproductive events taking place in the oviduct during the peri-ovulatory period

At the pre-ovulation period, a few hundreds to a few thousands of spermatozoa (Suarez, 2002; Suarez & Pacey, 2006; Suarez, 2007; Kölle et al., 2009) cross the utero-tubal junction and reach the isthmus, in which a subpopulation adhere to isthmic epithelial cells to form a sperm reservoir (Ferraz et al., 2017). When ovulation occurs (post-ovulation period), a cumulus-oocyte complex (COC) containing a matured oocyte (at the metaphase II stage of meiosis) is released from the dominant follicle and then captured by the fimbria, the finger-like projections that surrounds the end of infundibulum. After that, a matured COC will be transported to ampulla driven by ciliary beating and smooth muscle contraction (Hunter, 1988; Maillo et al., 2016). On the other side, around ovulation time, spermatozoa are released from the reservoir, undergo capacitation and migrate toward the ampulla (Suarez, 2002; Kölle et al., 2009). Under optimal conditions, only one capacitated spermatozoon fertilizes the oocyte (monospermic fertilization) resulting in a zygote composed of two haploid sets of chromosomes (diploid) from paternal and maternal contributions (Sepulveda-Rincon et al., 2016). Subsequently, the zygote (in bovine) further develops in the isthmic region up to the 8-16-cell. After the 16-cell stage, the blastomeres combine to each other thus increasing their intracellular contacts by means of intercellular junction formation (tight junctions and gap junctions; E-Cadherin, also known as Uvomorulin) contributes to the formation of a compact morula in which the boundaries between blastomeres can no longer be distinguished (Dale & Elder, 2020; Pohler et al., 2020). Following compaction, the morula stage embryo enters the uterus around day 5 after fertilization (Maillo et al., 2016; Saint-Dizier et al., 2019) (Fig. 2.5).

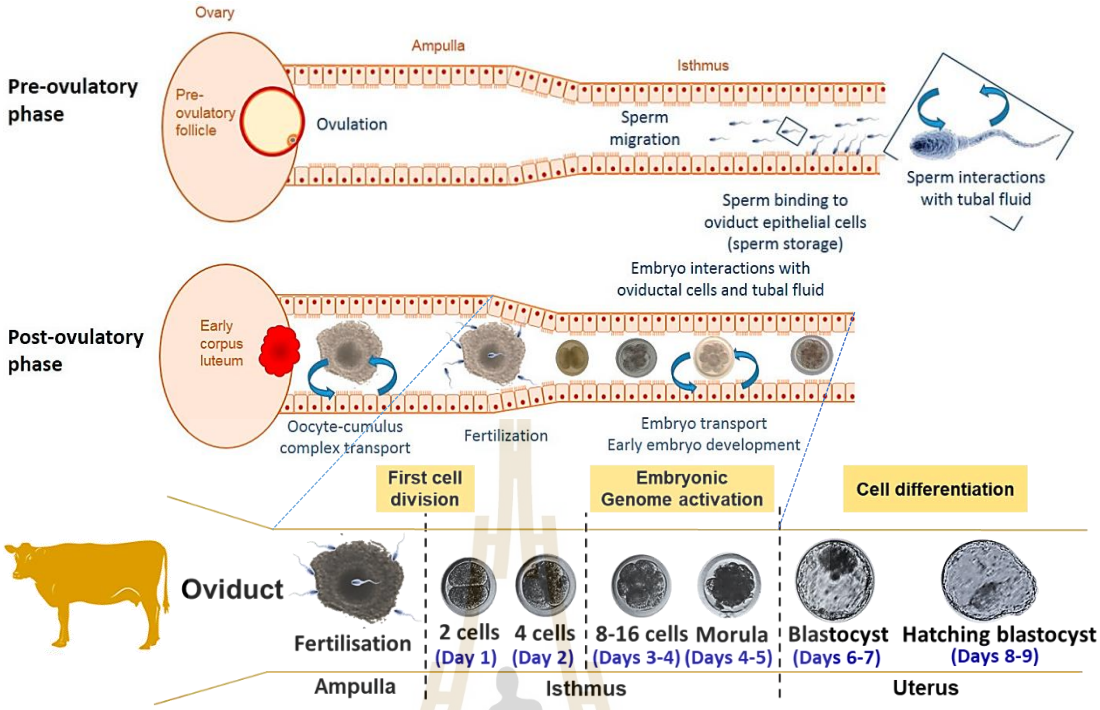

Figure 2.5 Reproductive processes at the peri-ovulatory period within the oviduct (Saint-Dizier et al., 2019) and a timing of bovine embryo development.

Around day 6 - After 6 days, the morula subsequently forms an early blastocyst containing a fluid filled growing cavity called blastocoele, followed by blastocyst hatching on days 8-9 (Fig. 2.5). The blastocyst comprises two cell types, the inner cell mass (ICM) and trophectoderm (TE) that are going to develop into the fetus and placenta, respectively (Piliszek & Madeja, 2018; Pohler et al., 2020). Blastocyst total cell number, ICM and the ICM:TE ratio, have been used widely for determining embryo quality *in vitro* as an indicator of embryo surviving potential and pregnancy rate after embryo transfer (Fleming et al., 2004). By which trophoblast cells have been suggested to play an important role in implantation and placentation (Ealy & Yang, 2009; Lopera-Vasquez et al., 2017b).

Taken together, the oviduct is the place of sperm storage, sperm capacitation, gametes transportation, fertilization and early embryo development up to the morula stage and transport to the uterus (Maillo et al., 2016; Saint-Dizier et al., 2019). All these early events of reproduction require the fine tuning of environment provided by the oviduct, controlled by hormonal and metabolic maternal status. The full success of *in vitro* embryo production depends on the ability to mimic this complex regulation *in vitro*.

1) Focus on the embryonic genome activation

The mitotic cell divisions of the embryo start in the oviduct. Mitosis is the division of somatic cells. It can be divided into interphase (G1, S and G2 phases), M phase (prophase, metaphase, anaphase, and telophase) and cytokinesis; cytoplasm division (Pollard et al., 2017). In cattle, at approximately 24 h after fertilization, the first cell division is achieved; one blastomere divides into two blastomeres (2-cell embryo) while the 4 and 8 cell stage embryos arise at 36-50 h and 56-66 h, respectively (Pohler et al., 2020). At the beginning of cleavage, the embryo employs maternal mRNAs and proteins that accumulated in the oocyte during oogenesis (Pavlok et al., 1993; Badr et al., 2007; Forde, 2018). The embryo is almost transcriptionally inactive, with only a few low noise transcriptions (Lee et al., 2014). At the 8-16-cell stage, the embryonic genome is activated (major EGA of bovine embryos) to initiate transcription and translation thus the embryo can use its mRNAs and proteins while maternal transcripts and proteins decrease and disappear (Badr et al., 2007; Lee et al., 2014; Forde, 2018; Loide et al., 2018; Pohler et al., 2020). The switch from maternal to embryonic transcripts is termed the maternal-to-embryonic transition (MET) or embryonic genome activation (EGA) (Loide et al., 2018) which is species-specific time (Badr et al., 2007) (Table 2.1). EGA is also mentioned as zygotic genome activation, ZGA (Piliszek & Madeja, 2018).

Minor embryonic transcription has been detected between the 1-cell to 4-cell stages in bovine embryos, thus implying an activity of transcription in zygotes and early embryos before major EGA (Memili et al., 1998; Meirelles et al., 2004; Badr et al., 2007; Tadros & Lipshitz, 2009; Pohler et al., 2020). An improper EGA may lead to a developmental delay or arrest (Schulz & Harrison, 2019; Dale & Elder, 2020; Zhou & Heald, 2023).

Table 2.1 Timing of pre-implantation embryo development in different mammalian species adapted from (Piliszek & Madeja, 2018). Days of post-oocyte insemination (D) and hours of post-insemination (h).

Species	Embryo stages					Major EGA
	Zygote	2-Cell	8-16 Cell	Morula	Blastocyst	
Hamster, mouse and rat	6-12 h	D1.5	D2.5	D3	D3.5	2-Cell
Rabbit	12 h	D1	D1.5	D2	D3-6	3-4 Cell
Pig	48 h	D1.5-2	D3-4	D4-5	D6-7	5-Cell
Sheep	24-36 h	D2.5	D3	D4-5	D7-8	8-16 Cell
Cow	20 h	D1.5-2	D3-4	D5	D7-9	8-16 Cell

Altogether, after *in vitro* fertilization, embryonic genome activation takes place at a species-specific time. It is a crucial event determining embryo fate. Inappropriate EGA may stop embryo development.

2.2 The embryo-maternal dialog in the oviduct: data from *in vivo* and *in vitro* studies

2.2.1 Embryo *in vitro* production and criteria to evaluate embryo quality

To produce embryo *in vitro* (Fig. 2.6), oocytes can be either retrieved from slaughterhouse ovaries or from donor cows using echo-guided transvaginal aspiration, named ovum pick-up (OPU) (Karadjole et al., 2010; Landeo et al., 2022). Embryos are routinely cultured for 7 days after insemination, then transferred in the uterus of a recipient cows at the late morula – blastocyst stage (Hansen, 2023).

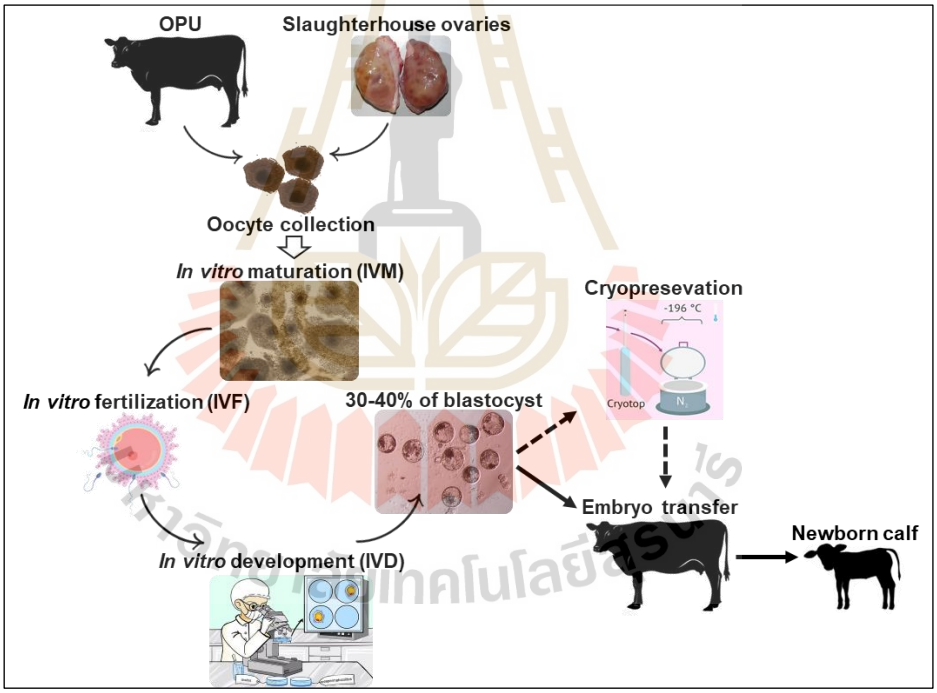

Figure 2.6 *In vitro* embryo production.

The evaluation of blastocysts on days 7-8 is routinely used prior to cryopreservation and embryo transfer (ET) to predict the success of pregnancy (Bo & Mapletoft, 2013; Gomez et al., 2021; Rabel et al., 2023). There are many criteria to evaluate embryo quality (Rabel et al., 2023), as shown in table 2.2.

Table 2.2 Evaluation of bovine blastocyst quality depending on oocyte origin and post-fertilization developmental conditions

Criteria	References
Total blastocyst cell number	Schmaltz-Panneau et al., 2015; Fabra et al., 2023
Blastocyst differential cell count (TE and ICM)	Knijn et al., 2003; Kocyigit & Cevik, 2016; Lopera-Vásquez et al., 2016; Asaadi et al., 2019
Blastocyst cryotolerance and thawing	Schmaltz-Panneau et al., 2015; Kocyigit & Cevik, 2016; Lopera-Vásquez et al., 2016; Asaadi et al., 2019; Fabra et al., 2023
Blastocyst gene expression	Cordova et al., 2014; Lopera-Vásquez et al., 2016; Asaadi et al., 2019
TUNEL labelling of apoptotic nuclei	Byrne et al., 1999; Matwee et al., 2000; Neuber et al., 2002; Knijn et al., 2003; Pomar et al., 2005; Antunes et al., 2010; Ghys et al., 2016
Lipid content	Pereira et al., 2007; Pereira et al., 2008; Cañón-Beltrán et al., 2020; Janati Idrissi et al., 2021; Dellaqua et al., 2023; Lipinska et al., 2023
Transcriptome profile by RNA Sequencing	Huang & Khatib, 2010; Driver et al., 2012; Graf et al., 2014; Zolini et al., 2020a; Zolini et al., 2020b
Proteomic profile	Talbot et al., 2010; Deutsch et al., 2014; Demant et al., 2015; Banliat et al., 2022a; Banliat et al., 2022b; Raes et al., 2023

2.2.2 Relative importance of the oocyte origin vs. post-fertilization culture conditions for blastocyst quality

It is well documented that *in vivo* produced blastocysts are of higher quality than their *in-vitro* produced counterparts in terms of morphology (Massip et al., 1995; Wright & Ellington, 1995; Rizos et al., 2002a), lipid content, transcriptomic and proteomic contents (Driver et al., 2012; Banliat et al., 2022a; Banliat et al., 2022b; Rabel et al., 2023), gene expression (Lonergan et al., 2003), cryotolerance (Enright et al., 2000; Rizos et al., 2002b) and pregnancy rate after transfer to recipient females, particularly after freezing/thawing (Enright et al., 2000; Maillo et al., 2016; Rodríguez-Alonso et al., 2020), highlighting the importance of the maternal environment during early steps of development.

Darker cytoplasm, lower density (Pollard & Leibo, 1994), high proportion of triglycerides and less lipids from other classes (I.M et al., 2000), swollen blastomeres (Van Soom et al., 1992), a more fragile zona pellucida (Duby, 1997), differences in

intercellular communication (Boni et al., 1999), and a higher incidence of chromosomal abnormalities (Viuff et al., 1999; Slimane et al., 2000) are the criterion used to distinguish morphology of *in vitro* produced embryos from *in vivo* embryos.

Rizos et al. (2002b) studied the effect of bovine oocyte maturation, fertilization or culture *in vivo* or *in vitro* on blastocyst yield and blastocyst quality, in terms of survival rate of vitrified-warmed blastocysts. **To examine the effect of oocyte maturation *in vivo* vs. *in vitro***, blastocysts were derived from i) immature oocytes from 2–6-mm follicles of slaughterhouse ovaries; ii) immature oocytes from > 6-mm follicles of slaughterhouse ovaries; iii) immature oocytes recovered *in vivo* by OPU just before the LH surge; and iv) *in vivo* matured oocytes recovered by OPU. Immature oocytes were subsequently IVM for 24 h. After IVM, oocytes from all 4 groups were subjected to IVF and IVC, respectively. No difference of cleavage rate was observed among 4 groups. In contrast, using *in vivo* matured oocytes (58.2%) resulted in higher blastocyst rate on day 8 than employing oocytes recovered just before the LH surge (39.2%) and oocytes from 2–6-mm follicles (38.9%). There were no differences between blastocysts developed from *in vivo* matured oocytes and oocytes from >6 mm follicles (46.5%). Moreover, survival rates of all vitrified-warmed blastocysts obtained from 4 different oocyte sources were less than 40% at 24 h and 20% at 72 h after warming. They concluded that oocytes matured *in vivo* are more competent than those matured *in vitro*. Oocytes derived from large follicles are more competent than those derived from small follicles. In addition, different sources of oocytes did not relate to different blastocyst quality. **To examine oocyte fertilization *in vivo* vs. *in vitro***, blastocysts were derived from i) *in vivo* matured oocytes recovered by OPU just before the LH surge followed by IVF and IVC; ii) *in vivo* matured oocytes fertilized *in vivo* by artificial insemination before recovering presumptive zygotes on day 1 by surgical technique for IVC; and iii) IVM/IVF/IVC (control). The results demonstrated that there was no difference of cleavage rate whether *in vivo* matured oocytes were fertilized *in vivo* (92.8%) or *in vitro* (87.3%). More blastocysts developed from *in vivo* fertilized oocytes (73.9%) than those from *in vitro* fertilized (58.2% and 39.2% for *in vivo* matured/IVF and IVM/IVF, respectively). Furthermore, higher blastocyst rate was acquired from *in vivo* matured/*in vitro* fertilized oocytes than *in vitro* matured oocytes. There were no differences of survival rates among all 3 groups, the results were similar to those of the effect of oocyte maturation. These findings demonstrated that the events around the time of fertilization may influence the developmental competence of the oocyte. **To examine the effect of embryo culture *in vivo* vs. *in vitro* (part 1)**, blastocysts

were derived from i) IVM/IVF/IVC; and ii) IVM/IVF/*in vivo* in the sheep oviduct. No difference of days 7-8 of blastocyst rates was observed between these 2 groups (34.1% vs 34.5% of day 8 blastocyst, for IVC and sheep oviduct, respectively;). Interestingly, presumptive zygote cultured in the sheep oviduct resulted in higher survival at all time points (24-48-72 h) and higher hatched blastocyst rate after vitrification followed by warming than those cultured *in vitro*. The results indicated that culture system can determine blastocyst quality. In addition, transient recipients (sheep) support the development of cattle embryos. **To examine the effect of embryo culture *in vivo* vs. *in vitro* (part 2)**, blastocysts were derived from i) *in vivo* matured oocytes fertilized *in vivo* by artificial insemination before recovering presumptive zygotes on day 1 by surgical technique for IVC; ii) *in vivo* matured oocytes fertilized *in vivo* by artificial insemination before recovering embryo on day 7 using OPU; and iii) IVM/IVF/IVC (control). There were no differences of cleavage and blastocyst rates of embryos produced from *in vivo* regardless of culture system (*in vitro* vs *in vivo* in cow oviduct). Moreover, low cleavage and days 6-8 of blastocyst rates were detected in *in vitro* control compared to *in vivo* groups. Blastocysts developed from *in vitro* culture regardless of zygote origin contributed to lower survival rate at all time points and lower hatched blastocyst rate after vitrification followed by warming than those culture *in vivo* (69.6 vs 0% and 1.8% survival at 72 h, for *in vivo* matured/fertilized/cultured, *in vivo* matured/fertilized/IVC and IVM/IVF/IVC, respectively). These findings suggested that oocyte origin determined oocyte developmental competence to the blastocyst stage. Overall, the method (*in vivo* vs. *in vitro*) used for oocyte maturation is crucial for blastocyst rate while the method used for embryo development is crucial for blastocyst quality.

In addition, attempts to evaluate bovine blastocyst quality by different methods are described in the following tables: Blastocyst cell count (total cell, TE and ICM) (Table 2.3), survival rate of blastocyst cryotolerance and thawing (Table 2.4), blastocyst gene expression (Table 2.5), TUNEL labelling of apoptotic nuclei (Table 2.6) and lipid content (Table 2.7).

Please note that only results obtained from the control group for each study were chosen for comparison; after IVF with oocytes matured *in vivo* or *in vitro*, embryos developed *in vivo* (after surgery transfer) or *in vitro* and complete *in vitro* (IVM-IVF-IVD) or complete *in vivo* production.

Table 2.3 Summary of blastocyst cell count (total cells, TE and ICM) depending on oocyte origin and post-fertilization developmental conditions.

References	Method of oocyte maturation		Method of embryo development		Method of complete IVM-IVF-IVD		Gas condition and T° during IVD	Blastocyst stage	Blastocyst cell count (Total cell, TE and ICM)			Unit
	In vivo	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*			Total cells	ICM	TE	
Knijn et al., 2003	+			+	IVM, IVF	IVD	7% O_2, 5% CO_2 and 88% N_2 at 39°C	Day 7	166 ± 11 (18)	130 ± 13 (8)	45 ± 4 (8)	Mean ± SEM (n)
	+			+	IVM, IVF	IVD		Expanded + Hatched blastocyst on day 7	205 ± 21 (15)	124 ± 13 (14)	78 ± 10 (14)	
		+		+		+	7% O_2, 5% CO_2 and 88% N_2 at 39°C	Day 7	114 ± 14 (5)	89 ± 14 (2)	45 ± 9 (2)	
		+		+		+		Expanded + Hatched blastocyst on day 7	160 ± 8 (23)	101 ± 6 (22)	62 ± 4 (22)	
Schmaltz-Panneau et al., 2015		+		+		+	5% O_2, 5% CO_2 and 90% N_2 at 38.8°C	Day 8	126.2 ± 7.8 (34)			Mean ± SEM (n)
Kocyigit & Cevik, 2016		+		+		+	5%O_2, 5% CO_2 and 90% N_2 at 38.5°C	Days 7-9 (Mix 100-160 µm in diameter)	82.58 (132)	18.86 (132)	63.72 (132)	Mean (n)
Lopera-Vásquez et al., 2016		+		+		+	5%O_2, 5% CO_2 and 90% N_2 at 38.5°C	Days 7-8	152.1 ± 4.7 (44)	47.7 ± 1.7 (44)	104.4 ± 4.2 (44)	Mean ± SEM (n)
Asaadi et al., 2019		+		+		+	5% CO_2 in air at 38.5°C	Expanded blastocyst on days 7 and 8	117.55 ± 2.01 (27)	35.01 ± 0.53 (27)	82.54 ± 1.65 (27)	Mean ± SEM (n)
Fabra et al., 2023		+		+		+	7% O_2, 5% CO_2 and 88% N_2 at 39°C	Day 8	54.54 ± 3.73 (30)			Mean ± SD (n)

Table 2.4 Summary of survival rate of blastocyst cryotolerance and thawing depending on oocyte origin and post-fertilization developmental conditions.

References	Method of oocyte maturation		Method of embryo development		Method of complete IVM-IVF-IVD		Gas condition and T° during IVD	Blastocyst stage	Survival rate of blastocyst cryotolerance and thawing						Unit
	In vivo	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*			3 h	4 h	5 h	24 h	48 h	72 h	
Schmaltz-Panneau et al., 2015		+		+		+	5% O_2, 5% CO_2 and 90% N_2 at 38.8°C	Days 7-8			43% (29)	18% (12)	9% (6)		% (n)
Kocyigit & Cevik, 2016		+		+		+	5% O_2, 5% CO_2 and 90% N_2 at 38.5°C	Days 7-9 (Mix 100-160 µm in diameter)				54.91% (130)			% (n)
Lopera-Vásquez et al., 2016		+		+		+	5% O_2, 5% CO_2 and 90% N_2 at 38.5°C	Day 7				54%		16.70%	%
Asaadi et al., 2019		+		+		+	5% CO_2 in air at 38.5°C	Expanded blastocyst on days 7 and 8		83.73 ± 1.5 (46)		52.57 ± 2.58 (29)	38.30 ± 3.08 (21)		Mean ± SEM (n)
Fabra et al., 2023		+		+		+	7% O_2, 5% CO_2 and 88% N_2 at 39°C	Day 7	68.18% (15)			18.18% (2)	54.54% (6)	9% (1)	% (n)
		+		+		+		Day 8	81.25% (26)			29.41% (5)	29.41% (5)	17.64% (3)	

Table 2.5 Summary of blastocyst gene expression depending on oocyte origin and post-fertilization developmental conditions.

References	Method of oocyte maturation		Method of embryo development		Method of complete IVM-IVF-IVD		Gas condition and T° during IVD	Blastocyst stage	Blastocyst gene expression: control vs treated groups		
	In vivo	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*			Upregulated genes	Downregulated genes	Similar with treated group (≤1)
Cordova et al., 2014		+		+		+	5% CO_2 in air at 38.8°C	Expanded blastocyst on day 8	*BAX*	*GLUT1*, *GPX1*, *DNMT3a*	*GLUT5*, *HIF1α*, *tp53*, *HSPA9B*, *SOD*
Lopera-Vásquez et al., 2016		+		+		+	5%O_2, 5% CO_2 and 90% N2 at 38.5°C	Day 7		*PAG1*	*PLIN2*, *ACACA*, *IFN-T*, *PLAC8*, *DNMT3A*, *TFAM*, *CX43*, *GPX1*, *MNSOD*, *GLUT1*, *GAPDH*, *G6PD*
Asaadi et al., 2019		+		+		+	5% CO_2 in air at 38.5°C	Expanded blastocyst on days 7 and 8	*IFN-T*		*BAX*, *P53*, *PLAC8*, *ATP1A1*, *AQP3*

Table 2.6 Summary of TUNEL labelling of apoptotic nuclei depending on oocyte origin and post-fertilization developmental conditions. Different superscripts indicate differences between origins (P = 0.05).

References	Method of oocyte maturation		Method of embryo development		Method of complete IVM-IVF-IVD		Gas condition and T° during IVD	Cultured media	Blastocyst stage	TUNEL labelling of apoptotic nuclei			Measurement	Unit
	In vivo	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*				Total cells	ICM	TE		
Neuber et al., 2002		+		+		+	5% CO_2 at 39°C	CR1aa + BSA	Day 6	2.3 ± 0.4 (4)	8.3 ± 4.8 (4)	1.4 ± 0.5 (4)	Dead cell index (DCI, total number of apopototic nuclei/total number of nuclei)	Mean ± SEM (n)
		+		+		+			Day 7	4.4 ± 0.9 (15)	11.7 ± 3.5 (15)	3.7 ± 0.7 (15)		
		+		+		+			Day 8	7.2 ± 2.1 (42)	18.2 ± 4.2 (42)	6.2 ± 2 (42)		
Knijn et al., 2003	+			+	IVM, IVF	IVD	7% O_2, 5% CO_2 and 88% N_2 at 39°C	SOF + BSA	Day 7	7.4 ± 0.8 (18)			Apoptotic index; cells displaying both TUNEL labeling and fragmentation and/or condensation of the nuclei relative to the total number of cells.	Mean ± SEM (n)
	+			+	IVM, IVF	IVD			Expanded + Hatched blastocyst on day 7	5 ± 0.8 (15)				
		+		+		+	7% O_2, 5% CO_2 and 88% N_2 at 39°C	SOF + BSA	Day 7	11.7 ± 5.1 (5)				
		+		+		+			Expanded + Hatched blastocyst on day 7	8.9 ± 1.3 (23)				
Pomar et al., 2005	+		+			·			Day 7	4.5 ± 3.1a (25)			TUNEL positive; apoptotic cells with DNA strand breaks	%Mean ± SD (n)
		+			+	+	7% O_2, 5% CO_2 at 39°C	SOF + BSA	Day 8	10.3 ± 10.9b (25)				
Ghys et al., 2016		+			+	+	5% O_2, 5% CO_2 and 90% N_2 at 39°C	SOF containing BSA, insulin, transferrin and selenium (BSA-ITS)	Expanded blastocyst on day 7		11.5 ± 1.2 (57)	9.8 ± 1 (57)	TUNEL-positive cells; DNA fragmentation	Mean ± SEM (n)
		+			+	+		BSA-ITS	Expanded blastocyst on day 7		11.2 ± 1.1 (55)	8.4 ± 0.9 (55)		
		+			+	+		SOF + 5% FCS	Expanded blastocyst on day 7		21.7 ± 1.4 (58)	14.5 ± 1.2 (58)		
		+			+	+		SOF + 5% FCS	Expanded blastocyst on day 7		17.9 ± 1.4 (69)	11.7 ± 0.8 (69)		

Table 2.7 Summary of lipid content depending on oocyte origin and post-fertilization developmental conditions.

References	Method of oocyte maturation		Method of embryo development		Method of complete IVM-IVF-IVD		Gas condition and T° during IVD	Cultured media	Blastocyst stage	Lipid content	Measurement
	In vivo	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*					
Pereira et al., 2007		+		+		+	5% O_2, 5% CO_2 and 90% N_2 at 39°C	SOF + PVA	Days 7/8	0% (Fat), 12.5% (Intermediate) and 87.5% (Lean)	Lipid content evaluation using Nomarsky microscopy evaluation
Cañón-Beltrán et al., 2020		+		+		+	5% O_2, 5% CO_2 and 90% N_2 at 38.5°C	SOF + 5% FBS	Day 7	0.39 ± 0.08 μm^2	lipid content by lipid droplet area in μm^2
Lipinska et al., 2023		+		+		+	5% O_2, 5% CO_2 and 90% N_2 at 39°C	SOF + fatty acid-free BSA	Expanded blastocyst	51 ± 22%	Lipid content in ICM per entire blastocyst (%)

Blastocyst cell count can reflect an embryo's ability for cell cycle progression (Kong et al., 2016). Therefore, it is frequently used to determine blastocysts competence. According to table 2.3, using *ex vivo*-collected embryos (Knijn et al., 2003) contributed to blastocyst with higher proportion of ICM cells than those using *in vitro* embryos. Although *in vitro*-produced bovine embryos are found to be really sensitive to chilling and cryopreservation, cryopreservation is still needed for widespread reproduction and conservation of elite animals using frozen-thawed embryos transfer (Ferré et al., 2020b). According to table 2.4, survival rate of *in vitro*-produced blastocyst after cryopreservation followed by thawing tended to decrease overtime. It is well documented that *in vitro*-produced embryos enable the study of gene expression. Nevertheless, even amongst *in vitro* themself, different culture conditions and stages of blastocyst can lead to different gene expression patterns (Table 2.5). Dead cell index measured by TUNEL in blastocyst increased from days 6 to 8 regardless of oocyte origin (Neuber et al., 2002; Pomar et al., 2005). Moreover, *ex vivo*-collected embryos (Knijn et al., 2003) resulted in less apoptotic index compared to *in vitro* embryos counterparts. In addition, FBS induced TUNEL-positive cells in ICM and TE of *in vitro*-produced blastocyst on day 7 (Table 2.6). There was evidence that *in vitro*-produced bovine embryos have high lipid accumulation compared to *in vivo* counterparts (de Andrade Melo-Sterza & Poehland, 2021). More lipid accumulation within embryo cytoplasm decreased cryotolerance, thus contributing to low quality of frozen-thawed embryos (Janati Idrissi et al., 2021). Hence lipid content became useful criteria determining embryo quality (Table 2.7).

2.2.3 Effect of co-culture with oviduct epithelial cells and oviduct secretions on embryo development and quality

The oviducts are not accessible on live animals without invasive surgery and their specific anatomy makes the oviductal lumen impossible to visualize. Therefore, oviduct epithelial cells (OEC) cultured *in vitro* have been used for a long time for supporting embryo development *in vitro*. They allowed the first success of blastocyst *in vitro* production, as well as first insights into early embryo-maternal communications. OECs have been isolated from oviductal lumen by enzymes (i.e. trypsin, collagenase) or mechanical scraping or squeezing before cell culture (Eyestone & First, 1989) and subjected to co-culture with embryo to create oviduct-like microenvironment.

Previous studies have shown that bovine embryos co-cultured with BOEC (bovine oviduct epithelial cells) or media conditioned by BOEC provided higher

blastocyst rates and quality than culture in the medium alone (Eyestone & First, 1989) (Table 2.8).

Cordova et al. (2014) studied different timings of BOEC (whole oviduct) co-culture with IVF-produced bovine embryos in terms of developmental rate and gene expression of day 8 expanded blastocyst using RT-qPCR. They found that embryos exposed to confluent BOEC for the first 4 days of culture (BE) reached higher blastocyst yields on days 6 and 7 compared to co-culture for 8 days (BOEC) and for the last 4 days of culture (BL) under 20% O_2. Despite blastocyst yields after co-culture with old cells BOEC for the first 4 days (Beo) were not different from control at any time, they were higher on days 7 and 8 when co-culture with young cells BOEC for the last 4 days (Bly).

In addition, the presence of cells induced changes in the embryos expression profile of genes known to be related to embryo quality, suggesting reduced apoptosis and increased capacity to struggle against oxidative stress. Under different timing of co-culture, the expression of genes involved glucose uptake (*GLUT1*, *GLUT5*), oxygen-induced transcription factor (*HIF1α*), apoptosis (*BAX*, *TP53*), stress (*HSPA9B*), DNA methylation (*DNMT3a*), lipid metabolism (*SCD*), and ROS protection (*GPX1*) were examined. *GLUT1*, *GPX1* and *DNMT3a* expression were upregulated in BOEC and BL compared to BE and control (embryos culture without cells under 20% O_2). Although *SCD* and *HSPA9B* expression were upregulated particularly in BOEC against all other groups, there was no difference in expression among BE, BL and control for *SCD* gene but between co-cultured groups and control for *HSPA9B* gene. The expression of *GLUT5* was noticed upregulated only in BOEC compared to BE. *HIF1α* expression was upregulated in co-culture groups except BE when compared to control. *TP53* expression was upregulated in BE compared to BL, whereas it was not different from BOEC and control. However, *Bax* proapoptotic gene expression was downregulated in all co-culture groups compared to control. In summary, BOEC improve the blastocyst rate and quality, reflected by blastocyst gene expression profile. The presence of BOEC during the first four days of the 8-days development is enough to produce these effects.

Schmaltz-Panneau et al. (2015) aimed to i) study effect of co-culture with BOEC (complete oviduct) on blastocyst yield and quality; ii) study effect of gas atmosphere on blastocyst yield in the presence or absence of BOEC; and iii) monitor the expression of genes in BOEC during culture related to oxidative stress (*Cu,Zn-SOD*, *GPX4*), steroid hormone receptors (*ESR2*, *PGR*), growth factors (*TGF-β1*, *IGF1*, *IGF2*, *FGF2*) and embryotrophic factors (*C3*, *OGP*, *SSP1*) via RT-qPCR (see below). They

demonstrated that co-culture with BOEC increased the blastocyst yield on days 7 and 8 in SOF. While in TCM199, co-culture with BOEC contributed to increase blastocyst yield, blastocyst total cell number (Hoechst staining) on day 8 and hatching blastocyst rate on day 8 when compared to culture without BOEC. Moreover, the presence of BOEC enhanced the survival rate of days 7–8 vitrified-warmed blastocysts after warming, at 24 and 48 h for embryos produced in both media compared to without BOEC and control (group of 25 embryos cultured in a 25 µL droplet of SOF under 5% O_2). Furthermore, under 20% O_2, co-culture with BOEC significantly improved blastocyst development and hatching rates in both media. This study has shown that BOEC in a co-culture system enhances bovine blastocyst formation and quality *in vitro* that could be attributed to embryotrophic secretions and the regulation of O_2 tension by this cell type.

Asaadi et al. (2019) examined the effects of frozen–thawed bovine ampullary epithelial cell (BAEC) and frozen–thawed bovine oviduct epithelial cells (BOEC, complete oviduct) on bovine embryo development under 20% O_2 and expanded blastocyst quality at days 7 and 8 including chemical-defined differential blastocyst staining (TE and ICM), cryotolerance and gene expression through RT-qPCR. For RT-qPCR, genes related to apoptosis (*BAX* and *P53*), implantation (*INF-T* and *PLAC8*), and cryotolerance (*ATP1A1* and *AQP3*) were analysed. There were 4 treatments: i) control, oocyte and embryo culture without cell during IVM and IVC; ii) AM, co-culture of oocytes with BAEC during the last 6 h of IVM; iii) OV, co-culture of embryos with BOEC for the first 4 days of IVC; and iv) AMOV, co-culture of oocytes with BAEC during the last 6 h of IVM then co-culture of embryos with BOEC for the first 4 days of IVC. They found that day 8 blastocyst yield was highest in AM against all other treatments, with no difference of cleavage rate between treatments. Survival rate of days 7-8 vitrified-warmed blastocysts after warming at 24 and 48 h and their 48 h-hatched blastocyst rate were higher both in AM and AMOV compared to control and OV. All co-culture groups increased TE and ICM of day 8 blastocysts with no difference among them compared to control. Furthermore, the expression levels of *PLAC8* and *AQP3* were highly upregulated in AMOV, followed by AM compared to control. While *ATP1A1* expression was highly upregulated in AMOV, followed by OV compared to control and AM. *BAX* expression was downregulated in the OV group than all other groups. *IFN-T* expression was downregulated in all co-culture conditions compared to control. *P53* expression was not different among groups.

If considering between OV and control, day 8 blastocyst yield was lower than for control. While survival rate of days 7–8 vitrified-warmed blastocysts after warming at 24 and 48 h and their 48 h-hatched blastocyst rate in OV was not different from control. However, TE and ICM were higher in OV than in control. For gene expression results, although *ATP1A1* expression was upregulated in OV than for control, *BAX* and *IFN-T* expression were downregulated. Moreover, the expression levels of *P53*, *PLAC8* and *AQP3* were similar between OV and control. In conclusion, co-culturing bovine oocytes with a frozen–thawed ampullary cell monolayer during the last 6 h of maturation increased blastocyst yield and quality.

Lopera-Vasquez et al. (2017b) evaluated the effect of OF supplementation during IVC on bovine embryo development and their blastocyst quality, in aspect of cryotolerance, chemical-defined differential day 8-blastocyst staining (TE and ICM) and gene expression of day 8 expanded blastocyst using RT-qPCR. The expression of genes associated with glucose metabolism (*SCL2A1*, *GAPDH*, *LDHA*), lipid metabolism (*LDLR*, *CYP51*, *FADS1*), epigenetics (*DNMT3A*, *IGF2R*, *UBE2A*) and water channels (*AQP3*) were determined. Supplementation with low OF concentrations at 1.25% and 0.625% in serum-free medium supported embryo development from days 7-9 as well as double increased survival rate of days 7-8 vitrified-warmed blastocysts after warming at 48 and 72 h compared to embryos cultured with serum. Embryo cultured with 1.25% and 0.625% OF for 8 days (days 1-9) resulted in higher blastocyst total cell number and TE than embryo cultured with and without serum. Although ICM of embryos cultured in the OF-supplemented group did not differ from serum-supplemented group, it was higher than in serum-free group. The expression levels of *SCLA1*, *LDLR*, *CYP51*, *FADS1*, *DNMT3A*, *IGF2R* showed to be upregulated in a 1.25% OF-supplemented group compared to a serum-containing group. However, the expression of these genes did not differ between OF-supplemented group and serum-free group. Nevertheless, only *AQP3* expression was found upregulated in a 1.25% OF-supplemented group than for with and without serum-supplemented-groups. There was no downregulated expression of any gene in the OF-supplemented group. Thus, supplementation with low concentrations of OF in serum-free culture medium during IVC has a positive effect on the development and quality of bovine embryos.

Hamdi et al. (2017) demonstrated the effects of both OF and UF (uterine fluid) supplementation during IVC on bovine embryo development at days 7-9 and blastocyst quality on days 7-8 including cryotolerance, ROS detection using

chloromethyl-2′-7′-dichlorodihydrofluorescein diacetate (CM-H2DCFDA) and gene expression through RT-qPCR.

OF was collected by gentle squeezing ipsilateral oviducts at post-ovulatory phase of ovarian cycle from the isthmus to ampulla. On the other hand, UF was collected by gentle squeezing ipsilateral oviducts at early luteal phase of ovarian cycle from internal opening of the cervix towards the uterotubal junction.

There were 5 groups: i) SOF+FCS (laboratory control), embryo culture in SOF supplemented with 5% FCS for 8 days (from days 1-9); ii) SOF+BSA (experimental control), embryo culture in SOF supplemented with 3 mg/mL BSA for 8 days; iii) OF+UF, embryo culture in SOF supplemented with 1.25% OF from days 1-4, then with 1.25% UF from days 4-9; iv) OF, embryo culture in SOF supplemented with 1.25% OF from days 1-4, then with SOF+BSA from days 4-9; and v) UF, embryo culture in SOF supplemented BSA from days 1-4, then SOF supplemented with 1.25% UF from days 4-9. Although high blastocyst yield on day 7 was observed in SOF+FCS compared to SOF+BSA and OF, it did not differ from those OF+UF and UF. Moreover, blastocyst yield on days 8-9 did not differ among groups. At 48 h and 72 h after warming, the survival rate of vitrified-warmed blastocysts in SOF+BSA, OF+UF and OF were similar, twice higher than SOF+FCS and were not different from blastocyst in UF. Moreover, blastocysts cultured in OF+UF, OF and UF resulted in lower ROS levels than for blastocysts from SOF+FCS. Nevertheless, only ROS levels in blastocyst from OF+UF were lower than SOF+BSA. For gene expression analysis, *DNMT3A* and *IGF2R* expression was upregulated in OF compared to SOF+FCS. In OF+UF and UF groups, the expression levels of *GPX1* and *CLIC1* were downregulated when compared to SOF+BSA and OF and SOF+FCS, SOF+BSA and OF, respectively. Summarily, supplementation with low concentration of OF and/or UF in serum-free culture medium during IVC supports embryo development and improves blastocyst quality. OF is associated with embryo DNA methylation control while UF may implicate in antioxidant activity (Hamdi et al., 2017).

Previous studies have reported positive effects of EVs derived from the OF (*in vivo* EVs or oEVs) and conditioned medium of BOEC (*in vitro* EVs) on embryo development. Supplementation of *in vitro* EVs provided positive effects on embryos in terms of gene expression using RT-qPCR, blastocyst cell number by chemical-defined differential staining and embryo cryosurvival (Lopera-Vásquez et al., 2016). Confocal microscopy and fluorescence-labelled oEVs showed that EVs can be uptaken by IVF-produced embryos and support blastocyst rate, hatching rate, and blastocyst cell number (Almiñana et al., 2017). oEVs derived from the isthmus region

had a positive effect on embryo quality in terms of cryotolerance and gene expression by RT-qPCR (Lopera-Vasquez et al., 2017a)

Altogether, *in vitro* models were still limited regarding the fact as follows. First, most *in vitro* models of OEC induce cell dedifferentiation or are technically challenging (See 2.3). Second, candidate genes with no exhaustive evaluation of changes in gene expression may not truly illustrate application of *in vitro* models. Third, EVs are difficult to isolate from very limited OF volume or from insufficient conditioned medium. Finally, more research on the molecular basis of embryo-maternal communication by transcriptomics and proteomics techniques are still required.

Table 2.8 Summary the effect of OECs, OF and oEVs on bovine embryo development rate. N = total number of cumulus-oocyte complexes; and n = total number of embryos. The different letters within column indicate significant differences (P<0.05).

Reference	Factor studies	Cultured conditions	N	% (n) of cleavage rate	% (n) of blastocyst rate			
				Day 2	Day 6	Day 7	Day 8	Day 9
Cordova et al. (2014)	Different timings of BOEC during IVC	Control	557	84^{a}	$6^{a, d, e}$ (31)	18^{a} (103)	16^{a} (88)	
		BOEC	343	$87^{a, b}$	$6^{d, e, f}$ (21)	26^{b} (89)	$30^{b, c}$ (104)	
		BE	534	$88^{b, c}$	14^{b} (74)	$32^{c, d}$ (173)	35^{b} (187)	
		BEo	175	$87^{a, c}$	2^{a} (4)	13^{a} (22)	16^{a} (28)	
		BL	455	84^{a}	$9^{c, f}$ (42)	24^{b} (110)	29^{c} (130)	
		BLy	200	$88^{a, c}$	3^{a} (5)	$27^{b, d}$ (54)	$31^{b, c}$ (62)	
Schmaltz-Panneau et al. (2015)	Effect of BOEC in two different culture mediums during IVC	Control	413	73 (303)		37^{a} (113)	51^{a} (155)	
		SOF	378	72 (273)		15^{b} (40)	27^{b} (74)	
		SOF-BOEC	385	79 (306)		22^{c} (67)	41^{c} (127)	
		TCM199	387	73 (218)		1^{d} (4)	10^{d} (27)	
		TCM199-BOEC	389	73 (284)		16^{bc} (46)	28^{b} (80)	
Asaadi et al. (2019)	Effect of frozen-thawed BAEC during IVM and frozen-thawed BOEC during IVC	Control	340	79.6 ± 0.8 (271)			33.1 ± 1^{b} (90)	
		AM	339	81.1 ± 0.8 (275)			38.9 ± 2.6^{a} (108)	
		OV	354	79.1 ± 0.8 (280)			24.2 ± 1.2^{c} (68)	
		AMOV	349	80.5 ± 0.7 (281)			26.3 ± 1.7^{c} (74)	
Lopera-Vasquez et al. (2017b)	Different concentrations of OF during IVC	C^{+} (with FCS)	872	86.7 ± 1.5 (747)		22.9 ± 1.2^{a} (198)	26.6 ± 1.2^{a} (226)	27.7 ± 1.9^{a} (236)
		C^{-} (without FCS)	927	88.8 ± 1.2 (823)		12 ± 1.7^{c} (96)	18.3 ± 1.2^{c} (164)	21.5 ± 1.4^{b} (195)
		2.5% OF	855	87.3 ± 1.1 (748)		13.9 ± 1.4^{bc} (110)	21.2 ± 1.4^{bc} (180)	22.7 ± 1.5^{b} (192)
		1.25% OF	964	89.1 ± 1.5 (855)		17.4 ± 1.5^{b} (163)	24.4 ± 1.7^{ab} (236)	27.5 ± 1.7^{a} (266)
		0.625% OF	1011	89.1 ± 1.3 (898)		16 ± 1.2^{b} (160)	22.3 ± 1^{b} (230)	27.5 ± 1.2^{a} (279)
Hamdi et al. (2017)	Different concentrations of OF and/or UF during IVC	SOF+FCS	400	83.5 ± 1.7 (330)		25.6 ± 2^{a} (100)	28.1 ± 2.4 (111)	29 ± 1.9 (115)
		SOF+BSA	430	88.6 ± 1.9 (380)		17.8 ± 3.1^{b} (75)	27.2 ± 3.2 (119)	30.6 ± 2.9 (129)
		1.25% OF+1.25% UF	460	86.8 ± 1.5 (399)		21.3 ± 1.8^{ab} (96)	29.5 ± 2.3 (139)	30.2 ± 2.5 (143)
		1.25% OF	479	85.5 ± 1.5 (410)		18.6 ± 1.5^{b} (87)	28.1 ± 1.5 (133)	29.5 ± 1.4 (142)
		1.25% UF	486	83 ± 1.4 (404)		21.5 ± 2.8^{ab} (98)	27.2 ± 3.1 (125)	28.2 ± 2.9 (131)

Table 2.8 Summary the effect of OECs, OF and oEVs on bovine embryo development rate. N = total number of cumulus-oocyte complexes; and n = total number of embryos. The different letters within column indicate significant differences (P<0.05). (Continue)

Reference	Factor studies	Cultured conditions	N	% (n) of cleavage rate	% (n) of blastocyst rate			
				Day 2	Day 6	Day 7	Day 8	Day 9
Almiñana et al. (2017)	Effect of fresh and frozen oEVs during IVC	Control	353	81 ± 3.6		33^{a} ± 3.1	34^{a} ± 2.7	23.8^{a} ± 4
		Fresh oEVs	332	73.6 ± 3.4		30.2^{a} ± 4	37.9^{ab} ± 2.6	35.2^{b} ± 4.9
		Frozen oEVs	332	76.6 ± 1.9		41^{b} ± 2.6	45.9^{b} ± 1.1	49.4^{b} ± 0.6
Lopera-Vasquez et al. (2017a)	oEVs isolated from ampulla and isthmus OF at different g-forces (10–100 K × g)	C^{+} (with FCS)	490	88.5 ± 2.2 (432)		$20.6 ± 2.1^{a}$ (99)	26.9 ± 1.9 (129)	29.6 ± 2.3 (143)
		C^{-} (with BSA)	566	88.1 ± 1.3 (498)		$13.4 ± 1.8^{b}$ (75)	24.6 ± 1.7 (139)	29.7 ± 2.5 (168)
		Ampulla-10K oEVs	514	89.6 ± 1.3 (460)		$12 ± 1.6^{b}$ (62)	26 ± 2.1 (133)	29.7 ± 2.1 (152)
		Ampulla-100K oEVs	462	88 ± 1.1 (406)		$13.8 ± 1.9^{b}$ (63)	25.3 ± 2.4 (116)	28.5 ± 2.2 (131)
		Isthmus-10K oEVs	557	88.6 ± 1.5 (494)		$13.7 ± 1.1^{b}$ (75)	24.6 ± 2.2 (135)	29.6 ± 1.9 (164)
		Isthmus-100K oEVs	549	89.1 ± 1.2 (488)		$14.3 ± 3^{b}$ (68)	26.1 ± 4.1 (126)	30.8 ± 4.5 (151)

2.2.4 Effect of developing embryos on gene expression in oviduct epithelial cells

As reported above, the oviduct epithelium changes gene expression in the developing embryo. Conversely, *in vitro* studies have shown that the developing embryos is also able to modify gene expression in OEC (Table 2.9).

Lee et al. (2002) compared the gene expression of mouse oviducts exposed to oocytes or early embryos through suppression subtractive hybridization (SSH). A group of 10-12 oocytes and embryos was transferred in contralateral oviduct and opposite side of oviduct in the same mouse, respectively. Oocytes and embryos then existed in the oviduct for 48 h before collecting flushed oviduct content for experiments. After SSH, 250 putative positive clones were screened out from the library. Of these, 97 putative positive clones were confirmed by reverse dot-blot analysis and subjected to DNA sequencing. Thirteen different subtracted oviductal clones (including OD200, OD13, OD25, OD163, OD182 and OD241; 6 clones from their prior study) out of 90 high-quality sequences were higher in the presence of embryos than in the oocytes. In which thymosin beta 4 (OD25) expression was upregulated. They reported that thymosin beta 4 may be involved in oviductal cell secretion. Moreover 11 clones out of 90 clones apart from 13 clones were novels. According to Reverse Northern blot analysis, there was 1.3- to 3.8-fold increase of transcripts' signals in oviducts exposed to embryos than for oocytes counterpart. In conclusion, early developing embryos but not oocytes induced changes of mouse oviduct gene expression (Lee et al., 2002).

Using a microarray analysis, **Schmaltz-Panneau et al. (2014)** determined the alteration of gene expression profile in BOEC (whole oviduct) at the end of culture (day 13) in response to the presence of developing bovine embryos during IVC for 8 days. They reported among 41 annotated transcripts, there were 33 differentially expressed genes (DEGs). Of these 32 genes were upregulated while one gene was downregulated (i.e. CTGF) in OECs after co-culture with embryos for 8 days. Highest upregulated DEGs related to antiviral and immune response were *IFI6*, *ISG15*, *MX1*, *IFI27*, *IFI44*, *RSAD2*, *IFITM1*, *EPSTI1*, *USP18*, *IFIT5*, and *STAT1*. Moreover, 12 GO biological processes terms of 32 DEGs were immune response, response to virus, ISG-protein conjugated, embryonic development, response to inorganic substance, mitotic cell cycle process, apoptosis, cell motion, positive regulation of signal transduction, ubiquitin-dependent protein catabolic process, generation of signal involved in cell-cell signalling and regulation of transcription. Of these, the top 3 categories were immune response, response to virus and ISG15-protein conjugation.

Furthermore, when consider the top 3 categories of canonical pathways, upregulated DEGs associated with recognition of bacteria and viruses (*OAS1*, *DDX58*, *EIF2AK2*), interferon regulatory transcription factor (IRF) (*DHX58*, *DDX58*, *STAT1*, *IFIT2*, *ISG15*) and interferon signalling (*OAS1*, *IFITM1*, *MX1*, *STAT1*) were detected. Seven candidate genes (*MX1*, *IFIT5*, *STAT1*, *ISG15*, *IFITM1*, *USP18*, and *OAS1*) was chosen to confirm the differential expression by RT-PCR, all their expression was upregulated in the presence of the embryo for 8 days compared to absence (control). According to immunostaining, STAT1 protein was found localized in the nucleus and perinuclear space in BOEC used to culture with embryos for 8 days compared to without (control). This finding suggested BOEC are able to modify their transcription in response to the presence of developing bovine embryos. Most of the upregulated genes were associated with antiviral and immune response stimulating by the interferon tau (IFNT) pathway.

Using RT-qPCR, **Schmaltz-Panneau et al. (2015)** did not observe any difference in mRNA levels of the 11 candidate genes in BOEC after co-culture with vs. without (controls) embryos for 8 days.

Maillo et al. (2015) examined the effect of the presence of single embryo or multiple embryos on the transcriptome of isthmic BOEC from ipsilateral oviducts in pregnant and cyclic heifers. Using microarray, they did not detect DEGs in isthmic BOEC exposed to a single 8-cell embryo and an unfertilized oocyte in the ipsilateral oviducts of pregnant and cyclic heifers, respectively. However, by RNA sequencing, 278 DEGS were detected in isthmic BOEC that have been transferred a group of 50 IVF-produced presumptive zygotes (per recipient) compared to BOEC that have been transferred media only (a sham transfer) to the ipsilateral oviducts of pregnant and cyclic heifers, respectively. Of these DEGs, 123 were upregulated while 155 were downregulated in pregnant heifers. For gene ontology biological processes, only upregulated genes related to cell division (*ROCK1*, *ROCK2*, *CCAR1*) and cytokinesis (*ROCK1*, *ROCK2*) were overrepresented, whereas most of the downregulated genes were associated with immune function. For gene ontology KEGG pathway, there were six pathways (vascular smooth muscle contraction, focal adhesion, regulation of actin cytoskeleton, TGF-beta signaling pathway, oocyte meiosis, and axon guidance) found in upregulated genes and four pathways (complement and coagulation cascades, viral myocarditis, arrhythmogenic right ventricular cardiomyopathy (ARVC) and ECM-receptor interaction) presented in downregulated genes. Ten candidate genes (*STK32A*, *SLC26A3*, *KERA*, *QRFPR*, *MCTP1*, *SOD3*, *PRELP*, *VAT1L*, *SOCS3* and *CCL20*) were chosen to validate RNA-Seq results via RT-qPCR. The expression of *SLC26A3*,

MCTP1, *STK32A*, *KERA*, *SOCS3*, *VAT1L*, *PRELP* and *SOD3* were upregulated in BOEC after transferring multiple embryos in the oviduct of pregnant than cyclic heifers. These results were consistent with RNA-Seq. Furthermore, when using the same candidate genes to confirm microarray results, none of the above upregulated genes except *KERA* were differentially expressed in BOEC exposed to a single embryo in the oviduct of pregnant heifers compared to cyclic heifers. In conclusion, the presence of multiple embryos (up to 50) in isthmic BOEC from ipsilateral oviducts alters BOEC transcriptome, but not in the presence of a single embryo (Maillo et al., 2015).

Rodríguez-Alonso et al. (2020) demonstrated the effects of oviduct anatomical region and the presence of an embryo on OF protein, amino acid, and carbohydrate composition using high-performance liquid chromatography-mass spectrometry (HPLC-ESI-MS/MS) system (proteomic analysis), high performance liquid chromatography (HPLC) and microfluorometric enzyme-linked assays, respectively. The results showed that OF derived from ipsilateral to the corpus luteum of isthmus and ampulla consequent to different OF composition. Consider day 3 post-estrus, lower lactate, glycine, and alanine and higher arginine concentrations were detected in OF derived from isthmus than for ampulla. Moreover, the presence of an embryo resulted in higher arginine, phosphoglycerate kinase 1, serum albumin, -1-antiproteinase and IGL@ protein concentrations in isthmus OF compared to ampulla OF. They concluded OF composition differed according to different oviduct regions and the presence of an early embryo.

Taken together, previous studies are still lacking OEC evaluation on physiology, metabolism, and cell dedifferentiation. Moreover, more research is required on transcriptomics, proteomics, and the impact of developing embryos on gene expression in OEC under challenging conditions (i.e. oxidative stress).

Table 2.9 Summary of changes in BOEC induced by developing embryo under *in vivo* and *in vitro* conditions.9

Reference	*In vivo/in vitro*	BOEC alteration
Lee et al. (2002)	*In vivo* (mouse)	Thirteen different subtracted oviductal clones (including OD200, OD13, OD25, OD163, OD182 and OD241; 6 clones from their prior study) out of 90 high-quality sequences were higher in the presence of embryos than in the oocytes. In which thymosin beta 4 (OD25) expression was upregulated. There was 1.3- to 3.8-fold increase of transcripts' signals in oviducts exposed to embryos than for oocytes counterpart.
Schmaltz-Panneau et al. (2014)	*In vitro*	After co-culture with embryos for 8 days, BOEC have shown to modify their transcriptomic profile measured by microarray in which 33 DEGs were detected, of these 32 genes were upregulated while one gene was downregulated. Most of the upregulated genes were associated with antiviral and immune responses stimulated by IFNT. All expression levels of 7 candidate genes (*MX1*, *IFIT5*, *STAT1*, *ISG15*, *IFITM1*, *USP18* and *OAS1*) in BOEC using real-time RT-PCR were upregulated in the presence of the embryo compared to absence during IVC for 8 days.
Schmaltz-Panneau et al. (2015)	*In vitro*	There was no significant difference of mRNA levels of all 11 candidate genes (*Cu, Zn-SOD*, *GPX4*, *ESR2*, *PGR*, *TGF-β1*, *IGF1*, *IGF2*, *FGF2*, *C3*, *OGP* and *SSP1*) in BOEC at freshly isolated cells, confluent cells and the end of culture regardless of the presence or absence of embryos, except for *ESR2*, *GPX4* and *SSP1* using RT-qPCR. Although the expression of ESR2 was downregulated in BOEC at the end of culture in SOF and TCM199 whether exposure with embryos or not compared to freshly isolated cells, *GPX4* was upregulated. In addition, *SSP1* expression was observed upregulated in BOEC from confluent cells to the end of culture in both media regardless of the presence or absence of embryos than freshly isolated BOEC.
Maillo et al. (2015)	*In vivo*	The presence of multiple embryos (up to 50) in isthmic BOEC of ipsilateral oviduct resulted in 278 DEGs between pregnant and cyclic heifers detected by RNA-Seq. However, in the presence of a single 8-cell embryo, no DEGs were observed through microarray.

2.3 Limitations of *in vitro* models of the oviduct epithelium to study embryo-maternal communication

So far, *in vitro* models resembling oviduct-like environments have been established to decipher early embryo-maternal dialogue and achieve higher embryo yield and quality. However, each *in vitro* model has its advantages and limitations, as summarized in figure 2.7.

2.3.1 OEC monolayers

OEC monolayers grown on plastic dishes revealed for the first time the existence of a real dialog between embryos and OEC during co-culture, providing increased embryo development rate and quality (Rizos et al., 2002b; Cordova et al.,

2014; Schmaltz-Panneau et al., 2015) as well as changes in OEC gene expression profiles as a result of the co-culture with embryos (Schmaltz-Panneau et al., 2014; Hamdi et al., 2019). However, OEC adherent to plastic rapidly dedifferentiate, lose morphological criteria of cell differentiation like cilia and secretory granules after 3 days in culture (Walter, 1995).

BOEC dedifferentiation after culture can be seen in the research of Schmaltz-Panneau et al. (2015) reported that at different time points of BOEC culture: i) freshly isolated cells (day 0); ii) cells at confluence (day 5); and iii) cells at the end of the culture (day13), *SOD*, *FGF2* and *TGF-β1* mRNA expression remained steady while *OGP*, *C3*, *PGR* and *ESR2* were clearly reduced at day 5 up and day 13 of culture compared to day 0. Nevertheless, *SSP1* and *GPX4* transcripts were slightly increased during culture, with a significant increase at day 13. Although *IGF1* and *IGF2* transcripts were detected throughout the culture, they tended to decrease at day 5 and then rose again at day 13. These findings thus suggest after 5 to 10 days in culture, OEC displayed a decrease in gene expression of steroid hormone receptors (*PGR*, *ESR2*) and oviduct-specific glycoprotein 1 (*OVGP1*) (Schmaltz-Panneau et al., 2015), one of the most abundant proteins found in the oviduct (Coy et al., 2008).

OEC monolayers grown on inserts in air-liquid interphase (ALI) have been developed to avoid cell dedifferentiation observed in the above system. The ALI system can be divided into 2 phases: cell proliferation for 7 days (d 0-7) regardless of species, then elimination of the supernatant (air interphase) to induce epithelial cell differentiation up to 21 days (d 8-21) in murine and porcine, or up to 28 days in bovine (d 8-28) (Chen et al., 2017). During the proliferation phase, OEC are seeded onto the apical compartment of the inserts and grown on a porous membrane with pore size of 0.4 µm, OEC are exposed to the medium from both the apical and basal compartments. In contrast, during the differentiation phase, only the basolateral side of the cells are submerged into the medium while the apical cell surface is exposed to air. This system maintains epithelial morphology more similar to the *in vivo* one, including ciliated cells, columnar shape and intercellular cohesion as well as the gene expression of steroid hormone receptors and *OVGP1* (Schoen & Chen, 2018). Moreover, this model allowed studying the formation of OF. However, the ALI culture of OEC takes long time to establish (three to four weeks, depending on the species), is technically challenging and so far, did not show any supporting effect on development of co-cultured bovine embryos (Chen et al., 2017; van der Weijden et al., 2017).

The oviduct secretions consist in a microfluidic environment with dynamic flows. OEC monolayers grown in an oviduct-on-a-chip are the combination of a three-dimensional (3D) printing technology equipped with microfluidics (Barton et al., 2020). Ferraz et al (2017) succeeded in applying this device for IVF and IVC, however, the cleavage rate and 8-16 cells formation were lower than optimised standard *in vitro* embryo production. Furthermore, numerous oocytes were lost in the system and embryos did not develop beyond the 8-16-cell stage (S. Le Gac, personal communication).

2.3.2 Oviduct organoids

OEC grown in a mixture close in composition to the extracellular matrix (ECM) have been recently reported (Bourdon et al., 2021; Lawson et al., 2023). Organoids are generated from single cells after enzymatic cell digestion and cultured in suspension in a complex and expensive medium (Matrigel®) containing WNT, TGFβ, BMP, ROCK and Notch activators or inhibitors (Lawson et al., 2023). Organoids clonal populations emerging from the proliferation of a single cell, they are capable of maintaining cell stemness, however their production is long and challenging (Schoen & Chen, 2018). In addition, the apical side of OEC is inside the organoid, making it impossible to interact with developing embryos. Nevertheless, nowadays there are techniques of micro-injection (Wilson et al., 2015; Williamson et al., 2018), flipping the organoids inside out (Co et al., 2021) or vigorous pipetting (van der Sanden et al., 2018) for apical exposure in order to assess the cells inside the sphere's cavity (Lawson et al., 2023).

2.3.3 OEC explants or aggregates in suspension culture

OEC aggregated collected from oviduct mucosa fragments and used the day of collection have been used to study sperm binding to the oviduct reservoir in mammalian species (De Pauw et al., 2002; Waberski et al., 2005; Henry et al., 2015; Schoen & Chen, 2018). Walter (1995) reported the first culture of free-floating bovine OEC forming vesicles and demonstrated that this culture could better maintain the cilia and secretory granules compared to confluent monolayers after 12 days in culture. Rottmayer et al. (2006) proposed a short time suspension culture of OEC aggregates for 24 h and evidenced a maintenance of their morphology and stable gene expression of *OVGP1* and steroid hormone receptors after 6 h of culture. However, the possibility of culturing OEC in suspension during the time needed for supporting embryo development, i.e., 7-9 days, has not been yet assessed. Moreover, OEC aggregates in suspension are not morphologically uniform in shape and size, which can be a source of variability between replicates, and thus, requires a proper

characterization and standardization to be able to use it for embryo co-culture purposes (Rottmayer et al., 2006).

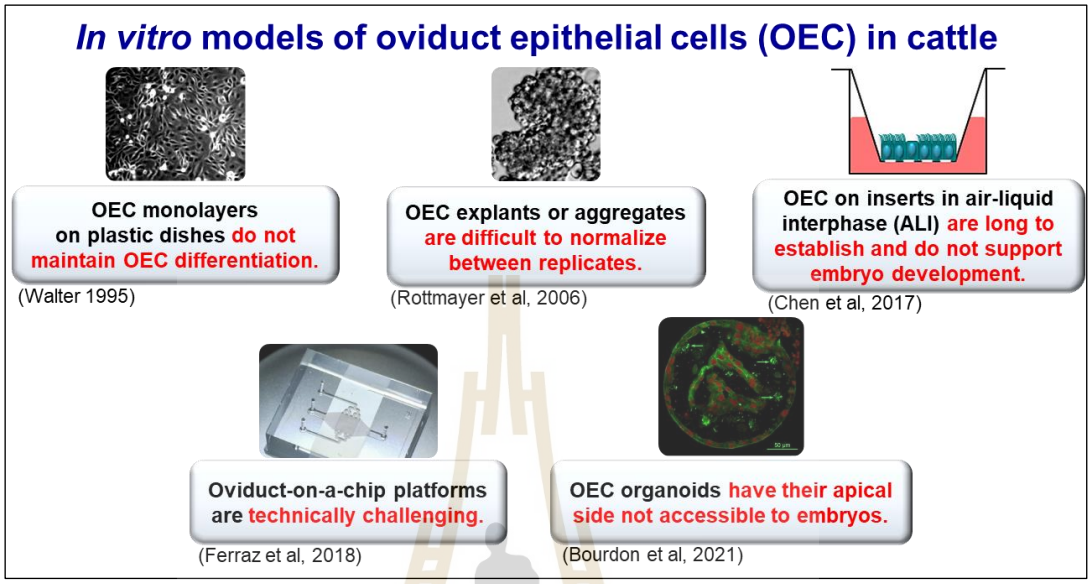

Figure 2.7 *In vitro* models of oviduct epithelial cells (OEC) in cattle.

2.4 Oxidative stress during *in vitro* embryo production and antioxidant effect of the oviduct

2.4.1 Origin and effects of ROS on embryo development during *in vitro* production

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are two major kinds of free radicals in biological systems (Agarwal et al., 2005; Ufer et al., 2014). Free radical species mention atoms and molecules containing unpaired electrons that tend to form pairs with other electrons to become stable, this makes free radical species unstable and highly reactive (Yoshikawa & Naito, 2002; Agarwal et al., 2005; Burton & Jauniaux, 2011). However, free radical species without unpaired electrons are termed as non-free radicals (Aranda-Rivera et al., 2022). The three major types of ROS are: superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2, non-free radical), hydroxyl ($OH^{\bullet}$) (Agarwal et al., 2005); they are also generated by embryo metabolism (Guérin et al., 2001). The RNS include nitric oxide ($NO^{\bullet}$); RNS major substrate, nitrogen dioxide (NO_2, non-free radical), peroxynitrite ($ONOO^-$, non-free radical) and others (Ufer et al., 2014; Moyinoluwa Comfort et al., 2022).

ROS at low levels (balance between ROS and antioxidants) act as second messengers in cell-signalling pathways to drive normal cell functions by regulating

survival, growth, proliferation, apoptosis, and others. On the other hand, high levels of ROS result in oxidative stress (imbalance between ROS and antioxidants) are detrimental to cells (Dennery, 2007; Ufer et al., 2014; H. Sies & D. P. Jones, 2020; Aranda-Rivera et al., 2022) (Fig. 2.8).

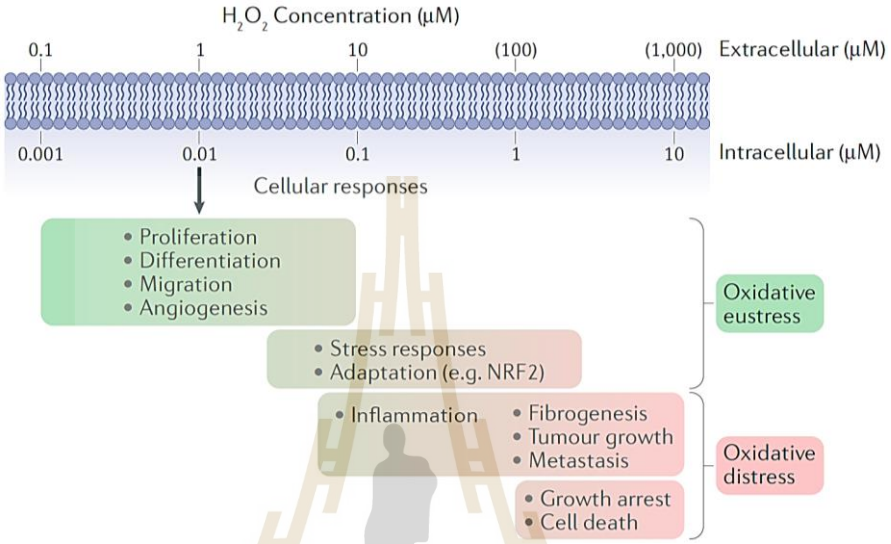

Figure 2.8 Estimated ranges of H_2O_2 concentration in oxidative stress with regard to cellular responses. Stress responses and adaptation occur at higher concentrations. Even higher exposure leads to inflammatory response, growth arrest and cell death by various mechanisms. Green and red colouring denotes predominantly beneficial (eustress) or deleterious responses (distress), respectively (Sies, 2017; Helmut Sies & Dean P. Jones, 2020).

Oxidative stress occurs when the levels of reactive oxygen species (ROS) and/or reactive nitrogen species (RNS) or other compounds (i.e. lipid radicals, amino acid radicals) are unable to be controlled by antioxidants, due to the overabundance of free radicals thus causing lipid peroxidation and DNA and protein damages in cells (Ufer et al., 2014; Aranda-Rivera et al., 2022; Moyinoluwa Comfort et al., 2022).

During pre-implantation embryo development, ROS can be generated from embryo metabolism and/or embryo surroundings (Guérin et al., 2001) or from *in vitro* culture (i.e. O_2 concentration, light, culture media) (Mauchart et al., 2023) and its level was shown to differ among embryo stages (Dalvit et al., 2005; Hajian et al., 2017) (Fig. 2.9). ROS are created as a result of ATP production by means of oxidative phosphorylation using pyruvate or oxaloacetate as a substrate (Harvey et al., 2002), most of them arises when electrons leak from the electron transport chain (ETC)

occurring in mitochondria (Fujii et al., 2005; Agarwal et al., 2012), and enzyme reactions including NADH oxidase (NOX) and xanthine oxidase (Guérin et al., 2001; Deluao et al., 2022).

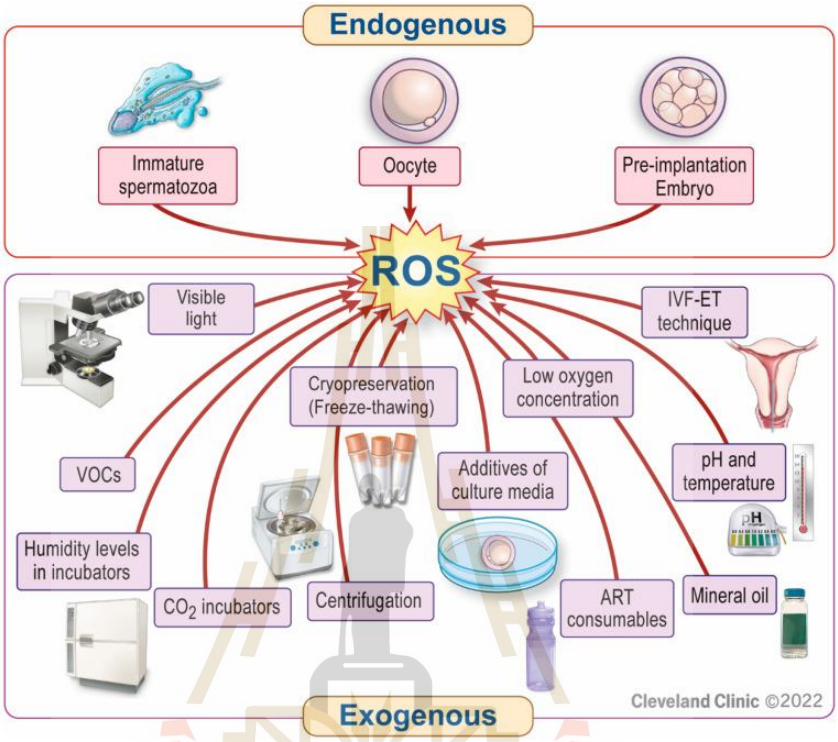

Figure 2.9 Factors responsible for increased ROS generation in an ART setting. ROS can be produced intracellularly from immature sperm, oocytes, or embryos. External sources or triggers of ROS production include inappropriately high or ultra-low oxygen tension, contamination of laboratory air, CO_2 incubators, or ART consumables (e.g., plastics, bisphenols) with volatile organic compounds (VOCs). In addition, centrifugation, visible light, temperature, humidity levels in incubators, mineral oil, additives of culture media, the *in vitro* fertilization (IVF)-embryo transfer (ET) technique, and cryopreservation of gametes or embryos also contribute to ROS generation in an ART setting (Agarwal et al., 2022).

In 1999, Iwata and colleagues found that allopurinol, an inhibitor of xanthine oxidase can reduce the peroxide in bovine embryos during *in vitro* production. ROS not only occurred from xanthine oxidase and hypoxanthine reactions but also are partly originated by high oxygen tension (5% CO_2 in air) and high glucose concentrations in the medium (Iwata et al., 1999). Thompson and

colleagues (2000) demonstrated that inhibition of oxidative phosphorylation using antimycin A and NaN3 inhibitors decreases ROS production in bovine embryos (Thompson et al., 2000). NADPH oxidase inhibitors incubated with mouse 2-cell embryos contribute to a concentration-dependent reduction in H_2O_2 generation (Nasr-Esfahani & Johnson, 1991).

Although ROS is essential for normal embryo development, overproduction of ROS causes embryo arrest, increased DNA damage, altering gene expression resulting in abnormal fetus (Deluao et al., 2022), mitochondrial alterations, ATP depletion, and apoptosis (Mauchart et al., 2023). A direct relationship between increased ROS and apoptosis was observed, contributing to fragmentation in human embryos (Yang et al., 1998; Lee et al., 2012; Mauchart et al., 2023). The increase of H_2O_2 was detected at 2-4-cell stage, during embryonic genome activation (ZGA) in mouse embryos cultured *in vitro* (Nasr-Esfahani et al., 1990), suggesting that ZGA is sensitive to ROS and may cause developmental arrest at the two-cell stage in mouse embryo (Harvey et al., 2002).

Yuan et al (2003) investigated an optimal oxygen concentration for bovine embryo culture *in vitro* in SOF for 9 days. Presumptive zygotes were subjected to culture under 4 different oxygen tensions: 2% O_2; 5% O_2; 20% O_2; and alternating O_2 tension, 20% O_2 for 3 days followed by 5% O_2 up to day 9 before access embryo development at days 3 (8-cell embryo), 6 (morula), 7 (blastocyst) and 9 (hatched blastocyst) and blastocyst quality on day 9 in addition to total cell number and apoptotic cells, both criteria were evaluated by TUNEL staining and *Bax* gene expression using RT-PCR. Embryos cultured under 20% O_2 resulted in the highest rate of 8-cell embryos compared to under 2% and 5% O_2, but lowest blastocyst and hatched blastocyst against the rest of 3 groups. The apoptotic cell ratio of embryos cultured under 20% O_2 was higher than for under 2%, 5% and alternating O_2, while the total cell number was lower when compared to 5% and alternating O_2. *Bax* expression was detected in only one embryo per each 5% and 20% O_2 cultured conditions. They suggested that 5% O_2 is optimal for bovine embryo culture in SOF without cell support (Yuan et al., 2003).

Amin et al (2014) studied the effects of two different oxygen tensions (5% and 20% O_2) on embryo development and the ability of preimplantation bovine embryos to activate the NF-E2-related factor 2 (NRF2)-mediated oxidative-stress-response pathway activity by means of ROS detection using 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA), mitochondrial activity detection by MitoTracker® Red CMXRos, blastocyst total cell number through Hoechst 33342

staining, cryotolerance, blastocyst immunofluorescence staining of NRF2 and KEAP1 (NRF2 inhibitor) proteins and embryo gene expression using real-time quantitative PCR. Genes associated with the NRF2 pathway (*NRF2*, *KEAP1*, *SOD1*, *CAT1*, *PRDX1*, *HMOX1*, *NQO1*, and *TXN1*) and lipid metabolism (*SREBF1*, *ACACA1*, *CPT2*, and *PPARA*α) were monitored. The results showed that embryos cultured under 20% O_2 had low blastocyst rates, low blastocyst total cell number and low mitochondrial activity, high ROS levels, high expression of nuclear NRF2 protein but less expression of cytoplasmic KEAP1 protein in blastocyst than those cultured at 5% O_2. Regarding gene expression analysis in pre-implantation embryos at different stages (2-, 4-, 8-, 16-cell and blastocyst), the expression of *NRF2*, *SOD1*, *CAT*, and *PRDX1* were upregulated while *KEAP1* was downregulated at 8-cell embryo up to blastocyst in embryos derived from 20% O_2 compared to 5% O_2. Higher expression levels of *HMOX1*, *NQO1* and *TXN1* were found upregulated at the 8- and 16-cell stages in embryos cultured at 20% O_2 compared to 5% O_2. In contrast, the expression of *SREBP1*, *ACACA1*, *CPT2*, and *PPARA*α were upregulated at various stages of development for embryos cultured at 20% O_2 compared to 5% O_2. For cryotolerance, hatching rate of vitrified-thawed competent blastocysts (embryos that reached the expanded-blastocyst stage at day-7 pi) was higher than those noncompetent blastocysts (embryos that reached the expanded-blastocyst stage at day-8 pi) regardless of oxygen tension. The findings of this study suggest that the activity of the NRF2-mediated oxidative stress response pathway is associated with the survival and developmental competence of bovine pre-implantation embryo cultured under 20% O_2 (Amin et al., 2014).

Leite et al (2017) examined the effects of two different oxygen tensions (5% and 20% O_2) on embryo development from days 7 to 9 and expanded blastocyst quality on day 7 in terms of ROS detection using 2′,7′-dichlorofluorescein (DCF) probe, differential staining (Hoechst 33342/ propidium iodide) of total cell number, ICM and TE and gene expression through RT-qPCR. Embryos cultured under 5% O_2 contributed to higher blastocyst rate from days 7 to 9, higher blastocyst total cell number, ICM and TE while lower ROS levels in embryos in comparison to under 20% O_2. For genes related to embryo development regulation, *CDX2*, *HSF1*, *KEAP1* and *OTX2* were found upregulated whereas *HAND1*, *MAPK1* and *NFkB2* were shown to be downregulated in embryos cultured under 5% O_2 compared to under 20% O_2. On the other hand, genes related to antioxidant response elements (*NFE2L2*, *ARO*, *CAT*, *GXP1*, *PRDX1*, *SOD1* and *SOD2*), response to cellular stress and DNA damage repair (*HSP90AA1*, *HSPD1* and *MORF4L2*) were upregulated in embryos cultured

under 20% O_2 against under 5% O_2. Considering genes related to cell proliferation, *HSF1*, *EGFR*, and *GSK3A* were upregulated whereas *MAPK1* was downregulated under 5% O_2 than for under 20% O_2, in which only *PLAC8* can be detected. For apoptosis-related genes, although *DDIT3* was upregulated under 5% O_2 compared to under 20% O_2, all *CASP3*, *HSPD1*, *BAX*, *MORF4L2* and *PLAC8* were downregulated. In conclusion, using 20% O_2 tension during IVD of bovine embryos affects embryo development and quality including alteration of the expression of genes related to embryo development regulation, oxidative and cellular stress response, DNA damage repair, cell proliferation and apoptosis (Leite et al., 2017).

2.4.2 Antioxidant effects of oviduct epithelial cells and secretions

Antioxidants define any compound that can delay or prevent the oxidation of substrate (Aranda-Rivera et al., 2022). They are classified into two types: enzymatic and non-enzymatic. Enzymatic antioxidants mention natural antioxidants (Agarwal et al., 2005) that function to break-down and remove free radicals; for example, superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), glutathione reductase (GR) and peroxiredoxins (Prxs) (Wu et al., 2013; Moyinoluwa Comfort et al., 2022).

SOD catalyzes the dismutation of superoxide ($O_2^{\bullet-}$) to oxygen (O_2) and hydrogen peroxide (H_2O_2) (Madkour, 2020). In mammalian cells, SOD consists of three isoenzymes (Fujii et al., 2005; Agarwal et al., 2012; Dasgupta & Klein, 2014; Madkour, 2020): i) SOD 1 (Cu, Zn-SOD) is located in cytosol and mitochondria, it requires copper (Cu) and zinc (Zn) as metal cofactors; ii) SOD 2 (Mn-SOD) is mainly found in mitochondria, it requires manganese (Mn); and iii) SOD 3 (extracellular SOD or EC-SOD) is located outside cells, it also needs Cu and Zn as cofactors. SOD 1 is a dimer, whereas SOD 2 and -3 are tetramers (Dasgupta & Klein, 2014). CAT is located in the peroxisome (Madkour, 2020). It is responsible for converting H_2O_2 (SOD byproduct) into water and oxygen and it needs iron as a cofactor (Dasgupta & Klein, 2014). The glutathione (GSH) family of enzymes includes glutathione peroxidase (GPx), glutathione S-transferase (GST) and glutathione reductase (GR) (Yao et al., 2002; Wang et al., 2021). GPx neutralizes H_2O_2 by taking hydrogens from two GSH molecules (act as a source of hydrogen) resulting in two H_2O and one GSSG (oxidized glutathione) (Pandey & Rizvi, 2010). GSSG which is, in turn, catalyzed by GR using NADPH obtained from pentose phosphate pathway as a source of hydrogen, thus GSH is recycled (Kellner et al., 2017; Adeoye et al., 2018; Wang et al., 2021). In addition, GPx can catalyze the reduction of organic hydroperoxides (-ROOH) into alcohol and water groups (Atika & Naouel, 2021) (Table 2.10).

Table 2.10 Enzymatic antioxidant defence reaction (Rahman & Biswas, 2006; Madkour, 2020).

Enzymatic antioxidants	Catalyzed reaction
Superoxide dismutase (SOD)	$2\ O_2^{\bullet-} + 2\ H^+ \rightarrow H_2O_2 + O_2$
Glutathione peroxidase (GPx)	$2\ GSH + H_2O_2 \rightarrow GSSG + 2\ H_2O$ $2\ GSH + ROOH \rightarrow GSSG + ROH + H_2O$
Catalase (CAT)	$2\ H_2O_2 \rightarrow 2\ H_2O + O_2$
Glutathione reductase (GR)	$GSSG + NADPH + H^+ \rightarrow 2\ GSH + NADP^+$
Peroxiredoxins (Prxs) (most)	$H_2O_2 + Trx(SH)_2 \rightarrow TrxS_2 + 2\ H_2O$

$O_2^{\bullet-}$, Superoxide anion; H^+, Proton; H_2O_2, Hydrogen peroxide; O_2, Oxygen; GSH, Glutathione; GSSG, Oxidized glutathione; H_2O, Water; ROOH, Organic hydroperoxides; ROH, Organic alcohol; NADPH, Reduced nicotinamide adenine dinucleotide phosphate; $NADP^+$, Oxidized nicotinamide adenine dinucleotide phosphate; $Trx(SH)_2$, Reduced thioredoxin; $TrxS_2$, Oxidized thioredoxin.

On the other hand, non-enzymatic antioxidants refer to natural or synthetic molecules, widely used as dietary supplements (Agarwal et al., 2005), that function to interrupt ROS chain reactions (Babula et al., 2012; Moyinoluwa Comfort et al., 2022). For example, vitamin A, C and E, pyruvate, albumin, GSH, taurine, hypotaurine, cysteamine (CSH), Zn, selenium (Se), betacarotene, and carotene (Guérin et al., 2001; Sharma & Agarwal, 2004; Agarwal et al., 2012; Moyinoluwa Comfort et al., 2022) are non-enzymatic antioxidants.

Embryos developed *in vivo* are protected against oxidative stress due to the low oxygen tension in the mammalian oviduct lumen (< 9% O_2) (Fischer & Bavister, 1993; Gardner, 1999; Harvey et al., 2007; Amin et al., 2014) and antioxidants naturally present in the follicular and oviductal fluids (i.e. vitamin A, C and E, pyruvate, taurine, GSH and cysteamine) (Deluao et al., 2022). By contrast, embryos produced *in vitro* rely on their own antioxidant defence mechanisms (Fig. 2.10) or antioxidants present in the culture medium (Deluao et al., 2022).

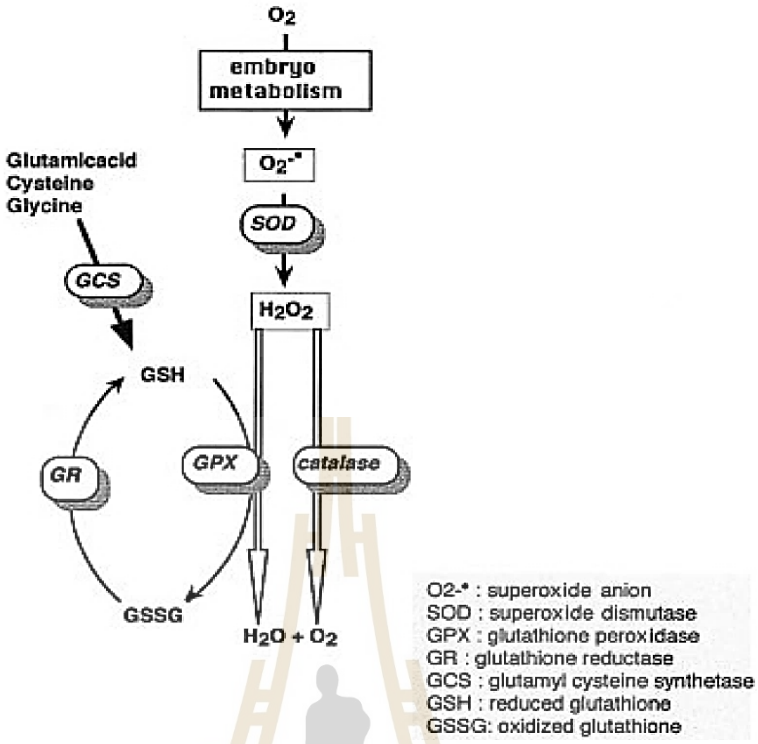

Figure 2.10 Enzymatic antioxidant defences in oocytes and embryos (Guérin et al., 2001).

Oviduct epithelial cells have been shown to secrete GSH, hypotaurine and taurine in the co-culture system (Guérin et al., 2001) (Fig. 2.11). Schmaltz-Panneau and colleagues demonstrated that Copper, zinc superoxide dismutase (Cu,Zn-SOD) and phospholipid hydroperoxide glutathione peroxidase (GPx-4) are two antioxidant enzymes expressed in BOEC (Schmaltz-Panneau et al., 2015). Cu,Zn-SOD functions to scavenge superoxide radicals in contrast to GPx-4 reduces lipid hydroperoxides and H_2O_2 which is derived from Cu,Zn-SOD reaction (Maas et al., 1976; Paria & Dey, 1990; Lapointe et al., 2005). The expression of Cu,Zn-SOD mRNA levels was detected throughout the culture as found in the oviduct and throughout the estrous cycle as found in the oviductal fluids (Lapointe & Bilodeau, 2003). While GPx-4 mRNA expression has been observed to increase at the beginning of co-culture (confluence; day 5) with steady levels up to the end of the end of embryo co-culture (day 13). Moreover, GPx-4 was suggested to be induced by high O_2 tension (5% CO_2) from culture. Thus, the success of blastocyst formation was partly from the beneficial effect of BOEC removal ROS by antioxidant enzymes (Schmaltz-Panneau et al., 2015) (Fig. 2.11). This finding can therefore be assumed that BOEC may act also as oxygen consumers and thus decrease the oxygen tension in the medium.

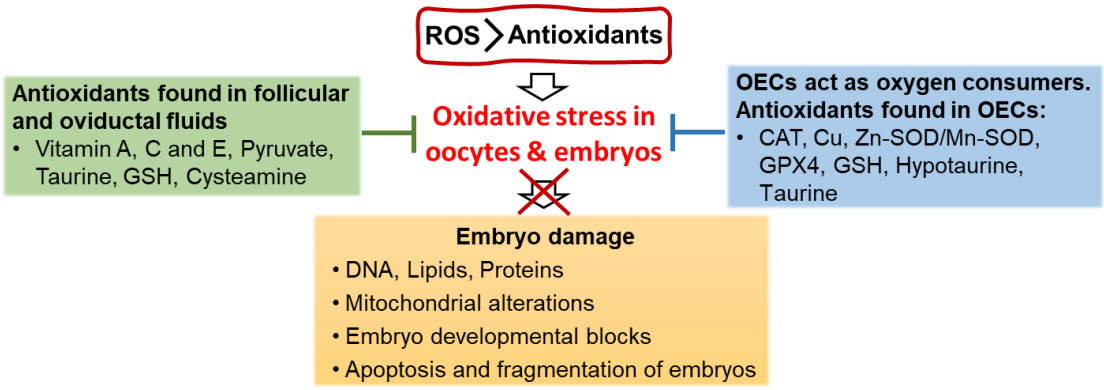

Figure 2.11 Effects of antioxidants found in OEC, FF (follicular fluid) and OF (Guérin et al., 2001; Schmaltz-Panneau et al., 2015; Deluao et al., 2022).

Nevertheless, the impact of oxidative stress on embryos in aspects of transcriptomics and the supportive effect of OEC protect against oxidative stress during co-culture with embryos require further investigation to gain insight on how oviductal environment is beneficial to developing embryo.

CHAPTER 3
CHARACTERIZATION OF OVIDUCT EPITHELIAL SPHEROIDS FOR THE STUDY OF EMBRYO–MATERNAL COMMUNICATION IN CATTLE

3.1 Abstract

Most *in vitro* models of oviduct epithelial cells (OEC) used thus far to gain insights into embryo–maternal communication induce cell dedifferentiation or are technically challenging. Moreover, although the presence of developing embryos has been shown to alter gene expression in OEC, the effect of embryos on OEC physiology remains largely unknown. Here, we propose a model based on bovine oviduct epithelial spheroids (OES) with specific shape and diameter (100–200 µm) criteria. The aims of this study were to i) determine the appropriate culture conditions of bovine OES cultured in suspension by evaluating their morphology, total cell number, viability, and activity of ciliated cells; ii) monitor gene expression in OES at the time of their formation (day 0) and over the 10 days of culture; and iii) test whether the vicinity of developing embryos affects OES quality criteria. On day 10, the proportions of vesicle-shaped OES (V-OES) were higher in M199/500 (500 µL of HEPES-buffered TCM-199) and synthetic oviduct fluid (SOF)/25 (25-µL droplet of SOF medium under mineral oil) than in M199/25 (25-µL droplet of M199 under mineral oil). The proportion of viable cells in V-OES was not affected by culture conditions and remained high (>80%) through day 10. The total number of cells per V-OES decreased over time except in SOF/25, while the proportions of ciliated cells increased over time in M199/500 but decreased in M199/25 and SOF/25. The movement amplitude of OES in suspension decreased over time under all culture conditions. Moreover, the gene expression of *ANXA1*, *ESR1*, *HSPA8*, and *HSPA1A* in OES remained stable during culture, while that of *PGR* and

OVGP1 decreased from day 0 to day 10. Last, the co-culture of developing embryos with OES in SOF/25 increased the rates of blastocysts on days 7 and 8 compared to embryos cultured alone, and increased the proportion of V-OES compared to OES cultured alone. In conclusion, M199/ 500 and SOF/25 provided the optimal conditions for the long-time culture of OES. The supporting effect of OES on embryo development and of developing embryos on OES morphology was

evidenced for the first time. Altogether, these results point OES as an easy-to-use, standardizable, and physiological model to study embryo–maternal interactions in cattle.

3.2 Introduction

The oviducts are paired ducts composed of three parts: the infundibulum, covering the ovary; the ampulla; and the isthmus, the narrower part, which finishes at the utero–tubal junction. Although the oviduct is a tiny part of the female genital tract, it endorses several major functions to secure the success of pregnancy (Li & Winuthayanon, 2017). At ovulation, the cumulus–oocyte complex (COC) is captured by the infundibulum, then driven by beating cilia to the ampulla, where it awaits fertilization. On the other side, spermatozoa travel through the female genital tract to the oviduct and bind to the ciliated epithelium of the isthmus until they are released toward the ampulla to meet the oocyte. If fertilization occurs, the newly formed embryo develops in the isthmus through the 8–16-cell to morula stages, when it enters the uterus (Maillo et al., 2016; Saint-Dizier et al., 2019).

Although *in vitro* embryo production has been successfully achieved in different species, it bypasses this oviduct milieu, resulting in low success in terms of blastocyst rate and capacity to give rise to healthy offspring (Review in: (Ferré et al., 2020)). Therefore, oviduct epithelial cells (OEC) cultured *in vitro* have been widely used to mimic the maternal environment and improve embryo development in different species, including rabbits (Carney et al., 1990), sheep (Gandolfi & Moor, 1987), goats (Yadav et al., 1998), mice, pigs, cattle, and humans (Li & Winuthayanon, 2017). Co-culturing with OEC has been shown to enhance blastocyst quality in terms of cell numbers, cryotolerance (Schmaltz-Panneau et al., 2015), and expression of target genes in cattle (Cordova et al., 2014), as well as pregnancy rates in humans (Yeung et al., 1992), red deer (Locatelli et al., 2005), and goats (Rodríguez-Dorta et al., 2007).

Two culture systems of OEC have mainly been used to support embryo development *in vitro*. The first and most documented one is OEC monolayers grown on plastic dishes. This model revealed for first time the existence of a real dialogue between embryos and OEC during co-culture, providing increased embryo development rate and quality (Rizos et al., 2002; Cordova et al., 2014; Schmaltz-Panneau et al., 2015), as well as changes in OEC gene expression profiles as a result of the co-culture with embryos (Schmaltz-Panneau et al., 2014; Hamdi et al., 2019). In addition, OEC-derived conditioned media supported embryo development (Mermillod et al., 1993), indicating that soluble factors are also involved. OEC

adherent to plastic rapidly dedifferentiate, however, losing morphological criteria of cell differentiation like cilia and secretory granules after 3 days in culture (Walter, 1995). Moreover, after 5–10 days in culture, OEC displayed a decrease in gene expression of steroid hormone receptors and oviduct-specific glycoprotein 1 (*OVGP1*) (Schmaltz-Panneau et al., 2015). The second system consists of OEC monolayers grown on inserts in air–liquid interphase (ALI). This system maintains epithelial morphology more similar to the *in vivo* ones, including ciliated cells, columnar shape, and intercellular cohesion, as well as the gene expression of steroid hormone receptors and *OVGP1* (Schoen & Chen, 2018). The ALI culture of OEC, however, takes long to establish (3–4 weeks, depending on the species), is technically challenging and so far has not shown any supporting effect on development of co-cultured bovine embryos (Chen et al., 2017; van der Weijden et al., 2017). More recently, vesicle-shaped organoids derived from oviduct epithelial stem cells have been reported (Bourdon et al., 2021; Lawson et al., 2023), but their production is long and challenging. In addition, the apical side of OEC is inside the organoid, making it difficult to interact with developing embryos.

The culture of OEC in suspension has been proposed as an alternative. Walter (1995) reported the first culture of free-floating bovine OEC forming vesicles and demonstrated that this culture could better maintain the cilia and secretory granules compared to confluent monolayers after 12 days in culture. Rottmayer et al. (2006) proposed a short time suspension culture of OEC aggregates for 24 h and evidenced the maintenance of their morphology and stable gene expression of OVGP1 and steroid hormone receptors after 6 h of culture. The possibility, however, of culturing OEC in suspension during the time needed for supporting embryo development, i.e., 7–9 days, has not been yet assessed. Moreover, OEC aggregates in suspension are not morphologically uniform in shape and size, a disparity that can be a source of variability between replicates; thus, they require proper characterization and standardization to be used for embryo co-culture purposes. Here, we propose a model based on oviduct epithelial spheroids (OESs), which differ from oviduct organoids in that they form rapidly (within 48–72 h) from isthmic mucosa fragments (IMF) containing an already-differentiated epithelium. We previously reported OESs with specific shape and size criteria (to avoid variability between replicates) as a good model to study sperm–oviduct interactions in cattle (Mahé et al., 2023). Here we proposed using OES for suspension co-culture with embryos. For this purpose, we based our model on the bovine species since it represents an excellent model for human reproduction (Polejaeva et al., 2016) and has been widely used to study the

early embryo–maternal interactions in the oviduct. Moreover, bovine oviducts are available from the slaughterhouse, allowing it to be used in experiments without further concerns about rare samples, breeding season, or ethics.

For the present study, we hypothesized that 1) OESs cultured in suspension may maintain well-differentiated OEC and stable gene expression for 10 days; 2) the density of OESs and culture medium composition may affect their quality in terms of morphology, cell viability, and activity of ciliated cells; and 3) the co-culture of OESs with developing embryos may support embryo development and OES quality. Therefore, the objectives in this study were to: i) determine the appropriate culture conditions of bovine OESs by evaluating their morphology, total cell number, viability, and activity of ciliated cells; ii) monitor gene expression in OESs at the time of their formation (day 0) and over the 10 days of culture under the best conditions; and iii) test whether the vicinity of developing embryos affects embryo development and OES quality.

3.3 Materials and methods

3.3.1 Materials

1) Chemicals and reagents

All chemicals and reagents were purchased from Sigma-Merck (St. Louis, MO, USA) unless otherwise stated.The following were used: phosphate-Buffered Saline (PBS; 1X, Eurobio Scientific, France, CS1PBS01-01), 4% paraformaldehyde (Santa Cruz Biotechnology, SC- 281692), Triton X-100 (9036-19-5), bovine serum albumin (BSA; A9647), Hoechst 33342 (B2261; 1 mg/mL), ethidium homodimer-1 (Invitrogen E1169, MA, USA), anti-cytokeratin (C2931), anti-acetylated tubulin (T7451), IgG1 (M9269), Alexa Fluor 488 goat anti-mouse IgG (Invitrogen A11001, MA, USA), Texas Red™ -X Phalloidin (Invitrogen T7471, MA, USA; 2 U/mL in methanol), mineral oil (ORIGIO Denmark), QIAGEN RNeasy Plus Micro Kit (Catalog no. 74034), carrier RNA (QIAGEN, 1068337), QuantiTect® Reverse Transcription Kit (QIAGEN, 1068337), iQTM SYBR® Green Supermix (Bio-Rad, 170- 8886), GenElute™ PCR Clean-Up Kit (NA1020).

2) Media used for cell culture and in vitro embryo production

The OES washing media was HEPES-buffered TCM-199 contained TCM-199 (Gibco 31150–022) and 25 mM HEPES (Gibco 15630-080). Two different media were used for the culture of OES. The **M199** medium was TCM-199 supplemented with 25 mM HEPES, 10% heat-inactivated fetal calf serum (FBS; Gibco A5256701) and 80 mg/L gentamycin (G1272). The **SOF** was synthetic oviductal fluid medium modified

according to Holm et al. (1999) and supplemented with 5% heat-inactivated FBS (MP Biomedicals, Ref: 2916749, batch MP5418).

The washing medium for oocyte collection (mPBS) was demineralized water with 8 g/L NaCl (S-7653), 0.2 g/L KCl (Prolabo 26764298), 0.2 g/L KH_2PO_4 (Prolabo 26936293), 1.43 g/L $Na_2HPO_4.2H_2O$ (Prolabo 28029292), 0.14 g/L $CaCl_2.2H_2O$ (Merck 2382), 0.2 g/L $MgCl_2.6H_2O$ (Merck 5833), 1 g/L D-glucose (S-8270), 0.036 g/L Pyruvate (P-4562), 50 mg/L gentamycin (G1272), 2 mg/L phenol red (P0290), and 0.5 mg/ L BSA (A9647), with osmolarity and pH adjusted to 280 mOsm and 7.4, respectively (Mermillod et al., 1993). The maturation medium was TCM-199 (M4530) supplemented with 5 IU/mL hCG, 10 IU/mL PMSG (PG600, Intervet), 19 ng/mL IGF-1, 2.2 ng/mL FGF, 10 ng/mL EGF, 5 µg/mL insulin, 5 µg/mL transferrin, 5 ng/mL selenium, 90 µg/mL L-Cystein, 100 µM β-mercaptoethanol, 75 µg/mL ascorbic acid, 720 µg/mL glycine, 0.1 mg/mL glutamine, and 110 µg/mL Pyruvate (Donnay et al., 2004; Schmaltz-Panneau et al., 2015). The sperm-washing medium was STL medium based on Tyrode medium supplemented with 25 mM bicarbonate (S5761), 10 mM lactate (L7900), 2.4 mg/mL HEPES (H3375), 6 mg/mL BSA (A9647), and 40 µg/mL gentamycin (G1272) (Schmaltz-Panneau et al., 2015; Lamy et al., 2017). The Tyrode medium was a mix of 1 L demineralized water with 6.666 g NaCl (Merck 6404), 240 mg KCl (Prolabo 26764298), 41 mg $NaH_2PO_4.H_2O$ (Merck 6346), 300 mg $CaCl_2.2H_2O$ (Merck 2382), 100 mg $MgCl_2.6H_2O$ (Merck 5833), and 2 mg/L phenol red (P0290) with osmolarity at 230 mOsm. The fertilization medium was Tyrode medium supplemented with 25 mM bicarbonate (S5761), 10 mM lactate (L7900), 1 mM pyruvate (S4562), 6 mg/mL BSA (A6003), 100 µg/mL heparin (Calbiochem Ref: 375 D95 batch B47089), and 40 µg/mL gentamycin (G1272) (Schmaltz-Panneau et al., 2015; Lamy et al., 2017).

3.3.2 Culture of bovine oviduct epithelial spheroids (OES)

Oviductal cell isolation and culture were conducted as described previously (Mahé et al., 2023). Briefly, pairs of oviducts and ovaries obtained from post-pubertal cows were collected at a local slaughterhouse (Vendôme, France) and transported at 4 °C to the laboratory. Pairs of oviducts at the peri-ovulatory phase of cycle (approximately day -2 to day +4 around ovulation time) were selected according to the morphology of the ovaries. The pre-ovulatory phase of the cycle was identified by a follicle at 11–20 mm in diameter and a small yellow-white *corpus albicans*. The post-ovulatory phase of the cycle was identified by a small *corpus heamoragicum* (<0.5 cm) and the *corpus albicans* from the previous cycle. For each culture, both oviducts from a pool of 2–3 cows were used. After removal of blood

vessels and connective tissue, the oviducts were cut at the ampullary–isthmic junction, when the oviduct diameter becomes smaller and with a more folded and thicker wall. Only the isthmic parts (around 6–8 cm long) of the oviducts were used. After a rapid dip in 70% ethanol and rinsing in 0.9% NaCl, the mucosa was expelled from the isthmic sections by squeezing with forceps into 10 mL of M199, vortexed for 1 min, then incubated at 38.8 °C for 10 min for cell sedimentation. Following the elimination of the supernatant containing cell debris and blood red cells, the pellet (around 1 mL) was resuspended in 10 mL of M199, and the vortex-sedimentation process was repeated. Finally, the pellet was diluted 10 times in the culture medium, and 50 µL of the resulting mixture containing isthmic mucosa fragments (IMFs) was added to 450 µL of M199 to reach a 100-fold final dilution. In the following, unless otherwise specified, "day 0" refers to the day of sorting and culture start of OES. On day -3, the IMFs were cultured in 4-well culture plates (Thermo Fisher Scientific, Denmark) at 38.8 °C in a humidified atmosphere containing 5% CO_2 in air. On day 0, a cavity appeared within the mucosa fragments, forming spheroids of various sizes and shapes, with the apical side of the epithelial cells oriented outward. Spheroids between 100 and 200 µm in diameter, homogeneous in shape and size, and exhibiting a cavity and ciliary beating outward, referred to as "vesicle-shaped OES" (V-OES; Fig. 3.1 and Movie 1) were selected using a mouth-operated drawn Pasteur pipette (Duran Wheaton Kimble, around 300 µm in diameter at the extremity; day 0) for characterization in different culture conditions until day 10 of culture (Experiment 1), for gene expression analysis (Experiment 2), or for co-culture with *in vitro*-produced bovine embryos (Experiment 3; see paragraph 2.8 for experimental design). All OESs, including those that had lost their cavities (collapsing OES), were kept in the culture medium through day 10 of culture.

Supplementary video related to this article can be found at https://doi.org/10.1016/j.theriogenology.2024.01.022

For gene expression analysis by RT-qPCR, the isthmic mucosa fragments (200/replicate) and V-OES at days 0, 3, and 10 of culture (200–400/replicate) were collected. V-OES between 100 and 200 µm in diameter were cultured in 1 mL of M199 (200–400 OES/mL) in a 60 × 15 mm culture dish (Falcon®, 353037). Half of the medium was renewed every 3 days, taking care not to remove any OES. Four biological replicates from 4 different cultures were used. All samples were collected with a minimum volume of medium in a 1.5 mL Ultra High Recovery Microcentrifuge Tube (STARLAB, E1415-2600, USA), immediately snap-frozen in liquid nitrogen, and stored at -80 °C before analysis.

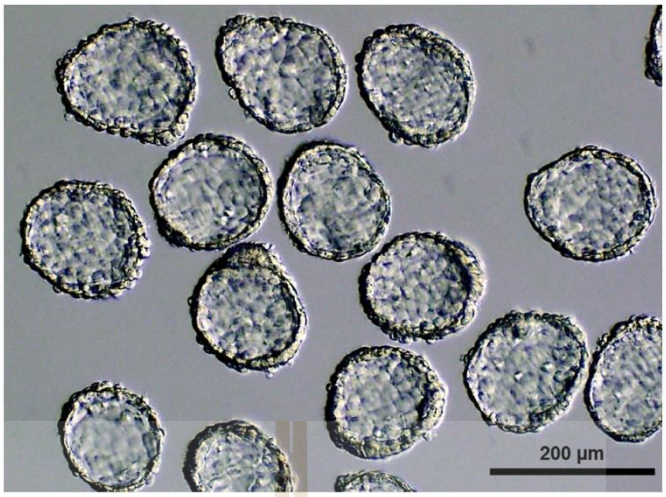

Figure 3.1 Bovine oviduct epithelial spheroids (OES) selected on day 0. Vesicle-shape spheroids containing a cavity, homogeneous in form and size (100–200 µm in diameter) and displaying outward ciliary beating were selected in this study.

3.3.3 Evaluation of OES morphology and straight-line velocity

For evaluation of OES morphology and movement, an inverted microscope (Nikon, Japan) equipped with a SPOT Insight Firewire 2 mega Sample Camera, the SPOT Advanced Software (version 4.5.9.1, USA), and a warm plate were used. Pictures of OES on days 0, 3, and 10 of culture were taken at 40× magnification. For motion analysis, groups of 20–25 OESs in suspension on days 0, 3, and 10 were gently placed at the center of the well and left there for 15 s for stabilization, then 15 successive pictures at 2-s intervals were taken at 40× magnification, allowing us to calculate the proportion of moving OESs and track the movement of individual OESs. All experiments were conducted by the same person to avoid an operator effect. The straight-line track of moving OESs (in pixels) was calculated from the first and last pictures at a 30-s interval using the TrackMate plugin in ImageJ software (version 1.54f) and expressed in µm/sec. The automated tracking of each OES was manually corrected using the TrackScheme function of the TrackMate plugin. Then, the mean straight-line velocity (in µm/sec) of moving OESs per condition was calculated.

3.3.4 Evaluation of cell viability

For the assessment of cell viability, V-OES collected on days 0, 3, and 10 of culture were washed twice in HEPES-buffered TCM-199, then incubated in 500 µL HEPES-buffered TCM-199 containing 2 µg/mL of Hoechst 33342 and 4 µM of ethidium homodimer-1 for 30 min in the dark at 38.8 °C in a humidified atmosphere

containing 5% CO_2 in air. Then, V-OES were washed and mounted on a glass slide (SuperFrost Plus™, Epredia, Germany) for observation under a confocal microscope (Zeiss LSM 700, Carl Zeiss, Oberkochen, Germany) at 200× magnification. Live (blue-nucleus) and dead (purple-nucleus) cells in whole individual V-OES were counted using the QuPath software (version 0.2.2; Fig. 3.4A). On day 10, V-OES and collapsed OES were also assessed for cell viability using the LIVE/DEAD cell viability assay (Invitrogen, MA, USA).

3.3.5 Immunostaining of oviduct epithelial spheroids

Immunostaining for pan-cytokeratin (a marker of epithelial cell intermediate filaments), vimentin (marker of stroma cells), and acetylated alpha-tubulin (marker of ciliated cells) was performed as previously described (Lopera-Vásquez et al., 2016; Ito et al., 2020). Briefly, V-OESs cultured in M199/500 were fixed and permeabilized (4% paraformaldehyde supplemented with 1% BSA and 0.25% Triton X-100, 30 min at 37 °C) on days 0, 3, and 10, washed 3 x in PBS + 1% BSA (PBS-BSA), and incubated in a blocking solution (10% goat serum in PBS-BSA, 30 min, room temperature). V-OESs were then incubated overnight at room temperature with the primary antibody (anti-cytokeratin, C2931; anti-vimentin, V6630, or anti-acetylated tubulin, T7451; final concentrations of 15.7, 28.5, and 2 µg/mL, respectively). The control V-OESs were incubated with IgG1 (M9269) at the same concentration as the primary antibody. After washing in PBS-BSA, the OESs were incubated with the secondary antibody coupled with Alexa Fluor 488 (A11001; final concentration at 1 µg/mL), counterstained with Hoechst 33342 and Texas Red™-X Phalloidin at a final concentration of 1 µg/mL and 2 mU/mL, respectively, for 3 h at room temperature under agitation in the dark. After washing in PBS-BSA, V-OESs were then mounted on a glass slide, observed under a confocal microscope at 200× magnification, and examined using the QuPath software (version 0.2.2).

3.3.6 *In vitro* embryo production (IVP)

1) Oocyte collection and in vitro maturation (IVM)

Bovine ovaries were collected from a local slaughterhouse (Vendˆome, France) and kept in 0.9% NaCl solution at 31–32 °C during transport (45 min) to the laboratory. Cumulus oocyte complexes (COCs) were aspirated from 3 to 6 mm follicles using an 18½-gauge needle connected with a suction pump. Immature oocytes enclosed in at least 3 layers of compacted cumulus cells with homogeneous cytoplasm were selected and washed twice in mPBS, then once in 1 mL of maturation medium. A group of 50–80 COCs were cultured in a 4-well plate (Thermo

Fisher Scientific REF179830, Denmark) containing 500 µL/ well of maturation medium for 22–23 h at 38.8 °C in a humidified atmosphere with 5% CO_2 in air.

2) In vitro fertilization (IVF)

The procedures were implemented as described previously by Schmaltz-Panneau et al. (2015). Briefly, a pool of frozen semen from 2 Holstein bulls of proven fertility was used for all IVF (0.25 mL straw). Straws were thawed in air for 10 s and submerged in 35 °C water for 30 s. Subsequently, frozen-thawed semen was transferred to the top of the Percoll density gradient (45/90%; Cytiva 17-0891-01), centrifuged at 700g for 20 min to retrieve the viable spermatozoa at the bottom, then centrifuged at 100g for 10 min in 5 mL of STL medium to eliminate Percoll. Sperm concentration was evaluated in Thoma cell and adjusted to 4 x 10^6 spermatozoa/mL with fertilization medium. Mature oocytes were washed once with 1 mL of fertilization medium and transferred to a 4-well plate (Thermo Fisher Scientific REF179830, Denmark) containing 250 µL of fertilization medium and 50–80 oocytes/well, and 250 µL of the sperm suspension were added to reach a final concentration of 2 x 10^6 spermatozoa/mL. The dishes were incubated for 18 h at 38.8 °C in a humidified atmosphere of 5% CO_2 in air. The day of fertilization was considered day 0.

3) Embryo culture (IVC)

After IVF, cumulus cells and attached sperm were removed from presumptive zygotes by vortex at moderate speed for 2 min in 2 mL of mPBS. Presumptive zygotes were then washed twice in mPBS, then once in the SOF medium. Next, groups of 25 presumptive zygotes were cultured in 25 µL droplets of SOF with 25 OES (SOF/25/E) at 38.8 °C for 8 days without medium changing under 5% CO_2 in air. The day of culture was considered day 1. Cleavage rates were evaluated on day 2 and blastocyst formation rates on days 6, 7, and 8 using an inverted microscope (Olympus IX70, Japan).

3.3.7 Gene expression analysis by RT-qPCR

RNA extraction was performed using the QIAGEN rNeasy Plus Micro kit according to the manufacturer's instructions and adding 0.01 µg/µL Poly-A carrier RNA (QIAGEN) into the cell lysates. RNA concentration and purity (A260/A280 ratios = 2.04-2.44) were examined using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Denmark). For each sample, 130 ng of total RNA were treated with DNase before reverse transcription (RT) in a final volume of 20 µL using the QuantiTect® Reverse Transcription kit following the manufacturer's instructions. The primers of 8 target genes (*ESR1, ESR2, PGR, OVGP1, ANXA1, VMAC, HSPA1A*, and

HSPA8) and 3 reference genes (*GAPDH, PPIA*, and *YWHAZ*) were designed using the Ensembl database (https ://www.ensembl.org/) and Primer3Plus (https://www.primer3plus.com/; see Table 3.1 for details).

For each gene, a standard curve was created by 1/10 serial dilutions of mucosa fragment cDNAs. The qPCR reactions were conducted in a final volume of 20 µL (1 µL of 6.5 ng/µL cDNA template, 0.25 µL of each primer at 10 µM, 8.5 µL of water, and 10 µL of iQTM SYBR® Green Supermix) using a BIO-RAD instrument (CFX Opus 96 Real-Time PCR System). Two technical replicates of each sample were performed under the following condition: 95 °C for 3 min, followed by 40 cycles at 95 °C for 30 s, 60 °C for 1 min, and 72 °C for 1 min. Only genes expressed with Cq < 32 were considered for expression analysis (Bustin et al., 2009). The normalization factor of the combination of reference genes (*GAPDH*, *PPIA*, and *YWHAZ*) obtained from geNorm algorithms (version 3.5) was used to calculate normalized relative gene expression using the **ΔΔ**Ct method, as previously described (Vitorino Carvalho et al., 2019).

Table 3.1 Primers used in the study. F: forward primer; R: reverse primer; bp: base pairs

Gene Symbol	Gene Name	Primer (5′–3′)	Accession Number	Amplicon size (bp)
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	F: ACCCAGAAGACTGTGGATGG R: ATGCCTGCTTCACCACCTTC	NM_001034034.2	245
PPIA	Peptidylprolyl isomerase A	F: GCATACAGGTCCTGGCATCT R: TTCTTGCTGGTCTTGCCATT	MK309342.1	192
YWHAZ	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta	F: ACTGGGTCTGGCCCTTAACT R: CTGCTTCAGCTTCGTCTCCT	MK396254.1	218
ESR1	Estrogen receptor 1	F: AGGGAAGCTCCTATTTGCTCC R: CGGTGGATGTGGTCCTTCTCT	NM_001001443.1	234
ESR2	Estrogen receptor 2	F: TGATGCTCCTGTCTCACGTC R: AGCCCTCTTTGCTCTCACTG	NM_174051.3	195
PGR	Progesterone receptor	F: GATGCTATATTTTGCGCCTGA R: CTCCTTTTTGCCTCAAACCA	NM_001205356.1	266
OVGP1	Oviductal glycoprotein 1	F: AAGAATGAGGCCCAGCTCAC R: TGCCGAAGATTTGGGGTCTC	NM_001080216.1	219
ANXA1	Annexin A1	F: ACCAGGAGCTATCCCCATCT R: AAAGAACATTGGCTGGCTTG	NM_175784.3	156
VMAC	Vimentin type intermediate filament associated coiled-coil protein	F: ATTGAGCGCCGCTTTAGAC R: CAGCCTGCAGACTCTGAACA	NM_001105371.1	107
HSPA1A	Heat shock protein family A (Hsp70) member 1A	F: AGGCGGACAAGAAGAAGGTG R: GTTACACACCTGCTCCAGCT	NM_203322.3	122
HSPA8	Heat shock protein family A (Hsp70) member 8	F: CGCAATGAATCCCACCAACA R: CCACCATGAAGGGCCAATGT	NM_174345.4	107

3.3.8 Experimental design

Experiment 1 characterized OES morphology, total cell number, viability, proportion of ciliated cells, and motion under 3 different conditions during 10 days of culture. For this purpose, groups of 25 OESs were allocated to one of the 3 following conditions at day 0: 1) 500 µL of M199 (M199/500); 2) a 25-µL droplet of M199 overlaid with mineral oil (M199/25); or 3) 25-µL droplet of SOF medium overlaid with mineral oil (SOF/25). In the M199/500 group, half of the fresh medium was renewed every 3 days. OESs were then evaluated for their morphology, total cell number, percentage of viable cells, percentage of ciliated cells, and motion (percentage of moving OES and straight-line velocity).

Experiment 2 evaluated the immunodetection of cytokeratin and vimentin in the V-OESs and of the candidate genes by RT-qPCR in IMF and V-OES during culture in M199. For this purpose, OESs were cultured in 1 mL of M199. This experiment was conducted only in M199, as it was the medium maintaining the best OES morphology and widely used for cell culture. Only V-OESs were included in the experiment to avoid bias due to the collapsing process.

Experiment 3 evaluated the effects of OES on blastocyst yield and of embryo co-culture on OES morphology, total cell number, viability, proportion of ciliated cells, and motion in SOF/25. For this purpose, on day 0, OES were cultured in 1 mL of M199 until day 2, then transferred into groups of 25 in 25-µL droplets of SOF overlaid with mineral oil (SOF/25) for 24 h. On day 3, groups of 25 OES in SOF/25 were cultured alone (OES control group) or with 25 presumptive *in vitro*-produced zygotes (SOF/25/E) for 8 days, i.e. up to day 10. A control group of 25 presumptive zygotes was cultured without OES in SOF/25 for 8 days (embryo control group). In both groups with embryos, the number of cleaved embryos was assessed on day 2 after IVF, and the numbers of blastocysts were assessed on days 6, 7, and 8 after IVF. In both groups with OES, the OES quality was evaluated on days 0, 3, and 10 using the same criteria as in Experiment 1.

3.3.9 Statistical analysis

Statistical analysis was performed using the GraphPad Prism software (version 8.1.1) and Rstudio (R version 4.3.0). The normality of the data was analyzed using the Shapiro-Wilk test. The effect of the different culture conditions on the proportion of V-OES, total cell number, cell viability, ciliated cells, moving OES, and straight-line velocity (µm/s) were analyzed by one-way ANOVA, followed when appropriate by Tukey's post-tests. The effects of OES on blastocyst yield on days 6, 7, and 8 and of the developing embryos on OES quality parameters on day 10 were

examined using Student t-tests. The RT-qPCR data were analyzed by t-tests (effect of spheroid formation on day 0) and one-way ANOVA followed by Tukey's post-tests (effect of culture time in OES). The cDNAs with a detection threshold beyond 32 Cq were excluded from the analysis. A p-value < 0.05 was considered significant. All data are presented as means ± standard error of the mean (SEM).

3.4 Results

3.4.1 Effect of culture conditions on OES morphology, total cell number, viability, proportion of ciliated cells, and motion

The proportions of V-OES, i.e. OES with a cavity, decreased over time in all culture conditions due to OESs collapsing (Fig. 3.2A) and the attachment of a few OESs at the bottom of the well. The proportion of floating OESs that kept their cavity (V-OES) did not differ between culture conditions on day 3 (70.0 ± 7.7%, 59.0 ± 5.3%, and 75.0 ± 5.7% in M199/500, M199/25, and SOF/25, respectively; Fig. 3.2B). On day 10, however, the proportion of V-OESs was significantly higher in M199/500 and SOF/25 (37.0 ± 3.4% and 27.0. ± 5.5%, respectively) than in M199/25 (13.0 ± 5%; P < 0.05).

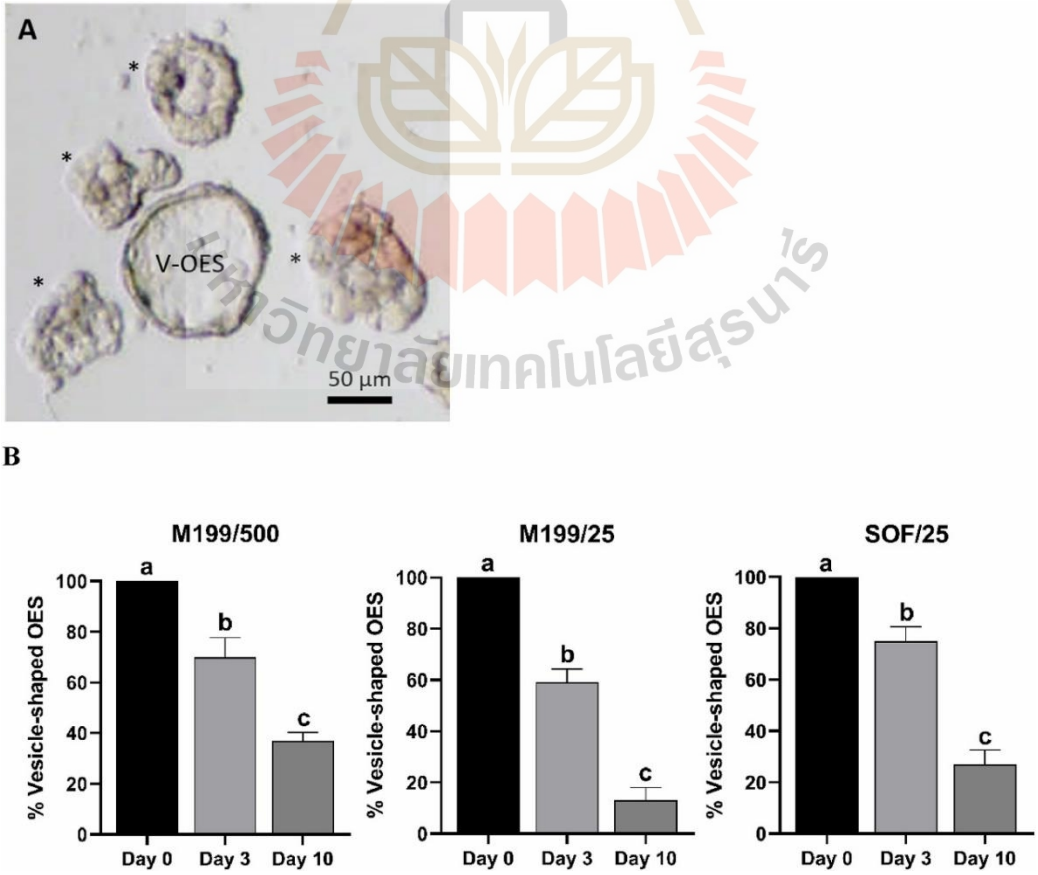

Figure 3.2 Proportions of OES keeping their cavity over time in three different culture conditions (Experiment 1). A, Representative picture of one vesicle-shape OES (V-OES) surrounded by four OES (asterisks) that lost their cavity on day 10 of culture in M199/500; B, Proportions of OES with a cavity on days 0, 3 and 10 of culture under three different culture conditions. Groups of 25 OES were cultured either in 500 µL of M199 (M199/500), 25-µL droplet of M199 under mineral oil (M199/25) or 25-µL droplet of SOF (SOF/25) for 10 days. Data are means ± SEM of 4 replicates (N = 100 OES/condition). The different letters on bars indicate significant differences between days ($P < 0.0001$).

The total number of cells per V-OES decreased over time in all culture conditions (Fig. 3.3; $p < 0.01$). On day 3, the total cell number per OES did not differ between culture conditions (means of 293.7 ± 19.1, 307.3 ± 21.8, and 268.4 ± 17.3 per V-OES for SOF/25, M199/25, and M199/ 500, respectively). On day 10, however, this number was significantly higher in SOF/25 (239.1 ± 18.1) than M199/500 or M199/25 (152.8 ± 10.7 and 195.2 ± 12.3, respectively; $p < 0.01$).

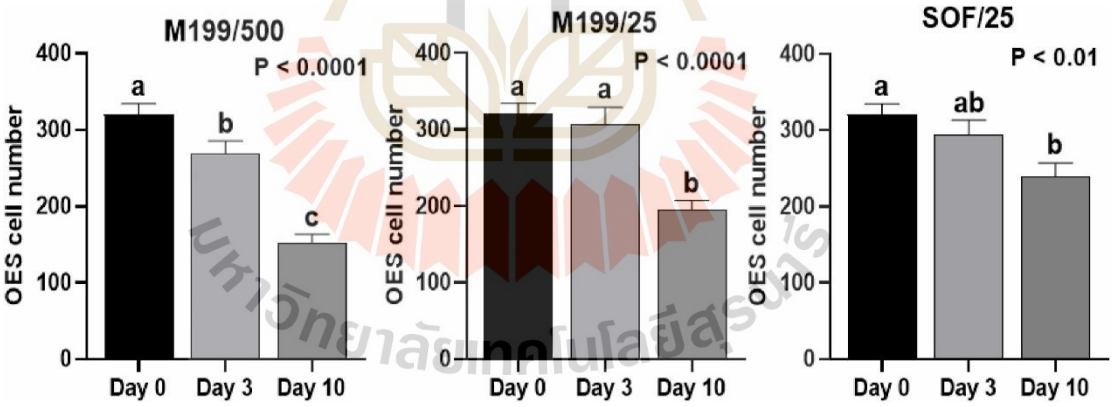

Figure 3.3 Total cell number in vesicle-shaped OES over time in three different culture conditions (Experiment 1). Groups of 25 OES were cultured either in 500 µL of M199 (M199/500), 25-µL droplet of M199 under mineral oil (M199/25) or 25-µL droplet of SOF under mineral oil (SOF/25) for 10 days. Data are provided as the mean ± SEM of 8 replicates (N = 53-87 OES/condition). The different letters on bars indicate significant differences between days.

The proportion of viable cells in V-OES decreased significantly over time (Fig. 3.4B; $p < 0.0001$) but remained high (>80%) through day 10 in all culture conditions. Furthermore, the proportion of viable cells in the V-OES did not differ between culture conditions on days 3 and 10.

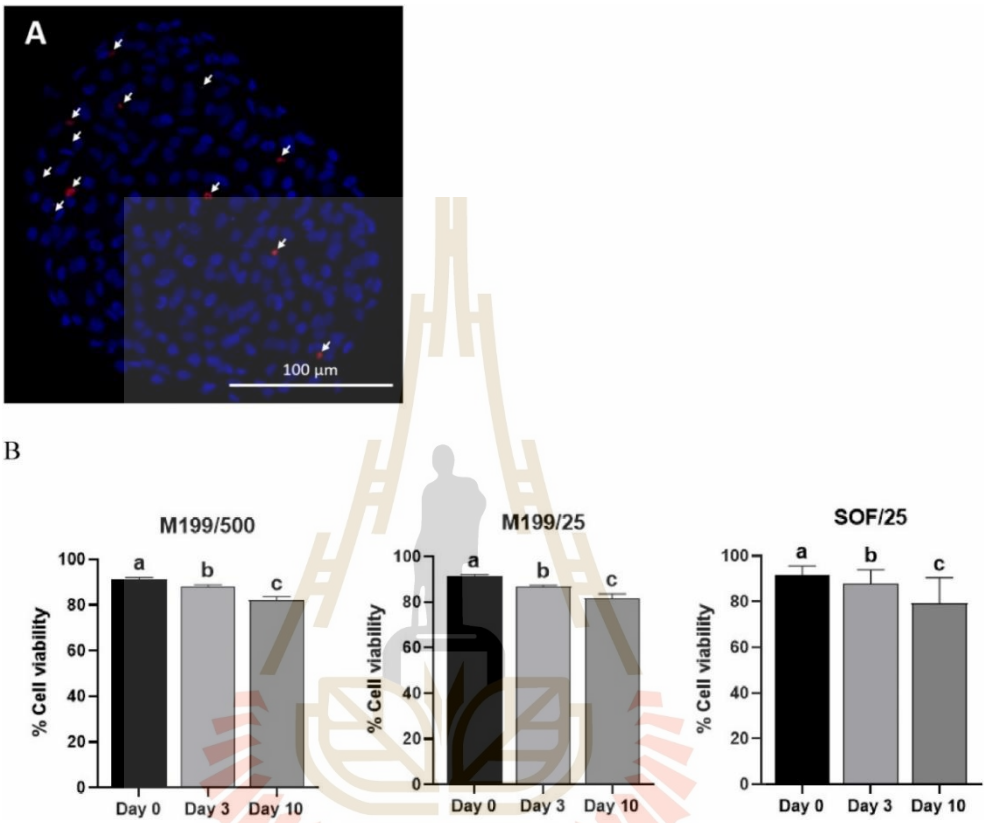

Figure 3.4 Cell viability in vesicle-shaped oviduct epithelial spheroid (OES) over time in three different culture conditions (Experiment 1). A, Representative picture of one vesicle-shaped oviduct epithelial spheroid after staining for assessment of cell viability. The nuclei of live cells appear in blue (stained with Hoechst) while nuclei of dead cells appear in purple (Hoechst + ethidium homodimer-1, arrows); B, Proportions of viable cells in vesicle-shaped OES at days 0, 3 and 10 of culture under three different culture conditions. Groups of 25 OES were cultured either in 500 µL of M199 (M199/500), a 25-µL droplet of M199 under mineral oil (M199/25) or a 25-µL droplet of SOF (SOF/25) for 10 days. Data are provided as the mean ± SEM of 4 replicates (N = 31-53 OES/condition). The different letters on bars indicate significant differences between days ($P < 0.0001$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

The proportions of ciliated cell in V-OES increased over time in M199/500 ($p < 0.01$) but decreased in M199/25 ($p < 0.05$) and SOF/25 ($p < 0.01$; Fig. 3.5). On day 3, the proportion of ciliated cells in V-OES did not differ between culture conditions. On day 10, however, this proportion was significantly higher in M199/500 (41.3 ± 4%) than M199/25 or SOF/25 (15.2 ± 4.3 and 13.9 ± 3%, respectively; $p < 0.0001$).

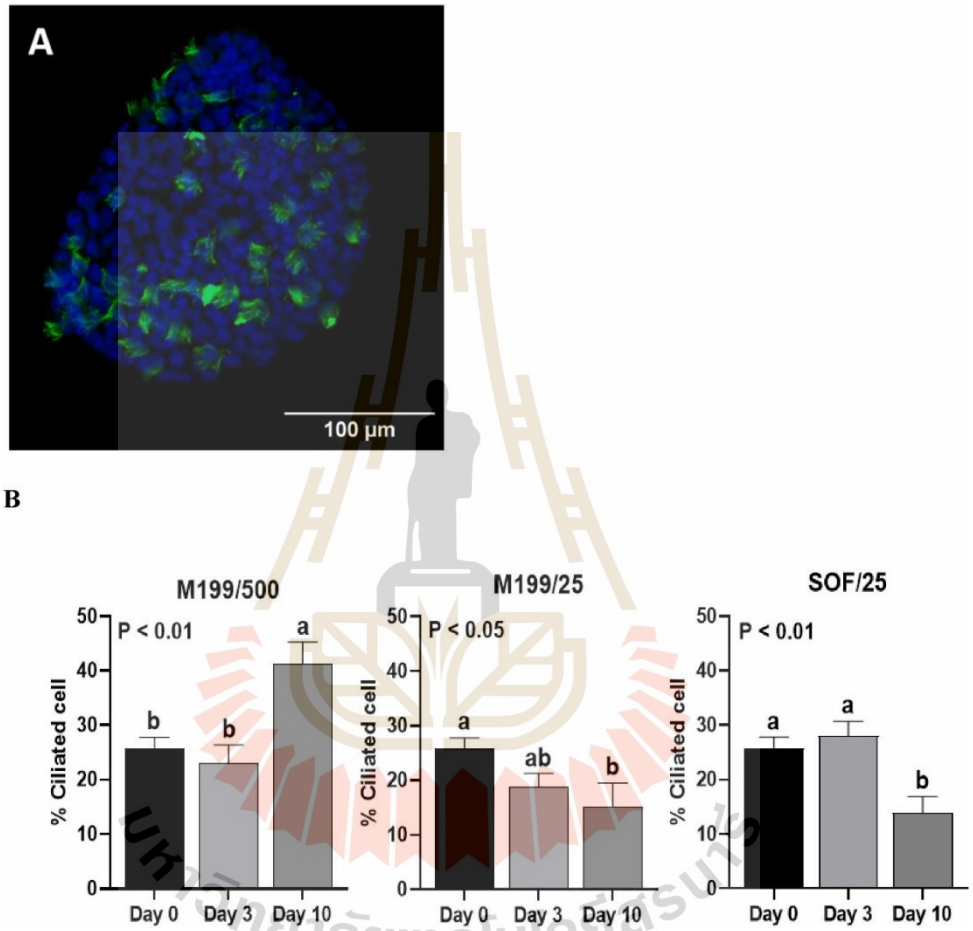

Figure 3.5 Immunostaining for alpha-tubulin and proportions of ciliated cells in vesicle-shaped oviduct epithelial spheroids (OES) over time in three different culture conditions (Experiment 1). A, Representative picture of one vesicle-shaped OES after immunostaining of acetylated alpha-tubulin for assessment of ciliated cells. B, Groups of 25 vesicle-shaped OES were cultured either in 500 µL of M199 (M199/500), a 25-µL droplet of M199 under mineral oil (M199/25) or a 25-µL droplet of SOF (SOF/25) for 10 days. Data are provided as the mean ± SEM of 4 replicates (N = 15-34 OES/condition). The different letters on bars indicate significant differences between days.

The proportions of moving OES due to ciliary beating remained high (>95%) in all culture conditions through day 10 of culture (Fig. 3.6a–c, and e; see Movie 1 for OES motion on day 0). Furthermore, the straight-line velocity of OES decreased over time (from 7.7 ± 0.6 μm/s on average on day 0 to 2.6 ± 0.5 μm/s on day 10; $p < 0.01$), with no difference between culture conditions (Fig. 3.6b–d, and f).

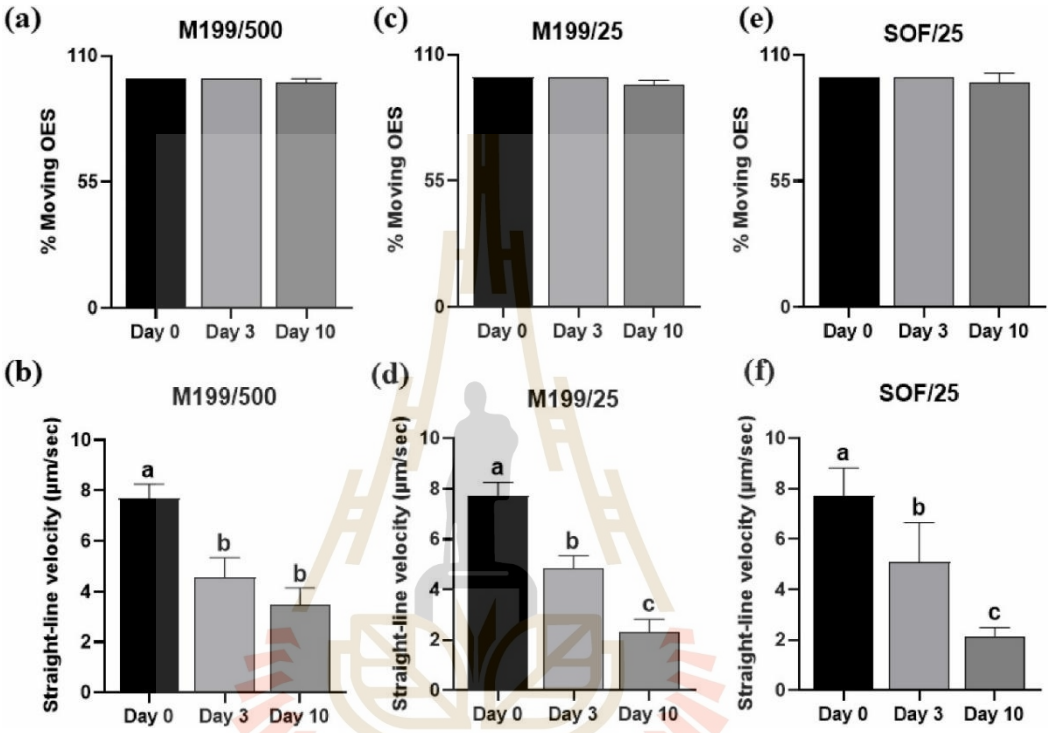

Figure 3.6 Proportions of moving OES and straight-line velocity over time in three different culture conditions (Experiment 1). Groups of 25 OES were cultured either in 500 μL of M199 (M199/500; a and b), 25-μL droplet of M199 under mineral oil (M199/25; c and d) or 25-μL droplet of SOF (SOF/25; e and f) for 10 days. Data are provided as the mean ± SEM of 4 replicates (N = 66-96 OES/condition). The different letters on bars indicate significant differences between days ($P < 0.01$).

3.4.2 Expression of cytokeratin, vimentin, and candidate genes in OES cultured in M199 medium

Cytokeratin, a marker of epithelial cells, was immunodetected in the cytoplasm of V-OES cells through day 10 of culture (Fig. 3.7A). The stromal marker vimentin was not detected through day 6 of culture, but a positive signal could be observed in OES cells on day 10 (Fig. 3.7B). The gene expression of *VMAC*

(a vimentin-type intermediate filament associated coiled-coil protein) and *ESR2* (estrogen receptor 2), as detected by RT-qPCR, was below the sensitivity threshold in the IMF and OES throughout the culture period. These 2 genes were not considered for statistical analysis.

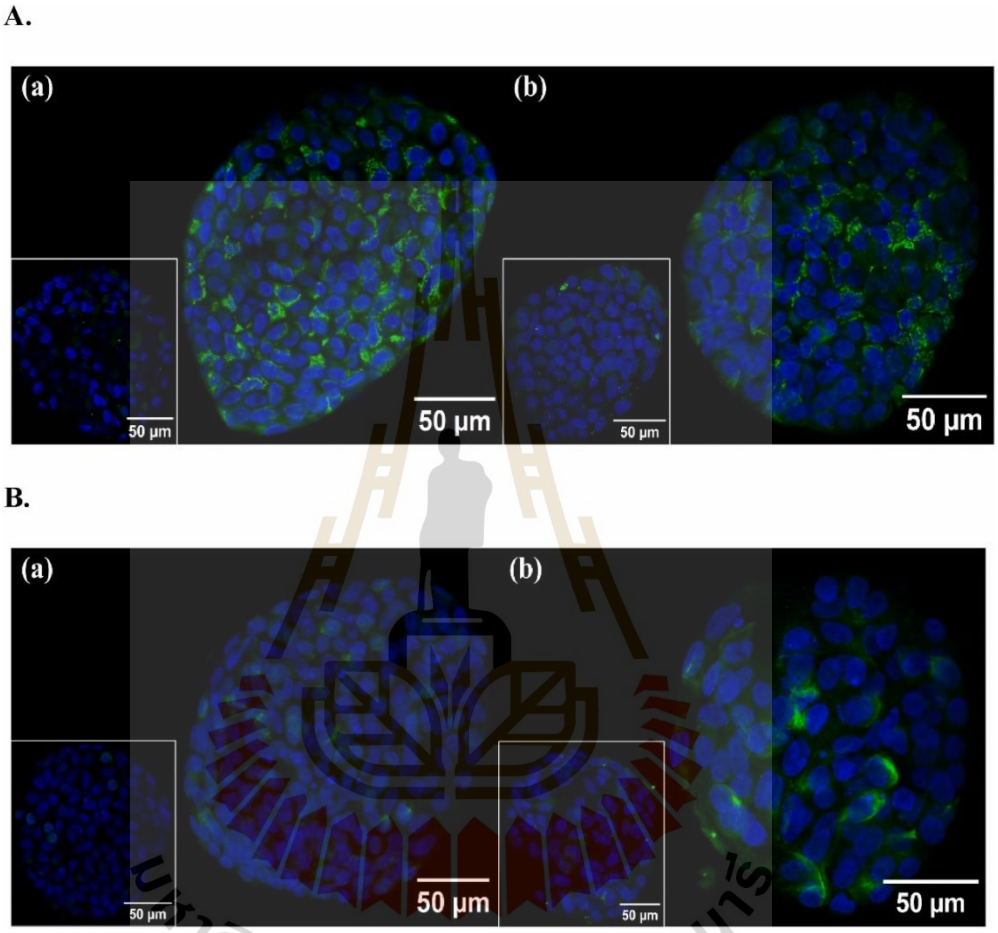

Figure 3.7 Expression of cytokeratin and vimentin in vesicle-shaped OES (Experiment 2). A, Immunostaining for cytokeratin (in green) at day 0 (a) and day 10 (b); B, Immunostaining for vimentin (in green) at day 0 (a) and day 10 (b). The nuclei appear in blue (stained with Hoechst). Inserts show the negative controls incubated with the IgG1 isotype of the primary antibody. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

When comparing the IMF and OES newly formed on day 0, a significant decrease in the expression of *ANXA1 (*annexin A1), *OVGP1* (oviductal glycoprotein 1), *ESR1* (estrogen receptor 1), *HSPA8* (heat shock protein family A (Hsp70) member 8),

and *HSPA1A* (heat shock protein family A (Hsp70) member 1A) was evidenced ($p < 0.05$), while the mRNAs for *PGR* (progesterone receptor) did not change significantly (Fig. 3.8). During OES culture, the gene expression of *ANXA1*, *ESR1*, *HSPA8*, and *HSPA1A* in OES remained stable through day 10, while *OVGP1* and *PGR* gene expression decreased after day 3 and reached lower levels on day 10 compared to day 0 ($p < 0.05$; Fig. 3.9).

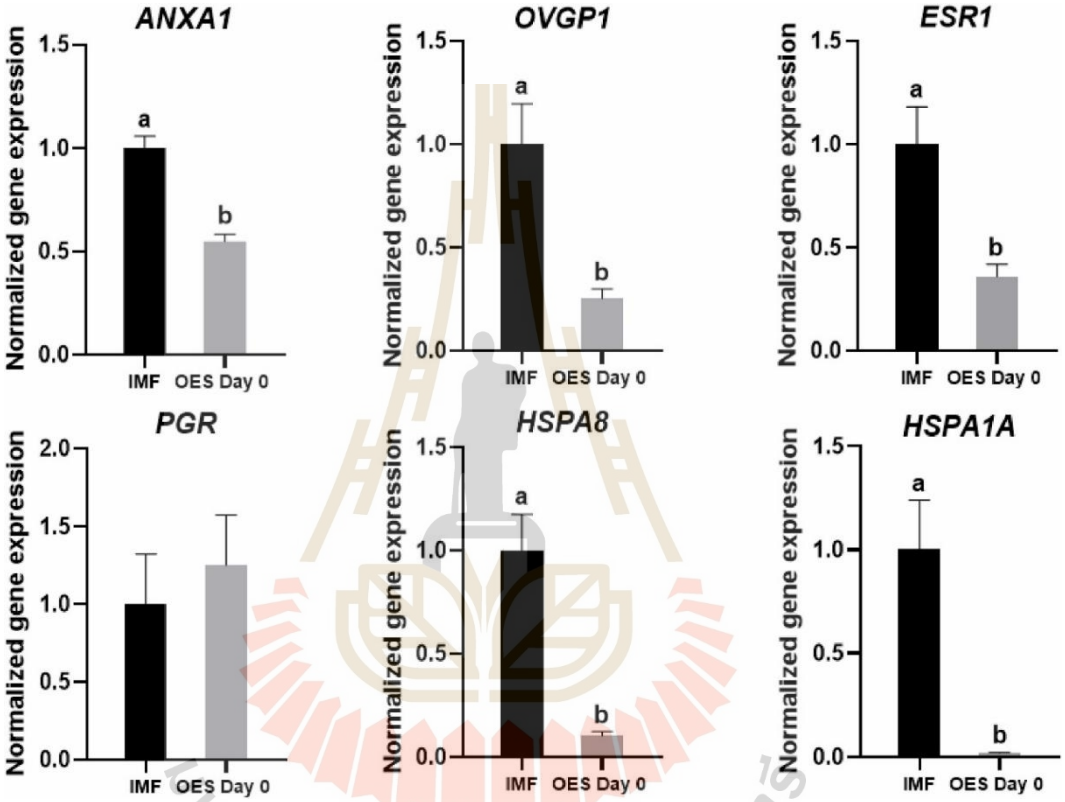

Figure 3.8 Impact of OES formation from isthmic mucosa fragments (IMF) on gene expression (Experiment 2). Gene expression of annexin A1 (*ANXA1*), oviductal glycoprotein 1 (*OVGP1*), estrogen receptor 1 (*ESR1*), progesterone receptor (*PGR*), heat shock protein family A (Hsp70) member 8 (*HSPA8*), and heat shock protein family A (Hsp70) member 1A (*HSPA1A*) was normalized according to *GAPDH*, *PPIA* and *YWHAZ* as reference genes. The different letters on bars indicate significant differences with a P-value < 0.05.

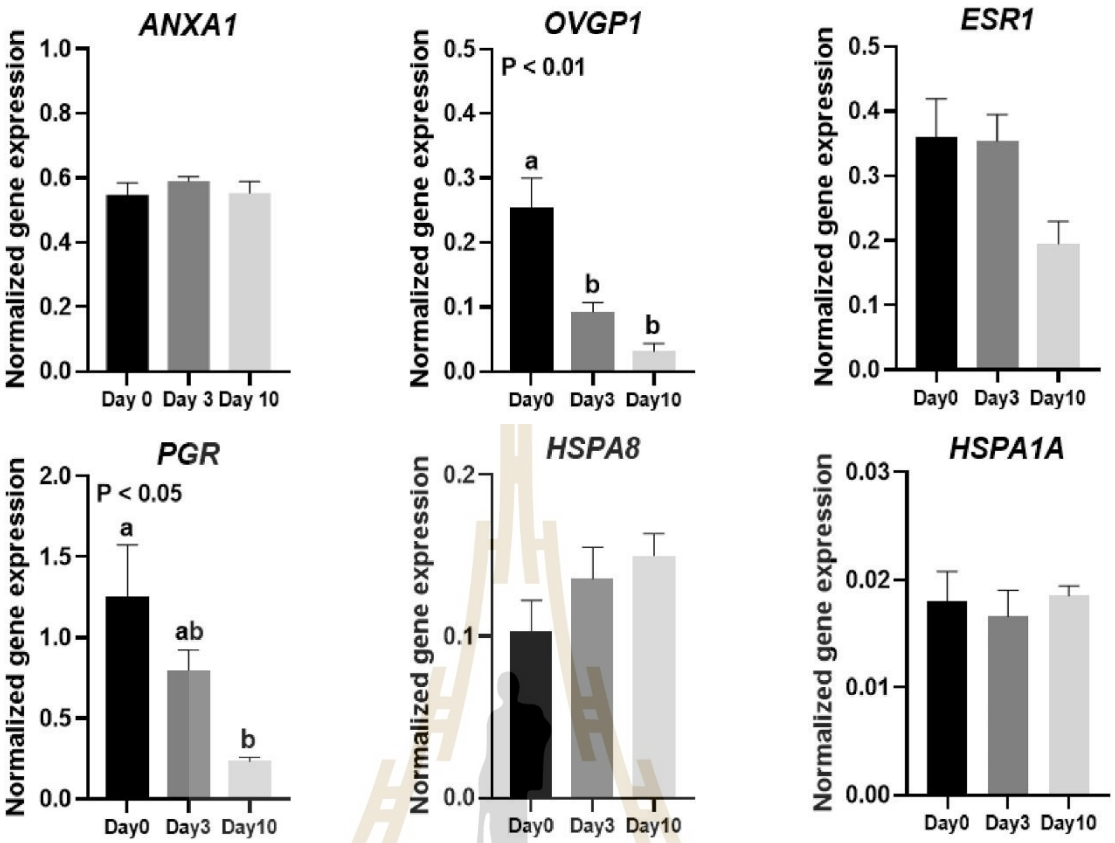

Figure 3.9 Impact of culture time on gene expression in OES (Experiment 2). Gene expression of annexin A1 (*ANXA1*), oviductal glycoprotein 1 (*OVGP1*), estrogen receptor 1 (*ESR1*), progesterone receptor (*PGR*), heat shock protein family A (Hsp70) member 8 (*HSPA8*), and heat shock protein family A (Hsp70) member 1A (*HSPA1A*) was normalized according to *GAPDH*, *PPIA* and *YWHAZ* as reference genes. The different letters indicate significant differences with a P-value < 0.05.

3.4.3 Effect of OES co-culture on embryo development

Preliminary experiments indicated that V-OESs and collapsed ones contained viable cells with apparent ciliary beating through day 10 of culture (data not shown). Groups of 25 presumptive zygotes were cultured in the presence or absence of 25 OES in the SOF/25 for 8 days after IVF. Compared to embryos cultured alone, the presence of OES significantly increased the blastocyst rates on days 7 and 8 after IVF ($p < 0.01$; Table 3.2).

Table 3.2 Effect of co-culture with OES on blastocyst yield in SOF/25 conditions.

	N	% (n) of cleavage rate	% (n) of blastocyst rate		
			Day 6	Day 7	Day 8
Control	790	72.8 ± 2.9 (580)	13.4 ± 1.9 (98)	17 ± 1.9[a] (132)	19.8 ± 2.4[b] (151)
+ OES	795	78.9 ± 2 (623)	16.1 ± 3.2 (123)	26 ± 2.3[b] (208)	31.8 ± 2.5[a] (248)

Percentage values are provided as the means ± SEM of 9 replicates. Different letters within one column indicate significant differences ($P < 0.01$, t-tests). N = total number of cumulus-oocyte complexes; and n = total number of embryos.

3.4.4 Effect of embryo co-culture on OES morphology, total cell number, viability, proportion of ciliated cells, and motion

Different parameters of OES were examined on day 10 after co-culture or not with embryo for 8 days in SOF/25. The presence of developing embryos during OES culture decreased the rate of OES collapsing. Indeed, the proportion of V-OES was significantly higher in the presence of embryos than in their absence (47.0 ± 5.3% *vs*. 27.0 ± 5.5%; $p < 0.05$, Fig. 3.10a). No other difference was found between groups concerning the total cell number per V-OES (239.2 ± 13.8 vs. 239.1 ± 18.1), percentage of live cells (78.4 ± 2.1 vs. 79.2 ± 1.7%), percentage of ciliated cells (13.7 ± 2.2 vs. 13.9 ± 3%), percentage of moving OES (97.3 ± 1.6 *vs*. 98 ± 2%), or their straight-line velocity (2.1 ± 0.4 vs. 2.1 ± 0.2 µm/s; Fig. 3.10b–f).

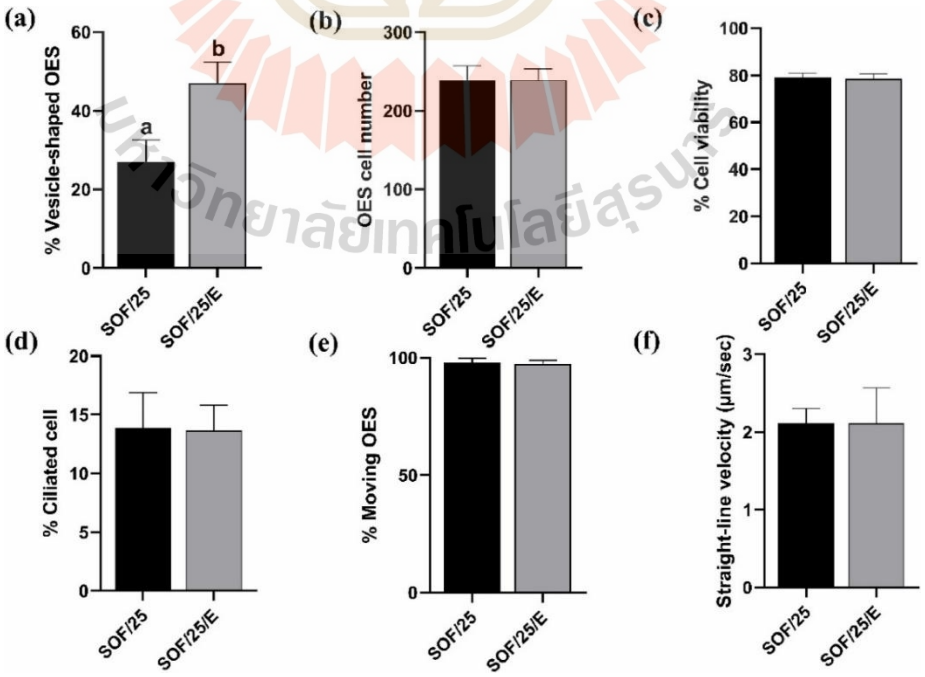

Figure 3.10 Effect of co-cultured embryos on OES morphology, total cell number, viability, proportion of ciliated cells and motion (Experiment 3).

Groups of 25 V-OES were cultured in SOF/25 alone or with 25 presumptive zygotes (SOF/25/E) for 8 days. At day 10, OES were evaluated for morphology (a), total cell numbers (b), cell viability (c), % ciliated cells (d), % moving (e) and straight-line velocity (f). Data are provided as the mean ± SEM of 4 replicates The different letters on bars indicate significant differences between groups ($P < 0.05$).

3.5 Discussion

The results of the present study show that OES represents a new *in vitro* alternative model over current ones for studying embryo–oviduct interactions since: 1) the OES contained well-differentiated OECs, 25% of which contained cilia at the time of their formation from IMF; 2) two different culture conditions (M199/500 and SOF/25) allowed the maintenance of cell morphology and viability suitable for embryo culture; 3) OES maintained stable expression of cytokeratin and several gene markers over 10 days of culture; and 4) OES cultured in SOF/25 supported embryo development, while embryos co-developing with OES helped to maintain the spheroid cavity.

3.5.1. Effect of culture conditions on OES morphology and cell physiology

The ability of OESs to maintain their cavity after forming from isthmic mucosa fragments was assessed by counting vesicle-shaped OESs (V-OESs), i.e. OES that did not collapse. The proportion of V-OES decreased up to day 10, regardless of the culture conditions. The mechanism involved in the formation of a cavity inside OES and the ability to maintain it over time remains unanswered at present. It might be related to the integrity of junction complexes at the basolateral part of the cells, avoiding liquid leaking outside the vesicle. Indeed, in the ALI culture system, the cells develop this cohesion progressively, increasing the electric resistance of the cultured epithelium (Chen et al., 2017). Therefore, it would be interesting to investigate the evolution of tight junctions during OES formation and culture.

Interestingly, the proportion of V-OES on day 10 was almost 3-fold higher in M199/500 than in M199/25, showing that the OES density per volume of medium (one OES per 20 µL vs. one OES per µL in M199/ 500 and M199/25, respectively) and the medium renewal (in the M199/ 500 only) affected the maintenance of the spherical morphology of OES. The proportion of V-OES on day 10 was higher in M199/500 than in M199/25, indicating a better ability to maintain OES shape at a low density. The absence of medium renewal in the M199/25 condition may induce excessive nutrient depletion, leading to a loss of cell cohesion and collapse of OES.

Although the total number of cells per V-OES decreased significantly over time in all culture conditions, the V-OESs kept more than 80% of the viable cells throughout the culture period. This suggests that the V-OESs eliminated dead cells during culture, probably by expulsion in the medium, as cell debris was observed in the culture medium on days 6 and 10 but not inside the V-OES cavity. At day 0, the OES selected for culture contained on average 300 cells, of which 25% contained cilia. Notably, this cell number and proportion of ciliated cells were constant among the experiments, offering a highly standardized and reproducible model. This proportion of ciliated cells is very similar to the proportion reported by Ito et al. (2016) in the epithelium of the isthmus from post-pubertal cows (20–40%). After 10 days in culture, the cell number per V-OES decreased on average to 225 cells (-75) in the SOF/25, of which 32 (14%) contained cilia, and to 150 cells (-150) in M199/500, of which 62 (41%) contained cilia. The decrease in the proportion of ciliated cells in V-OES indicates that the loss of cells in V-OES was at least in part due to the elimination of ciliated cells. The M199/500 condition, however, was much more favorable to the maintenance of ciliated cells in V-OES (9% of lost cells were ciliated) compared to the SOF/25 condition (57% of lost cells were ciliated), maybe due to the lower density and renewal of media in M199/500.

The functionality of the ciliated cells in V-OES was assessed through their capacity to move in suspension and straight-line velocity. While the proportions of moving OES remained very high (>95%) during the 10 days of culture, their straight-line velocity decreased over time in all culture conditions. The decrease in OES velocity was consistent with the reduction in the proportion of ciliated cells in the OES. This may also be due to a decrease in ciliary beat frequency over time in culture. Small 3D structures in suspension in a liquid medium naturally move due to Brownian motion (the random motion of a particle within a large set of smaller particles) but with no change over time. The straight-line velocity of OES, however, decreased over time in all culture conditions, probably due to the decrease in the proportion of ciliated cells, reinforcing the idea that the motion of OES was due to ciliary beating and not to random Brownian motion. To our knowledge, this is the first report of an easy-to-use method allowing the quantification of oviduct ciliary function. The ciliary beating of OECs has been previously evaluated by more complicated methods, such as fluorescent beads (Chen et al., 2013) or a digital videomicroscopic system (Kölle et al., 2009). Our results confirmed the existence of ciliated cells up to day 10 in V-OES, in accordance with Walter (1995), who evidenced the presence of ciliated cells in aggregates of OEC in suspension after 10 days of

culture using electron microscopy. Altogether, our data indicate that the culture conditions offering the best compromise for maintenance of OES quality were M199/500 and SOF/25.

3.5.2. Expression of candidate genes in the isthmic mucosa fragments (IMF) and OES

Vimentin encoded by *VMAC* is a fibroblast marker, which should not be expressed in OESs if they maintain their epithelial dedifferentiation without epithelial-to-mesenchymal transition (Kalluri & Weinberg, 2009). As expected, the immunodetection of cytokeratin confirmed that OES exclusively consisted of OECs.

The ability of OES to respond to estradiol-17β (E2) and progesterone signaling relies on the expression of estrogen receptor α and β (encoded by *ESR1* and *ESR2* genes) and progesterone receptors (encoded by *PGR* gene). The mRNAs coding for Erα (*ESR1*) were much more abundant than those coding for Erβ (*ESR2*), as previously reported by Ulbrich et al. (2003). Among the other candidate genes whose expression remains constant over time, annexin A1 (*ANXA1)* is located on the apical membrane of OECs and associated with sperm-binding at the oviduct sperm reservoir (Ignotz et al., 2007). Heat-shock protein family A (Hsp70) member 8 (HSPA8) is encoded by *HSPA8* gene and involved in sperm viability and fertilizing ability *in vitro* in pigs and cattle (Elliott et al., 2009). Finally, heat-shock protein family A (Hsp70) member 1A encoded by *HSPA1A* gene has been reported to be induced during heat stress in OECs (Rąpała et al., 2018).

By contrast, oviductal glycoprotein 1 (*OVGP1*) and PGR showed a significant decrease in their expression over time. OVGP1 is known to be involved in fertilization and early embryo development (Choudhary et al., 2019) as well as to reduce polyspermy by inducing zona hardening in pigs and cattle (Coy et al., 2008; Coy & Yanagimachi, 2015). PGR is normally highly expressed in the isthmic epithelium of cyclic and pregnant cows during the post-ovulatory period (Saint-Dizier et al., 2012). The decrease in OVGP1 and PGR expression over time is a limitation of the OES model. As the mRNA expression of both OVGP1 and PGR is up-regulated by E2 in the oviduct (Barton et al., 2020), their decrease might be overcome by the addition of reproductive steroid hormones to the OES culture medium.

Although a limited number of genes was evaluated in OES, the constant expression of most of them over time suggest no further effect of culture time on OEC gene expression, indicating that OESs are a suitable model for studies on embryo–maternal communication.

3.5.3. Effect of co-culture with embryos on OES morphology and cell physiology

Our data indicate a positive effect of OES on embryo development up to the blastocyst stage, as previously reported using OEC monolayers in cattle (Schmaltz-Panneau et al., 2015). Interestingly, the co-culture of OES with developing embryos increased the proportion of V-OES on day 10. To the best of our knowledge, this is the first report on such effect of embryos on OES morphology. This dialogue could be mediated by the release of factors into the media surrounding both embryos and OES. It could be speculated that the mechanisms underlying this effect on OES morphology are mediated by extracellular vesicles (EVs) derived from the embryo, in a similar way to the reported effect of oviduct extracellular vesicles (EVs) on embryo development (Almiñana et al., 2017; Bauersachs et al., 2020; de Alcântara-Neto et al., 2022). EVs are membrane-delineated vesicles that play a role in cell-to-cell communication by conveying nucleic acids (mRNAs, non-coding RNA), lipids, and proteins from producing cells to both nearby and long-distance target cells (Pavani et al., 2016). *In vivo*-derived oviduct EVs can be uptaken by *in vitro*-produced embryos (Almiñana et al., 2017), as well as primary confluent OEC after coincubation for 20 h (Pavani et al., 2016), and regulate the embryo transcriptome, as observed in cattle and porcine (Bauersachs et al., 2020; de Alcântara-Neto et al., 2022). In addition, it has been shown that developing embryos produce EVs that can cross the zona pellucida and were found in the culture medium (Pavani et al., 2016).

3.6 Conclusion

The OES culture in a large volume of M199 medium or in droplets of SOF medium provided the optimal conditions for OES *in vitro* culture. In SOF droplets, OESs supported blastocyst development. Furthermore, an effect of the developing embryos on OES morphology was evidenced, suggesting an effect of embryos on spheroid intercellular junctions that remain to be investigated. Altogether, it points to OES as an easy-to-use, easy-to-standardize, and physiological model to study early embryo–maternal interactions.

CHAPTER 4
OVIDUCT EPITHELIAL SPHEROIDS DURING *IN VITRO* CULTURE OF BOVINE EMBRYOS MITIGATE OXIDATIVE STRESS, IMPROVE BLASTOCYST QUALITY AND CHANGE THE EMBRYONIC TRANSCRIPTOME

4.1 Abstract

In vitro embryo production is increasingly used for genetic improvement in cattle but bypasses the oviduct environment and exposes the embryos to oxidative stress with deleterious effects on further development. Here we aimed to examine the effect of oviduct epithelial spheroids (OES) on embryo development and quality in terms of morphology and gene expression according to the co-culture time (up to embryonic genome activation at 8-16 cell stage *vs*. up to blastocyst stage) and oxygen tension (5% vs. 20%). Bovine presumptive zygotes produced by *in vitro* fertilization (day 0) using *in-vitro* matured oocytes were cultured in droplets of synthetic oviductal fluid (SOF) medium with or without (controls) OES for 4 or 7 days under 5% or 20% oxygen (4 treated and 2 control groups). Cleavage rates were evaluated on day 2 and blastocyst rates on days 7-8. Expanded blastocysts on days 7-8 were evaluated for total cell numbers and gene expression analysis by RNA-sequencing. Under 20% oxygen, blastocyst rates and total cell numbers were significantly higher in the presence of OES for 4 and 7 days compared to controls ($P<0.05$), with no difference according to the co-culture time. Under 5% oxygen, the presence of OES did not affect blastocyst rates but increased the number of cells per blastocyst after 7 days of co-culture ($P<0.05$). Both oxygen level and OES co-culture had a significant impact on the embryonic transcriptome. The maximal number of differentially expressed genes (DEGs) was identified after 7 days of co-culture under 20% oxygen. DEGs were involved in a wide range of functions, including lipid metabolism, membrane organization, intracellular transport and cell-cell junctions among the most significant ones. OES had beneficial effects on embryo development and quality under both 5% and 20% oxygen. Four days of co-culture were enough to obtain these effects, but maximal impact was observed after 7 days. This study evidence for the first time a modulation of the embryo-OES dialog according to the embryonic environment.

4.2 Introduction

The transfer of IVP embryos in cattle has drastically increased over the past 20 years and is now a major way to accelerate genetic progress for meat and milk production. According to the statistics of embryo production and transfer in domestic farm animals collected by the *International Embryo Transfer Society*, more than 1.5 million IVP bovine embryos were transferred worldwide in 2022 (Joao H. M. Viana, 2023). However, the success of IVP in farm animals is not optimal. The ability of cattle IVP embryos to survive after cryopreservation is much lower after *in vitro* than *in vivo* development (Enright et al., 2000; Rizos, Ward, et al., 2002) and recipient cows receiving IVP embryos have on average 10 to 40% less chance to become pregnant than after insemination (Enright et al., 2000; Maillo et al., 2016; Rodríguez-Alonso et al., 2020). A number of studies evidenced important differences between bovine *in vitro* and *in vivo* derived embryos in terms of morphology (Rizos, Fair, et al., 2002), ultrastructure (Abe et al., 1999; Crosier et al., 2001), lipid profiles (Janati Idrissi et al., 2021), gene expression (Lonergan et al., 2003; Driver et al., 2012; Gad et al., 2012) and proteomic contents (Banliat et al., 2022), highlighting the importance of the environment to which the oocyte then the embryo is exposed for future developmental competence.

Indeed, crucial events for the early bovine embryo take place in the oviduct: the first mitotic cell divisions, the switch from RNAs and proteins derived from the oocyte to those resulting from the zygote, called embryonic genome activation (EGA), occurring at the 8-16 cell stage in cattle as in humans, then the formation of first cell-cell junctions leading to morula compaction and cell lineage differentiation (Maillo et al., 2016). Then, around 5 days after fertilization, the embryo enters the uterus and reaches the blastocyst stage for further development. Previous transcriptomic (Gad et al., 2012) and proteomic (Banliat et al., 2022) data evidenced significant differences in the molecular dynamics during EGA between *in vivo* and *in vitro* derived embryos, suggesting that the oviduct environment has a significant impact on this key event. Furthermore, although the physiological oxygen tension in the oviduct lumen of most mammals range from 2 to 8% (Fischer & Bavister, 1993), a lot of laboratories culture embryos at atmospheric oxygen level (20%) for practical and budget reasons, causing oxidative stress and potential irreversible damage in developing embryos (Yuan et al., 2003; Karagenc et al., 2004; Yang et al., 2016; Leite et al., 2017).

Co-culture of embryos with monolayers of oviduct epithelial cells (OEC) under 20% oxygen has been shown to increase blastocyst production and quality

compared to controls without OEC in cattle (Clemente et al., 2008; Cordova et al., 2014; Schmaltz-Panneau et al., 2015). This positive effect may be due to a decrease in oxygen tension in the culture medium by OEC oxygen consumption, as well as direct positive effects of OEC secretion on embryo growth (Schmaltz-Panneau et al., 2015; Lopera-Vásquez et al., 2016; Almiñana et al., 2017; Kürüm et al., 2019) and gene expression (Pedersen et al., 2005; Clemente et al., 2008; Cordova et al., 2014), despite a partial dedifferentiation of OEC during culture (Schmaltz-Panneau et al., 2015). A previous comparison between 20% and 5% oxygen reported a higher blastocyst yield when embryos were co-cultured with OEC under 20% oxygen (Clemente et al., 2008), suggesting that OEC need high oxygen level to better support embryo development. In order to overcome the dedifferentiation of OECs in monolayers (Schmaltz-Panneau et al., 2015), we recently developed an *in vitro* model of oviduct epithelial spheroids (OES) that kept well-differentiated isthmic epithelial cells during the 8 days of embryo co-culture (Pranomphon et al., 2024). A positive effect of OES on blastocyst yield after 8 days of co-culture under 20% oxygen was evidenced (Pranomphon et al., 2024). However, the optimal time and oxygen level for *in vitro* embryo production by OES co-culture has not been determined. We hypothesized that OES supplementation up to the EGA, i.e. the first 4 days of culture, would be necessary and sufficient to obtain a supportive effect on embryo development and quality. Furthermore, although there is evidence that the oviduct secretions alter the embryo transcriptome (Bauersachs et al., 2020), most studies explored a limited number of candidate genes (Pedersen et al., 2005; Clemente et al., 2008; Cordova et al., 2014), calling for a bigger picture of the impact of OES on the embryonic transcriptome.

Therefore, the objectives of this study were to examine the effect of OES according to the co-culture time (4 vs 7 days) and oxygen levels (5% vs. 20%) on embryo development and blastocyst quality in terms of morphology and transcriptome using RNA-sequencing.

4.3 Materials and methods

4.3.1 Chemicals and Reagents

1) Chemicals and reagents

All chemicals and reagents were purchased from Sigma-Merck (Saint Louis, MO, USA), unless otherwise stated. Phosphate-Buffered Saline (PBS) (1X, Eurobio Scientific, France, CS1PBS01-01), 4% paraformaldehyde (Santa Cruz

Biotechnology, SC-281692), Triton X-100 (9036-19-5), bovine serum albumin (BSA; A9647), Hoechst 33342 (B2261; 1 mg/mL), mineral oil (ORIGIO Denmark), miRNeasy Tissue/Cells Advanced Micro Kit (#217684, Qiagen Basel, Basel, Switzerland), SMARTer® Stranded Total RNA-Seq Kit v3 - Pico Input Mammalian (#634485, Takara Bio Europe SAS, Saint-Germain-en-Laye, France).

2) Media used for cell culture and in vitro embryo production

The OES culture medium (**M199**) was TCM-199 supplemented with 25 mM HEPES, 10% heat-inactivated FBS (Gibco A5256701) and 80 mg/L gentamycin (G1272). The washing medium for oocyte collection (mPBS) was demineralized water with 8 g/L NaCl (S-7653), 0.2 g/L KCl (Prolabo 26764298), 0.2 g/L KH_2PO_4 (Prolabo 26936293), 1.43 g/L $Na_2HPO_4.2H_2O$ (Prolabo 28029292), 0.14 g/L $CaCl_2.2H_2O$ (Merck 2382), 0.2 g/L $MgCl_2.6H_2O$ (Merck 5833), 1 g/L D-glucose (S-8270), 0.036 g/L Pyruvate (P-4562), 50 mg/L gentamycin (G1272), 2 mg/L phenol red (P0290) and 0.5 mg/L BSA (A9647) with the osmolarity and pH adjusted at 280 mOsm and 7.4, respectively (Mermillod et al., 1993). The maturation medium was TCM-199 (M4530) supplemented with 5 IU/mL hCG, 10 IU/mL PMSG (PG600, Intervet), 19 ng/mL IGF-1, 2.2 ng/mL FGF, 10 ng/mL EGF, 5 µg/mL insulin, 5 µg/mL transferrin, 5 ng/mL selenium, 90 µg/mL L-Cystein, 100 µM β-mercaptoethanol, 75 µg/mL ascorbic acid, 720 µg/mL glycine, 0.1 mg/mL glutamine and 110 µg/mL Pyruvate (Donnay et al., 2004; Schmaltz-Panneau et al., 2015). The sperm washing medium was STL medium based on Tyrode medium supplemented with 25 mM bicarbonate (S5761), 10 mM lactate (L7900), 2.4 mg/mL HEPES (H3375), 6 mg/mL BSA (A9647) and 40 µg/mL gentamycin (G1272) (Schmaltz-Panneau et al., 2015; Lamy et al., 2017), Tyrode medium: mix 1 L of demineralized water with 6.666 g NaCl (Merck 6404), 240 mg KCl (Prolabo 26764298), 41 mg $NaH_2PO_4.H_2O$ (Merck 6346), 300 mg $CaCl_2.2H_2O$ (Merck 2382), 100 mg $MgCl_2.6H_2O$ (Merck 5833) and 2 mg/L phenol red (P0290) with the osmolarity at 230 mOsm. The fertilization medium was Tyrode medium supplemented with 25 mM bicarbonate (S5761), 10 mM lactate (L7900), 1 mM pyruvate (S4562), 6 mg/mL BSA (A6003), 100 µg/mL heparin (Calbiochem Ref: 375 D95 batch B47089) and 40 µg/mL gentamycin (G1272) (Schmaltz-Panneau et al., 2015; Lamy et al., 2017). The synthetic oviductal fluid medium used for cell culture and embryo development (**SOF**) was modified according to Holm et al. (1999) and supplemented with 5% heat-inactivated FBS (MP Biomedicals, Ref: 2916749, batch MP5418).

4.3.2 Culture of bovine oviduct epithelial spheroids (OES)

Oviductal cell isolation and culture were conducted as described previously (Mahé et al., 2023). Briefly, pairs of oviducts and ovaries obtained from post-pubertal cows were collected at a local slaughterhouse (Vendôme, France) and transported at 4 °C to the laboratory. Pairs of oviducts at the peri-ovulatory phase of cycle (approximately day -2 to day +4 around ovulation time) were selected according to the morphology of the ovaries. The pre-ovulatory phase of the cycle was identified by a follicle at 11-20 mm in diameter and a small yellow-white *corpus albicans*. The post-ovulatory phase of the cycle was identified by a small *corpus heamoragicum* (< 0.5 cm) and the *corpus albicans* from the previous cycle. For each culture, both oviducts from a pool of two to three cows were used. After removal of blood vessels and connective tissue, the oviducts were cut at the ampullary-isthmic junction and only the isthmic parts were used. After a rapid dip in 70% ethanol and rinsing in 0.9% NaCl, the mucosa fragments were expelled from isthmic sections with forceps into 10 mL of M199, vortexed for 1 min, then incubated at 38.8 °C for 10 min for cell sedimentation. Following the elimination of the supernatant containing cell debris and blood red cells, the pellet was resuspended in 10 mL of M199, and the vortex-sedimentation process was repeated. Finally, the pellet was diluted ten times in the culture medium and 50 µL of the resulting mixture was added to 450 µL of M199, to reach a 100-fold final dilution. The isthmic mucosa fragments were cultured for 72 h in four-well culture plates (Thermoscientific, Denmark) at 38.8 °C in a humidified atmosphere containing 5% CO_2 in air. After 72 h, a cavity appeared within the mucosa fragments, forming spheroids of various sizes and shapes, with the apical side of the epithelial cells oriented outward. The spheroids between 100 and 200 µm in diameter, homogeneous in shape and size and exhibiting a cavity and ciliary beating outward, referred as "OES" were selected, cultured in M199 medium at a density of 200 to 400 OES/mL for 2 days and then, transferred to culture droplet for 24 h before co-culture with presumptive zygotes.

4.3.3 *In vitro* oocyte maturation (IVM), fertilization (IVF) and embryo culture

IVM, IVF and *in vitro* culture were performed as previously described in Schmaltz-Panneau et al. (2015) with slight modifications. Bovine ovaries were collected at a local slaughterhouse (Vendôme, France) and kept in 0.9% NaCl solution at 31-32 °C for 45 min up to the laboratory. Cumulus oocyte complexes (COCs) were aspirated from 3-6 mm follicles using an 18½-gauge needle. COCs surrounded by three of more layers of compact cumulus and homogeneous cytoplasm were selected, washed twice in washing medium then once in 1 mL of

maturation medium. Groups of 50-80 COCs were cultured in 500 µL of maturation medium for 22-23 h at 38.8 °C in a humidified atmosphere of 5% CO_2 in air in a 4-well plate (Thermoscientific REF179830, Denmark). At the end of the maturation period, COCs were washed once in 1 mL of fertilization medium and transferred in groups of 50-80 into 4-well dishes containing 250 µL of fertilization medium per well. A pool of frozen semen from two Holstein bulls of proven fertility were used for all IVF (0.25-mL straw). Straws were submerged in a 35 °C water-bath for 30 sec then motile spermatozoa were obtained by centrifuging frozen-thawed semen on a Percoll density gradient (45/90%) at 700g for 20 min. Spermatozoa collected at the bottom of the 90% fraction were centrifuged in 5 mL of STL medium at 100g for 10 min, before counting spermatozoa in the pellet using a Thoma cell. Sperm concentration was adjusted to $4x10^6$ spermatozoa/mL with fertilization medium then 250 µL of this suspension were added to COCs to obtain a final concentration of $2x10^6$ spermatozoa/mL. Spermatozoa and COCs were co-incubated at 38.8 °C in a humidified atmosphere containing 5% CO_2 in air (IVF, day 0). After 18 h of co-incubation, the presumptive zygotes (day-1 embryos) were vortexed at moderate speed for 2 min in 2 mL of washing medium to eliminate cumulus cells, washed twice in washing medium then once in SOF medium supplemented with 5% FBS before being transferred in 25-µL droplets of the same medium overlaid with mineral oil at 38.8 °C. Groups of 25 presumptive zygotes were randomly allocated to one of the following experimental group: co-culture with 25 OES in a humidified atmosphere with 5% CO_2, 5% O_2 and 90% N_2 (low O_2 condition) up to day 5 (4dOES_5) or day 8 (7dOES_5) of culture, or in a humidified atmosphere with 5% CO_2 in air (high O_2 condition) up to day 5 (4dOES_20) or day 8 (7dOES_20) (Fig. 4.1). In both conditions, one group of 25 zygotes was cultured without OES as controls (C_5; C_20). In the 4dOES groups, OES were removed from the culture droplet with minimal volume of medium (<5 µL) using a glass pipette. The cleavage rates were evaluated on day 2 and blastocyst rates on days 6, 7, and 8 using an inverted microscope (Olympus IX70, Japan).

4.3.4 Assessment of blastocyst cell numbers

On days 7 and 8, subgroups of expanded blastocysts (around 150 µm in diameter) were fixed and permeabilized in 500 µL PBS with 4% paraformaldehyde (PFA), 1% BSA and 0.25% Triton X-100 for 30 min at 37 °C. After three washing in PBS + 1% BSA (PBS-BSA), embryos were stained in 1 µg/mL Hoechst 33342 for 1 h at room temperature under agitation in dark, rinsed and mounted on a glass slide. Embryos were observed at 200x magnification using a confocal microscope (Zeiss

LSM 700, Carl Zeiss, Oberkochen, Germany) and Z-stack images (2 to 7 per blastocyst) were acquired. The total number of nuclei per embryo was counted automatically using the Stardist plugin of the ImageJ software (version 1.54f). The counting of all nuclei and absence of double counting were checked visually and corrected manually when necessary.

4.3.5 RNA-seq analysis

Expanded blastocysts on days 7-8 of the six culture conditions (C_5, 4dOES_5, 7dOES_5, C_20, 4dOES_20 and 7dOES_20) were washed in washing medium, collected with minimum volume of medium in 1.5 mL Ultra High Recovery Microcentrifuge Tube (STARLAB, E1415-2600, USA), immediately stap-frozen in liquid nitrogen and stored at -80 ℃ until used for RNA extraction. Total RNA was isolated from pools of 8-12 blastocysts using the miRNeasy Tissue/Cells Advanced Micro Kit (#217684, Qiagen Basel, Basel, Switzerland) following the manufacturers' recommendations. RNA concentration and quality were measured on an Agilent 2100 Bioanalyzer (Agilent Technologies Schweiz AG, Basel, Switzerland) using the Agilent RNA 6000 Pico assay. The Agilent 2100 Bioanalyzer RNA integrity number (RIN) ranged from 9.3 to 10 (median= 9.9).

RNA-Seq library preparation was performed starting from 2 ng total RNA using the SMARTer® Stranded Total RNA-Seq Kit v3 – Pico Input Mammalian (#634485, Takara Bio Europe SAS, Saint-Germain-en-Laye, France). A pool of 36 libraries was run on one lane of an Illumina NovaSeq X plus 10B flow cell (Functional Genomic Center Zurich, https://fgcz.ch/). Paired-end 150 bp sequencing was performed and revealed between 21 and 36 million raw reads per library.

The Galaxy Europe server (https://usegalaxy.eu/ ("The Galaxy platform for accessible, reproducible and collaborative biomedical analyses: 2022 update," 2022)) was used to analyze the obtained sequence reads (FastQ files). Sequencing reads were processed using Trim Galore! (Galaxy version 0.6.7+galaxy0) with the parameters: remove 14 bp from the 3' end of read 1; remove 6 bp from the 3' end of read 2; trim low-quality ends from reads in addition to adapter removal (phred quality score threshold = 30); overlap with adapter sequence required to trim a sequence: 1; maximum allowed error rate: 0.1; discard reads that became shorter than 30 nt; remove 9 bp from the 5' end of read 1; remove 16 bp from the 5' end of read 2. Trimmed reads were mapped to the current bovine genome reference assembly (bosTau 9, ARS-UCD2.0) with HISAT2 (Galaxy version 2.2.1+galaxy1). Reads mapped to annotated features of the bovine genome were counted with the tool featureCounts (Galaxy Version 2.0.3+galaxy2). The latest NCBI GFF3 genome

annotation file was used (GCF_002263795.3_ARS-UCD2.0_genomic.gtf). The obtained read count table was filtered based on counts per million (cpm) cut- off 1.65 (corresponds to approx. 20 read counts) in at least 5 samples to remove reads with negligible read counts. Read count data were analyzed in R software (https://www.r-project.org) using the BioConductor package edgeR (Robinson et al., 2010). False discovery rate (FDR) was calculated to perform correction for multiple testing. RNA-seq data have been deposited at NCBI's Sequence Read Archive (SRA) under the BioProject accession ID PRJNAXXX.

Hierarchical clustering was performed to identify clusters of differentially expressed genes (DEGs) with similar expression profiles across experimental groups (Multiple Experiment Viewer, MeV v.4.8.1, https://sourceforge.net/projects/mev-tm4/) (Saeed et al., 2003). Functional annotation analysis for lists of DEGs was performed using Metascape (www.metascape.org) (Zhou et al., 2019). The webtool ToppCluster (https://toppcluster.cchmc.org/) (Kaimal et al., 2010) was used to generate a network of overrepresented functional terms obtained for DEGs.

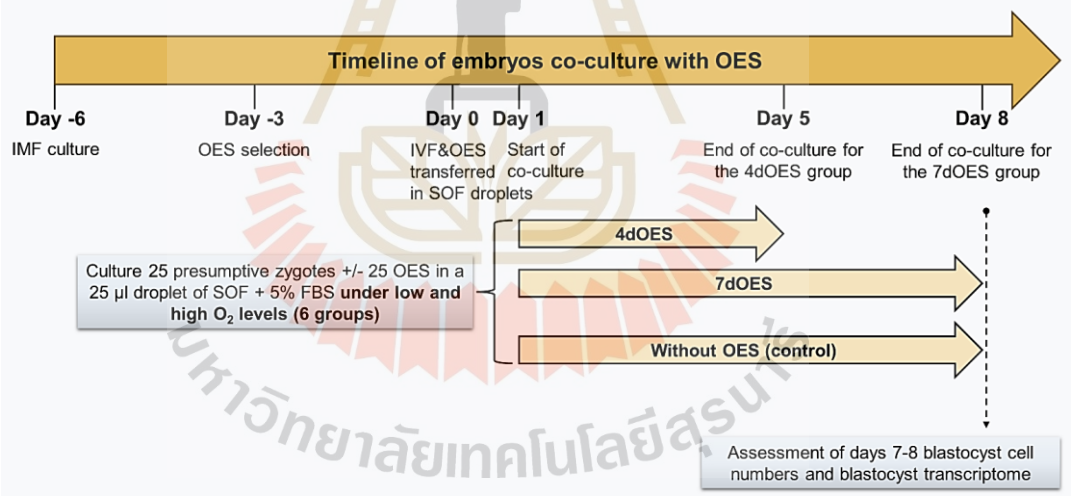

Figure 4.1 Experimental design of the study.

4.3.6 Statistical analysis

Statistical analysis was performed using the GraphPad Prism software (version 8.1.1) and the Rstudio (R version 4.3.3). The normality of data and homogeneity of variances were analysed using the Shapiro-Wilk and Levene tests, respectively. The rates of cleavage and blastocysts were compared among groups using one-way ANOVA followed when appropriate by Tukey's post-tests. Cleavage rates under low O_2 level (data distribution was not normal) were analysed using the Kruskal-Wallis test followed by Dunn's post-tests. The effect of oxygen level (5% vs.

20%) on cleavage and blastocyst rates was analysed using unpaired t tests with Welch's correction for unequal variances. A P-value < 0.05 was considered as significant. All data are presented as means ± SEM.

4.4 Results

4.4.1 Effect of oxygen level on blastocyst yield and cell number

Cleavage rates did not change among culture conditions (Fig. 4.2 and Tables 4.1-4.2). In the absence of OES, the blastocyst rate on day 8 and cell number per expanded blastocyst on days 7-8 were lower under 20% compared to 5% oxygen (P<0.05; Fig. S1 and Table S1). However, in the presence of OES, blastocyst rates and cell numbers were similar regardless of the oxygen level (Fig. S1B-C and Table S2-3). We then focused on the effect of OES compared to controls on embryo development and blastocyst quality in each oxygen condition.

4.4.2 Effect of OES and time of co-culture on blastocyst yield and cell number

Under 5% O_2, the culture of embryos with OES did not change the blastocyst rates on days 6-8 compared to controls without OES, whatever the time of co-culture (4 vs 7 days; Fig. 4.2A and Table 4.1). However, the mean cell number per expanded blastocyst co-cultured with OES for 7 days was higher than controls (102.6 ± 8.4 vs. 137.6 ± 10.8; P<0.05; Fig. 4.2A).

Under 20% O_2, OES increased the blastocyst rates on days 7 and 8 (P<0.01), with no difference between 4 and 7 days of co-culture (Fig. 4.2B and Table 4.2). Moreover, co-culture with OES increased the mean cell number per blastocyst compared to controls (P<0.0001). Comparison between times of co-culture evidenced that 7 days tended to have higher effect on blastocyst cell number than 4 days of co-culture (P=0.07; Fig. 4.2B).

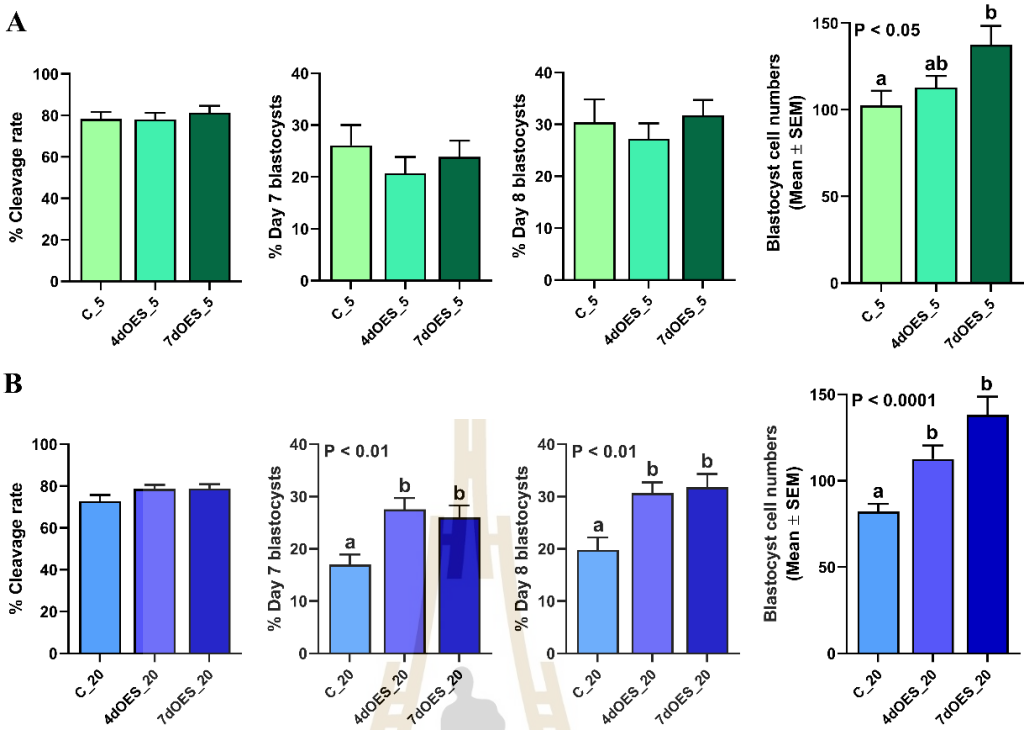

Figure 4.2 Effect of co-culture with OES on cleavage, blastocyst rates and total cell numbers. A, under 5% O_2 level. Different letters indicate significant differences (P<0.05; N= 507-657 COCs per condition for cleavage and blastocysts rates; N=20 blastocysts per condition for cell numbers). B, under 20% O2 level. Different letters indicate significant differences (P<0.01; N= 768-795 COCs per condition for cleavage and blastocysts rates; N=20 blastocysts per condition for cell numbers). C_5: 7 days culture with medium alone without OES at 5% O_2; 4dOES_5: 4 days co-culture with OES at 5% O_2; 7dOES_5: 7 days co-culture with OES at 5% O_2; C_20: 7 days culture with medium alone without OES at 20% O_2; 4dOES_20: 4 days co-culture with OES at 20% O_2; 7dOES_20: 7 days co-culture with OES at 20% O_2.

Table 4.1 Effect of co-culture with OES on blastocyst yield and quality under low O_2 level.

Time of co-culture	N	% (n) of cleavage rate	% (n) of blastocyst rate			Cell number per expanded blastocyst (n)
			Day 6	Day 7	Day 8	
Control	557	78.4 ± 3.2 (444)	24.3 ± 4.7 (136)	26.1 ± 3.9 (150)	30.4 ± 4.5 (177)	102.6 ± 8.4^{x} (20)
4 days	657	78 ± 3.3 (514)	15.9 ± 2.7 (110)	20.8 ± 3.1 (139)	27.3 ± 3 (181)	112.9 ± 6.7^{xy} (20)
7 days	507	81.4 ± 3.2 (417)	16.4 ± 2.8 (79)	23.9 ± 3.1 (117)	31.8 ± 3 (155)	137.6 ± 10.8^{y} (20)

Percentage values are provided as the means ± SEM of 8 replicates except blastocyst cell numbers (3 replicates). Different letters within one column indicate significant differences (P<0.05). N = total number of cumulus-oocyte complexes; and n = total number of embryos.

Table 4.2 Effect of co-culture with OES on blastocyst yield and quality under high O_2 level.

Time of co-culture	N	% (n) of cleavage rate	% (n) of blastocyst rate			Cell numbers in expanded blastocysts (n)
			Day 6	Day 7	Day 8	
Control	790	72.8 ± 2.9 (580)	13.4 ± 1.9 (98)	17 ± 1.9^{a} (132)	19.8 ± 2.4^{a} (151)	82.1 ± 4.5^{x} (20)
4 days	768	78.6 ± 2 (604)	16.8 ± 3.4 (136)	27.6 ± 2.2^{b} (215)	30.7 ± 2^{b} (236)	112.7 ± 7.8^{y} (20)
7 days	795	78.9 ± 2 (623)	16.1 ± 3.2 (123)	26 ± 2.3^{b} (208)	31.8 ± 2.5^{b} (248)	138.1 ± 10.5^{y} (20)

Percentage values are provided as the means ± SEM of 9 replicates except blastocyst cell numbers (3 replicates). Different letters within one column indicate significant differences (P<0.05). N = total number of cumulus-oocyte complexes; and n = total number of embryos.

4.3.3 Effect of co-culture with OES and oxygen level on blastocyst transcriptome

1) Global analysis

Principal component analysis of RNA-seq data (top 500 genes; FDR 1%) did not demonstrate major separation among samples, except the C_5 and 7dOES_20 groups, which were clearly separated (Fig. S2).

Hierarchical clustering of the DEGs obtained among the six groups of blastocysts is shown in Figure 4.3. The main differences were observed between groups co-cultured with OES (during 4 or 7 days) and controls, irrespective of oxygen level. In accordance, highest number of DEGs were identified for the comparisons between 7dOES and controls (911 and 1282 DEGs for 5% or 20% oxygen, respectively; FDR 1%; see Table 4.3 for details), followed by the 4dOES vs. controls comparisons (568 and 559 DEGs for 5% or 20% oxygen, respectively; FDR 1%). Furthermore, a total of 238 DEGs was identified for the comparison between 20% and 5% oxygen in controls, while this comparison evidenced 785 DEGs in blastocysts co-cultured with OES (FDR 1%; Table 4.3). Applying the same FDR cut-off (1%), only 5 DEGs were identified for the comparison between 4dOES and 7dOES (Table 4.3).

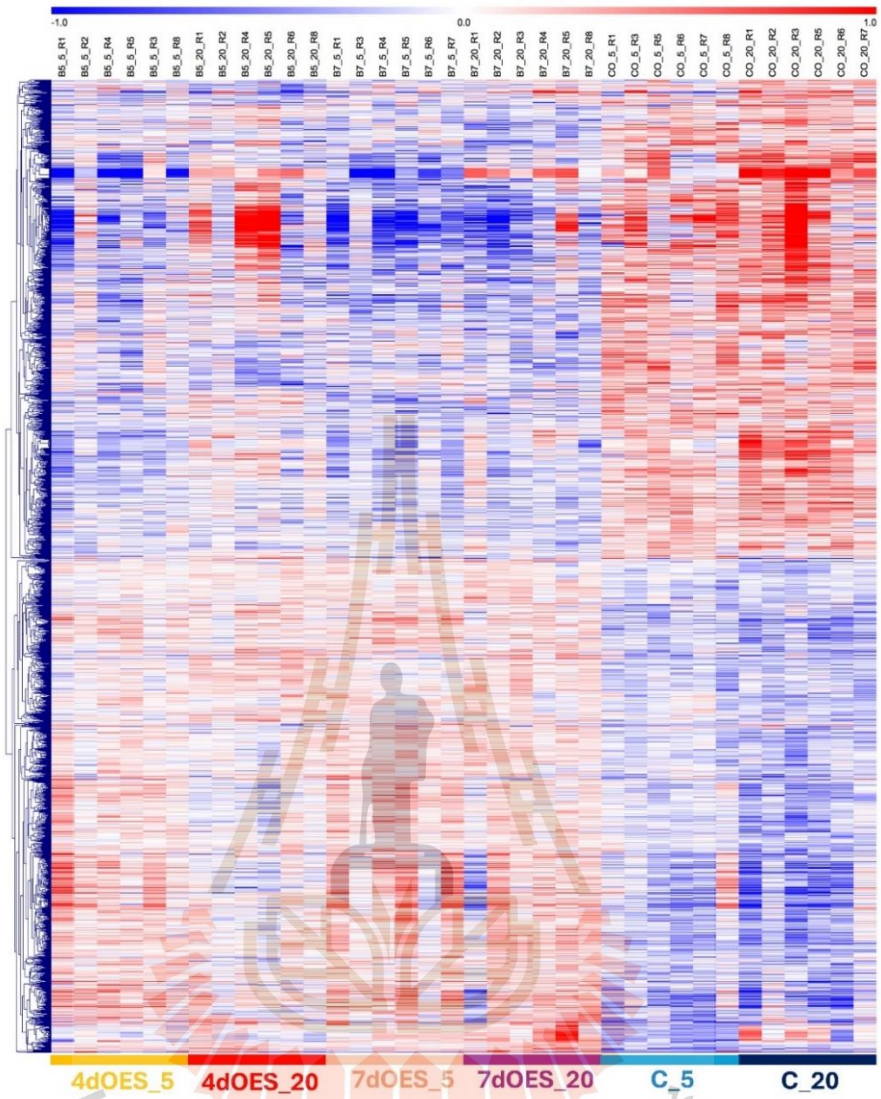

Figure 4.3 Heatmap of differentially expressed genes (DEGs) identified in expanded blastocysts according to culture conditions. Expanded blastocysts were co-cultured or not (controls) with OES for 4 or 7 days and under high or low oxygen tension. Each row represents one DEG (FDR 1%) while each column represents one pool of blastocysts (6 pools/condition). Mean-centered log2 intensity values were obtained (value of respective sample- mean of all samples) for DEGs across experimental groups. Hierarchical cluster analysis was carried out using Pearson correlation (MeV software). The blue color indicates lower expression than the mean of all samples whereas the red color indicates higher expression. Horizontal bars (bottom) indicate the six different culture conditions: yellow, 4dOES_5; red, 4dOES_20; pink, 7dOES_5; purple, 7dOES_20; blue, C_5; and dark blue, C_20.

Table 4.3 Numbers of up- and down-regulated genes and total number of DEGs according to comparison.

Comparison	Effect	FDR 10%			FDR 5%			FDR 1%		
		up	down	total	up	down	total	up	down	total
4dOES_5 vs. C_5	4 days co-culture				630	625	1255	286	282	568
7dOES_5 vs. C_5	7 days co-culture				949	925	1874	458	453	911
4dOES_20 vs. C_20	4 days co-culture				689	768	1457	308	251	559
7dOES_20 vs. C_20	7 days co-culture				1121	1240	2361	691	591	1282
7dOES_5 vs. 4dOES_5	7 or 4 days co-culture	0	2	2	0	2	2	0	2	2
7dOES_20 vs. 4dOES_20	7 or 4 days co-culture	127	165	292	29	27	56	3	5	8
7dOES vs. 4dOES	7 or 4 days co-culture	9	19	28	10	4	14	1	4	5
C_20 vs. C_5	% O_2				268	499	767	169	69	238
4dOES_20 vs. 4dOES_5	% O_2				411	439	850	153	125	278
7dOES_20 vs. 7dOES_5	% O_2				62	65	127	32	13	45
OES_20 vs. OES_5	% O_2				740	861	1601	358	427	785

DEGs were analyzed according to the presence of OES, time of co-culture, oxygen tension (effect) and FDR. up: number of DEGs with higher expression levels in first group of contrast (e.g., for 4dOES_5 vs. C_5 higher in 4dOES_5).

2) Global effect of OES on blastocyst transcriptome

Figure 4.4 illustrates the total numbers, distribution and overlap of DEGs (FDR 1%) for the four comparisons between co-cultured vs control blastocysts under 5% and 20% oxygen. Numbers of DEGs were higher after 7 days compared to 4 days of co-culture with OES under both oxygen conditions, with highest number of DEGs under 20% oxygen. Furthermore, a total of 1134 DEGs were specific to one comparison, the highest number and proportion of specific DEGs being observed for the 7dOES vs. control comparison (44%; 568/1282 vs. less than 31% for other comparisons).

To see more specifically which embryonic functions and cellular components were impacted by the presence of OES, an enrichment analysis of DEGs (up- and down-regulated together) was carried out using Metascape. Figure 4.5 shows the Metascape heatmap plot of the top 100 enriched clusters among the 4 comparisons. The heatmap illustrates that the majority of functional terms and pathways, including “metabolism of lipids”, “response to extracellular stimulus”, “response to oxygen levels”, “cell-cell junction”, “import into cell” and “transport of small molecules” were shared among all comparisons, i.e. impacted by OES co-cultured regardless of the oxygen level and time of co-culture. However, for most terms, the significance was higher for the 7dOES vs control under 20% oxygen compared to the 3 other comparisons. Furthermore, the biological processes related to “mitochondrial transport” and “amino acid metabolic process” were only enriched for DEGs under 20% oxygen (4dOES_20 and 7dOES_20 vs. C_20).

To have a better view of shared and specific functions of DEGs among the 4 comparisons, a functional network shown on Figure 4.6 was created using ToppCluster. The network evidenced that DEGs for the 7dOES vs. control comparison under 20% oxygen were specifically involved in numerous functions/pathways such as “regulation of phosphorylation”, “vesicle membrane” “regulation of apoptotic signalling pathways” and “endocytosis” among the most significant.

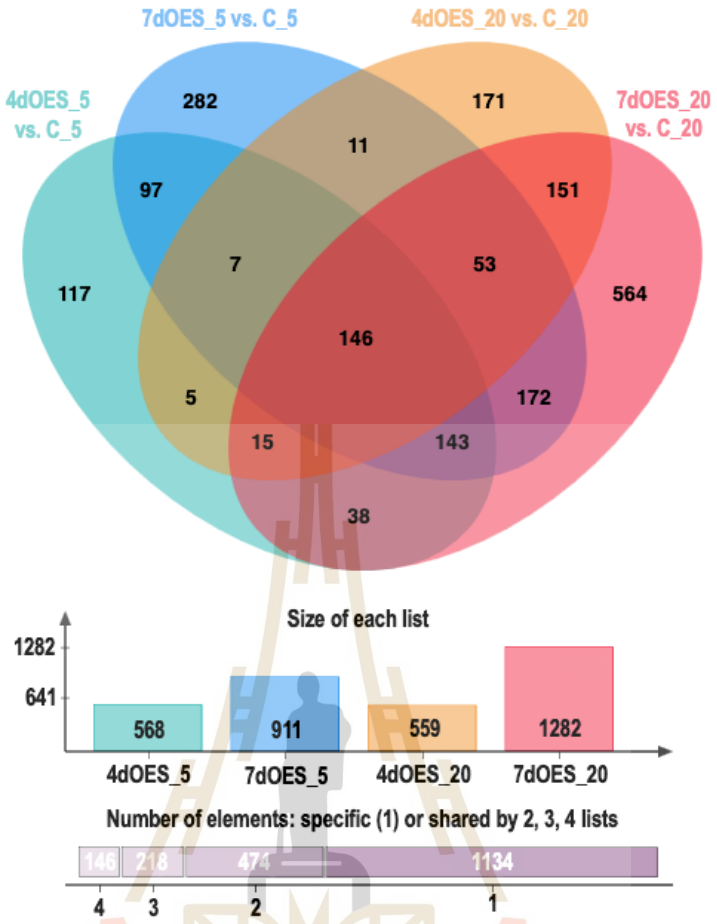

Figure 4.4 Venn diagrams showing the distribution of differentially expressed genes. DEGs s (FDR1%) of expanded blastocysts co-cultured with OES for 4 or 7 days compared to controls without OES under 5% and 20% oxygen were analyzed. The histogram below shows the number of identified DEGs for each comparison. The horizontal purple bar shows the sum of specific and shared DEGs between groups (bottom). 4dOES_5 vs. C_5: co-culture with OES for 4 days vs. controls under 5% oxygen; 7dOES_5 vs. C_5: co-culture with OES for 7 days vs. controls under 5% oxygen; 4dOES_20 vs. C_20: co-culture with OES for 4 days vs. controls under 20% oxygen; 7dOES_20 vs. C_20: co-culture with OES for 7 days vs. controls under 20% oxygen.

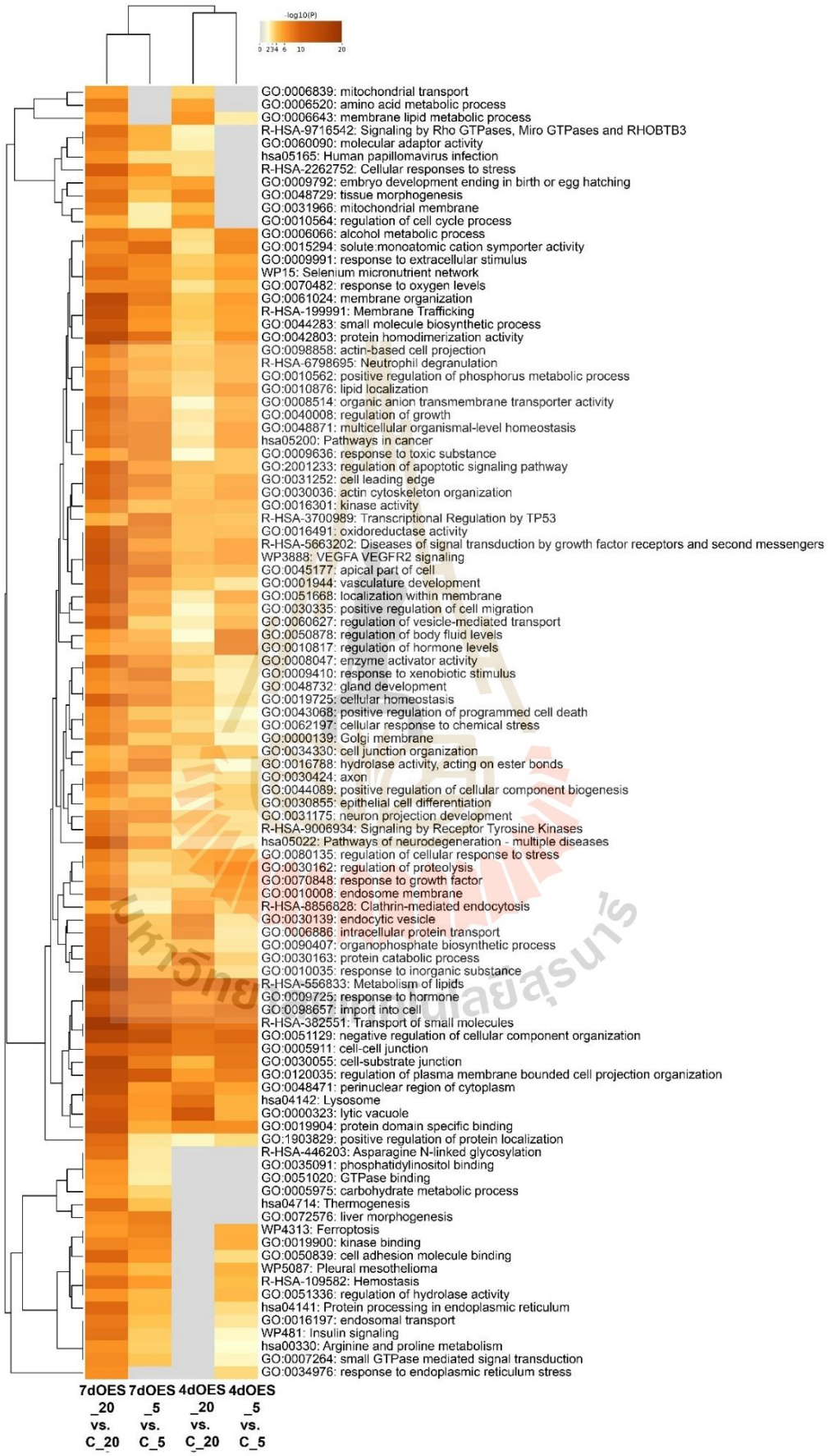
-log10(P)
0 2 3 4 6 10 20
GO:0006839: mitochondrial transport
GO:0006520: amino acid metabolic process
GO:0006643: membrane lipid metabolic process
R-HSA-9716542: Signaling by Rho GTPases, Miro GTPases and RHOBTB3
GO:0060090: molecular adaptor activity
hsa05165: Human papillomavirus infection
R-HSA-2262752: Cellular responses to stress
GO:0009792: embryo development ending in birth or egg hatching
GO:0048729: tissue morphogenesis
GO:0031966: mitochondrial membrane
GO:0010564: regulation of cell cycle process
GO:0006066: alcohol metabolic process
GO:0015294: solute:monoatomic cation symporter activity
GO:0009991: response to extracellular stimulus
WP15: Selenium micronutrient network
GO:0070482: response to oxygen levels
GO:0061024: membrane organization
R-HSA-199991: Membrane Trafficking
GO:0044283: small molecule biosynthetic process
GO:0042803: protein homodimerization activity
GO:0098858: actin-based cell projection
R-HSA-6798695: Neutrophil degranulation
GO:0010562: positive regulation of phosphorus metabolic process
GO:0010876: lipid localization
GO:0008514: organic anion transmembrane transporter activity
GO:0040008: regulation of growth
GO:0048871: multicellular organismal-level homeostasis
hsa05200: Pathways in cancer
GO:0009636: response to toxic substance
GO:2001233: regulation of apoptotic signaling pathway
GO:0031252: cell leading edge
GO:0030036: actin cytoskeleton organization
GO:0016301: kinase activity
R-HSA-3700989: Transcriptional Regulation by TP53
GO:0016491: oxidoreductase activity
R-HSA-5663202: Diseases of signal transduction by growth factor receptors and second messengers
WP3888: VEGFA VEGFR2 signaling
GO:0045177: apical part of cell
GO:0001944: vasculature development
GO:0051668: localization within membrane
GO:0030335: positive regulation of cell migration
GO:0060627: regulation of vesicle-mediated transport
GO:0050878: regulation of body fluid levels
GO:0010817: regulation of hormone levels
GO:0008047: enzyme activator activity
GO:0009410: response to xenobiotic stimulus
GO:0048732: gland development
GO:0019725: cellular homeostasis
GO:0043068: positive regulation of programmed cell death
GO:0062197: cellular response to chemical stress
GO:0000139: Golgi membrane
GO:0034330: cell junction organization
GO:0016788: hydrolase activity, acting on ester bonds
GO:0030424: axon
GO:0044089: positive regulation of cellular component biogenesis
GO:0030855: epithelial cell differentiation
GO:0031175: neuron projection development
R-HSA-9006934: Signaling by Receptor Tyrosine Kinases
hsa05022: Pathways of neurodegeneration - multiple diseases
GO:0080135: regulation of cellular response to stress
GO:0030162: regulation of proteolysis
GO:0070848: response to growth factor
GO:0010008: endosome membrane
R-HSA-8856828: Clathrin-mediated endocytosis
GO:0030139: endocytic vesicle
GO:0006886: intracellular protein transport
GO:0090407: organophosphate biosynthetic process
GO:0030163: protein catabolic process
GO:0010035: response to inorganic substance
R-HSA-556833: Metabolism of lipids
GO:0009725: response to hormone
GO:0098657: import into cell
R-HSA-382551: Transport of small molecules
GO:0051129: negative regulation of cellular component organization
GO:0005911: cell-cell junction
GO:0030055: cell-substrate junction
GO:0120035: regulation of plasma membrane bounded cell projection organization
GO:0048471: perinuclear region of cytoplasm
hsa04142: Lysosome
GO:0000323: lytic vacuole
GO:0019904: protein domain specific binding
GO:1903829: positive regulation of protein localization
R-HSA-446203: Asparagine N-linked glycosylation
GO:0035091: phosphatidylinositol binding
GO:0051020: GTPase binding
GO:0005975: carbohydrate metabolic process
hsa04714: Thermogenesis
GO:0072576: liver morphogenesis
WP4313: Ferroptosis
GO:0019900: kinase binding
GO:0050839: cell adhesion molecule binding
WP5087: Pleural mesothelioma
R-HSA-109582: Hemostasis
GO:0051336: regulation of hydrolase activity
hsa04141: Protein processing in endoplasmic reticulum
GO:0016197: endosomal transport
WP481: Insulin signaling
hsa00330: Arginine and proline metabolism
GO:0007264: small GTPase mediated signal transduction
GO:0034976: response to endoplasmic reticulum stress
7dOES_20 vs. C_20
7dOES_5 vs. C_5
4dOES_20 vs. C_20
4dOES_5 vs. C_5

Figure 4.5 Functional analysis of DEGs according to the presence of OES and co-culture time. Blastocysts co-cultured with OES for 4 or 7 days were compared with controls without OES in each oxygen group (5% and 20%; 4 comparisons). The Metascape heatmap shows the top-100 enriched clusters of both up- and down-regulated genes (FDR 1%). Each row indicates one cluster, and the orange-brown gradation reflects statistical significance (the darker the color, the more significant the p-value is). Gray color indicates a lack of significance. 7dOES_20 vs. C_20: co-culture with OES for 7 days vs. controls under 20% oxygen; 7dOES_5 vs. C_5: co-culture with OES for 7 days vs. controls under 5% oxygen; 4dOES_20 vs. C_20: co-culture with OES for 4 days vs. controls under 20% oxygen; 4dOES_5 vs. C_5: co-culture with OES for 4 days vs. controls under 5% oxygen.

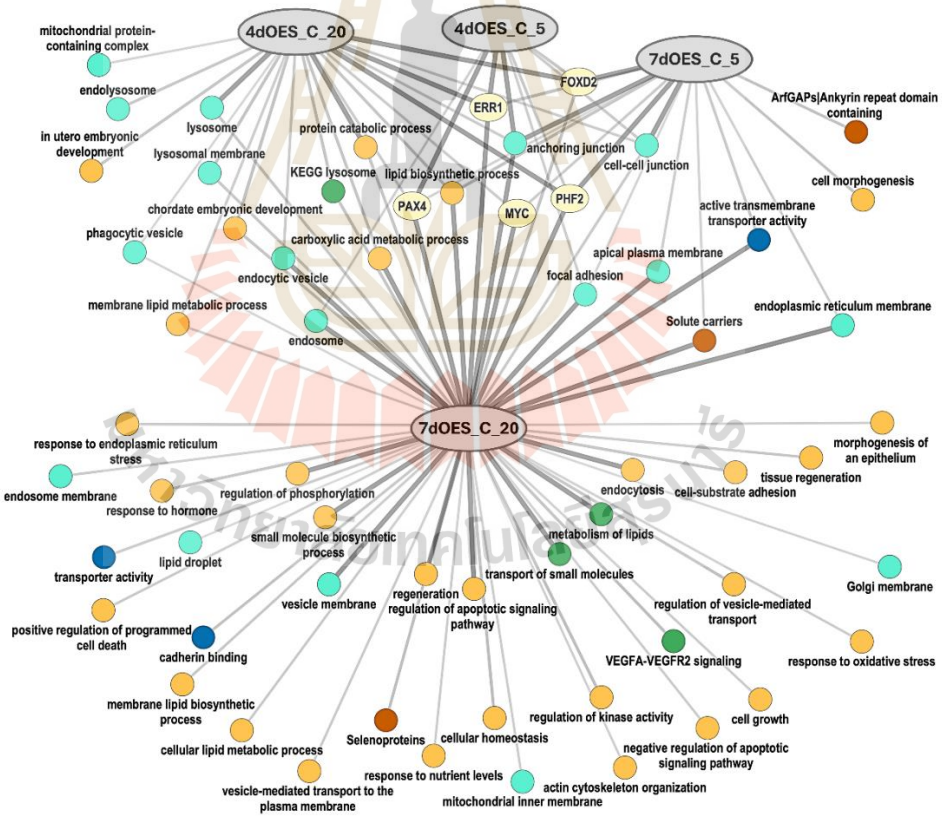

Figure 4.6 Network showing DEGs according to co-culture versus control at different oxygen tension. Expanded blastocysts co-cultured with OES for 4 or 7 days compared to controls without OES under 5% and 20% oxygen (4 comparisons) were analyzed. Both up- and down-regulated genes (FDR 1%) were considered. Nodes in dark blue (GO Molecular

functions), blue-green (GO cellular components), yellow (GO Biological processes), green (Pathways), brown (Gene Family) and light-yellow (Transcription factor binding site, TFBS) illustrates the enriched pathway and process terms associated with individual comparison and shared between comparisons in the network. The thickness of the line indicates higher significance. 4dOES_C_20: co-culture with OES for 4 days vs. controls under 20% oxygen; 4dOES_C_5: co-culture with OES for 4 days vs. controls under 5% oxygen; 7dOES_C_5: co-culture with OES for 7 days vs. controls under 5% oxygen; 7dOES_C_20: co-culture with OES for 7 days vs. controls under 20% oxygen.

3) Effect of co-culture time on blastocyst transcriptome

The functional enrichment analysis of DEGs (FDR 10%) obtained for the 4dOES vs. 7dOES comparison irrespective of oxygen level and under 20% oxygen is shown on Figure 4.7 (the 4dOES vs. 7dOES comparison under 5% oxygen evidenced almost no DEGs). Most enriched terms were obtained for the 4dOES vs. 7dOES comparison under 20% oxygen, for which the DEGs were mostly involved in functions such as “localization within membrane”, “import into cells”, “connective tissue development” and “chordate embryonic development” among the most significant.

4) Effect of oxygen level on blastocyst transcriptome

The functional enrichment analysis of DEGs (FDR 5%) obtained after comparison between 5% and 20% oxygen is illustrated in Figure 4.8 for the three groups of blastocysts (co-cultured with 4 days, 7 days and controls). Among the functions/pathways enriched in all groups were found the “Wnt signalling”, “regulation of cytoskeleton organization” “protein kinase activity” and “cell morphogenesis”, among others. Some functions were enriched specifically in embryos cultured alone, included “cell junction organization” and “regulation of protein stability”, and numerous functions/pathways were found only in blastocysts co-cultured with OES for 4 days, including “carbohydrate metabolite process”, “structural constituent of chromatin”, “nucleosomal DNA binding” and” signalling by interleukins”.

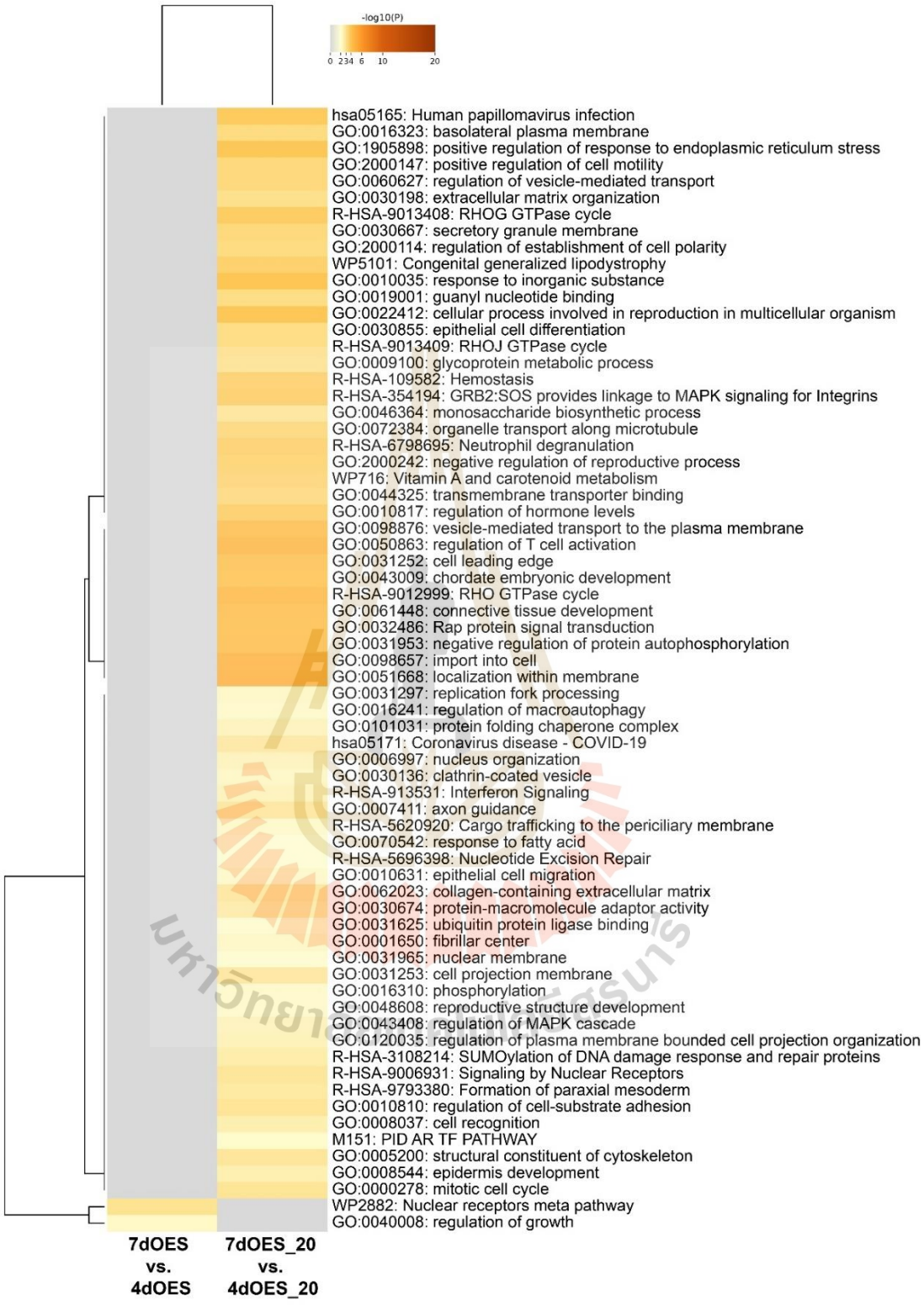

Figure 4.7 Functional analysis of DEGs according to the time of OES co-culture. Blastocysts co-cultured with OES for 4 days were compared with blastocyst co-cultured with OES for 7 days in each oxygen group (5% and

20% oxygen). The Metascape heatmap shows the top-100 enriched clusters. Each row indicates one cluster of both up- and down-regulated genes (FDR 10%), and the orange-brown gradation reflects statistical significance (the darker the color, the more significant the p-value is). Gray color indicates a lack of significance. 7dOES vs. 4dOES: co-culture with OES for 7 days vs. 4 days; 7dOES_20 vs. 4dOES_20: co-culture with OES for 7 days vs. 4 days under 20% oxygen.

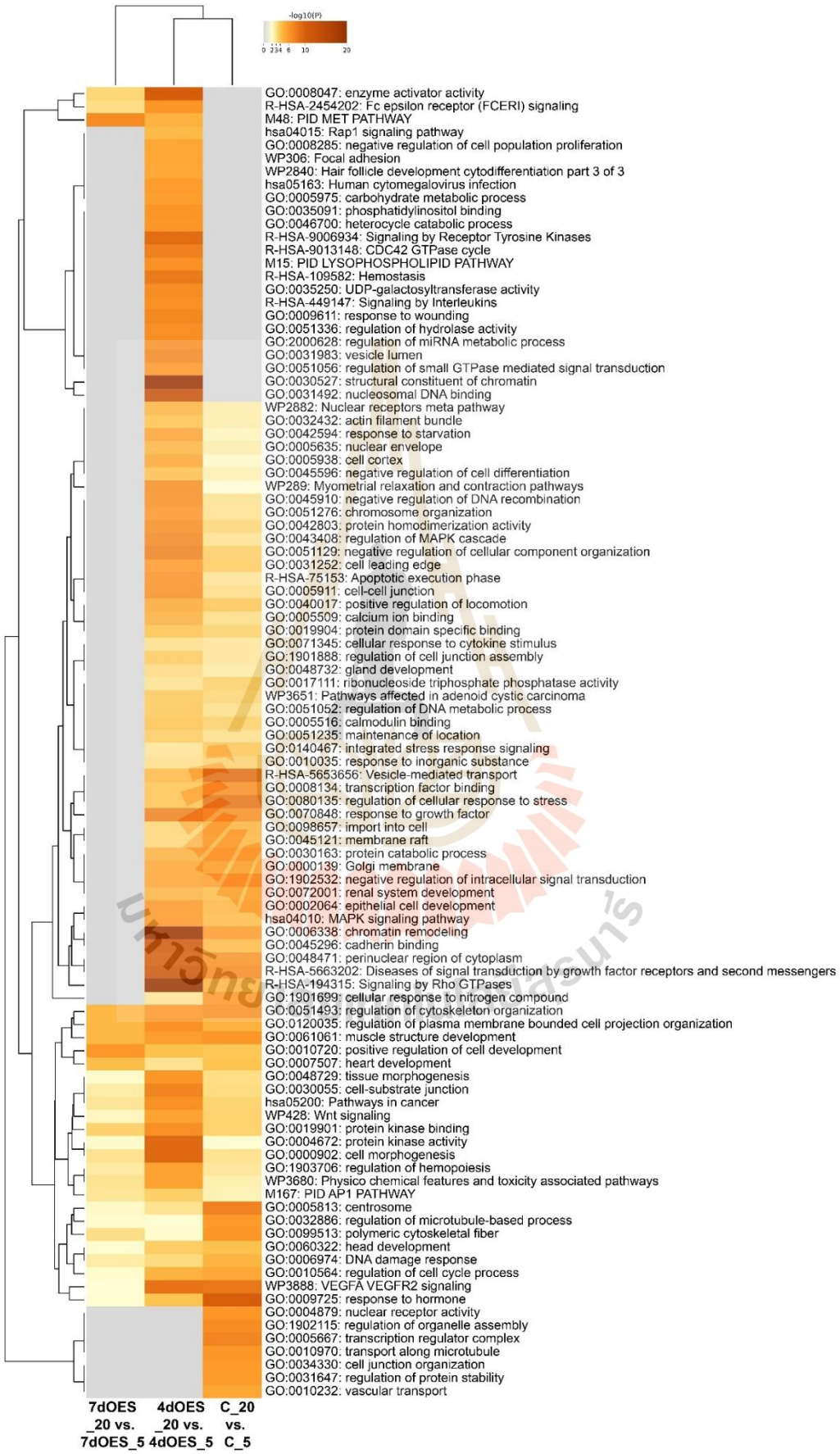
-log10(P)
0 2 3 4 6 10 20
GO:0008047: enzyme activator activity
R-HSA-2454202: Fc epsilon receptor (FCERI) signaling
M48: PID MET PATHWAY
hsa04015: Rap1 signaling pathway
GO:0008285: negative regulation of cell population proliferation
WP306: Focal adhesion
WP2840: Hair follicle development cytodifferentiation part 3 of 3
hsa05163: Human cytomegalovirus infection
GO:0005975: carbohydrate metabolic process
GO:0035091: phosphatidylinositol binding
GO:0046700: heterocycle catabolic process
R-HSA-9006934: Signaling by Receptor Tyrosine Kinases
R-HSA-9013148: CDC42 GTPase cycle
M15: PID LYSOPHOSPHOLIPID PATHWAY
R-HSA-109582: Hemostasis
GO:0035250: UDP-galactosyltransferase activity
R-HSA-449147: Signaling by Interleukins
GO:0009611: response to wounding
GO:0051336: regulation of hydrolase activity
GO:2000628: regulation of miRNA metabolic process
GO:0031983: vesicle lumen
GO:0051056: regulation of small GTPase mediated signal transduction
GO:0030527: structural constituent of chromatin
GO:0031492: nucleosomal DNA binding
WP2882: Nuclear receptors meta pathway
GO:0032432: actin filament bundle
GO:0042594: response to starvation
GO:0005635: nuclear envelope
GO:0005938: cell cortex
GO:0045596: negative regulation of cell differentiation
WP289: Myometrial relaxation and contraction pathways
GO:0045910: negative regulation of DNA recombination
GO:0051276: chromosome organization
GO:0042803: protein homodimerization activity
GO:0043408: regulation of MAPK cascade
GO:0051129: negative regulation of cellular component organization
GO:0031252: cell leading edge
R-HSA-75153: Apoptotic execution phase
GO:0005911: cell-cell junction
GO:0040017: positive regulation of locomotion
GO:0005509: calcium ion binding
GO:0019904: protein domain specific binding
GO:0071345: cellular response to cytokine stimulus
GO:1901888: regulation of cell junction assembly
GO:0048732: gland development
GO:0017111: ribonucleoside triphosphate phosphatase activity
WP3651: Pathways affected in adenoid cystic carcinoma
GO:0051052: regulation of DNA metabolic process
GO:0005516: calmodulin binding
GO:0051235: maintenance of location
GO:0140467: integrated stress response signaling
GO:0010035: response to inorganic substance
R-HSA-5653656: Vesicle-mediated transport
GO:0008134: transcription factor binding
GO:0080135: regulation of cellular response to stress
GO:0070848: response to growth factor
GO:0098657: import into cell
GO:0045121: membrane raft
GO:0030163: protein catabolic process
GO:0000139: Golgi membrane
GO:1902532: negative regulation of intracellular signal transduction
GO:0072001: renal system development
GO:0002064: epithelial cell development
hsa04010: MAPK signaling pathway
GO:0006338: chromatin remodeling
GO:0045296: cadherin binding
GO:0048471: perinuclear region of cytoplasm
R-HSA-5663202: Diseases of signal transduction by growth factor receptors and second messengers
R-HSA-194315: Signaling by Rho GTPases
GO:1901699: cellular response to nitrogen compound
GO:0051493: regulation of cytoskeleton organization
GO:0120035: regulation of plasma membrane bounded cell projection organization
GO:0061061: muscle structure development
GO:0010720: positive regulation of cell development
GO:0007507: heart development
GO:0048729: tissue morphogenesis
GO:0030055: cell-substrate junction
hsa05200: Pathways in cancer
WP428: Wnt signaling
GO:0019901: protein kinase binding
GO:0004672: protein kinase activity
GO:0000902: cell morphogenesis
GO:1903706: regulation of hemopoiesis
WP3680: Physico chemical features and toxicity associated pathways
M167: PID AP1 PATHWAY
GO:0005813: centrosome
GO:0032886: regulation of microtubule-based process
GO:0099513: polymeric cytoskeletal fiber
GO:0060322: head development
GO:0006974: DNA damage response
GO:0010564: regulation of cell cycle process
WP3888: VEGFA VEGFR2 signaling
GO:0009725: response to hormone
GO:0004879: nuclear receptor activity
GO:1902115: regulation of organelle assembly
GO:0005667: transcription regulator complex
GO:0010970: transport along microtubule
GO:0034330: cell junction organization
GO:0031647: regulation of protein stability
GO:0010232: vascular transport
7dOES_20 vs. 7dOES_5
4dOES_20 vs. 4dOES_5
C_20 vs. C_5

Figure 4.8 Functional analysis of DEGs according to oxygen tension. Blastocysts cultured under 5% oxygen were compared to blastocysts cultured under 20% oxygen in each culture group (4 days with OES; 7 days with OES; and controls without OES). The Metascape heatmap shows the top-100 enriched clusters. Each row indicates one cluster of both up- and down-regulated genes (FDR 5%), and the orange-brown gradation reflects statistical significance (the darker the color, the more significant the p-value is). Gray color indicates a lack of significance. 7dOES_20 vs. 7dOES_5: co-culture with OES for 7 days under 20% oxygen vs. under 5% oxygen; 4dOES_20 vs. 4dOES_5: co-culture with OES for 4 days under 20% oxygen vs. under 5% oxygen; C_20 vs. C_5: culture with medium alone without OES for 7 days under 20% oxygen vs. under 5% oxygen.

4.5 Discussion

The main results of this study are that: 1) OES mitigated the negative effect of air oxygen levels on embryo development and quality; 2) OES improved blastocyst total cell number under low and high oxygen levels; 3) the presence of OES up to the time of EGA (day 5 post-fertilization) was sufficient to support embryo development and blastocyst quality irrespective of the oxygen level; 4) both oxygen and OES co-culture had a significant impact on the embryonic transcriptome, maximal number of DEGs being identified under 20% oxygen; 5) the DEGs identified were involved in a wide range of functions, including lipid metabolism, membrane organization, intracellular transport and cell-cell junctions among the most significant ones.

4.5.1 OES improved embryo development and quality

Co-culture with OES significantly improved both embryo development and quality in terms of total cell numbers under high oxygen level. These results are consistent with previous studies that used OEC monolayers under 20% oxygen, in which blastocyst yields were improved on days 7-8 compared to embryos alone (Cordova et al., 2014; Schmaltz-Panneau et al., 2015; Pranomphon et al., 2024). Although we did not compare oxygen levels in the culture medium with or without OES, it is probable that this beneficial effect was partly due to oxygen consumption by OES in the culture medium, leading to an oxygen tension very close to the 2-8% in the oviduct lumen (Fischer & Bavister, 1993). Furthermore, the oviduct epithelium express antioxidant enzymes (glutathione peroxidase, superoxide dismutase and catalase) (Lapointe & Bilodeau, 2003; Schmaltz-Panneau et al., 2015) and antioxidant activities were measured in the oviduct fluid (Lapointe & Bilodeau, 2003). Among

proteins secreted in the oviduct fluid, 56 have been shown to interact with bovine IVP embryos (Banliat et al., 2020), among which annexins A1 and A2 with antioxidant activity (Harvey et al., 1995).There is evidence that OEC in co-culture or the supplementation of OEC-derived extracellular vesicles (EVs) in the culture medium increase the gene expression of enzymes involved in ROS scavenging, like GPX1 (Cordova et al., 2014) and SOD1 (Fang et al., 2022), in IVP embryos, with concomitant decrease in embryonic ROS levels (Fang et al., 2022). Thus, it could be speculated that under high oxygen level, OES are under optimal conditions to support embryo development through passive oxygen consumption, active antioxidant secretions as well as transfer of EV-mRNAs and proteins helping the embryos to mitigate the effect of oxidative stress.

In this study, total cell number per blastocyst was used as the main criteria determining embryo morphological quality as it has been positively correlated with pregnancy rates after embryo transfer in cattle (Hoelker et al., 2006; Ross et al., 2023). Overall, co-culture with OES under both oxygen levels significantly improved blastocyst growth, resulting in higher total cell numbers compared with embryos cultured alone. Our results are in line with previous reports in which the supplementation of the culture medium with low amounts of oviduct fluid (Lopera-Vasquez et al., 2017) or oviduct fluid-derived EVs (Almiñana et al., 2017) supported blastocyst rate and blastocyst cell number. The beneficial effect of OES could be the result of an inhibition of cell apoptosis in blastomeres, as previously shown for OEC monolayers in pigs (Fang et al., 2022) and cattle (Cordova et al., 2014), as well as a stimulation of cell mitosis. OECs in culture have been shown to secrete growth factors and embryotrophic proteins beneficial for embryo development (Tse et al., 2008), and to reduce the medium concentration of glucose and increase those of lactate and pyruvate, two metabolites important for early embryo development (Edwards et al., 1997).

4.5.2 OES for 4 days are enough, but 7 days are better to produce beneficial effects

In cattle, at the beginning of cleavage, the embryo employs maternal mRNAs and proteins and is almost transcriptionally inactive, with only minor transcription activity up to the 4-cell stage (Lee et al., 2014). However, at the 8-16-cell stage in cattle, the embryonic genome is activated (major EGA) to initiate transcription and translation so that the embryo produce its own mRNAs and proteins while maternal transcripts and proteins decrease and disappear (Badr et al., 2007; Lee et al., 2014; Pohler et al., 2020). Although the quality of the oocyte is

important for blastocyst yield, there is evidence that the quality of the environment to which the embryo is exposed after fertilization has a great impact on EGA and RNA profiles in the resulting blastocysts (Rizos, Ward, et al., 2002; Dalvit et al., 2005; Gad et al., 2012; Cordova et al., 2014; Lonergan & Forde, 2014; Rodríguez-Alonso et al., 2020). Prior research from our group showed that the presence of OEC monolayers for the first 4 days of *in vitro* development was sufficient to improve blastocyst rates in cattle (Cordova et al., 2014), yet with OEC partial dedifferentiation during co-culture (Schmaltz-Panneau et al., 2015). Here we validate the hypothesis that OES up to the EGA was enough to support both embryo development and quality. This short-term effect was particularly obvious under 20% oxygen as there was no difference in blastocyst yield and cell numbers between the 4dOES_20 and 7d0ES_20. On the same line, the number of DEGs obtained when considering the effect of co-culture time was very low (max 8 DEGs with FDR 1%, Table 4.3), showing that OES presence beyond the 16-cell stage had low impact on embryonic transcriptome. However, under 5% oxygen, 7 days of co-culture were necessary to observe a beneficial effect of OES on blastocyst cell numbers. Therefore, although 4 days of co-culture correspond to the physiological exposure of embryos to the oviduct environment *in vivo*, it appears more beneficial and practical to keep OES with embryos for 7 days.

4.5.3 OES differentially modulate the blastocyst transcriptome according to oxygen tension

Our results showed that the vicinity of OES during embryo culture had a significant impact on blastocyst RNA content. This impact on embryonic transcriptome increased with co-culture time and oxygen level, highest numbers of DEGs being identified after 7 days of co-culture under 20% oxygen. However, when considering the effect of OES (Fig. 4.5), most enriched terms were shared among the four comparisons (i.e. very few terms were specific to one comparison), with higher significance after 7 than 4 days in both oxygen levels. This shows that longer co-culture time did not change the activated pathways but mostly the scale of changes.

Among the enriched terms shared among the four comparisons, “lipid metabolism” was among the most significant ones. Bovine IVP embryos have high lipid accumulation and different phospholipid profiles (Janati Idrissi et al., 2021) compared to their *in vivo* counterparts (de Andrade Melo-Sterza & Poehland, 2021). These particularities are associated with lower cryotolerance of IVP embryos (Janati Idrissi et al., 2021). In accordance with our data, previous studies evidenced higher cryosurvival rates (Rizos, Ward, et al., 2002; Schmaltz-Panneau et al., 2015; Asaadi et

al., 2019) and changes in the expression of genes involved in lipid metabolism (Cordova et al., 2014; Lopera-Vasquez et al., 2017), when embryos were co-cultured with OECs or OEC-derived EVs.

In this present study, air oxygen level was used to induce oxidative stress on embryos and test the protective effect of OES, while the 5% oxygen tension was considered as physiological for embryos. Surprisingly, the "response to oxygen levels" term was enriched among DEGs in all blastocysts regardless of the oxygen level and time of co-culture. It thus appears that even a low oxygen level in the coculture system induced adaptative response from the embryos. The same pattern could be seen for "regulation of apoptotic pathways". Therefore, co-culture with OES during 7 days appear to help embryos to regulate ROS levels and apoptotic molecular pathways. One mechanism underlying the adaptative response of embryos may be the Wnt signalling pathway, which was enriched in the comparison between 20% and 5% oxygen. Wingless (Wnt) proteins are the peripheral proteins and lipid-anchored proteins on the surface of exosomes (Pegtel & Gould, 2019) in which their signalling mechanisms via ligand-receptor interactions are crucial for embryonic development (McGough & Vincent, 2016).

Another functional term emerging among the 4 days vs. 7 days comparison under 20% oxygen was "Interferon signaling". Interferon-tau (IFNT) is the pregnancy recognition signal secreted by the bovine blastocyst to inhibit luteolysis, allowing the maintenance of progesterone secretion by the corpus luteum (Kowalczyk et al., 2021). The embryonic transcription of interferon tau at early steps of embryonic development is thus crucial for pregnancy success. The enrichment in interferon signaling pathway is in agreement with findings from (Talukder et al., 2018) which demonstrated that co-culture of bovine embryos with OECs for 4 days induced the expression of the IFNT protein in morulas (Talukder et al., 2018).

Interestingly, numerous enriched biological functions and pathways were associated with "import into cell", "transport of small molecules", "regulation of vesicle-mediated transport", "endosomal transport", "membrane trafficking" and "endocytic vesicle" (Fig. 4.5 & 4.6). It can be assumed that OES secreted EVs in the culture medium, which conveyed RNAs through the zona pellucida to exert functional effects on embryos. In a previous study in which embryos were treated with oviduct fluid-derived EVs, upregulated DEGs compared to controls without EVs were related to "translation", "ATP metabolic process", "vesicle-mediated transport" and "signaling by WNT", while downregulated DEGs included "response to

endoplasmic reticulum stress", "apoptosis" and "intrinsic apoptotic signaling pathway in response to DNA damage" (Bauersachs et al., 2020).

4.6 Conclusion

In conclusion, this study reveals a considerable impact of OES on embryo development, blastocyst quality and gene expression under both 5% and 20% oxygen. Although 4 days of co-culture were enough to see beneficial effects of OES, maximal impact was seen after 7 days. This is the first in-depth analysis of transcripts and related functions modified by OES. Our results clearly show differential effects of OES on the embryonic transcriptome depending on oxygen tension and co-culture time, evidencing for the first time a modulation of the embryo-OES dialog according to the environment.

CHAPTER 5
OVERALL CONCLUSION

The mammalian oviduct provides an optimal environment for early embryo development. On the contrary, *in vitro* embryo production exposes the embryos to oxidative stress with deleterious effects on blastocyst development and quality. Although oviduct epithelial cells (OEC) have been used for a long time to improve development rate and quality of blastocysts produced *in vitro*, most *in vitro* models of OEC induce cell dedifferentiation or are technically challenging. In this study we propose a model based on oviduct epithelial spheroids (OES), which form rapidly (within 48–72 h) from isthmic mucosa fragments (IMF), containing an already-differentiated epithelium. Furthermore, we proposed to use OES to consume the O_2 in excess and produce ROS scavenger proteins, thus overcoming oxidative stress during *in vitro* embryo development. We summarised our findings as follows.

The OES culture in a large volume of M199 medium or in droplets of SOF medium provided the optimal conditions for OES *in vitro* culture. In SOF droplets, OESs supported blastocyst development. Furthermore, an effect of the developing embryos on OES morphology was evidenced, suggesting an effect of embryos on spheroid intercellular junctions that remain to be investigated. Altogether, it points to OES as an easy-to-use, easy-to-standardize, and physiological model to study early embryo–maternal interactions.

In conclusion, this study reveals a considerable impact of OES on embryo development, blastocyst quality and gene expression under both 5% and 20% oxygen. Although 4 days of co-culture were enough to see beneficial effects of OES, maximal impact was seen after 7 days. This is the first in-depth analysis of transcripts and related functions modified by OES. Our results clearly show differential effects of OES on the embryonic transcriptome depending on oxygen tension and co-culture time, evidencing for the first time a modulation of the embryo-OES dialog according to the environment.

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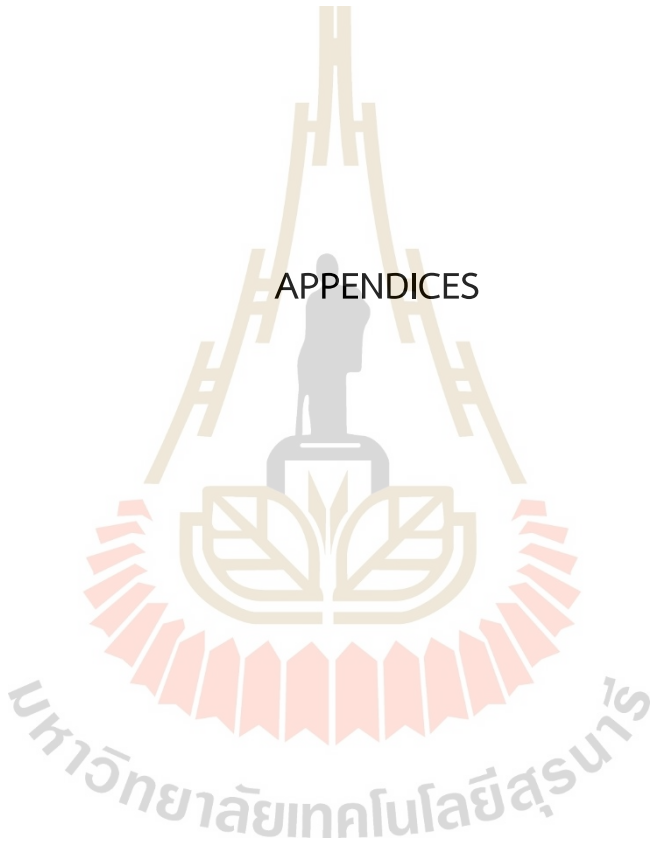
APPENDICES



APPENDIX A

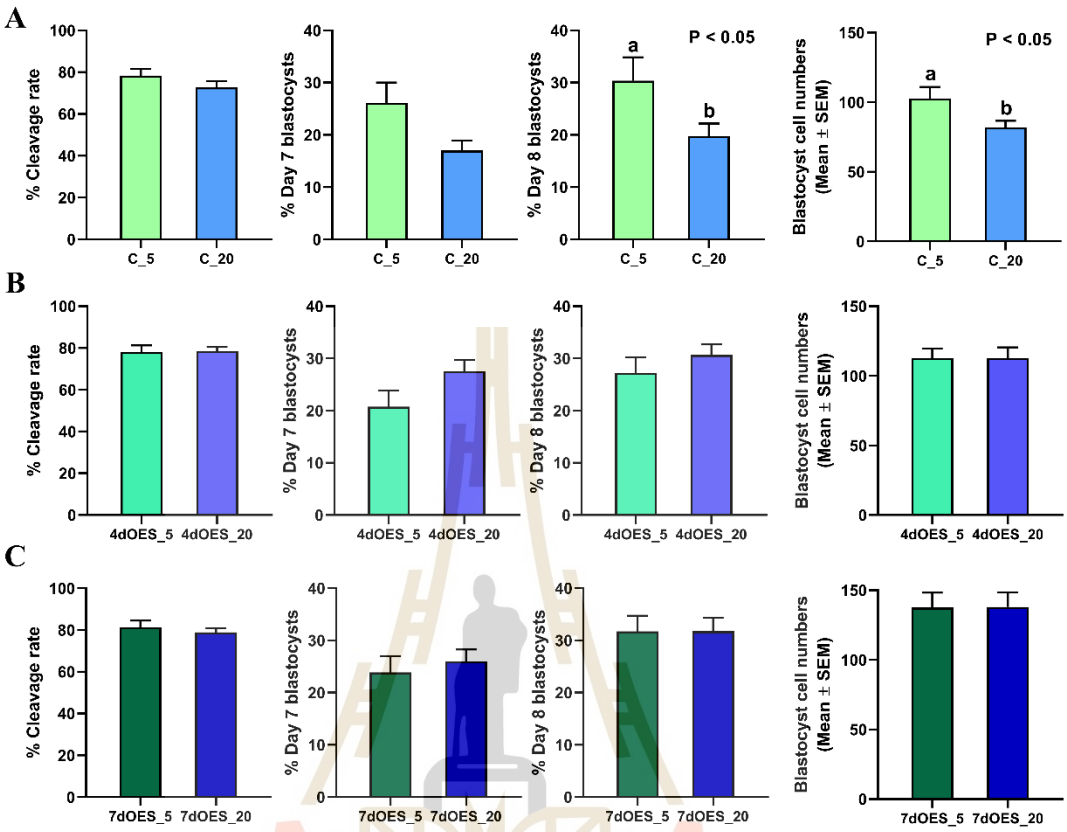


Figure S1 Effect of oxygen tension on cleavage, blastocyst rates and total cell numbers. A, in the absence of OES. B, in the presence of OES for 4 days. C, in the presence of OES for 7 days. Percentage values are provided as the means ± SEM of 8 replicates under 20% O_2, except blastocyst cell numbers (3 replicates in both oxygen tensions). Different letters indicate significant differences ($P<0.05$, unpaired t-test). C_5: 7 days culture with medium alone without OES at 5% O_2; C_20: 7 days culture with medium alone without OES at 20% O_2; 4dOES_5: 4 days co-culture with OES at 5% O_2; 4dOES_20: 4 days co-culture with OES at 20% O_2; 7dOES_5: 7 days co-culture with OES at 5% O_2; 7dOES_20: 7 days co-culture with OES at 20% O_2.

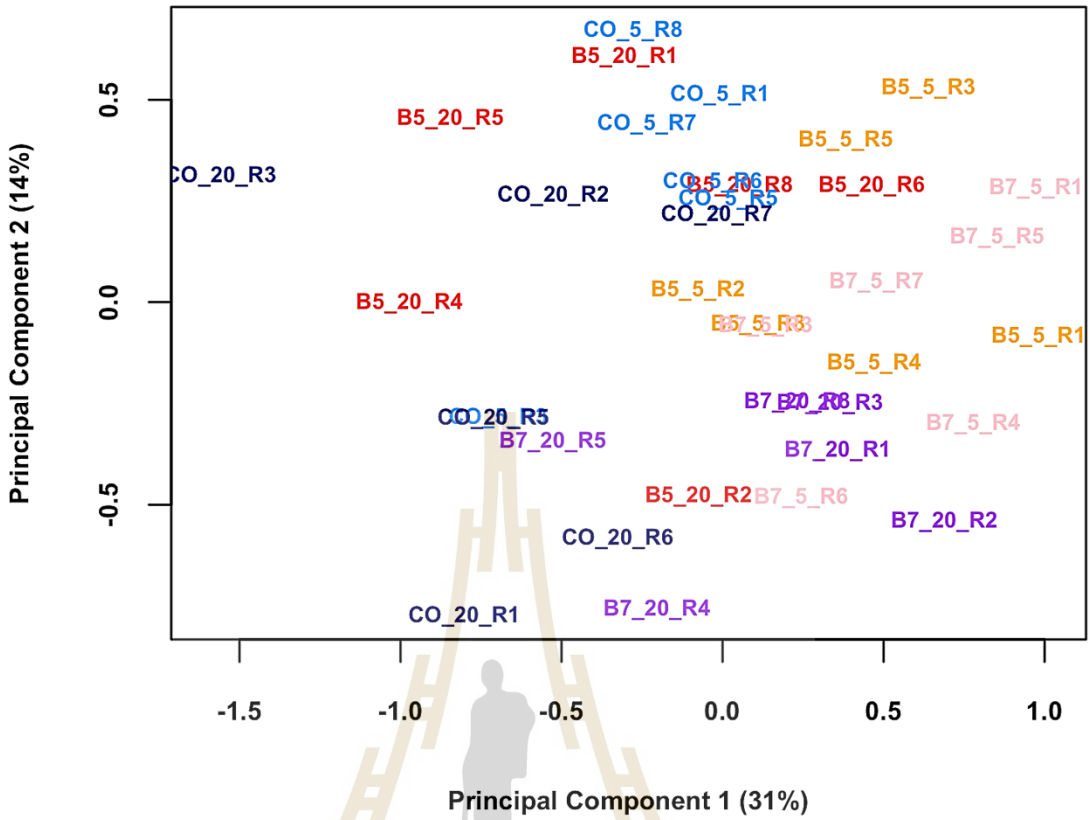


Figure S2 Multidimensional scaling (MDS) plot of RNA-seq data (top 500 genes; FDR 1%). RNA-seq data was carried out in expanded blastocysts co-cultured with OES for 4 or 7 days and under low or high oxygen tension. Each plot represents one pool of expanded blastocysts (6 pools/condition) and one color represents one condition. Blue: C_5, dark blue: C_20, yellow: 4dOES_5, red: 4dOES_20, pink: 7dOES_5, purple: 7dOES_20.

Table S1 Effect of oxygen tension on blastocyst yield in the absence of OES.

Control	N	% (n) of cleavage rate	% (n) of blastocyst rate			Cell numbers in expanded blastocysts (n)
			Day 6	Day 7	Day 8	
5% O_2	557	78.4 ± 3.2 (444)	24.3 ± 4.7 (136)	26.1 ± 3.9 (150)	30.4 ± 4.5^{a} (177)	102.6 ± 8.4^{x} (20)
20% O_2	790	72.8 ± 2.9 (580)	13.4 ± 1.9 (98)	17 ± 1.9 (132)	19.8 ± 2.4^{b} (151)	82.1 ± 4.5^{y} (20)

Percentage values are provided as the means ± SEM of 8 replicates under 5% O_2 and 9 replicates under 20% O_2, except blastocyst cell numbers (3 replicates in both

oxygen tensions). The different letters within one column indicate a significant ($P<0.05$, unpaired t-test). Except the rates of blastocysts on days 6 and 7 and blastocyst cell numbers were analyzed using unpaired t-test with Welch's correction (unequal variances). N = total number of cumulus-oocyte complexes; and n = total number of embryos.

Table S2 Effect of oxygen tension on blastocyst yield after co-culture with OES for 4 days.

Co-culture for 4 days	N	% (n) of cleavage rate	% (n) of blastocyst rate			Cell numbers in expanded blastocysts (n)
			Day 6	Day 7	Day 8	
5% O2	657	78 ± 3.3 (514)	15.9 ± 2.7 (110)	20.8 ± 3.1 (139)	27.3 ± 3 (181)	112.9 ± 6.7 (20)
20% O2	768	78.6 ± 2 (604)	16.8 ± 3.4 (136)	27.6 ± 2.2 (215)	30.7 ± 2 (236)	112.7 ± 7.8 (20)

Percentage values are provided as the means ± SEM of 8 replicates under 5% O_2 and 9 replicates under 20% O_2, except blastocyst cell numbers (3 replicates in both oxygen tensions). The rates of cleavage and blastocysts were analyzed using unpaired t-test ($P<0.05$). N = total number of cumulus-oocyte complexes; and n = total number of embryos.

Table S3 Effect of oxygen tension on blastocyst yield after co-culture with OES for 7 days.

Co-culture for 7 days	N	% (n) of cleavage rate	% (n) of blastocyst rate			Cell numbers in expanded blastocysts (n)
			Day 6	Day 7	Day 8	
5% O_2	507	81.4 ± 3.2 (417)	16.4 ± 2.8 (79)	23.9 ± 3.1 (117)	31.8 ± 3 (155)	137.6 ± 10.8 (20)
20% O_2	795	78.9 ± 2 (623)	16.1 ± 3.2 (123)	26 ± 2.3 (208)	31.8 ± 2.5 (248)	138.1 ± 10.5 (20)

Percentage values are provided as the means ± SEM of 8 replicates under 5% O_2 and 9 replicates under 20% O_2, except blastocyst cell numbers (3 replicates in both oxygen tensions). The rates of cleavage and blastocysts were analyzed using unpaired t-test ($P<0.05$). N = total number of cumulus-oocyte complexes; and n = total number of embryos.

Table S4 Candidate genes used for validation of RNA-seq results.

Gene Symbol	Gene Name
MUC13	Mucin 13, cell surface associated
UCHL1	Ubiquitin C-terminal hydrolase L1
FETUB	Fetuin B
COL17A1	Collagen type XVII alpha 1 chain
HSD3B1	Hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1
NOTCH1	Notch Receptor 1

CURRICULUM VITAE

Miss Thanya Pranomphon was born in Nakhon Pathom, Thailand on October 28th, 1992. She completed a bachelor's degree (B.Sc.) in Animal Sciences and Agricultural Technology from Silpakorn University Phetchaburi Information Technology Campus with first class honours in 2015. She received the scholarship from the Royal Golden Jubilee (RGJ) Ph.D. Programme in 2018 (grant number PHD/0032/2560) under the supervision of Assoc. Prof. Dr. Rangsun Parnpai and Dr. Pascal Mermillod. She had an opportunity to conduct the research at French National Research Institute for Agriculture, Food and Environment (INRAE) center Val de Loire-Physiologie de la Reproduction et des Comportements (PRC), 37380 Nouzilly, France for 2 years (2022-2024) under the supervision of Dr. Pascal Mermillod and Prof. Dr. Marie Saint-Dizier.

The research topic is "Effects of bovine oviduct epithelial spheroids during *in vitro* embryo culture on quality of embryo and transcriptomics pattern". The chapter 3 in this thesis was published in Theriogenology in January 2024 (https://doi.org/10.1016/j.theriogenology.2024.01.022). However, chapter 4 in this thesis is being prepared to be submitted in the Journal of Animal Science and Biotechnology.