P260

Determining maturation status of bovine and human *in vitro* derived oocytes from egg precursor cells by key morphological, genetic and metabolic indicators

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Introduction

- *In vitro* derived oocytes (IVDOs) can be defined as female gametes generated *in vitro* from already existing cells[1] displaying the defining characteristics of an oocyte. To date, approaches to maturing undifferentiated cells into oocytes have included the use of induced pluripotent stem cells, embryonic stem cells and egg precursor cells (also known as female germline stem cells, ovarian stem cells or oogonial stem cells).[2,3]
- Morphological characteristics, oocyte-related and germline-specific gene expression, and metabolic indicators are widely reported for the classification of endogenous oocytes and can be applied as initial success criteria for characterizing IVDOs. Further rigorous characterization, including the evaluation of DNA content, chromosome organization and recombination, as well as the birth of viable euploid offspring, are the proposed next steps for the classification of IVDOs and the evaluation of their potential.[4]
- IVDOs may provide a next-generation infertility treatment to help women produce healthy, young, fertilizable eggs without hormone stimulation injections.
- Egg precursor cells, which are ≤10 µm in diameter and located in the ovarian cortex, are one cell source under investigation. These cells are described as lineage specific, have been identified in multiple species (e.g., mouse, rat, cow and human) and can give rise to spontaneous IVDOs in culture (as defined by morphology and gene and protein expression).[2,3,5,6]
- The full potential of bovine and human egg precursor cell derived IVDOs is currently under investigation. In this current study, egg precursor cells were isolated from their respective ovarian cortices and cultured in defined model systems. The resultant IVDOs were morphologically, genetically and metabolically assessed to determine maturation status.

Objective

- To assess the maturation status of human and bovine IVDOs matured from egg precursor cells in a defined *in vitro* culture system.

Methods

Egg precursor cell isolation by fluorescence-activated cell sorting (FACS)

- Human ovarian tissue was obtained from postmortem donors 18–48 years of age (written informed consent for organ donation for research purposes provided). Tissue was sourced by Advanced Tissue Services (Phoenix, AZ, USA), Asterand Bioscience (Detroit, MI, USA) and the International Institute for the Advancement of Medicine (Edison, NJ, USA).
- Bovine ovarian tissue was collected from calves 4–10 months of age and sourced by ETCR (Rockwood, TN, USA).
- In preparation for cell sorting via FACS, human and bovine ovarian cortices were enzymatically and mechanically digested into single-cell suspensions. Cells were serum blocked and incubated with a proprietary monoclonal anti-DDX4 (DEAD box protein 4) antibody[7] and isolated using an LE-SH800Z (Sony Corporation, Tokyo, Japan) or a BD FACSAria II (BD Biosciences, Franklin Lakes, NJ, USA) cell sorter.

Egg precursor cell ➔ IVDO culture

- Egg precursor cells were aggregated with species-specific ovarian somatic cells and maintained in a defined culture system prior to IVDO isolation by microdissection.
- Cultures were maintained at 37°C (human) or 38.5°C (bovine) and 5% CO_2 in a humidified atmosphere, with 50/50 media exchanges every other day (human) or every day (bovine) for the duration of culture, and imaged twice weekly for gross morphological assessment.

IVDO characterization

Morphological characteristics and chromatin organization (microscopy)

- The maturation status of IVDOs was assessed by examining their morphological appearance using brightfield microscopy in accordance with the following morphological characteristics for endogenous oocytes: diameter (µm), presence of zona pellucida, germinal vesicle and/or polar body structures[8] (**Table 1**).

Table 1: Morphological classification of IVDO characteristics and definitions

Characteristic	Abbreviation	Definition
Zona pellucida	ZP	Presence of a transparent outer membrane
Germinal vesicle	GV	Presence of an enlarged circular depression in the cytoplasm containing a clear nucleolus
Polar body	PB	Presence of a small, round cell contained within the perivitelline space

- Hoechst 33342 dye (H; 10 µg/mL) was used to determine the nuclear chromatin arrangement in conjunction with propidium iodide (PI; 10 µg/mL) to assess cell viability.
- Images were captured using an IX73 inverted fluorescence microscope system equipped with a DP80 color camera (Olympus, Center Valley, PA, USA) or an Eclipse Ti-S inverted fluorescence microscope system equipped with a Photometrics CoolSNAP MYO color camera (Nikon, Melville, NY, USA).

Gene expression (reverse transcription quantitative polymerase chain reaction [qRT-PCR])

- Single-cell qRT-PCR was performed for the expression of the oocyte-expressed genes *FIGLA*, *LHX8*, *GDF9* and *ZP3* and the germline-specific genes *DDX4* and *DAZL*. The presence of each messenger RNA (RNeasy Micro Kit, Qiagen, Valencia, CA, USA) was assessed by single-cell qRT-PCR from individually isolated IVDOs.
- Amplification of the markers was performed using commercially available TaqMan primer/probes (Applied Biosystems, Foster City, CA, USA; **Table 2**) and a QuantStudio 6 thermocycler (ThermoFisher Scientific, Waltham, MA, USA). Gene expression was analyzed using C_T values to indicate the presence/absence of oocyte-related genes. β-actin was used as the reference gene.

Table 2: Oocyte-related and germline-specific genes assessed

			NCBI gene ID		TaqMan gene expression assay ID	
Type	Gene	Full name	Bovine	Human	Bovine	Human
Oocyte-related markers	*FIGLA*	Factor in the germline alpha	528891	344018	Custom	Hs01079386_m1
	LHX8	LIM homeobox 8	512385	431707	Custom	Hs00418293_m1
	GDF9	Growth differentiation factor 9	282574	2661	Bt03223996_m1	Hs00193364_m1
	ZP3	Zona pellucida glycoprotein 3	280964	7784	Bt03212153_m1	Hs0061023_m1
Germline-specific markers	*DDX4*	DEAD box protein 4	493725	54514	Bt03210246_m1	Hs00987130_m1
	DAZL	Deleted in azoospermia-like	530116	1618	Bt03255020_m1	Hs00154706_m1

NCBI, National Center for Biotechnology Information.

Metabolic (glucose-6-phosphate dehydrogenase [G6PD] activity)

- G6PD is an enzyme in oocyte cytoplasm that indirectly metabolizes Brilliant Cresyl Blue (BCB) dye. Immature oocytes have a high concentration of G6PD, enabling the blue dye to be converted into a colorless compound. As the oocyte matures, G6PD levels decrease, allowing the blue dye to penetrate and persist. As an indicator of oocyte maturation, this technique is frequently used for the selection of mature oocytes for the *in vitro* fertilization of large animal species.[9,10]
- Human IVDOs were incubated with 10 µM BCB (reconstituted in phosphate-buffered saline) for 10 minutes at 37°C in 5% CO_2 in a humidified atmosphere, washed in phosphate-buffered saline and immediately imaged for the presence or absence of BCB dye using an IX73 inverted fluorescence microscopy system equipped with a DP80 color camera (Olympus).
- Bovine IVDOs were incubated with 26 µM BCB (reconstituted in holding medium) for 30 minutes at 37°C on a hot plate, washed twice in holding medium and immediately imaged for the presence or absence of BCB dye using an EVOS XL Core inverted digital microscope system (ThermoFisher Scientific).

Results

Morphological characterization

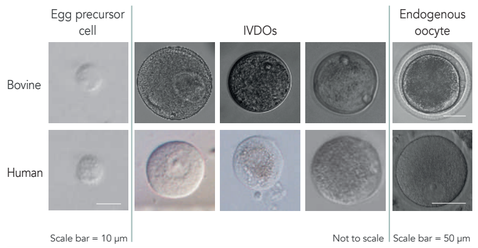


Figure 1: Morphological characteristics of bovine and human IVDOs. Examples of bovine and human egg precursor cell derived IVDOs compared with endogenous oocytes of the corresponding species.
The image of the human endogenous oocyte is reproduced with permission from the Atlas of Human Embryology – European Society of Human Reproduction and Embryology (ESHRE). Available at: http://atlas.eshre.eu/es/1454662357452400.

Gene expression profile

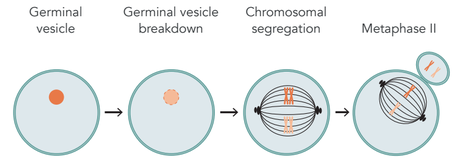
Table 3: Gene expression assessment of IVDOs[a]

		Bovine				Human			
Type	Gene	IVDO #1	IVDO #2	IVDO #3	Endog	IVDO #1	IVDO #2	IVDO #3	Endog
Oocyte-related markers	*FIGLA*								
	LHX8								
	GDF9								
	ZP3								
Germline-specific markers	*DDX4*								
	DAZL								

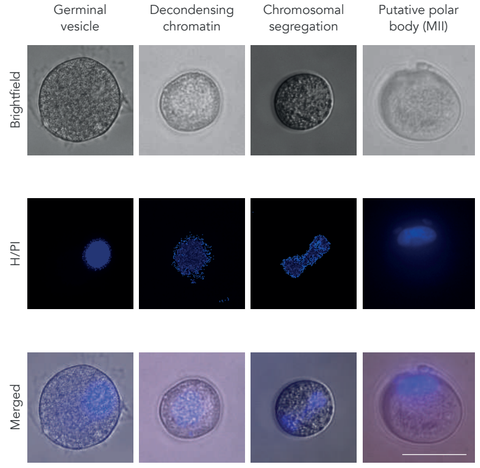
[a]Oocyte-related genes have been identified in IVDOs and endogenous oocytes by qRT-PCR. Green indicates marker gene expression, whereas orange indicates lack of marker gene expression.
Endog, endogenous oocyte.

Chromatin organization

A. Nuclear chromatin configurations during oocyte maturation



B. Chromatin configurations observed in bovine IVDOs



C. Chromatin configurations observed in human IVDOs

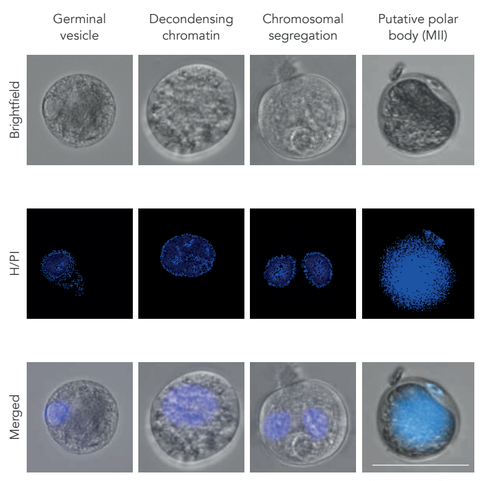


Figure 2: Observed chromatin configurations of bovine and human IVDOs.

Metabolic characterization

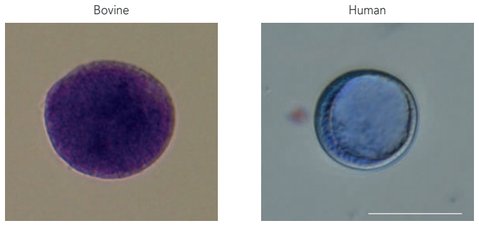


Figure 3: Metabolic evaluation with BCB. G6PD activity was assessed by incubating bovine and human IVDOs in BCB dye. The presence of BCB-positive IVDOs is indicative of cytoplasmic maturity.

Summary of IVDO characterization

Table 4: Comparison of human and bovine IVDOs with endogenous oocytes

	Endogenous		IVDO	
Criteria	Bovine	Human	Bovine	Human
Size (µm)	120	120	120	100
Morphology (GV/ZP/PB)	✓		✓	
Oocyte and germline gene expression	✓		✓	
G6PD metabolic activity	✓		✓	

Conclusions

- IVDOs generated from bovine and human egg precursor cells are similar to endogenous oocytes with respect to the 4 criteria investigated: size, morphology, gene expression and metabolic activity (**Table 4**).
- Culture systems in both bovine and human models support the *in vitro* maturation of egg precursor cells of ≤10 µm diameter into IVDOs that exhibit stage-appropriate morphological, genetic and metabolic hallmarks of maturing oocytes.
- The focus is now on cytoplasmic maturation and the synchronization of key events to ultimately generate IVDOs capable of generating euploid embryos and viable offspring.

Disclosures

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