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Review article

The characterization and therapeutic applications of ovarian theca cells: An update

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ABSTRACT

Theca cells perform a range of roles during folliculogenesis. So far, little is known about their recruitment process and function since early research has mainly focused on the interactions between granulosa cells and the oocyte, leaving theca cells unfairly forgotten in the understanding of ovarian physiology and pathogenesis. Given that research on theca cells has greatly emerged in recent years, this review of literature aims to discuss the established theoretical concepts with the most recent findings about theca cells' characterization and origins, *in vitro* culture applications as models for fertility preservation and pharmacological/toxicological studies, its importance in unraveling pathogenic pathways, and stem-cell-based bioengineering for hormonal replacement therapies. Isolation and *in vitro* culture techniques for theca cells have led to essential advancements in their characterization as a specific cell population. Unraveling the origins of theca cells during the *in vivo* differentiation process in the adult ovary will assist the development of hormonal replacement therapies, reestablishment of fertility, and treatments for diseases such as premature ovarian insufficiency and polycystic ovarian syndrome, which seem to be directly influenced by theca cells.

1. Introduction

In the adult ovary, theca cells perform a range of roles during folliculogenesis, enabling crosstalk with granulosa cells and the oocyte. They provide all androgens required for the development of the follicle and secretion of estrogens by granulosa cells. In atretic follicles, theca cells are usually the last cell population to go through apoptosis. In ovulating follicles, they differentiate into luteinizing cells to establish the corpus luteum. Even so, little is known about theca cell control of recruitment and function since most research has focused on the interactions between granulosa cells and the oocyte, leaving theca cells unfairly forgotten [1].

In contrast to granulosa cells and oocytes, the origins of these important cells still require additional study. Theca cells are probably recruited from the surrounding stroma, but from which specific cell population, we still cannot assure. In this sense, two leading theories are proposed: (1) they are present in the newborn ovary as putative theca cells or theca stem cells (TSCs), as demonstrated in mice, with a morphology very similar to fibroblasts and susceptible to steroidogenic *in vitro* differentiation, when induced by luteinizing hormone (LH) [2]; (2) they differentiate from two specific embryonic progenitor cells: *Wt1*$^{+}$ and *Gli1*$^{+}$ cells, which acquire theca lineage markers in response to

Abbreviations: TSC, the ca stem cells; LH, luteinizing hormone; CYP11A1, cytochrome P450 11A1; CYP17A1, cytochrome P450 17A1; StAR, steroidogenic acute regulatory protein; GSTA1, glutathione S-transferase A1; APOA1, apolipoprotein AI; FDX1, ferredoxin 1; C4BPB, c4b-binding protein; INHA, enoyl acyl carrier protein reductase; NME2, nucleoside diphosphate kinase 2; APOD, apolipoprotein D; APOC1, apolipoprotein C1; MEST, mesoderm specific transcript; WFDC1, WAP four-disulfide core domain protein 1; MATN2, matrilin-2; PTCH1, protein patched homolog 1; ACTA2, actin alpha 2; ADAMDEC1, ADAM-like decysin 1; CLD11, claudin-11; LOX, lysyl oxidase; OMD, osteoadherin; PDGFRα, platelet-derived growth factor receptor alpha; HSD3B1, hydroxy-delta-5-steroid dehydrogenase 3 beta 1; INSL3, insulin-like 3; LHCGR, luteinizing hormone/choriogonadotropin receptor; ALDH1A1, aldehyde dehydrogenase 1 family member a1; OGN, osteoglycin; POSTN, periostin; ASPN, asporin; TCF21, transcription factor 21; COL1A2, collagen type I alpha 2 chain; POI, premature/ primary ovarian insufficiency; PCOS, polycystic ovary syndrome; INSL3, insulin-like factor 3.

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specific paracrine signals [3,4]. Nevertheless, differentiated theca cells are not present in the ovary before folliculogenesis starts by puberty, when they are present surrounding follicles with two or more layers of granulosa cells [5,6].

In addition to committing to a complete understanding of ovarian physiology, the fact that theca cells have been "neglected" among the scientific community impairs advancements in the development of adequate systems for (1) *ex vivo* growth and development of follicles, (2) *in vitro* drug tests and evaluation of toxic compounds impact, and (3) development of an engineered ovary to restore fertility in patients who still cannot benefit from the techniques currently available. Thus, this review offers an overview of the literature on the established concepts and most recent studies about theca cells, regarding its characterization and origins, *in vitro* culture applications either as models for fertility preservation or pharmacological/toxicological studies, hormonal production aiming at new cellular therapies against ovarian failure, and its importance in unraveling pathogenic pathways.

2. Theca cell origin and characterization

The ovary, like many other organs, comprises two main compartments: the parenchyma, containing the functional or specialized tissue (follicles) that performs the gonad function, and the stroma, a supporting structure containing many other cell types that, by definition, are not ovarian follicles [7]. Embryologically, ovarian compartments have different origins: (1) oocytes migrate from the yolk sac to the primitive gonad crests by the 6th week of embryonic development; (2) stromal cells differentiate from the mesonephros' epithelial and mesenchymal cells, establishing the primitive sex cords. In the female primitive gonad, the surrounding epithelium, which differentiates from the peritoneal mesothelium, internalizes and encloses the primordial germ cells, establishing the follicular structures [8]. These, however, do not proliferate after birth, as they are activated during puberty. Throughout reproductive life, these follicles are recruited to allow continuous hormonal secretion and production of mature (MII) oocytes. For that, the follicle grows and differentiates, changing the oocyte, and granulosa cells and assimilating a new layer: the theca.

The follicular granulosa compound has, therefore, epithelial origin (cubic cells with a basal lamina delimiting the follicular surroundings). The theca cells, contrarily, have a mesenchymal origin.

Postnatally, among a population of incompletely characterized cells present in the stroma, it is generally agreed that some differentiate into theca cells, at least in part [9], by the moment primary follicles differentiate to the secondary stage of development. The term "theca interstitial cells" has been proposed to define these precursor cell populations [10]. Follicles in the advanced secondary stage show two distinct theca layers: theca externa, a well-vascularized layer comprising fibroblasts, blood vessels and muscle-like cells continuous to the ovarian stroma, and theca interna, a steroidogenic cell population [1]. The theca interna is essential to produce major endocrine regulatory factors, such as androgens, acting as substrates for the conversion to estrogens in granulosa cells [11]. The theca externa behaves rather as connective tissue and, therefore, serves as a structural component. In short, the theca interna and externa layers surrounding antral follicles (Fig. 1) have a crucial role in transporting nutrients and oxygen to the granulosa cells and the oocyte, as well as for the structural integrity of the follicle. Following ovulation of the oocyte of a large antral follicle, theca cells fulfill a role in the corpus luteum, which is formed after luteinization. This process is induced by a luteinizing hormone peak that activates signal-transduction pathways in granulosa and theca cells [12]. The corpus luteum has different cell phenotypes: large steroidogenic cells, derived from granulosa cells, and small steroidogenic cells, derived from the theca interna layer. As shown by our group, the latter can be stained with CYP17A1, also expressed in theca interna cells (Figs. 2 and 3). The granulosa-lutein cells perform a more remarkable progesterone synthesis and maintain estradiol secretion after ovulation. The theca-lutein cells, however, show a greater steroid production in the presence of human chorionic gonadotropin. These cells are the source of androgen precursors aromatized by granulosa-lutein cells, analogous to the mechanism of follicular steroidogenesis [13].

2.1. Isolation, in vitro culture and differentiation of theca stem and precursor cells

Characterizing theca cells is essential for investigating their normal function and is a solid base for understanding and solving various diseases related to that particular cell type. The ability to isolate the cells for their characterization is thus required.

One source for theca cell isolation in adult women is the follicular fluid from patients undergoing oocyte pick-up. Whereas, in this

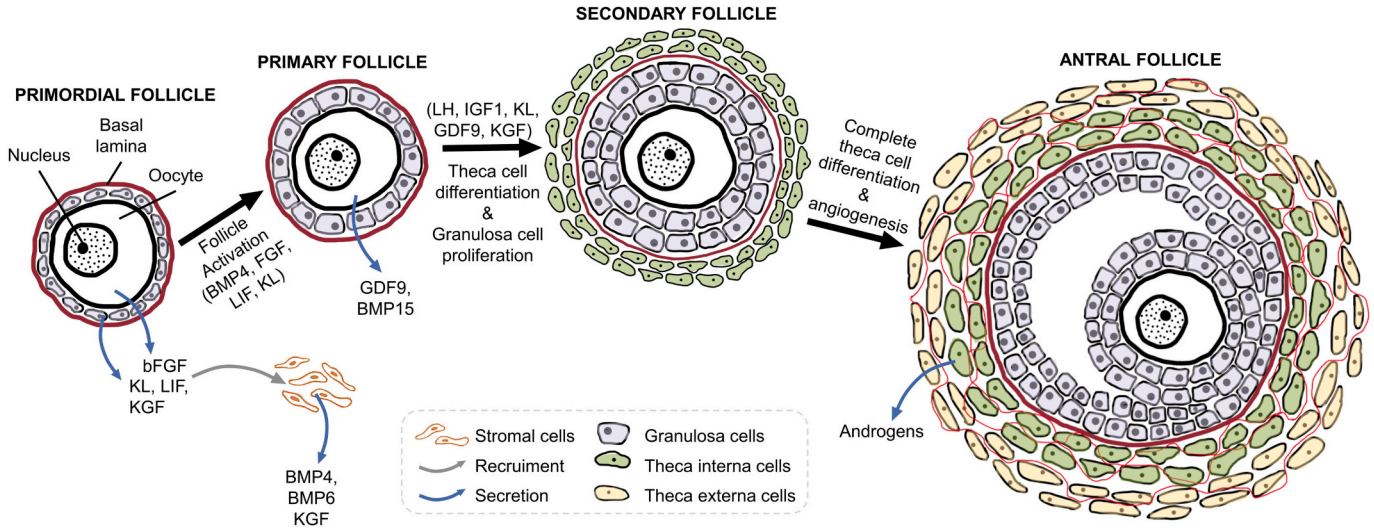

Fig. 1. Schematic representation of recruitment and differentiation of theca cells in mammals. Evidence suggests that TSCs reside in the ovarian stroma and are recruited by factors released from follicles after they are activated. Individual factors may be responsible for recruiting these cells, whereas others may promote differentiation and proliferation. bFGF: basic fibroblast growth factor; BMP4: bone morphogenetic protein 4; BMP6: bone morphogenetic protein 6; BMP15: bone morphogenetic protein 15; FGF: fibroblast growth factor; GDF-9: growth differentiation factor-9; IGF: insulin-like growth factor; LIF: leukemia inhibitory factor; KGF: keratinocyte growth factor; KL: kit ligand.

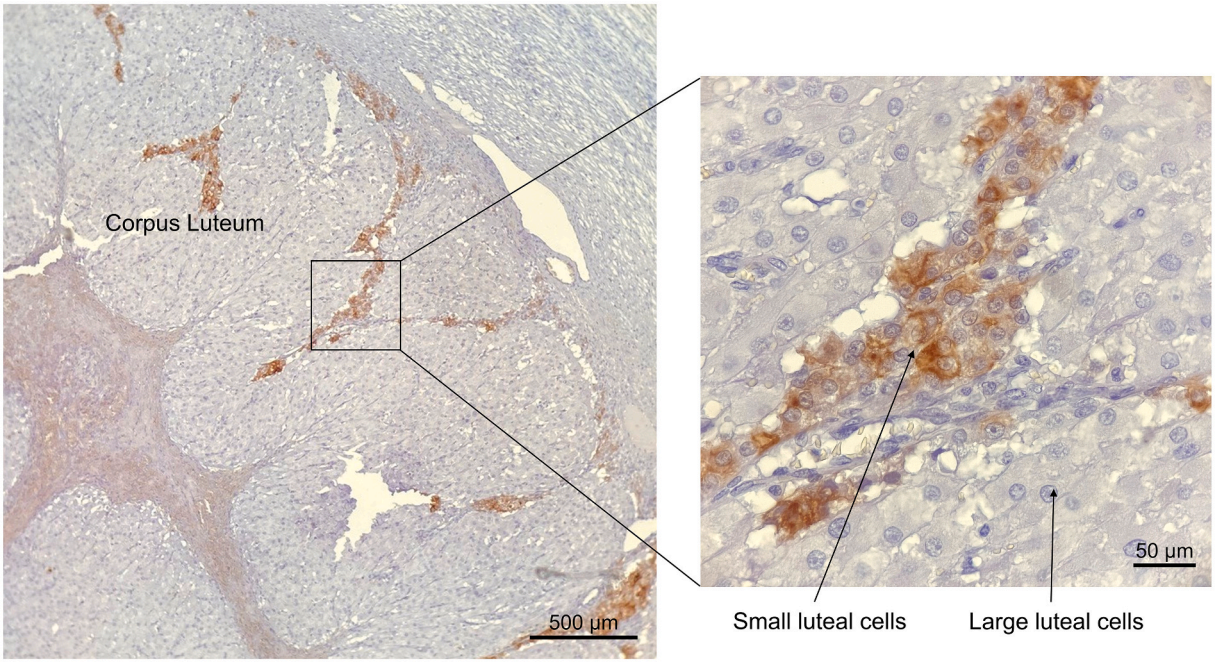

Fig. 2. CYP17A1 immunohistochemistry staining as a specific marker for the small luteal cells in the corpus luteum in the human ovary. Nuclei are counterstained with hematoxylin. The image and its enlargement have a scale bar of 500 µm and 50 µm respectively (Vlieghe et al., unpublished data).

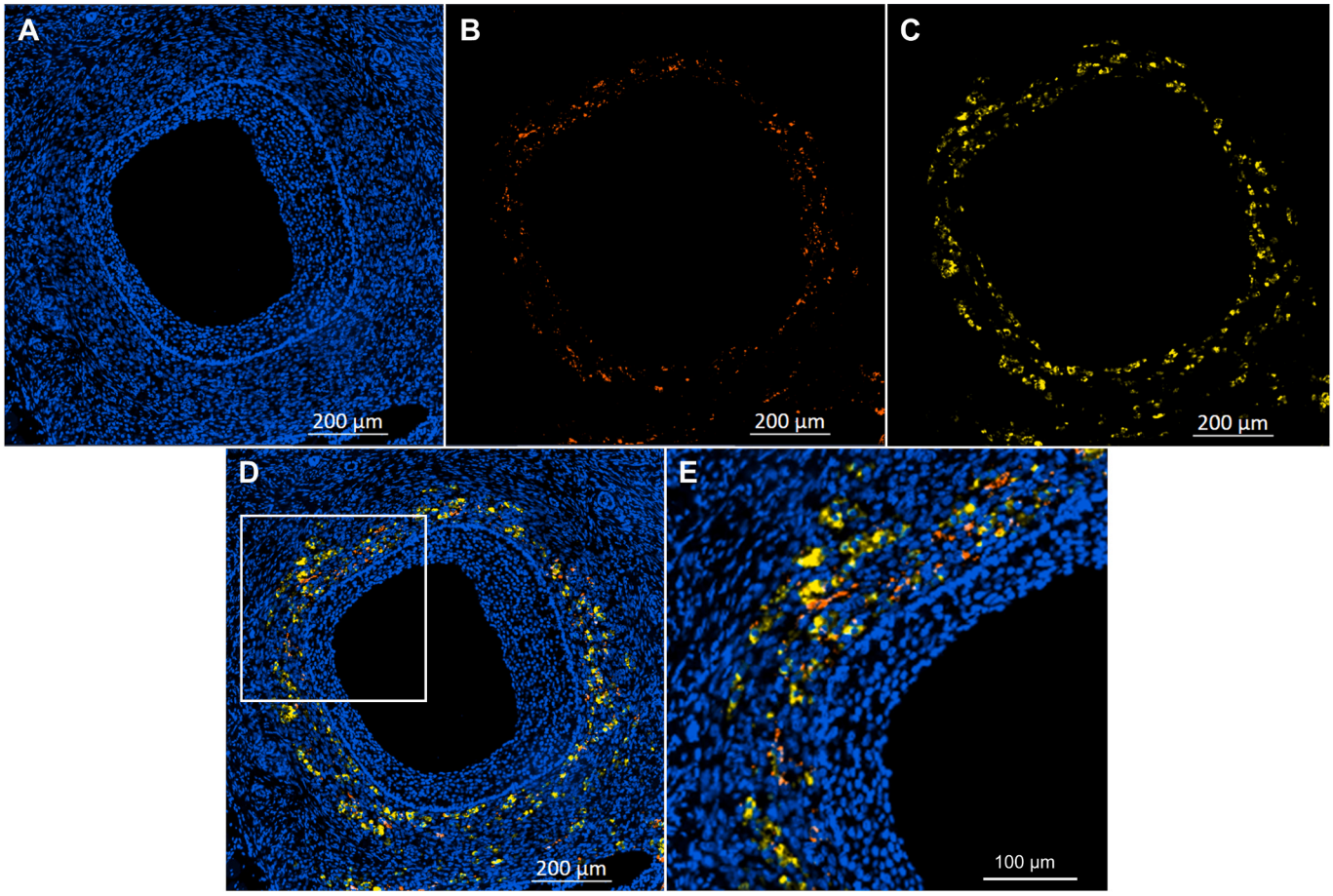

Fig. 3. Theca interna evidenced by CD13 staining as a specific marker for these cells. Fluorescence microscopy of DAPI (A), CD13 (B), and CYP17A1 (C) in an antral follicle from paraffin sections of human ovarian tissue, as well as the merged images (D) and the enlargement of the theca layer (white square in D) (E). Images (A-D) have a scale bar of 1:200 µm, and image (E) has a scale bar of 1:100 µm (Vlieghe et al., unpublished data).

procedure, not only theca cells but granulosa, epithelial, blood, and endothelial cells are harvested too. In this case, researchers have shown the feasibility of separating granulosa cells from the follicular fluid using Ficoll, Percoll and red blood cell lysing buffer methods as well as flow cytometry to select the leukocyte marker CD45 [14,15]. Focusing on the isolation of theca cells, our group applied a similar technique, replacing CD45 with a theca-specific marker [16]. Such surface markers are not well established yet for theca interna cells, but CD13 appears to be the most promising candidate (this will be discussed in further detail on the topic of specific markers of theca stem and progenitor cells). However, CD13 alone cannot assure pure isolation of theca cells. Therefore, setting up an *in vitro* culture is usually a convenient starting point for purifying or differentiating a specific cell population.

In human adult ovaries, Dalman et al. [17] showed the isolation of theca stem cells (TSCs) directly from small antral follicles easily located on the surface of the gonad. Briefly, human antral follicles measuring around 3–5 mm in diameter were punctured. After removal of the oocyte and granulosa cells, the remaining layer of theca cells was transferred into growth factor-supplemented culture media following enzymatic digestion into a single cell suspension. The authors described that such TSCs can differentiate into mesenchymal lineages (osteogenic, adipogenic, and chondrogenic) and theca progenitor cells *in vitro* [17].

Another option for isolating theca precursor cells directly from the ovary involves using ovaries from postmenopausal women. Indeed, our group has succeeded in laying down well-defined conditions for the isolation, differentiation, and culture of human theca interna cells from postmenopausal ovarian cells *in vitro*, allowing further research to be carried out [16].

The *in vitro* differentiation of hormone-producing theca interna cells was likewise shown in different species. In newborn mice, Honda et al. [18] enzymatically isolated theca stem cells from entire ovaries. On the other hand, Chen et al. [19] developed an enzyme-free method, starting from only a small ovarian biopsy of adult non-human primates. They cut the biopsy into small pieces and purified its cells using *in vitro* culture with an appropriate mix of hormones and growth factors to favor theca stem cells. The cells obtained after this culture are capable of self-renewal and differentiation into theca interna cells, improving follicle development and fertilization in a model of premature ovarian insufficiency (POI) upon transplantation into ovaries of non-human primates [19]. Furthermore, obtaining differentiated theca cells through an *in vitro* culture was shown in pigs [20,21], sheep [22] and cows [23].

2.2. Specific markers of differentiated theca cells

Along with the appearance of single-cell RNA sequencing analysis, two research groups succeeded in jointly elucidating the complete cell map of the human ovary of reproductive-aged women. These data allowed, for instance, the comparison of cells in the cortical [24] and medullary layers [25] of the ovary. Similar clusters of somatic cell types were found in both compartments. In addition, the data enabled the identification of all the different cell types in the ovary. These cells include theca cells, from the internal and external layers, as well as their potential progenitor cells, each with its specific set of markers (Table 1).

Although this map is critical due to its human origin, exciting data were also obtained from studies in mono-ovulatory species ovaries, such as cows and non-human primates. These may be an excellent reference for further description and identification of human theca cells, as these animals share high similarities with humans regarding folliculogenesis [26].

In bovine studies, a gene expression analysis showed that theca and granulosa cells are 92–95 % correlated. Genes exhibiting differential expression in theca cells are ACTA2, ADAM-like, decysin 1 (ADAMDEC1), claudin-11 (CLD11), lysyl oxidase (LOX), osteoadherin (OMD), platelet-derived growth factor receptor alpha (PDGFRα) [27], cytochrome P450 Family 11 Subfamily A Member 1 (CYP11A1), hydroxy-delta-5-steroid dehydrogenase, 3 beta 1 (HSD3B1), insulin-like 3

Table 1
Specific markers expressed in different types of theca cells, such as theca interna and externa cells, theca lutein cells and theca stem and progenitor cells.

Marker	Cell type specificity	Localization	Paper
Integrin subunit beta 1 (ITGB1 or CD29)	Theca stem cells	Membrane	[17]
CD44 molecule (Indian blood group) (CD44)	Theca stem cells	Membrane	[17]
5′-nucleotidase ecto (NT5E or CD73)	Theca stem cells	Membrane	[17]
Thy-1 cell surface antigen (CD90 or THY1)	Theca stem cells	Membrane	[17]
Endoglin (CD105 or ENG)	Theca stem cells	Membrane	[17]
NME/NM23 nucleoside diphosphate kinase 2 (NME2)	Theca progenitor cells	Cytosol, membrane	[25]
Apolipoprotein D (APOD)	Theca progenitor cells	Membrane, Secretion	[25]
Apolipoprotein C1 (APOC1)	Theca progenitor cells	Secretion	[25]
Mesoderm specific transcript (MEST)	Theca progenitor cells	Golgi apparatus, cytosol	[25]
WAP four-disulfide core domain 1 (WFDC1)	Theca progenitor cells	Secretion	[25]
Matrilin 2 (MATN2)	Theca progenitor cells	Secretion	[25]
Patched 1 (PTCH1)	Theca progenitor cells	Golgi apparatus	[25]
Actin alpha 2, smooth muscle (ACTA2)	Theca progenitor cells	Actin fillaments	[25]
Cytochrome P450 family 11 subfamily A member 1 (CYP11A1)	Theca interna cells	Mitochondria	[25]
Glutathione S-transferase alpha 1 (GSTA1)	Theca interna cells	Cytosol	[25]
Apolipoprotein A1 (APOA1)	Theca interna cells	Secretion, cytosol	[25]
Ferredoxin 1 (FDX1)	Theca interna cells	Mitochondria	[25]
Complement component 4 binding protein beta (C4BPB)	Theca interna cells	Golgi apparatus	[25]
Inhibin subunit alpha (INHA)	Theca interna cells	Golgi apparatus	[25]
Alanyl aminopeptidase, membrane (CD13 or ANPEP-N)	Theca interna cells	Membrane	[32]; [33]; [25]
Steroidogenic acute regulatory protein (StAR)	Theca interna cells	Mitochondria	[24]; [25]
Cytochrome P450 family 17 subfamily A member 1 (CYP17A1)	Theca interna cells	Endoplasmic reticulum	[24]; [25]
hormone/choriogonadotropin receptor (LHCGR)	Theca interna cells	Membrane	[25]
Insulin like 3 (INSL3)	Theca interna cells	Secretion	[25]
Perilipin 2 (PLIN2)	Theca interna cells	Lipid droplets	[25]
Collagen type I alpha 1 chain (COL1A1)	Theca externa cells	Secretion	[25]
Collagen type I alpha 2 chain (COL1A2)	Theca externa cells	Secretion	[25]
Collagen type III alpha 1 chain (COL3A1)	Theca externa cells	Secretion	[25]
Collagen type VI alpha 1 chain (COL6A1)	Theca externa cells	Secretion	[25]
Collagen type VI alpha 2 chain (COL6A2)	Theca externa cells	Secretion	[25]
NDRG family member 2 (NDRG2)		Membrane	[25]

(*continued on next page*)

Table 1 (*continued*)

Marker	Cell type specificity	Localization	Paper
	Theca externa cells		
Vacuole membrane protein 1 (VMP1)	Theca externa cells	Nucleoli, Endoplasmic reticulum	[25]
Fibroblast growth factor receptor 1 (FGFR1)	Theca externa cells	Membrane	[25]
Signal transducer and activator of transcription 3 (STAT3)	Theca externa cells	Cytosol	[25]
Collagen type VIII alpha 1 chain (COL8A1)	Theca externa cells	Secretion	[25]
Aldehyde dehydrogenase 1 family member A1 (ALDH1A1)	Theca externa cells	Cytosol	[25]
hydroxy-delta-5-steroid dehydrogenase, 3 beta 2 (HSD3B2)	theca lutein cells	Endoplasmic reticulum	[25]
Alanyl aminopeptidase, membrane (StAR)	theca lutein cells	Membrane	[25]
Cytochrome P450 family 11 subfamily A member 1 (CYP11A1)	theca lutein cells	Mitochondria	[25]
Cytochrome P450 family 17 subfamily A member 1 (CYP17A1)	theca lutein cells	Endoplasmic reticulum	[25]

(INSL3) and cytochrome P450 family 17 subfamily A member 1 (CYP17A1) [28]. Theca cells are usually clustered within the stromal cells when single-cell RNA sequencing is carried out. For this reason, another bovine study performed an RNA microarray analysis and revealed genes that were upregulated in the theca interna cells compared to the stromal cells. These genes were: insulin-like 3 (INSL3), luteinizing hormone/choriogonadotropin receptor (LHCGR), HSD3B1, CYP17A1, aldehyde dehydrogenase 1 family member a1 (ALDH1A1), osteoglycin (OGN), periostin (POSTN) and asporin (ASPN) [29]. One study looked even more closely at the details by performing transcriptome profiling of theca cells from healthy and antral atretic follicles. This revealed that the fate of theca cells from non-dominant atretic follicles is an arrest in the cell cycle rather than the occurrence of an apoptotic process [5].

In primates, a specific cluster of theca cells was not identified after a single-cell RNA sequencing analysis of the ovarian cortex and medulla. Nevertheless, a stromal cell cluster expressing transcription factor 21 (TCF21), collagen type I alpha 2 chain (COL1A2), and StAR, of which some cells, however, showed expression of theca cells markers StAR and CYP17, was identified [30].

Our group has been successfully using CD13 as a specific marker for theca interna cells (Table 1). As a zinc-dependent transmembrane ectopeptidase, CD13 or aminopeptidase N, can be expressed in hematopoietic cells and other cell types, such as fibroblasts and epithelial cells [31]. In the human ovary, this marker is shown to be specific on theca interna cells [32,33], and it is co-expressed with CYP17A1 as shown by our group (Fig. 3). As for CYP17A1, Asiabi et al. [34] showed the expression of other steroidogenic enzymes, such as CYP11A1 and StAR, in human theca interna cells.

2.3. *Specific markers of theca stem and progenitor cells*

In addition to the numerous research that envisions the characterization of theca cells, several studies are ongoing on identifying putative theca stem or progenitor cells. Cowan and Quirk [35] described the involvement of Gli1-expressing cells, identified within the mesenchyme of newborn ovaries in mice, in the development of theca layers of growing follicles. The authors still add that migration, proliferation and differentiation of such precursors are potentially coordinated by DHH and IHH secretion by granulosa cells of activated follicles.

Moreover, Dalman et al. [17] were able to obtain human theca stem cells and characterize them by the presence of the surface markers CD29, CD44, CD73, CD90, and CD105 and the absence of surface marker CD45/34 (Table1). Interestingly, a similar expression pattern was found on mesenchymal stem cells located in the placenta (CD29+, CD44+, CD73+, CD90+, CD105+ and CD34-, CD45-, HLA-DR-) [36]. However, the study of Dalman et al. [17] showed that the theca stem cells were different from bone marrow-derived mesenchymal stem cells.

In non-human primates, other markers (NESTIN, PDGFRα, and CD271), previously defined for stem Leydig cells [37–39], were used when isolating theca stem cells. Since theca and Leydig cells exhibit a similar expression pattern [9,40], these markers could also be used for theca stem cell characterization [19]. Nevertheless, their expression was not investigated in human theca stem cells yet. However, PDGFRα was shown to be differentially expressed by theca cells in a bovine study [27] and was found in the map of the human ovary as an expression marker in the stromal cell cluster [24]. Additional markers of a potential theca progenitor cell cluster were identified after single-cell RNA sequencing (Table 1) [25].

3. *In vitro* applications of theca cells

The gap in knowledge regarding the origins of theca cells has led the scientific community to develop *in vitro* systems to culture potential stem and differentiated theca cells. Designing a multistep technique for the complete *in vitro* development of female germ cells has been a challenge in humans and a crucial achievement in rodents [41,42]. In this sense, the use of theca cells in co-culture during oocyte growth allowed maturation, oocyte development, and blastocyst rates similar to growth factors-supplemented culture [43]. When applied as a feeder layer together with macrophages, theca cells improved the survival rates and *in vitro* growth of early murine follicles [10].

Co-culturing isolated mice follicles in a collagen gel matrix along with interstitial ovarian cells led to the formation of theca cell layers in its outermost part. In addition, such follicles showed higher growth rates than those cultured without interstitial cell co-culture [44]. The mentioned studies provide evidence of the potential source of theca cell differentiation: ovarian stromal/interstitial cells. They also confirm the importance of developing theca cell isolation and *in vitro* culture systems to allow further advancements in the ability to grow follicles in an *ex vivo* manner.

The culture of theca cells to develop a source of androgens has been investigated. One application of such experiments is to create an *in vitro* environment to test drugs or evaluate the impacts of specific compounds. For example, lipopolysaccharides produced from gram-negative bacteria infections inhibited steroid production in bovine theca cells [45]. Still, the use of melatonin in bovine theca cell culture showed that this hormone inhibits steroidogenesis and stimulates cell proliferation [46]. On the contrary, fibroblast growth factor 9 (FGF9) was recognized as a promoter of bovine theca cell proliferation and a suppressor of differentiation through the downregulation of steroidogenic genes such as *StAR* and *IGF2* [47].

Some *in vitro* studies on the ability of theca cell differentiation have uncovered their potential to give rise to other cell types. The study performed by Dalman et al. [17] demonstrated that human TSCs differentiate into adipocyte-, osteocyte-, and chondrocyte-like cells, confirming their plasticity and ability to redesign mesenchymal lineages, as well as theca progenitor cells. In sheep ovaries, TSCs were mechanically isolated from ovarian follicles and compared to mesenchymal stem cells, fibroblasts and embryonic pluripotent cells regarding their ability to differentiate into osteocyte-, adipocyte- and oocyte-like cells, confirming that such mesenchymal lineages have a potential to be differentiated *in vitro* from TSCs [22]. Swine theca-derived multipotent stem cells express mesenchymal cell surface markers (CD29, CD44 and CD90) [20,21], suggesting an *in vitro* differentiation capacity. This ability was confirmed by sequential changes in cells morphology and differentiation into oocyte-like cells, according to the expression of

GDF9B, C-MOS, DAZL, VASA, ZPC, SCP3 and STELLA [20,21].

Since folliculogenesis is a process where germ cells and two types of somatic cells interact *via* a paracrine mechanism, co-culture of theca and granulosa cells has proven its value in developing systems that elucidate follicular cell interactions. In mice, Honda et al. [18] isolated putative theca stem cells from neonatal mice ovaries. Such cells showed the ability to self-renew and differentiate into early precursors and steroidogenic cells (*in vitro* production of androstenedione). However, their differentiated morphology (oval shape, cytoplasmic lipid droplets and great availability of smooth endoplasmic reticulum) was only reached when co-culture with granulosa cells was performed [18]. The presence of granulosa cells within bovine ovarian stromal cells increased the incidence of lipid droplets and mitochondria, inducing androstenedione production in stromal cells, suggesting that granulosa cells induce functional differentiation and acquisition of LH responsiveness [23]. Goat ovarian cortical, medullary stromal and theca cells increased *in vitro* granulosa cells biosynthesis' of estradiol and progesterone [48]. In a similar study from the same group, the addition of granulosa cells from small antral follicles to the *in vitro* culture of theca cells increased levels of theca steroidogenesis, LH responsiveness, and *bcl-2* gene expression along with decreased apoptotic markers expression [49].

The co-culture system also implicates changes in the structure of cells and the system's morphology. According to Tajima et al. [50] bovine theca cells cultured alone are thin, flat and spindle-shaped, while in co-culture with granulosa cells, they appear more convex and densely packed, showing higher levels of steroid secretion.

Regarding the steroidogenic abilities, co-culture of theca and granulosa cells showed higher rates of testosterone and androstenedione in the spent medium than theca cells alone [51]. Also, increased mRNA expressions of steroidogenic enzymes (StAR, CYP11A1, CYP17A1, AND HSD3B2) in the co-cultured theca cells were observed [51].

3.1. Theca cells during cell death and proliferation

The role of theca cells in the control of apoptosis and proliferation of granulosa cells during bovine ovarian follicular development was studied by Tajima et al. [52]. In a collagen membrane co-culture system, they demonstrated that even though theca cells did not affect the proliferation of granulosa cells obtained from either small or large follicles, they regulated the fate of these cells throughout the follicular maturation process by secreting factors that suppress apoptosis [52].

In vitro culture of human ovarian cells has clarified some aspects of theca cell differentiation, their source and possibilities to be obtained *in vitro*. Asiabi et al. [16] isolated cells from the cortical layer of postmenopausal human ovaries, identifying a population of theca steroidogenic cells. The addition of different growth factors and companion granulosa cells in the culture system led to theca cell differentiation, according to the mRNA levels of LH receptor, StAR, CYP17A1, HSD3B1 and HSD3B2 [16]. This study was an essential step toward our knowledge of theca cell ontogenesis in the human ovary and contributes to developing *in vitro* systems for drug screening and understanding ovarian pathologies.

4. Theca cells used in POI and polycystic ovarian syndrome (PCOS)

As described in the previous sections, research on the origin of theca cells and their potential applications is finally emerging. Such applications could potentially aid patients suffering from ovarian pathologies. Two well-known conditions involving dysfunctional behavior of theca cells are POI and PCOS. In these cases, repairing or replacing theca cells could thus possibly offer treatment for the diseases. While exploring this possibility, the use of putative TSCs as a feasible therapy has also been investigated. In this section, we will thus shed light on the mechanisms causing POI and PCOS and the possible theca cell-related treatments for these conditions.

4.1. POI

POI is a condition leading to infertility in women before the age of 40. It has multiple causes, such as spontaneous causes (autoimmune, genetic or chromosomal) and chemo- or radiotherapy-related causes [53]. Generally, it results in a dysfunctional response of the follicles to gonadotropins, which is manifested by the low production of steroid hormones such as androstenedione, estrogen, and progesterone. Due to the known decrease of androgens, a dysfunction in theca cells was suggested to be the main cause of POI, although this proposal awaits confirmation [6,54]. Recently, more evidence was provided for the role of theca cells in POI. INSL3, produced primarily by theca cells in the ovary, is deficient in POI patients [55]. The currently existing treatment aims to normalize hormone levels. However, no or little benefits to improving fertility has been shown thus far [56]. Hence, studies have examined the ability of stem cells to restore ovarian function. Although different types of stem cells were used in animal models for this purpose [57], cell replacement therapy did not lead to the successful restoration of reproductive outcomes [58]. Therefore, it could be suggested that using resident ovarian stem cells might lead to a better result. For this reason, TSCs are the latest cell type investigated in the search for a treatment for POI. The use of TSCs to restore fertility was tested for the first time by autologous transplantation in mice. After transplantation, the TSCs could be differentiated into theca cells and layered around the basal lamina of the follicles, thereby showing their efficacy [18]. Recently, the autologous transplantation of TSCs was repeated in a non-human primate. This study also showed the differentiation capacity of these cells [19]. Moreover, after the fertilization of mature oocytes obtained from the TSC-treated ovary, the authors reported an increased fertilization and blastocyst formation rate. However, when transplanted and traced *in vivo*, these TSCs maintained their function for only three months [19]. Thus, theca cell therapy has the potential to improve the quality of follicles, ameliorating steroidogenesis in POI patients, but with a limited influence on long-term fertility preservation. What does remain to be investigated is the chromosomal constitution of the oocytes, as well as the possibility of implantation and progression of the pregnancy.

4.2. PCOS

Unlike POI, an excess of androgens or hyperandrogenism is the main feature in PCOS patients [59]. Hyperandrogenism in PCOS women causes increased LH levels and dysfunction of granulosa cells and is often accompanied by insulin resistance. Consequently, the mechanism responsible for the selection of one dominant follicle is disrupted, as well as ovulation, which will remain absent [60,61].

The exact cause of PCOS is still not clarified and can be related to many different factors. However, as in POI, the possibility that theca cells play a key role in the development of the condition was also demonstrated in PCOS. Using an *in vitro* culture system of PCOS theca cells, researchers found that steroidogenesis was increased in these cultured cells and that this increase was related to an augmentation in CYP17A1 and CYP11A1 [62]. Augmented CYP17A1 was also detected in the ovaries of PCOS women [63]. Recently, the multifunctional protein SET, involved in the control of cell apoptosis and cell cycle regulation, was found to be highly expressed in PCOS patients [64]. SET is commonly expressed in theca cells, and such expression is augmented in PCOS patients, being directly related to high rates of oocyte maturation and inhibited oocyte apoptosis, providing further clues on the pathologic pathway leading to PCOS understanding [64].

A prospective clue that PCOS is a polygenic disorder susceptible to modifications by epigenetic and environmental factors has been raised by genome-wide association studies [65]. As an example, Waterbury et al. [66] found an inverse relationship between ZNF217 mRNA and theca cell androgen synthesis, suggesting that *ZNF217* is part of the network of PCOS candidate genes, together with *DENND1A, LHCGR,*

YAP1, INSR, and others [65,67,68], regulating theca cell androgen production in patients with this condition.

As a definite cure for PCOS does not yet exist, current treatments aim to suppress the symptoms brought on by this condition. Often, contraceptive pills are recommended to normalize the menstrual cycle and the androgen levels. However, this is not suitable for women with a pregnancy wish. A lifestyle change is generally advised as the preferred treatment, which is highly recommended for PCOS patients with a pregnancy wish. This includes, for example, a special diet and frequent exercise in overweight patients, which reduces PCOS symptoms [69].

In searching for treatments for PCOS, Cadagan et al. [70] showed that the combination of insulin and LH treatment in both PCOS theca cells and non-PCOS theca cells could augment the expression of CYP17 as well as the expression of androgens. In addition, it was discovered that inhibition of the PI3K pathway in human PCOS theca cells decreased the expression of CYP17A1, suggesting a crucial role of this pathway and theca cells for the future treatment of PCOS [71]. Whether it is applicable to use transplantation of TSCs as an alternative treatment therapy remains to be investigated.

4.3. Limitations

While autologous TSC transplantation can potentially be an approach to improve fertility in POI and PCOS patients, it may have some limitations, such as the number of cells that can be produced from small biopsies of ovarian tissue. To surpass this obstacle, one can try the protocol for isolation and *in vitro* culture of TSCs developed by Chen et al. [19], which allows cells to exponentially proliferate while maintaining their stemness characteristics. Furthermore, while TSC may improve androgen-related symptoms in POI patients, symptoms associated with the decrease in estrogen remain unresolved. Depending on the exact cause of POI, the use of these cells should be carefully evaluated. In the most severe case of POI, when no follicles remain, TSC transplantation cannot be used.

Another alternative is the transplantation of theca cells differentiated *in vitro* [34]. However, it is possible that, similar to primary theca cells [72], *in vitro* differentiated cells rapidly lose their LH receptors during culture. Moreover, as these long-term cell cultures are not immortalized, the potential occurrence of cell senescence must be considered [73].

5. Theca cells in regenerative medicine

5.1. Transplantable engineered ovary

One future application of theca cells encompasses their function in the transplantable engineered ovary (TEO). This promising technique will hence be briefly discussed here. TEO is a potential fertility treatment, particularly for patients suffering from cancers contaminating the ovary [74]. Existing cancer treatments bear the risk of causing infertility. Therefore, several options have emerged in recent years to help these patients resume their fertility after recovery [75]. Yet, these techniques fall short in some particular cases. There is no applicable fertility preservation possibility for prepubertal girls who risk having malignant cells in the ovary and need urgent chemo- and/or radiotherapy. This is due to the risk of reintroducing the malignant cells in the patient when ovarian tissue is transplanted. Therefore, the TEO aims to provide a suitable fertility restoration option to these patients. An engineered ovary would consist of (1) preantral follicles isolated from a surgically removed ovary before cancer treatment, and (2) ovarian somatic cells isolated from a biopsy of the remaining ovary after cancer treatment, (3) encapsulated in a hydrogel that recreates the structural role of the ovary [76]. The follicles used in the TEO are in the primordial and primary stages since these can be found in considerable numbers in the ovarian cortex and have the highest survival rate after cryopreservation. These follicles correspond to more than 90 % of the follicular population in the ovary and are less vulnerable to cryopreservation-induced damage [77]. However, in this early stage of development, theca cells are not present yet. Therefore, theca cells must arise from ovarian cells under the influence of growing follicles.

Follicle isolation has been fine-tuned over the last few years, mainly by our group [78,79]. On the contrary, developing a suitable matrix that can sustain successful folliculogenesis remains challenging [80]. Our group has been focusing on the constitution of the natural polymer PEGylated fibrin, composed of PEG, fibrin and thrombin. This polymer could support the survival of human ovarian cells [81] and *in vitro* growth of preantral follicles [82]. It now remains to be tested for its follicle growth capacity, during which also the differentiation of theca cells takes place. If the theca cells cannot be recruited appropriately, they cannot fulfill their role in folliculogenesis. Moreover, it is essential that this recruitment can occur naturally and can be repeated throughout the woman's reproductive period. The latter condition was not met in the case of autologous transplantation of theca stem cells in POI monkeys, where the authors reported that theca stem cells could only remain in the ovary for up to three months [19].

5.2. Cell-based hormone replacement therapy

The control of symptoms caused by ovarian function loss has been primarily based on pharmacological hormone therapies, which usually consists of estrogen alone or in association with progesterone. However, such therapies have been shown to induce some detrimental side effects, such as stroke and cancer [83,84]. Thus, alternative treatments for ovarian failure have also been based on the development of cell-based methods. This has been accepted as a potential technique for controlling, among others, vasomotor symptoms, increased body weight, and osteoporosis outcomes of ovarian insufficiency.

The development of hormone-secreting effective granulosa and theca cell devices was first described by Liu et al. [85]. In their study, the authors isolated granulosa and theca cells from mice ovaries and enclosed them in microcapsules (Fig. 4). The devices were transplanted to ovariectomized mice, in which estradiol and progesterone physiological levels were detected for 60 days [85].

A transplantable multi-compartment biomimetic ovary has been recently proposed [86]. In this study, the authors described stable secretion of hormones in rats during 90 days of study, after implantation of devices containing encapsulated granulosa cells in an inner compartment surrounded by theca cells (Fig. 5), in which estradiol and progesterone were synthesized. More recently, the same group included bone marrow-derived stem cells in the constructs, which led to enhanced and sustained levels of ovarian steroids in the rat model [87]. Still, a layer-by-layer form of follicular spheroids connected to a microchannel network hydrogel recovered endocrine function in an ovariectomized mouse model [88].

Despite the fact that cell-based transplantable devices have not yet been approved for clinical use in humans, this is a promising technique with the potential to assist patients who do not adapt to conventional pharmacological treatments. Meanwhile, the best methods for developing the most adapted devices that impair immune reactions and allow ideal hormonal release levels are to be developed.

6. Perspectives

The process of folliculogenesis involves a dynamic process in which theca cells exert a pivotal role. Unraveling the origins of their *in vivo* differentiation process in the adult ovary will assist the development of hormonal replacement therapies, reestablish fertility and improve treatments for diseases like POI and PCOS, which seem to be directly influenced by theca cells. Such studies would also benefit from establishing a theca cell line, which, contrarily to granulosa cells, is not yet available. Thus, the only way to perform *in vitro* studies within this cell type is by isolation and primary culture, which impairs experimental autonomy, reliability, and repeatability. In this sense, if human

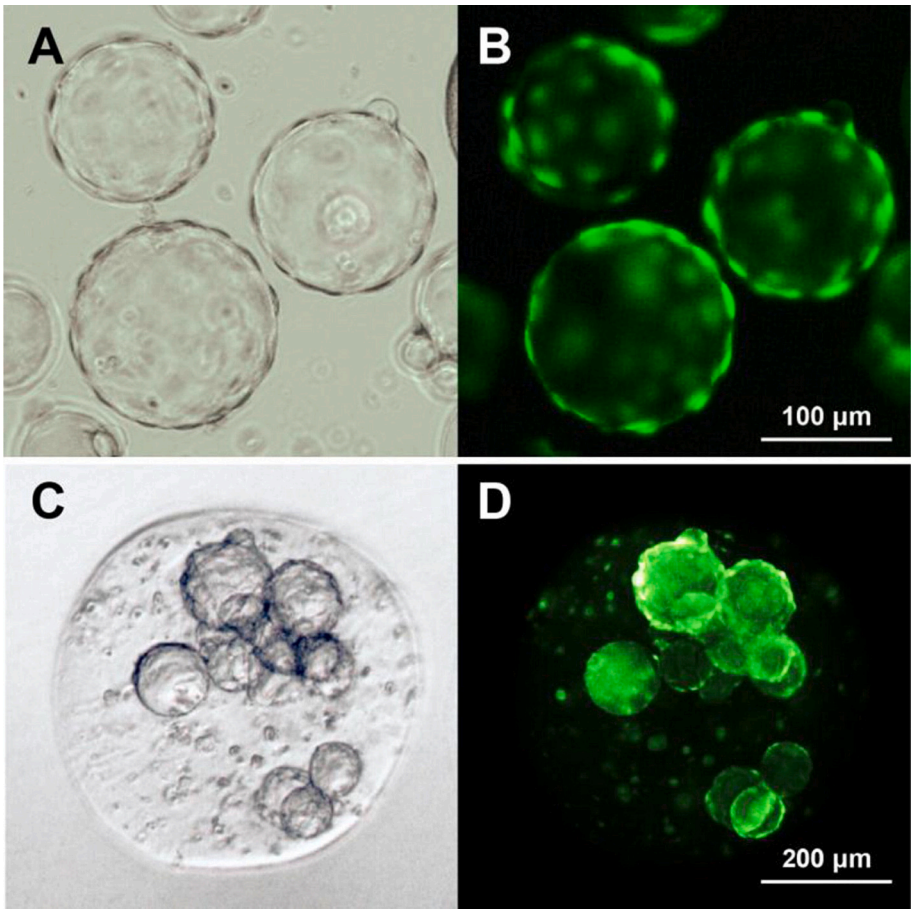

Fig. 4. Granulosa cells growing on microcarriers (A and B) and microencapsulated with theca cells (C and D) observed under light and fluorescence microscope after CFDA SE staining. Reprinted with permission from Liu et al. [85] © Elsevier (2022).

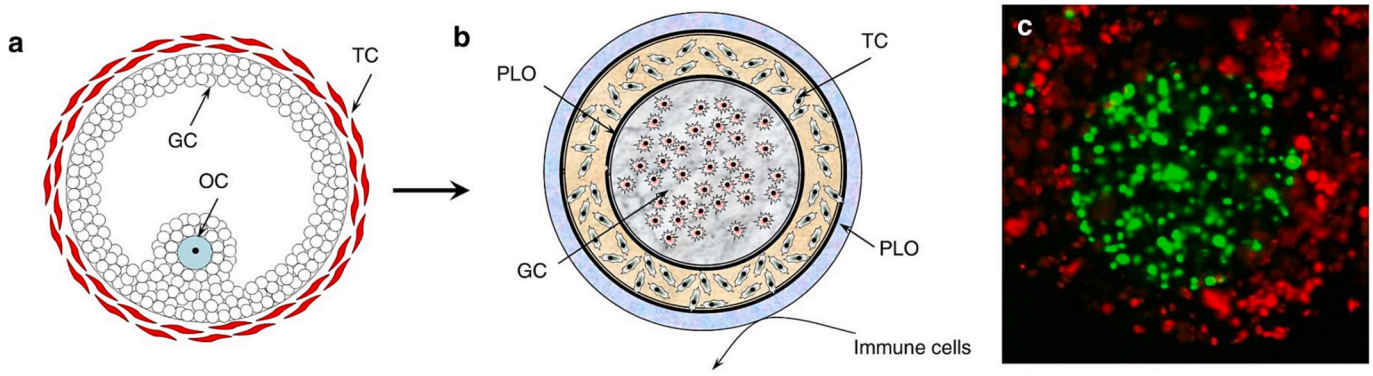

Fig. 5. Development of 3D encapsulated ovarian constructs. Schematic diagram of a native ovarian follicle (a) compared to the bioengineered ovarian construct (b). 3D-confocal images of the bioengineered ovarian construct (c), demonstrating the compartmentalization of different cells within the constructs as determined through the use of CellTracker green-labeled cells (granulosa) in the inner layer and CellTracker orange-labeled cells (theca) in the outer layer. Reprinted from Sittadjody et al. [86] with permission from the Creative Commons (www.creativecommons.org).

granulosa [89] and granulosa-lutein [90] immortalized cell lines have been ideally established, this is still a gap that impairs further advancements in the understanding of theca cells development and functionality.

CRediT authorship contribution statement

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Methodology: HV, ECRL, PA, CAA.
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No data was used for the research described in the article.

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