Chapter 6
Working Hypothesis and Challenges

Follicle rupture and CL formation can be compared with the breakdown of a skyscraper and its subsequent reconstruction with new architecture. Thus, an ingenious machinery is at work to manage a controlled tissue damage and its recovery for the next ovarian cycle. The INIM arises on the backs of the endocrine system. We suggest that the breakdown process is triggered by oxidative stress from the follicles inside, thus the signaling cascade goes from the granulosa towards the theca (Fig. 6.1). The process starts with ROS as by-products of steroidogenesis (Derouet-Humbert et al. 2005; Hanukoglu 2006; Yacobi et al. 2007), which is at its maximum in preovulatory follicles under the LH pulse. ROS is released into the follicular antrum and oxidizes nLDL to oxLDL. The oxLDL-dependent activation of LOX-1 in CK^{-} granulosa cells adds more ROS by a vicious internal feedback (Dandapat et al. 2007; Mehta et al. 2006). The preovulatory follicle turns into a structure under oxidative stress, which is in line with the findings and conclusions by others (Agarwal et al. 2003, 2005). At the culminating point, sensitive granulosa cells become damaged and release alarmins (Bianchi 2007; Rock et al. 2010). In particular, the blebs of apoptotic cells are reported to be enriched with oxidized lipids (Hartvigsen et al. 2009), which are recognized by PRRs. Our working hypothesis has a solid background, because LOX-1 and TLR4, which are receptors for oxLDL, have been found in human granulosa cells and oxLDL in the follicle fluid (Bausenwein et al. 2010; Duerrschmidt et al. 2006; Serke et al. 2009). In addition, 20–50% of dead cells were counted in fresh follicle harvests (Vilser et al. 2010). The alarmins from damaged cells could then mediate the release of soluble complement factors (C1q, C3a, C5a) as rapid danger sensors and transmitters of oxidative stress: the factors interact with specific complement membrane receptors (Köhl 2006a, b). Early outside signaling (from granulosa towards theca) within 12 h preferentially leads to the recruitment of neutrophils and macrophages, and to the breakdown of the follicle wall and oocyte expulsion (Fig. 3.3; Brännström and Enskog 2002). Early signaling is associated with IL-1β, IL-6 and TNF-α release into the follicular fluid similar to the cytokine profile from spleen cells in a thermal injury mouse model (Brännström et al. 1994a; Paterson et al. 2003). Late inside signaling (from theca towards granulosa after oocyte release) within another 12 h commands tissue remodeling

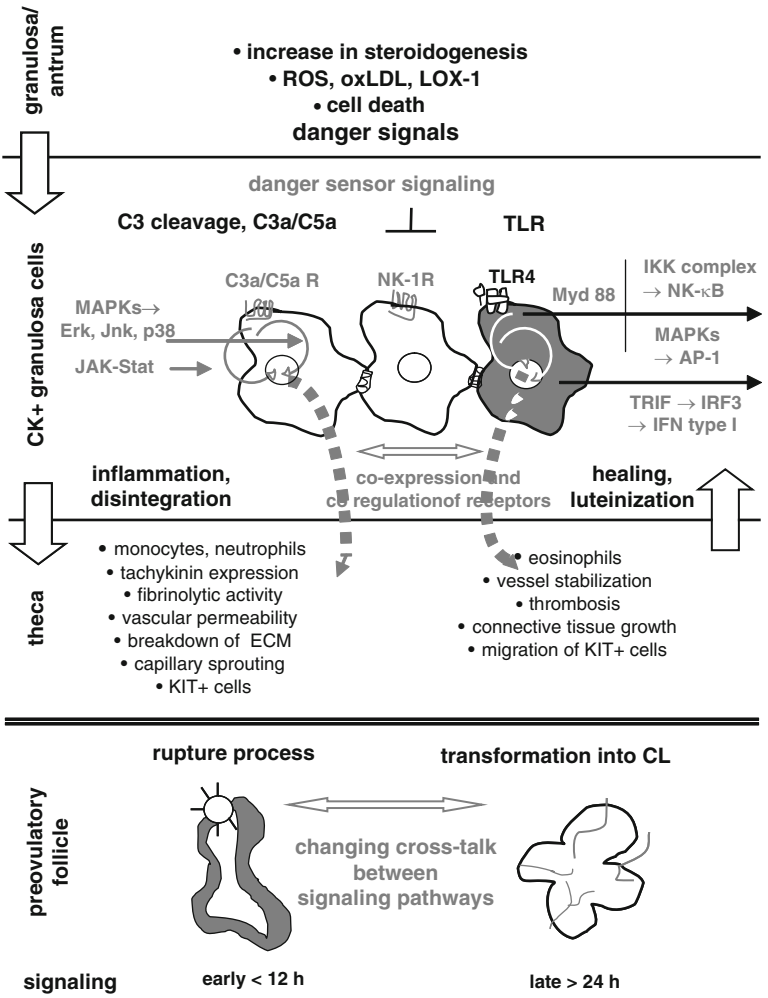

Fig. 6.1 Working hypothesis for inside-out signaling of CK^+ cells orchestrating follicular rupture and transformation into a CL as effector cells of INIM. The receptors for C3a and C5a as well as for tachykinin (NK-1R) could be co-expressed and co-regulated on CK^+ cells for the activation of the TLR4 pathway. The C3R-dependent pathways of MAPKs and of JAK-STAT lead to cell growth, differentiation cell death and pro-inflammatory events. The TLR4-related signaling activates MAPKs and IRAKs (interleukin-receptor-associated kinases) to generate immunoregulatory responses (both Myd88 dependent) through NF-κb and AP-1. The TLR4-related signaling also leads to anti-inflammatory events (TRIF-IRF3 dependent). The first sequence of interaction commands the inflammatory response with breakdown of the extracellular matrix (ECM), cell death, growth and cell differentiation under command of the AP-1 transcription factor. The second sequence governs immuno-response by dominant NF-κB and IFR-3 transcription factors for tissue repair. Pathways for disintegration and regeneration overlap, yet the dominance shifts with time. CK^+ cells built a microenvironment by tight junctions. Pathways are simplified. Theoretical associations are shown by the *gray broken line*

with cell proliferation, angiogenesis, eosinophil recruitment and connective tissue repair (Figs. 3.4–3.6; DiScipio and Schraufstatter 2007). The suggested turn in functional direction could be under the influence of the KIT–KIT ligand system being responsible for cell proliferation, migration and differentiation. A dense band of KIT-positive thecal cells with full-length KIT (160–135 kDa) developed before follicle rupture. Thereafter, KIT-positive cells formed a peripheral network among the luteinizing granulosa cells (Koch et al. 2009; Spanel-Borowski et al. 2007). The reappearance of CK^+ cells in preovulatory follicles and in regressing follicles (Fig. 4.2c–g) could be due to oxidative stress under the assumption that the CK^+ cells represent danger-sensing cells. They are likely to exert this role, because the CK^+ cells regulated TLR4, CD14 and ROS under oxLDL application in culture (Figs. 5.1–5.4), which is considered as a model of oxidative stress. The complement receptor cascade might be separate from TLR4 signaling in CK^+ granulosa cells. As an alternative mechanism, the complement receptor pathway might interact with TLR4 signaling (Hajishengallis and Lambris 2010; Hawlisch and Köhl 2006). Presently, the final outcome of the molecular cross-talk between the two ancient receptor systems is beyond imagination. The TLR4 system alone confers complex signaling pathways leading to a plethora of genes in control of immunoresponses and inflammatory cytokines (Kumar et al. 2009; Takeuchi and Akira 2010). The Myd88-dependent NF-kB pathway could be responsible for the peak values of TNF-α, IL1β and of IL-6 in preovulatory follicles (Adashi 1990; Brännström et al. 1994a) and MAPKs' signaling for cell growth and differentiation during angiogenesis and luteinization. The conversation between the complement cascade and the TLR4 pathway might be relevant for outside signaling, whereas a cross-talk between the pathways of TLR and the tachykinin-regulated NK-1R (Figs. 3.4–3.6) could be responsible for inside signaling (Fig. 6.1). This suggestion is a step forward in the debate on the role of tachykinins in inflammation and ovarian functions (Debeljuk 2006; O'Connor et al. 2004). One very much wants more details about a time-dependent change in pathway conversation. The surface epithelium can be discarded as influential tissue, because regular follicle rupture occurs in epithelium-denuded primate ovaries (Wright et al. 2010) and because of our findings on IOR (Figs 3.7 and 3.8).

It is no longer under debate whether leucocytes are incidental invaders into the preovulatory follicle wall or essential effectors (Adashi 1994; Brännström and Enskog 2002; Brännström et al. 1993, 1994b). Leucocyte accumulation and the physiological wound of the follicle wall before oocyte expulsion is part of an acute and sterile inflammatory reaction, which suddenly arises under the LH surge (Espey 1994; Medzhitov 2008, 2010a). More than 30 ovulation-specific genes are involved in two crucial signaling cascades: the progesterone receptor pathway for protease production (ADAMTS-1, cathepsin L) and the EGF-family for cumulus expansion by synthesizing the hyaluron-rich matrix (Espey 2006; Richards et al. 2002; Hernandez-Gonzalez et al. 2006). That these genes are predominantly in the granulosa cell layer reflects an inside-out process, which supports our hypothesis that oxidative stress in the follicle antrum represents the primary motor of the

ovulatory machinery. The inflammatory reaction of the ovulatory process seems part of an immune mechanism as first proposed (Richards et al. 2008). Comprehensive gene expression analysis of granulosa cells and cumulus cells depicted genes of the TLR family (*TLR2*, *4*, *8* and *9*) and the TLR adaptor molecules (*Cd14*, *C1q* and *Myd88*), as well as associated genes such as *CD34* and *pentraxin*. The long pentraxin3 belongs to the acute phase proteins, and acts as nodal point to stabilize the extracellular matrix of the cumulus complex (Bottazzi et al. 2006). Genes are translated to functional proteins in ovarian cells (Shimada et al. 2006, 2008). So far, these genes/proteins exclusively characterize immune cells. We here broaden the concept and consider the CK^+ granulosa cells as an ovary-specific institution to sense oxidative stress and to tailor immune responses in the preovulatory follicle and also in regressing antral follicles (Fig. 4.2e–g).

The question remains what happens with CK^+ granulosa cells after CL formation. The cells, which have orchestrated lesion and tissue repair for CL development, become unnecessary. The CL awaits another big task, which corresponds with the maintenance and then the removal of the CL. Luteolysis occurs rapidly in golden hamsters (Fig. 2.1b–e), and is delayed under the aspect of chronic inflammation in many other mammals (Fig. 2.2). The "corpus albicans", a hyalinized scar, is well known as the terminal stage of CL regression, which persists for several ovarian cycles. Our own findings and conclusions generated the concept that the steroidogenic CK^+ cells gradually switch off CK expression and become granulosa-like CK^- luteal cells, in culture termed type 5 cells (Fig. 6.2). The event might be due to changes of the endocrine micromilieu, in particular to high progesterone levels. That sex hormones govern the recruitment and function of antigen-presenting cells is shown for the female genital tract (Iijima et al. 2008). The change from steroidogenic CK^+ luteal cells to granulosa-like CK^- luteal cells remotely reminds one of immature DCs becoming mature DCs for migration to regional lymph nodes (Banchereau and Steinman 1998; Mellman and Steinman 2001). The converted cells postulated to be type 5 cells could cover different immune tasks in the CL of secretory or of regressing stages. The phenotype change provides the cellular condition to activate lymphocytes, and thus the connexion with adaptive immunity. It is remarkable that lymphocytes populate the bovine CL in increasing numbers comparing stages of development and regression (Bauer et al. 2001). When our theory holds true, type 5 represents the dock-gate to cell-mediated immune responses (Iwasaki and Medzhitov 2010).

The framework of our thoughts becomes more complex by including the presence of microvascular CK^+ cells (Figs. 4.3f–h, 5.6 and 5.7). Although they represent the minority in the intact bovine CL at the secretory stage, type 1 survives in long-term culture with maintenance of CK filaments and the formation of tight junctions and adherens junctions. As is explained under Sect. 5.2.2, type 1 could be the remote offspring of an endothelial precursor cell, which had been born in the aorta-gonado-mesonephros region (Dieterlen-Lièvre et al. 2006; Pouget et al. 2006; Zovein et al. 2008). In the embryo, it is the place where somite-derived angioblasts are generated and then migrate to various primitive

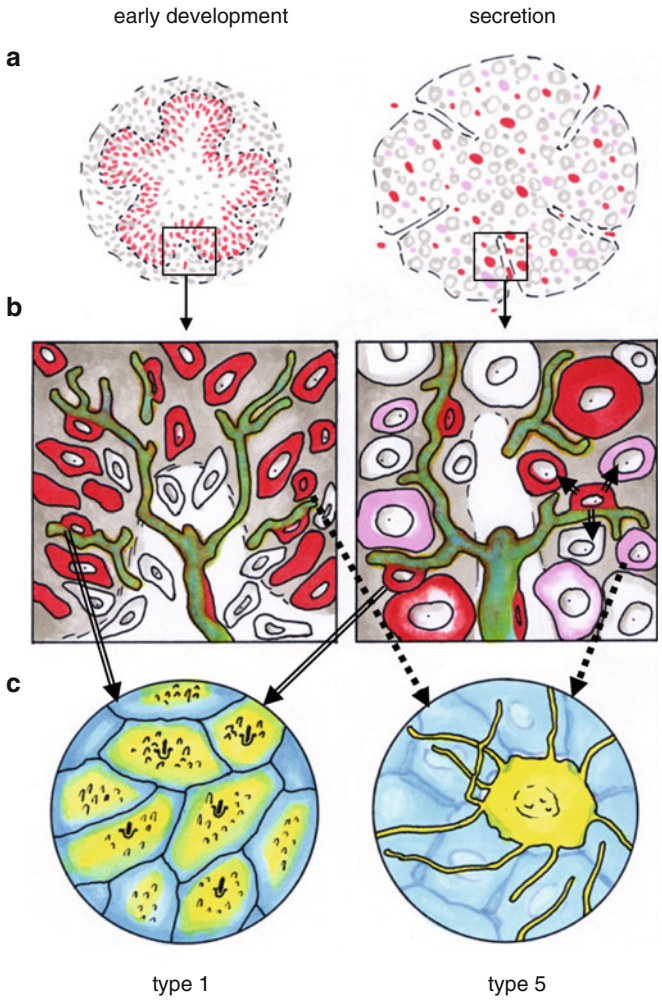

Fig. 6.2 Working hypothesis for the fate of CK^+ granulosa cells in the CL at the stage of development (*left*) and secretion (*right*). (a) for low magnification. At the early developmental stage, infoldings of the former granulosa cell layer generate a peripheral zone of CK^+ luteal cells (zonation). The former thecal layer, which forms the septum, rarely depicts a small CK^+ cell being an endothelial precursor cell. Zonation is absent at the stage of secretion. (b) for high magnification. At the stage of secretion, steroidogenic CK^+ cells of small and large size are ubiquitously distributed and demonstrate a decrease in CK intensity (red, pink and white cells) due to a switch-off in CK genes. The few CK^+ cells (in red), which are part of the microvessel (in green), show no difference in CK expression. (c) for cell culture. The switch-off in CK genes leads to the transition of steroidogenic CK^+ cells into granulosa-like cells of type 5 in culture (*broken arrows*). The microvascular CK^+ cells become type 1 cells in culture (*arrows*). They sense danger and signal it to steroidogenic CK^+ cells at the onset of luteolysis (*short open arrows*, secretory stage). Theoretical associations are *open and broken arrows in black*. *Drawn by R. Spanel*

organ systems and likely also to the near-by genital ridge. Such an endothelial precursor cell probably resides in the adult ovary, becomes part of the angiogenic event during CL formation (Fig. 6.2) and is seen in immunostained sections in the established microvascular bed of the CL in the secretory stage. There, the CK^+ microvascular type could sense hypoxic changes at the onset of morphological luteolysis and communicate the upcoming danger with the granulosa-like CK^- luteal cells/type 5 cells.

Although appealing because so many new aspects, we know very well that our novel concept requires a lot of work before the sovereign authority of INIM becomes generally accepted in ovarian biology. The dogma that sex hormones and intra-ovarian regulators (cytokines, chemokines and growth factors) are orchestrated by FSH and LH remains untouched. Signals of the endocrine system and of the local immune system coexist and probably interact. Our meticulous characterization of different phenotypes derived from preovulatory follicles and CL is considered as an essential basis for future experiments. The advantage is that the characterized cells are obtained from bovine and human ovaries. Cow ovaries have comparable ovarian cycles and are thus ideal models for human ovaries. Experiments should address the question whether CK^+ cells are involved in the generation and inhibition of the complement cascade in the preovulatory follicle. Details on the TLR signaling cascade and the modulation by co-regulating pathways are needed. Immunologic profiles of cytokine/chemokine secretion of surface molecule expression (MHC I and II, TLR members, and lipoprotein receptors such as CD14, CD36, and LOX-1 under TNF-α and IFN-γ treatment) might establish type 1 and 5 as immunocompetent cells. It is necessary to learn which form of cell death, whether autophagy, apoptosis or necrosis, is preferred by individual phenotypes when kept under oxidative stress such as oxLDL treatment. Such findings will broaden our understanding of follicular atresia with and without apoptotic bodies in preantral/antral follicles (Fig. 3.2; Van Wenzel et al. 1999). That survival autophagy plays a role in the maintenance of the CL has been published (Del Canto et al. 2007; Gaytn et al. 2008), yet the molecular network remains unclear. Most importantly, CK^+ granulosa cells should be experimentally converted into type 5 cells and the influence of KIT-positive thecal cells studied in culture. The in vitro findings then have to be validated with animal models, for example, by targeting the adaptor molecule Myd88. The inhibition of Myd88 in addition to the TIR domain more efficiently blocks the damage-induced acute inflammation in mice than inhibition of TLR signaling (Chen et al. 2007; Rock et al. 2010). This could be one reason why TLR4-gene deficient mice are fertile (Richards et al. 2008).

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Footmarks of Innate Immunity in the Ovary and Cytokeratin-Positive Cells as Potential Dendritic Cells

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