Chapter 18
Telocytes in Inflammatory Gynaecologic Diseases and Infertility

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18.1 Introduction

Infertility is a common disease in women of reproductive age. It can be divided into anatomical and functional disease. Anatomically, oviduct and uterine diseases, such as severe endometriosis, tubal ectopic pregnancy, para-tubal adhesion after pelvic inflammatory diseases, uterine septum, intrauterine adhesion, etc., frequently cause infertility. On the other hand, among the functional reasons, immune-mediated fertility problems and related diseases, such as autoimmune diseases, early stage of endometriosis, pelvic inflammatory disease and salpingitis, were the predominant diseases in the clinic. Generally, the female reproductive tract must maintain a unique immune micro-environment, in order to tolerate the semi-allogeneic sperm and foetus and protect against harmful pathogens [1, 2]. Further researches showed that immunocytes such as monocyte and macrophage, which were important multifunctional players in local peritoneal immune response in endometriosis or pelvic inflammatory disease, and their dysfunction or uncontrolled augmentations in quantity and/or activation might not only change smooth muscle motility, microcirculation and pelvic pain in endometriosis [3] but also lead to immune-mediated fertility problems, such as miscarriage, tubal infertility and tubal ectopic pregnancy [4, 5].

Endometriosis (EMs) is a kind of aseptic inflammatory, ischemic, oestrogen-dependent disease with many clinical manifestations [6, 7]. It was characterized by the presence of endometrium outside the uterine cavity and affects an estimated 8–10 % of women of reproductive age in industrialized countries [6, 8]. Generally,

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massive monocyte/macrophage and lymphocyte aggregation, which overproduce cytotoxic substance, such as inflammatory factors (iNOS, COX-2), oxidative stress (LPO) and estradiol, was the most frequent pelvic micro-environment changes in EMs [7–9]. Then, EMs causes many abnormalities either in local anatomy or reproductive endocrine and immunologic micro-environment disorders, subsequently leading to various clinical symptoms, such as chronic pelvic pain, hypermenorrhoea, dysmenorrhoea and sub- or infertility [8].

Current knowledge regarding EMs-related fertility disorders remains insufficient. Generally, in women with obvious macroscopic anatomical changes of the pelvis, mechanical interference will be adversely affected by oocyte pickup, transport and tubal peristalsis. However, women with absence of macroscopic pelvic alterations also complained of unexplained functional sub- or infertility [8], of which, many steps of the fertilization process were believed to be disturbed by inflammatory peritoneal fluid which were thought to exert direct cytotoxic effects or release various cytokines/enzymes into the pelvic milieu, such as ovulatory dysfunction, altered folliculogenesis, sperm phagocytosis, luteal-phase defects, impaired fertilization, defective implantation and early embryo growth [10]. Interestingly, the phenomenon of impaired utero-tubal sperm transport in early-stage EMs and adenomyosis, strongly suggested that "tubal motility disorder" might be a potential cause for EMs-associated tubal factor sub- or infertility [11]. We suggested that tubal dysperistalsis might develop from a subset of clinically affected known or unknown oviduct cells that were functionally dysregulated, probably due to aforementioned toxic pelvic micro-environment.

Acute pelvic inflammatory disease (PID) is a serious, infectious disease of the upper female genital tract, which frequently causes severe pelvic damage and leads to fertility problems among women of reproductive age, such as tubal ectopic pregnancy and tubal factor infertility [12]. Generally, PID was caused by ascending polymicrobial infection, particularly sexually transmitted organisms, such as *Chlamydia trachomatis*, mycoplasma and *Neisseria gonorrhoeae* or vaginal dysbacteriosis. Clinically, acute PID can be divided into tubo-ovarian abscess, pelvic peritonitis, acute endometritis and acute salpingitis (AS) which was the most important component of PID spectrum. AS can cause severe anatomical damage of a subset of oviduct cells, manifested by oviduct acute edema, pyosalpinx, obstruction of the lumen, peritubal adhesion, interstitial fibrosis and rigidity of the wall [12]; frequently induced oviduct dysfunction, such as chronic tubal spasm, immunologically mediated mechanism in *Chlamydia trachomatis* infection. All of these changes were suggested as the underlying mechanisms for AS-induced female fertility problems. In our opinion, the clinical sequelae of fertility problems were developed from a subset of AS-affected oviduct cells that were structurally and functionally dysregulated, including the widely known ciliated cells and various types of interstitial cells such as smooth muscle cells, fibroblasts, etc. All of these cell components play definite roles and were indispensable in a successful reproductive process. Nevertheless, besides the well-known impair of classically described oviduct cells, other damages that simultaneously occurred within the oviduct wall, which also structurally and functionally affect oviduct fertility capacity, are worthy of further investigation.

With recent progress in electron microscope, telocytes (TCs), previously known as interstitial Cajal-like cells (ICLC), were identified as a distinct interstitial cell component by Popescu et al., in interstitial space of a wide variety of cavitary and non-cavitary human and mammalian organs, including non-pregnant and pregnant myometrium, endometrium, oviduct, placenta, etc. (www.telocytes.com). TCs have particular ultrastructure which can be clearly distinguished from classical interstitial cell of Cajal (ICC), fibroblasts, etc. Morphologically, TCs have a small piriform-/spindle-/triangular-shaped cell body, with two to five extremely long and slim telopodes (Tps), which contained thin segments (podomers) and dilated segments (podoms). By their Tps, TCs provide visible "short-distance" direct structural support for homocellular or heterocellular junctions between themselves and other type of cells. And based on their distribution, identification, immunophenotype and ultrastructure features in different normal organs/tissues, TCs were supposed to play an essential role in the maintenance of structural and functional integrity, by intercellular signalling, spreading slow waves generated by the pacemaker ICC, involved in local tissue homeostasis/angiogenesis, nurse stem cells (SCs) and mediated tissue repair/remodelling, immunoregulation or immunosurveillance, etc. On the other hand, increasing number of reports described "long-distance" indirect intercellular contacts for TCs, such as through chemical [13–15], paracrine/juxtacrine signalling [13, 14, 16–19], extracellular vesicles (EVs) [14, 16–20] [15, 21–30], sex hormone [14, 16, 31–33] and/or microRNAs [23, 34–37]. These paracrine effects were believed to play important roles in function-specific intercellular communication and regulate activity of "long-distance" neighbouring cells.

Recently, disease-induced TC damage was reported in fibrotic lesions of skin, cardiac, ulcerative colitis, Crohn's disease and gallstone disease, and multiple potential pathophysiological roles have been speculated for TCs [37–42]. Nevertheless, as a new type of interstitial cell, the exact alterations of TC population in disease-affected oviduct tissue, such as in aseptic inflammatory disease of EMs, and infectious AS; and potential involvement in structural and functional abnormalities of oviduct, further engagement in oviduct fertility capacity or adverse reproductive outcome (sub- or infertility), need more detailed evidence.

On the other hand, increasing studies showed that both in normal and disease-affected tissues, TCs developed heterocellular junctions with various immunocytes and potentially modulated their activities, such as macrophages, mast cells, lymphocytes, etc. [1, 26–28, 38–41, 43–53]. In particular, in inflammatory-affected rat oviduct tissue [45, 46], TCs connected to the activated immunocytes, including mononuclear cells, mast cell, eosinophils and neutrophils. All of these studies supposed that TCs might act as active players or "data suppliers" and involved in local immunoregulation/immunosurveillance [26, 28, 50, 51]. Progressive damage and loss of TCs might impair intercellular communication or immune homeostasis in immunoinflammatory process, such as multiple autoimmune, chronic inflammatory and fibrotic disorders [26, 38–41, 44–46, 51]. We speculated that through direct junctional complexes or possibly indirect paracrine messages, TCs might potentially involve in immunological signal presenting and/or transduction, influence and contribute to subsequent immunoresponse and then change normal physiological

process and lead to immune-mediated gynaecologic diseases or reproductive abnormalities. Nevertheless, currently, no reliable cytological evidences are available for this hypothesis.

Regarding the aforementioned questions, we have designed researches and provided related evidence in the following three aspects. We believed that such knowledge will be helpful to elucidate pathophysiological role of TCs in female genital tract disease and fertility problems, with the aim of providing a potential target for genetic, pharmaceutical and clinical interventions.

18.2 In Vivo TC Damage, Molecular Mechanisms and Potential Impact in Fertility in EMs-Affected Rat Oviduct Tissue

Women with EMs frequently complained with unexplained fertility problems. The recently identified TCs were found to participate in the maintenance of structural and functional integrity of female oviduct, but so far whether TCs were involved in EMs-affected oviduct tissue and potentially influence female fertility capacity remains to be elucidated.

TCs were studied in an oviduct EMs model and in sham control Sprague-Dawley rat, respectively, together with determination of inducible nitric oxide synthase (iNOS), COX-2, lipid peroxide (LPO) and estradiol. Briefly, the autotransplantation rat model of oviduct EMs was surgically constructed by removing uterine horn and transplanted towards both surfaces of contralateral mesosalpinx, with endometrial side adjacent to the arteries that irrigate the oviduct [54, 55]. Rats received the same surgery with removal of the uterine horn and blank sutures, without any tissue masses, serving as the sham control. Then, EMs-affected oviduct segment with grade III ectopic endometriotic vesicles (larger than 4 mm) was harvested at 2 months [56]. Proceed for an integrated technique of haematoxylin and eosin staining, in situ immunohistochemistry (IHC), double-labelled immunofluorescence IHC staining and transmission electron microscopy (TEM) observation.

After confirming by routine pathologic observation for disease-affected and disease-unaffected oviduct tissues (Fig.18.1), in situ IHC was performed on consecutive sections from sham oviduct, and presumably TCs with special morphology and immunophenotype were observed: perivascular stellate-shaped cells with prolonged cell body and double-positive expression for CD34/vimentin (Fig. 18.2a, b) and negative for c-kit (Fig.18.2c).

Double-labelled fluorescent IHC confirmed the existence of typical TCs in sham group (Fig.18.3a), with characteristic appearance: one or more extremely long/thin cellular prolongations located around perivascular space, with specific immunophenotype of CD34 positive/vimentin positive/c-kit negative, consistent with in situ IHC (Fig.18.2), while in EMs-affected oviduct tissue, cell populations with typical TC morphology and immunophenotype significantly decreased and were sparse or even completely undetectable ($P = 0.000$; Fig. 18.3b, c).

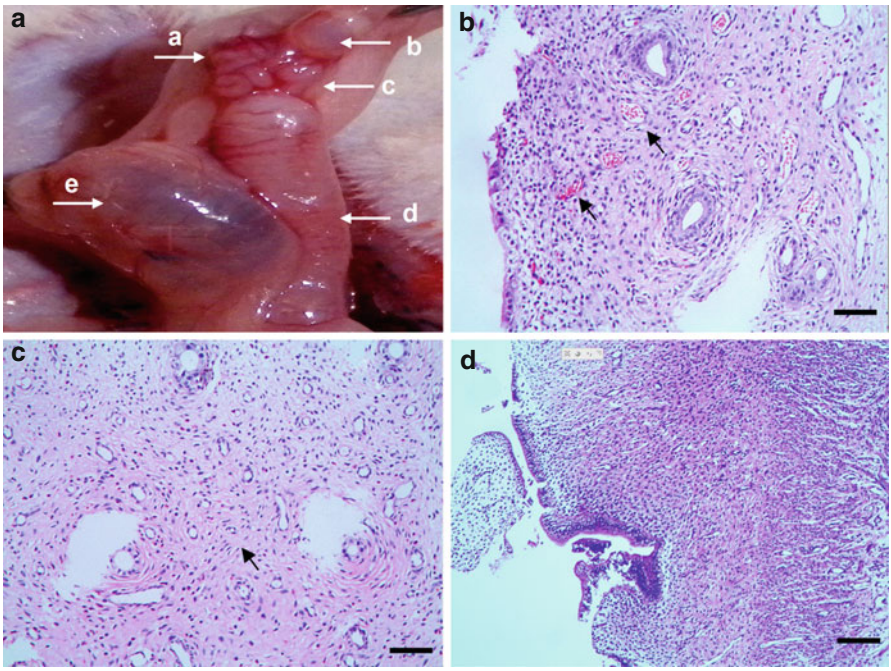


Fig. 18.1 Macroscopical and microscopical view of EMs-affected rat oviduct, scale bar = 100 μm. (**a**) Ectopic endometrial vesicles (>4 mm in diameter), located in mesosalpinx under naked eyes in study group. (**a**) ovary; (**b**) ectopic vesicle; (c) oviduct; (**d**) uterine horn; (**e**) oviduct swelling and edema after removal of the opposite uterine horn. (**b**) Inflammatory tissue reaction in EMs-affected oviduct, manifested by hyperplasia and disturbance of capillaries (*black arrows*). (**c**) Chronic inflammation and interstitial fibrosis, manifested by hyperplasia capillaries with excessive interstitial lymphocyte infiltration and fibre contents (*black arrow*) in EMs-affected oviduct wall. (**d**) Nearly normal oviduct tissue from the sham control (Yang et al. [45] adapted with permission)

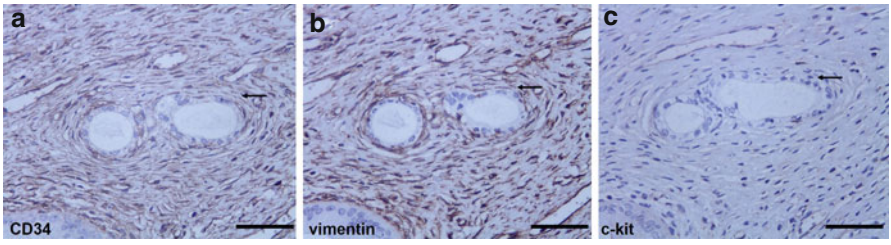


Fig. 18.2 Single-labelled in situ IHC on serial sections from the sham control (*black arrow*); scale bar = 200 μm. (**a**) Perivascular TC-like CD34 (+) cells with slender cell body and prolongations. (**b**) TC-like CD34 (+) vimentin (+) cells. (**c**) c-Kit (−) in corresponding site of serial slides (Yang et al. [45] adapted with permission)

Meanwhile, quantitative analysis by using single-labelled fluorescent IHC suggested that in EMs-affected oviduct tissue, the contents of iNOS, COX-2, LPO and estradiol were elevated significantly than in the sham group, respectively (all $P < 0.01$; Fig. 18.4a–d), thus suggestive of intra-tubal inflammation and ischaemia state.

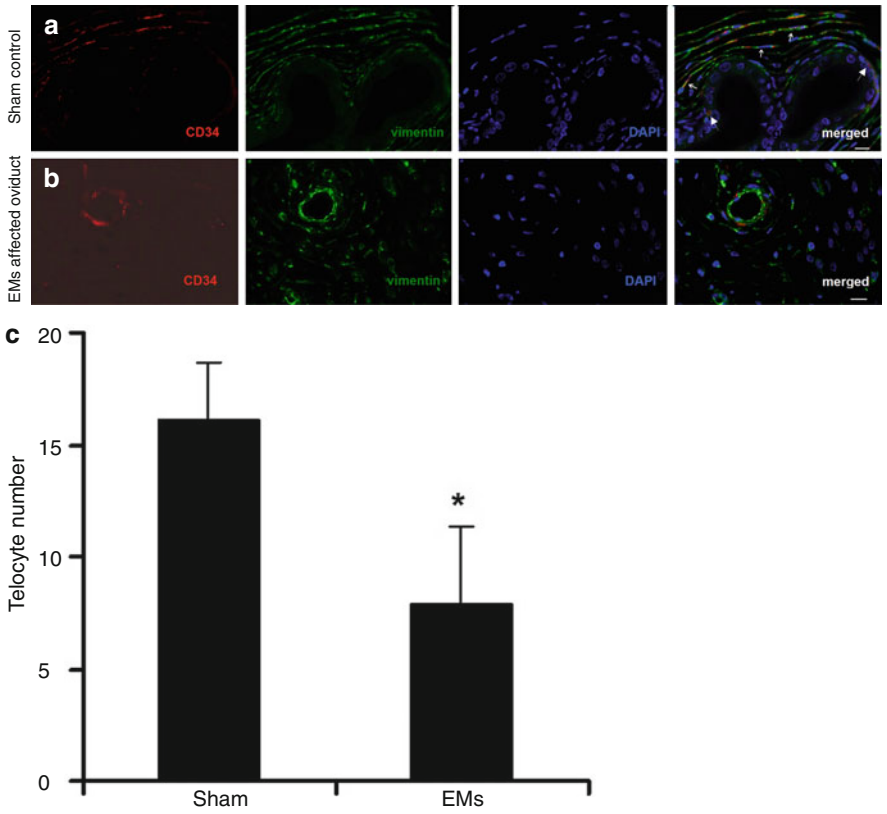


Fig. 18.3 Double-labelled immunofluorescence IHC confirmed TCs with typical morphology and immunophenotype of CD34 (+)/vimentin (+); c-kit-negative images not shown; scale bar = 20 μm. (**a**) In sham control, CD34 (*red*) overlying vimentin (*green*) both in cellular body and telopodes (Tps) (solid *arrows*) around perivascular space (CD34-positive capillary cells, indicated by *dotted arrows*). (**b**, **c**) In EMs-affected oviduct, TCs with intact structure were scarce or even undetectable, decreased significantly in number. *$P<0.05$. Error bars = SD (Yang et al. [45] adapted with permission)

Ultrastructure observation in sham oviduct confirmed that normal TCs display typical ultrastructure features: a slender piriform/spindle/triangular cell body, with one or more extremely long, thin, very sinuous telopodes (Tps), alteration with thin (podomers) and thick segments (podoms), stretched from cell bodies to different directions. In addition, a rich amount of organelles, such as mitochondria, rough endoplasmic reticulum, cytoskeletal elements, caveolae and microvesicles, is located within podoms (Fig. 18.5). TCs are distributed around the perivascular space; two or three layers of TCs formed almost a complete sheath with Tps and surrounded the vascular endothelial cells, with homocellular or heterocellular junctions among Tps or fibrocytes and pericytes (Fig. 18.5a). Moreover, TCs also scattered among smooth muscle cells (SMC), with heterocellular junctions between them, and microvesicles are contained in Tps and synaptic cleft (Fig. 18.5b).

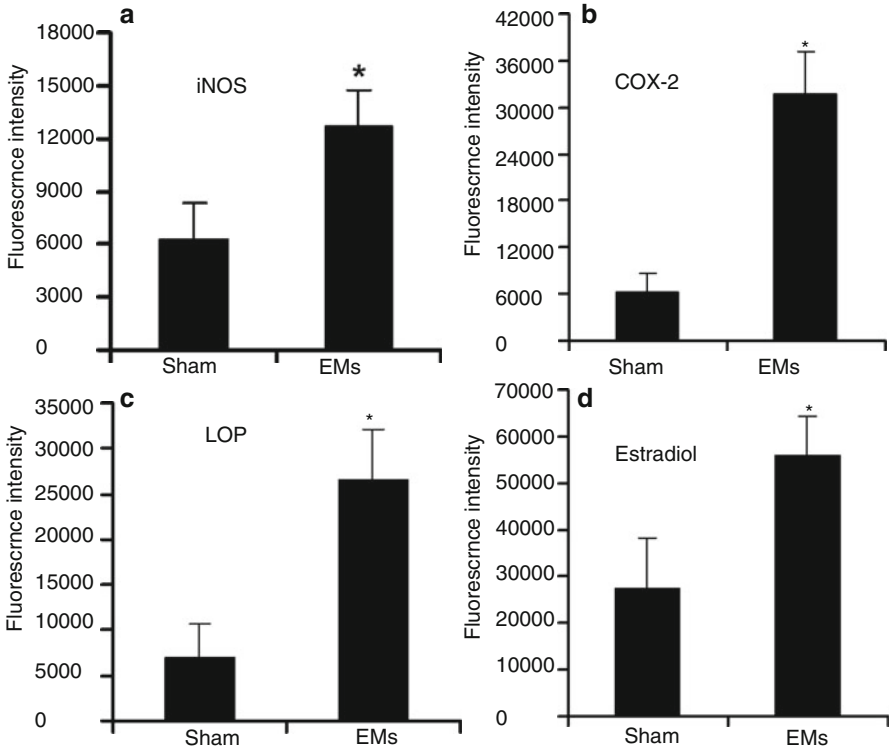


Fig. 18.4 iNOS (**a**), COX-2 (**b**), LPO (**c**) and estradiol (**d**) increased significantly in EMs-affected oviduct tissue, as compared to sham control. $*P<0.05$. Error bars = SD (Yang et al. [45] adapted with permission)

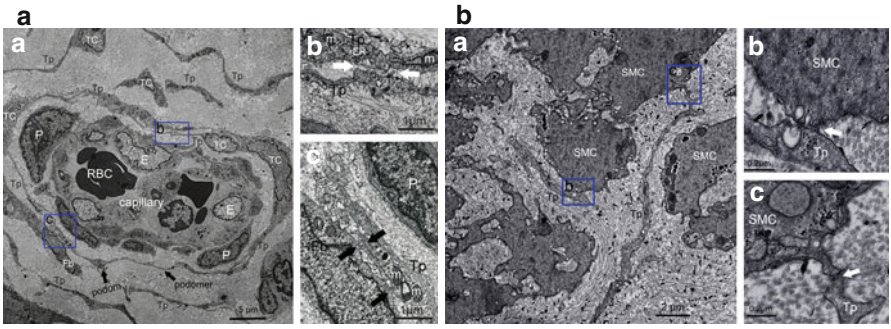


Fig. 18.5 Normal TCs. (**a**) Perivascular TCs. (**a**) By Tps (podom and podomer, *black arrows*), TCs completely enwrap vascular endothelial cells (E) and pericyte (P). Mitochondria (M), rough endoplasmic reticulum (rER). (**b** and **c**) Higher magnifications of the *blue boxed* areas within "a": homocellular and heterocellular junctions between TCs (*white arrows* in "b") and fibrocyte (Fb; *black arrows* in "c"). (**b**) TCs among smooth muscle cells (SMCs). (**a**) Heterocellular junctions between Tp and SMC. (**b** and **c**) Higher magnifications of the blue boxed areas within "a": extracellular vesicles in synaptic cleft (*white arrows*) (Yang et al. [45] adapted with permission)

However, in EMs-affected oviduct tissue, which is confirmed by typical ectopic endometriotic glands (Fig. 18.6a), extensive ultrastructural damage or complete loss of TCs was observed (Fig. 18.6b–e), such as loss of organelles, swollen cell nucleus and mitochondria, cytoplasmic vacuolization, endoplasmic reticulum dilatation and swollen

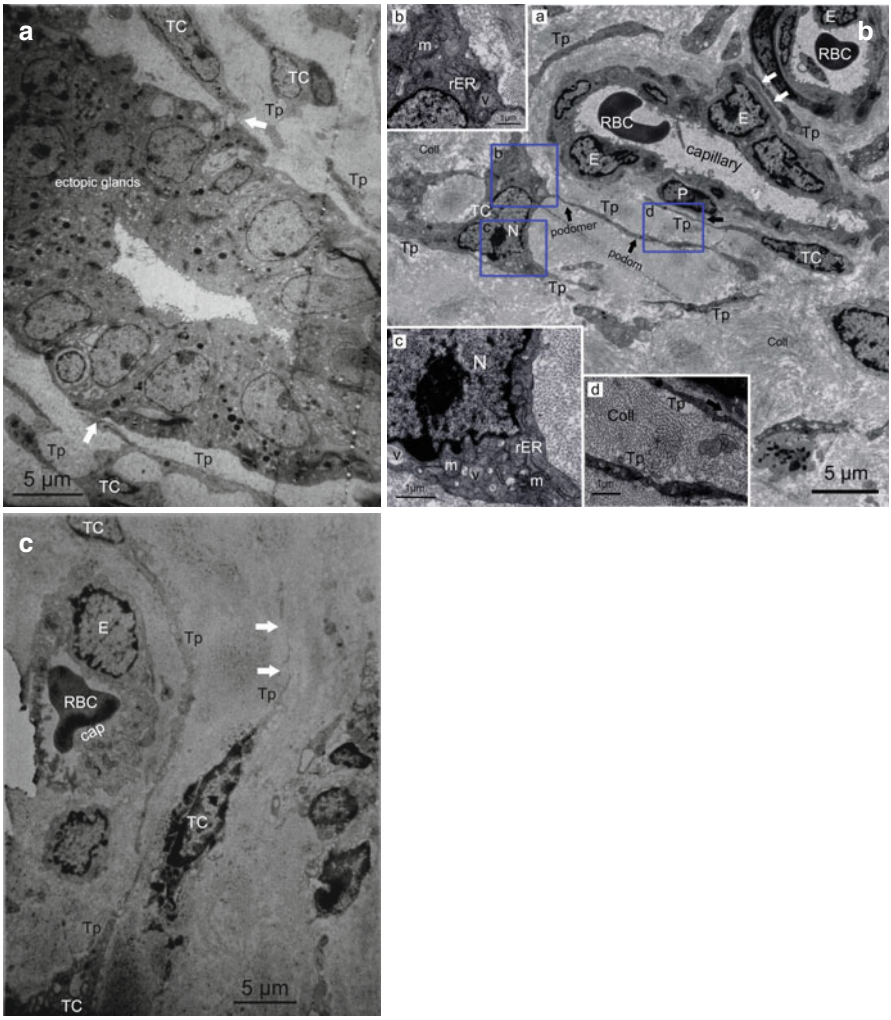


Fig. 18.6 TC damage in EMs-affected oviduct tissue. (**a**) Ectopic endometriotic glands with dense secretory granules, with close contact to Tps (*white arrows*). (**b**) Perivascular TC damage and tissue fibrosis, with nearly normal endothelial cells (*E*). (**a**) Disintegration of TC network, with swollen cell junctions (*white arrows*). (**b–d**) Higher magnifications of the *blue boxed* areas; (**b** and **c**) cellular organelles damage, cell nucleus (*N*) and mitochondria (*m*) swollen, dilatation of rough endoplasmic reticulum (rER), cytoplasmic vacuolization (*v*). (**d**) Excessive amount of collagen fibres (Coll) embedded by damaged Tps. (**c**) Perivascular TC damage (*white arrows*), with endothelium (*E*) damage. (**d**) Heterocellular junctions between slightly damaged TCs and mast cells (MC; *white arrows*), together with tissue fibrosis (collagen fibril, Coll). (**e**) Damaged TCs nursed a group of putative normal stem cells (SC) to develop a possible SC niche, with heterocellular junctions between them (*white arrows*) (Yang et al. [45] adapted with permission)

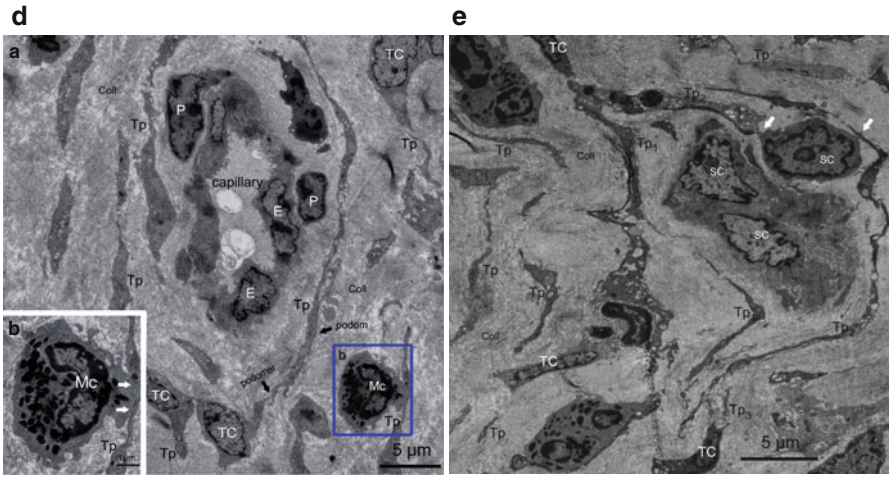


Fig. 18.6 (continued)

cell junctions. Interestingly, TC damage with normal endothelial cells and SC, nearly normal TCs with severely damaged microvessels, abundant collagen fibres and tissue fibrosis can also be observed (Fig. 18.6b, d, e). In addition, TCs developed heterocellular synapse to mast cells (MCs) (Fig. 18.6d) and stem cell (SC) (Fig. 18.6e), suggesting potential participation in local immunoregulation and stem cell-mediated tissue repair.

This successfully constructed rat model displays ectopic endometriotic vesicles macroscopically and inflammation microscopically, with overproduced iNOS, COX-2, LPO and estradiol in oviduct tissue. This kind of rat model resembled clinic physiopathology of EMs and thus suitable for TCs and infertility research.

Classically, oviduct stromal cells are mainly composed of myocyte and immunocytes such as dendritic cells, macrophages, mast cells, plasma cells, eosinophils and lymphocytes, plus interstitial cells of Cajal (ICC) as pacemaker for smooth muscles [57]and fibroblasts as effector cells for tissue fibrosis [40]. Each of them was essential in reproductive process. However, we reported for the first time that the newly discovered TCs participate in the maintenance of structural and functional integrity of normal oviduct tissue. And in EMs-affected oviduct, we found inflammation and reactive oxygen species-induced extensive TC damage and demonstrated a broad involvement of TCs with tissue fibrosis in EMs-affected oviduct wall and parallel trends of TC damage with the severity of EMs.

Although the exact roles of TCs are supposed merely based on their distribution and intercellular connections, oviduct TC damage might cause (i) dysregulation of intercellular signalling, such as impaired immunoregulation/immunosurveillance, attenuated intercellular signalling and oviduct contractility; (ii) impaired stem cell-mediated tissue repair/regeneration and remodelling of interstitial fibrosis; and (iii) derangement of 3-D interstitial architecture, which were structural support for intercellular signalling, tissue repair/remodelling and homeostasis. All in all, the newly identified TCs provide a new explanation for structural and functional oviduct disease.

In the future, animal models with TC network depletion will be more helpful to clarify what exactly happened on the pathway, by which TCs mediate cell interactions

with other structural components of oviduct, and unveil the real functional consequences of TC damage on reproductive process. In addition, comparative high-throughput analysis for TCs between disease-affected and disease-unaffected tissues would provide more insights into potential roles of TCs in oviduct pathophysiology. On the other hand, whether uterine TCs simultaneously underwent cell damage and cause uterine dysfunction may be another critical topic. Interestingly, as accompanied by normal endothelial and SC, the mechanism of why TCs were seemingly less tolerant in the disturbed pelvic milieu needs further elucidation. Finally, whether TC transportation (together with or without stem cells), rebuilding TC network, can promote regeneration and reparation of disease-affected tubal damage, oviduct fibrosis and fertility disorders is worthy of future research.

18.3 In Vivo Ultrastructure Damage of TCs in Acute Salpingitis-Affected Rat Oviduct Tissues

Acute salpingitis (AS) is a kind of acute pelvic inflammatory diseases; it causes severe damage to a subset of classically described cells lining in the oviduct wall and finally leads to interstitial fibrosis and tubal factor sub- or infertility. However, with recent increasing reports regarding TC damage in various disease-affected tissues, potential involvement and pathophysiologic role of TCs in AS-induced tubal factor fertility problems remain unknown.

In order to get AS-affected rat oviduct tissues, the rat model of AS was established, the injection of bacterial liquid (0.1 ml, 2×10^7 E. coli) into both sides of oviduct lumens was performed only after entering the abdominal cavity via lower midline trans-abdominal incision under aseptic conditions. The open surgery was done before injection [58]. The same amount of sterile saline instead of bacterial liquid served as the sham control. Then, 7 days later, both sides of oviducts were obtained for HE, IHC, immunofluorescence IHC and TEM observation.

AS was confirmed by acute inflammation within oviduct tissues under HE staining, by massive neutrophil infiltration under CD177 immunostaining (Fig. 18.7a–d) and simultaneously by single-labelled immunofluorescence IHC and quantitative analysis, which showed significant overproduced inflammatory factors (iNOS, COX-2) (both $P=0.000$; Fig. 18.7e–f).

TC immunodiagnosis was performed by double-labelled immunofluorescence IHC and confirmed the existence of rich amount of CD34/vimentin double-positive TCs around perivascular space of the sham group. TCs showed typical cell body and two or more extremely long/thin prolongations, as well as double immunofluorescence in its full length (Fig. 18.8a). While there were no co-expression of vimentin and c-kit in cell bodies or prolongations of TCs (images not shown). Nevertheless, in sections from AS-affected oviduct tissues, CD34/vimentin double-positive TCs with intact structure significantly decreased and were sparse or completely absent ($P=0.000$; Fig. 18.8b, c).

Ultrastructure observation under TEM identified normal TCs by their characteristic ultrastructure features (Fig. 18.9a, b). TCs enwrap the whole capillaries with

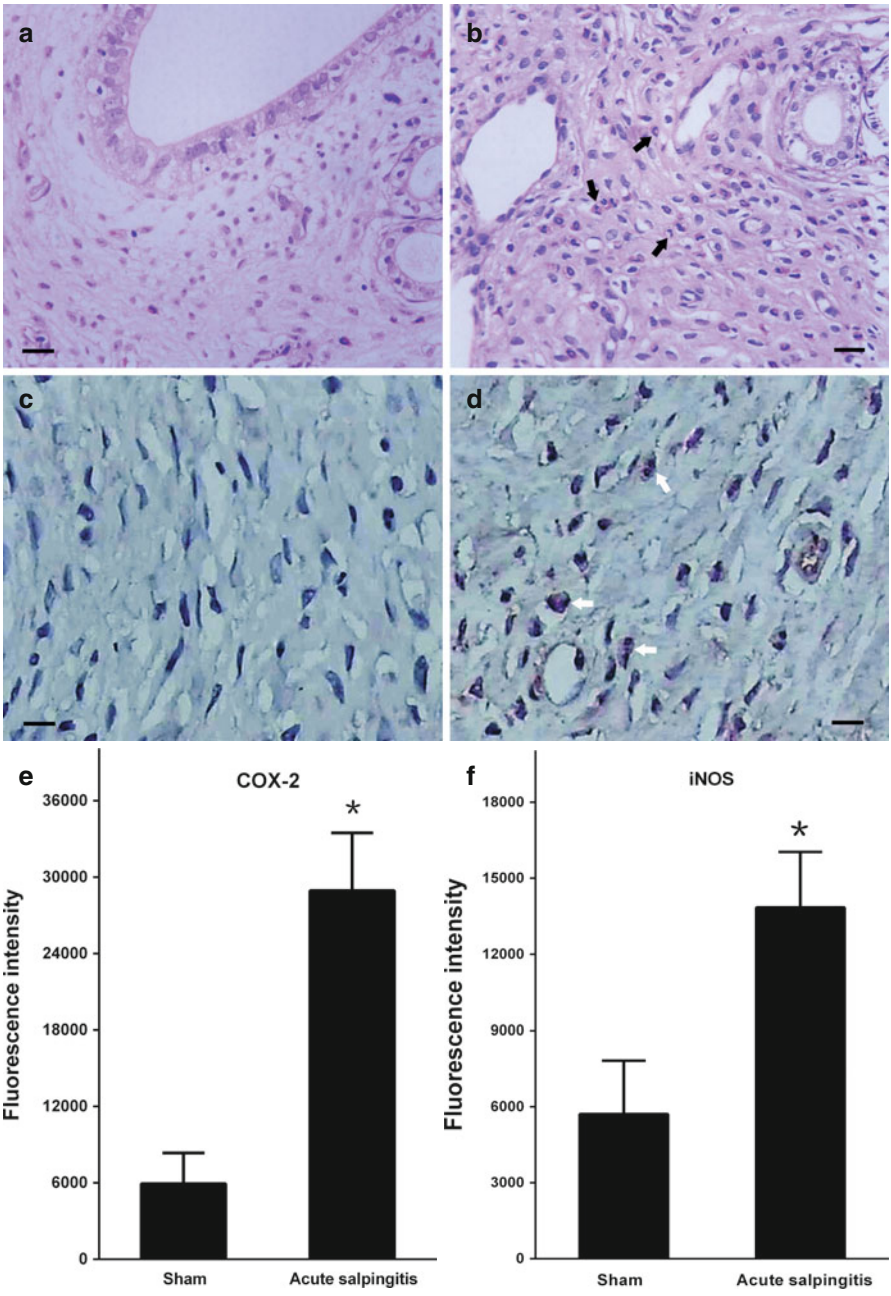


Fig. 18.7 Oviduct inflammation confirmed by HE staining (**a**, **b**, scale bar = 40 μm), IHC (**c**, **d**, scale bar = 20 μm) and single-labelled immunofluorescence IHC and quantitative analysis (e, f). (**a**, **c**) Normal oviduct tissues, with no signs of inflammatory cells infiltration indicated by negative CD177 IHC. (**b**, **d**) Acute inflammation in AS-affected oviduct tissues showed signs of SMC and capillary swelling, interstitial congestion, exudation, oedema and interstitial fibrosis (**b**, *black arrows*) and massive neutrophil infiltrations, suggested by strong positive CD177 IHC (**d**, *white arrows*). (**e**, **f**) COX-2 (e) and iNOS (f) increased significantly in AS-affected oviduct tissue, as compared to sham control. *$P < 0.05$. Error bars = SD (Yang et al. [46] adapted with permission)

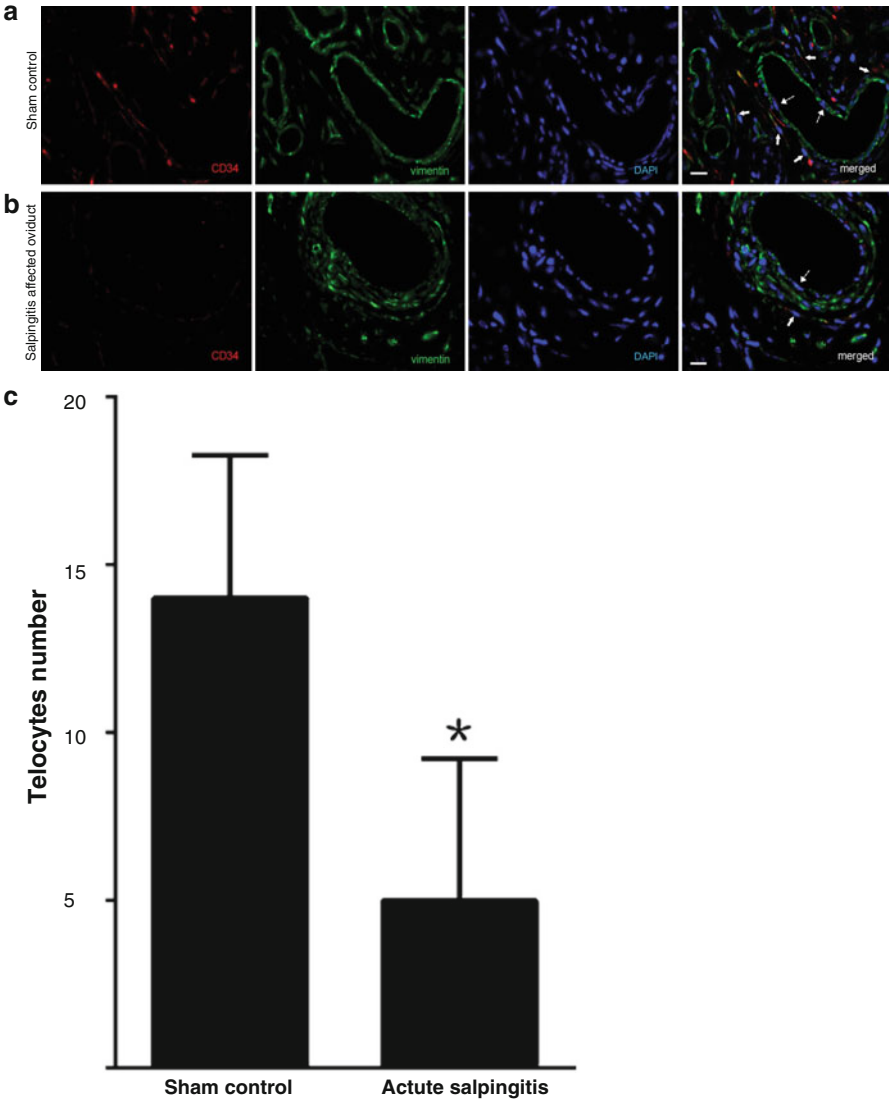


Fig. 18.8 TCs with typical morphology and double-positive CD34 (+)/vimentin (+) were confirmed by double-labelled immunofluorescence IHC. c-Kit-negative images not shown; scale bar = 20 μm. (**a**) In sham control, CD34 (*red*) overlying vimentin (*green*) in whole length of TCs (*solid arrows*). (**b** and **c**) In AS-affected oviduct tissues, CD34/vimentin double-positive TCs with well-defined nuclei were less densely stained (solid arrows), significantly decreased in cell number. *$P<0.05$, error bars = SD (Yang et al. [46] adapted with permission)

long Tps or scattered in SMC bundles in mucosa and muscular layer of the sham oviduct, with complex homocellular and heterocellular junctions with adjacent cell components, thus organizing a unique 3-D network, providing mechanical support

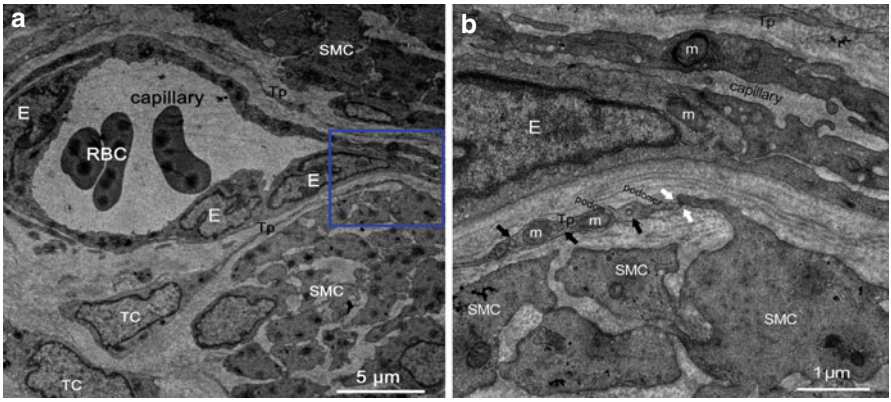


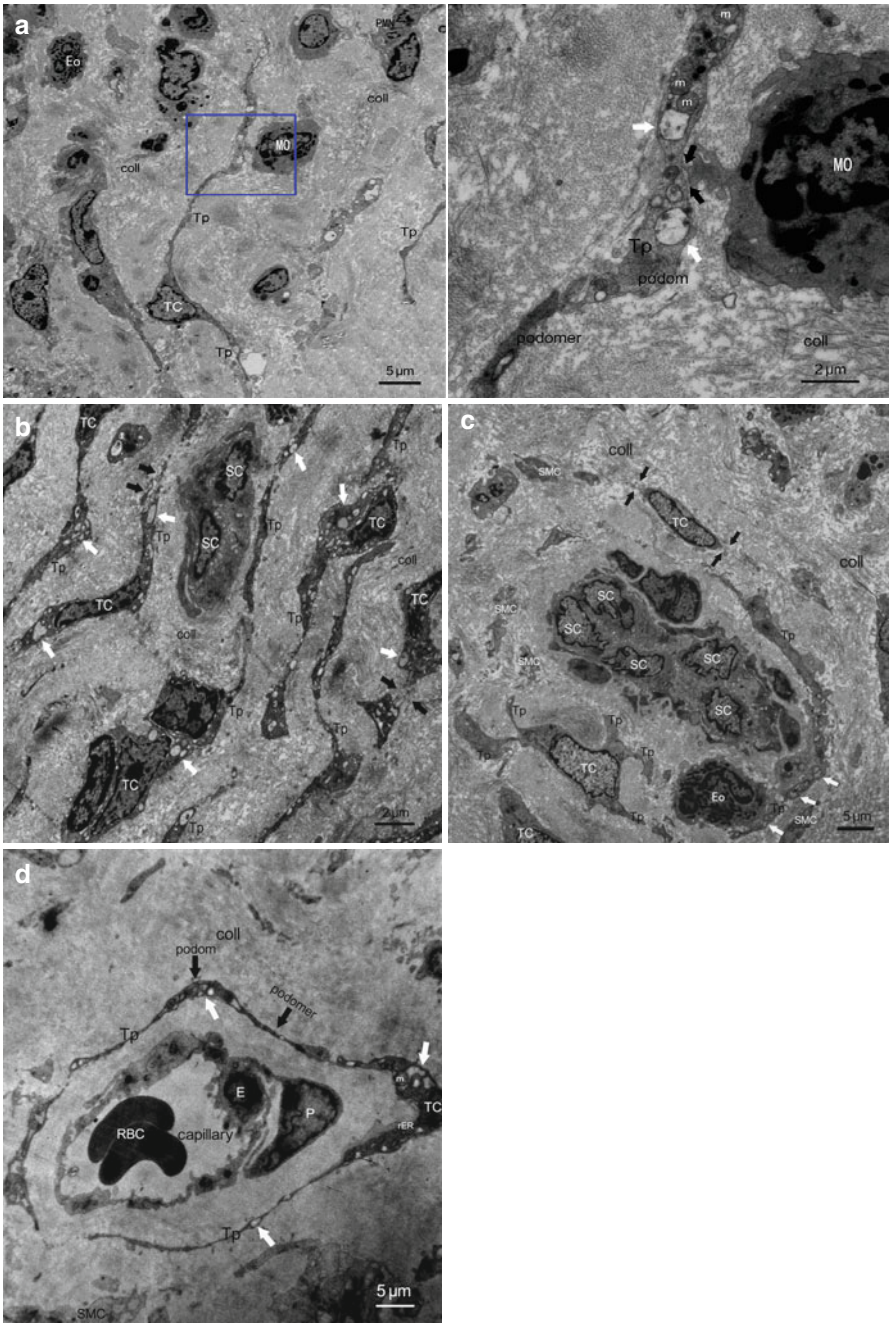
Fig. 18.9 Normal TCs in perivascular space or among smooth muscle bundles. *RBC* red blood cells, *E* vascular endothelial cells, *m* mitochondria, *rER* rough endoplasmic reticulum. (**A**, **a**) By Tps, which was composed of podoms and podomers, TCs surrounded and developed heterocellular contact with SMCs (*white arrows*) in perivascular space. (**b**) Higher magnification of the *blue boxed* area; homocellular contacts (*black arrow*) between TCs and heterocellular connections with *E* (*white arrows*). (**B**, **a**) Perivascular TCs scattered among SMCs. (**b**) Higher magnification of the *blue boxed* area: abundant mitochondria (*m*) and microvesicles (*black arrows*) within podom and heterocellular junctions between Tps and SMCs (*white arrows*) (Yang et al. [45] adapted with permission)

or structural basis for interstitial compartment and communicating and potentially affecting any of nearby different cell types.

Nevertheless, in AS-affected oviduct tissues, TCs displayed multiple ultrastructural damage both in cellular body and Tps, together with obvious TC loss, disrupted TC-SC niches (TC-SCNs) and excessive collagen content (Fig. 18.10). Furthermore, impaired homocellular or heterocellular junctions connected by TCs and adjacent cells also cause dearrangement of interstitial 3-D network. And especially, TCs can also be observed to connect with the activated immunocytes with dense secretory granules (mononuclear cells, MO; eosinophils, Eo; Fig. 18.10a, c) and thus might be involved in local immunoregulation and functionally affect (repression or activation) local immune state.

These results provided the first evidence that oviduct TCs displayed inflammatory-induced extensive ultrastructure damage, with obvious cell loss, decrease or almost complete absence in the successfully constructed AS-affected oviduct tissues, which were confirmed by acute inflammation, interstitial fibrosis, numerous neutrophils infiltration and overproduced iNOS and COX-2.

Similarly, in the first part of this chapter, the aseptic-based inflammation also causes TC damage in EMs-affected oviduct tissues [45], herein strengthening the broad involvement of TC damage in infection-based inflammatory AS-affected oviduct tissues. Nevertheless, although none of the supposed pathophysiologic functions have yet been proven definitive for oviduct TCs, based on their distinct ultrastructure, distribution and immunophenotype, we suggested that TC damage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might consequently lead to structural and reproductive functional abnormalities of oviduct, probably via the following mechanisms:

(i) Ultrastructure damage, such as loss of multiple subcellular organelles within cellular body and Tps, will subsequently cause decreased synthesis of energy and substances, presumably, e.g. TC-specific genome, microRNAs and proteomic profiles, ion channels, neurotransmitter and cytoskeletal elements, which were all key elements for complex functions and activities of TCs, such as electrophysiology, dynamics of Tps in terms of adherence, spreading/extension and ramification, etc.

(ii) Swollen or loss of heterocellular (planar or point) junctions between Tps and nearby cells, e.g. capillaries, SMCs, activated immunocytes and SCs, will inevitably decrease TC-mediated intercellular signalling and proposed functions, such as tissue repair/regeneration, homeostasis/angiogenesis, immunoregulation, neurotransmission/muscular contraction, etc.

(iii) Loss of strategic position and disintegrating of extracellular 3-D architecture which resulted from TC damage, will change correct organization or mechanical support for different structural components of interstitium; disturb cell migration or chemotaxis (SCs, activated immunocytes, etc.); impair intercellular signal transduction, coordination and integration between TCs and adjacent cells; and finally cause interstitial fibrosis. Interestingly, although previous studies suggested that TC loss might occur before the onset of fibrotic reconstruction of the intestinal wall [41], now it still lacks molecular evidence to tell whether TC loss happened before the onset of oviduct fibrosis or it is just being merely a consequence of the fibrotic process.

(iv) Finally, a special intra-tubal immune state was essential for a successful fertilization, early embryonic development and transportation. However, heterocellular junctions between impaired TCs and the activated immunocytes (MO, Eo) might affect local immune state or inflammatory response by either repression or activation of secretion of various cytotoxic cytokines/enzymes. Subsequently they will likely to cause immune-mediated early pregnancy failure, such as intra-tubal sperm phagocytosis, impaired fertilization, defective early embryo growth, abnormal transportation and implantation.

Fig. 18.10 TC damage in AS-affected oviduct tissues, with tissue fibrosis. (**A**, **a**) Degenerated Tps developed heterocellular contact with activated mononuclear cells (MO), which contained dense secretory granules, together with eosinophil (Eo) and neutrophil (PMN) infiltration. (**b**) Higher magnification of the blue boxed area: degenerated Tps connected to activated MO (*black arrows*), with swollen mitochondria (m) and vacuoles (*white arrows*) in Tps. (**B**, **C**) Intercellular contacts became wider or disappeared (*black asterisks*) between a group of putative stem cells (SCs) and damaged TCs and Tps (*black arrows*), between Tps and activated eosinophils (Eo) containing dense secretory granules (*white asterisk*) in disrupted TC-SC niches. Swollen mitochondria (m), cytoplasmic vacuolization (*white arrows*) in Tps and dissolution of SMCs can also be observed. (**D**) Perivascular TCs and Tps, endothelial cell (*E*) and pericyte (*P*) damage: swollen mitochondria (m), rough endoplasmic reticulum (rER) dilatation and cytoplasmic vacuolization (*white arrows*) (Yang et al. [45] adapted with permission)

Nevertheless, future work should take the following into consideration, such as the underlying molecular and cellular mechanisms for TC damage in the setting of different oviduct diseases and downstream pathway of TC involvement in differentiation/activation of fibroblasts/fibrocytes and accompanied fibrosis, in local inflammatory process/immunoregulation and possibly in immune-mediated early pregnancy failure.

18.4 In Vitro Immunomodulation of Mouse Peritoneal Macrophages by Uterine TCs

The first two parts of this chapter describe in vivo heterocellular junctions between TCs and various adjacent immunocytes, including mononuclear cells, mast cells, eosinophils and neutrophils, both in normal and inflammatory-affected oviduct tissue in SD rat model, suggesting potential involvement of TCs in local immunoregulation or immunosurveillance. Therefore, we hypothesized that through direct heterocellular junctions or indirect paracrine effects, TCs might have the ability to influence the activity of immunocytes and then be involved in local inflammatory process or in immune-mediated various reproductive abnormalities.

Nevertheless, so far, no reliable in vitro cytological evidence is available to support the proposed immunoregulation/immunosurveillance role for TCs. The aim of this part of study is to confirm the aforementioned in vivo findings, by evaluating in vitro paracrine effect of uterine TCs on mouse peritoneal macrophages (pMACs). Therefore, mouse pMACs were designed to coculture with uterine TC-conditioned media (TCM) for 48 h, followed with study of in vitro morphology, viability and cytokines/enzyme production by pMACs [59]. Meanwhile, applying the same amount of DMEM/F12 or lipopolysaccharide (LPS) instead of TCM served as negative and positive controls, respectively.

To harvest uterine TCs, mice uterine tissues were used for primary culture, and in vitro TC identification was carried out by methylene blue staining, mitochondrial labelling and double immunofluorescence cytochemistry. And then uterine TCs were clearly identified based on its typical morphology: small bipolar or multipolar cell body with extremely long Tps, which composed of podomers and podoms; and by Tps, TCs formed homocellular contacts (Fig. 18.11a–c). In addition, active energy metabolism was indicated by mitochondria labelling (Fig. 18.11d). Furthermore, double-positive CD34/vimentin was obvious both in the cell body and its alternating thick and thin segments of Tps (Fig. 18.11e).

Morphological study of pMACs demonstrated that after 48 h of coculture with TCM, DMEM/F12 or LPS (0.5 μg m L-1), obvious activation/immunoresponse of pMACs was elicited by TCM, in contrast to overstimulation or cell death by LPS exposure and no sign of activation by DMEM/F12 (Fig. 18.12a–c). Further quantitative cell viability assay of pMACs by a cell counting kit 8 (CCK-8) indicated significant activation of pMACs by TCM and LPS, as compared to DMEM/F12 (Fig. 18.12d) (both $P<0.05$), with no significant difference between TCM and LPS ($P>0.05$), thus verifying obvious cell morphological differences among three groups.

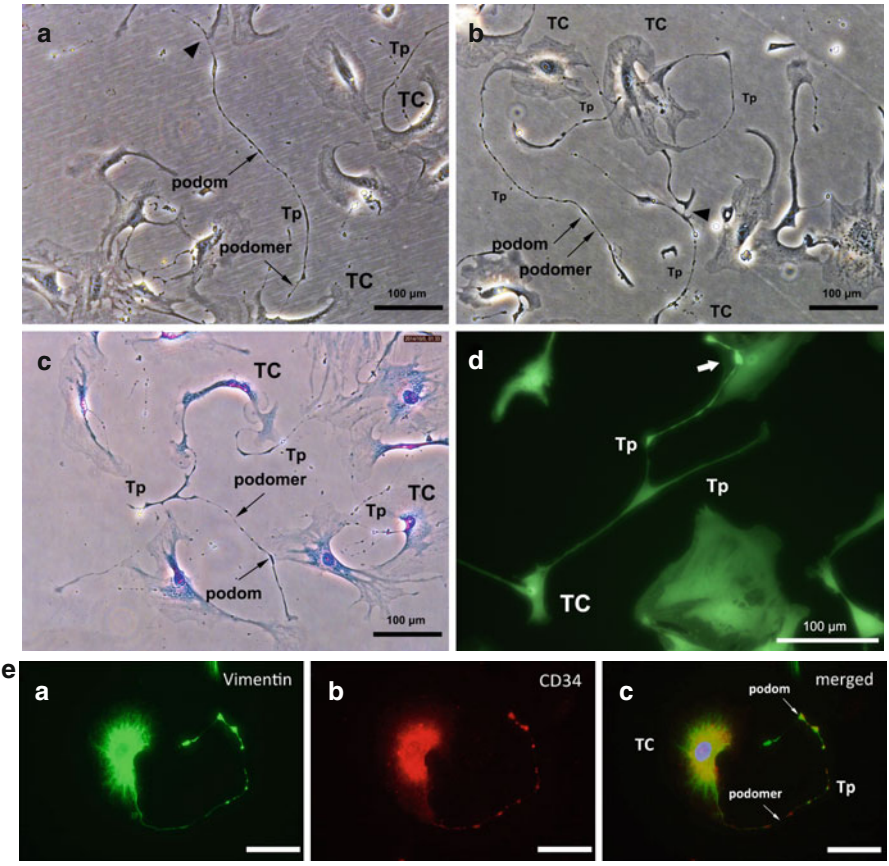

Fig. 18.11 Typical TCs in primary culture from the mouse uterus demonstrated characteristic cell body and Tps, alternating with podoms and podomers that developed homocellular network (*black arrowhead*). (**a** and **b**) Phase-contrast microscopy. (**c**) Methylene blue staining under phase-contrast microscopy. (**d**) MitoTracker Green staining indicated extensive fluorescence and cell metabolism around intercellular connections between Tps and other cells (*white arrow*). (**e**) Typical TC morphology with immunophenotype of CD34 (+)/vimentin (+)/c-kit (−) indicated by double-labelled immunofluorescence IHC. Scale bar = 50 μm (Chi et al. [59] adapted with permission)

Quantitative analysis of a panel of pMAC-derived cytokines/enzyme showed that interleukin-6 (IL-6) and iNOS were significantly elevated in TCM-treated pMACs; tumour necrosis factor a, IL1-R1 and IL-10 were also significantly, but slightly, up-regulated (all $P<0.05$; Fig. 18.13a, b). Meanwhile, no changes were observed for transforming growth factor-b1, IL-1b, IL-23a and IL-18 (all $P>0.05$; Fig. 18.13b).

These data provided preliminary in vitro evidence and support our hypothesis that TCs are not merely innocent bystanders in the interstitial compartment but are instead potential functional active players in local immunoregulation or immunosurveillance. TCs display the ability to trigger the activation of pMACs and potentially induce a subsequent in vitro immune response, manifested by morphology and viability alterations, and secretion of multiple cytokines/enzyme, likely

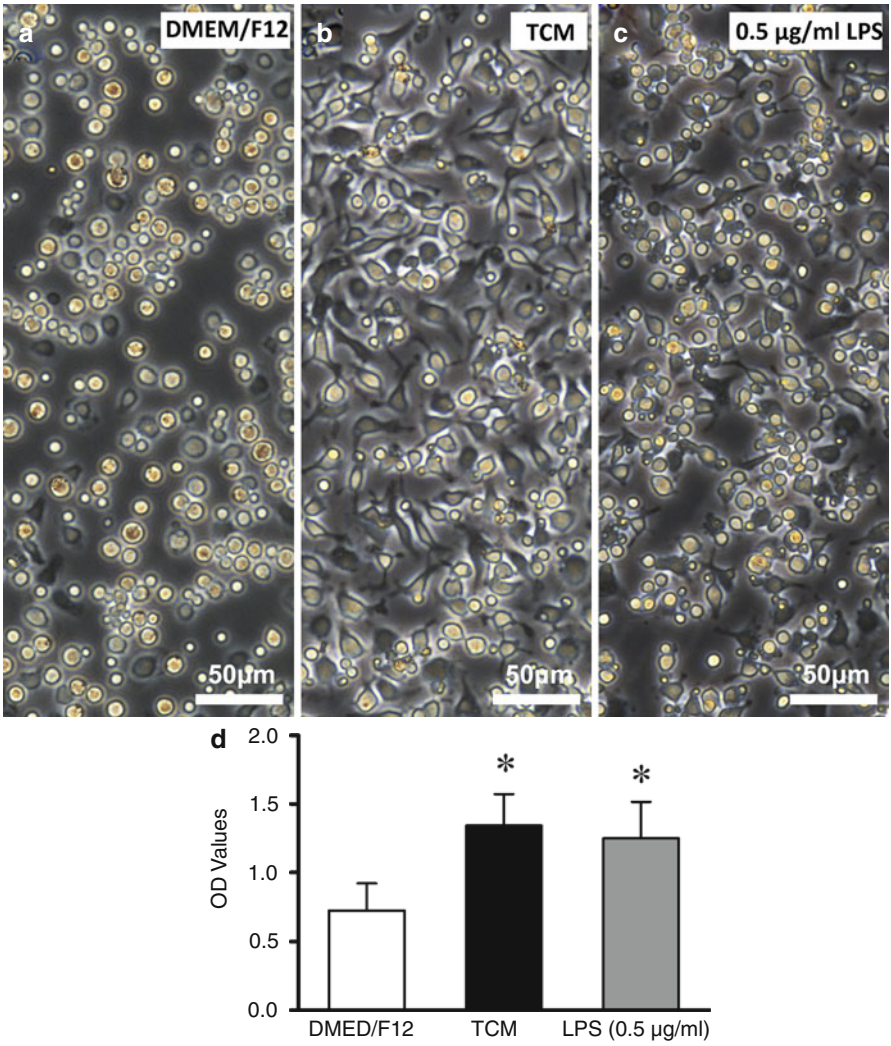

Fig. 18.12 Morphology and viability changes of mouse pMACs after exposure to TCM, DMEM/F12 or LPS (0.5 μg mL-1) for 48 h. (**a**) No signs of cell activation in DMEM/F12 group, manifested by normal morphology with regular round shape, abundant clear cytoplasm and wider intercellular spaces. (**b**) Moderate activation/immune response in TCM group, manifested by obvious morphological changes, including polyhedron shape, large and sufficient pseudopodia, abundant granules within the cytoplasm and narrow intercellular spaces. (**c**) Excessive activation in LPS group, manifested by irregular, doublet or multiple shapes and cell death, indicated by cell membrane blebbing, cell body atrophy and nuclear condensation or fragmentation. (**d**) TCM and LPS both significantly activated pMACs (*$P<0.05$ versus DMEM/F12), with no significant difference between TCM and LPS ($P>0.05$), error bars = SD (Chi et al. [59] adapted with permission)

through indirect paracrine effects. Such ability was similar to but slightly weaker than the classical stimulus, LPS.

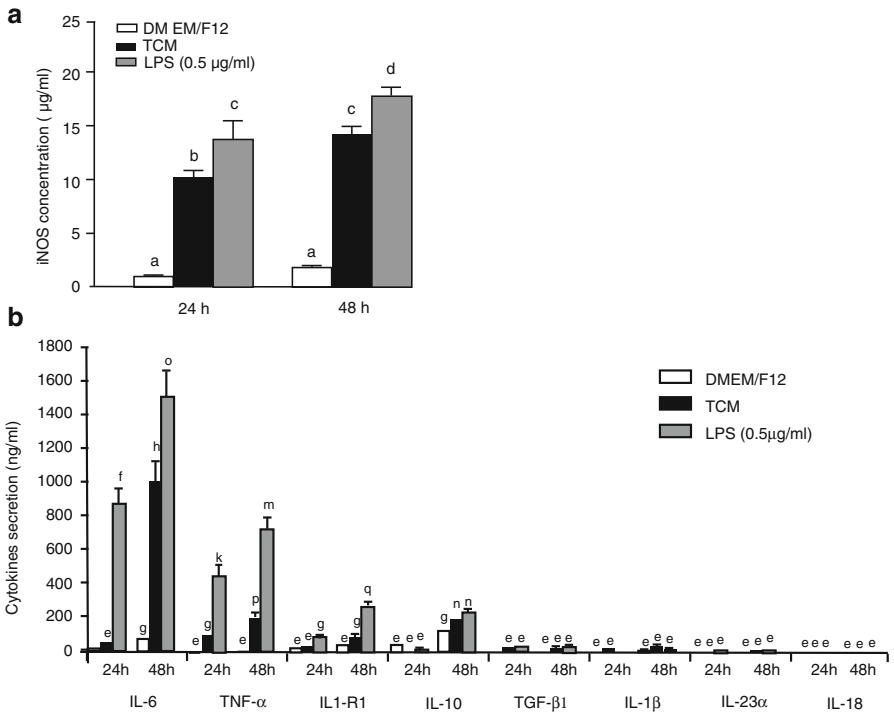

Fig. 18.13 ELISA analysis of nine pMACs-related cytokines/enzymes after exposure to TCM, DMEM/F12 or LPS (0.5 μg mL-1) for 24 and 48 h, respectively. The bars that share a common letter represent either non-significant difference ($P > 0.05$), or their values were too low for any biological behaviour. (**a** and **b**) iNOS and IL-6 were overproduced significantly, together with TNF-a, IL1-R1 and IL-10 slightly, but significantly increased in TCM and LPS groups, as compared to DMEM/F12 (all $P < 0.05$), either at 24 or 48 h, by one-way ANOVA, followed by significant differences between DMEM/F12 and test values by Dunnett's test (all $P < 0.05$) and significant differences within TCM and LPS groups at the 24 and 48 h time points, respectively, by *t*-test (all $P < 0.05$). (**b**) No significant changes in TGF-b1, IL-1b, IL-23a and IL-18 were observed (all $P > 0.05$) (Chi et al. [59] adapted with permission)

We believe that TCM inducing the panel of elevated cytokines/enzymes might play important roles in reproduction. iNOS is an enzyme catalysing the production of nitric oxide (NO) from L-arginine. iNOS produces large quantities of NO upon stimulation, such as by pro-inflammatory cytokines (e.g. interleukin-1, tumour necrosis factor alpha and interferon gamma). iNOS has been suggested to participate in host immunity, antimicrobial and antitumour activities as part of the oxidative burst of macrophages. Furthermore, it has been proposed that excessive NO generated through increased iNOS production may decrease tubal ciliary beats and smooth muscle contractions and thus affect embryo transport, which may consequently result in tubal ectopic pregnancy and tubal factor infertility and probably also in uterine contractility disorders [60].

Moreover, high IL-6 levels might cause improper endometrial environments and implantation failure. And overexpression of TNF-a not only stimulates the pathological

production of NO but also causes toxicity in multiple reproductive processes, such as implantation failure and immune-mediated abortion. Also, up-regulation of IL-1R1 by TCM might cause abnormal peri-implantation state and finally lead to failure of blastocyst implantation. Moreover, overproduction of IL-10, which was essential for a successful pregnancy, might lead to an adverse pregnancy outcome.

Generally, TCs release three types of extracellular vesicles (EVs): exosomes, ectosomes and multivesicular bodies. EVs contain many secretomes and transfer complex multimolecular biological messages from Tps [14–30, 61]. Paracrine secretion mediated cell proliferation, differentiation and tissue repair has been proposed for TCs in many studies. Specifically, uterine TCs can shed or released EVs and/or exosomes from Tps [16, 20, 25]. TCs from other organs/tissues also release soluble mediators, containing IL-6, VEGF and nitric oxide [34, 50, 61]; and IL-6, VEGF, macrophage inflammatory protein 1a (MIP-1a), MIP-2 and monocyte chemoattractant protein 1 (MCP-1), IL-2, IL-10, IL-13 and growth-related oncogene/keratinocyte-derived chemokine (GRO-KC) were significantly expressed in the secretome of cultured rodent cardiac TCs [61]. These multiple paracrine mediators might be essential for intercellular communication (long distance) and regulate the activity of neighbouring immunocytes, thus achieving the immunoregulation/immunosurveillance roles of TCM. However, behind the general concepts of paracrine effects, we still wondered what were the underlying cellular and molecular mechanisms. The exact pathway (nuclear factor kappa B, NF-kB, or else) and/or complex networks, which are responsible for the crosstalk between pMACs and TCM, maintenance of pMAC activation and subsequent overproduction of the panel of cytokines/enzymes, remain to be fully elucidated.

More questions still need to be answered, whether this immunomodulation role is only specific for uterine TCs or exists widely in other organs/tissues. And whether the observed near-infrared low-level laser stimulation of human myometrium TC growth [62] might also provide a potential choice for TC-associated abnormalities related to local reproductive immunity remains to be determined. Last but not least, to confirm this in vitro result, animal models will be more helpful to better clarify or strengthen in vivo functional consequences of the proposed immunoregulation/immunosurveillance roles of TCs. Then we can forward clinical applications of TCs in immune-mediated fertility problems and other related diseases.

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