difference in the apoptotic cell numbers between the ceftriaxone and saline sham operation groups. Our results suggest that ceftriaxone did not have significant neurotoxic effects on the developing brain, probably because manipulation of glutamate transporter activity by ceftriaxone is thought to subtly modulate glutamatergic excitatory transmission compared to NMDA receptor antagonists.[38]

Neuroprotective preconditioning, which was first described by Murry et al[39] in 1986, is a process by which a tissue is rendered more tolerant to a subsequent lethal insult, such as ischemia.[40] Tolerance to injury can be achieved by pretreatment with a sublethal stress or chemicals, which is believed to trigger a cascade of endogenous adaptive mechanisms resulting in the development of tolerance. To date, several models of preconditioning using neonatal animals have been developed,[41,42] some of which show long-lasting neuroprotection.[43] Although erythromycin and minocycline are also reported to be neuroprotective against H-I animal models,[23,24] GLT-1 upregulation by ceftriaxone preconditioning seems to be rational for immature brain as a safe and effective prophylactic approach to reduce H-I damage. However, because severe hypoxia at birth leading to neonatal HIE is rare and generally unpredictable for most term neonates, this study has a limitation as to the clinical feasibility in perinatal medicine. Although we focused on the preinsult treatment of ceftriaxone in this study, we would like to examine the possibility of ceftriaxone treatment shortly after H-I insult in a future work.

In this study, we used P7 rats as a model of "term" neonatal HIE. However, NMDA receptors are also present on developing oligodendrocytes,[44] which predominate in the cerebral hemispheres (corpus callosum and cortex) between P1 and P5 rats.[45] Additionally, excitotoxicity plays a significant role in the pathogenesis of periventricular leukomalacia in premature infants,[46] in which diffuse injury of developing oligodendrocytes with subsequent hypomyelination is the major neuropathological feature.[45] Therefore, ceftriaxone preconditioning may also be effective not only for term but also for preterm brains, which have a higher risk of encephalopathy.

In conclusion, we have shown that ceftriaxone preconditioning upregulated GLT-1 expression without histological neurotoxicity and reduced brain damage in a P7 rat H-I model. Our results and those of several studies suggest that ceftriaxone preconditioning may have a greater potential if applied to the prevention of neonatal encephalopathy. Further studies are necessary to determine the long-term neuroprotective effects and safety in order to establish a new prophylactic strategy for human neonates in clinical perinatal medicine.

Authors' Note

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Declaration of Conflicting Interests

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EXPERT REVIEWS

Clinical implication of recent advances in our understanding of IL-17 and reproductive immunology

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The identification of a novel helper T (Th)-cell subset, the IL-17-producing Th (Th17) cells, has provided new insight into our understanding of the molecular mechanisms of reproduction. IL-17 has an important role in induction of the protective immune response against extracellular bacteria or fungal pathogens. Th17 cells seem to participate in successful pregnancy processes. Th17 cells also play a pivotal role in pathogenesis of endometriosis, miscarriage, preterm labor and preeclampsia. Recent data show the reciprocal development of pathways between Th1/Th17 subsets and between Th17/Treg subsets, and the imbalance of Th17/Treg development has been reported in recurrent pregnancy loss and preeclampsia.

Keywords: abortion • endometriosis • IL-17 • pregnancy • preterm labor

In the 1980–1990s, Wegmann *et al.* proposed that maternal tolerance toward fetal alloantigens was explained by the predominant Th2-type immunity during pregnancy [1]. Other reports support this idea that predominant Th2-type immunity protects the fetus from maternal Th1 cell attack [2,3]. However, this simplistic characterization did not fully explain the immunopathogenesis of abortion/miscarriage [4,5]. Indeed, Th2-dominant immunity was reported in recurrent miscarriage cases and, even in combined absence of IL-5, IL-9 and IL-13, these mice show normal pregnancy, even in allogeneic pregnancy [6]. However, this work did not include IL-10 – a Th2-type cytokine that has been reported to be of importance in pregnancy [7–10]. Th1 cells are believed to be involved in the pathogenesis of autoimmune disease, but neutralization of IFN-γ resulted in more pronounced tissue injury and worsening of symptoms in mice with experimental autoimmune encephalitis [11]. Furthermore, deletion of the p35 subunit of IL-12, a model for depletion of Th1-type immunity, enhanced collagen-induced arthritis [12]. However, IL-23- or IL-17-defective mice have reduced susceptibility to autoimmune or chronic inflammatory diseases [13,14]. The expression of IL-17 appears to be increased in human autoimmune diseases such as multiple sclerosis, rheumatoid arthritis and psoriasis [15–17]. It has recently been clarified that the newly described Th17 subset defined by secretion of IL-17 plays an important role for induction of autoimmune disease. Th17 cells play an important role in enhancing host protection against extracellular pathogens and fungi that are not efficiently cleared by Th1 and Th2 responses [18,19].

At the same time, it has been revealed that Th cells are regulated by regulatory T (Treg) cells [20,21]. Treg cells are potent suppressors of inflammatory immune responses, and are essential in preventing allograft rejection. Treg cells play pivotal roles for maintenance of allografted pregnancy in mice and humans [22–24]. Therefore, the classic Th1/Th2 paradigm of effector T-cell differentiation has been recently expanded by the discovery of another lineage of T-helper cells that include Th17 and Th9 cells [25], and immunoregulatory T cells, termed Treg cells. We should therefore discuss again the reproductive events from the view points of the new Th1/Th2/Th17 and Treg paradigms.

Differentiation of Th1, Th2, Th17, Th9, Tr1 & Treg cells from naive CD4⁺ T cells

Th1 cell differentiation is triggered by the presence of IL-12, and upregulation of transcription

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factor T-bet induces the differentiation of Th1 cells, which produce IFN-γ and TNF-α/β and are involved in cell-mediated immunity and allograft rejection (Figure 1). Th17 cell differentiation occurs via the synergistic activation by TGF-β and IL-6 in mice or IL-1β and IL-6 or IL-1β and IL-23 in humans (Figure 1) [17,26]. IL-23 is produced by activated antigen-presenting cells, and IL-23 promotes proliferation of IL-17-producing cells. IL-23 is important to maintain the Th17 phenotype. Recent data demonstrated that microRNA (small, conserved and noncoding RNA) modulate the development of Th17 cells (Figure 1) [27]. Ets-1, the prototype member of the Ets family of transcription factors, is a negative regulator of Th17 cell differentiation [28]. Interestingly, microRNA326 inhibits the induction of Ets-1, resulting in induction of Th17 cells. In addition, microRNA326 downregulates Ets-1 expression in humans [29]. Th17 cells are involved in host protection against Gram-negative bacteria and fungi [18,19], induction of autoimmune diseases [7,12–17] and transplant rejection [30–33]. It is very interesting that reciprocal developmental pathways exist between the Th1/Th17 subset and Th17/Treg subset (Figure 1) [15–17]. When proinflammatory cytokines such as IL-1β and/or IL-6 and TGF-β are present, these Th17/Treg progenitor cells differentiate into Th17 cells. Recent data demonstrated that TGF-β1 was highly expressed by Th17 cells and acted in a predominantly autocrine manner to maintain Th17 cells *in vivo* [34]. On the other hand, a high TGF-β concentration induces the differentiation to Treg cells (Figure 1). Stimulation by IL-12 induces Th1 polarization; and, conversely, stimulation by IL-1β, IL-6 or IL-23 induces Th17 polarization. These data show that Th1/Th2/Th17 and Treg lineages are associated with each other, and that they are able to convert to other lineages based on environmental factors [35–38]. Recently, very important findings that Treg cells promote Th17 cell development *in vivo* have been reported [39,40]. Treg cell depletion resulted in a reduced frequency of Th17 cells, correlating with reduced inflammatory skin responses [40]. Upregulation

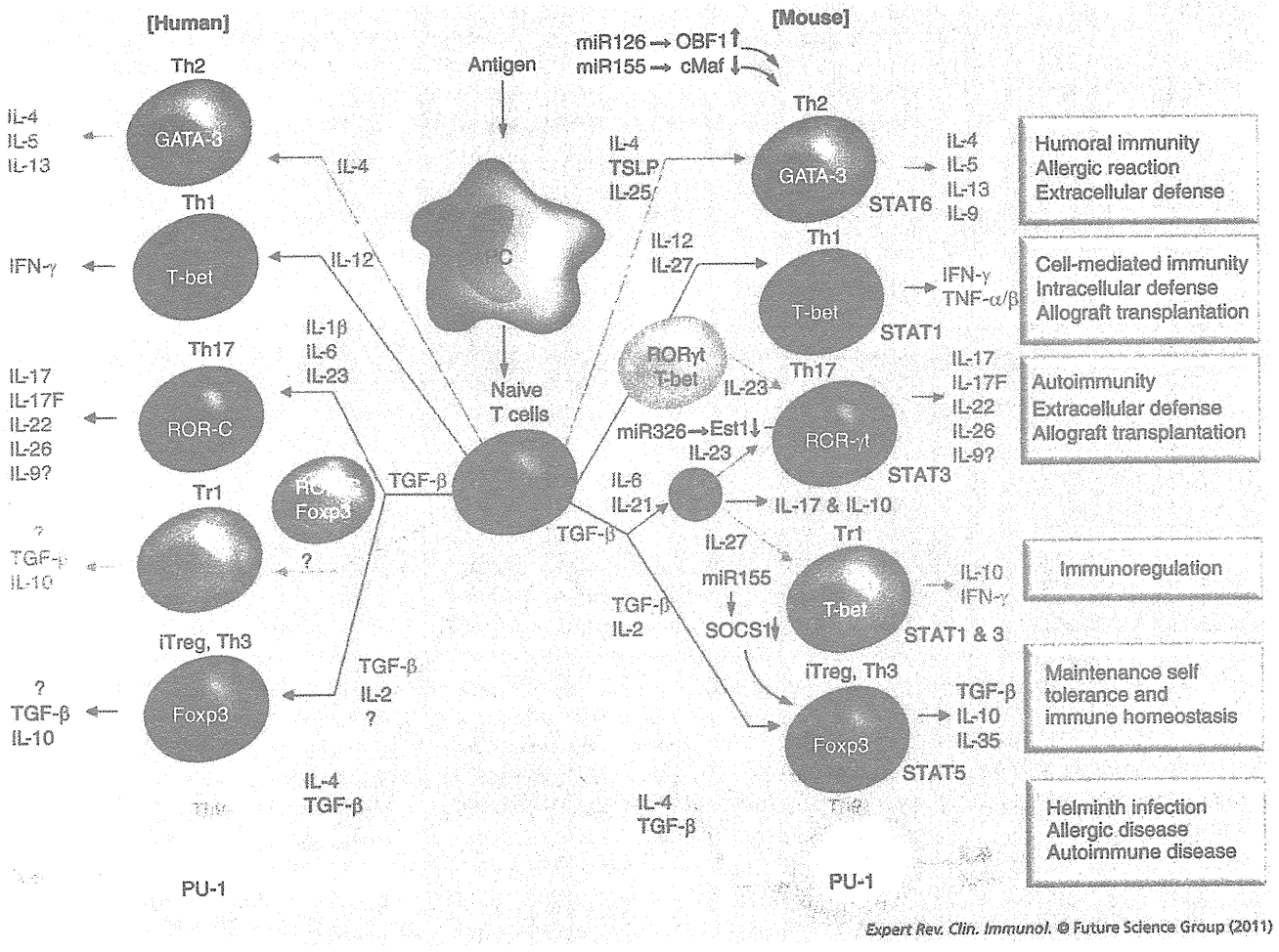

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Figure 1. Differentiation of Th1, Th2, Th3, Th9, Th17, Tr1 and Treg cells from naive CD4+ T cells. Most of the essential transcription factors for each T-helper cell lineage are similar between human and mouse, but the environmental cytokines profile that induces the differentiation to each cell lineage is different between human and mouse. Interestingly, reciprocal developmental pathways between the Th1/Th17 subset and Th17/Treg subset have been reported. The cytokine profile could determinate the differentiation of these progenitor cells into Th1 cells or Th17 cells or Treg cells. Essential cytokines for the differentiation of T-helper cells are shown in red text, and supporting cytokines for their differentiation are shown in green text.

of IL-17 secretion in responding CD4+ T cells was dependent on consumption of IL-2 by Treg cells at early time points, both *in vitro* and *in vivo* [39]. Importantly, Treg cells induced IL-17 secretion, resulting in enhanced fungal clearance and recovery from fungal infection during an oral *Candida albicans* infection in mice [40]. These findings suggest that Treg cells have a capacity to fight infections in addition to their classical role in maintaining tolerance or immunohomeostasis. In healthy conditions, this balance is well coordinated, but imbalance of this differentiation is observed in some pathological conditions [38,41]. Addition of TGF-β and IL-4 during T-cell activation induces Th9 cells, which are involved in helminth infection, allergic disease and autoimmune diseases. Recent data show that both IL-9+ and IL-17+ cells are found *in vivo* [37]. Th17 cells producing IL-9 may co-exist and interact with Th9 cells. Therefore, it is very important to re-evaluate the Th1/Th2/Th17/Th9/Tr1 and Treg cell balance in reproduction.

Role of Th17 cells

Th17 cells produce IL-17A (IL-17), IL-17F, IL-22 and TNF-α. The IL-17 family is composed of IL-17A, IL-17B, IL-17C, IL-17D, IL-17E (IL-25) and IL-17F. The IL-17A homodimer (IL-17A/A); IL-17A heterodimer for IL-17F (IL-17A/F) or the IL-17F homodimer (IL-17F/F) bind to the IL-17 receptor (IL-17R)A and IL-17RC complex (Figure 2). IL-17 is produced by CD4+ Th17 cells, some populations of CD8+ T cells, natural killer (NK) cells, γδT cells, neutrophils, eosinophils and monocytes; however, the main producer is Th17 cells. IL-17RA is highly expressed on hematopoietic cells and lowly expressed on osteoblasts, fibroblasts, endothelial cells and epithelial cells. Human IL-17RC binds human IL-17RA with high affinity, but mouse IL-17RC does not bind mouse IL-17 (Figure 2). The signal is transuded by the Act 1-dependent pathway or Act 1-independent pathway (Figure 2) [42,43]. These signal pathways induce the production of proinflammatory cytokines and chemokines and induce the recruitment of neutrophils.

Th17 cells play an important role in host defense to protect against extracellular bacteria, *Mycobacterium tuberculosis* and fungus [18,19]. When pathogens invade the mucosa, IL-17 is produced by Th17 cells, γδT cells and NK cells, and induces the production of CXC chemokines, G-CSF, and antimicrobial protein such as β-defensin 2, HBD2, lipocalin and calgranulins, resulting in protection from bacterial infection. Th17 cells appear at the site of inflammation with rapid kinetics, and bridges the gap between innate and adaptive immunity. It was believed that autoantigen-specific Th1 cells cause organ-specific autoimmune diseases. Additionally, many data have indicated that Th17 cells induce inflammation and tissue damage by inducing autoimmune tissue inflammation both in experimental animals and in humans [7,12–19]. Thus, a sequential involvement of Th1 cells and Th17 cells play an important role in the pathophysiology of autoimmune diseases.

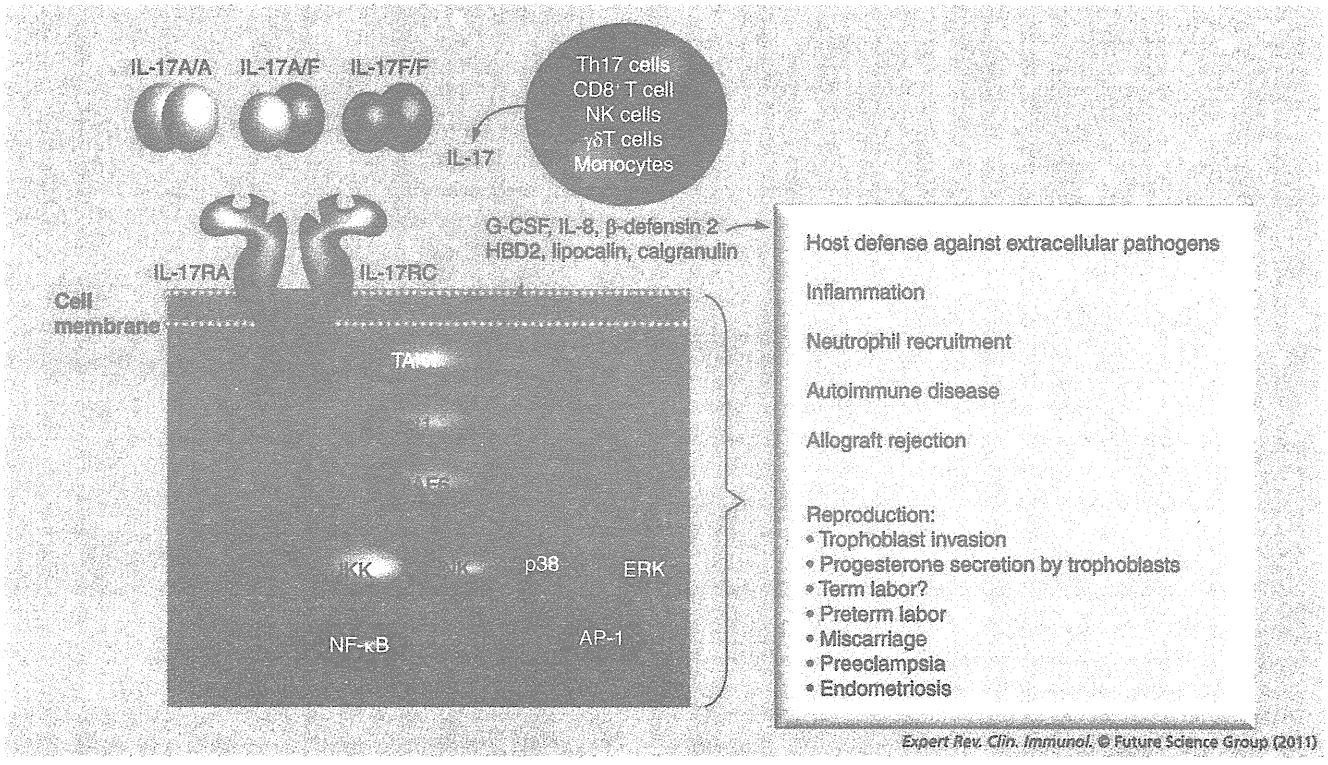

Figure 2. IL-17 and IL-17 receptor signal pathway. IL-17A homodimer (IL-17A/A), IL-17A heterodimer for IL-17F (IL-17A/F) and IL-17F homodimer (IL-17F/F) bind to IL-17 receptor A and IL-17 receptor C complex. This signal is transduced by IKK–NF-κB, JNK, p38MAPK or ERK pathways. IL-17 also plays important roles in host defense against extracellular pathogens, inflammation, neutrophil recruitment, autoimmune diseases, allograft rejection and reproduction.
IL-17R: IL-17 receptor; NK: Natural killer.

Another important role for IL-17 is that Th17 cells may contribute to allograft rejection [30–33]. Neutralization of IL-17 significantly improved graft survived in a rat cardiac allograft model [44], and the upregulation of IL-17 and IL-23 was observed at the site of rejection and draining lymph nodes [45]. Furthermore, accumulated neutrophils at the graft induced by IL-17 could induce damage in the grafted organ [46]. Although these data suggest a role for IL-17 in allograft rejection, the direct involvement of Th17 cells has not been proved.

IL-17 in the pathogenesis of endometriosis

Endometriosis is a common gynecologic disorder, affecting at least 10% of reproductive-age women, and is characterized by the outgrowth of endometrial tissue outside the uterus cavity. The condition results in severe pelvic pain, and the prevalence of pelvic endometriosis is greater in infertile than fertile women. Multiple lines of evidence suggest that inflammation and immune responses play a pivotal role in the pathogenesis of endometriosis [47,48]. Production of various inflammatory cytokines such as IL-6, IL-8, IL-1, TNF-α, CCL5 (RANTES) and CCL2 (MCP-1) has been reported in endometriosis. Hirata *et al.* first showed the presence of Th17 cells in the peritoneal fluid of endometriotic women by flow cytometry and the presence of IL-17A-producing cells in endometriotic cells by immunohistochemistry [49]. Interestingly, they showed that IL-17 enhanced IL-8 secretion from endometriotic stromal cells (ESCs). Inhibitors of p38MAPK, p42/44 MAPK, and JNK suppressed secretion of IL-8 from IL-17-activated ESCs, suggesting that MAPK plays an important role in IL-8 production. Furthermore, a cooperative effect of IL-17 and TNF-α on IL-8 secretion by ESCs was also observed. IL-17 also enhanced the expression of cyclooxygenase-2 (*COX-2*) mRNA and the proliferation of ESCs. These data clearly demonstrate that IL-17 stimulates inflammatory responses and proliferation of ESCs, suggesting a role for IL-17 in the pathogenesis of endometriosis.

IL-17 in normal pregnancy

Most of the IL-17-producing cells are $CD4^+$ T cells in the peripheral blood and deciduas [50–52]. Our group reported that Th17 levels in peripheral blood lymphocytes did not change during normal pregnancy [50], but Santner-Nanan *et al.* reported that the frequency of Th17 cells in peripheral blood in the late pregnancy period was decreased compared with that in nonpregnant women [53]. We should clarify whether Th17 cell levels are stable during pregnancy or decrease in the late pregnancy period.

The proportion of $IL\text{-}17^+$ decidual lymphocytes is significantly higher than that of peripheral blood lymphocytes [50]. *IL-17* mRNA is expressed in purified cytotrophoblasts and syncytiotrophoblasts, placental macrophages (Hofbauer cells) and extravillous trophoblasts [54]. Ostojic *et al.* reported that $IL\text{-}17^+$ cells were localized in the glands and in the basal proliferative stromal cells at days 6.5, 8.5, 9.5 and 10.5 in mice [55]. Our group showed that IL-17-stained cells were restricted in lymphocytes, and staining for IL-17A was not observed in villous trophoblasts, extravillous trophoblasts, endometrial gland cells and ESCs [51]. We have previously shown that recombinant IL-17 inhibits staining for IL-17 and, therefore, specificity for immunostaining should be elucidated. *IL-17R* mRNA and protein are observed in JEG-3 human choriocarcinoma cells [56]. IL-17 also increases the invasive capacity of JEG-3 cells but has no effect on the proliferation of JEG-3 cells [56]. In addition, IL-17 increases progesterone secretion by JEG-3 cells but has no effect on hCG secretion [57]. These findings suggest that IL-17 plays some role in the establishment of pregnancy. Serum IL-17 levels increase in the third trimester in healthy women with term labor [58], suggesting that increased IL-17 might induce inflammation and labor. We should evaluate whether production of IL-17 by Th17 cells increases or whether the population of Th17 cells increases in the labor period.

IL-17 in the pathogenesis of miscarriage

The involvement of Th17 cells in transplant rejection has been reported [30–33]. The fetus is an allograft to the maternal host, and therefore it is of interest to study whether Th17 cells increase in miscarriage cases. Wang *et al.* reported that the proportion of Th17 cells in the peripheral blood and decidua was significantly higher in inevitable abortion, that is, a progressive stage of abortion in unexplained recurrent spontaneous abortion (RSA) compared with women with a normal early pregnancy [59]. Furthermore, the expression of not only IL-17 but also the Th17-inducing cytokine, IL-23, and essential transcription factor for Th17, RORc, in peripheral blood and decidual tissue in unexplained RSA was significantly higher than in normal pregnancy subjects [59]. Reciprocal differentiation between Th17 cells and Treg cells has been reported, and a decrease in Treg cells in unexplained RSA has been reported [23,60,61]. Depletion of Treg cells in the early pregnancy period induces implantation failure and abortion in allogeneic murine pregnancy [62]. Wang *et al.* reported an inverse relationship between the number of Th17 and Treg cells in the peripheral blood and decidua [59]. This finding is also supported by Liu *et al.* [63]. Recent data show that estrogen and placental protein 14 induce Treg cell differentiation and reduce the secretion of IL-17 [64,65]. On the other hand, proinflammatory cytokines, such as IL-6 and IL-1β, augment the differentiation of Th17 cells. The balance between these factors could influence the Th17/Treg cell balance during pregnancy [38]. Wang *et al.* also reported that $CD4^+CD25^+$ Tregs in healthy controls inhibited IL-17 secretion by stimulated $CD4^+$ T cells, and this regulation was disturbed in unexplained RSA cases [66]. Cell-to-cell contact is necessary for this suppression. High-dose TGF-β and intermediate doses of IL-10 inhibited the secretion of IL-17 by stimulated $CD4^+$ T cells [66]. This finding supports the paper by Cao *et al.* that showed that Treg cells isolated from the joints of rheumatoid arthritis cases could inhibit the secretion of IL-17 from effector T cells [67]. However, another group reported that Treg cells suppress the proliferation of Th1 and Th2 clones but not Th17 cells, and IL-17 secretion could not be suppressed by Treg cells [68,69]. Persistent Toll-like receptor (TLR) or IL-6 stimulation suppresses Treg function; therefore, chronic inflammation may reduce Treg cell function. Further studies are necessary to clarify the function of Treg cells in unexplained RSA cases. We have also reported that the number of decidual $IL\text{-}17^+$ cells increased in inevitable

abortion and the number of neutrophils was well correlated with the number of IL-17$^+$ cells [52]. Importantly, the population of Th17$^+$ cells did not change in the decidua of missed abortion cases that did not show vaginal bleeding, uterine cramping or cervical dilation [52]. These findings suggest that increased Th17 cells might not be the cause, but rather, the effect of miscarriage.

IL-17 in the pathogenesis of preterm labor

Chorioamnionitis is a major risk factor for preterm delivery. Chorioamniotic tissues are characterized by an intense infiltration of maternal neutrophils. Plasma IL-17 levels at 15–27 weeks of gestation are not significantly elevated in preterm labor with chorioamnionitis [70]. We have collected amniotic fluid in preterm labor cases at 22–34 weeks of gestation. Amniotic fluid levels of IL-8 and IL-17 in preterm delivery cases are significantly higher than those in term delivery cases, and these cytokine levels are especially high in the progressive stage of chorioamnionitis [51]. A positive correlation between amniotic IL-8 level and amniotic IL-17 level was observed. We detected IL-17$^+$CD3$^+$ T cells in chorionic plates in preterm labor cases with chorioamnionitis, and flow cytometrical examination demonstrated that most IL-17-producing cells were CD4$^+$ Th17 cells. Interestingly, immunostained IL-7R-positive cells were observed in human amniotic mesenchymal (HAM) cells but not in human amniotic epithelial (HAE) cells. Neither IL-1β or IL-17 alone affect the secretion of IL-8 in both HAM and HAE cells, but TNF-α increases IL-8 secretion significantly in both cells. Interestingly, IL-17 significantly augments the TNF-α-induced secretion of IL-8 in a dose-dependent manner. IKK inhibitor, p38 MAPK inhibitor, JNK1/2 inhibitor and ERK1/2 inhibitor effectively suppress the IL-8 secretion by IL-17- and TNF-α-stimulated HAM cells, and inhibition by IKK inhibitor is greatest [51], suggesting that the IKK–NF-κB pathway is the most critical for the signal transduction by both IL-17 and TNF-α. These findings suggest that not only macrophage- or fibroblast-derived proinflammatory cytokines, but also T-cell-derived IL-17 plays an important role in the induction of inflammation at the feto–maternal interface in preterm delivery.

IL-17 in the pathogenesis of preeclampsia

Preeclampsia (PE) occurs in 3–5% of pregnancies and is a major cause of maternal and neonatal mortality. These patients present with high blood pressure and proteinuria in the second half of pregnancy. Chronic inflammation, shallow extravillous trophoblast invasion to the uterine spiral artery, poor angiogenesis with an excess amount of soluble Flt-1 and endoglin, and inadequate tolerance are believed to be involved in the pathogenesis of PE [71–73]. Interestingly, the size of peripheral blood Treg cells is decreased in PE [52,74,75], but the suppressive activity of Treg cells is not disturbed [52]. Santner-Nanan *et al.* reported that the size of peripheral blood Th17 cells decreased in normal pregnancy, but this change was not observed in PE cases [53]. However, our group reported that the size of peripheral blood Th17 cells was stable during pregnancy [50], and therefore further studies are needed to clarify this discrepancy. Jianjun *et al.* reported that the expression levels of *FOXP3* mRNA, a master gene for Treg differentiation, were decreased in both peripheral blood mononuclear cells and decidua, and that *T-bet* mRNA expressed in Th1 cells and *RORc* mRNA expressed in Th17 cells were significantly increased, suggesting that decreased Treg cells and a predominant Th17- and Th1-type immunity are present in PE [76].

It has been reported that soluble endoglin, an inhibitor of TGF-β-receptor signaling, is high before the onset of PE [73]. Increased soluble endoglin may inhibit the TGF-β signal, resulting in the reduction of Treg cells and the expansion of Th17 cells. Therefore the chronic inflammation theory, endothelial dysfunction theory, poor angiogenesis theory and immunomal adaption theory in the pathophysiology of PE might be linked by imbalanced differentiation of Treg cells and Th17 cells [77]. Predominant Th1-type immunity has been reported in PE [78–81]. Interestingly, Th1-type immunity could induce inflammation [78], therefore increased production of inflammatory cytokines such as IL-6 or IL-1β might induce Th17 cell differentiation from Th17/Treg progenitor cells. Recently, we reported that the amount of angiogenetic factor VEGF in peripheral blood NK cells and T cells was decreased [82]. Also, the amount of galectin 1, which induces tolerance, in peripheral blood CD4$^+$ T cells, CD8$^+$ T cells and NK cells was down regulated in PE [83]. Granulysin plays an important role for the induction of apoptosis of extravillous trophoblasts in miscarriage cases [84]. Intracellular granulysin in T cells was dramatically decreased in PE, but intracellular granulysin in NK cells did not change [85], suggesting that granulysin-positive T cells might attack extravillous trophoblasts, resulting in dysregulation of vascular remodeling. Predominant Th1 and Th17 immunity and decreased Treg cells might disturb the tolerance, resulting in rejection of the fetus.

Expert commentary

In this article, we have reviewed the role of IL-17 in reproduction. IL-17 increases the invasive capacity of the choriocarcinoma cell, JEG-3, and IL-17R is expressed on extravillous trophoblasts [56], suggesting that Th17 cells might play very important roles for implantation and placentation. IL-17 also increases progesterone secretion by JEG-3 cells, which is an essential factor for maintenance of pregnancy [57]. More information is needed to clarify which IL-17 plays important roles in the establishment of pregnancy.

Many studies have demonstrated that Th17 cells play crucial roles in host defense [15–19], and IL-17 induces the host defense molecules such as β-defensin 2, HBD2, lipocalin and calgranulin on the mucosal membrane (Figure 2). Therefore, IL-17 could play important roles for preventing pathological infection in reproductive organs, although this has not been reported. Further studies are needed to clarify the role of IL-17 on the defense mechanisms in reproductive organs; if excessive IL-17 is produced or both TNF-α and IL-17 are produced at the inflammation site, IL-8 production and *COX-2* expression are dramatically enhanced, causing neutrophil recruitment and tissue damage. These phenomena are observed in endometriosis and preterm labor [49,51]. As another important finding, the imbalance of Th17/Treg cells

is observed in miscarriage and PE [53,58,60,76,77]. We need to clarify the mechanism that explains why the imbalance of Th17/Treg cells is present in these cases. Additionally, we should examine the Th1/Th2/Th3/Th17 and Treg cell balance in the normal pregnancy state and abnormal pregnancy state such as miscarriage, preterm labor or PE. These systemic analyses have not been reported yet in reproductive biology. We may discover new biological mechanism in reproduction based on these new paradigms.

Five-year view

In the next 5 years, we expect progress in the clarification of reproduction from the view point of the new Th1/Th2/Th3/Tr1/ Th17 and Treg cell paradigms. These new findings are very important to determine the pathophysiology of infertile, endometriosis, unexplained recurrent pregnancy loss, preterm labor or PE, and this information will be useful to find new therapies for these diseases. Furthermore, we should reanalyze the new Th1/Th2/Th3/Tr1/Th17 and Treg paradigms in pregnant women complicated with autoimmune diseases.

We should study the function of IL-17 in normal reproduction events such as ovulation, implantation and pregnancy. And finally, we should compare the function of IL-17 between normal pregnancy and complicated pregnancy such as miscarriage, PE and preterm labor.

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Key issues

- The Th1/Th2 paradigm has now developed into the new Th1/Th2/Th3/Tr1/ Th17 paradigm.
- T-helper cells are regulated by Treg cells.
- Reciprocal development pathways between Th1/Th17 subsets and between Th17/Treg cell subsets have been reported.
- Imbalanced differentiation of Treg cells and Th17 cells has been reported in preeclampsia and unexplained recurrent pregnancy loss.
- IL-17 play important roles in host defense and the establishment of pregnancy, but excessive IL-17 induces severe inflammation causing tissue damage in endometriosis, miscarriage, preterm labor and preeclampsia.
- IL-17 and TNF-α activate NF-κB and MAPK pathways and induce IL-8 secretion in amniotic mesenchymal cells and endometriotic stromal cells.
- Both innate immune cells and IL-17-producing T cells promote inflammation in reproductive organs.

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Original article

Evaluation of Ability for Basic Movement Scale for Children Type T (ABMS-CT) for disabled children

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Abstract

Background: The objective of this pilot study was to test the validity and reliability of a new scale, the Ability for Basic Movement Scale for Children Type T (ABMS-CT).

Methods: Forty-nine pediatric patients with disabilities (aged 1.00–15.17 years; 29 males and 20 females) participated in this prospective study. To prove the validity and reliability of the ABMS-CT, subjects were administered the ABMS-CT by two physicians. In addition to the ABMS-CT score, data on age, diagnosis, and results of the Functional Independence Measure for Children (WeeFIM) were recorded.

Results: Spearman's rank correlation coefficient analysis showed that the ability to perform basic movements according to the individual scores for each item on the ABMS-CT and the total scores of the ABMS-CT correlated significantly with the total scores of the motor and cognitive WeeFIM, respectively ($r = 0.753$–0.892, $p = 0.0001$). The five items on the ABMS-CT had appropriate internal consistency (Cronbach's $\alpha = 0.966$). Inter-rater reliability analysis indicated that the "oral and facial area", "hands and fingers", "one leg", "both legs", and "stairs" items on the ABMS-CT had almost perfect reliability ($\kappa = 0.854$–0.925).

Conclusion: This study provides evidence for the validity and reliability of the ABMS-CT with regard to assessment of the functional ability for complex movements in disabled pediatric patients even if they can walk independently.

Keywords: Pediatric patients with disability; Ability for Basic Movement Scale for Children Type T (ABMS-CT); Rehabilitation; Complex basic movements

1. Introduction

We previously reported evidence of the validity and reliability of the Ability for Basic Movement Scale for Children (ABMS-C) [1]. High correlations were shown between the Gross Motor Function Classification System (GMFCS) score and all items on the ABMS-C, and test-retest reliability of each task was established using a κ coefficient. Based on these results, we believe that the ABMS-C is appropriate to evaluate the functional ability of pediatric patients to make basic movements. On the other hand, the ABMS-C is insufficient to evaluate more complex basic movements in children in early childhood but after infancy who can walk independently. Therefore, we developed a new scale, the Ability for Basic Movement Scale for Children Type T (ABMS-CT) to evaluate the ability of pediatric patients to perform complex basic movements. The objective of this pilot study was to test the validity and reliability of the ABMS-CT by assessing the relationship between

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the inter-rated ABMS-CT score and the scores for activities of daily life as assessed by the Functional Independence Measure for Children (WeeFIM) at a medical examination prior to rehabilitation services. In 1987, the Functional Independence Measure (FIM) was adapted for use in pediatrics by a multidisciplinary team of physicians, nurses, and therapists [2]. The resulting scale, known as WeeFIM, is a measure of functional abilities and the need for assistance that is associated with various levels of disability in children 6 months to 7 years old age. It can also be used with children well beyond the age of seven when delays in functional performance are evident. The WeeFIM is being most widely used in the area of pediatric rehabilitation medicine. Therefore, in assessing the validity and reliability of the ABMS-CT, we determined whether results of the ABMS-CT and the WeeFIM were comparable.

2. Material and methods

2.1. Subjects

From March to April 2011, 49 pediatric patients with motor and/or cognitive impairment at the National Center for Child Health and Development received an examination prior to rehabilitation services. There were 29 males and 20 females, and their median age was 4.50 (range 1.00–15.17 years) years. Of the 49 patients, nine had cerebral palsy, seven spinal bifida, seven traumatic brain injury, five chromosomal abnormality, three delayed language, three pervasive developmental disorders, two a hearing disorder, two hydrocephalus, two Chiari malformation, two encephalitis, one osteogenesis imperfecta, one epilepsy, and five had other conditions. All of the patients had some delay in motor and/or cognitive development.

2.2. Tests

Following admission, all study patients received an examination in preparation for rehabilitation. As part of that examination, we assessed their ability to perform basic movements at the bedside by using the ABMS-CT and functional abilities by the WeeFIM. The five-item ABMS-CT consists of items on "oral and facial area", "hands and fingers", "one leg", "both legs", and "stairs". These five items are assessed as follows: oral and facial area, determination of whether the patient can move the tongue, lips, and cheeks according to instructions; hands and fingers, patient is instructed to hold up all five fingers, then fold one finger at a time; one leg, determination whether the patient can stand on one leg for over 5 s and hop on one leg; both legs, patient is asked to stand on tiptoe with both feet for more than 1 s, jump forward with two feet together, and skip; and stairs, patient is asked to climb stairs. Fig. 1 shows details of the instructions given during the evaluations and the ABMS-CT scoring system. The ABMS-CT is a very simple scale to assess complex basic movements that small children can perform, but its purpose is not to assess age-appropriate movements. Thus, we did not group study patients according to age.

The WeeFIM utilizes the same items and rating scale as the Adult FIM. The 18 items are organized into six subscales of self-care, sphincter control, transfers, locomotion, communication, and social cognition. Each item is scored on a seven-level ordinal scale ranging from complete independence to need for total assistance. Children in level 7 can perform activities with complete independence (timely, safely); children in level 6 can perform activities with modified independence (assistive device needed); children in level 5 can perform activities with modified dependence (supervision or setup); children in level 4 can perform activities with modified dependence (minimal assistance, subject participation = 75% +); children in level 3 can perform activities with modified dependence (moderate assistance, subject participation = 50% +); children in level 2 are almost completely dependent in performing activities (maximal assistance, subject participation = 25% +); and children in level 1 are completely dependent in performing activities (total assistance, subject = 0% +).

In this study, we recorded the total scores of the motor WeeFIM and cognitive WeeFIM. The total scores of the motor WeeFIM consist of scores for the subscales of self-care, sphincter control, transfers, and locomotion. The total scores for the cognitive WeeFIM consist of scores for communication and social cognition.

2.3. Data analysis

Using Spearman's rank correlation coefficients, we examined the strength of the association between the ABMS-CT grade and the total scores of the motor WeeFIM and cognitive WeeFIM, respectively, in all participants. In addition, 42 of the 49 subjects were retested by the ABMS-CT by another physician on the same day. Internal consistency of the five items comprising the ABMS-CT was checked by Cronbach's coefficient alpha (Cronbach's α). Inter-rater reliability for each task was established using a kappa (κ) coefficient. Data were analyzed using SPSS 12.0 J software (SPSS Japan, Inc., Tokyo, Japan).

3. Results

Spearman's rank correlations among complex basic mobility as measured by the ABMS-CT, functional ability as measured by the WeeFIM and age for the 49 study subjects are shown in Table 1. Spearman's rank

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grade	0	1	2	3
Oral and facial area	cannot move lips as instructed 	can stick tongue straight out 	can pucker lips 	can puff out right and left cheek alternately 
Hands and fingers	cannot move fingers as instructed 	can hold up one finger 	can hold up two fingers 	can fold one finger at a time 
One leg	cannot stand on one leg 	can stand on one leg for under 5 seconds 	can stand on one leg for 5 seconds or more 	can hop on one leg 
Both legs	cannot stand on tiptoe with both feet for over 1 second 	can stand on tiptoe with both feet for over 1 second 	can jump forward with two feet together 	can skip 
Stairs	cannot climb stairs 	can climb stairs using the handrail with 2 feet per step 	can climb stairs using the handrail with one foot per step 	can climb stairs without using the handrail with one foot per step 

Fig. 1. The Ability for Basic Movement Scale for Children Type T (ABMS-CT).

correlation coefficient analysis showed that the total scores of the motor WeeFIM and cognitive WeeFIM both significantly correlated with the scores for each ABMS-CT item and the total score of the ABMS-CT (Table 1). The five items on the ABMS-CT had appropriate internal consistency (Cronbach's $\alpha = 0.970$), and results of analysis of inter-rater reliability for the ABMS-CT indicated almost perfect reliability for all five items ($\kappa = 0.868$–0.931) (Table 2).

4. Discussion

Previously, we reported evidence for the evaluative value of the ABMS-C with regard to functional ability in disabled pediatric patients [1]. In that study, the scale did not include a grade for a function that was appropriate for assessing development in children who can walk independently. We considered that information on the degree of ability of children with a disability to perform complex basic movements in an environment other than the bedside would be valuable in understanding their physical functional ability. In the field of pediatric rehabilitation, to assess the motor function of children with disability, the GMFCS, gross motor function measure (GMFM), WeeFIM, pediatric evaluation of disability inventory (PEDI), and Bailey motor developmental scale are very well known. Morris and Bartlett [3] reported that the GMFCS has had, and continues to

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Table 1
Correlation between ABMS-CT scores, WeeFIM scores, and age Spearman's rank correlation with WeeFIM.

Variable	Median	Range	With motor WeeFIM	With cognitive WeeFIM	P
			r	r	
Basic mobility (ABMS-CT)	($n = 49$)				
Oral and facial area	2.00	0–3	0.846	0.827	0.0001
Hands and fingers	2.00	0–3	0.878	0.863	0.0001
One leg	1.00	0–3	0.826	0.794	0.0001
Both legs	1.00	0–3	0.799	0.753	0.0001
Stairs	2.00	0–3	0.869	0.833	0.0001
Total scores of ABMS-CT	8.00	0–15	0.892	0.854	0.0001
Age, years	4.50	1.00–15.17	0.709	0.599	0.0001
Motor WeeFIM	59.00	0–91	–	0.858	0.0001
Cognitive WeeFIM	19.00	0–35	0.858	–	0.0001

ABMS-CT, Ability for Basic Movement Scale for Children Type T; WeeFIM, Functional Independence Measure for Children.

Table 2
Inter-rater reliability of each ABMS-CT item by two physicians with kappa coefficient in 42 pediatric patients.

Inter-rated ABMS-CT	Physician	($n = 42$)		Reliability
		Median	Range	κ
Oral and facial area	A	1.00	0–3	0.895
	B	1.50	0–3	
Hands and fingers	A	1.00	0–3	0.854
	B	1.50	0–3	
One leg	A	0.00	0–3	0.925
	B	0.00	0–3	
Both legs	A	0.00	0–3	0.897
	B	0.50	0–3	
Stairs	A	2.00	0–3	0.863
	B	1.50	0–3	

ABMS-CT, Ability for Basic Movement Scale for Children Type T; κ, kappa coefficient.

have, a major effect on the health care of children with cerebral palsy. The number of citations of the GMFCS has been increasing every year, and the classification system has had good acceptance internationally and across the spectrum of health professionals for use in research design and clinical practice by providing a system for clearly communicating children's gross motor function. On the other hand, Kondo et al. [4] examined the reliability of the Japanese version of the GMFCS and using a Delphi survey determined the opinions of experts on the clinical use of the GMFCS. They concluded, as suggested by Rosenbaum et al. [5], that reliability for levels 3 and 4 in the GMFCS was somewhat lowered because the level 3 description for ages 4–6 years indicated a relatively lower level than what is usual in this age group. In a systematic literature review of assessment measures, Ketelaar et al. [6] concluded that only two evaluative assessment measures, the GMFM and the PEDI, fulfill the criteria for reliability and validity with respect to responsiveness to change in a child's condition. After testing the reliability of the WeeFIM in 37 non-disabled children and 30 children with disabilities, Ottenbacher et al. [7] found this instrument to be reliable. They also reported that the WeeFIM instrument had the ability to document change in functional abilities over a 1-year period in 173 children with chronic disabilities [8]. However, these instruments are not commonly used in actual examinations by pediatricians because they are difficult to administer in the usual clinical practice.

It is difficult to administer the rather specialized scales used in rehabilitation medicine. Our group developed the ABMS-C and ABMS-CT to make available an instrument that could easily assess a patient's abilities to perform the basic movements used in daily life at a young age. One of more useful points of the ABMS-CT in comparison with other scales such as the WeeFIM is that we can easily record the child's ability to perform five different complex basic movements and thereafter can monitor the progress of that child's ability to perform each individual movement. There were high correlations between the motor and cognitive WeeFIM score and all items on the ABMS-CT (Table 1). Also, inter-rater reliability for each task was established using a κ coefficient (Table 2). Based on these results, we believe that the ABMS-CT is appropriate to evaluate the ability of pediatric patients who can walk to make complex basic movements.

Several limitations of this study should be mentioned. Although the WeeFIM was developed to assess pediatric

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patients 6 months to 7 years old age, some of the 49 patients examined by ABMS-CT in this study were more than 7 years of age. Another limitation is that none of the participants underwent the ABMS-CT examination at home in an environment compatible with daily life but at a rehabilitation gym in our hospital. Sometimes it is necessary to perform assessments of patients in situations other than at the hospital. In future research on the use of the ABMS-CT, it is necessary to consider means to utilize the instrument in evaluating patients in the home environment. Also, it will be necessary to accumulate further cases and to consider further revision of this assessment tool.

Acknowledgments

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nature genetics

Dynamic CpG island methylation landscape in oocytes and preimplantation embryos

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Elucidating how and to what extent CpG islands (CGIs) are methylated in germ cells is essential to understand genomic imprinting and epigenetic reprogramming[1–3]. Here we present, to our knowledge, the first integrated epigenomic analysis of mammalian oocytes, identifying over a thousand CGIs methylated in mature oocytes. We show that these CGIs depend on DNMT3A and DNMT3L[4,5] but are not distinct at the sequence level, including in CpG periodicity[6]. They are preferentially located within active transcription units and are relatively depleted in H3K4me3, supporting a general transcription-dependent mechanism of methylation. Very few methylated CGIs are fully protected from post-fertilization reprogramming but, notably, the majority show incomplete demethylation in embryonic day (E) 3.5 blastocysts. Our study shows that CGI methylation in gametes is not entirely related to genomic imprinting but is a strong factor in determining methylation status in preimplantation embryos, suggesting a need to reassess mechanisms of post-fertilization demethylation.

Because DNA methylation in oocytes occurs in meiotically arrested cells[3,7], it represents a uniquely informative system for investigating requirements and mechanisms of *de novo* methylation. These mechanisms, especially at CGIs, are poorly understood, mainly because of the very limited number of methylated CGIs identified so far in germ cells. To obtain genome-wide information on DNA methylation in oocytes, as well as in sperm, we performed reduced representation bisulphite sequencing (RRBS) using a protocol optimized for low amounts of DNA (**Supplementary Fig. 1**). RRBS combines the base-pair resolution and quantitative assessment of bisulphite sequencing with high enrichment for CGIs[8,9]. The fidelity of the method was shown by detection of the expected methylation of known maternal germline differentially methylated regions (DMRs) at imprinted loci (**Supplementary Fig. 2**).

CpG methylation overall, and in CGI and repetitive element contexts, showed a dynamic profile during oocyte growth: 0.5% of all CpGs assessed by RRBS were highly methylated in day 5 oocytes (≥80% methylation), 11.3% in day 20 germinal vesicle and 15.3% in ovulated metaphase II (MII) oocytes. CpG methylation was lower overall in mature oocytes than in sperm (24.9% of CpGs were highly methylated in sperm), consistent with previous observations on repetitive elements[10]; methylation in a CGI context, irrespective of location with respect to genes, was markedly lower in sperm (**Fig. 1a** and **Supplementary Figs. 3a,b,4**). Using a threshold for scoring CGIs that reads should cover ≥10% of the CpGs per CGI (see Online Methods for a full account), we obtained information on ~15,000 (~65%) of the extended set of CGIs recently identified by CXXC affinity purification plus deep sequencing (CAP-Seq)[11] and identified 1,062 methylated CGIs (≥75% methylation) in mature oocytes (**Fig. 1b,c** and **Supplementary Table 1**). By extrapolation, there may be ~1,600 fully methylated CGIs in mature oocytes. Of interest, we found that the CGIs associated with the major promoters of *Dnmt3b* and *Dnmt1* (the *Dnmt1s* promoter) were methylated (**Fig. 1d,e**). Eighty-nine CGIs identified as methylated in MII oocytes were not fully methylated in germinal vesicle oocytes, showing that CGIs acquire methylation at different rates during oocyte growth, as reported for germline DMRs[12,13] (**Supplementary Table 2**). In sperm, we identified 185 fully methylated CGIs, 58 of which were methylated exclusively in sperm and 100 of which were also methylated in mature oocytes (27 of the CGIs methylated in sperm were not informative in mature oocyte datasets) (**Fig. 1b,c** and **Supplementary Table 1**). For subsequent analyses, we considered CGIs scored with ≥75% methylation as fully methylated and those with ≤25% methylation as unmethylated; this definition does not exclude that CGIs scored between these cutoffs have methylation.

Having identified the extent of CGI methylation in gametes, we tested whether they had distinctive sequence properties. In comparison with unmethylated CGIs, higher proportions of CGIs methylated in oocytes and sperm were intragenic (**Supplementary Fig. 3a,c**), similar to findings of CGI methylation in somatic tissues[11,14,15]. CGIs methylated in oocytes and sperm were shorter than unmethylated CGIs, with lower GC content and CpG density (**Supplementary Fig. 5a–d**). These properties may reflect the enrichment in intragenic CGIs, which are shorter and less CpG dense (data not shown), and we also observed this trend when comparing methylated intragenic and promoter CGIs (**Supplementary Fig. 5e**). Contrary to

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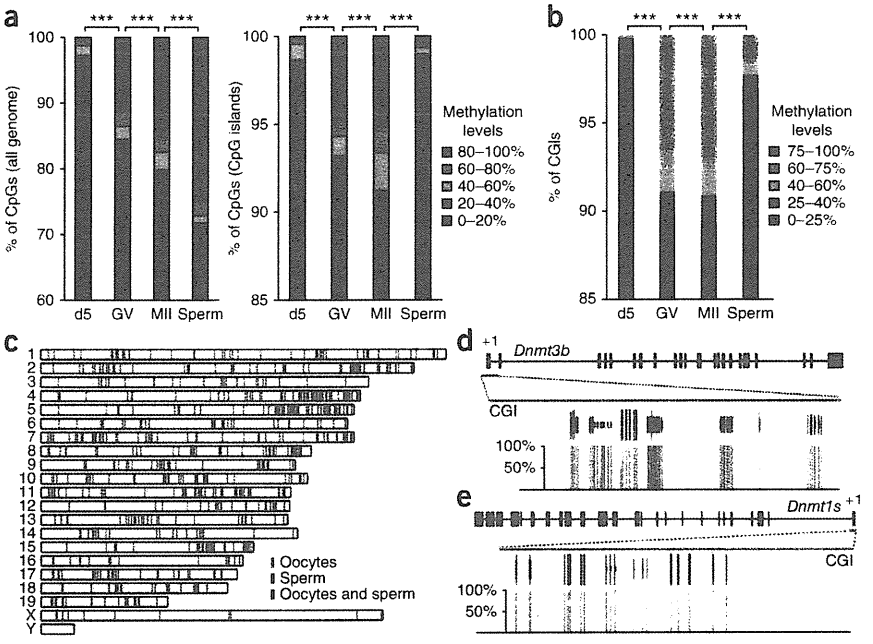

Figure 1 DNA methylation landscape in oocytes and sperm determined by RRBS. (**a**,**b**) Distribution of CpG methylation levels across the genome (**a**, left), within CGIs (**a**, right) and CGI methylation (**b**) in immature (day 5), mature (germinal vesicle and MII) oocytes and sperm (***$P < 0.001$, χ^2 test). The number of CpGs and CGIs analyzed is indicated in **Supplementary Figure 1b**. (**c**) Chromosome distribution of the 1,062 CGIs methylated in oocytes and 185 CGIs methylated in sperm (with 100 CGIs in both). (**d**) CpG methylation levels (percentage of all cytosines called methylated) at the *Dnmt3b* and *Dnmt1s* promoter CGIs in germinal vesicle oocytes. The gray vertical lines represent the sequencing read depth of individual cytosines; below, the percentage methylation of the corresponding CpGs is represented by colored vertical lines.

reports implicating tandem repeats in DMR methylation[16], the content of this repetitive element class was similarly low in germline methylated and unmethylated CGIs (**Supplementary Fig. 5f**). A systematic analysis of sequence properties performed using EpiGRAPH[17] identified sequence attributes enriched in methylated CGIs (**Supplementary Table 3**) but was not able to predict the methylation status of CGIs based on these. A key observation implicating intrinsic sequence properties in *de novo* methylation of DMRs is the tetrameric association of DNMT3A and DNMT3L, the factors responsible for DMR methylation[4,5,18], which results in the two DNMT3A catalytic sites being separated by a spacing corresponding to 8–10 bp of DNA. This spacing was described as the dominant CpG periodicity unit of maternal germline DMRs[6]. Notably, we did not observe any differences in the CpG periodicity of CGIs methylated in oocytes or sperm, or unmethylated CGIs (**Supplementary Fig. 5g**). Overall, these results indicate that CGIs methylated in gametes do not appear to have strong discriminating sequence features.

We next examined whether CGIs methylated in oocytes depend on DNMT3A and DNMT3L by performing RRBS on germinal vesicle oocytes genetically depleted in these factors. Both *Dnmt3a*$^{-/-}$ and *Dnmt3L*$^{-/-}$ oocytes showed a gross, genome-wide reduction in CpG methylation, including at repetitive elements and CGIs independent of their genic location (**Fig. 2a** and **Supplementary Fig. 6**). Of 654 CGIs methylated in *Dnmt3a*$^{+/+}$ oocytes with coverage in *Dnmt3a*$^{-/-}$ oocytes, the vast majority (96%) were unmethylated in *Dnmt3a*$^{-/-}$ oocytes (**Fig. 2b**). We also had information on 301 methylated CGIs in *Dnmt3L*$^{+/+}$ oocytes and their *Dnmt3L*$^{-/-}$ counterparts; similarly, 92% of CGIs were unmethylated in *Dnmt3L*$^{-/-}$ oocytes (**Fig. 2c**). Notably, the CGIs remaining methylated (≥75%) in *Dnmt3L*$^{-/-}$ oocytes were also highly methylated in *Dnmt3a*$^{-/-}$ oocytes, sperm and day 5 oocytes, suggesting that they are incompletely demethylated during primordial germ-cell reprogramming[19] (**Supplementary Table 1**). Overall, this shows the genome-wide role of DNMT3A and DNMT3L in CGI methylation beyond genomic imprinting.

Recent reports highlight the link between active transcription and DNA methylation. We previously showed that transcription across the DMRs of the imprinted *Gnas* locus is required for their methylation in oocytes[12]. The H3K4 demethylase KDM1B, which is associated with active gene bodies, is required for methylation of a subset of DMRs in oocytes[20,21]. In addition, interaction of DNMT3A and DNMT3L with chromatin is inhibited by H3K4 methylation[22,23], whereas DNMT3A binds H3K36me3 (ref. 24), a transcriptional elongation mark. To explore the general relationship between transcription and CGI methylation in oocytes, we undertook mRNA-Seq in day 10 oocytes (onset of *de novo* methylation) (**Supplementary Fig. 7**). This showed that methylated CGIs annotated as intragenic were more likely to be within

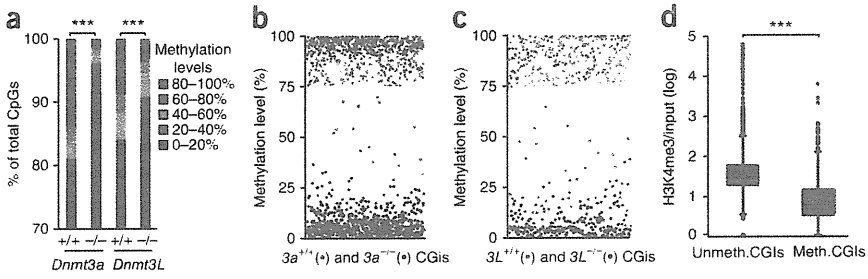

Figure 2 Mechanism of DNA methylation establishment in oocytes. (**a**) Distribution of CpG methylation levels across the genome in *Dnmt3a*$^{-/-}$ and *Dnmt3L*$^{-/-}$ oocytes and their wild-type counterparts ($^{+/+}$); the number of CpGs analyzed is indicated in **Supplementary Figure 1b** (***$P < 0.001$, χ^2 test). (**b**,**c**) Methylation levels of CGIs in *Dnmt3a*$^{-/-}$ and *Dnmt3L*$^{-/-}$ oocytes; only those CGIs for which methylation was ≥75% in the corresponding wild-type oocytes are shown. (**d**) Overall correlation between H3K4me3 enrichment determined in day 15 oocytes by ChIP-seq and methylation status (meth., methylated; unmeth., unmethylated) of CGIs (all CGIs irrespective of genomic location). Each group is shown as a box plot (plotted using default settings in SPSS) with the median values shown as thick horizontal lines. The box covers the twenty-fifth to seventy-fifth percentiles, and the whiskers outside the box extend to the highest and lowest value within 1.5 times the interquartile range. Points outside the whiskers are outliers. The difference in the median values between groups was tested using the Mann-Whitney U test (***$P < 0.001$).

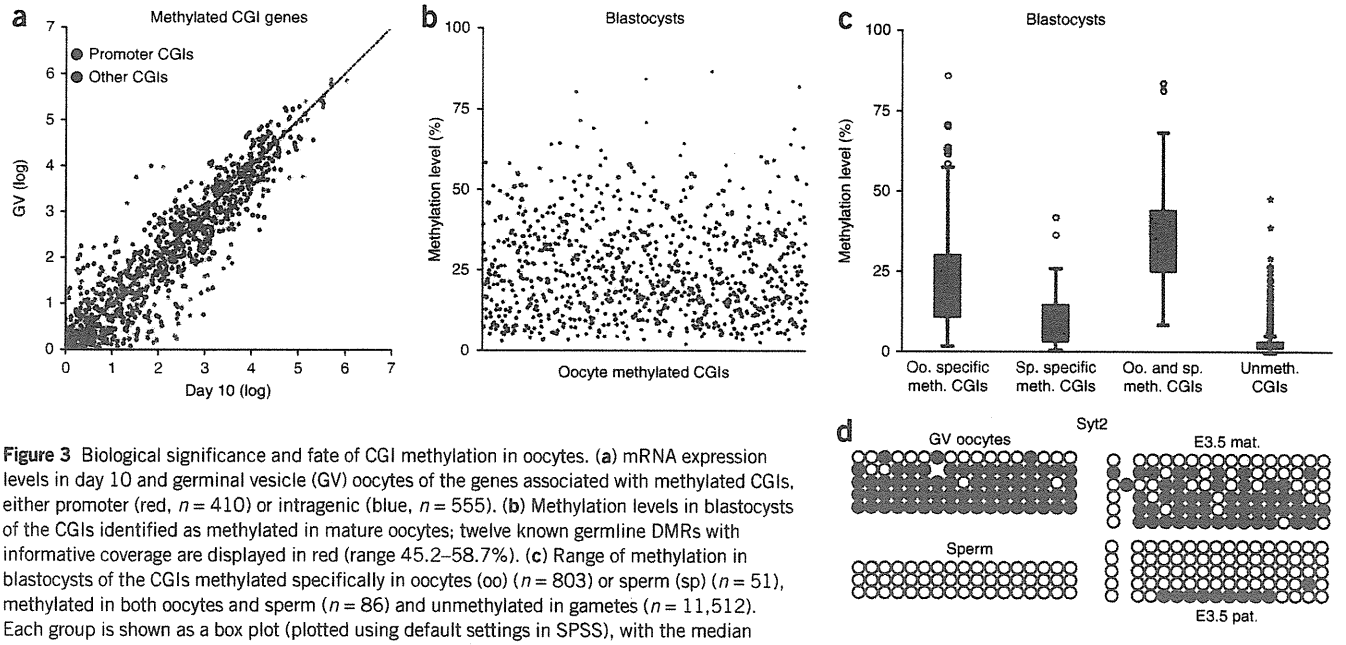

Figure 3 Biological significance and fate of CGI methylation in oocytes. (**a**) mRNA expression levels in day 10 and germinal vesicle (GV) oocytes of the genes associated with methylated CGIs, either promoter (red, $n = 410$) or intragenic (blue, $n = 555$). (**b**) Methylation levels in blastocysts of the CGIs identified as methylated in mature oocytes; twelve known germline DMRs with informative coverage are displayed in red (range 45.2–58.7%). (**c**) Range of methylation in blastocysts of the CGIs methylated specifically in oocytes (oo) ($n = 803$) or sperm (sp) ($n = 51$), methylated in both oocytes and sperm ($n = 86$) and unmethylated in gametes ($n = 11,512$). Each group is shown as a box plot (plotted using default settings in SPSS), with the median values shown as thick horizontal lines. The box covers the twenty-fifth to seventy-fifth percentiles, and the whiskers outside the box extend to the highest and lowest value within 1.5 times the interquartile range. Points outside the whiskers are outliers, with asterisks indicating those >3 times outside the interquartile range. (**d**) Bisulphite sequencing in germinal vesicle oocytes, sperm and C57BL/6J × CAST/Ei hybrid E3.5 blastocysts of the *Syt2* CGI. We discriminated bisulphite sequence profiles from the maternal (mat.) and paternal (pat.) alleles in blastocysts by polymorphisms between C57BL/6J and CAST/Ei. Open circles represent unmethylated CpGs, and filled circles represent methylated CpGs.

transcription units active in oocytes compared to unmethylated intragenic CGIs (75% versus 28%, $P < 0.001$, χ^2 test). Furthermore, methylated CGIs overlapping annotated promoters were more frequently within overlapping transcripts compared to unmethylated promoter CGIs (39% versus 8%, $P < 0.001$, χ^2 test). In addition, mRNA-Seq identified alternative, upstream promoters for 35% of the methylated CGIs compared to 10% of unmethylated CGIs ($P < 0.001$, χ^2 test) (**Supplementary Fig. 7**). These observations strengthen an association between location within active transcription units and probability of methylation. We also performed H3K4me3 ChIP-Seq in day 15 oocytes (early phase of *de novo* methylation) (**Supplementary Fig. 7**). This revealed that CGIs methylated in oocytes were relatively depleted in H3K4me3: 6.4% of methylated CGIs had significant H3K4me3 enrichment compared with 60.9% of unmethylated CGIs (13% versus 65% for CGIs associated with annotated promoters, 2% versus 34% for intragenic CGIs and 4.5% versus 36% for intergenic CGIs) (**Fig. 2d**; $P < 0.001$, Mann-Whitney U test). H3K4me3 enrichment at a large proportion of intragenic and intergenic unmethylated CGIs has been described in other cell types, including embryonic stem cells[11].

Having identified a large set of methylated CGIs in mature oocytes, we asked what the biological role of such methylation is. The function of genes associated with methylated CGIs as assessed by gene ontology analysis is diverse (data not shown). To assess the impact of methylation on gene expression in oocytes, we compared expression in fully grown germinal vesicle oocytes with day 10 growing oocytes by mRNA-Seq but did not observe marked differences in expression levels of transcripts associated with methylated CGIs, including those with methylated promoter CGIs (**Fig. 3a**). This suggests that most such transcripts accumulate during oocyte growth before the onset of methylation or that these genes are transcribed from alternative, unmethylated promoters. Such alternative promoters may ensure stringent control of these genes by oocyte-specific factors or environment, and this control might also necessitate that their 'somatic' promoters are silenced. If CGI methylation does not contribute appreciably to regulation of the maternal mRNA store in oocytes, it may nevertheless have important repercussions for expression of the associated genes after fertilization.

Following fertilization, methylation is comprehensively reprogrammed (except for imprinted genes): the maternal genome is passively demethylated between the zygote and morula, and the paternal genome is actively demethylated in the zygote, which is followed by the establishment of new methylation landscapes[2]. To evaluate the importance of gamete-derived CGI methylation, we performed RRBS on blastocysts (E3.5). This was validated by the expected degree of methylation at 12 known maternal germline DMRs (range 45.2–58.7%). Consistent with genome-wide erasure, there was a substantial reduction in the proportion of methylated CpGs (≥60%) across the genome or within CGIs compared with gametes (**Fig. 3b** and **Supplementary Fig. 8a**). Crucially, a minority of CGIs methylated in germ cells showed complete protection from demethylation: only ~15% of CGIs methylated in oocytes retained ≥40% methylation in blastocysts (**Fig. 3b,c**). This substantial post-fertilization reprogramming suggests that most CGI methylation in oocytes and sperm is unrelated to imprinting and argues that maintenance of methylation in preimplantation embryos is a decisive factor in imprinting.

However, we observed that most CGIs methylated in oocytes showed greater levels of methylation in blastocysts than expected if they were fully subject to passive demethylation, by which methylation should be <2% by the 32-cell stage. This was striking, as very few CGIs are methylated in blastocysts (**Fig. 3b,c** and **Supplementary Fig. 8b,c**). To examine the degree to which gametic methylation is a factor in CGI methylation in preimplantation embryos, we looked at the dependence of methylation in blastocysts on prior methylation in gametes. Of 280 CGIs showing intermediate methylation levels (25–40%) in blastocysts, the vast majority (234, or 83%, $P < 0.001$, χ^2 test) were fully methylated in MII oocytes (including 27 CGIs methylated in both

oocyte and sperm) (**Supplementary Fig. 8d**). In contrast, less than 0.5% of CGIs unmethylated in both gametes are methylated ≥25% in blastocysts (**Fig. 3c** and **Supplementary Table 1**). To investigate whether CGI sequence influences the likelihood of maintaining methylation, we checked how the properties of CGIs highly methylated in MII oocytes (≥75%) differed according to methylation level in blastocysts. For most parameters, the differences were minor, but there was a tendency for CGIs retaining higher levels of methylation to be shorter and to be intragenically located (**Supplementary Fig. 8e,f**). To validate the CGI methylation allele specifically, we examined a selection of CGIs in C57BL/6J × Cast/Ei hybrid embryos by conventional bisulphite sequencing. As exemplified by the *Syt2* locus, the CGI is fully methylated in oocytes, and the maternal allele partially retains methylation in blastocysts (**Fig. 3d** and **Supplementary Fig. 9**). For CGIs specifically methylated in sperm, there was less evidence for substantial maintenance of methylation in blastocysts (**Fig. 3c** and **Supplementary Fig. 9**). These findings extend observations of researchers from a previous study, who, using MeDIP-chip analysis of promoter methylation in preimplantation embryos, identified some nonimprinted sequences that resist demethylation in preimplantation development[25]. Thus, CGI methylation status in gametes strongly predisposes toward methylation in blastocysts, either by incomplete post-fertilization demethylation of methylated CGIs or because some legacy of gametic methylation instructs their re-methylation in a subpopulation of cells. By either mechanism, mosaicism of CGI methylation patterns between blastomeres is predicted to arise. This does not exclude a contribution of *de novo* methylation, as some CGIs unmethylated in gametes have become methylated in blastocysts (**Fig. 3c**, **Supplementary Fig. 8d** and **Supplementary Table 1**), including genes involved in trophectoderm development[26].

In conclusion, we reveal the extent and dynamics of CGI methylation in oocytes; this provides an important reference by which to judge future studies on mechanisms of *de novo* methylation in germ cells. A comprehensive account of the differential CGI methylation in male and female gametes is also a prerequisite for defining the full repertoire of imprinted genes and the mechanistic basis of parent-of-origin expression effects in somatic tissues. We also describe an unexpectedly complex fate of gamete-derived methylation after fertilization. Rather than a binary choice, with DMRs characterized by absolute maintenance and other gametic methylation comprehensively lost through active demethylation or lack of maintenance during the first cleavage divisions, our analysis suggests a greater diversity of methylation choices. This diversity might lead to the establishment of epigenetic mosaicism within the early embryo, which might have the potential to influence first-lineage specification[27].

URLs. Bareback, http://www.bioinformatics.bbsrc.ac.uk/projects/; SeqMonk, http://www.bioinformatics.bbsrc.ac.uk/projects/.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturegenetics/.

Accession codes. All sequencing files have been deposited in the Sequence Read Archive under the study accession number ERP000689 (European Nucleotide Archive).

Note: Supplementary information is available on the Nature Genetics website.

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AUTHOR CONTRIBUTIONS

S.A.S. designed the study, performed RRBS, mRNA-Seq, direct BS-PCR experiments, data analysis and wrote the manuscript. S.-i.T. contributed to direct bisulphite sequencing PCR experiments and performed oocyte collections. F.K. and S.R.A. performed CpG methylation calls, general Illumina sequence alignments and data analysis. N.R. performed ChIP-Seq experiments. N.C. analyzed data. A.S.-P. performed statistical analysis. S.S. and K.H. provided *Dnmt3L* wild-type and knockout oocytes. G.K. designed and supervised the study and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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