been investigated. Although the copy numbers of these regions were not thoroughly validated with other methods; such as, quantitative PCR, according to DGV, most of the CNVRs identified here have been reported in previous studies, indicating that they should be observed by other methods or techniques. Because our identification strategy was based on a microarray technique, it is inevitable that errors would have occurred. Besides routine data processing, we also carefully curated the data by examining the B allele frequencies and signal intensities ($\log R$ ratio) for each CNVR using the GenomeStudio software (Illumina) (Figure 2). We found that many implausible calls were situated in regions with high G + C contents; for example, in subtelomeric regions. All of them were copy number gain-type CNVs rather than copy number loss-type CNVs. Although further research is required, it is important to note that CNVRs tend to be detected in those regions by SNP microarrays. Even if such CNVRs are false positives, our data set is still useful for screening large numbers of candidate CNVs.

It is unclear whether CNVs are selectively neutral on the basis of genetic drift, but they are certainly distributed throughout all human populations. Using the genotypes of mitochondrial DNA and Y chromosome, geneticists and anthropologists have surmised various intriguing scenarios about the history of humans.[37–40] However, these genetic materials have been transmitted exclusively through maternal and paternal lineages, respectively. In contrast, the CNVs reported here occur in the more extensive remaining genome regions; that is, on autosomes or the X chromosome. Therefore, they have acted some times as maternal alleles at and at other times as paternal alleles. They might also have been subjected to crossingover. CNV data from various parts of the world are essential to substantiate these hypothetical scenarios.

Chromosomal anomalies are found with conventional cytogenetic techniques in approximately half of all early sporadic miscarriages.[41] It is possible that miscarriages and pregnancy losses are also caused by submicroscopic chromosomal changes, including CNVs. Twenty-eight CNVs have been reported as candidate miscarriage-related variations when instances of recurrent pregnancy loss were examined by Rajcan-Separovic *et al.*[42] When 17 Caucasian and three African-American couples with recurrent pregnancy losses and their miscarriage samples were examined, CNVs that may have been related to miscarriages were reported.[42] They reported 11 novel CNVs in miscarriage samples and three in the parent samples and suggested that these CNVs were probably mutations causing susceptibility to miscarriage. Of the 11 CNVs in the miscarriage samples, one on chromosome 12 (130 060 706–130 430 847 in hg18) and another one on chromosome X (6 498 521–8 091 951) overlapped with our data set. Whereas the first one on chromosome 12 was up to 370 kb in length and encompassed the *GPR133* gene, the corresponding variable region in our data set is much shorter and includes no known genes. The *GPR133* gene encodes one of the orphan G-protein-coupled receptors, but its function is unknown.[43] It is possible that this receptor protein has a role in several signal-transduction pathways via classical receptor/G-protein interactions. Therefore, the CNV mentioned above may be a variant that causes miscarriage. However, one of the CNVs on chromosome X is consistent with our data set, suggesting that it is a commonly observed variant. In fact, Rajcan-Separovic *et al.*[42] tried to define the common CNVs using a collective repository in the DGV, but insufficient phenotypic information was available to refine the data. Taking these observations together, it seems that to define a set of common CNVs, it will be necessary to collect a large number of control data that focus on a specific phenotype; such as, normal parity in this case.

The Japanese are an admixture of ancient Asian populations that inhabited regions outside the Japanese Archipelago. We investigated the similarities among the CNVRs detected in various populations and noted that around 15% of Japanese CNVRs overlap those of other populations (Table 2). It has been suggested that the number of overlapping CNVs is influenced by the number of subjects. For instance, Japanese and Tibetan data showed dissimilarity because of the limited number of Tibetan subjects. Although the sample sizes of the Korean and Chinese populations are smaller than those of the European and African populations, similarities between the Japanese and other East Asian populations were similar to those of the European and African populations. This probably suggests strong similarities between the Japanese and other East Asian populations.

Previous studies have predominantly targeted European and African populations, but CNVs have been observed at different frequencies or copy numbers in different populations; for example, variations in the salivary amylase gene.[44] Many CNVs; such as, those at the *AMY1* locus, may be associated with diabetes, asthma, hypertension, allergy and other diseases of affluence in each ethnic group. Although CNVRs may result from the accumulation of tolerable structural mutations in the course of an ethnic history, they could start to influence the population's susceptibility to disease once its lifestyle is altered. The allelic frequencies of SNPs and short indels in each population have recently been documented.[45] The complete documentation of the CNVRs in each ethnic group is similarly important. The development of an innovative method to achieve this; such as, one involving next-generation sequencing and informatics, is another challenge.

CONFLICT OF INTEREST

The authors received no financial support from Illumina KK and the company had no role in the study design. The authors declare no conflict of interest.

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TOPIC HIGHLIGHT

WJSC 6th Anniversary Special Issues (2): Mesenchymal stem cells

Differentiation of mesenchymal stem cells into gonad and adrenal steroidogenic cells

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Abstract

Hormone replacement therapy is necessary for patients with adrenal and gonadal failure. Steroid hormone treatment is also employed in aging people for sex hormone deficiency. These patients undergo such therapies, which have associated risks, for their entire life. Stem cells represent an innovative tool for tissue regeneration and the possibility of solving these problems. Among various stem cell types, mesenchymal stem cells have the potential to differentiate into steroidogenic cells both *in vivo* and *in vitro*. In particular, they can effectively be differentiated into steroidogenic cells by expressing nuclear receptor 5A subfamily proteins (steroidogenic factor-1 and liver receptor homolog-1) with the aid of cAMP. This approach will provide a source of cells for future regenerative medicine for the treatment of diseases caused by steroidogenesis deficiencies. It can also represent a useful tool for studying the molecular mechanisms of steroidogenesis and its related diseases.

Key words: Steroid hormone; Adrenal; Gonad; Steroidogenic factor-1; Liver receptor homolog-1; Mesenchymal stem cells; Differentiation

Core tip: Stem cells can be a potential source of cells for regenerative medicine for diseases caused by steroidogenesis deficiency. Among various stem cell types, mesenchymal stem cells have the potential to differentiate into steroidogenic cells both *in vivo* and *in vitro*. This system can also provide a powerful tool for studying the molecular mechanisms of steroidogenesis and its related diseases.

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INTRODUCTION

In mammals, steroid hormones are produced from cholesterol mainly in adrenal glands and gonads. Steroid hormones are essential for glucose metabolism, the stress response, fluid and electrolyte balance, sex differentiation and reproduction via binding to cognate receptors in target tissues. Therefore, a steroidogenesis abnormality can often be life threatening. Congenital adrenal hyperplasia (CAH) is one of the most common disorders caused by deficiency of any enzyme involved in steroidogenesis in adrenal glands[1,2]. Impaired cortisol and aldosterone pro-

duction increases adrenocorticotropic hormone (ACTH) secretion from the pituitary gland, leading to adrenal hyperplasia and accumulation of adrenal androgens. Female patients are prenatally virilized because of excess androgen and neonates of both genders may suffer from a life-threatening Addisonian crisis. Steroid hormone deficiency also occurs in aging people by hypogonadism. In males, testosterone concentrations decline with age, causing various clinical symptoms such as obesity and hypertension[3-6]. Postmenopausal women often suffer from osteoporosis caused by estrogen deficiency[7,8]. Hormone replacement therapy has been well established for the treatment of such patients, although they require hormone replacement for their entire lifetime. In addition, these patients suffer from various side effects (liver and kidney damage, immune system dysfunction) and risks associated with long-term replacement therapy (cancer). Therefore, another therapy is needed to resolve these problems. Stem cells represent an innovative tool for tissue regeneration and gene therapy, which could possibly solve these problems. In this review, we provide an overview of differentiation and regeneration of steroidogenic cells using mesenchymal stem cells (MSCs), preceded by a description of the development of steroidogenic organs. We also describe molecular events, such as coactivator function and epigenetic modifications, which occur during differentiation.

DEVELOPMENT OF STEROIDOGENIC ORGANS AND NUCLEAR RECEPTOR 5A SUBFAMILY

Steroidogenesis begins with conversion of cholesterol into pregnenolone in mitochondria by the P450 side chain cleavage enzyme (P450scc/CYP11A1/Cyp11a1), a rate-limiting enzyme in the synthesis of all steroid hormones. Thereafter, various hormones are synthesized by tissue-specific P450 hydroxylases and hydroxysteroid dehydrogenases[9,10]. Although adrenal glands and gonads produce various steroid hormones in adult life, they have a common developmental origin, a so-called adrenogonadal primordium (AGP) that mainly originates from the intermediate mesoderm and is localized on the coelomic epithelia of the developing urogenital ridge[11-13]. As development proceeds, AGP separates into two distinct populations, adrenocortical and gonadal primordia, characterized by the existence of chromaffin cell precursors and primordial germ cells, respectively, which originate and migrate from other germ layers. During differentiation, adrenal glands and gonads synthesize tissue-specific steroid hormones by specific expression patterns of steroidogenic enzymes.

Steroidogenic factor-1 (SF-1, also known as Ad4BP) is one of the earliest markers of the appearance of AGP[11,14]. Because SF-1 knockout mice fail to develop adrenal glands and gonads, SF-1 represents a master regulator of the development of these organs[15-17]. SF-1/Ad4BP is also important for steroidogenesis by regulating the transcription of steroidogenic genes. SF-1/Ad4BP was originally discovered by Keith Parker and Ken Morohashi as a transcription factor that binds to the Ad4 sequence in promoter regions of all cytochrome P450 steroid hydroxylase genes for transactivation[18,19]. They concluded from the expression of SF-1 in steroidogenic cells and its regulation of all steroid hydroxylase genes that SF-1 is a determinant factor in cell-specific expression of steroidogenic enzymes. In addition to steroidogenic enzymes, diverse groups of SF-1 target genes, such as other steroidogenic genes, pituitary hormones and cognate receptors, and sex differentiation-related genes have been identified thus far[17,20,21]. SF-1 belongs to the nuclear receptor (NR) superfamily. NRs are lipophilic ligand-dependent and independent transcription factors and essential for various physiological phenomena[22,23]. A large number of family members have been identified from invertebrate to mammals. There are a total of 48 family members on the human genome. They share a common structural organization: zinc finger DNA-binding domain and a carboxyl-terminal ligand-binding domain. The NR superfamily can be broadly divided into four classes based on their characteristics (steroid hormone receptors, RXR heterodimers, dimeric orphan receptors and monomeric orphan receptors). SF-1 is categorized into monomeric orphan receptors, although Ingraham and colleague argued the possibility that phosphatidylinositols are ligands for SF-1[24]. SF-1 is very similar to liver receptor homolog-1 (LRH-1). LRH-1 was originally identified in the liver[25] and is known to function in metabolism, cholesterol and bile acid homeostasis by regulating the transcription of a number of genes[26-29]. In addition to the liver, LRH-1 is highly expressed in tissues of endodermal origin. It is also expressed in gonads and involved in steroidogenesis; in particular, its ovarian expression levels are the most abundant among tissues[30]. These factors constitute one of the NR subfamilies and are designated as NR5A proteins (Table 1, SF-1 is NR5A1 and LRH-1 is NR5A2). SF-1 and LRH-1 have various common characteristics, such as binding sequences, target genes and cofactors[24,31-38].

Consistent with its role in steroidogenesis, SF-1 expression is detected in adults in three layers of the adrenal cortex (zona reticularis, zona fasciculata and zona glomerulosa), testicular Leydig and Sertoli cells, ovarian theca, granulosa cells and, to a lesser extent, in the corpus lutea[39,40]. In the corpus lutea, LRH-1 rather than SF-1 is highly expressed and is important for progesterone production[36,41,42]. LRH-1 is also expressed in testicular Leydig cells[12,43,44].

SF-1 knockout mice die shortly after birth because of adrenal insufficiency and exhibit male-to-female sex reversal in external genitalia[15]. These phenotypes are caused by the complete loss of adrenal glands and gonads. Although the initial stages of adrenal and gonadal development occur in the absence of SF-1, they regress and disappear during the following developmental stage.

Table 1 Summary of the characteristics of steroidogenic factor-1 and liver receptor homolog-1

Nuclear receptor	Expressing tissues	Function	Phenotypes of knockout mice
SF-1/ Ad4BP/ NR5A1	Testis, ovary, adrenal,	Steroidogenesis Sex differentiation Energy homeostasis	Adrenal and gonadal agenesis Sex reversal in external genitalia Impaired expression of pituitary gonadotropins Abnormality of ventromedial hypothalamic nucleus
LRH-1/ NR5A2	Ovary, testis, liver, pancreas, intestine, early embryo	Steroidogenesis Ovulation Bile acid synthesis Glucose metabolism	Embryonic lethal around E6.5-7.5 d

Because gonads disappear prior to male sexual differentiation, the internal and external urogenital tracts of SF-1 knockout mice are of the female type, irrespective of genetic sex. Heterozygous SF-1 knockout mice show decreased adrenal volume associated with impaired corticosterone production in response to stress[45-47], whereas transgenic overexpression of SF-1/Ad4BP increases adrenal size and ectopic adrenal tissue in the thorax[48,49]. Total SF-1 disruption in mice demonstrated that SF-1 is crucial for the determination of steroidogenic cell fate *in vivo*. It has also been shown in Leydig cell and granulosa cell-specific knockout (LCKO and GCKO, respectively) models that SF-1 plays important roles in steroidogenesis following the development of steroidogenic organs. In LCKO mice, testicular steroidogenic acute regulatory protein (StAR) and Cyp11a1 expression is impaired, indicating a defect in androgen production[50]. Consistent with this hypothesis, the testes fail to descend (an androgen-dependent developmental process) and are hypoplastic. In GCKO mice, the ovaries are hypoplastic, adults are sterile and ovaries show reduced numbers of oocytes and lack corpora lutea[51]. Gonadotropin-induced steroid hormone production are also markedly reduced in this model.

LRH-1 knockout mouse embryos die around E6.5-7.5 d[52,53]. Moreover, heterozygous and GCKO models revealed the importance of LRH-1 in steroidogenesis[41,54,55]. In heterozygous Lrh-1-deficient male mice, testicular testosterone production is decreased along with the expression of steroidogenic enzymes and the development of sexual characteristics[54]. In addition, GCKO mice are infertile because of anovulation with impaired progesterone production[41]. It has also been demonstrated that LRH-1 has a broader role beyond steroidogenesis in these cells as they fail to luteinize.

Although SF-1 and LRH-1-deficient models revealed a common function in gonadal steroidogenesis, both factors cannot compensate for the deficiency of the other factor, even in cells expressing both factors. These facts indicate that even although SF-1 and LRH-1 control transcription by binding to the same response sequences, each has selective actions on the pattern of gene expression in the development of steroidogenic cells and steroidogenesis.

DIFFERENTIATION OF MSCS INTO STEROIDOGENIC CELLS

In an early study, forced expression of SF-1 has been shown to direct differentiation of murine embryonic stem cells (ESCs) toward the steroidogenic lineage and then Cyp11a1 mRNA was expressed after the addition of cAMP and retinoic acid[56]. However, the steroidogenic capacity of these cells is very limited and they do not undergo *de novo* synthesis because progesterone is the only steroid hormone produced in the presence of the exogenous substrate, 20α-hydroxycholesterol. In addition, major differences between these differentiated cells and natural steroidogenic cells have been shown in cholesterol delivery and the steroidogenic pathway, including deficiencies of StAR (cholesterol delivery protein from the outer to inner mitochondrial membrane in steroidogenic cells) and steroidogenic enzymes, except for Cyp11a1 and Hsd3b1[56-58]. It is also very difficult to isolate clones expressing SF-1 from ESCs and induced pluripotent stem cells[37,57,59] because SF-1 (and LRH-1) overexpression is cytotoxic to these cells. These studies clearly indicate that SF-1 initiates the fate-determination program of the steroidogenic lineage in stem cells, although it is not completed in pluripotent stem cells.

Based on these results, we focused on MSCs[57], multipotent adult stem cells that have been shown to differentiate into mesodermal lineages, such as adipocytes, chondrocytes, osteoblasts and hematopoietic-supporting stroma, both *in vivo* and *ex vivo*[60-63]. Furthermore, MSCs are able to generate cells of all three germ layers, at least *in vitro*. Although MSCs were originally discovered in bone marrow (BM-MSCs)[60,64-66], they have also been isolated from various origins, such as fat, placenta, umbilical cord blood and other tissues[62,63,67-69]. In addition to their multipotency, MSCs have attracted considerable interest for use in cell and gene therapies because they can be obtained from adult tissues and suppress immune responses[70,71]. Indeed, their therapeutic applicability has been assessed in some cases and particularly in bone tissue engineering[72,73].

Induction of MSC differentiation into steroidogenic cells in vivo and in vitro

To investigate the potential of MSCs to differentiate into steroidogenic cells, BM-MSCs from GFP-transgenic rats were transplanted into prepubertal testes (Figure 1A)[57]. In testes, there are two different steroidogenic populations, fetal and adult Leydig cells[74-76]. Even although the cells in these two populations share a common characteristic of producing androgen, they are different in their origin, ultrastructure, lifespan, steroidogenic pathway and its regulation. Fetal Leydig cells have multiple origins and

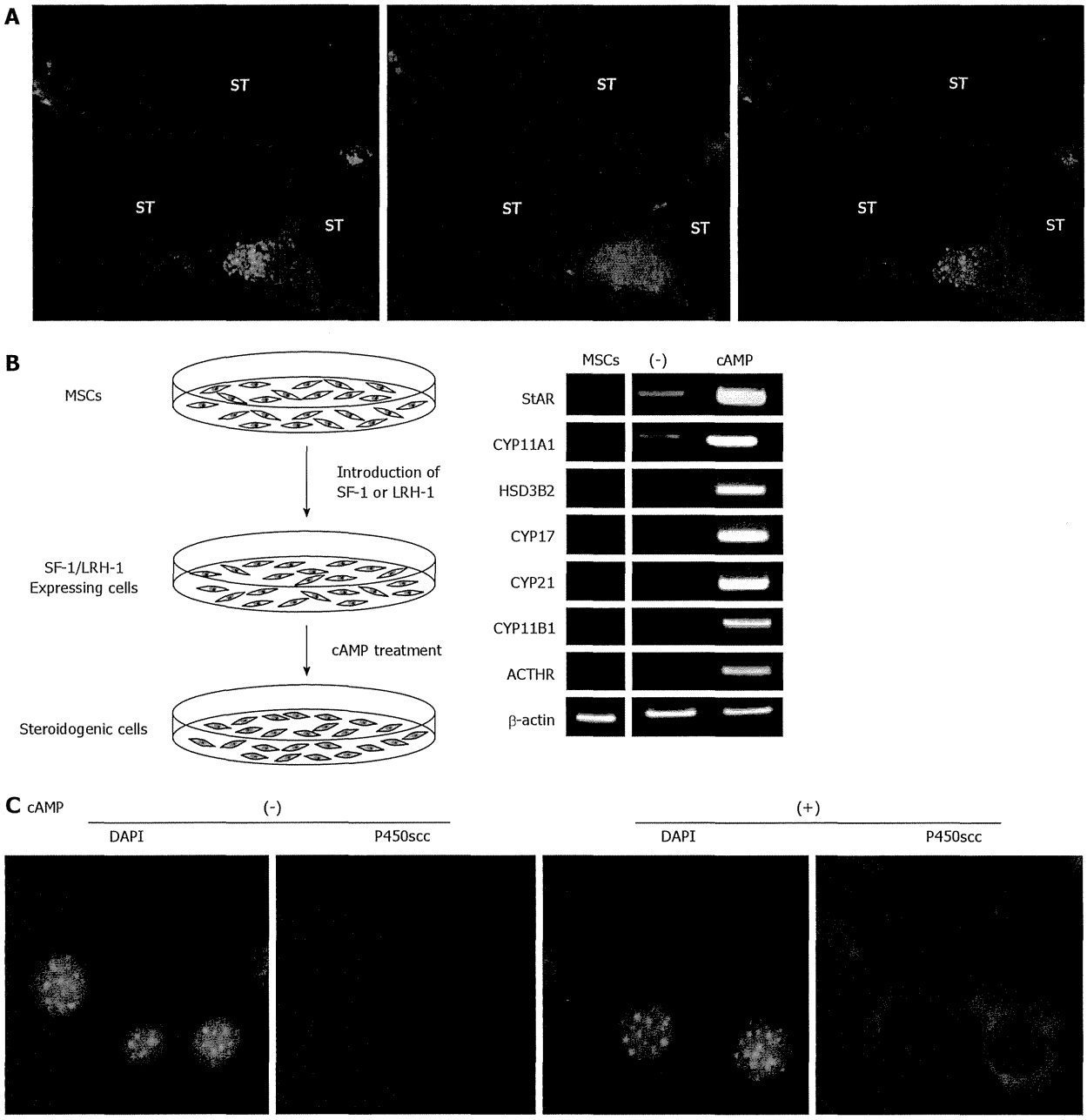

Figure 1 Differentiation of mesenchymal stem cells into steroidogenic cells. A: Transplantation of GFP-positive MSCs into prepubertal testis. Double staining of frozen sections from the testis 5 wk after MSC transplantation with anti-GFP and anti-P450scc antibodies; B: Protocol for generating steroidogenic cells from MSCs, and gene expression pattern of steroidogenic cells derived from hBM-MSCs; C: Fluorescence images of DAPI staining and P450scc immunostaining of SF-1 introduced BM-MSCs cultured with or without cAMP. ST: Seminiferous tubule. MSC: Mesenchymal stem cell.

appear in the interstitial space to induce sex differentiation just after the formation of the testis cord. Adult Leydig cells, which originate from mesenchymal precursor cells present in the testicular interstitium, appear to induce puberty. During the postnatal period, fetal Leydig cells are replaced by adult Leydig cells in prepubertal testis. Therefore, it should be possible to use transplanted BM-MSCs in such conditions *in vivo*. Indeed, after 3 wk, transplanted GFP-positive cells were located in the interstitium and expressed various steroidogenic enzymes for androgen production (P450scc/Cyp11a1, 3β-HSD I and Cyp17). These results indicate that MSCs have the capacity to differentiate into steroidogenic Leydig cells *in vivo*.

Although these data suggest that the injected stem cells differentiated into Leydig cells, the apparent stem cell plasticity may also be explained by possible cell-nuclear fusion between donor and recipient cells. However, purified murine BM-MSC lines spontaneously differentiate into steroidogenic cells *in vitro*[57]. A human *CYP11A1* promoter-driven GFP reporter, which consisted of a 2.3-kb fragment that drives reporter gene expression selectively in adrenal and gonadal steroidogenic cells[77],

Table 2 Properties of steroidogenic cells derived from mesenchymal stem cells induced by steroidogenic factor-1/liver receptor homolog-1 and cAMP

Cells	Origin	SF-1/LRH-1	Produced	Properties of differentiated cells
KUM9	Mouse Bone marrow	Plasmid	Testosterone	Testicular leydig cells
hMSC-TERT-E6/7	Human Bone marrow	Plasmid	Cortisol	Adrenal fasciculata cells
		Retrovirus	Cortisol	
UE7T-13	Human Bone marrow	Retrovirus	Testosterone, cortisol	Fetal adrenal-like cells
UE6E7T-12		Retrovirus	Testosterone, cortisol	
UE6E7T-11		Retrovirus	Testosterone, cortisol	
UCB408E6E7T-33	Human Umbilical cord blood	Retrovirus	Progesterone cells	Ovarian granulosa-luteal cells

has been transfected into BM-MSCs to detect cell populations committed to the steroidogenic lineage. In some transfected cell lines, GFP fluorescence was detected in very small populations that were also positive for Cyp11a1. Further analysis showed that these cells expressed several Leydig cell markers, including 3β-HSD type I and VI and luteinizing hormone (LH) receptor. These observations further support the *in vivo* findings that MSCs have the capacity to differentiate into steroidogenic cells, even under the isolated condition. Therefore, part of population of MSCs can spontaneously differentiate into steroidogenic cells *in vitro*. Interestingly, SF-1 expression was also detected in the GFP-positive cells.

Differentiation of MSCs into steroidogenic cells induced by SF-1 and LRH-1

The above mentioned results strongly suggest that SF-1 can effectively direct the differentiation of MSCs into the steroidogenic lineage. Indeed, MSCs completely differentiate into steroidogenic cells and show their phenotype after stable expression of SF-1 (using plasmids or retroviruses) and cAMP treatment (Figure 1B)[36,37,44,57,78,79]. SF-1 by itself induces morphological changes in BM-MSCs, such as the accumulation of numerous lipid droplets, although these cells hardly express steroidogenic enzyme genes or produce steroid hormones at detectable levels. However, SF-1 expressing cells strongly become positive for CYP11A1/Cyp11a1 after cAMP treatment (Figure 1C). These cells express many other steroidogenesis-related genes (*SR-BI*, *StAR*, *3β-HSD* and other P450 steroid hydroxylases) and autonomously produce steroid hormones, including androgen, estrogen, progestin, glucocorticoid and aldosterone. Notably, this approach differentiates human BM-MSCs into high cortisol-producing cells in response to ACTH, which are very similar to fasciculata cells in the adrenal cortex (Figure 1B). Adenovirus-mediated transient expression of SF-1 also differentiates BM-MSCs into steroidogenic cells with the capacity of *de novo* synthesis of various steroid hormones[80-84]. After transplantation into animal models, these MSC-derived steroidogenic cells can improve symptoms of steroid hormone deficiencies caused by adrenalectomy. However, as mentioned above, these methods are not applicable to ESCs, embryonal carcinoma cells and terminally differentiated cells, such as fibroblasts and adipocytes[37,57,81]. These results indicate that MSCs are suitable stem cells for differentiation of steroidogenic cells. This hypothesis is supported by the fact that after predifferentiation into MSCs, ESCs can also be subsequently differentiated into steroidogenic cells using SF-1[37].

As in the case of SF-1, introduction of LRH-1 (using retroviruses) into BM-MSCs with the aid of cAMP induced the expression of steroidogenic enzymes and differentiation into steroid hormone-producing cells[44]. Expression of SF-1 was never induced in LRH-1-transduced cells and vice versa. Therefore, LRH-1 could act as another master regulator for determining the MSC fate to the steroidogenic lineage. This phenomenon is likely to represent a situation of active progesterone production in human corpus luteum; LRH-1 is highly expressed, whereas SF-1 is expressed at very low levels[36,42].

MOLECULAR MECHANISMS OF DIFFERENTIATION

Steroidogenic cells derived from various MSCs and their properties

In addition to BM-MSCs, various MSC types have been differentiated into steroidogenic cells by the above mentioned methods. However, their steroidogenic properties markedly vary and depend on the derivation tissues and species (Table 2)[36,42,57,83,84]. For example, hBM-MSCs differentiated into cortisol-producing adrenocortical-like cells and umbilical cord blood (UCB)-derived MSCs differentiated into granulosa luteal-like cells, which produced high levels of progesterone[36,57]. Gondo *et al*[83] also reported that steroidogenic profiles of adipose tissue-derived MSCs were markedly different from those of BM-MSCs prepared from the same mouse. However, the cell differentiation fate was consistent in each MSC. These findings suggest that the steroidogenic properties of the differentiated cells depend on the characteristics of the originating MSCs.

To determine the difference between BM-MSCs and UBC-MSCs, the fluctuations in gene expression were investigated by a DNA microarray[36,85]. Among the identified genes, peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) was expressed only in UBC-MSCs at relatively high levels. Consistent with these re-

sults, the expression of PGC-1α was observed in ovarian granulosa cells. Overexpression of PGC-1α in granulosa cells induced the genes essential for progesterone synthesis, whereas knockdown of PGC-1α in granulosa cells attenuated the expression of these genes. These results demonstrate that PGC-1α represents one of the important factors for progesterone production in luteinized granulosa cells.

Epigenetic regulation during differentiation

Differentiation of stem cells into specialized cells can be viewed as a process in which epigenetic changes result in alterations in genes expressed by the cell as it becomes more specialized[86,87]. Thus, stem cell differentiation is a process that involves a series of epigenetic changes in the genome: histone and DNA modifications cause chromatin structural changes and affect the profiles of gene expression. In fact, such epigenetic modifications contribute to the induction of steroidogenesis-related genes when MSCs differentiate into steroidogenic cells[44,88-90].

The histone code hypothesis predicts that post-translational modifications of histone tails, alone or in combination, function to direct specific and distinct DNA-templated programs[91]. Histone acetylation is a positive marker of transcription, while histone methylation correlates with transcriptional activation (H3K4, H3K36) and repression (H3K9, H3K27) that are dependent on their amino acid residues[92]. In hMSCs-derived steroidogenic cells, H3K27 acetylation and H3K4 dimethylation (active enhancer markers) increased in the regulatory regions of some steroidogenesis-related genes (glutathione S-transferase A and ferrodoxin reductase) after the introduction of SF-1[89,90]. Conversely, histone eviction, which has been reported in actively transcribed genes[93], took place on the promoter and the enhancer regions of the *StAR* gene[88]. Because these modifications occurred around the SF-1 binding sites, recruitment of SF-1 to the regulatory regions is likely to induce recruitment of various transcriptional regulators and histone modifiers, which in turn alter chromatin structure and lead to the expression of steroidogenesis-related genes.

In addition to histone modifications, DNA methylation at cytosine residues of the dinucleotide sequence CpG, which induces gene silencing, is essential for differentiation and development[94,95]. In MSC-derived steroidogenic cells, the DNA methylation status changes in the promoter regions of some steroidogenic genes during differentiation[44]. In undifferentiated hBM-MSCs, the *CYP11A1* promoter region is hypomethylated, whereas the *CYP17A1* promoter region is highly methylated. In SF-1/LRH-1-introduced MSCs during cAMP treatment, this condition was almost completely unchanged in the *CYP11A1* promoter region, whereas the *CYP17A1* promoter region was progressively demethylated. These methylation patterns of the *CYP11A1* and *CYP17A1* promoters closely paralleled the induction patterns of both genes by cAMP. There is a time lag associated with the induction of steroidogenic enzymes by cAMP treatment in SF-1/LRH-1-introduced MSCs[44,57]. The order of induction of the enzymes is similar to the sequential order of the steroid hormone synthesis pathway; upstream enzymes (CYP11A1 and 3β-HSD) were rapidly induced at earlier time points (6-12 h), whereas downstream enzymes (CYP17A1 and CYP11B1) were induced at later time points (24-48 h). Because this time lag disappeared by treatment with a demethylating agent, the status of DNA methylation in the promoter regions could be important for regulating the expression of steroidogenic enzymes in MSCs.

CONCLUSION

It is clear that SF-1 represents a master regulator, not only for the development of steroidogenic organs, but also for steroidogenesis following organogenesis. LRH-1 is also important for steroidogenesis in gonads. In addition, SF-1 and LRH-1 direct differentiation of non-steroidogenic stem cells into steroidogenic cells. Among the various stem cell types, MSCs are suitable stem cells for the differentiation of steroidogenic cells. After pre-differentiation into MSCs, pluripotent stem cells can also be subsequently differentiated into steroidogenic cells using SF-1. These cells may provide a source for regenerative and gene therapies, although various problems should be resolved in future studies. It is essential to delineate the conditions that allow the directed differentiation into specific steroidogenic lineages with the characteristics of testicular Leydig cells, ovarian granulosa and theca cells, as well as various types of adrenocortical cells (reticularis, fasciculata and glomerulosa). In addition, it is necessary to establish methods for inducing SF-1 and LRH-1 expression in stem cells without gene transfer. Further studies are required for the realization of regeneration of steroidogenic tissues.

MSC-derived steroidogenic cells also provide opportunities for investigating various phenomena involved in differentiation of steroidogenic cells and steroidogenesis. In addition to the molecular mechanisms of differentiation described herein, the conservation and evolution of the androgen metabolic pathway (11-ketotestosterone production) between teleost fish and mammals has been revealed[78,96]. Genome-wide analyses of differentiated cells identified novel target genes regulated by SF-1 and LRH-1[89,90,97,98]. In addition, they contributed to the elucidation of one of the causes of steroidogenesis disorders[99-101]. Thus, progression of these studies is also important for the understanding of steroidogenesis and its related disorders.

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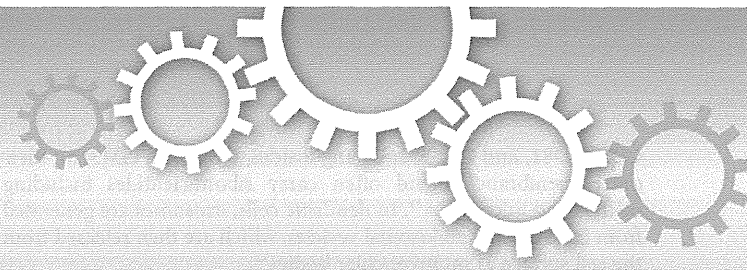

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Absence of CD9 reduces endometrial VEGF secretion and impairs uterine repair after parturition

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In mammals, uterine epithelium is remodeled cyclically throughout adult life for pregnancy. Despite the expression of CD9 in the uterine epithelium, its role in maternal reproduction is unclear. Here, we addressed this issue by examining uterine secretions collected from patients undergoing fertility treatment and fertilization-competent *Cd9*$^{-/-}$ mice expressing CD9-GFP in their eggs (*Cd9*$^{-/-}$*TG*). CD9 in uterine secretions was observed as extracellular matrix-like feature, and its amount of the secretions associated with repeated pregnancy failures. We also found that the litter size of *Cd9*$^{-/-}$*TG* female mice was significantly reduced after their first birth. Severely delayed re-epithelialization of the endometrium was then occurred. Concomitantly, vascular endothelial growth factor (VEGF) was remarkably reduced in the uterine secretions of *Cd9*$^{-/-}$*TG* female mice. These results provide the first evidence that CD9-mediated VEGF secretion plays a role in re-epithelialization of the uterus.

In humans, remodeling events that occur naturally in the uterus, namely menstruation and parturition, have features in common with tissue injury and repair in other tissues[1,2]. During such remodeling in the uterus, the human endometrium undergoes the menstrual cycle[1], whereas most other mammals are subjected to the estrous cycle[2]. In humans and mice, the endometrium commonly grows to a thick and blood vessel-rich glandular layer, providing the optimal environment for implantation of blastocysts in the uterine tissue[3]. The endometrium consists of a columnar epithelium and connective tissues that vary in thickness by hormonal control, and undergoes extensive epithelial turnover throughout adult female life[3]. The endometrium also prevents adhesion between the opposing walls of muscular layers in the uterus, termed the myometrium, thereby maintaining the patency of the uterine cavity[3].

In mice, the estrous cycle is divided into two ovarian phases, follicular and luteal phases[2]. The follicular phase is the period of ovarian follicle development consisting of proestrus and estrus stages, whereas the luteal phase is the period of corpus luteum formation and function comprising metestrus and diestrus stages[2]. The uterus is distended during proestrus and estrus stages because of the increase in uterine vascular permeability and accumulation of uterine secretions. This distention decreases in the midestrus stage, and it is no longer observed during the diestrus stage. The four stages of the estrous cycle are easily distinguished by the vaginal smear test[4].

CD9 gene encoding a 24-kDa protein is transcribed in all types of mammalian cells[5]. This protein is localized on the cell membranes and partly on endosomes, and it is expected to be involved in cell-cell adhesion, because CD9 associates with integrin family[5]. CD9 is also known as a motility-related protein 1 (MRP-1), which plays a role in suppressing tumor metastasis[6]. The pregnancy-specific glycoproteins (Psg) are secreted hormones encoded by multiple genes in rodents and primates, and the only Psg receptor identified is CD9[7]. Although Psg proteins are associated predominantly with endothelial cells lining vascular channels in the decidua, maternal CD9 is not essential for successful pregnancy[7]. CD9 belongs to a membrane protein family, collectively termed "tetraspanin", which encompasses 35 members in mammals, such as CD9, CD37, CD53, CD63, CD81, CD82, and CD151[5].

Nano-sized microvesicles, termed exosomes, are released from various cell types and play a role in transferring cellular materials from cell to cell[8]. They contain heat shock proteins, HSP70 and HSP90, present tetraspanins,

CD9, CD81, and CD63, and gangliosides, GM1 and GM3, on their outer membrane[9,10], and often carry ribonucleotides including mRNA and microRNA[11]. In dendritic cells, exosomes are generated from intraluminal endosomal vesicles, which are then released from the cell surface as multivesicular bodies[12].

CD9 plays a crucial role in sperm-egg fusion, and *Cd9*-deficient eggs are unable to fuse with sperm[13–15]. Moreover, CD9-containing exosome-like vesicles, namely egg exosomes, are released from eggs and transferred to the sperm head to facilitate sperm-egg fusion[16].

Some evidence in somatic cells and eggs has indicated the presence of two forms of exosomes, the former with intact lipid bilayers[8] and the latter without typical lipid bilayers[16,17]. In the endometrium, CD9 is predominantly distributed in the lateral membranes of epithelial cells[18]. However, in $Cd9^{-/-}$ female mice, no overt abnormalities have been reported in uterine function for pregnancy. Here, we focus on the role of CD9 in the uterus, more specifically endometrial epithelial cells.

Results

Extracellular presence of CD9 in mouse uterine secretions. In mice, CD9-containing exosomes are present in the extracellular region of eggs[16,17]. Hence, we considered that CD9 might be present extracellularly in the inner cavity of the uterus. Because the inner cavity is filled with uterine secretions during estrus, we first carried out immunostaining for CD9 in the endometrium at the estrus stage in 8–9-week-old C57BL/6N female mice (Fig. 1a). When we used anti-mouse CD9 mAb raised against the extracellular loop of CD9[19], CD9 was intensely expressed on the epithelial layers, and its intensity in the inner layer was stronger than that in the outer layer. Furthermore, in the outer layer, CD9 was localized at the basolateral region, but not the apical region (Fig. 1b).

Next, we estimated the amount of CD9 in the uterine secretions collected from mice at each stage of the estrus cycle by immunoblotting with anti-mouse CD9 mAb. As depicted in Fig. 1c, CD9 was detected at all four stages and the amount of CD9 in the estrus stage was strikingly higher than that in other stages (Fig. 1d, Supplementary Fig. 1a). Furthermore, to remove blood and epithelial cells, the uterine secretions were centrifuged and then subjected to immunoblotting (Supplementary Fig. 1b). Despite the removal of cells, CD9 was strongly detected in the supernatant (Supplementary Fig. 1c). Moreover, when we carried out immuno-electron microscopic analysis of CD9 in the uterine secretions, the gold particles conjugated to the anti-CD9 mAb reacted with extracellular matrix-like structures (Fig. 1e, g) that were distinct from typical exosomes with lipid bilayers (arrows in Fig. 1f). As depicted in Fig. 1h, these results suggest that extracellular CD9 is present in the uterine cavity and the amount of CD9 increases in a female reproductive cycle-dependent manner.

Correlation between the secreted CD9 and recurrent implantation failure (RIF) in patients. In humans, CD9 is expressed in the endometrial epithelium and localized on the cell membrane[20], but its extracellular presence is unknown. Therefore, we carried out immunoblotting for CD9 in human uterine flushing collected from the uterine cavity of subfertile patients. CD9 was detected in some samples (Fig. 2a, Supplementary Fig. 2a), but the total protein concentration in samples did not correlate with the amount of CD9 (Supplementary Fig. 2b). These results suggest that CD9 is present either in a soluble extracellular form or on exosomes in uterine fluid, and that patients can be divided into CD9-positive and CD9-negative groups. Therefore, we determined whether the absence of CD9 contributed to any clinical features in patients.

We examined the correlation between the presence of CD9 in uterine flushing and RIF. Two patient groups were recruited; those with RIF (37.1 ± 3.2 years old) and a control within 6 months since their first visit at clinics with complaint of infertility (36.4 ± 4.5 years old) (Supplementary Table 1a). The CD9-negative rate in patients with RIF (65.2%, n = 115) was significantly higher than that in the control (44.6%, n = 56) (Fig. 2b, Supplementary Table 1a). When the RIF patients were separated into those with or without a history of dilatation and curettage (D&C), the CD9-negative rate was significantly higher in patients with a history of D&C (78.1%, n = 32) than those without a history of D&C (53.1%, n = 49) (Fig. 2c, Supplementary Table 1b). Furthermore, when the RIF patients were separated into those with thin endometrium at the mid luteal phase (<8.5 mm by transvaginal ultrasound) or those with normal-width endometrium (≥8.5 mm), the CD9-negative rate was significantly higher in the RIF patients with thin endometrium (81.8%, n = 22) than those with normal endometrium (49.3%, n = 69) (Fig. 2d, Supplementary Table 1c). These results suggest that lack of CD9 might be associated with insufficient repair of endometrial injury and consequent thin endometrium. Moreover, we studied if the presence of CD9 in uterine flushing influences on the prognosis of the RIF patients. When the RIF patients were classified into four groups based on the presence of CD9 in uterine flushing and endometrial thickness at the mid luteal phase, the miscarriage rate for the RIF patients with CD9 (−) and thin endometrium was the highest (66.7%, n = 9), compared to those with CD9 (+) and thin endometrium (50.0%, n = 6), those with CD9 (+) and normal endometrium (42.9%, n = 7), and those with CD9 (−) and normal endometrium (15.0%, n = 20) (Fig. 2e, Supplementary Table 1d). As depicted in Fig. 2f, the absence of extracellular CD9 in the uterine cavity might be linked to insufficient repair of endometrial injury, endometrial thinning, and implantation failure.

Delayed repair of $Cd9^{-/-}$ endometrial epithelium. To explore the role of CD9 in the endometrium, we examined $Cd9^{-/-}$ female mice that are severely subfertile because of a strongly reduced ability of egg fusion with sperm. We previously generated fertilization-competent $Cd9^{-/-}$ mice expressing GFP-tagged CD9 (CD9-GFP) only in their eggs ($Cd9^{-/-}TG$)[16]. As depicted in Fig. 3a, after mating $Cd9^{-/-}TG$ female mice with $Cd9^{-/-}$ male mice, we quantitated the resulting litter size. Concomitantly, $Cd9^{+/+}$ female mice were mated with $Cd9^{-/-}$ male mice as a control. In general, the litter size of mice exhibits an age-dependent reduction, but the reduction of the litter size of $Cd9^{-/-}TG$ mice did not depend on their age (Fig. 3b). Therefore, we rearranged the litter size in a new order depending on parturition times (Fig. 3c). Although, the first litter size of $Cd9^{-/-}TG$ mice was comparable to that of $Cd9^{+/+}$ mice (7.2 ± 0.2 vs. 8.1 ± 0.3), the second litter size of $Cd9^{-/-}TG$ mice was significantly reduced compared with that of $Cd9^{+/+}$mice (2.9 ± 0.7 vs. 8.6 ± 0.4; $P < 0.001$). Similarly, the third litter size showed a significant reduction ($Cd9^{-/-}TG$ vs. $Cd9^{+/+}$; 2.8 ± 0.9 vs. 7.8 ± 0.8; $P < 0.001$). Moreover, the fourth litter size was reduced for both $Cd9^{-/-}TG$ and $Cd9^{+/+}$ mice (1.8 ± 0.9 vs. 2.4 ± 1.5). Thus, because the litter size reduction was dependent on parturition in $Cd9^{-/-}TG$ female mice, despite the expression of CD9-GFP in eggs[16], CD9 might also act in the endometrial epithelium.

Therefore, we focused on the process of endometrial repair after the first parturition in $Cd9^{-/-}TG$ female mice. As depicted in Fig. 3a, $Cd9^{-/-}TG$ female mice were mated with $Cd9^{-/-}$ male mice and their uteri were isolated after the first parturition. Concomitantly, $Cd9^{+/+}$ female mice were mated with $Cd9^{-/-}$ male mice. The endometrium of $Cd9^{+/+}$ mice at day 0 after parturition was sectioned and stained with H&E, revealing that the epithelial layer was separated from fetus-attached sites (upper panels in Fig. 3d). In $Cd9^{+/+}$ mice, the endometrium had fragile connective tissues, but was completely sealed by the epithelial layer at day 2 (middle panels in Fig. 3d). On the other hand, in the endometrium of $Cd9^{-/-}TG$ mice at day 5, the stromal layers were exposed on the inner surface of the uterus at the site of fetal attachment and the opposing walls were attached in the myometrium. These features resulted in a partial loss of patency in the uterine cavity (lower panels in Fig. 3d). Further observation at

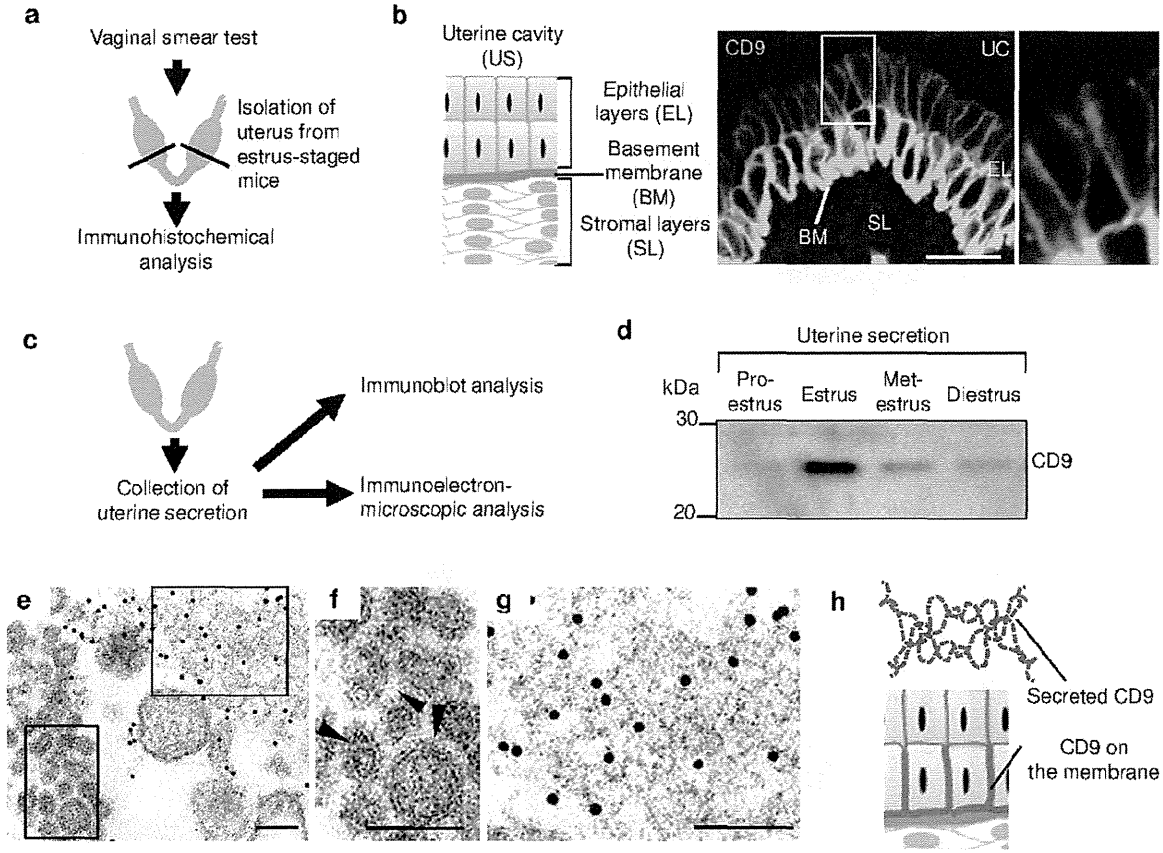

Figure 1 | Detection of extracellular CD9 in uterine secretions. (a), Experimental schematic for immunostaining the endometrial epithelium with an anti-CD9 mAb. After estrus-stage mice were identified by a vaginal smear test, the uterus was isolated and examined by immunohistochemical analysis. (b), As depicted from the left, the endometrium includes epithelial layers (EL), a basement membrane (BM), and stromal layers (SL). The endometrial epithelium was incubated with the anti-CD9 mAb and then an Alexa Fluor 488-labeled secondary antibody. UC, uterine cavity. Arrowheads, CD9-reduced apical regions. Scale bar, 20 μm. (c), Experimental schematic for immunoblotting of uterine secretions at each stage of the estrous cycle, and immuno-electron microscopic analysis of uterine secretions at the estrus stage. (d), Immunoblotting of uterine secretions collected from each stage of the estrous cycle. (e), Immuno-electron microscopic images of uterine secretions at the estrus stage. (f) and (g), Enlarged images of boxes in (e). Scale bars, 100 nm. (h), Schematic of the two types of CD9.

day 21 revealed that the endometrium was completely sealed and the connective tissues were recovered in $Cd9^{+/+}$ mice (upper panels in Fig. 3e). However, in $Cd9^{-/-}TG$ mice, the endometrium was not sealed by the epithelial layer, even at the same day, and the uterus had still lost patency (middle and lower panels in Fig. 3e). Based on these results, CD9 deficiency might reduce the motility and proliferation of epithelial cells in the uterus.

In vitro regenerative ability of the Cd9$^{-/-}$TG endometrial epithelium. To examine the regenerative ability of the $Cd9^{-/-}$ endometrial epithelium, we carried out a conventional *in vitro* wound healing assay. As depicted in Fig. 3f, after the uterus was isolated from estrus-stage $Cd9^{-/-}TG$ female mice, its inner cavity was treated with collagenase to collect and culture the epithelial cells. Concomitantly, epithelial cells were isolated from $Cd9^{+/+}$ female mice for culture. In wound healing assays from 0 to 17.5 hours, the wounds were significantly wide at 3.5 and 17.5 hours in cultures of $Cd9^{-/-}TG$ mouse epithelial cells compared with those in cultures of $Cd9^{+/+}$ mouse epithelial cells (3.5 h: 0.82 ± 0.04 vs. 0.60 ± 0.01, $P < 0.007$; 17.5 h: 0.46 ± 0.05 vs. 0.00 ± 0.00, $P < 0.001$; the wound width at 0 h was assigned a relative value of 1) (Fig. 3g, h). This result suggests that the regenerative ability of $Cd9^{-/-}$ endometrial epithelial cells is low compared with that of $Cd9^{+/+}$ cells.

Cell adhesion-related proteins in the Cd9$^{-/-}$TG endometrium. Repair of the endometrial epithelium is regulated by various physiological events such as cell-cell adhesion, cell motility, and cell growth[1]. In cell-cell adhesion, cadherins and integrins are known to be involved in endometrial repair[21]. In addition, CD98 is a multifunctional type II glycoprotein involved in amino acid transport, cell fusion, and integrin-dependent cell spreading[22]. CD98 is thought to function as a receptivity determinant, because its expression is undetectable outside of the implantation window[20]. According to these previous studies, we examined the localization of integrin α6 and β1, and CD98 in the $Cd9^{-/-}$ *TG* endometrium. Integrin α6 was normally localized at the basolateral membrane in $Cd9^{-/-}TG$ mice similar to that in $Cd9^{+/+}$ mice (upper panels in Supplementary Fig. 3). Basolateral and apical membrane localization of integrin β1 was also unchanged in the $Cd9^{-/-}$ endometrial epithelium (lower panels in Supplementary Fig. 3). Furthermore, the basolateral localization of E-cadherin was indistinguishable between $Cd9^{-/-}TG$ and $Cd9^{+/+}$ mice (upper panels in Supplementary Fig. 4). In addition, localization of CD98 at the apical and basal membrane was unaltered in $Cd9^{-/-}TG$ mice compared with that in $Cd9^{+/+}$ mice (lower panels in Supplementary Fig. 4). Thus, these cell adhesion-related proteins act normally in the $Cd9^{-/-}TG$ endometrial epithelium.

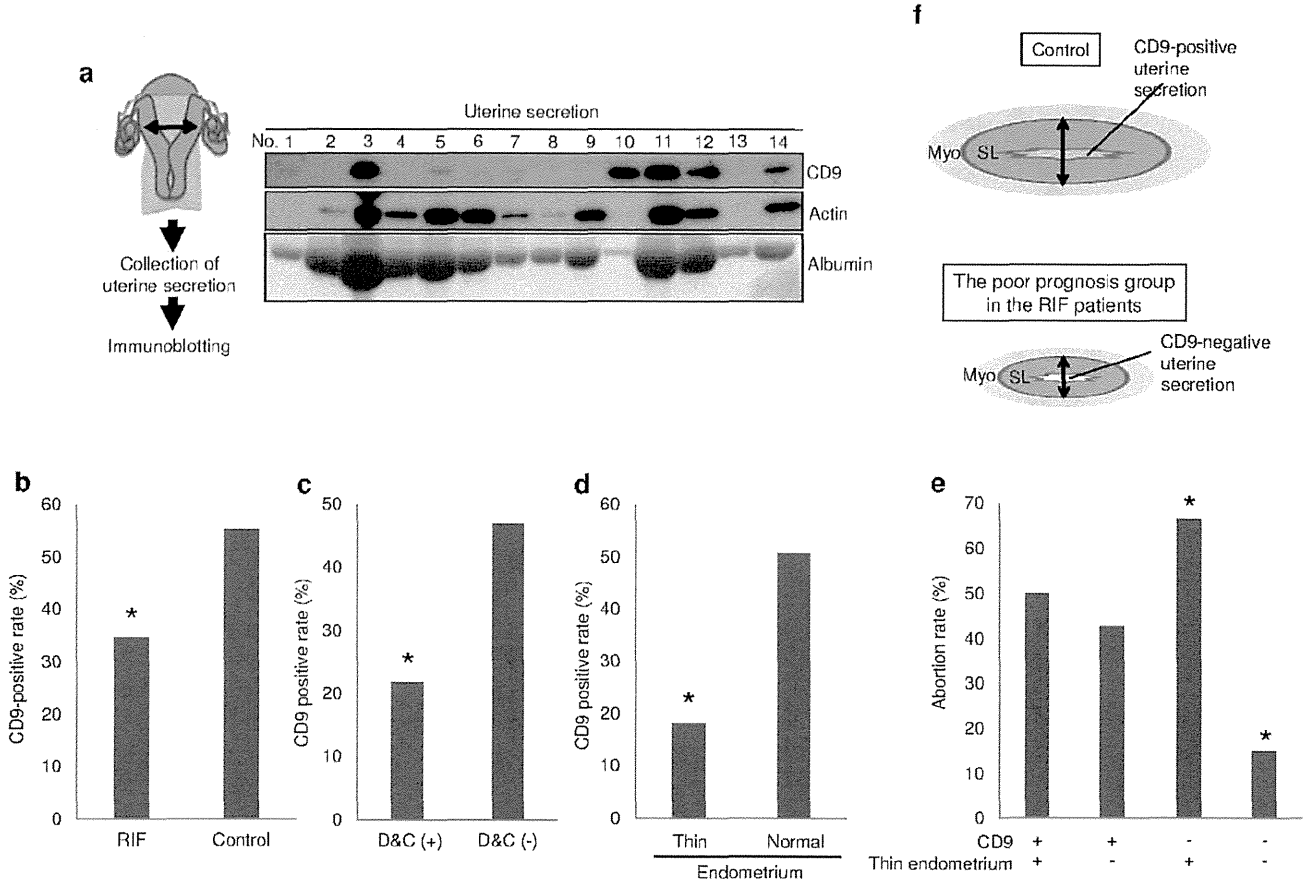

Figure 2 | Immunoblotting of uterine secretions collected from patients undergoing fertility treatment. (a), Uterine secretions were collected from the uterine cavity and examined by immunoblotting. Human samples were immunoblotted with anti-CD9 and anti-β-actin-mAbs, and stained with Coomassie brilliant blue for detection of albumin. The number of samples is shown. (b), The presence of CD9 in uterine flushing in recurrent implantation failure (RIF) patients (n = 115) and the control patients (n = 56). A significant difference among the mean values with asterisk was observed ($P < 0.05$). (c), The association of the CD9 presence with a history of dilation and curettage (D&C) in the RIF patients (D&C[+], n = 22; D&C[−], n = 49). A significant difference among the mean values with asterisk was observed ($P < 0.05$). (d), The association of the CD9 presence with endometrial thickness in the RIF patients. When endometrium width measured by vaginal ultrasound was less than 8.5 mm at the mid luteal phase, the endometrium was categorized as thin endometrium (n = 22). When the width was 8.5 mm or more than that, the endometrium was categorized normal-width endometrium (n = 69). A significant difference among the mean values with asterisk was observed ($P < 0.05$). (e), The association of the CD9 presence with prognosis (miscarriage rates) in the RIF patients. The RIF patients were classified into four groups: those with CD9 (+) and normal endometrium (n = 7), those with CD9 (+) and thin endometrium (n = 6), those with CD9 (−) and normal endometrium (n = 20), and those with CD9 (−) and thin endometrium (n = 9). A significant difference among the mean values that have different superscripts was observed ($P < 0.05$). The endometrial thickness was measured by ultrasound and magnetic resonance imaging at the site indicated with the line in (a), and double-headed arrows in (f). (f), Schematic explanation of a hypothetical relationship between the absence of CD9 and endometrial thinning in the RIF patients.

Cytokines in uterine secretions. In general, tissue remodeling is tightly controlled in a spatiotemporal manner by a complex network of regulators including cytokines[23]. Because immune cells are recruited into the endometrium during repair[24], there is the possibility that CD9 deficiency may directly or indirectly affect the amounts of cytokines in uterine secretions.

Therefore, we first estimated the amounts of cytokines of interest in the uterine secretions using a multiplex suspension array (Supplementary Fig. 5a). The interleukin (IL)-1 family is known to initiate inflammatory responses and play an important role in the physiology of the human endometrium[25]. To explore the possible involvement of IL-1 in uterine repair, we compared the amounts of IL-1α and IL-1β between *Cd9*$^{-/-}$*TG* and *Cd9*$^{+/+}$ mice. The amount of IL-1α in *Cd9*$^{-/-}$ *TG* mice was slightly decreased compared with that in *Cd9*$^{+/+}$ mice (65.2 ± 5.0 vs. 90.1 ± 11.7 pg/ml; $P = 0.017$) (Supplementary Fig. 5b). On the other hand, there was no difference in the amount of IL-1β between *Cd9*$^{-/-}$*TG* and *Cd9*$^{+/+}$ mice (38.4 ± 2.6 and 46.3 ± 4.9 pg/ml) (Supplementary Fig. 5b). These results imply a reduction in the amount of IL-1α, but sustained total activity of the IL-1 family.

IL-2 is necessary for growth, proliferation, and differentiation of T cells. Makkar et al.[26] reported that a low pregnancy rate is associated with increased expression of IL-2 in the endometrium. To evaluate the involvement of the IL-2 family in endometrial regeneration, we estimated the amounts of this family of cytokines, namely IL-2, IL-7, IL-9, and IL-15. The results show that the amounts of these cytokines in *Cd9*$^{-/-}$ *TG* mice were comparable to those in *Cd9*$^{+/+}$ mice (IL-2: 9.0 ± 1.3 and 8.6 ± 1.0 pg/ml; IL-7: 4.4 ± 0.3 and 4.9 ± 0.5 pg/ml; IL-15: 20.2 ± 2.8 and 21.7 ± 1.5 pg/ml) (Supplementary Fig. 5c). On the other hand, the amount of IL-9 was slightly reduced in *Cd9*$^{-/-}$*TG* mice compared with that in *Cd9*$^{+/+}$ mice (202.7 ± 8.4 vs. 269.2 ± 23.5 pg/ml; $P = 0.031$) (Supplementary Fig. 5c). Therefore, the amounts of IL-2, IL-7, IL-9, and IL-15 are maintained in the endometrium.

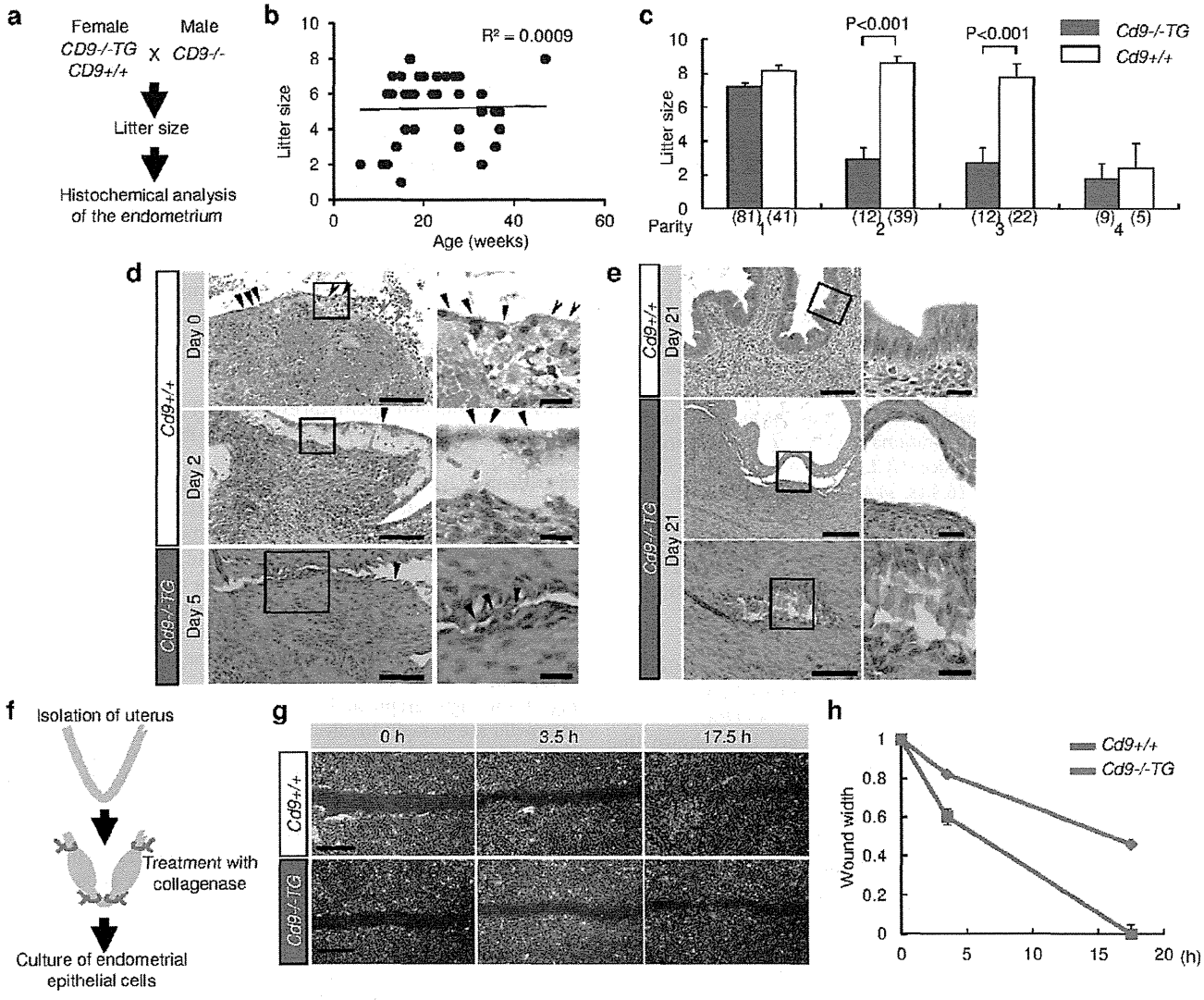

Figure 3 | Reduction of endometrial repair in *Cd9*$^{-/-}$*TG* mice. (a), Experimental schematic for examining the litter size of *Cd9*$^{-/-}$*TG* female mice. (b), Age-independent reduction of the litter size of *Cd9*$^{-/-}$*TG* mice. (c), Reduction of the litter size dependent on parturition in *Cd9*$^{-/-}$*TG* mice. Parenthesis indicates the number of examined mice. Values are the mean ± SEM. (d) and (e), Histochemical analysis of endometrial repair in *Cd9*$^{-/-}$*TG* and *Cd9*$^{+/+}$ mice after parturition (the day of parturition = day 0). (f), Experimental schematic for *in vitro* wound healing assays of the endometrial epithelium of *Cd9*$^{-/-}$*TG* mice. After the uterus was isolated from *Cd9*$^{-/-}$*TG* mice at the estrus stage, collagenase was injected to the intrauterine cavity to collect the epithelial cells. (g), Wounded epithelial cells after scratching the monolayer with a pipette tip. Scale bars, 150 μm. (h), Graph of the wound width of wounded epithelial cells. Values are the mean ± SEM.

IL-3 stimulates the differentiation of hematopoietic stem cells into myeloid and lymphoid progenitor cells, and functions in implantation[27]. Macrophage colony-stimulating factor (M-CSF) is involved in the proliferation, differentiation, and survival of monocytes, macrophages, and bone marrow progenitor cells, and a low serum level of M-CSF is associated with unexplained abortion[28]. In the present study, the amounts of IL-3 and M-CSF in the uterine secretions of *Cd9*$^{-/-}$*TG* mice were comparable to those in *Cd9*$^{+/+}$ mice (IL-3: 3.2 ± 0.1 and 3.1 ± 0.1 pg/ml; M-CSF: 10.8 ± 0.9 and 9.1 ± 0.8 pg/ml) (Supplementary Fig. 5d, e).

Interferon-γ (IFN-γ) is involved in the initiation of uterine vascular modification and maturation of uterine natural killer cells[29]. IL-10 has pleiotropic effects in immunoregulation and inflammation, and inhibits the synthesis of pro-inflammatory cytokines such as IFN-γ, IL-2, and IL-3[30]. In this study, the amounts of IFN-γ and IL-10 in *Cd9*$^{-/-}$*TG* mice were comparable to those in *Cd9*$^{+/+}$ mice (IFN-γ: 6.0 ± 2.7 and 4.6 ± 0.7 pg/ml; IL-10: 55.8 ± 36.3 and 16.4 ± 1.5 pg/ml) (Supplementary Fig. 5f).

IL-12 stimulates the production of IFN-γ in T and natural killer cells[31]. The amount of IL-12 in *Cd9*$^{-/-}$*TG* mice was significantly reduced compared with that in *Cd9*$^{+/+}$ mice (20.9 ± 1.7 vs. 31.4 ± 2.4 pg/ml; $P < 0.001$) (Supplementary Fig. 5g).

Neutrophils are present in large numbers and play an important role during endometrial repair[32]. Granulocyte colony-stimulating factor (G-CSF) stimulates the survival, proliferation, and differentiation of neutrophil precursors and mature neutrophils[33]. The amount of G-CSF was reduced in *Cd9*$^{-/-}$*TG* mice compared with that in *Cd9*$^{+/+}$ mice (761.3 ± 88.6 vs. 4610.4 ± 1955.3 pg/ml; $P = 0.025$) (Supplementary Fig. 5h). However, the number of uterine neutrophils in HE sections was comparable between *Cd9*$^{-/-}$*TG* and *Cd9*$^{+/+}$ mice.

Collectively, these results suggest that the immune system is maintained in the intrauterine cavity of *Cd9*$^{-/-}$*TG* mice, but there might be an impairment of the function of neutrophils.

Chemokines in uterine secretions. C-C motif chemokine-ligand 4 (CCL4) is released from neutrophils[34]. C-X-C motif chemokine

ligand 5 (CXCL5) is a strong chemoattractant for neutrophils[34]. To explore the involvement of these chemokines in the retarded repair of the endometrial epithelium in *Cd9*$^{-/-}$*TG* mice, we examined their amounts in uterine secretions using multiplex suspension arrays as depicted in Supplementary Fig. 5a. As a result, the levels of both chemokines was reduced in uterine secretions of *Cd9*$^{-/-}$*TG* mice (*Cd9*$^{-/-}$*TG* vs. *Cd9*$^{+/+}$; CCL4: 31.3 ± 4.6 vs. 45.1 ± 2.4 pg/ml, $P < 0.001$; CXCL5: 54.3 ± 4.4 vs. 96.0 ± 9.0 pg/ml, $P < 0.001$) (Supplementary Fig. 6a). This result reinforced the idea that neutrophils might be involved in the retarded endometrial repair in *Cd9*$^{-/-}$*TG* mice.

We further quantified the amounts of the following four chemokines in uterine secretions. CCL11, (also called eotaxin), CCL3, (also called macrophage inflammatory protein-1α), and CXCL1 (also called keratinocyte-derived chemokine) are involved in neutrophil recruitment[35]. CCL2, also called monocyte chemotactic protein-1, acts in neutrophil migration[35]. Unexpectedly, the amounts of these chemokines in the uterine secretions of *Cd9*$^{-/-}$*TG* mice were comparable to those of *Cd9*$^{+/+}$ mice (CCL11: 493.4 ± 130.0 vs. 554.9 ± 99.5 pg/ml; CCL3: 65.2 ± 16.4 vs. 44.4 ± 1.7 pg/ml; CXCL1: 23.4 ± 1.1 vs. 23.8 ± 1.5 pg/ml; CCL2: 16.0 ± 1.3 vs. 14.1 ± 2.6 pg/ml) (Supplementary Fig. 6b). These results indicate that neutrophil recruitment is maintained in the uterine secretions of the *Cd9*$^{-/-}$TG mice. Therefore, the immune system functions normally in the uterine secretions of *Cd9*$^{-/-}$*TG* mice.

VEGF and matrix metalloproteinases in uterine secretions of Cd9$^{-/-}$TG mice. VEGF, also called VEGF-A, is a well-known angiogenic factor that is essential for embryonic vasculogenesis and postnatal angiogenesis[36]. Considerable evidence have shown that VEGF also has non-angiogenic functions such as anti-apoptosis and vascular remodeling[37]. To explore the possible involvement of VEGF in uterine repair, we estimated the quantity of VEGF at the estrus stage in uterine secretions of *Cd9*$^{-/-}$TG mice using multiplex suspension arrays as explained in Supplementary Fig. 5a. The results show that the quantity of VEGF was significantly reduced in uterine secretions of *Cd9*$^{-/-}$*TG* mice compared with that in *Cd9*$^{+/+}$ mice (5.4 ± 0.5 vs. 16.6 ± 3.6 pg/ml; $P < 0.001$) (Fig. 4a). To confirm this result, we carried out immunoblotting for VEGF in the uterine secretions of *Cd9*$^{-/-}$*TG* and *Cd9*$^{+/+}$ mice. We detected VEGF in uterine secretions of *Cd9*$^{+/+}$ mice, but not in those of *Cd9*$^{-/-}$*TG* mice (Fig. 4b Supplementary Fig. 7). Three different sized-bands were detected in the uterine secretions of *Cd9*$^{+/+}$ mice. Because VEGF proteins preferentially form homodimers, these were predicted to be the monomer, dimer, and tetramer forms of VEGF as 21, 42, and 84 kDa, respectively, as described previously[38] (Fig. 4b, Supplementary Fig. 7). Matrix metalloproteinases (MMPs) play a role in endometrial repair, especially MMP2, MMP3, MMP7, and MMP11[1]. Tissue inhibitors of MMPs (TIMPs) are also involved in endometrial repair[1], indicating the importance of balanced activities of MMPs and TIMPs. Therefore, we carried out immunoblotting for MMP2, MMP3, MMP7, MMP11, and TIMP1 (Fig. 4b). The levels of MMP2, MMP7, and MMP11 in the uterine secretions of *Cd9*$^{-/-}$*TG* mice were comparable to those of *Cd9*$^{+/+}$ mice. On the other hand, the level of MMP3 was increased in *Cd9*$^{-/-}$*TG* mice compared with that in *Cd9*$^{+/+}$ mice. Furthermore, TIMP1 was detected in uterine cavity of *Cd9*$^{-/-}$*TG* mice, although its expression level was approximately half of that of *Cd9*$^{+/+}$ mice. Because TIMP1-heterozygotes female mice did not show severe phenotypes during fertilization and implantation[39], we considered that the reduced amount of TIMP1 would not affect fertility of *Cd9*$^{-/-}$*TG* mice. These results indicated that the levels of MMPs and TIMP1 in the uterine secretions were different between *Cd9*$^{-/-}$*TG* and *Cd9*$^{+/+}$ mice, but the total MMP activity was balanced in the uterine secretions of *Cd9*$^{-/-}$*TG* mice.

To further examine VEGF expression in the endometrial epithelium of *Cd9*$^{-/-}$*TG* and *Cd9*$^{+/+}$ mice, we carried out immunohistochemical analysis. As shown in Fig. 4c, the uteri of estrus-stage mice were double immunostained for VEGF and CD9, and counterstained with DAPI. The endometrial epithelial cells showed strong expression of VEGF compared with that in the stromal cells of both *Cd9*$^{-/-}$*TG* and *Cd9*$^{+/+}$ mice (Fig. 4c). In contrast, VEGF was present in the intrauterine cavity of *Cd9*$^{+/+}$ mice, but not in that of *Cd9*$^{-/-}$*TG* mice (arrows in Fig. 4c). From the immunohistochemistry, the uterine epithelium displayed elevated expression of VEGF receptor1, Flt-1, but not VEGF receptor2, Flk-1 (Supplementary Fig. 8a). Moreover, as the result from the immunoprecipitation with anti-CD9 antibody followed by blotting with anti-VEGF, the direct interaction between CD9 and VEGF was observed in the uterine fluid collected from *Cd9*$^{+/+}$ mice (Supplementary Fig. 8b). These results suggested that VEGF may be secreted from the endometrial epithelium in a CD9-dependent manner.

VEGF secretion and microvilli extension. To examine the morphological features affecting VEGF secretion, the endometrial epithelium of *Cd9*$^{-/-}$*TG* and *Cd9*$^{+/+}$ mice was examined by electron microscopy. Uteri were isolated from mice staged at estrus or metestrus. In *Cd9*$^{+/+}$ mice at the estrus stage, microvilli were formed on the apical membrane of the endometrial epithelium (hollow arrowheads in upper left panel of Fig. 4d). Similarly, microvilli were formed in the endometrial epithelium of *Cd9*$^{-/-}$*TG* mice, but their length was significantly shorter (arrowheads in lower left panel of Fig. 4d and left graph in Fig. 4e). On the other hand, at the metestrus stage, the short microvilli were present in the endometrial epithelium of both *Cd9*$^{-/-}$*TG* and *Cd9*$^{+/+}$ mice (Supplementary Fig. 9 and right graph in Fig. 4e). In differentiated Caco-2 cells, microvilli are thought to effectively increase the surface area of the cells and are useful for secretion of extracellular vesicles[40]. Because the length of microvilli was significantly shorter at the estrus stage in *Cd9*$^{-/-}$*TG* mice compared with that in *Cd9*$^{+/+}$ mice, we considered that the ability for secretion would be strikingly reduced in the endometrial epithelium of *Cd9*$^{-/-}$*TG* mice. Actually, the uterine cavity of *Cd9*$^{-/-}$*TG* mice was extremely narrow at the estrus stage compared with that of *Cd9*$^{+/+}$ mice (Supplementary Fig. 10), and it was difficult to collect a certain amount of uterine fluid from *Cd9*$^{-/-}$*TG* mice.

Intrauterine treatment of Cd9$^{-/-}$TG endometrial epithelium with VEGF. To explore the contribution of VEGF to the delay of endometrial repair in *Cd9*$^{-/-}$*TG* mice, we injected VEGF into the uterine cavity of *Cd9*$^{-/-}$*TG* mice. As depicted in Fig. 5a, at 1 week after parturition, the VEGF-linked microparticles were injected into the upper region of the left uteri and BSA-linked one as a control were injected into right uteri from the ventral view. The blue microparticles were retained in the uterine cavity even at 1 week after injection (Fig. 5b). To investigate the effects of VEGF treatment, the uteri were sectioned and stained with H&E. In VEGF-treated uteri, the endometrium was fully re-epithelialised (hollow arrowheads in left panels of Fig. 5c). On the other hand, in BSA-treated uteri, the epithelial layers remained limited and the stromal layers were exposed on the endometrium, and the debris including microparticles, decidualised tissues and immune cells was attached to the stromal layers without the epithelium (right panels of Fig. 5c). We counted the number of re-epithelialized regions where fetus had been implanted, and found the re-epithelialization in most VEGF-treated sites, but not in BSA-treated sites (67.5% for VEGF, $n = 8$; 0% for BSA, $n = 13$) (Fig. 5d). In addition, we measured the endometrial thickness and found that the thickness was significantly reduced in *Cd9*$^{-/-}$*TG* mice compared with that in *Cd9*$^{+/+}$ mice (174.5 ± 13.8 μm for VEGF, $n = 15$; 108.0 ± 15.2 μm for BSA, $n = 17$; $P = 0.003$) (Fig. 5e). As depicted in Fig. 5f, our results collectively suggest that the reduction of re-epithelialization in the endometrium of *Cd9*$^{-/-}$*TG* mice is reversed by VEGF treatment, thereby thickening the endometrium. Thus, we propose a novel mechanism

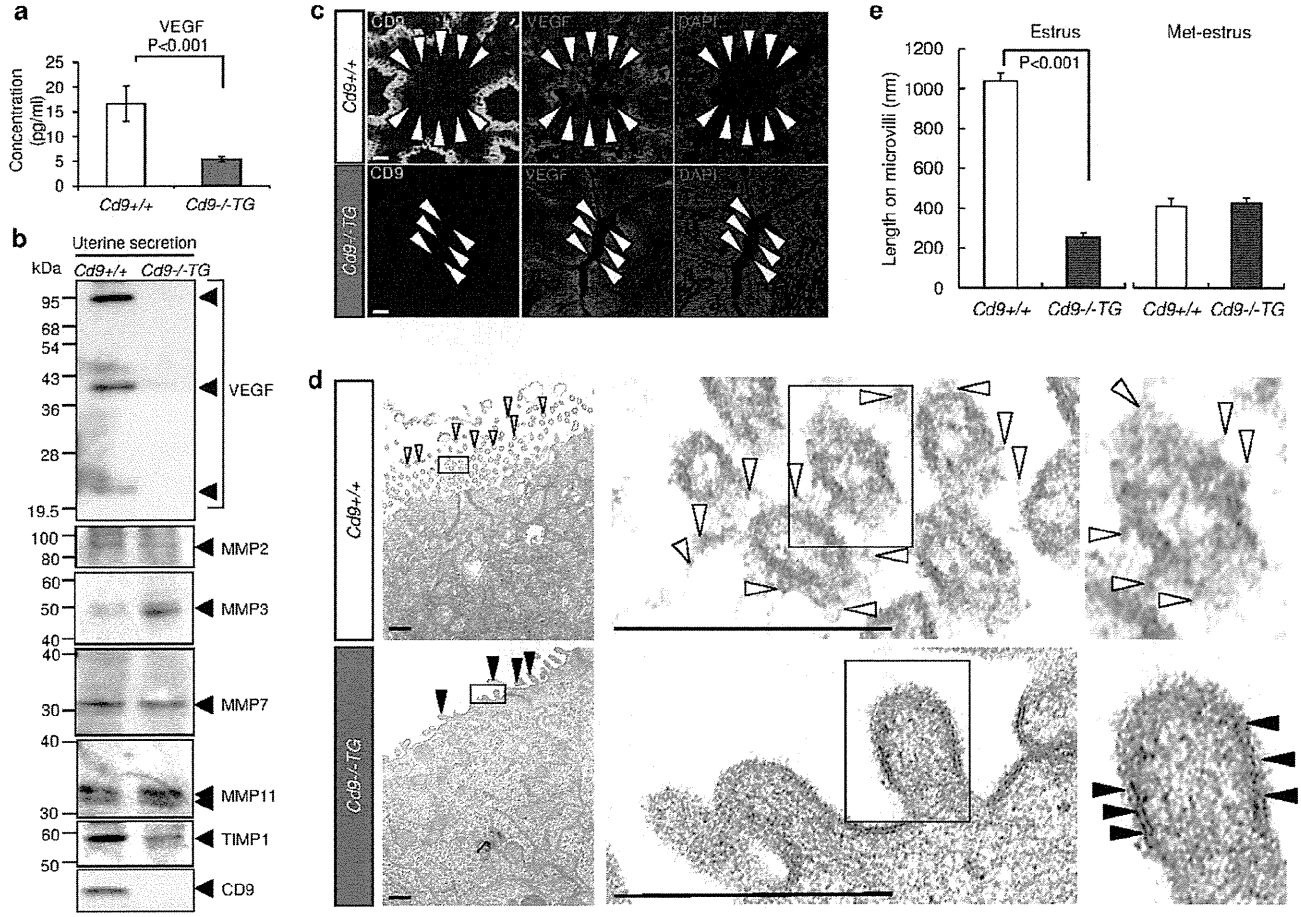

Figure 4 | Decrease of VEGF in uterine secretions of *Cd9*$^{-/-}$*TG* mice. (a), Comparison of the amount of VEGF in uterine secretions of *Cd9*$^{-/-}$*TG* mice at the estrus stage using a multiplex suspension array. (b), Immunoblotting of uterine secretions at the estrus stage in *Cd9*$^{-/-}$*TG* and *Cd9*$^{+/+}$ mice. (c), Immunohistochemical observation of the endometrial epithelium in *Cd9*$^{-/-}$*TG* and *Cd9*$^{+/+}$ mice. White arrowheads indicate the uterine cavity. Scale bars, 20 μm. (d), Electron microscopic images of endometrial epithelial cells in *Cd9*$^{-/-}$*TG* and *Cd9*$^{+/+}$ mice. Left panels, endometrial epithelial cells of *Cd9*$^{-/-}$*TG* and *Cd9*$^{+/+}$ mice at the estrus stage. Middle panels, enlarged images of the boxes in the left panels. Right panels, enlarged images of the boxes in the middle images. Hollow arrowheads indicate the secreted materials in *Cd9*$^{-/-}$*TG* mice. Arrowheads indicate the outer membrane consisting of lipid bilayers in *Cd9*$^{+/+}$ mice. Scale bars, 500 nm. (e), Length of microvilli. Left graph, endometrial epithelial cells at the estrus stage. Right graph, endometrial epithelial cells at the metestrus stage. Values are the mean ± SEM.

of endometrial repair, in which CD9 promotes secretion of VEGF from endometrial epithelial cells and aids the retention of VEGF on the uterine surface (Fig. 5f).

Discussion

The endometrial epithelium undergoes cyclic repair throughout the female reproductive life, but it also undergoes extensive repair because of tissue destruction caused by parturition, both of which are essential for maintenance of mammalian uterine function for pregnancy[2]. Endometrial epithelialization also prevents pathological adhesion between the opposing walls of the endometrium to eventually maintain the space in the uterine cavity required for embryo implantation and subsequent growth[2]. However, there is limited understanding of the mechanism of endometrial repair. Using VEGF blockade, Fan et al.[41] demonstrated that VEGF plays a critical role in early angiogenesis during postmenstrual endometrial repair in both mice and primates. They observed dramatic inhibition of re-epithelialization after VEGF blockade during endometrial repair[41]. The present study provides the first evidence for CD9-mediated VEGF secretion that contributes to endometrial repair (Fig. 5g).

As shown in Fig. 1 and Supplementary Fig. 1, the CD9-containing structure collected from the uterine cavity had no detectable plasma membrane, which was similar to the structure of egg exosomes[16]. Recently, Ng et al.[42] reported that endometrial exosomes including CD9 were observed in the uterine cavity of women. They demonstrated that exosomes of the size of 50–100 nm are present and contain specific miRNAs. These data support our findings that mouse uterine fluid at estrous contains exosome-like particles that are positive for the tetraspanin CD9. In other tissues, CD9 is also detected in extracellular fluid, cerebrospinal fluid from children with acute lymphoblastic leukemia43. Although CD9 is beginning to be recognized as an exosome marker[8,44], the change of the CD9-localization from membrane into the extracellular milieu is largely uncertain. In malignant or transformed cells, it has been known that the release of glycoproteins from the cell membrane is an active process named shedding[43,45,46]. Because CD9 has a potential N-glycosylation site in its extracellular loop, it has been suggested that CD9 is released from cells as one of these glycoproteins[43]. To our knowledge, however, there is no evidence that CD9 is actually glycosylated, and the molecular size of CD9 detected in the uterine was definitely consistent with its non-glycosylated size (24 kDa) (Figs. 1, 2 and 4). In previous paper, we observed the microvilli and the exosomes including CD9 in perivitelline space between egg plasma membrane and zona-pellucida in matured eggs of *Cd9*$^{+/+}$ mice, but not in that of

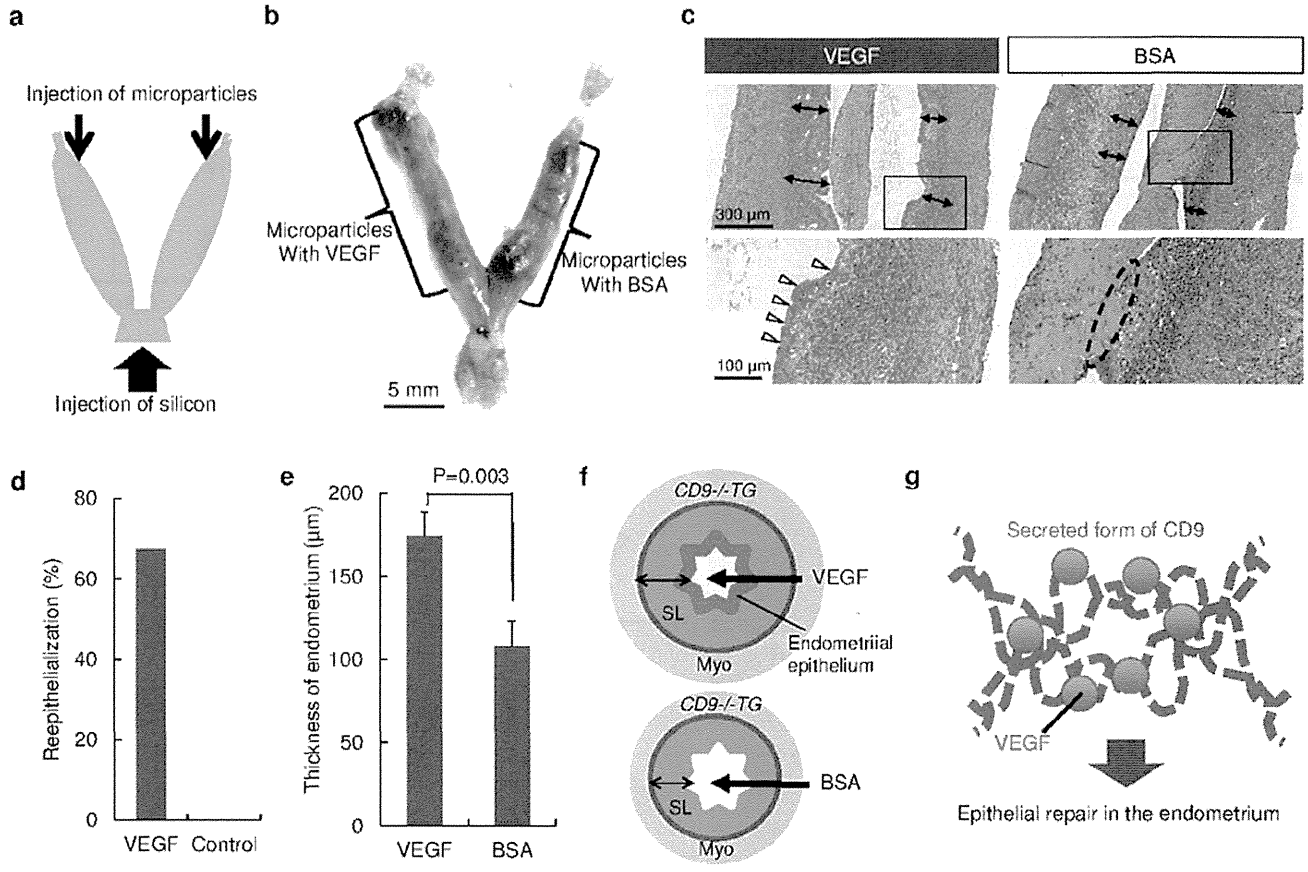

Figure 5 | Endometrial epithelium repair by VEGF treatment in *Cd9*$^{-/-}$*TG* mice. (a), Experimental schematic for treatment of the endometrial epithelium of *Cd9*$^{-/-}$*TG* mice with VEGF. (b), Ventral view of the horns of a uterus isolated from a *Cd9*$^{-/-}$*TG* mouse at 1 week after VEGF-linked microparticles were injected into the uterine cavity. Scale bar, 5 mm. (c), Endometrial epithelium treated with VEGF or BSA as a control. Lower panels, enlarged images of boxes in the upper panels. Double-headed arrows indicate the endometrial thickness. Hollow arrowheads indicate the re-epithelialized layers. The dotted circle indicates the fused site of endometrial stromal layers without epithelium with a mixture of microparticles and immune cells. Scale bars, 300 μm in upper panels and 100 μm in lower panels. (d), The rate of re-epithelialized sites in the *Cd9*$^{-/-}$*TG* endometrium treated with VEGF or BSA. (e), Thickness of the endometrium treated with VEGF or BSA. Values are the mean ± SE. (f), Schematic model of re-epithelialization of the endometrium treated with VEGF. The endometrial thickness was measured at the site indicated with double-headed arrows. SL, stromal layers; Myo, myometrium. (g), Schematic model of CD9 and VEGF secretion from epithelial cells. CD9-mediated VEGF release contributes to endometrial re-epithelialization.

Cd9$^{-/-}$ mice and unmatured oocytes of *Cd9*$^{+/+}$ mice[16]. These results indicate that a more specific mechanism controls the extracellular release of CD9, which is presumably related to the internal concentration of hormones depending on the female sexual cycle, as shown in Fig. 1d.

Because the reduction of the litter size of *Cd9*$^{-/-}$*TG* mice was not correlated with age, we considered that their endometrium was repaired during the estrous cycle, even in the absence of CD9 (Fig. 3c). In contrast, because the endometrial cavity of *Cd9*$^{-/-}$*TG* mice was narrowed, there may be a common mechanism underlying the endometrial repair, which works in both cyclic repair and repair after parturition. There are at least two possible mechanisms: (1) a mechanism regulated by MMPs and integrins as described previously[1] and (2) a mechanism regulated by CD9-mediated VEGF secretion. Both mechanisms would act in endometrial repair, but CD9-mediated VEGF secretion would contribute more to the endometrial repair after parturition than the cyclic repair.

In conclusion, we found that 1) a reduction of the amount of CD9 in uterine secretions and thinning of the endometrial thickness are correlated with the failure rate of pregnancy in patients; 2) CD9 deficiency causes reduced endometrial repair after parturition, leading to a reduction of fecundity; 3) CD9-mediated exocytosis plays a role in VEGF secretion.

Methods

Antibodies and chemicals. For immunohistochemistry and immunoblotting, a rat anti-mouse CD9 monoclonal antibody (mAb) (clone KMC8) and mouse anti-human CD9 mAb (clone ALB6) were purchased from BD Biosciences (San Jose, CA), and a mouse anti-mouse vascular endothelial growth factor (VEGF) mAb (clone RM0009-2G02) was purchased from Abcam. For immunohistochemistry, rat anti-integrin α6 and β1, and CD98 mAbs (clones GoH3, KMI6, and H202-141, respectively) were purchased from BD Biosciences, a rat anti-E-cadherin mAb (clone DECMA-1) was purchased from Sigma-Aldrich, and a rabbit anti-HIF-1α polyclonal antibody was purchased from Novus Biologicals. Secondary antibodies for immunohistochemistry were Alexa Fluor 488- and 546-conjugated IgGs purchased from Molecular Probes. Horseradish peroxidase-conjugated secondary antibodies (Sigma-Aldrich) were used for immunoblotting. Nuclei were counterstained with 4′, 6-diamidino-2-phenylindole (DAPI) (WAKO Pure Chemical Industries).

Immunoblotting of uterine secretions. Two patient groups were recruited from the outpatient department of the Fertility Clinic Tokyo (Tokyo, Japan) and St. Women's Clinic, Tokyo, Japan (Saitama, Japan). One group included 115 women with recurrent implantation failure (RIF). Patients in this group had received transfers of good quality embryos at least twice, but had not achieved pregnancy. A blastocyst with a grade higher than BB according to Gardner's criteria[47] was categorized as "good quality" in this study. The other group was a control including 56 women who had first visited the clinics with complaint of infertility within 6 months before the