Induction of steroidogenic cells from adult stem cells and pluripotent stem cells

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Abstract. Steroid hormones are mainly produced in adrenal glands and gonads. Because steroid hormones play vital roles in various physiological processes, replacement of deficient steroid hormones by hormone replacement therapy (HRT) is necessary for patients with adrenal and gonadal failure. In addition to HRT, tissue regeneration using stem cells is predicted to provide novel therapy. Among various stem cell types, mesenchymal stem cells can be differentiated into steroidogenic cells following ectopic expression of nuclear receptor (NR) 5A subfamily proteins, steroidogenic factor-1 (also known as adrenal 4 binding protein) and liver receptor homolog-1, with the aid of cAMP signaling. Conversely, these approaches cannot be applied to pluripotent stem cells, such as embryonic stem cells and induced pluripotent stem cells, because of poor survival following cytotoxic expression of NR5A subfamily proteins. However, if pluripotent stem cells are first differentiated through mesenchymal lineage, they can also be differentiated into steroidogenic cells via NR5A subfamily protein expression. This approach offers a potential suitable cells for future regenerative medicine and gene therapy for diseases caused by steroidogenesis deficiencies. It represents a powerful tool to investigate the molecular mechanisms involved in steroidogenesis. This article highlights our own and current research on the induction of steroidogenic cells from various stem cells. We also discuss the future direction of their clinical application.

Key words: Steroid hormone, Steroidogenic factor-1/adrenal 4 binding protein, Liver receptor homolog-1, Stem cells, Transcription
Development of steroidogenic organs

In adult, adrenal glands and gonads are the primary organs that produce steroid hormones for maintaining homeostasis. Although each organ produces different kinds of steroid hormones during adult life, they are developed from a common origin, the adrenogonadal primordium (AGP), at early developmental stage [7-9]. The AGP is derived from the mesodermal lineage and is localized on the coelomic epithelia of the developing urogenital ridge. As development proceeds, AGP separates into two distinct populations, the adrenocortical and gonadal primordia, characterized by the existence of chromaffin cell precursors and primordial germ cells, respectively. These cells are subsequently differentiated into organs that produce tissue-specific steroid hormones under the control of ACTH and gonadotropins.

In steroidogenic cells, steroidogenesis begins in the mitochondria with conversion of cholesterol into pregnenolone by the P450 side chain cleavage enzyme (P450scc, CYP11A1/Cyp11a1), which is a rate-limiting enzyme involved in synthesis of all steroid hormones [1]. Thereafter, the various hormones are synthesized by tissue-specific P450 hydroxylases and hydroxysteroid dehydrogenases. Likewise, as common origin of both organs during development, steroidogenic pathway is common during early steps of tissue-specific hormone synthesis in adrenal and gonadal organs. However, this process is separated by tissue-specific enzymes to produce corticosteroids and sex steroids.

Steroidogenic factor-1 (SF-1)/adrenal 4 binding protein (Ad4BP), is a key transcription factor that enables steroidogenesis to occur in the adrenal glands and gonads [10-13]. SF-1/Ad4BP is important for steroidogenesis by regulating the transcription of steroidogenesis-related genes, such as cytochrome P450 steroid hydroxylases, hydroxyl dehydrogenases, cholesterol deliverers and electron transporters [14]. In addition, because SF-1/Ad4BP-knockout mice fail to develop gonads and adrenal glands, SF-1/Ad4BP represents a master regulator of the development of these organs [13, 15-17]. SF-1/Ad4BP belongs to the nuclear hormone receptor (NR) 5A subfamily with liver receptor homolog-1 (LRH-1) [14, 18, 19]. Although LRH-1 is known to be abundantly expressed in tissues of endodermal origin (liver, pancreas and intestine), it is also expressed in adrenal glands and gonads. In particular, its expression levels in the ovary are much higher than in all other tissues [20]. Because LRH-1 shares various characteristics with SF-1/Ad4BP, such as binding sequences, target genes, and cofactors, with SF-1/Ad4BP, the likelihood of its involvement in steroidogenesis is high. In fact, LRH-1-knockout mice demonstrate that LRH-1 is necessary for progesterone and testosterone production in ovarian granulosa-lutein cells [21-23] and testicular Leydig cells [24], respectively.

Differentiation of mesenchymal stem cell into steroidogenic cells

Based on their mesodermal developmental origin, our group focused on the ability of bone marrow-derived mesenchymal stem cells (BM-MSCs) to undergo steroidogenesis [25]. MSCs can be easily isolated from a number of adult tissues and possess the ability to differentiate into various mesodermal lineages, such as osteoblasts, chondrocytes and adipocytes [26-28]. Although originally discovered in the bone marrow [29], MSCs are also present in the mesenchyme of almost all organs, and represent a promising source for various cell therapies [30, 31]. Owing to their multipotency, paracrine potential and immune tolerance properties following transplantation, they are widely applicable in the field of tissue regeneration [30-32]. In fact, successful clinical therapeutic interventions using MSCs have been made in bone regeneration [33] and cardiac repair [34, 35], to name a few.

We first investigated whether MSCs had the potential to differentiate into steroidogenic cells both in vivo and in vitro. Experimentations were approached through transplantation studies and promoter-sorting analyses using MSCs isolated from rodent bone marrow. Because Leydig cells in the adult testes are known to differentiate until puberty from non-steroidogenic stem cells associated with seminiferous tubules [36-38], it was deemed possible to successfully transplant BM-MSCs into this in vivo environment. After 3 weeks, the transplanted BM-MSCs had colonized the interstitium, and whereby they expressed various steroidogenic enzymes similar to endogenous Leydig cells. In addition, some of the purified murine BM-MSC lines spontaneously differentiated into steroidogenic cells in vitro.

Using a human CYP11A1 promoter-driven green fluorescence protein (GFP) reporter, consisting of a 2.3-kb fragment that selectively drives reporter gene
Steroidogenic cells from stem cells

Steroidogenic cells from stem cells similar to adult Leydig cells. Consistent with the gene expression profile, these cells mainly produced progesterone and testosterone.

Using the same approach, human BM-MSCs have been shown to differentiate into cortisol-producing cells in response to ACTH, resulting in cells that are very similar to fasciculata cells in the adrenal cortex. Yanase et al. have demonstrated that adenovirus-mediated transient expression of SF-1 in mouse and human BM-MSCs results in their differentiation into steroidogenic cells [41-43]. In addition to MSCs derived from bone marrow, MSCs from other tissue sources have been successfully differentiated into steroidogenic cells using these methods [44-46]. However, their steroidogenic properties vary markedly and depend on the derivation of tissues and species (Table 1). Conversely, these methods have not been successful using ES cells, embryonal carcinoma cells, and terminally differentiated cells such as fibroblasts and adipocytes [25].

![Fig. 1](image)

**Fig. 1** Mesenchymal stem cells (MSCs) were efficiently differentiated into steroidogenic cells by the stable expression of SF-1/Ad4BP or LRH-1, with the aid of cAMP. This method induced the differentiation of all MSCs into Cyp11a1-positive steroidogenic lineage cells.
for MSCs are not applicable to pluripotent stem cells, because SF-1/Ad4BP overexpression is cytotoxic to these cells [48]. Milbrandt et al. also reported that ES cells did not autonomously produce steroid hormones by ectopic expression of SF-1, even though they expressed some steroidogenic enzymes [49]. This finding could relate to the function of endogenous LRH-1 in pluripotent stem cells. LRH-1 is abundantly expressed in pluripotent stem cells and involved in the transcriptional regulation of central transcriptional factors, such as Oct-3/4 and Nanog, which are essential for maintaining the pluripotent and undifferentiated status [50, 51]. LRH-1 can enhance the reprogramming efficiency [52] and replace Oct-3/4 among the four essential factors (Yamanaka factors) in the production of iPS cells [53, 54]. Similar to their functions in steroidogenesis, SF-1 and LRH-1 have the potential to regulate Oct-3/4 expression [51], and thus the induction of somatic cells into iPS cells [54, 55]. Consequently, ectopic or overexpression of these factors in pluripotent stem cells can cause elevation of Oct-3/4 expression levels. It is well-known that the level of expression of Oct-3/4 determines the ES fate [56]. A less than twofold increase in Oct-3/4 expression causes differentiation of ES cells into primitive endoderm and mesoderm, whereas repression of Oct-3/4 expression induces loss of pluripotency and causes cell de-differentiation into the trophectoderm. This strongly suggests that the plu-

## Differentiation of ES cells into adrenocortical-like cells

Transplanted MSC-derived steroidogenic cells could improve symptoms in patients with steroid hormone deficiencies. However, MSCs derived from patients with congenital genetic deficiencies should not be used because the resulting autologous cells would contain the same genetic defects. The limited proliferative potential of adult stem cells is also an issue for regenerative therapy. Pluripotent stem cells, such as ES cells and induced pluripotent stem (iPS) cells, are able to self-renew indefinitely and maintain the ability to differentiate into all cell types. Thus, these stem cells offer the possibility to produce steroidogenic cells not only for regenerative medicine, but also for gene therapy, whereby defective alleles can be corrected by gene-specific targeting. However, same approaches for MSCs are not applicable to pluripotent stem cells, because SF-1/Ad4BP overexpression is cytotoxic to these cells [48]. Milbrandt et al. also reported that ES cells did not autonomously produce steroid hormones by ectopic expression of SF-1, even though they expressed some steroidogenic enzymes [49]. This finding could relate to the function of endogenous LRH-1 in pluripotent stem cells.

LRH-1 is abundantly expressed in pluripotent stem cells and involved in the transcriptional regulation of central transcriptional factors, such as Oct-3/4 and Nanog, which are essential for maintaining the pluripotent and undifferentiated status [50, 51]. LRH-1 can enhance the reprogramming efficiency [52] and replace Oct-3/4 among the four essential factors (Yamanaka factors) in the production of iPS cells [53, 54]. Similar to their functions in steroidogenesis, SF-1 and LRH-1 have the potential to regulate Oct-3/4 expression [51], and thus the induction of somatic cells into iPS cells [54, 55]. Consequently, ectopic or overexpression of these factors in pluripotent stem cells can cause elevation of Oct-3/4 expression levels. It is well-known that the level of expression of Oct-3/4 determines the ES fate [56]. A less than twofold increase in Oct-3/4 expression causes differentiation of ES cells into primitive endoderm and mesoderm, whereas repression of Oct-3/4 expression induces loss of pluripotency and causes cell de-differentiation into the trophectoderm. This strongly suggests that the plu-

### Table 1: Summary for the induction of steroidogenic cells from stem cells

| Cells          | Origin               | Produced steroid hormone | Properties of differentiated cells | Methods of induction | References |
|----------------|----------------------|--------------------------|-----------------------------------|----------------------|------------|
| MSCs           | Rat Bone marrow      | Testosterone (?)         | Testicular Leydig cells           | Transplantation      | [25]       |
| KUM9 (MSCs)    | Mouse Bone marrow    | Testosterone             | Testicular Leydig cells           | Promoter sorting SF-1/Ad4BP (plasmid) | [25, 67]   |
| hMSC-TERT-E6/7 (MSCs) | Human Bone marrow | Cortisol                 | Adrenal fasciculata cells         | SF-1/Ad4BP (plasmid) SF-1/Ad4BP (retrovirus) LRH-1 (retrovirus) LRH-1 (adenovirus) | [25, 60, 64] |
| UE77-13 (MSCs) | Human Bone marrow    | Testosterone, Cortisol   | Fetal adrenal-like cells          | SF-1/Ad4BP (retrovirus) SF-1/Ad4BP (adenovirus) LRH-1 (retrovirus) LRH-1 (adenovirus) | [70, 60, 64] |
| UCB408E67T-33 (MSCs) | Human Umbilical cord blood | Progesterone           | Ovarian granulosa-luteal cells   | SF-1/Ad4BP (retrovirus) | [45]       |
| EBRTcH3 (ESCs) | Mouse Embryo         | Corticosterone           | Adrenal fasciculata cells         | SF-1/Ad4BP (knock-in) | [48]       |
Steroidogenic cells from stem cells

ripotency and indefinite proliferation of ES cells are disturbed by overexpression of NR5A family proteins via Oct-3/4 expression. This would likely cause difficulties in the induction of steroidogenic cells from pluripotent stem cells by NR5A subfamily members.

We next hypothesized that pluripotent stem cells could differentiate into steroidogenic cells following expression of NR5A subfamily proteins after first being induced into MSCs [48]. To demonstrate this hypothesis, we used a murine ES cell line, carrying a tetracycline (Tc)-repressible transgene at the ROSA26 locus [57]. SF-1 cDNA was integrated into the ROSA-TET locus by a knock-in method, and drug-resistant clones were selected (Fig. 2). Withdrawal of Tc from the culture medium resulted in the expression of SF-1 within 48 h, even though the ES cells maintained an undifferentiated state and did not express steroidogenesis-related genes. However, after leukemia inhibitory factor (LIF) removal from the culture medium, the ES cells ceased to proliferate and died within several days. These results are in agreement with the findings as described above.

Fig. 2 Induction of steroidogenic cells from ES cells using the Tet-off system. ES cells were differentiated into adrenocortical-like cells by SF1/Ad4BP expression, only after pre-differentiation into MSCs.
We then induced MSCs from ES cells under the presence of Tc. First, ES cells were cultured on collagen IV-coated dishes and treated with pulse exposures to retinoic acid (RA), as described by Era et al. [58]. Various mesenchymal lineage molecular markers were induced by RA treatment, indicating that the ES cells had successfully differentiated into mesenchymal cells including MSCs. These cells were cultured further without RA and Tc for 3 days. In contrast to undifferentiated ES cells, the differentiated cells were able to survive following SF-1 expression in the absence of LIF. In these cells, SF-1 expression resulted in the expression of various steroidogenesis-related genes such as StAR, Cyp11a1, Hsd3b1, Cyp17a1, Cyp21a1, Cyp11b1 and Acthr. The gene expression pattern was similar to that in adrenocortical cells, namely fasciculata cells. Consistent with the gene expression profile, corticosterone was the most abundantly secreted steroid hormone from these cells. These results indicate that ES cells can be differentiated into steroidogenic cells by SF-1 via the mesenchymal lineage.

In support of this hypothesis, Sonoyama et al. demonstrated that human ES and iPS cells can be differentiated into cortisol-producing cells following expression of SF-1, but only after their pre-differentiation into mesodermal cells [59]. Jameson et al. also reported that SF-1-transfected ES cells differentiate into steroidogenic cells only under specific culture conditions to form embryoid bodies, although these cells predominantly produced sex steroids [59].

**Steroidogenic cells derived from various MSCs and their properties**

MSC-derived steroidogenic cells also provide opportunities for investigating the process steroidogenesis. Using these cells, novel SF-1/Ad4BP-regulated genes, including 5-aminolevulinic acid synthase 1 [60], ferredoxin 1 [61], ferredoxin reductase [62], glutathione S-transferase A family [63] and ovarian isoform of LRH-1 [20] have been identified by combined DNA microarray and promoter tiling array analyses. In adrenal and gonadal steroidogenic cells, expression of these genes is regulated by direct binding of SF-1/Ad4BP to the promoter and enhancer regions. It has also been demonstrated that epigenetic changes such as DNA methylation and histone modification are involved in expression of these and other steroidogenic genes during cell differentiation [47, 64]. We also revealed the cooperation of SF-1/Ad4BP and transcriptional regulators, peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) and CCAAT-enhancer-binding protein-beta (C/EBPβ), in steroidogenesis. PGC-1α acts as a powerful co-activator for NR5A subfamily proteins and stimulates the progesterone production in ovarian granulosa cells, although it is inhibited by DAX-1 without gonadotropin stimulation [45, 65]. C/EBPβ binds to the promoter regions of some steroidogenic genes and enhances steroid hormone production with SF-1/Ad4BP [66].

In addition to these molecular mechanisms of steroidogenesis, stem cell-derived steroidogenic cells have been used to highlight the conservation and evolution of 11-ketotestosterone production between teleosts and mammals (human and mouse) [67, 68]. These studies also revealed involvement of the androgen/androgen receptor pathway in the induction of ovulation-related genes, cyclooxygenase-2 and amphiregulin, in periovulatory granulosa cells [69]. Furthermore, MSC-derived steroidogenic cells have contributed to analysis of transcriptional regulation of the cytochrome P450 oxidoreductase gene, POR, and its deficiency [70, 71]. Thus, stem cell-derived steroidogenic cells represent important tools for the progression of studies in steroidogenesis and its related disorders.

**Summary**

Of the various stem cell types, MSCs are suitable for differentiation into steroidogenic cells by SF-1/Ad4BP expression. Pluripotent stem cells can also be differentiated into steroidogenic cells, but only after pre-differentiation into MSCs prior to SF-1/Ad4BP expression. Both cell types represent candidates for regenerative and gene therapies, although a number of issues need to be resolved through future studies prior to their clinical application. For example, it is essential to define the conditions required for directed differentiation into specific steroidogenic lineages, including 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 to induce steroidogenic cells from stem cells without gene transfer. Aside from regenerative medicine, MSC-derived steroidogenic cells offer a tool for investigating the process of steroidogenic cell differentiation and steroidogenesis. To date, they have...
contributed to identifying one of the main causes of steroidogenesis disorders. Thus, progression of these studies is critical for the understanding of steroidogenesis and its related disorders.

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