Pediatric Surgery International (2019) 35:1353–1361
https://doi.org/10.1007/s00383-019-04561-7

ORIGINAL ARTICLE

An intra-amniotic injection of mesenchymal stem cells promotes lung maturity in a rat congenital diaphragmatic hernia model

Shohei Takayama[1,2] · Kohei Sakai[1] · Shigehisa Fumino[1] · Taizo Furukawa[1] · Tsunao Kishida[2] · Osam Mazda[2] · Tatsuro Tajiri[1]

Accepted: 12 September 2019 / Published online: 26 September 2019

Abstract

Purpose We aimed to evaluate the effect of human mesenchymal stem cells (hMSCs) on congenital diaphragmatic hernia (CDH) by intra-amniotic injection in a rat CDH model.

Methods Nitrofen (100 mg) was administered to pregnant rats at E9.5. hMSCs (1.0×10^6) or PBS was injected into each amniotic cavity at E18, and fetuses were harvested at E21. The fetal lungs were classified into normal, CDH, and CDH-hMSCs groups. To determine the lung maturity, we assessed the alveolar histological structure by H&E and Weigert staining and the alveolar arteries by Elastica Van Gieson (EVG) staining. TTF-1, a marker of type II alveolar epithelial cells, was also evaluated by immunohistochemical staining and real-time reverse transcription polymerase chain reaction.

Results The survival rate after intra-amniotic injection was 72.1%. The CDH-hMSCs group had significantly more alveoli and secondary septa than the CDH group ($p<0.05$). The CDH-hMSCs group had larger air spaces and thinner alveolar walls than the CDH group ($p<0.05$). The medial and adventitial thickness of the pulmonary artery in the CDH-hMSCs group were significantly better ($p<0.001$), and there were significantly fewer TTF-1-positive cells than in the CDH group ($p<0.001$).

Conclusion These results suggest that intra-amniotic injection of hMSCs has therapeutic potential for CDH.

Keywords Congenital diaphragmatic hernia · Mesenchymal stem cell · Intra-amniotic injection · Fetal therapy · Lung hypoplasia · Nitrofen

Introduction

Congenital diaphragmatic hernia (CDH) is a disease characterized by a diaphragmatic defect and protrusion of abdominal content into the thoracic cavity, leading to lung hypoplasia and immaturity [1]. Although the treatment of patients with CDH has been improving with the introduction of the current therapeutic approaches, including fetal tracheal occlusion, gentle ventilation strategy, and extracorporeal membrane oxygenation, the mortality rate remains high in infants with severe CDH [1–3]. Therefore, it is desirable to combine novel therapies targeting the facilitation of fetal and postnatal lung growth, including new drugs, along with regenerative medicine, cellular therapy, and other modalities.

Mesenchymal stem cells (MSCs) are widely used in experimental research for cell-based therapy. MSCs can be isolated from mesenchymal tissue, including bone marrow, muscle, adipose tissue, and lungs, and have the ability to differentiate to multiple lineages [4]. MSCs have also a low antigen-presenting capacity due to their low expression of MHC class II cell surface markers and are considered to have a low immune response [5]. Several studies have demonstrated the efficacy of MSCs transplantation in treating models of lung injury, such as lung fibrosis, emphysema, and hyperoxic lung injury [6–8].

Intravascular injection is often selected for fetal therapy using MSCs, although it can cause complications such as immunodeficiency, inflammation, and microvascular embolism [5, 9]. Furthermore, the maternal blood is separated from the fetus blood by the blood–placental barrier, so the

✉ Shohei Takayama
taka0815@koto.kpu-m.ac.jp

[1] Department of Pediatric Surgery, Kyoto Prefectural University of Medicine, 465 Kawaramachi-Hirokoji, Kamigyo-ku, Kyoto 602-8566, Japan

[2] Department of Immunology, Kyoto Prefectural University of Medicine, Kyoto, Japan

potential for migration of MSCs delivered via intravascular administration is unclear.

During the gestation period, the fetus takes in amniotic fluid to the pulmonary alveoli by fetal breathing movement (FBM) to encourage lung maturity [10]. In this study, we focused on the intrauterine amniotic fluid and hypothesized that the intra-amniotic injection of MSCs might allow MSCs to migrate to the pulmonary alveoli through FBM. The present study therefore aimed to evaluate the therapeutic effect of human MSCs (hMSCs) on CDH via their intra-amniotic injection in a rat CDH model.

Materials and methods

hMSCs

MSCs derived from the adipose tissue of a Caucasian female (KURABO, Japan) were cultured using a Stem Life™ MSC Comp Kit (KURABO) with 1% (v/v) penicillin and streptomycin (Nacalai Tesque, Japan) in 5% CO_2/95% humidified air at 37 °C. The cells were trypsinized and harvested at about 70–80% confluency to receive the intra-amniotic injection. All experiments used low-passage hMSCs for in vivo studies (passages 6–8).

Nitrofen-induced CDH rat model

All experimental procedures and protocols for the animals conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Committee for Animal Research of Kyoto Prefectural University of Medicine (Code No. M28-529).

Timed-pregnancy Sprague–Dawley (SD) rats (Shimizu Laboratory, Japan) were housed in separate cages under normal room temperature and light conditions with free access to food and water. Pregnant rats were anesthetized with isoflurane (Pfizer, Japan) and administered 100 mg of herbicide nitrofen (2,4-dichorophenyl-p-nitrophenyl ether; WAKO Chemical, Japan) dissolved in 1 ml of olive oil via an orogastric tube at E9.5, whereas control rats received the same dose of olive oil without nitrofen. Even with nitrofen, not all fetuses developed CDH, so those without CDH were excluded from the analysis.

The intra-amniotic injection was performed at E18. Pregnant rats were anesthetized with isoflurane and maintained with pentobarbital (Kyoritsu Seiyaku, Japan). After midline laparotomy, the uterine horn was exteriorized, and the number of fetuses was counted. A 30-G needle with a 300 µL syringe (Becton–Dickinson and Company, Japan) was inserted into each amniotic fluid cavity, and hMSCs were injected near the fetus mouth, carefully avoiding the placenta, umbilical cord, and fetus itself (Fig. 1a, b). In addition, insertion near the innermost fetuses was avoided in order to prevent premature birth. As a sham treatment, the pregnant rats that received solvent at E9.5 were also injected with PBS.

The fetuses were harvested by Caesarean section and weighed at E21. After counting the number of surviving fetuses, the peritoneal cavities of each fetus were opened, and diaphragmatic defects were confirmed by a visual inspection. Only the fetuses with left diaphragmatic defects were used for the analysis. The fetuses were classified into the following three groups based on their findings: (1) the Normal group, including rats treated with PBS instead of hMSCs after olive oil exposure; (2) the CDH group, including rats treated with PBS instead of hMSCs after nitrofen exposure; and (3) the CDH-hMSCs group, including rats treated with hMSCs after nitrofen exposure. The fetal lungs were harvested, and a section of the left lungs was immersed in paraformaldehyde (PFA) solution. The rest of the left lungs was also weighed, and the lung-to body weight ratio (LBWR; calculated as the weight of the bilateral lungs [mg]/body weight [g]) was measured. The lungs were then frozen in liquid nitrogen. In order to detect hMSCs migration into the pulmonary alveoli, we also harvested the fetus from another pregnant rat at E19 and performed immunohistochemical staining (IHC) via the procedure described below.

Lung morphometry

After tracheal injection of 4% PFA under the constant pressure of 20 cm H_2O, the fetal trachea was ligated. The expanded lungs were immersed in 4% PFA for 24 h and then embedded in paraffin. Each sample was cut into 4-µm-thick sections and deparaffinized for further morphometric and immunohistochemical analyses. Fetal lungs without sufficient expansion were excluded from the analysis.

Tissue sections stained with H&E were used for pulmonary alveoli assessment, which included radial alveolar count (RAC), pulmonary alveolar wall thickness (AWT), and mean linear intercept (Lm). For the RAC assessment, a perpendicular line was drawn from the junction of the terminal bronchioles and respiratory epithelia to the nearest visceral pleura, and all airspaces on the line were counted in 10 sections per fetus [11]. The AWT was measured using lines drawn at 90° angles across the narrowest 20 sections per field (10 fields per fetus). The Lm was measured as the average distance between the alveolar walls in five sections per field (5 fields per fetus) [12]. Tissue samples stained with Weigert's were used for a secondary septa assessment for the elastin deposition. Five fields were measured, and their values were averaged in each group. Tissue samples stained with Elastica van Gieson (EVG) were used for the pulmonary artery (PA) assessment based on the proportionate medial thickness (%MT) and the proportionate

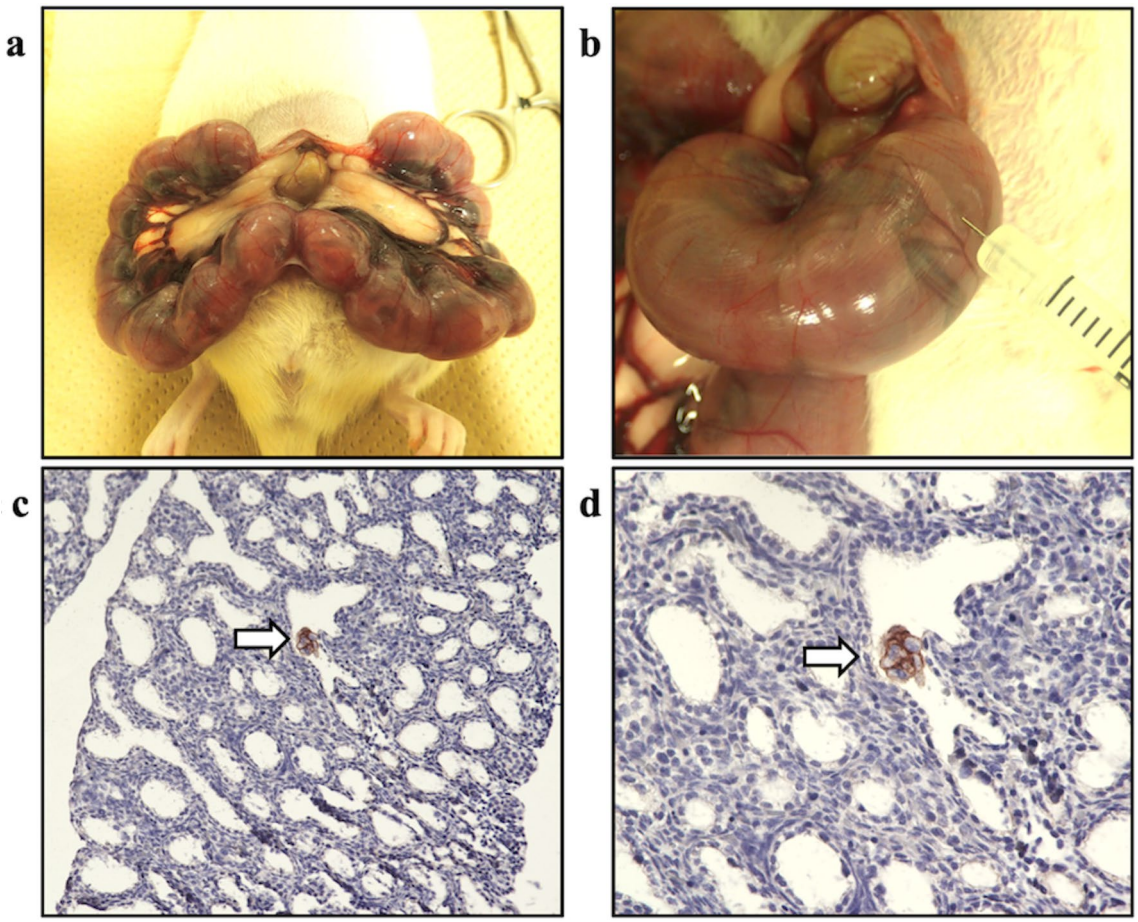

Fig. 1 Intra-amniotic injection of hMSCs. **a**, **b** hMSCs were carefully injected into the intra-amniotic cavity with a 30-G needle at E18. **c**, **d** Representative micrograph of CD90 immunohistochemically stained lung section. (**c** ×200 original magnification, **d** ×400). hMSCs were taken into the pulmonary alveoli at E18

adventitia thickness (%AT) according to the following formulae: %MT = 2 × medial wall thickness/external diameter; %AT = adventitial wall thickness/external diameter [13]. In each sample, only small PAs (external diameter: 30-100 μm) were measured in an approximately circular shape (minimum diameter/maximum diameter × 100 > 50%). These analyses were performed blindly with a pathologist.

Immunohistochemical staining

To detect the migration of hMSCs into the pulmonary alveoli histologically, we performed IHC staining with antibodies to CD90/Thy1 (1:200, ab133350; Abcam, UK). The slides were incubated with the appropriate secondary antibody followed by DAB Chromogen reagent (DAKO, USA) and counterstained with hematoxylin. IHC staining using an antibody against thyroid transcription factor-1 (TTF-1) (1:200, mouse, ab212886; Abcam) for the type II alveolar epithelial cell (AEC-II) assessment. Images were obtained with a microscope BZ-X700 (Keyence, Japan), and the number of TTF-1-positive cells was counted and their values averaged in each group.

Real-time reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from each lung ($n = 8$) using RNeasy Mini kit (Qiagen, Germany) and reversed transcribed using ReverTra Ace® qPCR RT Master Mix (Toyobo, Japan) according to the manufacturer's instructions. Real time RT-PCR of TTF-1 was performed using an SYBR green qPCR assay (Takara, Japan) according to the recommended protocol. The expression of β-actin was used as an endogenous control. The primers were used as follows: Forward, AAATTTGGGGGTCTTTCTGG, Reverse, AGAGTGCATCCACAGGGAAG.

Statistical analyses

Data were expressed as mean ± standard error of the mean. All data were analyzed statistically using Student's *t* test with the StatMate V software program (ATMS, Japan). Statistical significance was defined as a *p* value of less than 0.05.

Results

Intra-amniotic cavity injection of hMSCs

The overall survival rate of the intra-amniotic injection of hMSCs ($n=117$) or PBS ($n=132$) in a rat nitrofen injected model was 72.1%, and there was no significant difference in the survival between the hMSCs injection group and the PBS injection group (68.66% ± 8.29% vs. 75.63% ± 7.89%, respectively, $p=0.55$). hMSCs immunohistochemically stained with CD90 were confirmed in the pulmonary alveoli at E19 (Fig. 1c, d). hMSCs were observed in the junction of the terminal bronchioles and respiratory epithelia diffusely.

Incidence of CDH and lung body weight ratio

The incidence of CDH in this study was 36.4% ($n=48/132$) in the CDH group and 46.2% ($n=54/117$) in the CDH-hMSCs group. There was no significant difference in the lung body weight ratio between the CDH group and the CDH-hMSCs group (13.97 ± 0.50 vs. 14.00 ± 0.55, respectively, $p=0.94$).

The morphological assessment of the pulmonary alveoli

For the alveolar assessment, the RAC, AWT, Lm, and elastin deposition were measured (Figs. 2, 3). The RAC was significantly lower in the CDH group than in the normal group (2.43 ± 0.14 vs. 5.55 ± 0.21, $p<0.001$) and significantly higher in the CDH-hMSCs group than in the CDH group (2.93 ± 0.10 vs. 2.43 ± 0.14, $p<0.05$) (Fig. 2d).

The CDH group was characterized by interstitial thickening due to immature lung development, resulting in a thicker AWT and a narrower Lm than in the normal group (AWT: 23.23 ± 1.03 vs. 9.04 ± 0.35, $p<0.001$; Lm: 23.83 ± 1.31 vs. 47.62 ± 1.57, $p<0.001$). In contrast, in the CDH-hMSCs group, the abnormal interstitial thickness was improved, and the AWT was thinner and the Lm wider than in the CDH group (AWT: 19.37 ± 1.16 vs. 23.23 ± 1.03, $p<0.05$; Lm: 32.19 ± 2.03 vs. 23.83 ± 1.31, $p<0.01$) (Fig. 2e, f). Elastin deposition was noted on the tips of the secondary septa. This deposition in the CDH group was significantly less than in the normal group (4.86 ± 0.62 vs. 11.52 ± 1.16, $p<0.001$). The elastin deposition in the CDH-hMSCs group was significantly greater than in the CDH group (7.26 ± 0.86 vs. 4.86 ± 0.62, $p<0.05$) (Fig. 3d).

The morphological assessment of the PAs

For the assessment of the PAs, we measured the thickness of the tunica media and tunica adventitia (Fig. 4a–c). The tunica media and tunica adventitia in the CDH group were significantly thicker than in the normal group (tunica media: 29.25 ± 1.01 vs. 16.50 ± 1.93, $p<0.001$; tunica adventitia: 39.04 ± 2.67 vs. 22.25 ± 2.72, $p<0.001$). In contrast, both tunicae in the CDH group became thinner with hMSCs treatment (tunica media: 29.25 ± 1.01 vs. 20.50 ± 1.77, $p<0.001$; tunica adventitia: 39.04 ± 2.67 vs. 24.85 ± 1.40, $p<0.001$) (Fig. 4d, e).

The assessment of AECs-II

The number of TTF-1-positive cells on IHC in the CDH group was significantly greater than in the normal group (614.86 ± 19.27 vs. 433.75 ± 7.06, $p<0.001$) (Fig. 5a–d). Conversely, the number of TTF-1-positive cells in the CDH-hMSCs group ($n=10$) was less than that observed in the CDH group (451.12 ± 78.43 vs. 614.86 ± 19.27, $p<0.001$). On real-time RT-PCR, the expression of TTF-1 (TTF-1/β-actin) in the CDH group was greater than in the normal group (1.49 ± 0.12 vs. 1.00 ± 0.05, $p<0.001$). There were no significant differences between the CDH-hMSCs and CDH groups (1.25 ± 0.05 vs. 1.49 ± 0.12, $p=0.08$), but the gene expression in the CDH-hMSCs group tended to be lower than that in the CDH group, similar to the protein levels of TTF-1 (Fig. 5e).

Discussion

The present study demonstrated that the intra-amniotic injection of hMSCs promoted lung growth and maturity in a rat CDH model. The therapeutic effect of MSCs on CDH has been previously reported. Yuniartha et al. mentioned that MSCs derived from fetal lung parenchyma promoted lung maturity by transplantation via the uterus vein [14]. Pederiva et al. reported that amniotic fluid stem cells rescued nitrofen-induced hypoplastic lungs in coculture [15]. The intravascular injection route is often selected for fetal therapy using MSCs, but this approach has the potential to cause complications, such as immunodeficiency, inflammation, and microvascular embolism [5, 9]. Therefore, we selected the intra-amniotic injection route in order to reduce the side effects potentially caused by the intravascular injection of MSCs in this study. It has been also previously reported that the intra-amniotic injection at E13 has a therapeutic effect in

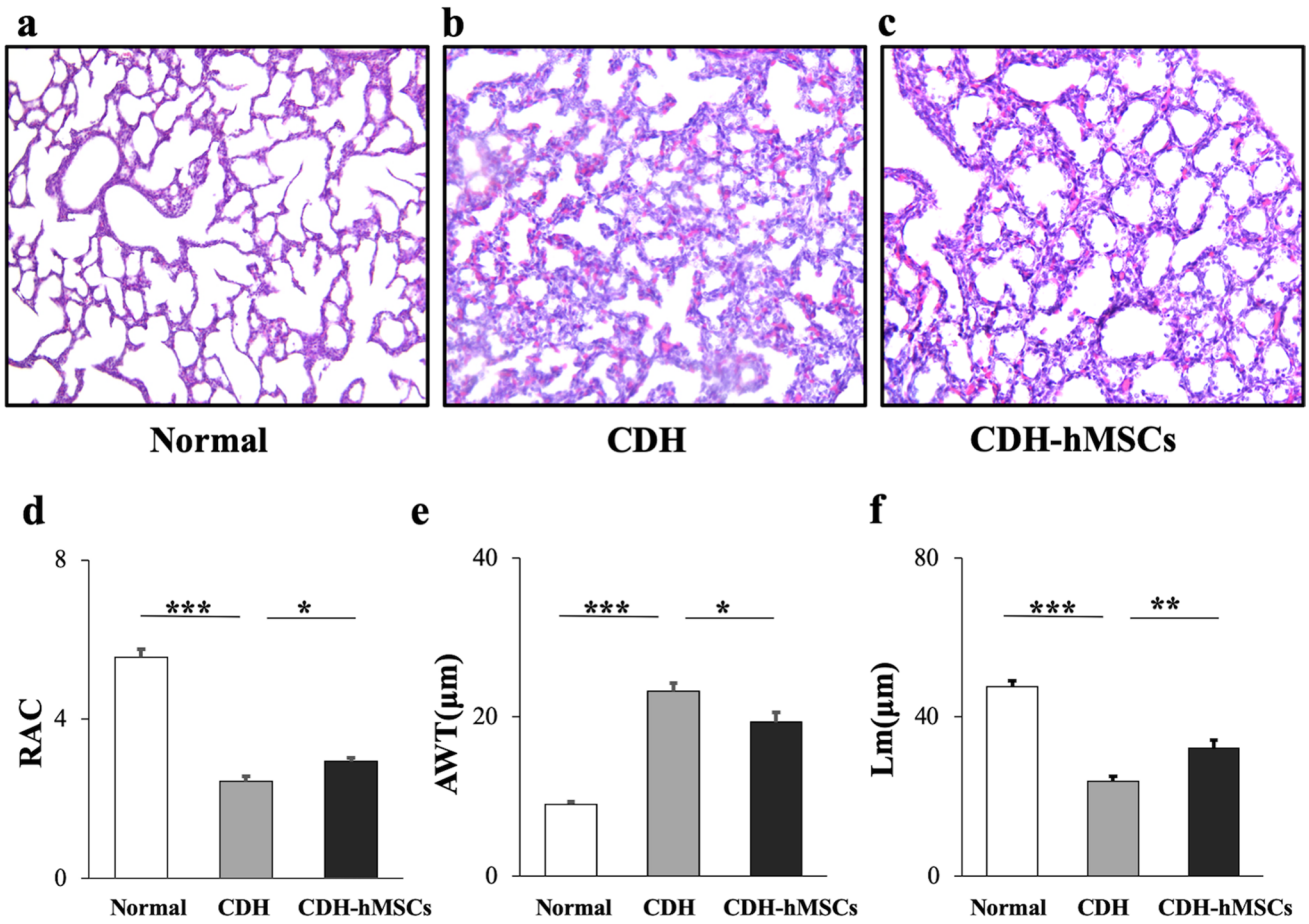

Fig. 2 The morphological assessment of the pulmonary alveoli by H&E staining. **a–c** Micrographs show representative images of the H&E-stained lung sections from the normal group (**a**), CDH group (**b**), and CDH-hMSCs group (**c**) at ×100 original magnification. **d** The radial alveolar count (RAC) in the CDH group was significantly lower than in the normal group ($p < 0.001$). The RAC in the CDH-hMSCs group was significantly greater than in the CDH group ($p = 0.009$). **e** The alveolar wall thickness (AWT) in the CDH group was significantly greater than in the normal group ($p < 0.001$). The AWT in the CDH-hMSCs group was significantly lower than in the CDH group ($p = 0.023$). **f** The mean linear intercept (Lm) in the CDH group was significantly smaller than in the normal group ($p < 0.001$). The Lm in the CDH-hMSCs group was significantly larger than in the CDH group ($p = 0.003$). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

an explant in vivo CDH model, but to our knowledge, there have been no previous reports describing the therapeutic effect in an in vivo study [16].

In this study, we confirmed that fetal rats could take in hMSCs with amniotic fluid at E18. Rat FBM reportedly starts from E16 and becomes active from E18 [10]. Therefore, E18 was presumed to be the best timing for the intra-amniotic injection. In addition, amniocentesis is a common procedure as a prenatal diagnostic test in clinical practice, being typically performed at 16-18 weeks when the fetal lung is developing in humans, which corresponds to E18 in a rat model.

Our previous study showed that prenatal treatment with administration of bombesin increases the LBWR [17]. However, the present study found no significant difference in the LBWR between the CDH and CDH-hMSCs groups. We assumed that this result was due to the surgical invasion related to the intra-amniotic injection. In a previous study, a midline laparotomy was made, and an osmotic pump containing bombesin was implanted in a pregnant rat. Conversely, the present surgical procedure was complicated and more invasive, involving midline laparotomy, exteriorization of the uterine horn, and intra-amniotic injection in both the CDH and CDH-hMSCs groups.

Pathologic abnormalities of CDH are characterized by decreased alveolar branching, mesenchymal thickening, and defective secondary septation. Similar changes are seen in the nitrofen-introduced CDH model, but the mechanism underlying these pathological changes is poorly understood. It is hypothesized that nitrofen inhibits retinal aldehyde dehydrogenase 2 (RALDH-2), resulting in the suppression of retinoic acid (RA) production [15]. The suppression of the RA production also downregulates several growth factors, such as bone morphogenetic proteins (BMP), Shh proteins,

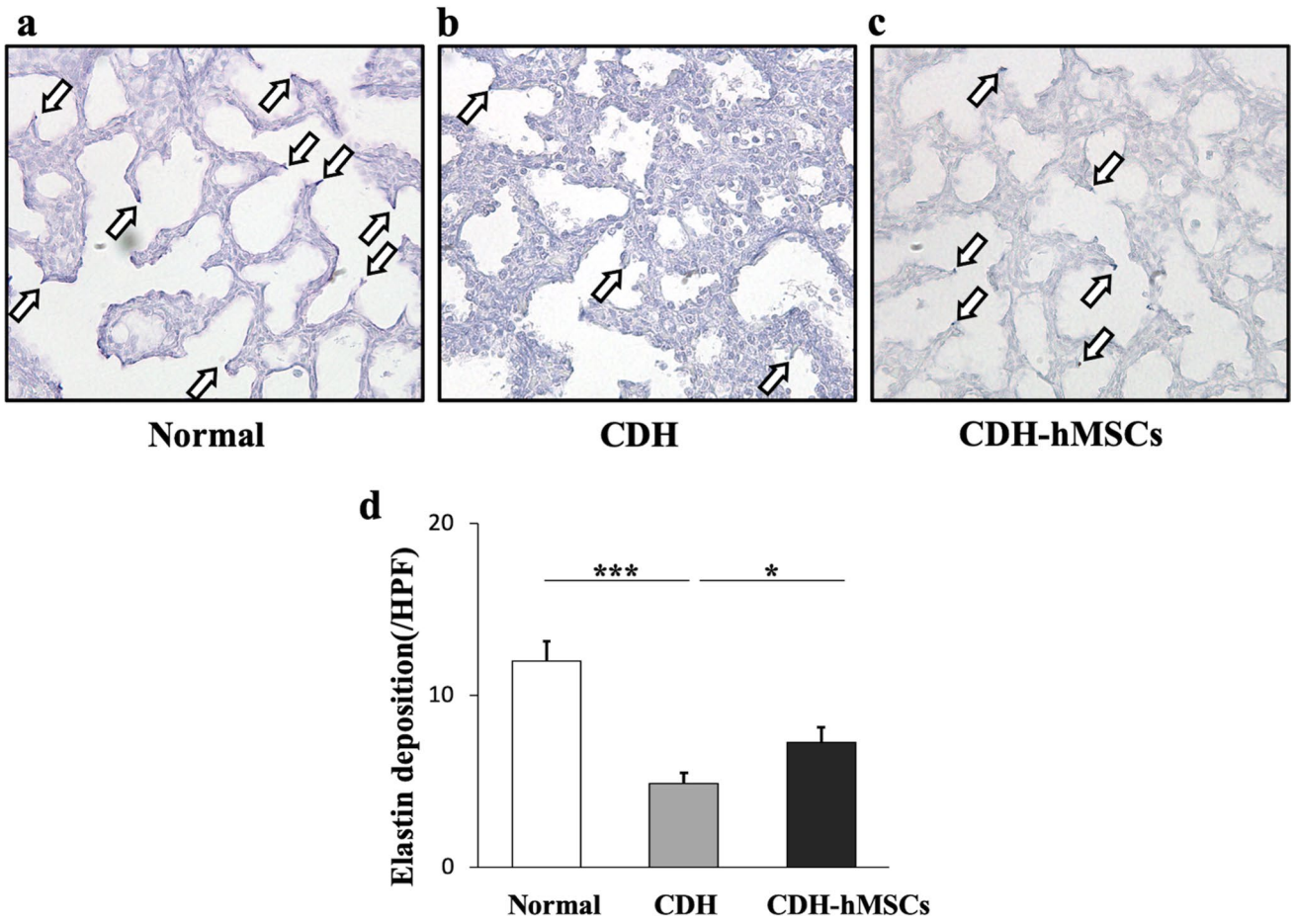

Fig. 3 The morphological assessment of the pulmonary alveoli by Weigert's elastin-staining. **a**–**c** The representative micrograph images of the Weigert's elastin-stained lung sections from the normal group (**a**), CDH group (**b**), and CDH-hMSCs group (**c**) at ×400 original magnification. White arrows indicate elastin deposition. **d** The elastin deposition in the CDH group was significantly less than in the normal group ($p < 0.001$). The elastin deposition in the CDH-hMSCs group was significantly greater than in the CDH group ($p = 0.036$). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

and fibroblast growth factor (FGF). As BMP plays a critical role in airway branching morphogenesis, the downregulated BMP results in a decreased number of alveoli. It has been hypothesized that mesenchymal thickening is caused by the diminished apoptosis of fibroblasts and the suppression of fibroblast proliferation via Shh signaling, such as the activation of kinesin family-7 and Forkhead box-1 [18, 19]. In addition, impaired Shh signaling inhibits the migration of alveolar myofibroblasts, which is essential for secondary septation, resulting in defective secondary septation [18].

In the present study, we observed a significant increase in the RAC, AWT thinning, Lm expansion and greater elastin deposition by the intra-amniotic injection of hMSCs than in the CDH group. The RAC, AWT/Lm, and elastin deposition have been used as indicators of lung maturity, mesenchymal thickening, and secondary septal formation, respectively [11, 12, 20, 21]. Our results showed that the intra-amniotic injection of hMSCs improved fetal pulmonary morphological abnormalities. Furthermore, our results showed that the PA wall became thinner following the intra-amniotic injection of hMSCs than in the CDH group. Mesenchymal thickening has been shown to lead to developmental abnormalities in pulmonary blood vessels [18]. Adventitial thickening of the PA may increase the vascular bed volume and inhibit vasodilation in CDH. Furthermore, medial thickening of the PA may increase the smooth muscle layer and pulmonary vascular resistance. Therefore, tunica media and adventitia thinning is assumed to help reduce pulmonary persistent hypertension in neonates [22]. Given these previous findings, the intra-amniotic injection of hMSCs is suggested to potentially prevent postnatal pulmonary persistent hypertension in neonates.

TTF-1 is a marker of AECs-II, which have been considered a progenitor of type 1 alveolar epithelial cells (AECs-I) [23]. The AEC-II numbers are increased while the AEC-I numbers are decreased in hypoplastic lungs [18, 24]. Our

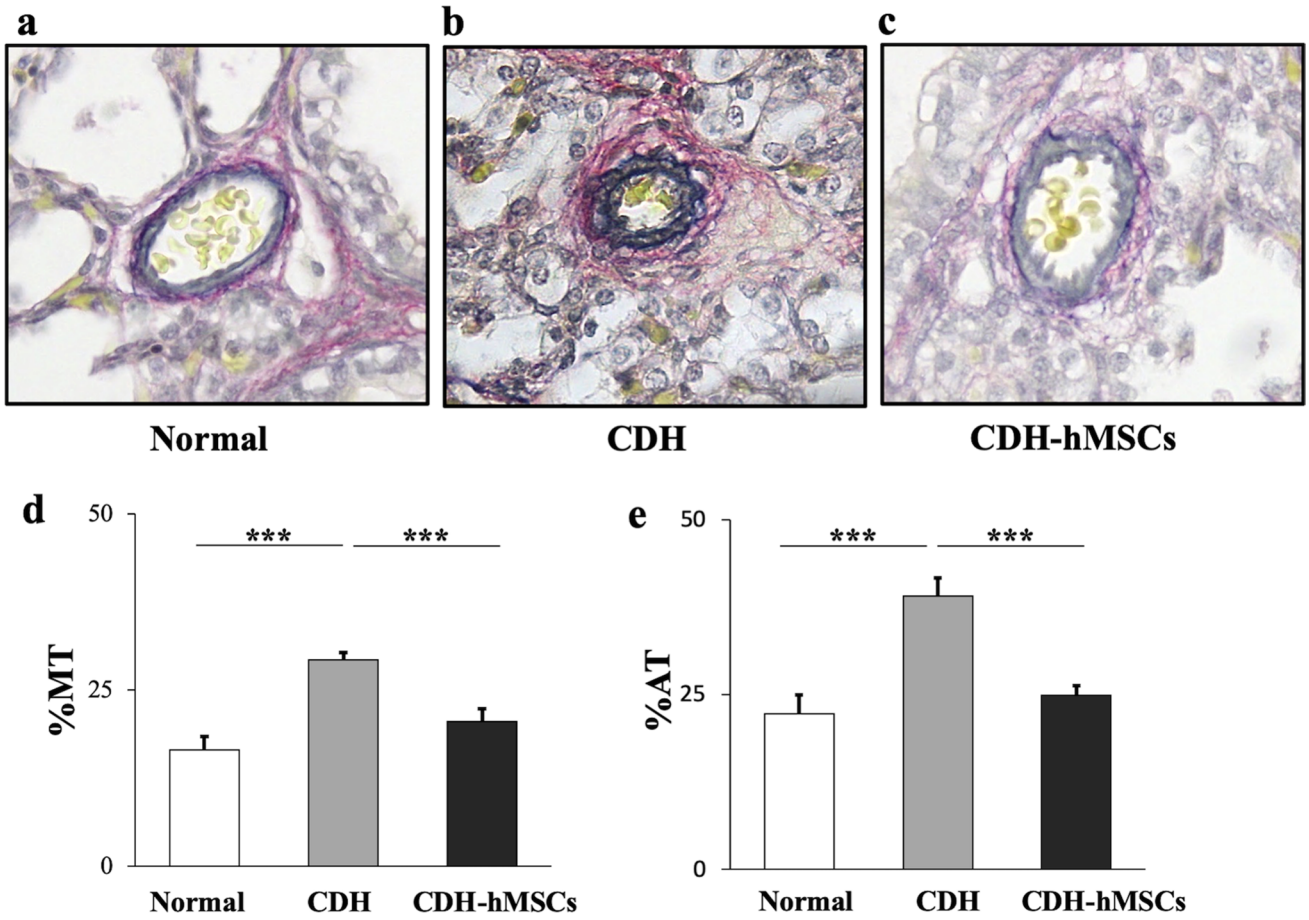

Fig. 4 The morphological assessment of the pulmonary arteries by Elastica Van Gieson staining. **a**–**c** The representative micrograph images of the Elastica Van Gieson-stained lung sections from the normal group (**a**), CDH group (**b**), and CDH-hMSCs group (**c**) at ×400 original magnification. **d** The proportionate medial thickness (%MT) in the CDH group was significantly higher than in the normal group ($p < 0.001$). The %MT in the CDH-hMSCs group was significantly lower than in the CDH group ($p < 0.001$). **e** The proportionate adventitial thickness (%AT) in the CDH group was significantly higher than in the normal group ($p < 0.001$). The %AT in the CDH-hMSCs group was significantly lower than in the CDH group ($p < 0.001$). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

previous studies have shown that TTF-1 levels decreased with lung maturation and confirmed that TTF-1 can be used as an indicator of lung maturation [17]. Our present study also confirmed that the AEC-II count was increased in CDH and decreased by the intra-amniotic injection of hMSCs (for both protein and gene expression). This result showed that the intra-amniotic injection of hMSCs also promoted the maturation of alveolar epithelial cells.

In our experiment, a detailed molecular biological analysis of the effects of MSCs was not performed. MSCs have been shown to secrete several growth factors, including tumor growth factor-β (TGF-β), hepatocyte growth factor (HGF), and FGF, in an experimental CDH rat model [25]. Ahn et al. also reported that the therapeutic effect of MSCs was mediated by paracrine mechanisms in a rat experimental model using MSC-derived exosomes [8]. The intra-amniotic injection of hMSCs is thus assumed to be affected the lung development through a similar paracrine mechanism.

In conclusion, the intra-amniotic injection of hMSCs improved the morphological abnormalities and promoted fetal lung maturity in a rat nitrofen-induced CDH model. The intra-amniotic injection of hMSCs may be a therapeutic approach for improving lung hypoplasia in patients with fetal CDH. In the future, it will be necessary to investigate the paracrine effect mediated by growth factors, such as FGF, HGF, and VEGF, in order to clarify the therapeutic mechanism underlying the intra-amniotic fluid injection.

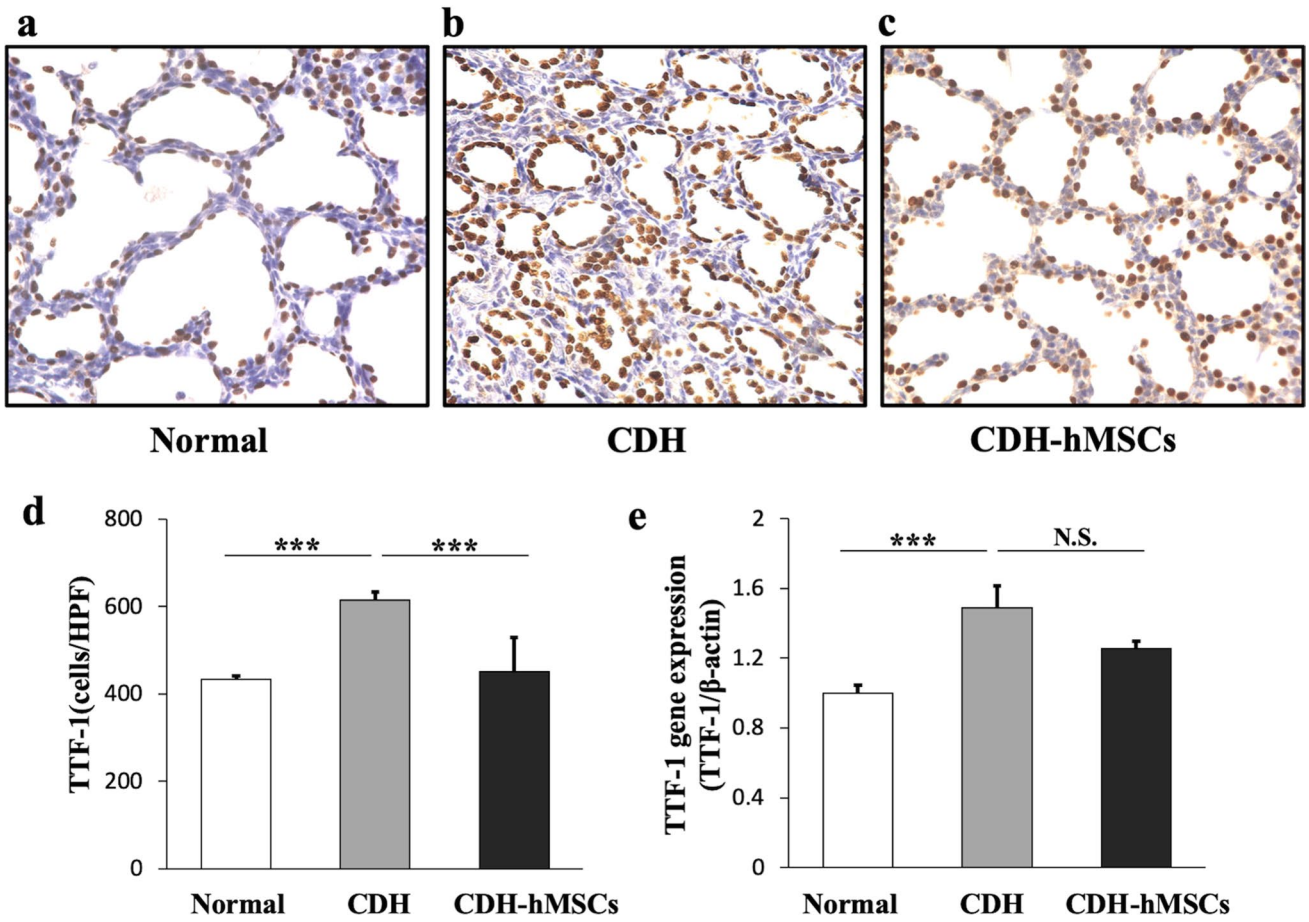

Fig. 5 The assessment of the TTF-1 expression in alveolar epithelial cells type II. **a**–**c** The representative micrograph images of TTF-1 IHC-stained lung sections from the normal group (**a**), CDH group (**b**), and CDH-hMSCs group (**c**) at ×200 original magnification. **d** The number of TTF-1-positive cells in the CDH group was significantly greater than in the normal group ($p < 0.001$). The number of TTF-1-positive cells in the CDH-hMSCs group was lower than in the CDH group ($p < 0.001$). **e** The gene expression of TTF-1 was determined by RT-PCR. The expression of TTF-1 in the CDH group was greater than in the normal group ($p < 0.001$). The expression of TTF-1 in the CDH-hMSCs group was lower than in the CDH group ($p = 0.08$). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

Acknowledgements This work was supported in part by Grant-in-Aid for Exploratory Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (MEXT KAKENHI grant number 15K10926 [TF]). The English used in this manuscript was reviewed by Brian Quinn (Editor-in-Chief, Japan Medical Communication).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

References

1. Chandrasekharan PK, Rawat M, Madappa R, Rothstein DH, Lakshminrusimha S (2017) Congenital diaphragmatic hernia—a review. Matern Health Neonatol Perinatol 3:6. https://doi.org/10.1186/s40748-017-0045-1
2. Kimura O, Furukawa T, Higuchi K, Takeuchi Y, Fumino S, Aoi S et al (2013) Impact of our new protocol on the outcome of the neonates with congenital diaphragmatic hernia. Pediatr Surg Int 29(4):335–339. https://doi.org/10.1007/s00383-012-3242-z
3. Ruano R, Yoshisaki CT, da Silva MM, Ceccon ME, Grasi MS, Tannuri U et al (2012) A randomized controlled trial of fetal endoscopic tracheal occlusion versus postnatal management of severe isolated congenital diaphragmatic hernia. Ultrasound Obstet Gynecol 39(1):20–27. https://doi.org/10.1002/uog.10142
4. Delens L, Jouret F, Detry O, Beguin Y, Krzesinski JM (2014) The role of mesenchymal stromal cells in solid organ transplantation. Rev Med Suisse 10(439):1540–1543 **(1538)**
5. Vanover M, Wang A, Farmer D (2017) Potential clinical applications of placental stem cells for use in fetal therapy of birth defects. Placenta 59:107–112. https://doi.org/10.1016/j.placenta.2017.05.010
6. Moroncini G, Paolini C, Orlando F, Capelli C, Grieco A, Tonnini C et al (2018) Mesenchymal stromal cells from human umbilical cord prevent the development of lung fibrosis in immunocompetent mice. PLoS One 13(6):e0196048. https://doi.org/10.1371/journal.pone.0196048

7. Li Y, Gu C, Xu W, Yan J, Xia Y, Ma Y et al (2014) Therapeutic effects of amniotic fluid-derived mesenchymal stromal cells on lung injury in rats with emphysema. Respir Res 15:120. https://doi.org/10.1186/s12931-014-0120-3
8. Ahn SY, Park WS, Kim YE, Sung DK, Sung SI, Ahn JY et al (2018) Vascular endothelial growth factor mediates the therapeutic efficacy of mesenchymal stem cell-derived extracellular vesicles against neonatal hyperoxic lung injury. Exp Mol Med 50(4):26. https://doi.org/10.1038/s12276-018-0055-8
9. Furlani D, Ugurlucan M, Ong L, Bieback K, Pittermann E, Westien I et al (2009) Is the intravascular administration of mesenchymal stem cells safe? Mesenchymal stem cells and intravital microscopy. Microvasc Res 77(3):370–376. https://doi.org/10.1016/j.mvr.2009.02.001
10. Kobayashi K, Lemke RP, Greer JJ (2001) Ultrasound measurements of fetal breathing movements in the rat. J Appl Physiol 91(1):316–320. https://doi.org/10.1152/jappl.2001.91.1.316
11. Cooney TP, Thurlbeck WM (1982) The radial alveolar count method of Emery and Mithal a reappraisal 2–intrauterine and early postnatal lung growth. Thorax 37(8):580–583. https://doi.org/10.1136/thx.37.8.580
12. Pua ZJ, Stonestreet BS, Cullen A, Shahsafaei A, Sadowska GB, Sunday ME (2005) Histochemical analyses of altered fetal lung development following single vs. multiple courses of antenatal steroids. J Histochem Cytochem 53(12):1469–1479. https://doi.org/10.1369/jhc.5A6721.2005
13. Roubliova XI, Deprest JA, Biard JM, Ophalvens L, Gallot D, Jani JC et al (2010) Morphologic changes and methodological issues in the rabbit experimental model for diaphragmatic hernia. Histol Histopathol 25(9):1105–1116. https://doi.org/10.14670/HH-25.1105
14. Yuniartha R, Alatas FS, Nagata K, Kuda M, Yanagi Y, Esumi G et al (2014) Therapeutic potential of mesenchymal stem cell transplantation in a nitrofen-induced congenital diaphragmatic hernia rat model. Pediatr Surg Int 30(9):907–914. https://doi.org/10.1007/s00383-014-3576-9
15. Pederiva F, Ghionzoli M, Pierro A, De Coppi P, Tovar JA (2013) Amniotic fluid stem cells rescue both in vitro and in vivo growth, innervation, and motility in nitrofen-exposed hypoplastic rat lungs through paracrine effects. Cell Transplant 22(9):1683–1694. https://doi.org/10.3727/096368912X657756
16. Di Bernardo J, Maiden MM, Hershenson MB, Kunisaki SM (2014) Amniotic fluid derived mesenchymal stromal cells augment fetal lung growth in a nitrofen explant model. J Pediatr Surg 49(6):859–865. https://doi.org/10.1016/j.jpedsurg.2014.01.013
17. Sakai K, Kimura O, Furukawa T, Fumino S, Higuchi K, Wakao J et al (2014) Prenatal administration of neuropeptide bombesin promotes lung development in a rat model of nitrofen-induced congenital diaphragmatic hernia. J Pediatr Surg 49(12):1749–1752. https://doi.org/10.1016/j.jpedsurg.2014.09.015
18. Montalva L, Zani A (2019) Assessment of the nitrofen model of congenital diaphragmatic hernia and of the dysregulated factors involved in pulmonary hypoplasia. Pediatr Surg Int 35(1):41–61. https://doi.org/10.1007/s00383-018-4375-5
19. Kugler MC, Joyner AL, Loomis CA, Munger JS (2015) Sonic hedgehog signaling in the lung. From development to disease. Am J Respir Cell Mol Biol 52(1):1–13. https://doi.org/10.1165/rcmb.2014-0132TR
20. Umeda S, Miyagawa S, Fukushima S, Oda N, Saito A, Sakai Y et al (2016) Enhanced pulmonary vascular and alveolar development via prenatal administration of a slow-release synthetic prostacyclin agonist in rat fetal lung hypoplasia. PLoS One 11(8):e0161334. https://doi.org/10.1371/journal.pone.0161334
21. Boucherat O, Benachi A, Barlier-Mur AM, Franco-Montoya ML, Martinovic J, Thebaud B et al (2007) Decreased lung fibroblast growth factor 18 and elastin in human congenital diaphragmatic hernia and animal models. Am J Respir Crit Care Med 175(10):1066–1077. https://doi.org/10.1164/rccm.200601-050OC
22. Shehata SMK, Sharma HS, van der Staak FH, van de Kaa-Hulsbergen C, Mooi WJ, Tibboel D (2000) Remodeling of pulmonary arteries in human congenital diaphragmatic hernia with or without extracorporeal membrane oxygenation. J Pediatr Surg 35(2):208–215. https://doi.org/10.1016/s0022-3468(00)90011-3
23. Herriges M, Morrisey EE (2014) Lung development: orchestrating the generation and regeneration of a complex organ. Development 141(3):502–513. https://doi.org/10.1242/dev.098186
24. Takayasu H, Nakazawa N, Montedonico S, Sugimoto K, Sato H, Puri P (2007) Impaired alveolar epithelial cell differentiation in the hypoplastic lung in nitrofen-induced congenital diaphragmatic hernia. Pediatr Surg Int 23(5):405–410. https://doi.org/10.1007/s00383-006-1853-y
25. Di Bernardo J, Maiden MM, Jiang G, Hershenson MB, Kunisaki SM (2014) Paracrine regulation of fetal lung morphogenesis using human placenta-derived mesenchymal stromal cells. J Surg Res 190(1):255–263. https://doi.org/10.1016/j.jss.2014.04.013