REVIEW

Human Amniotic Fluid Stem Cells: Therapeutic Potential for Perinatal Patients with Intractable Neurological Disease

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Mesenchymal stem cells (MSCs) have generated great interest in the fields of regenerative medicine and immunotherapy because of their unique biological properties. Among MSCs, amniotic fluid stem cells (AFS) have a number of characteristics that make them attractive candidates for tissue engineering and cell replacement strategies, particularly for perinatal medicine. If various neonatal conditions, including birth asphyxia, preterm birth, and congenital abnormalities, which result in long-lasting severe impairments, could be predicted during pregnancy, it would allow collection of small samples of amniotic fluid cells by amniocentesis. *In vitro* culture of these autologous AFS during pregnancy would make them available for use soon after birth. Hypoxic-ischemic encephalopathy (HIE) and myelomeningocele (MMC) are neonatal conditions that cause permanent neurological disability, for which the treatment options are extremely limited. Experiments using animal models of HIE and MMC and human clinical trials have demonstrated that MSCs, including AFS, have beneficial effects on the central nervous system through paracrine influences, indicating that autologous AFS treatment may be applicable for intractable neurological diseases, including HIE and MMC, during the perinatal period. In this review, we focus on recent research related to the therapeutic potential of AFS for perinatal neurological diseases such as HIE and MMC. (DOI: 10.2302/kjm.2017-0019-IR; Keio J Med 67 (4) : 57–66, December 2018)

***Keywords*: mesenchymal stem cells, amniotic fluid stem cells, perinatal medicine, hypoxic-ischemic encephalopathy, myelomeningocele**

Introduction

Cellular therapy has evolved quickly over the past decade, with valuable experience gained in both preclinical research and clinical trials. One group of adult stem cells, mesenchymal stem cells (MSCs), has generated great interest in the fields of regenerative medicine and immunotherapy due to their unique biological properties.[1] MSCs can be purified from various tissues, including adipose tissue, heart, Wharton's jelly, dental pulp, peripheral blood, and umbilical cord blood, among others. These cells can be expanded *in vitro*, which allows their numbers to rapidly increase for subsequent application *in vivo*. Among MSCs, amniotic fluid stem cells (AFS) have a number of characteristics that make them attractive candidates as a potential source of cells for tissue engineering and cell replacement strategies, especially for perinatal medicine.[2–4]

AFS fulfill the criteria for MSCs,[5] and express the pluripotency markers Nanog, Oct-4, and SOX-2,[2,4] and the embryonic stem cell markers CD117, SSEA-4, TRA-1–60, and TRA-1–81.[3,6,7] AFS are not ethically controversial; in fact, the excess amniotic fluid collected during routine clinical amniocentesis that is not used for genetic research is normally discarded. Moreover, AFS lack tumorigenicity when implanted in immuno-deficient mice.[8]

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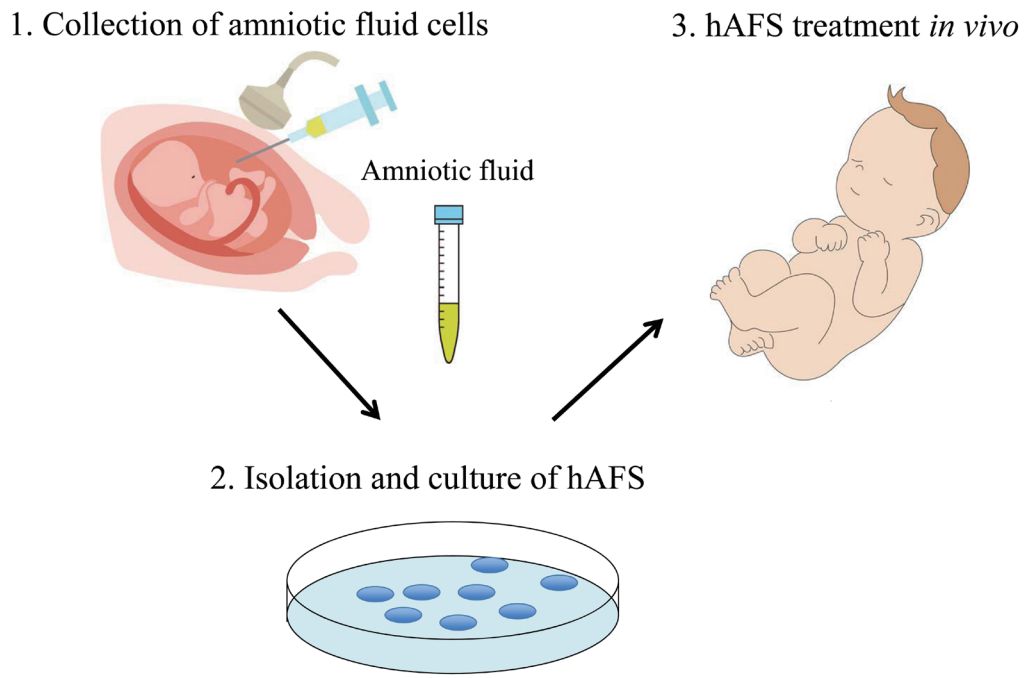

Fig. 1 Autologous AFS therapy for perinatal disease.
Prediction of neonatal complications during pregnancy would allow the collection of a small sample of amniotic fluid cells by amniocentesis (1), and expansion of autologous AFS *in vitro* during pregnancy (2), which could be ready to use therapeutically immediately after birth or even during pregnancy (3). hAFS, human amniotic fluid stem cells.

These characteristic features of AFS are potentially advantageous in the context of therapeutic applications during the perinatal period.

AFS have potential for use in autologous stem cell treatment for a variety of neonatal complications, including birth asphyxia, preterm birth, and congenital abnormalities, which often result in long-lasting severe impairments and are difficult to treat effectively. Currently, the therapeutic options for affected neonates are extremely limited. Preparation of an adequate amount of autologous AFS to treat neonates with these conditions would require only a small amount of amniotic fluid cells collected by amniocentesis during pregnancy, a procedure that entails minimal risk (**Fig. 1**). In this review, we discuss MSC-based treatments, including the use of autologous AFS, for a variety of neonatal complications, particularly those affecting the central nervous systems.

Amniotic Fluid: A Novel Source of Stem Cells

The amnion is a sac that contains the developing embryo, surrounded by the chorion and yolk sac. Along with enveloping the amniotic fluid, the amnion protects the fetus against trauma and against infectious and toxic agents.[9] The composition and volume of amniotic fluid change during gestation, partly in response to fetal development. During the first half of gestation, the composition of amniotic fluid is dependent on the osmotic gradient developed as a result of sodium and chloride transport across the amniotic membrane and fetal skin. In the second half of gestation, amniotic fluid also contains fetal respiratory secretions, urine, and excrement. Consequently, amniotic fluid cells are a heterogeneous population that varies with gestational age.[10,11]

When grown in culture, amniotic fluid cells can be separated into adherent cells, dividing colony-forming cells, and nonadherent cells. Based on their morphological and growth characteristics, amniotic fluid cells can be classified into three types: epitheloid E-type cells, amniotic fluid-specific AF-type cells, and fibroblastic F-type cells (**Fig. 2**). AF-type and E-type cells both appear at the beginning of cultivation.[2,10,11] AF-type cells persist during the cultivation process, whereas the number of E-type cells soon decreases significantly. E-type cells are thought to derive from fetal skin and urine, AF-type cells from fetal membranes and trophoblasts, and F-type cells from fibrous connective tissue and dermal fibroblasts. AF-type cells produce estrogen, human chorionic gonadotropin, and progesterone, which suggests that they originate from trophoblast tissue. In contrast, F-type cells are considered to originate from mesenchymal tissue.[10,11] Approximately 1% of the cells in cultures

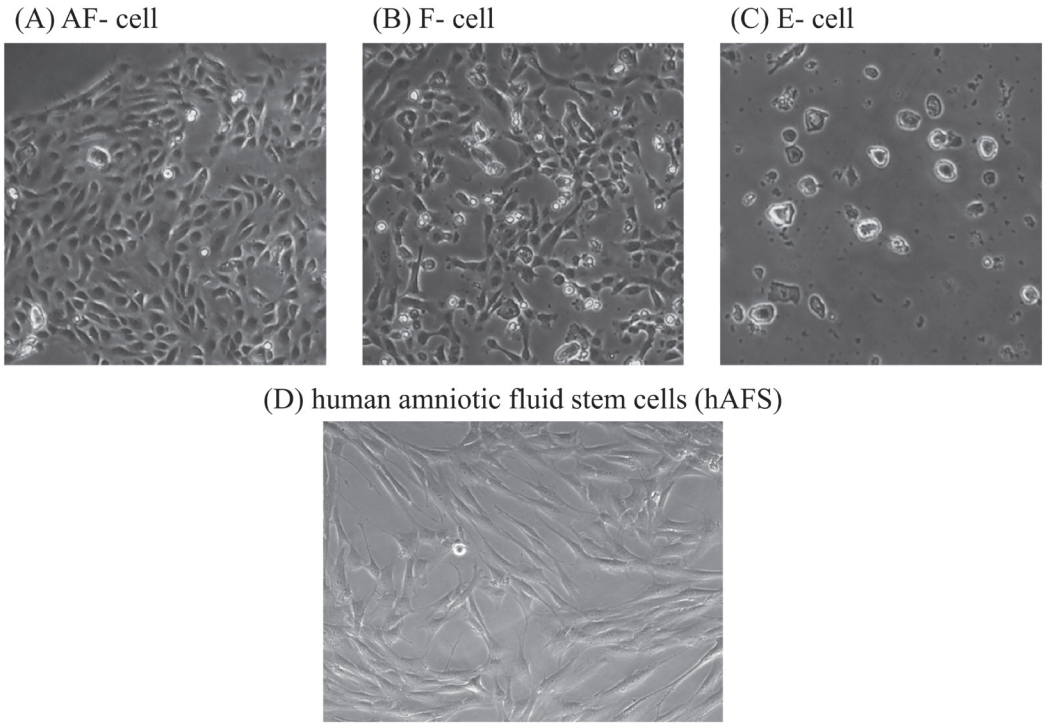

Fig. 2 Morphological characteristics of amniotic fluid cells and AFS.
Morphological characteristics of human amniotic fluid cells and those after CD117 selection: (A) amniotic fluid-specific AF-type cells, (B) fibroblastic F-type cells, (C) epitheloid E-type cells, and (D) human amniotic fluid stem cells (hAFS).

of human amniocentesis specimens express the surface antigen CD117.[3]

Numerous publications have described methods for isolating AFS from amniotic cells.[2–4] To date, a number of different protocols for AFS cultivation have been developed. Culture protocols can mainly be divided into two categories: those that select for the cell surface marker CD117 (**Fig. 2**) and those that isolate fibroblast-like colonies.[3] AFS can be isolated from amniotic fluid samples collected at any gestational age; however, those isolated from first trimester amniotic fluid are thought to be more primitive, whereas mid- or late-trimester AFS may still retain relevant therapeutic characteristics.

Based on their morphology, phenotypes, and *in vitro* differentiation potential, AFS fulfill the criteria for MSCs, as defined by the International Society for Cellular Therapy.[5] AFS are negative for the hematopoietic markers CD45 and CD34, and positive for the mesenchymal markers CD29, CD73, CD90, and CD105. The surface marker profile of AFS and the expression of the pluripotency markers Nanog, Oct-4, and SOX-2,[2,4] and the embryonic stem cell markers CD117, SSEA-4, TRA-1–60, and TRA-1–81,[3,6,7] suggest that AFS represent an early stage of cell differentiation; i.e., they may possess multipotency or even pluripotency. These characteristics of AFS are likely to be advantageous for eventual therapeutic applications, particularly for diseases occurring in the perinatal period.

Autologous AFS Therapy for Perinatal Disease

For the treatment of perinatal disease, AFS have advantages as a stem cell source compared to other sources, such as bone marrow, adipose tissue, and umbilical cord blood. Although MSCs, isolated mainly from the bone marrow, have been extensively studied in a variety of experimental models over the past decade,[1] allotransplantation of MSCs raises clinical concerns during the neonatal period. Prediction during pregnancy of a variety of neonatal complications, such as birth asphyxia, preterm birth, and congenital abnormalities, which result in long-lasting severe impairments, would allow the collection of small samples of amniotic fluid cells by amniocentesis. This procedure would be followed by expansion of autologous AFS *in vitro* during pregnancy to produce cells that could be ready to use therapeutically soon after birth or even during pregnancy (**Fig. 1**). Regarding the route of AFS transplantation, local injection and intra-amniotic fluid administration are assumed for fetuses, whereas local injection, intravenous administration, and intra-

peritoneal administration would be suitable for neonates. Therefore, autologous AFS therapy could represent a new therapeutic option for neonates with various diseases.

The Application of Autologous AFS for Neurological Diseases: Neuroprotection and Repair

Mechanisms of neuroprotection and repair

MSCs have demonstrated ability to modulate the neuronal environment and improve its function.[1,12] Although this is partially due to their direct differentiation into neuronal cells, including neurons and oligodendrocytes, the principal mechanism induced by MSC treatment is the release of trophic factors. These trophic factors differ according to their origin (e.g., bone marrow, adipose tissue, umbilical cord blood, placenta, and Wharton's jelly), culture conditions (e.g., type of medium used), and preconditioning by hypoxia, inflammation, and drugs.[13]

AFS can differentiate into neurons *in vitro* (**Fig. 3**, unpublished data) and *in vivo*[3]; however, it is not clear that replacement of lost cells is the mechanism underlying the improvements observed after their transplantation into animal models of nervous system injury, as described below. Despite the limited evidence for neuronal differentiation of AFS *in vivo*, a number of studies have demonstrated beneficial effects of AFS transplantation in rodent models of central nervous system diseases. These findings suggest that AFS can enhance host neuron survival through a paracrine mechanism, stimulate endogenous repair through the recruitment of progenitor cells, and promote neuronal outgrowth, thereby rendering the perilesional environment less toxic and more favorable to regeneration by modulating neuroinflammatory immune responses.[12]

Supporting this hypothesis, it has been shown that AFS secrete neurotrophic factors, including brain-derived neurotrophic factor, glial cell line-derived neurotrophic factor, nerve growth factor, neurotrophin-3, and several cytokines and chemokines. Although the precise mechanism remains to be determined, these neurotrophic factors have a synergistic paracrine effect in modulating the neuronal environment and improving its function.[12]

Experimental studies

When rat AFS were implanted at sites of traumatic nerve injury, beneficial effects on the nervous system were observed, and these effects were mediated via paracrine mechanisms.[14–16] Although implanted AFS did not survive for more than a month, the improvement of sciatic nerve motor function was maintained in this model. Beneficial effects of AFS transplantation have been also demonstrated in stroke models.[17,18] Intraventricular and intravenous administration of AFS derived from rodents and humans in a rodent middle cerebral artery occlusion model resulted in improved stroke neurological severity scores, as well as improvements in cognitive and motor function. These improvements probably resulted from paracrine effects that acted to restore cellular function in the injured brain.[17–20] These data suggest that AFS therapy could have therapeutic potential for neurological diseases in neonates with hypoxic-ischemic encephalopathy (HIE) and myelomeningocele (MMC).

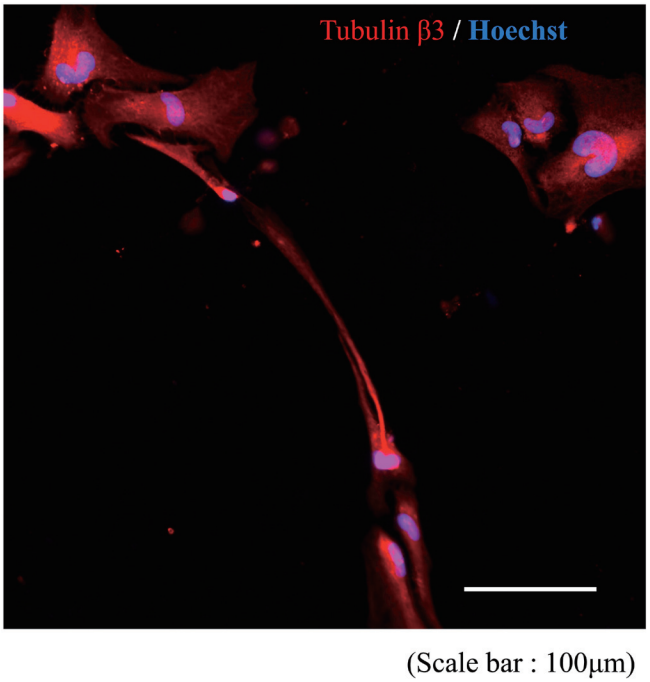

Fig. 3 Neurogenic differentiation potential in AFS.
Human AFS were cultured in neurogenic differentiation medium. After 3 weeks of culture with this medium, we observed cells with a typical neuronal morphology. They expressed tubulin-β3 as a definitive neuronal marker.

The Therapeutic Potential of Autologous AFS for Neonatal HIE

Clinical features of neonatal HIE and treatment options

HIE remains a major cause of cerebral palsy (CP) in newborn infants, occurring in about 2 per 1000 term live births and in almost 60% of very low birth weight newborns.[21,22] Up to 25% of survivors have permanent neurological deficits, including CP, mental retardation, learning disability, and chronic epilepsy.[23,24]

Although the underlying causes and exact timing of brain injuries in HIE infants are unknown, there may be a severe intrapartum hypoxic-ischemic insult at birth caused by placental abruption, umbilical cord prolapse,

or prolonged labor. However, there may also be precipitating antenatal risk factors including preeclampsia, antenatal bleeding, or fetal growth restriction.[25–27]

At present, treatment options for neonatal HIE are extremely limited. Recent clinical trials have shown that hypothermia has modest effects on outcome.[28,29] Hypothermia can be effective in neonates with a gestational age of ≥36 weeks diagnosed with moderate to severe HIE; however, neurodevelopmental deficits persist in 40%–50% of patients, even after hypothermia treatment. Because of the similar pathophysiological symptoms of neonatal HIE and adult stroke, stem cell-based therapies, such as MSC transplantation, which are currently being tested in stroke, may prove to be successful in neonatal HIE.[30]

Stem cell therapy for HIE: Preclinical studies

Experimental clinical and animal studies have begun to elucidate the utility of stem cell-based therapies to prevent or repair perinatal brain injury. Stem cell treatments for neonatal HIE have used neural stem cells, embryonic stem cells, bone marrow-derived MSCs, umbilical cord blood cells (UCBC), and induced pluripotent stem cells.[31–36] Such treatments have, for the most part, been effective in conferring significant neuroprotection, neuroregeneration, and improvement of functional outcomes.[31–36] However, in addition to the efficacy of stem cell-based therapies, it is important to reflect on ethical concerns, accessibility, and the abundance of stem cells for clinical applications.

Accordingly, MSC-based therapies using UCBC transplantation are clinically emerging for the treatment of neonatal HIE. UCBC is a rich source of stem cells, including hematopoietic stem cells and MSCs. The effectiveness of intraperitoneal UCBC injection for rat neonatal HIE was first reported in 2006.[37] Since then, a number of animal studies have examined the effects UCBC transplantation following HIE, predominantly in newborn rodents. Experiments using the Rice-Vannucci animal model have reported positive results following UCBC transplantation, including decreased reactive gliosis, increased tissue repair, cognitive improvements, amelioration of injury-related effects in the primary somatosensory cortex, and enhancement of endogenous neural stem cell proliferation via Hedgehog signaling.[38,39]

Clinical studies

Several human clinical trials are currently in progress to examine the potential therapeutic benefits of stem cells for neonatal HIE using UCBC and other MSCs. In **Table 1**, we list nine clinical trials identified through the ClinicalTrials.gov database using the following search terms, "UCB + neonatal HIE" and "stem cell + neonatal HIE", combined with a filter to limit results to studies focused on children (birth to under 17 years).

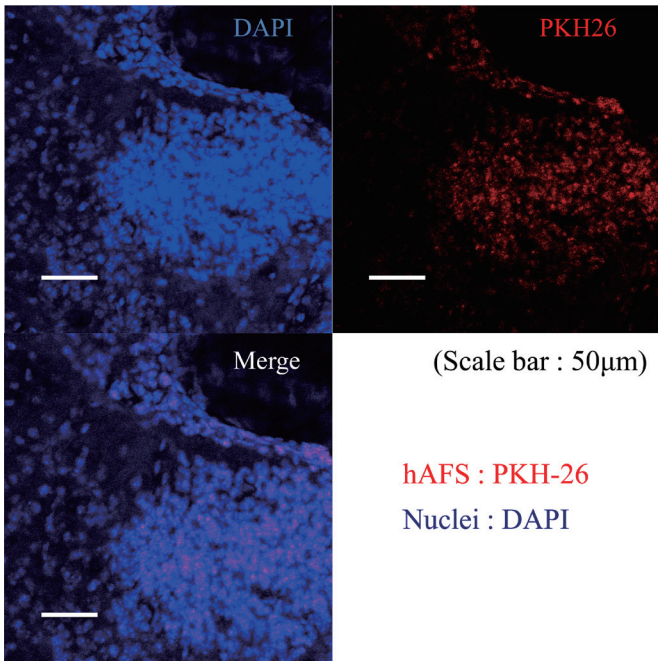

Fig. 4 AFS migrated to the injured brain after intranasal transplantation.
Representative images of the brain section 24 hours after intranasal transplantation of hAFS labeled with PKH-26 (red). Sections were stained with DAPI (blue) to visualize all nuclei.

With regard to UCBC, two clinical studies have been completed and there is one report to date of autologous UCBC transplantation tested in a phase I trial of 23 infants with HIE who met the inclusion criteria and received concurrent therapeutic hypothermia.[40] In this study, non-cryopreserved autologous UCBC was feasible for use in infants with HIE. No significant adverse reactions, cardiorespiratory compromise, or infections occurred as a result of the transfusions.

Therapeutic potential of human AFS for neonatal HIE

Although there is increasing evidence from *in vitro* and *in vivo* studies that MSCs have multiple beneficial effects on neonatal HIE, as described above, the therapeutic potential of AFS remains to be determined. We raise the intriguing possibility of neuroprotective therapies using autologous AFS for the newborn brain identified as at risk of CP following neonatal HIE. Therefore, we are investigating the therapeutic effect of human AFS using a neonatal HIE mouse model. In this model, human AFS migrated to the brain after transplantation (**Fig. 4**, unpublished data) and reduced the progression of motor deficits by preserving ipsilateral brain volume. This finding sug-

Table 1. Clinical trials for stem cell therapy using umbilical cord blood cells and/or mesenchymal stem cells for neonatal hypoxic-ischemic encephalopathy

Status	Study title	Intervention	Location	Phase
Completed	Cord Blood Neonatal Hypoxic-ischemic Encephalopathy	Autologous cord blood	Duke University, United States	Phase 1
Recruiting	Multisite Study of Autologous Cord Blood Cells for Hypoxic-Ischemic Encephalopathy (HIE)	Infusion of autologous cord blood	Duke University, United States	Phase 2
Recruiting	Autologous Cord Blood Cell Therapy for Neonatal Encephalopathy	Autologous umbilical cord blood cells	Neonatal Encephalopathy Consortium, Japan	Phase 1
Recruiting	Cytokines Associated with Cell Therapy for Neonatal Encephalopathy	Autologous cord blood cell therapy	Neonatal Encephalopathy Consortium, Japan	
Recruiting	Neuroprotective Effect of Autologous Cord Blood Combined with Therapeutic Hypothermia Following Neonatal Encephalopathy	Autologous cord blood in combination with hypothermia	Children's Hospital of Fudan University, China	Phase 1 Phase 2
Completed	Autologous Cord Blood Cells for Brain Injury in Term Newborns	Autologous cord blood	National Medical Research Council (NMRC), Singapore	Phase 1
Not yet recruiting	Autologous Cord Blood and Human Placental-Derived Stem Cells in Neonates with Severe Hypoxic-Ischemic Encephalopathy (HPDSC+HIE)	Autologous human placental-derived stem cells (HPDSC) and autologous cord blood	New York Medical College, United States	Phase 2
Not yet recruiting	Neonatal Hypoxic Ischemic Encephalopathy: Safety and Feasibility Study of a Curative Treatment with Autologous Cord Blood Stem Cells (NEOSTEM)	Autologous cord blood stem cell	Assistance Publique Hopitaux de Marseille, Marseille, France	Phase 1 Phase 2
Recruiting	Neural Progenitor Cell and Paracrine Factors to Treat Hypoxic-Ischemic Encephalopathy	Neural progenitor cell and paracrine factors	Navy General Hospital, China	

gests that human AFS could be a novel autologous treatment option for neonatal CP (unpublished data).

Advantages of AFS treatment versus UCBC treatment for neonatal HIE

Although a lack of evidence exists on the effectiveness of AFS compared with UCBC in neonates with HIE, AFS has definitive advantages over UCBC. First, autologous AFS can be immediately administered if severe fetal hypoxemia occurs at birth as long as the AFS are prepared before delivery. Second, there is time to prepare a sufficient amount of AFS via cell culture before delivery. Third, preconditioning, including hypoxic and spheroid culture, and drug stimulation, can enhance the therapeutic effect of AFS. In contrast, UCBC therapy cannot be performed if sufficient umbilical cord blood is not collected at birth. Furthermore, UCBC culture and/or preconditioning is impossible if cell therapy is needed immediately after birth. Although further investigation is needed to determine the advantages of AFS versus UCBC for neonatal HIE, AFS treatment could be a novel treatment option in this patient population.

Therapeutic Potential of Autologous AFS for Myelomeningocele: Perspective on *in utero* Fetal Stem Cell Therapy

Clinical features and perspective of fetal therapy

Myelomeningocele (MMC) is a congenital malformation with complex physical and neurodevelopmental symptoms for which there is no cure. MMC often results in life-long impairment, including loss of sensorimotor function of the lower extremities, skeletal deformity, bladder and bowel incontinence, ventriculomegaly, and the Chiari II malformation. Although the cause of MMC remains poorly understood, primary failure of either neural tube or mesenchymal closure at the caudal neuropore in the embryonic period results in exposure of vulnerable neural elements to the intrauterine environment (first hit). Without protective tissue coverage, secondary destruction of the exposed spinal cord by chemical and mechanical injury may occur throughout gestation (second hit).[41,42]

Theoretically, *in utero* interventions for the protection of the spinal cord could prevent the second hit, but not the primary injury. Recently, the multicenter prospective randomized Management of Myelomeningocele

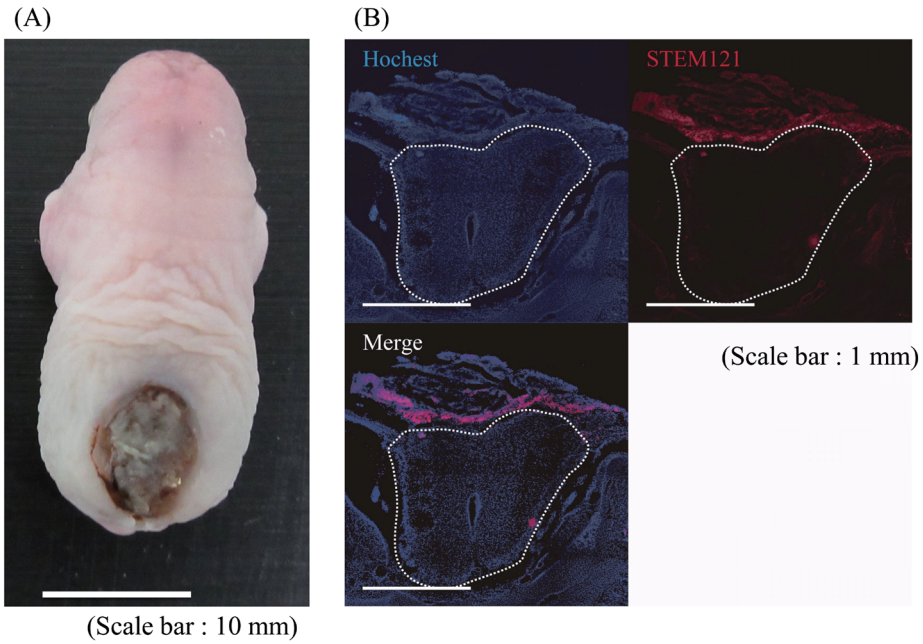

Fig. 5 hAFS engrafted on the surface of the defective spinal cord of fetal MMC.
(A) Representative images of fetal MMC successfully treated with intra-amniotic injection of hAFS. (B) Representative images of STEM 121 staining of spinal cord section. Dotted line shows the spinal cord.

Study (MOMS) clearly demonstrated that fetal surgery for MMC repair led to a decreased rate of shunting at 12 months of age, reversal of hindbrain herniation, and improved outcomes, including the ability to walk at 30 months of age. However, fetal surgery was associated with risk of maternal complications and preterm birth.[43] To overcome these limitations, *in utero* stem cell therapy could provide a practical, minimally invasive approach for fetal MMC treatment.

Stem cell therapy for fetal MMC

Fetal stem cell therapy is a treatment option for a variety of birth defects. Early *in utero* treatment is considered to be beneficial or critical for effective treatment, particularly in fetal MMC. Li and Yuan et al. demonstrated for the first time that direct injection of bone marrow MSC in retinoic acid-induced rat fetal MMC could ameliorate sensory and motor neuron deficiency by differentiation into neurons *in situ* and reduction of spinal neuron apoptosis.[44] They also showed that intrinsic epidermal growth factor, fibroblast growth factor (FGF)-2, FGF-8, and FGF-20 might affect the *in vivo* fate of transplanted MSCs.[45]

Intra-amniotic administration is another route for stem cell delivery to the spinal cord in fetal MMC, and this approach is associated with minimal risk to the mother and fetus. Lee et al., reported enhancement of the reclosure capacity by intra-amniotic injection of human embryonic stem cells in surgically induced spinal open neural tube defects in chick embryos.[46,47] The study revealed the possibility of using human embryonic stem cells in prenatal management of human neural tube defects, demonstrating enhanced reclosure induced by the bridging effect required to fill the injury site, resulting in protection of injured tissue and promotion of regeneration.

Therapeutic potential of human AFS for MMC

Although the therapeutic potential of human AFS remains to be determined in a fetal MMC model, intra-amniotic injection of rat AFS had some beneficial impact in a retinoic acid-induced rat fetal MMC model. Allotransplantation of rat AFS led to the development of variable degrees of coverage of the defect with a primitive form of skin and minimized the Chiari-II malformation. In this study, AFS were engrafted, not in a primitive form of skin, but in the bone around the defect.[48–51]

Using this model, we investigated the therapeutic effects of intra-amniotic injection of human AFS. Consistent with the previous report using rat cells described above, human AFS also promoted skin coverage of the lesion, protected neurons, and reduced astrogliosis via a paracrine mechanism. However, in contrast to the results of allotransplantation of rat AFS, human-derived cells remained on the surface of the spinal cord in our study, rather than in the bone around the defect (**Fig. 5**, unpub-

lished data). Further investigation is needed to clarify the reasons for these observed differences.

Conclusion

Persistent neurological deficits in neonates with perinatal brain injury and congenital neurological malformations remain an enormous social problem, despite substantial research efforts. Stem cell-based therapies have generated promising results in experimental models of neonatal neurological disease. Although the translation of treatment options into clinically applicable therapies will be a complex task, greater understanding of human AFS gleaned through the continuous advancement of stem cell research, both in the laboratory and in clinical trials, will eventually lead to improved treatments for neurological deficits in neonates with brain injury and congenital abnormalities.

Acknowledgments

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Conflicts of Interest

The authors have no conflicts of interest to declare.

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