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Critical Review

The Potential of Amniotic Membrane/Amnion-Derived Cells for Regeneration of Various Tissues

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Abstract. Regenerative medicine is a new field based on the use of stem cells to generate biological substitutes and improve tissue functions, restoring damaged tissue with high proliferability and differentiability. It is of interest as a potential alternative to complicated tissue/organ transplantation. Recently, amnion-derived cells have been reported to have multipotent differentiation ability, and these cells have attracted attention as a cell source for cell-transplantation therapy. The amnion possesses considerable advantageous characteristics: the isolated cells can differentiate into all three germ layers; they have low immunogenicity and anti-inflammatory functions; and they do not require the sacrifice of human embryos for their isolation, thus avoiding the current controversies associated with the use of human embryonic stem cells. Moreover, we developed human amniotic cell-sheets using a novel culture surface coated with a non-cytotoxic, temperature-responsive elastic protein-based polymer. We also generated a "hyper-dry-amnion", which has already been applied clinically in the ophthalmological field. Compared to cryopreserved fresh amnion, "hyper-dry-amnion" is easy to handle and has started to bring good results to patients. These materials from the amnion are also expected to open a new field in tissue engineering. Thus, amnion, which had been discarded after parturition, has started to be appreciated as an attractive material in the field of regenerative medicine. In this review, the most recent and relevant clinical and experimental data about the use of amniotic membrane and cells derived from it are described.

Keywords: amniotic membrane, amnion-derived cell, dried amnion, regenerative medicine, multipotency

I. Introduction

Recently, in addition to the current therapeutic modalities, such as medical therapy, surgery, organ transplantation, and mechanical assist devices, regenerative medicine is being focused on as a potential alternative to complicated tissue/organ transplantation. Regenerative medicine is a new field based on the use of stem cells to generate biological substitutes and improve tissue functions. Three essential factors are necessary: stem cells, which retain the capacity to renew themselves and may be able to restore damaged tissue with high proliferability and differentiability; the scaffolds that support them; and growth and differentiation factors.

Stem cells isolated from adults or developing embryos (embryonic stem (ES) cells) are currently thought to be a source of cells for regenerative medicine. However, despite their therapeutic potential, both adult and ES cells present a number of challenges associated with their clinical application and are thus not in general use yet (1). For example, although adult stem cells can be directly isolated from the patient and are therefore immunologically compatible with the patient, they are generally hard to isolate and grow in culture; and moreover, transplantation of a sufficient number of cells to adult tissue needs a large-scale cell supply. In contrast, human ES cells can proliferate very fast in culture and differentiate into cells of all adult tissues, but additional

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research is required to control the growth and to overcome the risks of tumor formation by undifferentiated ES cells and graft rejection. It is also necessary to resolve the ethical issues surrounding the use of materials from embryos. Therefore, much attention is now focused on obtaining a novel source of cells.

Recently, the multipotent differentiation ability of amnion-derived cells has been reported and these cells have attracted a lot of attention as a cell source for cell transplantation therapy. The amnion-derived cells have considerable advantages that will be described in detail later: they can differentiate into all three germ layers; they have low immunogenicity and anti-inflammatory functions; and they do not require the sacrifice of human embryos for their isolation, thus avoiding the current controversies associated with the use of human ES cells. The amniotic membrane has already been applied in medicine, for example, in burn lesion treatment, surgical wound covering to avoid collusion (2), and ocular surface reconstitution, although uncertainty remains regarding the mechanism of its effects.

II. Basic structure and function of amniotic membrane

The amniotic membrane is a tissue of fetal origin and is composed of three major layers: a single epithelial layer, a thick basement membrane, and an avascular mesenchyme (3). There are no nerves, muscles, or lymphatics in the amnion. It is adjacent to the trophoblast cells and lines the amniotic cavity. It can be easily separated from the underlying chorion, with which it never truly fuses at the cellular level. The amnion obtains its nutrition and oxygen from the surrounding chorionic fluid, the amniotic fluid, and the fetal surface vessels.

One of the basic functions of the amniotic membrane is to provide the developing embryo with protection against desiccation and an environment for suspension in which the embryo can grow without distortion by pressure from surrounding structures. The amnion also plays an important role during parturition. In the initiation and maintenance of uterine contraction, prostaglandins play a pivotal role. The amniotic epithelium is not only one of the main sources of prostaglandins, especially prostaglandin E_2 (4), but also expresses prostaglandin-biosynthesis enzymes such as phospholipase, prostaglandin synthase, and cyclooxygenase (5). Moreover, these enzymes are regulated by human chorionic gonadotropin (hCG), the receptors of which are found on the amniotic epithelium (6).

Amniotic epithelium is metabolically highly active throughout gestation, and it is also responsible for regulating the pH of the amniotic fluid, keeping it constant at about 7.10.

From a structural perspective, the amniotic cells are connected to each other by numerous desmosomes; however, tight junctions occluding the lateral intercellular spaces and thus limiting paracellular transport can not be observed between the cells (7). Consequently, the intercellular clefts may represent an effective route for paracellular transfer of macromolecules. The basal lamina contains large quantities of proteoglycans, rich in heparan sulfate, that may serve as a permeability barrier to amniotic macromolecules (8). Wolf et al. reported that in the amniotic epithelium, the specialized arrangement of intracellular cytoskeletal filaments such as actin, α-actinin, spectrin, ezrin, cytokeratins, vimentin, and desmoplakin indicates their role in the structural integrity and modulation of cell shape as well as in junctional permeability (9).

Laminin is one of the main components of the basement membrane and it critically contributes to cell differentiation, cell shape and movement, maintenance of tissue phenotypes, and promotion of tissue survival via cell surface receptors such as integrins and dystroglycans (10). Therefore, we characterized the laminin subunit chains in the human amnion (manuscript in preparation). Laminin has several heterotrimeric isoforms composed of an α-, a β-, and a γ-chain; and each type of chain has several types of subunit chains: $\alpha 1-5$, $\beta 1-3$, $\gamma 1-3$. Laminin is produced and secreted from adjacent epithelial cells, and therefore the gene expression of laminin subunit chains in human amniotic epithelial cells (HAE) was investigated by reverse transcription-polymerase chain reaction (RT-PCR). We detected the expression of laminin subunits $\alpha 2$, $\alpha 3$, $\alpha 5$, $\beta 1$, $\beta 2$, $\beta 3$, $\gamma 1$, and $\gamma 2$ chains by RT-PCR and found that the mRNA expression of the subunit chains in the basement membrane paralleled the immunohistochemical staining for these subunit chains. These findings suggested that the basement membrane of the human amnion contains a broad spectrum of laminin isoforms, laminin-2, -4, -5, -6, -7, -10, and -11. These findings will provide clues not only for understanding the physiological roles of the amnion and HAE cells, but also for applying this tissue in the tissue engineering field.

III. Advantages of amniotic membrane/amnion-derived cells as materials for regenerative medicine

1. Pluripotency of amnion-derived cells

Regarding differentiation potential, it is expected that the amniotic membrane might maintain pluripotent properties. Developmentally, the inner cell mass of the blastocyst, from which ES cells are derived, gives rise to

both the epiblast (from which the embryo is derived) and the hypoblast (from which the yolk sac is derived). From this epiblast (embryonic ectoderm) the amniotic epithelial layer (amniotic ectoderm) is derived on about the 8th day after fertilization, while the mesenchymal cells (amniotic mesenchyme) are from extraembryonic mesoderm of the primitive streak (11). Considering that the epiblast also gives rise to all of the germ layers of the embryo, amniotic epithelial cells are also expected to give rise to three germ layers. Moreover, the amniotic epithelial layer is derived before gastrulation. Considering that pluripotent embryonal carcinoma cells can only be generated from cells derived before gastrulation (12), indicating the importance of gastrulation in the differentiation and specification of cell fate, it is expected that amniotic epithelial cells might maintain the plasticity existing in the cells in the pregastrulation embryo (13). Previously, several reports, including ours, demonstrated that both HAE cells and human amniotic mesenchymal cells (HAM cells) express several stem cell markers such as octamer-binding transcription factor (OCT)-4, which is specifically expressed in ES cells and germ cells; GATA-4, which is a marker of definitive embryonic and visceral (extra-embryonic) endoderm; hepatocyte nuclear factor-3β (HNF-3β), which is a marker of definitive endoderm; nestin, which is an intermediate protein and a neural stem cell-specific marker; and nanog (13 – 15). These facts suggest that not only HAE cells but also HAM cells possess pluripotency.

2. Anti-inflammatory and low immunogenic characteristics of amniotic membrane/amnion-derived cells

Amniotic membranes/amnion-derived cells have been considered to be suitable tissue or cells for allotransplantation, based on their anti-inflammatory effects and low immunogenicity. Much evidence supporting these ideas has been accumulated.

As to anti-inflammatory effects, Hao et al. reported that both HAE and HAM cells express various anti-angiogenic and anti inflammatory proteins such as interleukin (IL)-1 receptor antagonist; tissue inhibitors of metalloproteinase (TIMPs)-1, -2, -3, -4; and IL-10 (16). Amniotic membrane stromal matrix markedly suppressed lipopolysacharide-induced upregulation of both IL-1α and -1β in human corneal limbal epithelial cells cultivated on it (17). In addition, amniotic membrane stromal matrix also suppressed DNA synthesis and subsequent differentiation of myofibroblasts obtained from human cornea and limb, through suppressing the TGF-β signaling system. The authors noted that these actions would explain in part not only the antiscarring results of amniotic membrane transplantation but also why fetal wound healing is scarless (18). HAE cells expressed mRNA of tumor necrosis factor (TNF)α, Fas ligand, TNF-related apoptosis-inducing ligand (TRAIL), TGF-β, and macrophage migration-inhibitory factor (MIF) (19). The supernatants from HAE cell cultures inhibited the chemotactic activity of neutrophils and macrophages toward macrophage inflammatory protein 2, reduced the proliferation of T- and B-cells after mitogenic stimulation, and induced apoptosis of T and B cells, but not of corneal epithelial cells (19), and also suppressed corneal neovascularization and migration of major histocompatibility complex (MHC) class II+ antigen-presenting cells (APCs) in cauterized mouse corneas (20). As to antibacterial properties, human amniotic membrane reduces bacterial counts and promotes healing of infected wounds (21).

As to the low immunogenicity, clinical signs of acute rejection were not observed when amniotic membrane was transplanted into volunteers (22). The expression level of MHC class I antigens is still controversial. Although it was reported that HLA-A, -B, -C, and -DR were not detected in cultured amniotic epithelium (23), the detection of class I antigen in almost all cells in the amniotic membrane has been reported since then (24, 25). In contrast, class II antigen was expressed only in some fibroblasts in human amniotic membrane (25).

Several studies examined the fate of amniotic membrane grafts. Wang et al. (26) studied allogeneic GFP+ mouse amniotic membrane grafts heterotopically transplanted in the eye. Kubo et al. studied xenotransplanted human amniotic membrane in the eye of rats (25). These experiments showed that the fate or allogenicity of amniotic epithelial cells depended on the graft site. For example, grafts implanted in the anterior chamber or intracornea survived longer than those transplanted on the ocular surface. The authors mentioned that because of the short period of viability of donor-derived amniotic epithelial cells grafted on the ocular surface, these cells had already lost viability and thus were unable to display enough antigens to represent a target for effector CD4+ and CD8 + T cells. As a result, long-term memory of sensitization was not acquired (26). In addition, from the observation that amniotic cells disappeared without a rejection reaction, the authors speculated that the short existence of donor-derived AE cells on the ocular surface might be due to the process of apoptosis (25, 26). Actually, both HAE (19, 27) and HAM (25) cells were reported to express Fas ligand, which may be easily released from apoptotic amniotic cells as the soluble form, and may exert an immunosuppressive effect.

Fetal nonclassic HLA-G (class Ib antigen) is also expressed in the human amniotic membrane (24, 25).

Because the HLA-G molecule has low polymorphism compared to with class Ia antigen, aggression against the fetus is not easily initiated by HLA-G expression in the fetal-maternal interface. Kubo et al. noted that the expression of HLA-G in the amniotic membrane implies two possibilities for the host immune system. First, HLA-G may play the role of a tolerogenic peptide, and the host lymphocytes or dendritic cells may be inactivated by HLA-G's binding to inhibitory receptors. Secondly, HLA-G may be recognized by certain T cells because CD8 can bind to HLA-G, and these cells may have a suppressor function (25). The expression of these immunosuppressive and immunoregulatory factors may also explain it in part. Thus, the problem of immune rejection could be overcome by the use of amniotic membrane.

However, it was also reported that in presensitized recipient mice and recipients that underwent repeated amniotic epithelial implantation, graft survival was markedly shorter than in normal recipients, suggesting that fresh allogeneic mouse amniotic epithelium expressed immunogenicity (26). Therefore, at present, amniotic membrane and the cells derived from it seem to be suitable tissue for transplantation because of their anti-inflammatory and low immunogenic characteristics; however, in clinical applications, the risk of immunogenicity depending on the transplantation-site or of rejection, occurring after repeated amniotic membrane transplantation from the same donor, should not be ignored.

3. Non-tumorigenicity

There was no evidence of tumorigenicity in humans when isolated amniotic cells were transplanted into human volunteers to examine their immunogenicity or into patients in an attempt to correct lysosomal storage diseases (22, 28, 29).

However, trisomy mosaicism in the amnion has been reported, especially in amniotic epithelial cells. Robinson reported that in 16 (48%) of 33 cases of prenatally diagnosed trisomy mosaicism, trisomy was confirmed to be present by molecular analysis of the amnion, although the trisomy was absent from most fetal tissues (30). In the cases in which trisomy mosaicism in the amnion was not noticed because the fetal tissue was normal, whether or not some biological effects would arise in clinical application of these amnions is unclear. Further studies are necessary to clarify this.

4. Little ethical problems with usage

Because the amniotic membrane is discarded after parturition, it is easy to obtain *without* harming mothers or babies and would thereby overcome the ethical issues associated with the use of ES cells. However, it is still in the possession of the mother, so the use of human amniotic membrane had been approved by the Ethics Committee of each Institution and written informed consents had been obtained from the mothers. Based on these considerations, human amniotic membrane /amnion-derived cells are considered to be a useful biological material and also a novel cell source for cell transplantation.

IV. Isolation and cultivation of amniotic cells

The human amnion was mechanically peeled from the chorion of a placenta obtained from an uncomplicated Cesarean section with informed consent. The study and the use of the amnion were approved by the Ethics Committee of the University of Toyama, School of Medicine. HAE and HAM cells were isolated by sequential trypsin and collagenase digestion as described previously (14). Briefly, the amnion was washed in phosphate-buffered saline (PBS) and then cut into pieces in PBS containing 0.03% hyaluronidase (Sigma-Aldrich Co., St. Louis, MO, USA) and 0.025% deoxyribonuclease I (DNase I, Sigma-Aldrich Co.). The resulting minced amnion was digested with 0.2% trypsin (Sigma-Aldrich Co., Steinheim, Germany) in Dulbecco's Modified Eagle's Medium (DMEM; Sigma, Irvine, UK) and incubated at 37°C in an Incubate Box M-260F (Taitec Co., Saitama) with stirring at 100 rpm for 30 min. Thereafter, the medium was removed, and the tissue was minced again and incubated at 37°C in DMEM with trypsin and stirred at 400 – 600 rpm for 30 min. The mixture was then poured over gauze to separate the dispersed amnion epithelial cells from the tissue pieces. The dispersed epithelial cells were collected by centrifugation and suspended in DMEM supplemented with heat-inactivated 10% FCS (Gibco BRL, Grand Island, NY, USA) and 1% antibiotic-antifungal solution (Gibco BRL) and then seeded into culture dishes (Greiner Bio-One GmbH, Frickenhausen, Germany) at a concentration of 3.0×10^4 cells/cm^2 (Fig. 1). Incubation was carried out at 37°C under 5% CO_2 in air. Trypsinization of the amnion was repeated several times until no more HAE cells were obtained. The yield of amniotic epithelial cells obtained from one amniotic membrane was 8×10^6/tissue to 50×10^6/tissue.

Mesenchymal cells were isolated from the amniotic tissue pieces after the removal of epithelial cells. The tissue pieces were placed in DMEM with collagenase (0.75 mg/ml) (Wako, Osaka) and DNase I (0.075 mg/ml) and were incubated at 37°C with stirring at 600 rpm for 60 min. The dispersed mesenchymal cells

were collected by filtration through gauze and centrifugation. The yield of mesenchymal cells was approximately 1×10^6/tissue. It was also reported that the epithelial: mesenchymal cell ratio was 4.3:1 at preterm (15 cases at 23 – 36 weeks) and 7.8:1 at term (27 cases) (31).

V. Characterization

The amniotic epithelial cells express not only epidermal markers such as CA125 (32) and general epithelial markers such as cytokeratins, but also express vimentin (33, 34). Human amniotic mesenchymal cells are derived from the fetal mesoderm (11) and are positive for CD44 and desmin, as well as vimentin. Vimentin-positive cells made up 97.5% of the total cells tested in cultured HAM cells, while 3.6% of the total HAM cells expressed the phenotype CK19+/vimentin+, indicating coexpression of epithelial and mesenchymal cell markers (35). These facts suggest that the amnion-derived cells have not completely differentiated into epithelial or mesenchymal cells. Another explanation from the embryological point of view is that the epithelial-mesenchymal transition (EMT) may occur in the amniotic membrane (35).

After culturing with bromodeoxyuridine (BrdU) for 24 h, 66% – 82% of HAM cells were found to be BrdU-positive, suggesting that they have proliferative potency (35). Nakajima et al. studied telomerase activity in rat aminiotic cells that were thought to be equivalent to human amniotic epithelial cells. Telomerase activity was higher in the cells isolated from fetuses in the middle stage (day 13.5 to 15.5) than in the late stage (day 17.5 to 21.5) of gestation (36). However, in vitro, under conditions without any growth factors except 10% FCS, HAE cells stop proliferating and settle into senescence after 3 or 4 passages.

Erythropoietin (EPO) and EPO-receptor (EPO-R) are expressed in HAE cells. EPO stimulates the differentiation, proliferation, and survival of erythroid precursors via binding to the EPO-R, and its production is regulated by the concentration of oxygen in the blood. However, EPO production in HAE cells was not stimulated by hypoxia (37). Ogawa et al. reported that EPO synthesis in HAE-SV40 cells, which were immortalized by transfection with simian virus 40 large T antigen, is stimulated by progesterone, but not by 17β-estradiol. Progesterone, as a transcriptional regulator, is mediated by two distinct forms of the progesterone receptor (PR): PR-A and PR-B. PR-A is an N-terminal truncated form of PR-B. In the human term amnion cells, PR-B is a transcriptional activator of progesterone–responsive genes, whereas PR-A is a transdominant repressor of PR-B-mediated transcription (38). PR-B was detected in both HAE cells and HAE-SV40 cells by Western blotting, indicating that EPO synthesis might be regulated by progesterone not only in HAE-SV40 cells but also in HAE cells (39). However, the functions of EPO in the amniotic membrane are still unknown.

VI. Differentiation-potential of amniotic membrane-derived cells (Fig. 2)

1. Hepatocytes

For treatment of fatal liver diseases, such as fuluminant hepatitis and severe congenital liver failure, hepatocyte transplantation has been expected to be a novel method. However, many questions have remained regarding this approach, especially concerning donor cells. In 2000, hepatic stem cells were identified from the developing mouse liver (40), and hepatic stem cells were suggested to be a good candidate as a transplantable cell source. Non-hepatic lineage cells that can differentiate into hepatocytes have also been suggested to be potentially useful for hepatocyte transplantation. Bone marrow-derived cells were shown to differentiate into hepatocytes both in vivo (41) and in vitro (42) in rats, as well as mouse embryonic stem cells (43). In humans, embryonic stem cells (44), bone marrow-derived cells (45), and cord blood cells (45) have been shown to be possible candidate cell sources for hepatocyte transplantation, although in the case of bone marrow-derived cells and cord blood cells, the efficiency of differentiation into hepatocytes was low and in some cases γ-irradiation was necessary.

To evaluate whether human HAE cells can be used as a cell source for hepatocyte transplantation, we analyzed hepatic gene expression of HAE cells (15). RT-PCR analysis demonstrated that freshly isolated HAE cells expressed albumin and α1-antitrypsin (α1-AT) (serum proteins), cytokeratin 18 (cytoskeletal protein gene), glutamine synthetase and carbamoyl phosphate synthetase-1 (ammonia metabolism-related genes), phosphoenolpyruvate carboxykinase (gluconeogenesis-related gene), and cytochrome P450 (CYP) 2D6 and CYP3A4 (drug metabolisum-related genes). After cultivation, HAE cells started to express α-fetoprotein (α-FP), transthyretin, tyrosine aminotransferase (TAT), and CYP2CP. These data are consistent with other reports (46, 47). HAE cells did not express ornithine transcarbamylase, glucose-6-phosphatase, or tryptophan 2,3-dioxygenase.

In order to clarify the regulatory system for the expression of these genes, we investigated the gene expression of transcription factors such as HNF-1α, -1β, -3α, -3β, -3γ, -4α, or -6, GATA-4, and C/EBP-α that are

essential for liver development and hepatocyte function. Among these genes, HNF-3γ and C/EBP-α were expressed in HAE cells (15). HNF-3γ, one of the isoforms of transcription factor HNF-3, induces the expression of albumin, α-FP, α1-AT, and transthyretin. C/EBP-α controls liver functions such as glycogen storage (48) and the gene expression of albumin, α-FP, transthyretin, and TAT. These transcription factors may regulate the expression of the hepatocyte-related genes noted above. In addition, albumin and glycogen were detected by immunohistochemistry in almost all cultivated HAE cells, which is consistent with the previous report that albumin production was also observed in half of the adherent amniotic cells in rats (36). Moreover, the secretion of albumin into the culture medium was detected by ELISA in amnion organ culture [4.055 ± 1.532 ng albumin/μg genomic DNA per 24 h ($n = 6$)], although the level of secretion was lower than that of HepG2 [44.21 ± 5.14 ng/μg per 24 h ($n = 4$)] (49). Based on these observations, it was assumed that HAE cells have typical functions of hepatocytes such as albumin production and glycogen storage.

However, although we stimulated HAE cells for 10 days with growth factors that contribute to liver regeneration and/or liver development: hepatocyte growth factor (HGF) and/or fibroblast growth factor (FGF)-2 and/or oncostatin M (OCM) in combination with dexamethasone, we could not induce the gene expression of ornithine transcarbamylase, glucose-6-phosphatase, and tryptophan 2,3-dioxygenase.

To investigate whether the amnion possesses hepatocyte-related functions in vivo, pieces of amnion tissue was transplanted into SCID mice and their survival and secretion of albumin were studied in vivo (15). Following transplantation into the peritoneal cavity of mice, the amnion survived for 2 weeks, with metabolizing ability against MTT and secretion of albumin until 7 days after transplantation. These observations suggest that transplantation of HAE cells and/or amnion tissue could be a novel therapeutic strategy for treatment of hepatic diseases; however, short survival in the transplantation site remained one of the problems to be solved.

Takahashi et al. reported the possibility of rat amniotic epithelial cells as a gene carrier for hepatic tissue. Amniotic epithelial cells from day 18.5 – 20.5 fetuses were transfected with adenoviral AdlacZ and harvested to inject into fetal rat liver of the syngeneic strain (day 18.5 – 20.5). The transformed cells formed a cellular mass and survived for up to 14 days after birth. They suggested that the use of amniotic epithelial cells as a gene carrier would result in long-term expression of exogeneous genes in the liver (49).

2. Cardiomyocytes

Cellular cardiomyoplasty is a major method for the treatment of ischemic injury to cardiomyocytes, which results in heart failure, because adult cardiomyocytes do not regenerate sufficiently. Because of the great interest in finding suitable cell sources for cellular cardiomyoplasty, the suitability of a wide range of cell types has been examined, including ventricular cardiomyocytes, skeletal myoblasts, embryonic stem cells, and adult somatic stem cells. Each cell type has its own advantages, but there are still many problems associated with their use, for example, cell number, immune rejection, tumorigenesis, and ethical issues (50).

We examined the potential of HAM cells to differentiate into cardiomyocytes in response to growth factor treatment and assessed whether they could integrate into heart explants and undergo cardiac differentiation in coculture experiments (51).

RT-PCR analysis demonstrated that freshly isolated HAM cells expressed cardiac-specific transcription factor GATA4; cardiac-specific genes such as myosin light chain (MLC)-2a, MLC-2v, cTnI, and cTnT; and the α-subunits of the cardiac-specific L-type calcium channel (α1c) and the transient outward potassium channel (Kv4.3). After stimulation with basic fibroblast growth factor (bFGF) or activin A, HAM cells expressed Nkx2.5, a specific transcription factor for cardiomyocytes, and a cardiac-specific marker, atrial natriuretic peptide (ANP). Nkx2.5 is expressed earliest in heart development and regulates the expression of several transcription factors not only in the developing stage but also in the latter stage of differentiation to cardiomyocytes (52). Nkx2.5 can also transactivate the ANF gene synergistically with the zinc finger transcription factor GATA-4 (53). In addition, the cardiac-specific gene α-myosin heavy chain (α-MHC) was expressed in HAM cells after treatment with activin A, but not with bFGF (51), consistent with the previous report showing α-MHC expression during cardiac myogenic differentiation programmed by activin A (54). In contrast, after stimulation by activin A or bFGF for 7 days, HAE cells did not express cardiac genes such as Nkx2.5, ANP, or α-MHC, suggesting that the characteristics of HAE and HAM are different.

The genes of ANP, MLC-2a, MLC-2v, GATA-4, α-MHC, Nkx2.5, cTnT, cTnI, α1c, and Kv4.3 were expressed in the human ES-cell-derived cardiomyocytes (55, 56). ANP, MLC-2a, MLC-2v, GATA-4, and Nkx2.5 were also expressed in the cardiomyocytes derived from both mouse marrow stromal cells (57) and mouse adipose cells (58).

Coculture experiments confirmed that HAM cells were able to both integrate into cardiac tissues and

differentiate into cardiomyocyte-like cells. In vivo after xenotransplantation into myocardial infarcts in rat hearts, HAM cells survived in the scar tissue for at least 2 months and differentiated into cardiomyocyte-like cells. These results showed that in vivo some endogeneous factors could induce cardiac differentiation of HAM cells (51).

In our culture system, beating cells were not observed in cardiomyocyte-like cells derived from HAM cells. Beating cardiomyocytes derived from ES cells and bone-marrow cells have been verified both in mice and humans (55 – 57). Although several methods have been reported for generating beating cardiomyocytes, and 5-azacytidine is one of the major differentiation factors, 5-azacytidine-stimulated HAM cells did not express NKX2.5, ANP, or α-MHC genes. Different inducers may be required for the differentiation into myocardiocytes, depending on the cell source.

From the perspective of electrophysiological properties, bone marrow mesenchymal stem cell derived cardiomyocytes have been reported to be useful as biopacemakers (59). However, little is known about the electrophysiology and chronotropism of stimulated HAM cells expressing ion channels, such as α1c and Kv4.3. Thus, further study is necessary in order to induce HAM cell-derived cardiomyocytes to contract spontaneously.

These studies suggested that HAM cells possess some characteristics of cardiomyocytes and the possibility of being a suitable cell source for cellular cardiomyoplasty, although their properties and potential are not yet completely understood.

3. Chondrocytes

We tried to analyze whether HAM cells have characteristics of chondrocytes and the possibility of their being a suitable cell source for cartilage repair (J.P. Wei, submitted manuscript). HAM cells expressed chondrocyte-related genes, including SOX-9, SOX-5, SOX-6, bone morphogenetic proteins (BMP)-2 and -4, as well as BMP receptors. In vitro, collagen type II and aggrecan were expressed after the induction of chondrogenesis with BMP-2. In vivo, HAM cells that were transplanted into non-cartilage tissue of mice with BMP-2 or implanted with collagen-scaffold into the defects generated in a rat's bone underwent morphological changes with deposition of collagen type II. These results showed that HAM cells have the potential to differentiate into chondrocytes in vitro and in vivo, suggesting that they have therapeutic potential for the treatment of damaged or diseased cartilage.

4. Pancreatic β-cells

For the treatment of type I diabetes mellitus, whole pancreas or islet transplantation can prevent the progression of secondary complications, controlling blood glucose level. However, it is always limited by the shortage of transplantation materials.

As alternative strategies for supplying β-cells, the stimulation of existing β-cells or the induction of embryonic stem cells, as well as pancreatic or nonpancreatic adult stem/progenitor cells, to differentiate into functional β-cells were reported.

It was reported that through 5 steps of induction via embryonic bodies or nestin-positive cells, mouse ES cells could differentiate into insulin-producing cells, but not into mature β-cells (60, 61). The efficiency of induction to insulin-producing cells was up-regulated by expression of TGF β2 or pdx-1, important factors for the development of the pancreas (62). The induction of insulin-producing β-cells from human ES cells was also reported (63).

Although putative adult islet stem/progenitor cells have been assumed to exist in the ductal epithelium, islet (64) and exocrine cells, it is still controversial whether new islets are derived from pluripotent stem cells or from de-/trans-differentiation of preexisting differentiated cells. Suzuki et al. reported that glucagon-like peptide (GLP) 1-(1-37) induces insulin production in developing and, to a lesser extent, adult intestinal epithelial cells in vitro and in vivo. This process, mediated by up-regulation of the Notch-related gene ngn3 and its downstream targets, is involved in pancreatic endocrine differentiation (65).

We assessed the possibility of inducing HAE cells to differentiate into insulin-producing cells by culturing with 10 mM nicotinamide for up to 4 weeks (14).

In vitro, after stimulation with nicotinamide, cultivated HAE cells expressed insulin mRNA. In vivo, HAE cells normalized the blood glucose level in streptozotocin-induced diabetic mice several weeks after implantation. The distributions of human cells and human insulin secretion in mouse tissue were studied by immunohistochemistry for antihuman-specific β2-microglobulin and anti-human-specific insulin. They showed the same location in mouse tissue.

However, 20% of diabetic mice, which became normoglycemic after HAE-implantation, returned to the hyperglycemic 6 weeks after the implantation, indicating that this process is reversible. This might depend in part on the half-life of the implanted cell clusters. Therefore, we are now investigating the possibility of elongating the half-life of implanted cells by using immortalized HAE cells transfected with insulin-producing genes. The responsiveness of transplanted

HAE-cells to "glucose-stimulated insulin secretion" is also one of the remaining issues to be studied. For the treatment of diabetes with β-cell grafts derived from amniotic membrane, it will be necessary to demonstrate stimulus-secretion coupling after obtaining enriched or homogeneously cultured β-cells, especially terminally differentiated, mature β-cells. Although the mechanism regulating the terminal differentiation of β-cells from insulin-expressing cells remains unknown, several important factors have been reported such as large Maf family (66), HB9 (67), or snail family (68). Based on these findings, the efficiency of transplantation of HAE cells for the therapy of diabetes can be improved.

5. Neuronal cells

Both HAE cells and HAM cells have characteristics of neural progenitor cells. Some differentiation markers for neural stem, neuron, and glial cells are reported to be expressed in HAE cells such as the synthesis and release of acetylcholine (69), catecholamines, and dopamine (70), neurotrophic factors (71), activin, and noggin (72).

HAM cells express the phenotype of neuroglial progenitor cells (35). When subjected to a neural cell differentiating protocol, HAM-cell bodies extended long bipolar or complex multipolar processes. Nestin (87.7% of total cells tested) and Musashi1 (93.1%) were expressed in undifferentiated cultured HAM cells, and their positively stained cells increased in number slightly after induction. Musashi 1 is an evolutionally conserved marker for the central nervous system (CNS) progenitor cells, including neural stem cells (73). Thus, this study demonstrated that undifferentiated HAM cells were already "primed for neural differentiation" (35, 74). Considering that these nestin-positive cells showed positive immuoreactivity to BrDU and vimentin, neuronal progenitor cells exist in HAM cells.

Undifferentiated HAM cells were also stained by anti-TuJ 1, -neurofilament (NF)-M, and -glial fibrillary acidic protein (GFAP), and their positively stained cells increased significantly in number after induction. Tuj1 is a very early marker for postmitotic neurons that have just finished their final cell division (75), whereas NF-M expression is associated with the initiation of neuritogenesis, neural process outgrowth, and assumption of the characteristic mature neuronal morphology (76). GFAP, a classical astrocyte marker, is also expressed in neuroglial precursor cells (74). These results indicated that HAM cells can be differentiated into neuroglial phenotypes by using an optimized differentiation protocol (35).

Studies of intracerebral grafting of HAE cells for the treatment of a mouse model of Parkinson's disease showed that HAE cells have an advantage for use in Parkinson's disease, because they can synthesize and release catecholamine and neurotrophic factors, such as nerve growth factor, neurotrophin-3, and brain-derived neurotrophic factor (70, 71).

6. Auditory systems

In the auditory system, stem cells are present in the inner ear and in spiral ganglion tissue (77) and are capable of giving rise to hair cells and neurons. The vast majority of the cases of hearing impairment are caused by a loss of hair cells, the sensory cells in the inner ear that transduce sounds into neural signals. Fewer instances of deafness are due to auditory neuropathy, where the VIIIth cranial nerve is degenerated or absent. However, regeneration may not occur because the stem cell population decreases after birth (78). Therefore, cell transplantation may be the best option for therapeutic repair of the damaged cochlea.

Corrales et al. showed that neural progenitors derived from murine ES cells, when transplanted into the cochlea nerve trunk in a gerbil model with nearly complete loss of the afferent innervation of cochlear hair cells, survived and differentiated into neurons that appeared to respond to cues guiding neurite outgrowth by sending out processes toward denervated hair cells in the organ of Corti (79). This study showed the regrowth of the neuronal processes of the replaced neurons toward hair cells in vivo and augments the in vitro observation of growth toward hair cells (80).

In these reports, neural regeneration or reinnervation was the focus of attention, and local ion transportation and cochlea fibrocyte-regeneration were not investigated. Cochlear fibrocytes are also crucial components of the inner ear homeostasis and are related to its defects resulting from various causes such as mutation of connexin (Cx) 26 that leads to hearing loss. We studied the possibility of using HAE cells for the treatment of hearing loss (81).

In in vitro studies, cultivated HAE cells expressed Cx26 on the cell membranes and nuclei and also expressed Na^+-K^+-ATPase on the cell membranes and cytoplasm. In addition, a scrape-loading lucifer yellow dye transfer assay revealed cell-to-cell communication capacity through the gap junction between the cultured HAE cells. In vivo, HAE cells transplanted in the guinea-pig cochlea were located predominantly at the supralimbal region, supraspiral region in the scala vestibule, and subcentral region of spiral ligament in the scala tympani. They could survive there for at least 3 weeks, expressing Cx26 and Na^+-K^+-ATPase, which are known to be expressed in cochlear fibrocytes, indicating that HAE cells were acting as functional fibrocyte-like cells. The cochlear lateral wall fibrocytes

have been reported to maintain the local ion homeostasis that is crucial for hearing. Studies of transcellular recycling routes of the potassium ion in the cochlea indicated that Cx26 and Na^+-K^+-ATPase were involved in the ion transportation (82). However, it is still controversial whether these characteristic features of transplanted cells in that study were the result of trans-differentiation or fusion of cells, and functional recovery of hearing should also be evaluated using an animal deafness model. However, these findings did suggest that application of HAE cells in transplantation therapy would be one of the therapeutic strategies for various inner ear diseases.

VII. Isolation of stem cells in the amniotic membrane

Transplantation of a sufficient number of cells to adult tissue needs a large-scale cell-supply. If we could isolate stem cells from the amnion, it might be possible to efficiently induce the differentiation of amnion-derived cells to specific cell types, and a small number of cells might be sufficient for transplantation.

It is known that HAE cells express surface markers, such as Oct-4 and nanog, normally present on embryonic stem and germ cells. However, it has not been clarified whether the major population of HAE cells possesses pluripotent characteristics or whether only stem cells possess them.

Several methods have been tried to isolate stem cells from adult tissue. One of them is isolation of the side population (SP) fraction. SP cells, regardless of their tissue origin, are identified by their unique fluorescence-activated cell sorting (FACS) profile. Red (>675-nm) and blue (440 – 460-nm) emissions from the vital fluorescent DNA dye Hoechst-33342 have been used to define a small subset of cells, called the side population (SP) cells. SP cells are enriched in stem cells and have the ability to actively efflux the Hoechst-33342 (83). This efflux is the result of the expression of members of the ATP binding cassette (ABC) transporter super-family, such as the breast cancer resistance protein (BCRP)/ABCG2 and multidrug resistance gene (MDR1) in the SP cells. Expression of ABCG2 is a characteristic also observed in ES cells, suggesting that expression of this protein may represent a general marker of stem cells. We detected 0.01% of HAE cells in the SP-fraction (manuscript in preparation). These HAE SP cells showed higher ability to form colonies compared to non-sorted cells, and they were completely lost in the presence of verapamil, a blocker of BCRP protein.

Miki et al. reported that under high-density culture conditions without other cell-derived feeder layers, amniotic epithelial cells form spheroid structures that retain stem cell characteristics. They maintain Oct-4 expression, do not express telomerase, and are nontumorigenic upon transplantation, as well as having the potential to differentiate in vitro into all three germ layers-endoderm (liver, pancreas), mesoderm (cardiomyocytes), and ectoderm (neural cells) (13). Their spheroids contain 10% stem cell marker-positive cells, expressing Oct-4, SSEA-3, TRA1-60, or TRA1-81. However, as the authors commented, long-term self-renewal ability and single-cell clonal analysis will be necessary before describing them as stem cells. Further work will thus be required.

VIII. Human amniotic cell sheet usage in the field of tissue engineering

Usually, cell transplantation has been performed by injection of cell suspensions. However, direct injection of dissociated cells makes it difficult to control the graft size, location, and functions of the differentiated cells; and thus it is not sufficient for replacing congenital defects. To overcome these problems, cell sheets have been developed, using noninvasive cell-manipulation techniques such as a temperature-responsive culture-surface-system (84, 85). In this system, poly-*N*-isopropylacrylamide (PNIPAM) was widely studied and used as a coating material; however, its cytotoxic properties could not be completely eliminated (86). Therefore we developed a novel culture surface coated with a noncytotoxic, temperature-responsive elastic protein-based polymer (87). It is also highly biocompatible and imperceptible (the polymer can be injected into the tissue and disappears within 2 weeks without leaving any signs of its presence). This unique temperature-responsive polymer switches from hydrophilic surfaces below its transition temperature (29°C) to forming a hydrophobic surface above this temperature. Primary cultured HAE or HAM cells could adhere, spread, and proliferate on this novel culture surface similarly to those on noncoated tissue culture surfaces at 37°C. Upon reducing the temperature to 4°C, the coating surface of the culture dish melted and the primary HAE or HAM cell sheet could detach from the coated surface, using a polyvinylidene difluoride (PVDF) membrane for support. The recovered cell sheet could be transferred to re-adhere and to re-proliferate on another surface of a culture dish. In this system, in contrast to enzymatic digestion, only the interaction between adhesion proteins and the material surface is disrupted. The cells detach from the substratum together as a continuous cell sheet with membrane-proteins and intact adhesion-proteins when the temperature is decreased. This new

technique of cell sheet formation will be useful in tissue engineering, as well as in cell sheet transplantation.

IX. Clinical application of amniotic membrane

1. In the field of ophthalmology

Transplantation of human amniotic membrane in ocular disorders was introduced into ophthalmology more than 60 years ago. Since 1995, amniotic membrane transplantation has been successfully applied for ocular surface reconstruction in patients with a variety of ocular surface diseases, including chemical or thermal burns (88); persistent corneal ulcers of different etiologies (89); Stevens-Johnson syndrome; pterygium (90, 91); symptomatic bullous keratopathy; removal of tumor, scar, or adhesion; ocular cicatricial pemphigoid; and other causes leading to limbal stem cell deficiency. These studies reported that ocular surface inflammation was markedly reduced in the area covered by amniotic membrane.

There are three types of applications for human amniotic membrane transplantation in ocular surface disorders (92): 1) Grafting: By transplanting amniotic membrane on corneal or scleral stroma, proper proliferation and differentiation of the ocular surface epithelium are promoted. 2) Patching: Amniotic membrane may be used to cover the inflamed ocular surface, especially when associated with epithelial defects. It is also useful in the acute phase of chemical/thermal burns or Stevens-Johnson syndrome. 3) Stuffing: Deep corneal/scleral ulcerations or small perforations refractory to conventional medical therapy can be treated by stuffing small pieces of amniotic membrane into the stromal defects, followed by amniotic membrane grafting and patching over the area. Amniotic membrane is used not only as a substitute but also as a scaffold upon which cells can migrate and regenerate, forming new and healthy tissue. In addition to these applications, amniotic membrane, on which limbal stem cells or their progeny have been cultivated in vitro, can also be used as a graft.

A variety of characteristics, such as promoting re-epithelialization, decreasing inflammation and fibrosis, and inhibiting angiogenesis, make amniotic membrane useful in clinical applications. Another advantage of amniotic membrane for use on the ocular surface is that it is an avascular tissue.

Inhibition of neovascularization during corneal surface reconstruction is important. Regarding the antiangiogenic activity of human amniotic membrane, pigment epithelium-derived factor (PEDF), which localizes in the basement membrane of amniotic membrane, is reported to play a major role in inhibiting endothelial cell growth in the cornea (93). PEDF inhibited the proliferation of human umbilical vein endothelial cells and bovine retinal capillary endothelial cells, while it promoted the proliferation of bovine cornea epithelial cells.

Although much evidence has been accumulated, there is still a lack of scientific evidence based on randomized comparative studies to prove that the use of amniotic membrane is better than other alternative therapies for ocular surface reconstruction (94). Based on such studies, appropriate indications for the therapies using amniotic membranes will be clarified, and thus it will be possible to give the best therapy to each patient. In addition, these studies may help unravel other new applications of amniotic membrane transplantation.

2. Hyper dry amnion

At present, in most cases, cryopreserved amniotic membrane tissue has been used clinically for the membrane grafts. The immunogenicity of cryopreserved tissue is generally thought to be less than that of fresh tissue. However, ≥50% of AE cells, cryopreserved for 2 months, remained viable and able to grow in culture (25), and Akle et al. reported that low-grade inflammatory responses were observed when viable amniotic epithelial cells were present (22), suggesting that live amniotic membrane is immunogenic.

To overcome these problems, we developed a novel dried amniotic membrane (Hyper-dry-amnion), using far-infrared rays and microwaves, in addition to γ-irradiation for sterilization (M. Okabe, manuscript in preparation: Fig. 3). When we made it dry, the boiling temperature could be decreased under the low-air-pressure condition. Thus we could decrease the degradation of tissue-protein as far as possible.

We have already started to apply this "Hyper-dry-amnion" in outpatient clinics with the informed consent of patients, with the approvement of the Ethics Committee of the University of Toyama, School of Medicine. Compared to cryopreserved amnion, which can be preserved for less than 3 months at −80°C, "Hyper-dry-amnion" can be preserved at room temperature without time limitation. Because it is very easy to handle it, the operation-time was shortened in the clinical application in the case of recurrent pterygium, and the clinical outcomes for these diseases might have been improved.

However, there is still a lack of appropriate indications or scientific evidence based on randomized comparative studies to prove that its use is better than other alternative therapies, and we are now accumulating evidence on this point. In the very near future, we will be able to report our findings and conclusions.

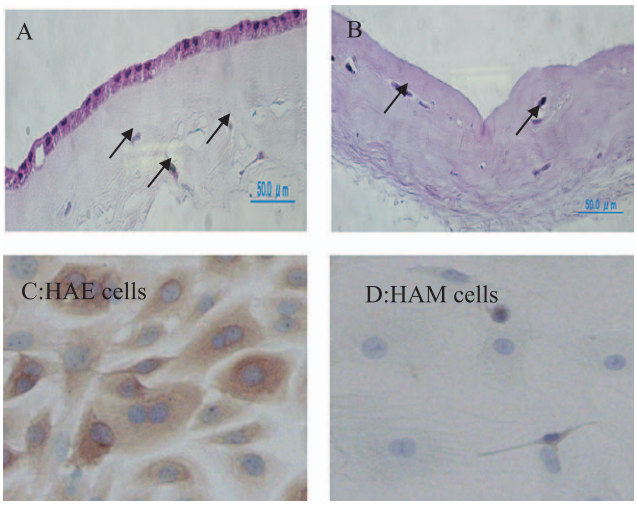

Fig. 1. Isolation of human amniotic cells. In the amnion before trypsinization, both epithelial cells and mesenchymal cells are observed (A: HE staining). After trypsinization, only epithelial cells are detached (B: HE staining) and collected (C: cytokeratin 19-positive HAE cells). HAM cells are cytokeratin 19-negative (D). Arrows show amniotic mesenchymal cells (A and B).

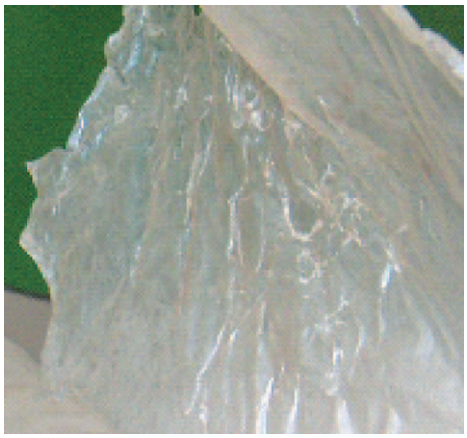

Fig. 3. Novel dried amniotic membrane (Hyper-dry-amnion) was developed using far-infrared rays and microwaves, in addition to γ-irradiation for sterilization (M. Okabe, manuscript in preparation). The boiling temperature could be decreased under the low-air-pressure condition. Thus, we could decrease the degradation of tissue-protein as much as possible.

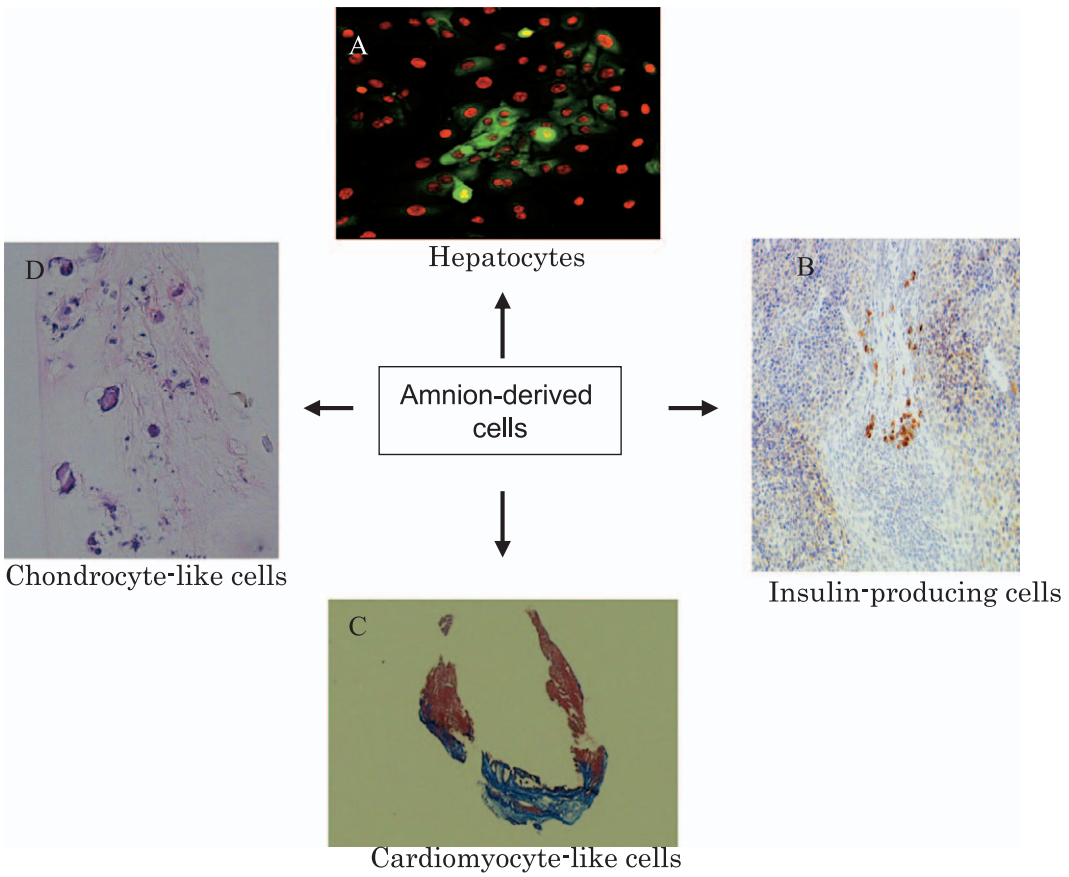

Fig. 2. The isolated amnion-derived cells were capable of differentiation into multiple cell types, including hepatocytes, expressing albumin (A: FITC-labeled anti-human albumin staining, S. Takashima 2004: ref. 15), insulin-producing cells (B: αHuman insulin staining, J.P. Wei 2003: ref. 14), cardiomyocyte-like cells (C: Masson-trichrome staining), and chondrocytes (J.P. Wei, submitted for publication).

X. Summary

Based on the above analyses, it was shown that amnion-derived cells have the potential to differentiate into all three germ layers: endoderm (liver, pancreas), mesoderm (cardiomyocytes), and ectoderm (neural cells), both in vitro and in vivo. Their ability to integrate into the tissue and respond to the degenerated environment suggests that amnion-derived cells retain the plasticity to respond to these potential signals from the nearest environment. These data suggest that amnion-derived cells are a useful cell source for developing therapeutic strategies such as cell transplantation, gene therapy, and artificial organs to treat various disorders. If stem cells are identified in these amnion-derived cells, their usage in regenerative medicine will be more effective.

Much clearer characterization of amniotic membrane and the cells derived from it will enable a wide range of new applications in which amniotic membrane/amnion-derived cells can play a key role in reconstruction of damaged tissue. Hyper-dry-amnion and cell sheets will also be attractive materials in the field of tissue engineering.

References

1 Smith S, Neaves W, Teitelbaum S. Adult stem cell treatments for diseases? Science. 2006;313:439.
2 Trelford JD, Trelford-Sauder M. The amnion in surgery, past and present. Am J Obstet Gynecol. 1979;134:833–845.
3 Benirschke K, Kaufman P. Pathology of the human placenta. New York: Springer-Verlag; 2000. p. 273–281.
4 Okazaki T, Casey ML, Okita JR, MacDonald PC, Johnston JM. Initiation of human parturition. XII. Biosynthesis and metabolism of prostaglandins in human fetal membranes and uterine decidua. Am J Obstet Gynecol. 1981;139:373–381.
5 Bryant-Greenwood GD, Rees MC, Turnbull AC. Immunohistochemical localization of relaxin, prolactin and prostaglandin synthase in human amnion, chorion and decidua. J Endocrinol. 1987;114:491–496.
6 Toth P, Li X, Lei ZM, Rao CV. Expression of human chorionic gonadotropin (hCG)/luteinizing hormone receptors and regulation of the cyclooxygenase-1 gene by exogenous hCG in human fetal membranes. J Clin Endocrinol Metab. 1996;81:1283–1288.
7 King BF. Cell surface specializations and intercellular junctions in human amniotic epithelium: an electron microscopic and freeze-fracture study. Anat Rec. 1982;203:73–82.
8 King BF. Related distribution and characterization of anionic sites in the basal lamina of developing human amniotic epithelium. Anat Rec. 1985;212:57–62.
9 Wolf HJ, Schmidt W, Drenckhahn D. Immunocytochemical analysis of the cytoskeleton of the human amniotic epithelium. Cell Tissue Res. 1991;266:385–389.
10 Akashi T, Miyagi T, Ando N, Suzuki Y, Nemoto T, Eishi Y, et al. Synthesis of basement membrane by gastrointestinal cancer cell lines. J Pathol. 1999;187:223–228.
11 Enders AC, King BF. Formation and differentiation of extraembryonic mesoderm in the rhesus monkey. Am J Anat. 1988;181:327–340.
12 Diwan SB, Stevens LC. Development of teratomas from the ectoderm of mouse egg cylinders. J Natl Cancer Inst. 1976; 57:937–942.
13 Miki T, Lehmann T, Cai H, Stolz DB, Strom SC. Stem cell characteristics of amniotic epithelial cells. Stem Cells. 2005; 23:1549–1559.
14 Wei JP, Zhang TS, Kawa S, Aizawa T, Ota M, Akaike T, et al. Human amnion-isolated cells normalize blood glucose in streptozotocin-induced diabetic mice. Cell Transplant. 2003;12: 545–552.
15 Takashima S, Ise H, Zhao P, Akaike T, Nikaido T. Human amniotic epithelial cells possess hepatocyte-like characteristics and functions. Cell Struct Funct. 2004;29:73–84.
16 Hao Y, Ma DH, Hwang DG, Kim WS, Zhang F. Identification of antiangiogenic and antiinflammatory proteins in human amniotic membrane. Cornea. 2000;19:348–352.
17 Solomon A, Rosenblatt M, Monroy D, Ji Z, Pflugfelder SC, Tseng SCG. Suppression of interleukin 1(alpha) and interleukin 1(beta) in human limbal epithelial cells cultured on the amniotic membrane stromal matrix. Br J Ophthalmol. 2001;85:444–449.
18 Tseng SCG, Li DQ, Ma X. Suppression of transforming growth factor-beta isoforms, TGF-receptor type II, and myofibroblast differentiation in cultured human corneal and limbal fibroblasts by amniotic membrane matrix. J Cell Physiol. 1999;179:325–335.
19 Li H, Niederkorn JY, Neelam S, Mayhew E, Word RA, McCulley JP, et al. Immunosuppressive factors secreted by human amniotic epithelial cells. Invest Ophthalmol Vis Sci. 2005;46:900–907.
20 Kamiya K, Wang M, Uchida A, Amano S, Oshika T, Sakuragawa N, et al. Topical application of culture supernatant from human amniotic epithelial cells suppresses inflammatory reactions in cornea. Exp Eye Res. 2005;80;671–679.
21 Talmi YP, Sigler L, Inge E, Finkelstein Y, Zohar Y. Antibacterial properties of human amniotic membranes. Placenta. 1991;12:285–288.
22 Akle CA, Adinolfi M, Welsh KI, Leibowitz S, McColl I. Immunogenicity of human amniotic epithelial cells after transplantation into volunteers. Lancet. 1981;2:1003–1005.
23 Adinolfi M, Akle CA, McColl I, Fensom AH, Tansley L, Connolly P, et al. Expression of HLA antigens, beta 2-microglobulin and enzymes by human amniotic epithelial cells. Nature. 1982;295:325–327.
24 Hammer A, Hutter H, Blaschitz A, Mahnert W, Hartmann M, Uchanska-Ziegler B, et al. Amnion epithelial cells, in contrast to trophoblast cells, express all classical HLA class I molecules together with HLA-G. Am J Reprod Immunol. 1997;37:161–171.
25 Kubo M, Sonoda Y, Muramatsu R, Usui M. Immunogenicity of human amniotic membrane in experimental xenotransplantation. Invest Ophthalmol Vis Sci. 2001;42:1539–1546.
26 Wang M, Yoshida A, Kawashima H, Ishizaki M, Takahashi H, Hori J. Immunogenicity and antigenicity of allogeneic amniotic epithelial transplants grafted to the cornea, conjunctiva, and anterior chamber. Invest Ophthalmol Vis Sci. 2006;47:1522–1532.
27 Runic R, Lockwood CJ, LaChapelle L, Dipasquale B, Demopoulos RI, Kumar A, et al. Apoptosis and Fas expression in human fetal membranes. J Clin Endocrinol Metab. 1998;

83:660–666.
28 Scaggiante B, Pineschi A, Sustersich M, Andolina M, Agosti E, Romeo D. Successful therapy of Niemann-Pick disease by implantation of human amniotic membrane. Transplantation. 1987;44:59–61.
29 Sakuragawa N, Yoshikawa H, Sasaki M. Amniotic tissue transplantation: clinical and biochemical evaluations for some lysosomal storage diseases. Brain Dev. 1992;14:7–11.
30 Robinson WP, McFadden DE, Barrett IJ, Kuchinka B, Penaherrera MS, Bruyere H, et al. Origin of amnion and implications for evaluation of the fetal genotype in cases of mosaicism. Prenat Diagn. 2002;22:1076–1085.
31 Ochsenbein-Kolble N, Bilic G, Hall H, Huch R, Zimmermann R. Inducing proliferation of human amnion epithelial and mesenchymal cells for prospective engineering of membrane repair. J Perinat Med. 2003;31:287–294.
32 Nanbu Y, Fujii S, Konishi I, Nonogaki H, Mori T. CA 125 in the epithelium closely related to the embryonic ectoderm: the periderm and the amnion. Am J Obstet Gynecol. 1989;161:462–467.
33 Beham A, Denk H, Desoye G. The distribution of intermediate filament proteins, actin and desmoplakins in human placental tissue as revealed by polyclonal and monoclonal antibodies. Placenta. 1988;9:479–492.
34 Wolf HJ, Schmidt W, Drenckhahn D. Immunocytochemical analysis of the cytoskeleton of the human amniotic epithelium. Cell Tissue Res. 1991;266:385–389.
35 Sakuragawa N, Kakinuma K, Kikuchi A, Okano H, Uchida S, Kamo I, et al. Human amnion mesenchyme cells express phenotypes of neuroglial progenitor cells. J Neurosci Res. 2004;78:208–214. Erratum in: J Neurosci Res. 2005;79:725.
36 Nakajima T, Enosawa S, Mitani T, Li XK, Suzuki S, Amemiya H, et al. Cytological examination of rat amniotic epithelial cells and cell transplantation to the liver. Cell Transplant. 2001;10:423–427.
37 Ogawa A. Animal cell technology: basic & applied aspects, Vol.12, Dordrecht: Kluwer Academic Publishers; 2002.
38 Pieber D, Allport VC, Bennett PR. Progesterone receptor isoform A inhibits isoform B-mediated transactivation in human amnion. Eur J Pharmacol. 2001;427:7–11.
39 Ogawa A, Terada S, Sakuragawa N, Masuda S, Nagao M, Miki M. Progesterone, but not 17beta-estradiol, up-regulates erythropoietin (EPO) production in human amniotic epithelial cells. J Biosci Bioeng. 2003;96:448–453.
40 Suzuki A, Zheng Y, Kondo R, Kusakabe M, Takada Y, Fukao K, et al. Flow-cytometric separation and enrichment of hepatic progenitor cells in the developing mouse liver. Hepatology. 2000;32:1230–1239.
41 Petersen BE, Bowen WC, Patrene KD, Mars WM, Sullivan AK, Murase N, et al. Bone marrow as a potential source of hepatic oval cells. Science. 1999;284:1168–1170.
42 Oh SH, Miyazaki M, Kouchi H, Inoue Y, Sakaguchi M, Tsuji T, et al. Hepatocyte growth factor induces differentiation of adult rat bone marrow cells into a hepatocyte lineage in vitro. Biochem Biophys Res Commun. 2000;279:500–504.
43 Hamazaki T, Iiboshi Y, Oka M, Papst PJ, Meacham AM, Zon LI, et al. Hepatic maturation in differentiating embryonic stem cells in vitro. FEBS Lett. 2001;497:15–19.
44 Rambhatla L, Chiu CP, Kundu P, Peng Y, Carpenter MK. Generation of hepatocyte-like cells from human embryonic stem cells. Cell Transplant. 2003;12:1–11.
45 Danet GH, Luongo JL, Butler G, Lu MM, Tenner AJ, Simon MC, et al. C1qRp defines a new human stem cell population with hematopoietic and hepatic potential. Proc Natl Acad Sci U S A. 2002;99:10441–10445.
46 Sakuragawa N, Enosawa S, Ishii T, Thangavel R, Tashiro T, Okuyama T, et al. Human amniotic epithelial cells are promising transgene carriers for allogeneic cell transplantation into liver. J Hum Genet. 2000;45:171–176.
47 Davila JC, Cezar GG, Thiede M, Strom S, Miki T, Trosko J. Use and application of stem cells in toxicology. Toxicol Sci. 2004;79:214–223.
48 Wang ND, Finegold MJ, Bradley A, Ou CN, Abdelsayed SV, Wilde MD, et al. Impaired energy homeostasis in C/EBP alpha knockout mice. Science. 1995;269:1108–1112.
49 Takahashi N, Enosawa S, Mitani T, Lu H, Suzuki S, Amemiya H, et al. Transplantation of amniotic epithelial cells into fetal rat liver by in utero manipulation. Cell Transplant. 2002;11:443–449.
50 Hughes S. Cardiac stem cells. J Pathol. 2002;197:468–478.
51 Zhao P, Ise H, Hongo M, Ota M, Konishi I, Nikaido T. Human amniotic mesenchymal cells have some characteristics of cardiomyocytes. Transplantation. 2005;79:528–535.
52 Tanaka M, Chen Z, Bartunkova S, Yamasaki N, Izumo S. The cardiac homeobox gene Csx/Nkx2.5 lies genetically upstream of multiple genes essential for heart development. Development. 1999;126:1269–1280.
53 Kasahara H, Usheva A, Ueyama T, Aoki H, Horikoshi N, Izumo S. Characterization of homo- and heterodimerization of cardiac Csx/Nkx2.5 homeoprotein. J Biol Chem. 2001;276:4570–4580.
54 Logan M, Mohun T. Induction of cardiac muscle differentiation in isolated animal pole explants of Xenopus laevis embryos. Development. 1993;118:865–875.
55 Kehat I, Kenyagin-Karsenti D, Snir M, Segev H, Amit M, Gepstein A, et al. Human embryonic stem cells can differentiate into myocytes with structural and functional properties of cardiomyocytes. J Clin Invest. 2001;108:407–414.
56 Mummery C, Ward-van Oostwaard D, Doevendans P, Spijker R, van den Brink S, Hassink R, et al. Differentiation of human embryonic stem cells to cardiomyocytes: role of coculture with visceral endoderm-like cells. Circulation. 2003;107:2733–2740.
57 Makino S, Fukuda K, Miyoshi S, Konishi F, Kodama H, Pan J, et al. Cardiomyocytes can be generated from marrow stromal cells in vitro. J Clin Invest. 1999;103:697–705.
58 Planat-Benard V, Menard C, Andre M, Puceat M, Perez A, Garcia-Verdugo J-M, et al. Spontaneous cardiomyocyte differentiation from adipose tissue stroma cells. Circ Res. 2004;94:223–229.
59 Tomita Y, Makino S, Hakuno D, Hattan N, Kimura K, Miyoshi S, et al. Application of mesenchymal stem cell-derived cardiomyocytes as bio-pacemakers: current status and problems to be solved. Med Biol Eng Comput. In Press 2007.
60 Lumelsky N, Blondel O, Laeng P, Velasco I, Ravin R, McKay R. Differentiation of embryonic stem cells to insulin-secreting structures similar to pancreatic islets. Science. 2001;292:1389–1394. Erratum in: Science. 2001;293:428.
61 Moritoh Y, Yamato E, Yasui Y, Miyazaki S, Miyazaki J. Analysis of insulin-producing cells during in vitro differentiation from feeder-free embryonic stem cells. Diabetes. 2003;52:1163–1168.
62 Miyazaki S, Yamato E, Miyazaki J. Regulated expression of pdx-1 promotes in vitro differentiation of insulin-producing cells

from embryonic stem cells. Diabetes. 2004;53:1030–1037.
63 D'Amour KA, Bang AG, Eliazer S, Kelly OG, Agulnick AD, Smart NG, et al. Production of pancreatic hormone-expressing endocrine cells from human embryonic stem cells. Nat Biotechnol. 2006;24:1392–1401.
64 Dor Y, Brown J, Martinez OI, Melton DA. Adult pancreatic beta-cells are formed by self-duplication rather than stem-cell differentiation. Nature. 2004;429:41–46.
65 Suzuki A, Nakauchi H, Taniguchi H. Glucagon-like peptide 1 (1-37) converts intestinal epithelial cells into insulin-producing cells. Proc Natl Acad Sci U S A. 2003;100:5034–5039.
66 Nishimura W, Kondo T, Salameh T, El Khattabi I, Dodge R, Bonner-Weir S, et al. A switch from MafB to MafA expression accompanies differentiation to pancreatic beta-cells. Dev Biol. 2006;293:526–539.
67 Wang J, Elghazi L, Parker SE, Kizilocak H, Asano M, Sussel L, et al. The concerted activities of Pax4 and Nkx2.2 are essential to initiate pancreatic beta-cell differentiation. Dev Biol. 2004;266:178–189.
68 Rukstalis JM, Ubeda M, Johnson MV, Habener JF. Transcription factor snail modulates hormone expression in established endocrine pancreatic cell lines. Endocrinology. 2006;147:2997–3006.
69 Sakuragawa N, Misawa H, Ohsugi K, Kakishita K, Ishii T, Thangavel R, et al. Evidence for active acetylcholine metabolism in human amniotic epithelial cells: applicable to intracerebral allografting for neurologic disease. Neurosci Lett. 1997;232:53–56.
70 Kakishita K, Elwan MA, Nakao N, Itakura T, Sakuragawa N. Human amniotic epithelial cells produce dopamine and survive after implantation into the striatum of a rat model of Parkinson's disease: a potential source of donor for transplantation therapy. Exp Neurol. 2000;165:27–34.
71 Uchida S, Inanaga Y, Kobayashi M, Hurukawa S, Araie M, Sakuragawa N. Neurotrophic function of conditioned medium from human amniotic epithelial cells. J Neurosci Res. 2000;62:585–590.
72 Koyano S, Fukui A, Uchida S, Yamada K, Asashima M, Sakuragawa N. Synthesis and release of activin and noggin by cultured human amniotic epithelial cells. Dev Growth Differ. 2002;44:103–112.
73 Kaneko Y, Sakakibara S, Imai T, Suzuki A, Nakamura Y, Sawamoto K, et al. Musashi1: an evolutionally conserved marker for CNS progenitor cells including neural stem cells. Dev Neurosci. 2000;22:139–153.
74 Woodbury D, Reynolds K, Black IB. Adult bone marrow stromal stem cells express germline, ectodermal, endodermal, and mesodermal genes prior to neurogenesis. J Neurosci Res. 2002;69:908–917.
75 Nakamura Y, Yamamoto M, Oda E, Yamamoto A, Kanemura Y, Hara M, et al. Expression of tubulin beta II in neural stem /progenitor cells and radial fibers during human fetal brain development. Lab Invest. 2003;83:479–489.
76 Carden MJ, Trojanowski JQ, Schlaepfer WW, Lee VM. Two-stage expression of neurofilament polypeptides during rat neurogenesis with early establishment of adult phosphorylation patterns. J Neurosci. 1987;7:3489–3504.
77 Li H, Liu H, Heller S. Pluripotent stem cells from the adult mouse inner ear. Nat Med. 2003;9:1293–1299.
78 Oshima K, Grimm CM, Corrales CE, Senn P, Martinez Monedero R, Geleoc GS, et al. Differential distribution of stem cells in the auditory and vestibular organs of the inner ear. J Assoc Res Otolaryngol. 2007;8:18–31.
79 Corrales CE, Pan L, Li H, Liberman MC, Heller S, Edge AS. Engraftment and differentiation of embryonic stem cell-derived neural progenitor cells in the cochlear nerve trunk: growth of processes into the organ of Corti. J Neurobiol. 2006;66:1489–1500.
80 Kondo T, Johnson SA, Yoder MC, Romand R, Hashino E. Sonic hedgehog and retinoic acid synergistically promote sensory fate specification from bone marrow-derived pluripotent stem cells. Proc Natl Acad Sci U S A. 2005;102:4789–4794.
81 Yuge I, Takumi Y, Koyabu K, Hashimoto S, Takashima S, Fukuyama T, et al. Transplanted human amniotic epithelial cells express connexin 26 and Na-K-ATPase in the inner ear. Transplantaion. 2004;77:1452–1454.
82 Spicer SS, Schulte BA. Evidence for a medial K+ recycling pathway from inner hair cells. Hearing Res. 1998;118:1–12.
83 Goodell MA, Brose K, Paradis G, Conner AS, Mulligan RC. Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. J Exp Med. 1996;183:1797–1806.
84 Okano T, Yamada N, Sakai H, Sakurai Y. A novel recovery system for cultured cells using plasma-treated polystyrene dishes grafted with poly(N-isopropylacrylamide). J Biomed Mater Res. 1993;27:1243–1251.
85 Shiroyanagi Y, Yamato M, Yamazaki Y, Toma H, Okano T. Transplantable urothelial cell sheets harvested noninvasively from temperature-responsive culture surfaces by reducing temperature. Tissue Eng. 2003;9:1005–1012.
86 Vihola H, Laukkanen A, Valtola L, Tenhu H, Hirvonen J. Cytotoxicity of thermosensitive polymers poly(N-isopropylacrylamide), poly(N-vinylcaprolactam) and amphiphilically modified poly(N-vinylcaprolactam). Biomaterials. 2005;26:3055–3064.
87 Zhang H, Iwama M, Akaike T, Urry DW, Pattanaik A, Parker TM, et al. Human amniotic cell sheet harvest using a novel temperature-responsive culture surface coated with protein-based polymer. Tissue Eng. 2006;12:391–401.
88 Kim JC, Tseng SC. Transplantation of preserved human amniotic membrane for surface reconstruction in severely damaged rabbit corneas. Cornea. 1995;14:473–484.
89 Lee SH, Tseng SC. Amniotic membrane transplantation for persistent epithelial defects with ulceration. Am J Ophthalmol. 1997;123:303–312.
90 Prabhasawat P, Barton K, Burkett G, Tseng SC. Comparison of conjunctival autografts, amniotic membrane grafts, and primary closure for pterygium excision. Ophthalmology. 1997;104:974–985.
91 Shimazaki J, Shinozaki N, Tsubota K. Transplantation of amniotic membrane and limbal autograft for patients with recurrent pterygium associated with symblepharon. Br J Ophthalmol. 1998;82:235–240.
92 Shimazaki J. Clinical application of amniotic membrane transplantation. Jpn J Ophthal Surgery. 2002;15:25–29.
93 Shao C, Sima J, Zhang SX, Jin J, Reinach P, Wang Z, et al. Suppression of corneal neovascularization by PEDF release from human amniotic membranes. Invest Ophthalmol Vis Sci. 2004;45:1758–1762.
94 Gomes JA, Romano A, Santos MS, Dua HS. Amniotic membrane use in ophthalmology. Curr Opin Ophthalmol. 2005;16:233–240.