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Amnion-derived cells as a reliable resource for next-generation regenerative medicine

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ABSTRACT

The placenta is composed of the amnion, chorionic plate, villous and smooth chorion, decidua basalis, and umbilical cord. The amnion is a readily obtainable source of a large number of cells and cell types, including epithelium, mesenchyme, and endothelium, and is thus an allogeneic resource for regenerative medicine. Endothelial cells are obtained from large arteries and veins in the amniotic membrane as well as the umbilical cord. The amnion-derived cells exhibit transdifferentiation capabilities, including chondrogenesis and cardiomyogenesis, by introduction of transcription factors, in addition to their original and potential phenotypes. The amnion is also a source for production of induced pluripotent stem cells (AM-iPSCs). The AM-iPSCs exhibit stable phenotypes, such as multipotency and immortality, and a unique gene expression pattern. Through the use of amnion-derived cells, as well as other placenta-derived cells, preclinical proof of concept has been achieved in a mouse model of muscular dystrophy.

1. Text

The human placenta is comprised of cells that are obtained from amnion, chorion, umbilical cord, and decidual endometrium [1,2]. Among these placental components, the amnion serves as a suitable raw materials for regenerative therapy products due to the large number of cells [3]. Endothelium, epithelium, and mesenchymal stroma from the amnion have been successfully isolated, propagated and phenotyped. In this review, we describe the characteristics and potential of amnion-derived propagated or reprogrammed cells from the viewpoints of regenerative medicine.

2. Derivation of amniotic cells

The amniotic membrane is an avascular thin membrane (about 100 μm thick) and is part of the placenta in the uterus of a pregnant woman. The membrane covers the fetus and holds the amniotic fluid. It is composed of epithelium, the basement membrane, and a compact layer; amniotic ectoderm originates from the epiblast (inner cell mass) embryologically. As the amniotic membrane is often treated as medical waste after childbirth, opportunity exists for it to be used as a source of many types of cells. Amnion, villous chorion and decidua can easily be manually separated (Fig. 1A–G). We cut amniotic membrane into 5-mm squares (Fig. 1H and I) and evenly arrange them in dishes with culture medium containing 10% fetal bovine serum. After incubation for one week, cells start to proliferate from the tissue pieces which have adhered to the bottom of the dish, and soon, the cells become confluent. Alternatively, the amnion is digested with trypsin, collagenase, or dispase for cell isolation and subsequent cell cultivation. The propagated cells with fibroblast-like morphology are designated as amnion-derived cells at passage 0 in our experimental setting (Fig. 1J and K).

3. Characteristics of amniotic cells

Mesenchymal stromal cells (MSCs) from the amniotic membrane can easily be propagated ex vivo [4–7]. Amniotic MSCs show fibroblast-like morphology in culture, while amniotic epithelial cells and decidua-derived cells exhibit a cobblestone-like morphology. Amniotic MSCs proliferate for more than 20 population doublings, but then reach senescence. Their limited lifespan in culture suggests a dynamic cell character. Amniotic MSCs obtained before 10 population doublings are generally used for practical reasons. The amniotic membrane is a rich cellular source of MSCs: Approximately 2×10^6 cells are available for use 3 days after the primary culture of 1 g tissue samples from the

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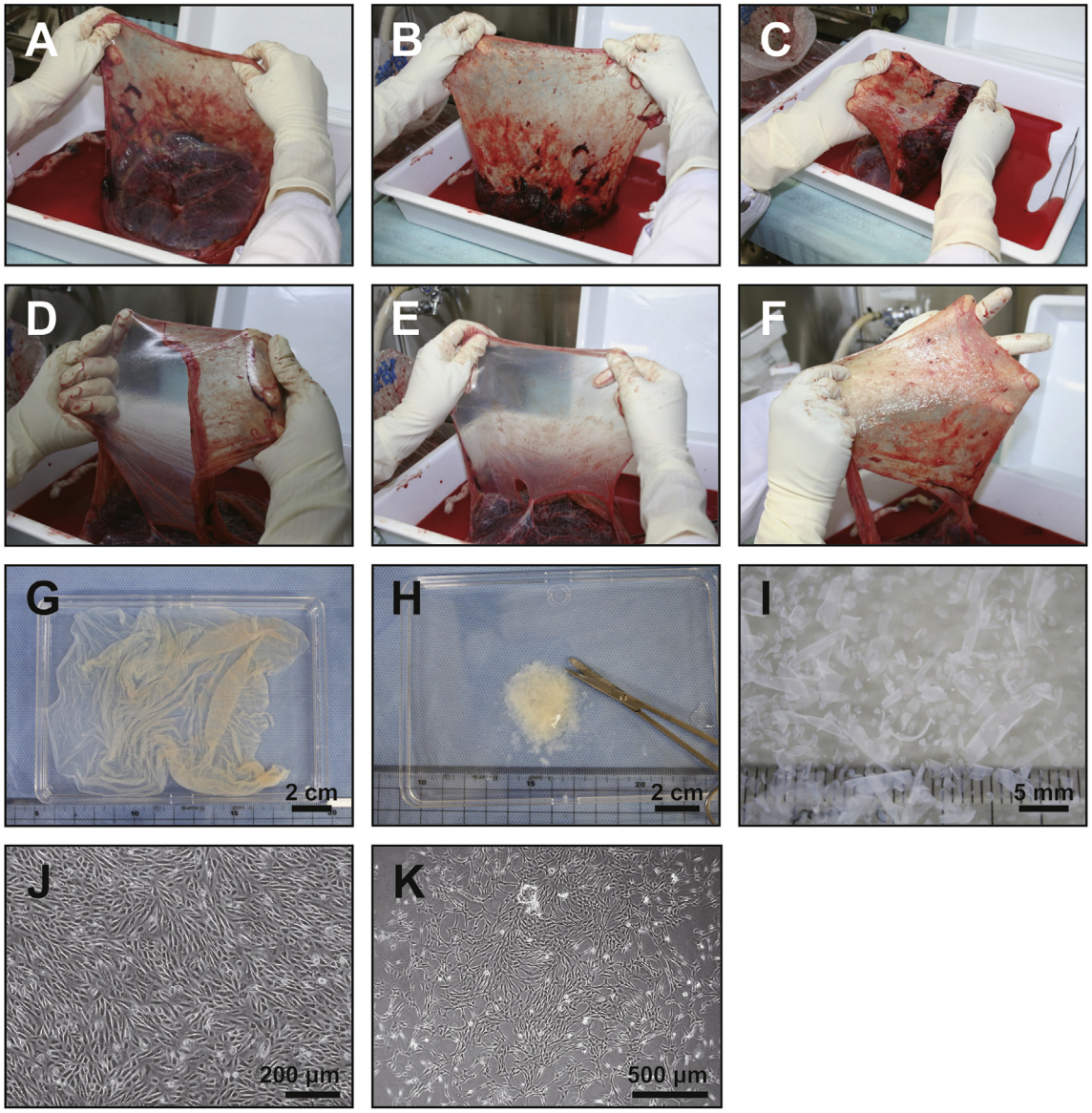

Fig. 1. Isolation of human amniotic cells. A. Separation of amniotic membrane from human placenta. The fetal membrane, viewed from the fetal side. B. The amniotic membrane, viewed from the maternal side. C. Separation of the fetal membrane from the placenta. D. Separation of the amniotic membrane from fetal chorion and maternal decidua. This panel is reproduced from Sugawara et al., Fig. 1A [53]. E. Fetal amniotic membrane. F. Fetal chorion and maternal decidua. G. Preparation of amniotic membrane for cultivation. To generate amniotic cells, the amnion was washed in PBS supplemented with penicillin-streptomycin and Amphotericin B. H. Amnion was cut into pieces approximately 5 mm^2 in size. This panel is reproduced from Sugawara et al., Fig. 1B [53]. I. Magnification of scattered amniotic membrane. J. Morphology at passage 4 of human amnion-derived cells (AM936EP). K. Morphology at the time of first passage of the human amniotic epithelium-derived cells (AM933EP-ep). Morphology of AM933EP-ep is fibroblast-like.

amniotic membrane.

Propagated MSCs express CD10, CD13, CD29, CD44, CD55, CD59, CD73, CD90, CD105, and CD166, but not hematopoietic cell markers (e.g., CD14, CD34, CD45, CD117, and CD309) [4,7,8]. Lack of hematopoietic cell markers suggests that amniotic MSCs are depleted of hematopoietic cells after ex vivo propagation. From the viewpoint of the cell surface markers, amniotic MSCs exhibit a heterogeneous pattern at early passages, but develop a homogeneous pattern in later passages. Amniotic MSCs have a distinct expression pattern of the HOX gene family: they express the HOX B genes, such as HOX B2, B6, B7, and B8. MSCs also express other epithelial markers (cytokeratins), a mesenchymal marker (vimentin), neuronal markers, and stem cell markers (Oct-4/3, c-kit, SSEA4) [7,9]. MSCs differentiate into adipoblasts, osteoblasts, and chondroblasts, and exhibit myogenic, angiogenic, pancreatic, cardiogenic, hepatocytic cells, and neurogenic potential in vitro [4,10–12].

4. Regenerative medicine/cell-based therapy with amniotic cells

Placental cells and amniotic fluid-derived cells have been shown to aid in recovery of murine genetic diseases and ameliorate animal models of birth defects, including Duchenne muscular dystrophy, osteogenesis imperfecta, and myelomeningocele [4,8,10,13,14]. Placental cell implantation results in the expression of dystrophin through fusion of donor cells with recipient cells to restore dystrophic muscles [8,15] (Fig. 2A). The dystrophin gene in the donor cells is transactivated by myogenic transcription factors in the donor myocytes after fusion (Fig. 2B, C, D). Other types of cells may be useful for the treatment of genetic muscular diseases, however, placental cells are ideal because of the ability to obtain a large number of cells with broad differentiation potential. The efficacy of placental cells in Duchenne muscular dystrophy is exerted via fusion of the donor and recipient cells; in contrast, efficacy in heart failure is due to cytokines produced from the donor

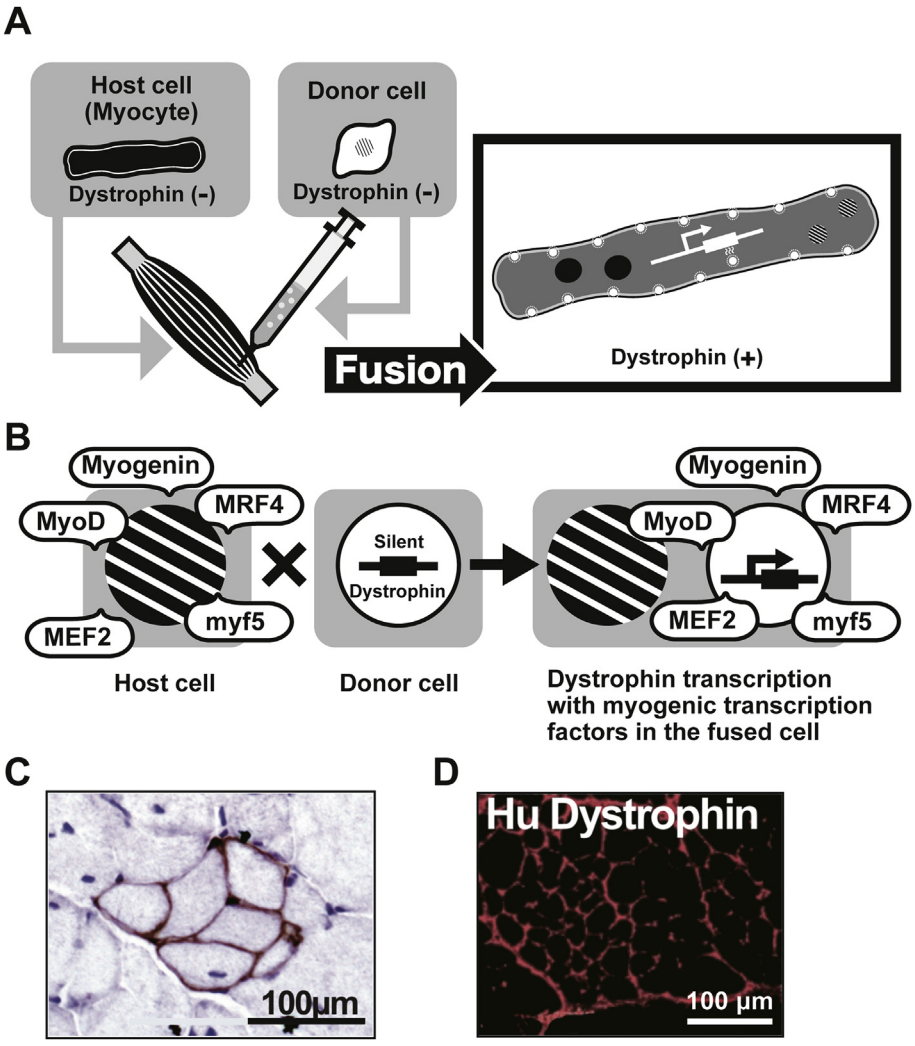

Fig. 2. Regenerative medicine using placental cells. A. Scheme for placental cell implantation into dystrophic host myocytes. The dystrophin gene from the donor cells is expressed shortly after fusion. B. Mechanism of the dystrophin gene expression in the fused cell. No dystrophin protein is expressed in myocytes of the mdx mouse, a model for Duchenne muscular dystrophy. The dystrophin gene in the donor cells is transcribed using myogenic transcription factors from the host myogenic cells. C. Conferral of dystrophin to mdx myocytes by human placenta-derived cells. Immunohistochemistry shows dystrophin protein after implantation of amniotic cells [8]. D. Conferral of dystrophin to mdx myocytes by human placenta-derived cells. Immunohistochemistry shows human dystrophin in the dystrophic muscle after implantation of placental endothelium [15]. Hu Dystrophin: human dystrophin (red).

cells [16,17]. Placental cells can differentiate into cardiomyocytes and reverse ischemic cardiac diseases [12,18–20]. High myogenic/cardiomyogenic potential of amniotic cells may be a reflection of the intrinsic cellular potential of extraembryonic mesoderm.

In addition to diseases of skeletal muscle and heart muscle, placental cells have been used experimentally for other diseases. In animal models, placental cells contribute to recovery from fibrotic diseases, including liver fibrosis and pulmonary fibrosis [21,22]. Amniotic MSCs improve reproductive and ovarian function through direct injection [23]. Placental MSCs demonstrate efficacy in experimental animal models of ischemic brain disease, spinal cord injury, Parkinson's disease, Alzheimer's disease and cerebral ischemia through angiocrine growth factors, cytokines and/or neurotrophic factors secreted from placental MSCs [10,24–27]. MSCs can also be used as a component of three-dimensional skin and engineered heart tissue [14,28–30].

5. Immunomodulation with amnion-derived MSCs

MSCs have a modulatory effect on the immune system which depends on the cells' origin [31,32]. Amnion-derived MSCs possess a higher differentiation potential than terminally differentiated somatic cells and are also less likely to result in immune rejection [15,33] due to the expression of low levels of HLA class I (HLA-A, B, C) antigens and their lack of HLA class II (HLA-DR) antigens. Interestingly, amniotic cells do express HLA-G, which blocks the immune reaction [15,28,33]. Thus, amniotic cells are useful in regenerative medicine and are suitable for cell banking because (1) large quantities of cells are available, (2) they possess a high differentiation potential, (3) they exhibit few genetic mutations, and (4) they are less likely to be rejected by the immune system. In addition, the placental MSCs show a higher level of immunomodulation compared with MSCs from adipose tissue and bone marrow [31,32].

6. Direct reprogramming of amniotic cells

Chondrocytes and scleral cells share the characteristic of chondrogenic potential, and both contribute to chondrogenesis in vitro and in vivo [28,33]. Placenta-derived cells do not show such potential, and protocols for simple proliferation and differentiation do not generate chondrogenic cells. We previously employed a transdifferentiation strategy and redirected cell differentiation [34]. By use of multiple cartilage-specific genes, we successfully introduced direct conversion from placenta-derived cells to chondrocytes, opening an avenue for generation of cartilage from placenta [28]. Amniotic cells have a strong capacity to transdifferentiate into cardiomyocytes [7,12]. Placental cells are therefore a useful source of myocardial cells that can be used for cell transplantation therapy for intractable heart disease, a condition that is currently difficult to treat [35]. Chorionic plate cells also have cardiomyogenic potential due to constitutive expression of cardiomyocyte-specific genes, and they start to beat synchronously after the appropriate co-cultivation system.

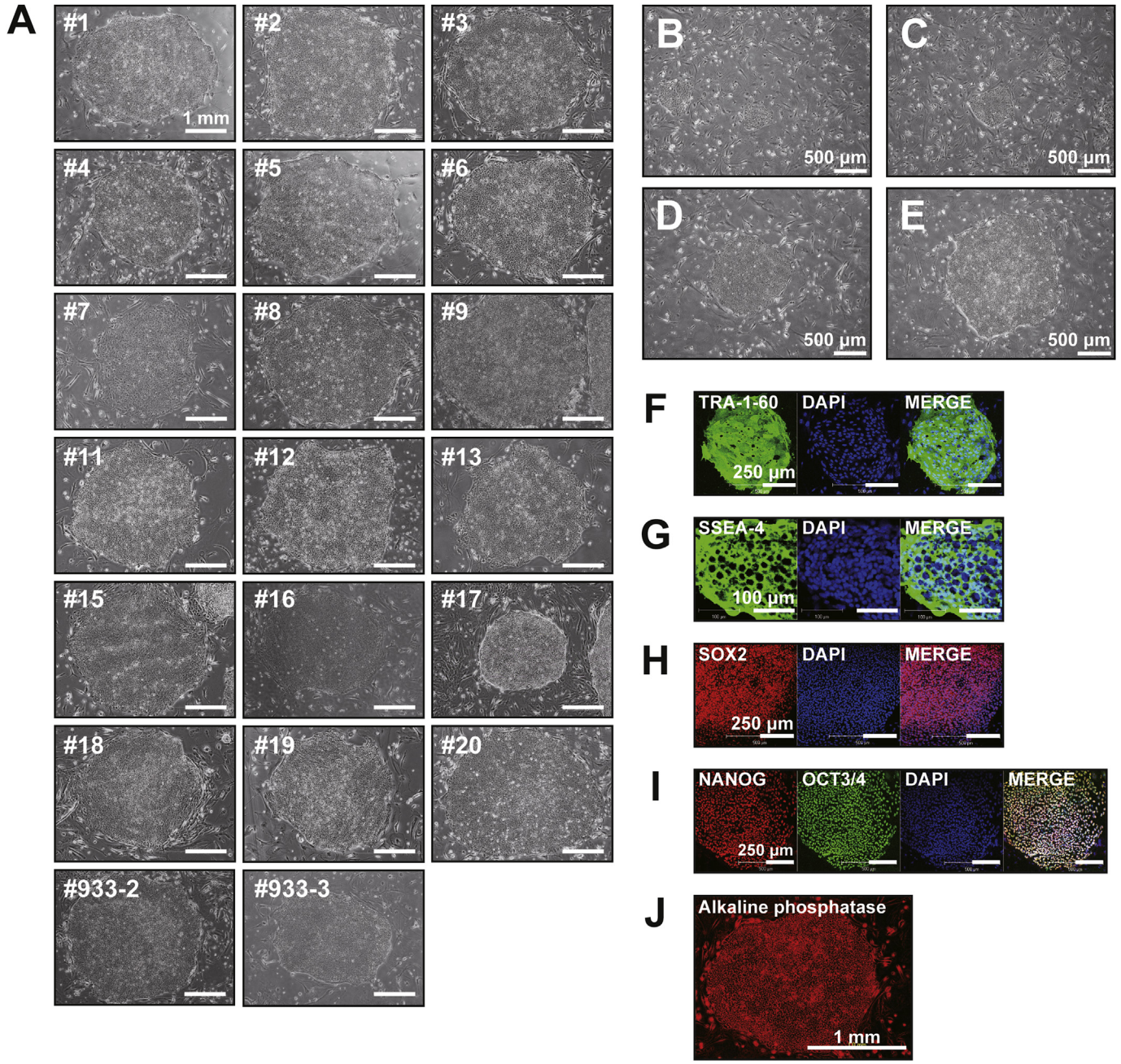

Fig. 3. Generation of iPSCs from amnion. A. Morphology of AM-iPSCs generated from amniotic cells. AM-iPSC#1 to #20 were generated from AM933EP-ep. iPSC#933–2 and iPSC#933–3 were generated from AM933EP-ep. B. Growth process of AM-iPSC#3. Morphology of post-passage day 1. C. Growth process of AM-iPSC#3. Morphology of post-passage day 3. D. Growth process of AM-iPSC#3. Morphology of post-passage day 5. E. Growth process of AM-iPSC#3. Morphology of post-passage day 7. F. Immunocytochemistry of AM-iPSC#6 with an antibody to the cell surface marker TRA-1-60. G. Immunocytochemistry of AM-iPSC#6 with an antibody to the cell surface marker SSEA-4. H. Immunocytochemistry of AM-iPSC#6 with an antibody to SOX2. I. Immunocytochemistry of AM-iPSC#6 with an antibody to OCT3/4 (green) and NANOG (red). J. Alkaline phosphatase stain of AM-iPSC#6.

7. Generation of iPSCs from amniotic cells

iPSCs have been established from murine and human amniotic cells (AM-iPSC) [36,37]. Generation of iPSCs involves reversion to a pluripotent state, a kind of dedifferentiation [38,39]. This process involves lineage-switching back to a branch point and out again in a different direction [40]. AM-iPSC colonies exhibit so-called “primed” phenotypes, and their morphology during the growth process is very similar to that of ESCs (Fig. 3A–E). AM-iPSCs are immunocytochemically positive for markers of undifferentiation, i.e. *TRA-1-60*, *SSEA4*, *SOX2*, *OCT3/4* and *NANOG*, and histochemically positive for alkaline phosphatase (Fig. 3F–J). AM-iPSCs exhibit multipotency in vivo, and maintain normal karyotypes and chromosomal stability after long cultivation periods [41–43], i.e., more than 60 passages (Fig. 4A–D). Hierarchical cluster analysis of gene expression data of AM-iPSCs, MRC5-iPSCs and ESCs shows that AM-iPSCs are positioned close ESCs, but not to parental amniotic cells (Fig. 4E). ESCs and AM-iPSCs indeed show similar expression patterns of undifferentiation marker genes such as *OCT3/4*, *SOX2*, *KLF4*, *c-MYC*, and *TERT*. Venn diagrams illustrate the number of probes whose expression change more than five-fold between the iPSCs and their parental cells (Fig. 4F and G). During the reprogramming of amniotic cells to iPSCs, 1294 genes are up-regulated and 1449 are down-regulated. Gene ontology analysis shows that the up-regulated genes are cell cycle-related, such as *LZTS1*, *CDKN1C* and

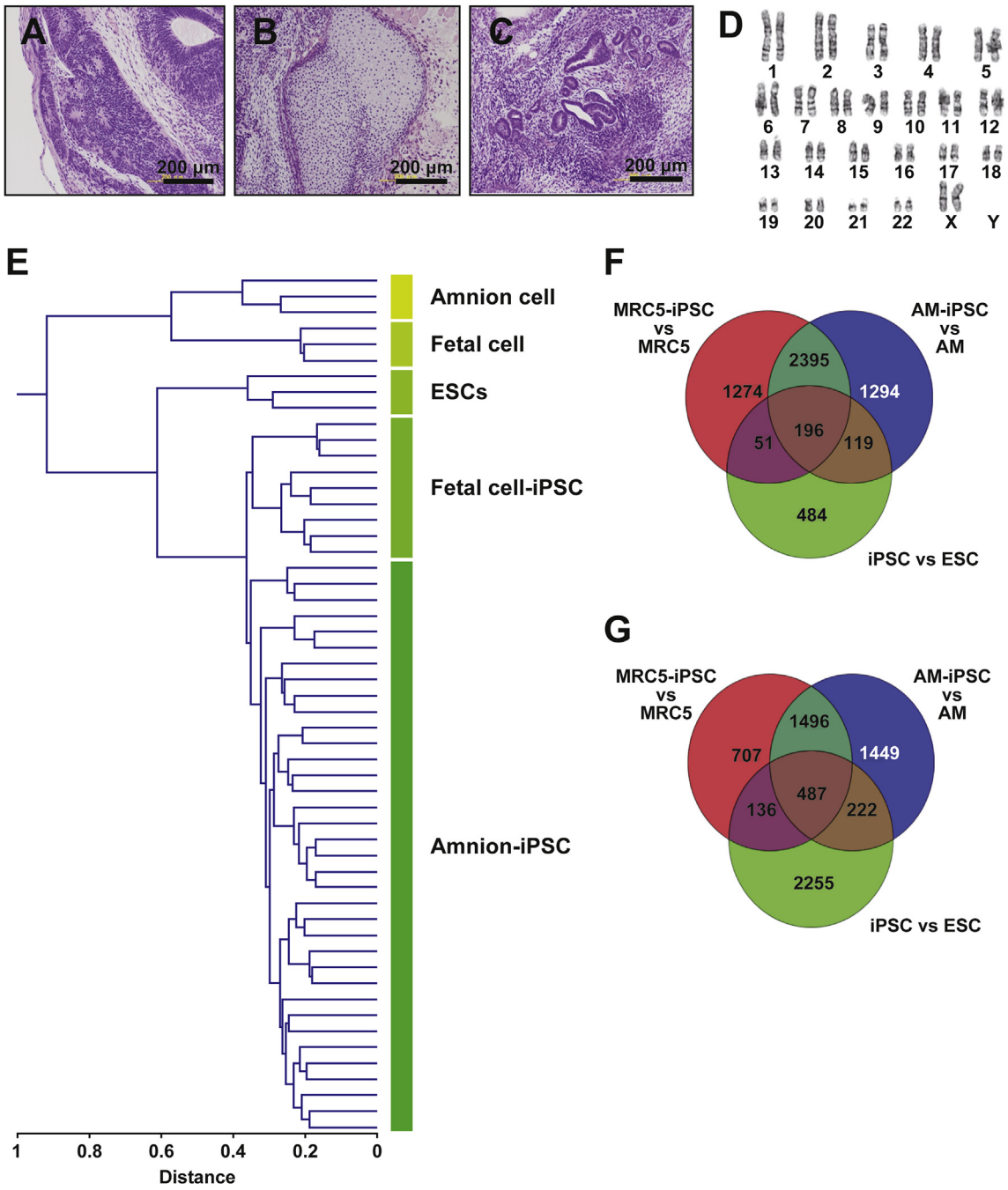

Fig. 4. Characterization of iPSCs generated from amnion. A. Neural tissue (ectoderm) in the teratoma generated by subcutaneous injection of AM-iPSC#2. AM-iPSC#2 was implanted into the subcutaneous tissue of the immunodeficient mouse (NOD/ShiJic-scid Jcl). Differentiation into three germ layers was observed. All other lines differentiated into three germ layer systems. B. Cartilage in the teratoma. C. Intestinal epithelium (endoderm) in the teratoma. D. Karyogram of AM-iPSC#7 showing 46, XX. E. Hierarchical clustering analysis based on gene expression. Parental AM936 cells (Amniotic cell), MRC5 cell (Fetal cell), MRC5 iPSCs (Fetal cell-iPSC), AM936 iPSCs (Amnion-iPSCs). Amnion-iPSCs are located close to the ESCs. F. Venn diagrams showing number of the probes whose expression increased more than five-fold between the iPSCs and their parental cells. The number of probes that increased in AM-iPSCs, compared with amniotic cells (AM), is 1294 (shown in blue). We conducted gene ontology (GO) analysis on probes whose expression changed between cell types, and extracted GO terms. For the analysis, GeneSpring GX (ver. 11.5) was used. MRC5-iPSC: iPSC lines generated from MRC5, AM-iPSC: iPSC lines generated from amniotic cells. G. Venn diagrams showing number of the probes whose expression decreased more than five-fold between the iPSCs and their parental cells. The number of probes that increased in AM-iPSCs, compared with amniotic cells (AM), is 1449 (shown in blue).

E2F8, and the down-regulated genes are cell membrane-associated, such as *PAPPA*, *PIG2* and *PALMD*. Reprogramming to AM-iPSCs decreases expression of most of HLA genes, such as HLA-A, -B, –C, -E, –F and -G, more than five-fold. It is also noteworthy that iPSCs resemble ESCs in gene expression and epigenetics during cultivation [44,45].

In conclusion, we believe that for many cases of degenerative and genetic diseases, the placenta is a readily available, reliable source of allogeneic cells in addition to other sources including bone marrow, adipose tissue, ligament, and cartilage [46–52]. Because amniotic cells have a broad differentiation potential, few genetic mutations, and unique expression patterns of HLA antigens, they are a promising resource for regenerative medicine. Amniotic cell isolation and subsequent

propagation is suitable for industrial scale-up for a sustainable supply of large quantities of affordable, quality-controlled cells.

Competing financial interests

The authors declare no competing financial interests.

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