Human Mesenchymal Stem Cells Form Purkinje Fibers in Fetal Sheep Heart

Judith A. Airey, PhD; Graça Almeida-Porada, MD, PhD; Evan J. Colletti, BS; Christopher D. Porada, PhD; Jason Chamberlain, BS; Matthew Movsesian, MD; John L. Sutko, PhD; Esmail D. Zanjani, PhD

Background—We have investigated the usefulness of a model of cardiac development in a large mammal, sheep, for studies of engraftment of human stem cells in the heart.

Methods and Results—Adult and fetal human mesenchymal stem cells were injected intraperitoneally into sheep fetuses in utero. Hearts at late fetal development were analyzed for engraftment of human cells. The majority of the engrafted cells of human origin formed segments of Purkinje fibers containing exclusively human cells. There were no differences in engraftment of human mesenchymal stem cells from adult bone marrow, fetal brain, and fetal liver. On average, 43.2% of the total Purkinje fibers in random areas (n = 11) of both ventricles were of human origin. In contrast, ∼0.01% of cardiomyocytes were of human origin.

Conclusions—Human mesenchymal stem cells preferentially engraft at high levels in the ventricular conduction system during fetal development in sheep. These findings raise the possibility that stem cells contribute to normal development of the fetal heart. (Circulation. 2004;109:1401-1407.)

Key Words: stem cells ■ embryology ■ Purkinje fibers

S

stem cell therapy may provide an important modality for treating cardiac pathologies. Many studies have analyzed incorporation of stem cells in damaged or injured myocardial tissue. However, important issues exist relating to engraftment of stem cells under normal conditions, particularly during development of the heart, as would occur during in utero stem cell therapy. Questions concern the phenotype and biochemical and functional properties of cardiac cells formed from stem cells. Such studies have not been possible because of low levels of stem cell engraftment and use of small-animal models. Therefore, we investigated engraftment of human mesenchymal stem cells (hMSCs) during development in the hearts of a large mammal, sheep. The human–sheep chimeric model of stem cell engraftment is a well-established large-mammal model in which stem cells are injected into the fetus. Using this model, previous work in our and other laboratories has determined that engrafted human stem cells can be detected in a number of tissues in both prenatal and postnatal sheep.1–3

Methods

In Utero Transplantation Into Sheep Fetuses

Fetal sheep (bred and housed at the University of Nevada Agricultural Experimental Station) were injected intraperitoneally with hMSCs as described previously.4,5 Fetuses were injected between 55 and 62 days of gestation, before development of an immune response. Although it is thought that MSCs do not generate a significant immune response when injected into immune-competent fetuses, this window was chosen to allow future comparison with other sources of stem cells that are known to generate an immune response. Hearts were analyzed at 115 to 131 days of gestation. All procedures were in accordance with institutional guidelines.

Isolation of hMSCs

Heparinized human bone marrow was obtained from healthy donors after informed consent. Low-density bone marrow mononuclear cells were separated by a Ficoll-Hypaque density gradient (1.077 g/mL) (Sigma), washed twice in Iscove’s modified Dulbecco’s media (Gibco), and then enriched for Stro-1 cells by magnetic cell sorting (Miltenyi Biotec). Stro-1 cells were plated at low density in MSC medium (Poesites). Human fetal brain and liver (18 to 22 weeks gestational age) were purchased from Advanced Bioscience Resources. Fetal tissues were homogenized to yield single-cell suspensions, and Stro-1 cells were isolated and cultured as described for bone marrow mononuclear cells.

Retroviral Vectors

DsRed Vector

The coding region for DsRed was mutagenized and excised from pDsRed (Clontech) by polymerase chain reaction to obtain the coding region with 5′ flanking sequence containing the EcoR1 site and a novel Xho1 in the 3′ flanking sequence. The purified
polymerase chain reaction product was ligated into pMSCV-Neo (Clontech) that had been digested with EcoR1 and Xho1. The plasmid was transformed into DH5-α cells and selected with ampicillin.

**GFP Vectors**
The MLV-Neo-CMV-GFP and pHR-CMV-GFP, both pseudotyped with VSV G protein, were a kind gift from Dr Donald Kohn.

**Transduction of hMSCs**
Subconfluent cultures of hMSCs were transduced for 48 hours with either a DsRed or GFP vector diluted in serum-free QBSF/60 medium (Quality Biological) containing 8 μg/mL protamine sulfate (Lyphomed), with changes of fresh medium/supernatant every 12 hours. Transduced cells were selected with G418, when vectors encoding the neomycin resistance gene were used.

**Immunocytochemistry and Histochemistry**
Thin strips of tissue dissected from both atria, both ventricles, and the intraventricular septum were pinned slightly stretched and submerged in ice-cold PBS containing 4% paraformaldehyde for 20 minutes. The strips were cut into 5-mm cubes and then incubated in fresh fixative for 1 hour. After cryoprotection in increasing sucrose concentrations to 20%, tissue was incubated in 2 parts 20% sucrose, 1 part OCT compound for 1 hour, then embedded in fresh solution by rapid freezing in isopentane cooled in liquid nitrogen. Cryosections 7 to 10 μm thick were adhered to VectaBond coated slides (Vector) and blocked in 10% normal serum from the species source of the secondary antibody or antibodies. Primary antibodies were against the following proteins: HSP27 (27-kDa heat-shock protein) (Stressgen), GFP and DsRed (Clontech), neuron-specific phosphotransferase II (NPT II) (Cortex Biochemical), HNK-1 (lei-7) (ATCC), protein gene product 9.5 (PGP 9.5, ubiquitin C-terminal hydroxylase) (Ultracalce and Biogenes), connexin43 (Chemicon), dystrophin (Novocastra), ryanodine receptor, sarcomeric myosin (heavy chain), and slow isoform of the Ca2+-ATPase (SERCA2) (Developmental Studies Hybridoma Bank). For single labeling, the secondary antibody was conjugated to Alexa 488. For double labeling with antibodies from different species, the secondary antibody was conjugated to Alexa 568, 594, or 647. For double labeling with antibodies to different species, the secondary antibody was conjugated to Alexa 488. Human and sheep hearts, permitting a side-by-side comparison of the levels of expression and distribution of these proteins in the engrafted human hearts and the surrounding sheep hearts. Engrafted human hearts and adjacent sheep myocardial cells have similar levels of expression and the same patterns of distribution as these proteins. Data for SERCA2 and the ryanodine receptor are shown in Figure 2. As can be seen in Figure 2E, the ryanodine receptor has the punctate distribution typical of cardiac muscle, consistent with localization of this protein at junctions.

**Engrafted hMSCs Form Purkinje Fibers**
As can be seen in the figures, the majority of the engrafted cells formed ribbon-like aggregates of large cells with a distinctive morphology. The aggregates of human cells were found in the ventricles but not the atria, with no mixing of sheep and human cells detected within aggregates of engrafted human cells. To determine whether engrafted human cells had a heart-cell phenotype, tissue sections were probed with antibodies against proteins expressed in cardiac cells, including SERCA2, sarcomeric myosin (heavy chain), dystrophin, and the ryanodine receptor. These antibodies have broad species specificity and react with proteins expressed in both human and sheep hearts, permitting a side-by-side comparison of the levels of expression and distribution of these proteins in the engrafted human hearts and the surrounding sheep hearts. Engrafted human cells and adjacent sheep myocardial cells have similar levels of expression and the same patterns of distribution as these proteins. Data for SERCA2 and the ryanodine receptor are shown in Figure 2. As can be seen in Figure 2E, the ryanodine receptor has the punctate distribution typical of cardiac muscle, consistent with localization of this protein at junctions.

**Results**

**hMSCs Engraft in Fetal Sheep Heart**
To determine whether human cells engrafted, a human-specific antibody against HSP27 was used. In preliminary studies (data not shown), this antibody was found to react with normal and failing human heart but not with either fetal or adult sheep heart. By use of this antibody, significant numbers of cells of human origin were identified in the septum and both ventricles of sheep hearts. Very few human cells were detected in the atria. To confirm that cells were human, hMSCs from all 3 sources were transduced with retroviral vectors encoding either of the fluorescent proteins, GFP or DsRed. Double labeling showed that cells expressing GFP, DsRed, or anti-NPT II (for vectors having neomycin resistance) were positive for HSP27 (Figure 1). These data clearly demonstrate that human cells engraft in fetal sheep heart and confirm the human specificity of the anti-HSP27 antibody used in these studies.

**Engrafted hMSCs Form Purkinje Fibers**
As can be seen in the figures, the majority of the engrafted cells formed ribbon-like aggregates of large cells with a distinctive morphology. The aggregates of human cells were found in the ventricles but not the atria, with no mixing of sheep and human cells detected within aggregates of engrafted human cells. To determine whether engrafted human cells had a heart-cell phenotype, tissue sections were probed with antibodies against proteins expressed in cardiac cells, including SERCA2, sarcomeric myosin (heavy chain), dystrophin, and the ryanodine receptor. These antibodies have broad species specificity and react with proteins expressed in both human and sheep hearts, permitting a side-by-side comparison of the levels of expression and distribution of these proteins in the engrafted human hearts and the surrounding sheep hearts. Engrafted human cells and adjacent sheep myocardial cells have similar levels of expression and the same patterns of distribution as these proteins. Data for SERCA2 and the ryanodine receptor are shown in Figure 2. As can be seen in Figure 2E, the ryanodine receptor has the punctate distribution typical of cardiac muscle, consistent with localization of this protein at junctions.

Purkinje fibers are located in the septum and ventricles of the heart, forming the electrical conduction system essential for coordinated contraction of the ventricles. The morphology of the ribbon-like aggregates of the engrafted human cells is similar to that of Purkinje fibers. Purkinje fiber cells are larger and less cylindrical than cardiomyocytes and are particularly obvious in ungulates, such as sheep. Consistent with being specialized to rapidly conduct action potentials, Purkinje fiber cells express some proteins typically found in neurons, including HNK-1 and PGP 9.5. Double-labeling studies with either anti-GFP, anti-DsRed, or anti-HSP27 and either anti-HNK-1 or anti-PGP 9.5 show that in addition to muscle-specific proteins, engrafted human cells express both HNK-1 and PGP 9.5 (Figure 3). As seen in Figure 3D, nerves near the Purkinje fiber are labeled with anti-HNK-1, showing that, as expected, HNK-1 is expressed in neuronal tissue but not ventricular cardiomyocytes. In both control and chimeric fetal hearts, there were ribbon-like aggregates of cells that did not stain with the HSP27 antibody, did not express DsRed or GFP, but were positive for HNK-1 and PGP 9.5. This indicates that these cell aggregates are normal structures in the developing fetal sheep heart and not an artifact of the introduction of human stem cells.

Gap junctions are important for the electrical conduction between cardiac cells and are necessary for normal heart function. Therefore, we determined the level of expression and distribution of connexin43 in normal and chimeric hearts. Connexin43 has been determined to be expressed in neonatal human heart, but there is little information about connexin43 distribution in the Purkinje system during late fetal...
mammalian development. As shown in Figure 4, connexin43 has a punctate distribution in both human and sheep Purkinje fiber cells, being localized at the cell surface but not at exterior surfaces of the fibers where the cells contact the connective tissue sheath. The adjacent working myocardium has a different connexin43 distribution. The distinctive pattern of connexin43 expression is further evidence that the engrafted hMSCs form Purkinje fiber cells, indicating that the

Figure 1. Human hMSCs engraft in fetal sheep hearts. A, GFP fluorescence in a section of right ventricle from a fetal sheep injected with hMSCs from adult bone marrow expressing GFP, showing that hMSCs engraft in sheep hearts. B to G, Pairs of images of double-stained sections demonstrating that anti-HSP27 antibody staining occurs in cells that express fluorescent proteins and NPT II. B is anti-GFP, D is anti-DsRed, F is anti-NPT II and C, E, and G are anti-HSP27. B and C are left ventricle, and C to G are right ventricle. B through E are fetal brain hMSCs, and F and G are fetal liver hMSCs. Bars=30 μm.

Figure 2. Engrafted human cells have a cardiac muscle phenotype. Serial sections of right ventricle demonstrating that engrafted human cells express cardiac cell proteins with localization patterns typical of differentiated cardiac cells. A and C are anti-HSP27, B is anti-SERCA2, C is anti-NPT II, and D is anti-ryanodine receptor. E is a higher magnification of part of D. Bars=50 μm in A and B and 30 μm in C and D.
human cells are at the same developmental stage as the sheep Purkinje fiber cells and that the human cells may be electrically coupled.

Purkinje fiber cells contain high levels of glycogen. To confirm further that engrafted human cells form Purkinje fiber cells, sections were stained with PAS and hematoxylin. As can be seen in Figure 5, A and D, both sheep and human Purkinje fibers are strongly stained by PAS, indicating high levels of glycogen. Comparison of the histochemical staining with paired serial sections double labeled with anti-HSP27 and PGP 9.5 shows that hMSCs form Purkinje fibers. In Figure 5, D through F, the blood vessel close to the Purkinje

Figure 3. Vast majority of engrafted human stem cells are Purkinje fiber cells. A to D, Colocalization of staining for human HSP27 and PGP 9.5 and HNK-1, 2 neuronal proteins expressed in Purkinje fiber cells. A and C are anti-HSP27, B is anti-PGP 9.5, and D is anti-HNK-1. In D, nerves near Purkinje fiber are recognized by HNK-1 antibody. Bars=50 μm.

Figure 4. Human and sheep Purkinje fiber cells have same distribution of connexin43. A and B, control sheep; C through F, chimeric sheep; A, C, and E, anti-HSP 27; and B, D, and F, anti-connexin 43. Bars=30 μm.
fiber is negative for both HSP27 and PGP 9.5. Nerves that are adjacent to the vessel are positive for PGP 9.5. These data confirm further that engrafted human cells are Purkinje fiber cells and not vascular structures.

**Level of Engraftment of hMSCs**

Human cells were present in hearts from 20 of 21 fetuses injected with hMSCs. In the 1 negative fetal sheep heart, human cells were not detected in other tissues tested, so it is presumed that there was no engraftment in this fetus. Tissue sections obtained at random from different areas of both ventricles from different fetuses injected with hMSCs from the different sources of hMSCs, that contained at least 10 areas of Purkinje fibers, were analyzed for levels of engraftment. Figure 6 shows montages of 2 sections. The human areas represented an average of 43.2% of the total Purkinje fiber area (n=11; SD, ±16.5%; range, 21.1% to 74.1%). The presence of human cells in sections from different regions of the ventricles suggests that human cells engraft throughout the Purkinje system. The observation that hMSCs from both adult and fetal sources, as well as from different tissues, preferentially form Purkinje fibers at similar levels indicates that there is no difference in engraftment potential of hMSCs from different sources in the developing fetal heart. Cardiomyocytes of human origin were detected in both atria and ventricles at low levels (≈0.01%).

**Discussion**

This study is the first to demonstrate engraftment of stem cells into Purkinje fibers. One previous study analyzed engraftment of hMSCs from adult bone marrow in heart using the human–sheep chimeric model. In that study, human cells were not detected in hearts from the majority of sheep analyzed at different times postnatally. The few cells detected in positive sheep were located in working myocardium and not in Purkinje fibers. A possible reason for this difference is that the antibody and primers used were against human β2 microglobulin. Major histocompatibility complex antigens are either not expressed or expressed at levels below detection in normal, nondiseased cardiac muscle cells. Therefore, this human cell marker may be difficult to detect in heart in the absence of inflammation. HSP27 was used in the present study as a human cell marker, because it is expressed at significant levels in normal nonstressed heart.

Engrafted hMSCs preferentially formed part of the Purkinje fiber conduction system. An explanation for this phenomenon is that the Purkinje fiber system may be in a phase of expansion when human stem cells were introduced into the fetus, and therefore an environment or niche promoting differentiation into a Purkinje fiber phenotype may exist. This is consistent with findings of plasticity in stem cells, in which in some circumstances the local milieu may be a more important determinant of the differentiated phenotype of engrafting stem cells than the source of cells. An alternative explanation for the predominance of human Purkinje fiber cells is that hMSCs used in this study preferentially differentiate into certain cell types. If this is the case, different lineages of cells may engraft and form different ratios of cell types within the heart.

Currently, uncertainty exists about whether engrafted stem cells transdifferentiate or fuse with cells of the recipient animal. In a mouse model of liver injury, regeneration was shown to occur by fusion of donor hematopoietic stem cells with recipient liver cells; however, in another murine...
model, cell fusion was not detected in the pancreas. Because the human–sheep chimeric model is a developmental model and fusion probably does not play a significant role in normal fetal cardiac development, hMSCs would have to be more fusogenic than native sheep MSCs for fusion to be the mechanism of human cell engraftment. The clustering of human cells is consistent with transdifferentiation, and aggregates of human Purkinje fiber cells could be a result of 2 possibilities. First, stem cells may home to the developing conduction system; however, some mixing of sheep and human cells might be expected in this scenario. Second, stem cells, or cells derived from the stem cells, may divide within the heart. Whether there is cell fusion in the human–sheep chimeric model and whether human cells divide within the heart is currently being investigated.

An important question that now needs to be addressed concerns whether human Purkinje fiber cells are functional in sheep heart. Because a high proportion of Purkinje fibers in chimeric sheep are human, and cardiac proteins, such as the ryanodine receptor, are expressed and correctly localized, it is likely that the engrafted human cells are functional. The similar expression pattern of connexin43 in sheep and human Purkinje fibers suggests that human Purkinje fiber cells are coupled electrically. The dispersed distribution of connexin43 is consistent with findings that the final mature distribution of gap junctions in cardiomyocytes does not occur until 6 to 7 years of age and that the conduction velocity in Purkinje fibers increases during maturation of neonatal canine heart. Taken together, the high percentage of human Purkinje fibers in chimeric fetal hearts and the observation that chimeric and control sheep exhibit similar fetal growth patterns, birth rates, neonatal and adult viabilities, and ability to reproduce suggest that human cells are integrated in both a structurally and functionally competent manner in sheep hearts. Studies are currently under way to confirm that chimeric sheep have normal cardiac function, whether the human cells are electrically coupled, and whether they have an electrophysiological profile typical of Purkinje fiber cells.

If engrafted human stem cells form functional Purkinje fibers, the human–sheep chimeric model will provide a system for investigation of functional characteristics of Purkinje fibers, as well as a source of human Purkinje fiber cells at different stages of development. In addition, using stem cells from individuals having known mutations in cardiac proteins, such as ion channels, will provide a paradigm in which the consequences of these mutations in Purkinje fiber cells can be analyzed.

The human–sheep chimeric model may also provide unique opportunities for investigating development of the fetal heart. Cell lineage marking in avian and murine models has indicated that in embryonic heart, Purkinje fiber cells and cardiomyocytes share a common multipotent working cardiomyocyte precursor, with Purkinje fiber cells being more closely related to nearby cardiomyocytes than more distant Purkinje fiber cells. Our results could suggest that in sheep (and possibly other large mammals), there may be a different developmental pattern from that in chicks and mice, because it would be expected that if there are cardiomyocyte Purkinje fiber precursors, human cardiomyocytes would have been detected in the vicinity of human Purkinje fibers. An alternative explanation is that cardiomyocytes are Purkinje fiber precursors in embryonic heart, but later, during fetal development, stem cells are recruited into the Purkinje fiber system. The finding that an average of 43.2% of Purkinje fiber cells are derived from human stem cells introduced via intraperitoneal injection suggests that circulating stem cells could serve as a source of Purkinje fiber cells during a second phase of expansion, thereby contributing to normal growth and development of the fetal heart. If correct, these observations will have implications for treatment of heart conditions in utero and possibly in some premature births.

In conclusion, the present report contains several unique findings. hMSCs engraft in fetal sheep heart, with the majority of the engrafted cells forming a single differentiated cardiac cell type, Purkinje fiber cells. Within the Purkinje system, there is a high level of engraftment (~40%) of human cells, and unlike most other reports of stem cell engraftment in which engrafted cells occur singly, cells of human origin were present in aggregates without intermixing with sheep cells. Different sources of hMSCs, adult bone marrow, fetal brain, and fetal liver, gave similar levels of engraftment. The high levels of engraftment of circulating stem cells in fetal hearts raises the possibility that stem cells may contribute to the growth and development of the heart during normal mammalian development.

Acknowledgments
This work was supported by National Institutes of Health grants HL-53677, AR-45112 (Drs Airley and Sutko), HL-70566 (Dr Almeida-Porada), HD-40228 (Dr Porada), HL-49042, HL-52955, and DK-51427 (Dr Zanjani); NASA grant NRA00-HEDS (Dr Almeida-Porada); Medical Research Funds from the Department of Veterans Affairs (Dr Movsesian); and National Science Foundation grant EPS0132556 (Dr Sutko).

References
Human Mesenchymal Stem Cells Form Purkinje Fibers in Fetal Sheep Heart
Judith A. Airey, Graça Almeida-Porada, Evan J. Colletti, Christopher D. Porada, Jason Chamberlain, Matthew Movsesian, John L. Sutko and Esmail D. Zanjani

Circulation. 2004;109:1401-1407; originally published online March 15, 2004;
doi: 10.1161/01.CIR.0000124222.16321.26
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2004 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/109/11/1401

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation is online at:
http://circ.ahajournals.org//subscriptions/