Engineering Patient-Specific Valves Using Stem Cells Generated From Skin Biopsy Specimens

David L. Simpson, PhD, Brody Wehman, MD, Yekaterina Galat, BS, Sudhish Sharma, PhD, Rachana Mishra, PhD, Vasiliy Galat, PhD, and Sunjay Kaushal, MD, PhD

Division of Cardiac Surgery, University of Maryland Medical School, Baltimore, Maryland; and Developmental Biology Program, iPS and Human Stem Cell Core Facility, Children's Memorial Research Center, Northwestern University, Feinberg School of Medicine, Chicago, Illinois

Background. **Pediatric patients requiring valve replacement will likely require reoperations due to a progressive deterioration of valve durability and limited repair and growth potential. To address these concerns, we sought to generate a biologically active pulmonary valve using patient-specific valvular cells and decellularized human pulmonary valves.**

Methods. **We generated induced pluripotent stem cells (iPSCs) by reprogramming skin fibroblast cells. We then differentiated iPSCs to mesenchymal stem cells (iPCSs-MSCs) using culture conditions that favored an epithelial-to-mesenchymal transition. Next, decellularized human pulmonary heart valves were seeded with iPCS-MSCs using a combination of static and dynamic culture conditions and cultured up to 30 days.**

Results. **The iPSCs-MSCs displayed cluster of differentiation CD105 and CD90 expression exceeding 90% after four passages and could differentiate into osteocytes, chondrocytes, and adipocytes (n = 4). Consistent with an MSC phenotype, iPSCs-MSCs lacked expression of CD45 and CD34. Compared with bone marrow MSCs, iPSCs-MSC proliferated more readily by twofold but maintained a gene expression profile exceeding 80% identical to bone marrow MSCs. In repopulated pulmonary valves compared with decellularized pulmonary valves, immunohistochemistry demonstrated increased cellularity, α-smooth muscle actin expression, and increased presence of extracellular matrix components, such as proteoglycans and glycosaminoglycans, suggesting sustained cell function and maturation.**

Conclusions. **Our results demonstrate the feasibility of constructing a biologically active human pulmonary valve using a sustainable and proliferative cell source. The bioactive pulmonary valve is expected to have advantages over existing valvular replacements, which will require further validation.**

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Previous attempts to generate a heart valve using synthetic, biodegradable scaffolds have been limited by the lack of maturation of the extracellular matrix structure affecting in vivo performance [1–4]. Use of synthetic biomaterials also introduces the potential risk for inflammatory responses [5]. A promising alternative is the use of decellularized allogeneic tissues. These tissues are decellularized by enzymatic (protease or nuclease, or both) processing or the use of surfactants with the end point of removing all of the cells and nuclear material to avoid adverse immune responses [5–7]. In addition, this process preserves the extracellular matrix to allow for sufficient biomechanical strength and maintenance of the native matrix composition [7–10].

Previous reports have shown decreased immunogenicity and increased durability with the use of decellularized heart valves [7, 11]. There was also neovasculature formation within the tissue and lining of the pulmonary valve (PV) leaflet (PVL) with endothelial cells (ECs) after transplantation [12]. Unfortunately, the lack of living cells minimized tissue growth because there was incomplete engraftment of endogenous valvular cells after transplantation [13, 14]. Repopulation of decellularized allografts with multipotent stem cells may help overcome these limitations.

Several groups have used bone marrow mesenchymal stem cells (BM-MSCs) to tissue-engineer pulmonary valves due to their plasticity and ability to form nonmuscle myosin and vimentin-positive cells [15]. However, MSCs have limited proliferation capacity, which is even further compromised in older or diseased patients. In addition, other types of cells, such as smooth muscle cells and fibroblasts, exist within the structures located between the endothelial linings of the PVL, which are defined as the fibrosa, spongiosa, and ventricularis [16]. Consequently, an alternative cell source that maintains a higher level of stemness and produces a higher variety of

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Address correspondence to Dr Kaushal, University of Maryland Medical Center, Division of Cardiac Surgery, 110 S Paca St, 7th Flr, Baltimore, MD 21201; e-mail: skaushal@smail.umaryland.edu.

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Abbreviations and Acronyms

Ac-LDL	=	acetylated-low density lipoprotein
α-SMA	=	α-smooth muscle actin
BM-MSCs	=	bone marrow mesenchymal stem cells
CD	=	cluster of differentiation
DAPI	=	4′,6-diamidino-2-phenylindole
DePV	=	decellularized pulmonary valve
DMEM	=	Dulbecco Modified Eagle's Medium
EC	=	endothelial cell
GAG	=	glycosaminoglycans
hESCs	=	human embryonic stem cells
iPSCs	=	induced pluripotent stem cells
iPSCs-ECs	=	induced pluripotent stem cells-endothelial cells
iPSCs-MSCs	=	induced pluripotent stem cells-mesenchymal stem cells
LDL	=	low-density lipoprotein
MSC	=	mesenchymal stem cell
PV	=	pulmonary valve
PVL	=	pulmonary valve leaflet
SRS	=	static-rotary-static
vWF	=	von Willebrand factor

cell types is preferred for the engineering of valvular tissue.

The recent development of techniques to reprogram fibroblast cells into pluripotent stem cells, termed induced (i)PSCs, has important implications in disease biology and regenerative medicine [17]. Advantages to using these cells are their histocompatibility with the patient and bypassing the need for human embryonic tissues, thus avoiding the ethical dilemma regarding the use of embryonic tissue. Recently, iPSCs have been shown to differentiate into many cellular lineages, including MSCs [18] and ECs [19].

In the current study, we combined iPSC technology with tissue engineering to create a patient-specific heart valve. Using cells derived from human skin biopsy specimens, we reprogrammed skin fibroblasts to a state of induced pluripotency and further differentiated these iPSCs into MSCs. We sought to demonstrate that as the primary cell source for seeding a decellularized valve, MSCs derived from iPSCs (iPSCs-MSCs) could differentiate into a variety of relevant cell types and proliferate faster than conventional BM-MSCs, thus introducing a novel approach to heart valve tissue engineering.

Material and Methods

This study was approved by the Children's Memorial Hospital Institutional Review Board.

Cell Culture and Cell Differentiation

Human skin cells were obtained from American Type Culture Collection (ATCC, Manassas, VA) and Coriell Cell Repositories (Camden, NJ). We developed several original iPSC lines from human skin cells with the help of genetic manipulations. (Specifically: the SR2 iPSC line was derived from MRC-5 fibroblasts [ATCC] and DSV1, DSV2 iPSC clones from AG06872 fibroblasts [Coriell] by overexpressing Oct4, Sox2, Nanog, and cMyc using retroviral vectors [pMXs-cMyc, pMXs-Klf4, pMXs-hOct3-4 or pMXs-Sox2]; Addgene, Cambridge, MA). The retroviral vectors were produced by transient transfection of 293T cells. The fibroblasts were incubated in the viral supernatants containing 5 μg/mL polybrene (Sigma-Aldrich, St. Louis, MO) for 4 hours to increase transduction efficiency. The transduced cells were then incubated at 37°C in 5% CO_2 in a humidified atmosphere for 2 to 3 weeks until development of the pluripotent clones. After isolation, the clones were grown in StemPro medium (Invitrogen, Carlsbad, CA) on a Matrigel substrate (BD Bioscience, San Jose, Calif). The cultures were split mechanically using the StemPro EZ Passage tool (Invitrogen).

To differentiate iPSCs to MSCs, embryoid body media containing knockout Dulbecco Modified Eagle Medium (DMEM; Invitrogen), knockout serum replacement, L-glutamine, and antibiotics was added to establish a differentiating culture (Fig 1A). Afterward, EGM 2-MV growth media (Lonza, Basel, Switzerland) was added for 25 days. Cells were passaged and replated to confluence. The iPSCs-MSCs were expanded to passage 1 to passage 7 and were maintained in EGM-2MV endothelial growth media at 37°C in 5% CO_2.

To differentiate iPSCs into ECs (iPSCs-ECs), iPSC lines were cultured on OP9 murine stromal cells. Endothelial cluster of differentiation $CD34^+CD31^+CD43^-$ cells were isolated in two steps. First, $CD34^+$ cells were positively selected on a magnetic column (Milteneyi Biotec Inc, San Diego, CA), and then separated from hematopoietic $CD34^+CD43^+$ progenitors by adherent culture [20].

Characterization of iPS-MSCs and iPS-ECs

FLOW CYTOMETRY. The iPSCs-MSC phenotype was measured by monitoring the expression of CD105, CD90, CD45, and CD31 over several passages. The iPSCs-MSCs were lifted with 0.25% trypsin-ethylenediaminetetraacetic acid and fixed using 4% paraformaldehyde for 15 minutes on ice. Next, cells were stained with the appropriate primary antibodies for 30 minutes in room air. When necessary, fluorescent-conjugated secondary antibodies were added afterwards for 30 to 60 minutes at 4°C (Santa Cruz Biotechnology; Santa Cruz, CA). Cells were washed in a 0.3% bovine serum albumin/phosphate-buffered saline solution and analyzed by flow cytometry to define populations.

PROLIFERATION. Proliferation capacity was measured by monitoring the incorporation of 5-ethynyl-2′-deoxyuridine (Invitrogen), a nucleoside analog to thymidine that is incorporated into DNA during synthesis. The iPSCs-MSCs were pulsed with 10 mmol/L 5-ethynyl-2′-deoxyuridine for 48 hours directly after passaging. Afterwards cells were fixed using the Click-iT fixative (Invitrogen) for 15 minutes at room temperature. Next, cells were permeabilized with Triton X-100 and stained using the Click-iT cocktail mixture for 30 minutes at room temperature. Afterwards, cells were washed with 1% bovine serum albumin/phosphate-buffered saline and used for flow cytometry. Positive controls using BM-MSCs (Lonza) were run in parallel.