

# Recovery of Multipotent Progenitors from the Peripheral Blood of Patients Requiring Extracorporeal Membrane Oxygenation Support

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**Rationale:** Studies have demonstrated that bone marrow-derived cells can be recruited to injured lungs through an unknown mechanism. We hypothesize that marrow progenitors are mobilized into the circulation of patients with cardiac and/or respiratory failure, and may then traffic to and incorporate into the sites of tissue injury. **Objectives:** To determine whether progenitor populations are increased in the blood of patients with severe acute cardiorespiratory failure placed on extracorporeal membrane oxygenation (ECMO).

**Methods:** Mononuclear cells from ECMO, umbilical cord, and control blood samples were evaluated in colony-forming assays for hematopoietic, mesenchymal, and epithelial cells. Progenitors were identified by proliferative and differentiative capacities, and confirmed by the expression of lineage-specific markers.

**Measurements and Main Results:** Significantly higher levels of hematopoietic progenitors were observed in ECMO (n = 41) samples than neonatal intensive care unit (n = 16) or pediatric intensive care unit controls (n = 14). Hematopoietic progenitor mobilization increased with time on ECMO support. Mesenchymal progenitors (MSC) were recovered from 18/58 ECMO samples with rapid sample processing (< 4 h) critical to their recovery. MSC were not recovered from normal controls. ECMO-derived MSC had osteogenic, chondrogenic, and adipogenic differentiation potential. The recovery of MSC did not influence survival outcome (61%). Epithelial progenitors were observed in eight ECMO samples but not in control samples. Their presence was associated with a lower survival trend (38%).

**Conclusions:** Hematopoietic, mesenchymal, and epithelial progenitors were mobilized into the circulation of patients on ECMO. This may reflect a response to severe cardiopulmonary injury, blood-foreign surface interactions with the ECMO circuit, and/or hemodilution.

**Keywords:** extracorporeal membrane oxygenation; hematopoietic progenitors; mesenchymal progenitors; epithelial progenitors; circulating progenitors

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## AT A GLANCE COMMENTARY

### Scientific Knowledge on the Subject

Studies have demonstrated graft-host chimerism in injured heart or lungs after hematopoietic stem cell or solid organ transplantation. The mechanism for the tissue chimerism is unknown.

### What This Study Adds to the Field

Hematopoietic, mesenchymal, and epithelial progenitor populations can be identified in the blood of patients with acute severe cardiorespiratory failure who require extracorporeal membrane oxygenation (ECMO) support. Circulating hematopoietic progenitor frequency in patients undergoing ECMO was higher than in ICU control subjects. Circulating hematopoietic progenitor frequency in patients undergoing ECMO suggests that progenitor levels are increased in the setting of severe disease.

Experimental studies have shown that bone marrow progenitor cells can incorporate into the lung and heart. These studies have demonstrated the engraftment of several different donor cell populations into lung and heart injury models and/or the contribution of these cell populations to injury repair with reduced inflammation (1–11). Furthermore, this process occurs rarely in steady state (recently reviewed in Reference 12). Donor cell incorporation into recipient lungs (13–15) and heart (16) has also been observed after opposite-sex clinical hematopoietic stem cell transplantation. Microchimerism has been observed in human solid organ recipients with host-derived cells incorporated into lung and heart allografts, with higher level of chimerism in areas of inflammation than in uninjured tissue in these allografts (16–19). Although these studies demonstrated that circulating cells can incorporate into injured nonhematopoietic tissues the mechanism and clinical significance were unclear.

One possible mechanism of circulating progenitor cell engraftment into injured solid tissues is the recruitment of cells from the bone marrow or other tissues into the circulation in response to mediators released into the circulation by the injured tissues. In support of this theory, hematopoietic and mesenchymal progenitors migrate *in vitro* toward mediators such as SDF-1 $\alpha$  (20), IL-8 (21, 22), IL-6, and bFGF (23), which are released into the circulation (24) and found in the bronchoalveolar fluid in patients with lung injury (25–27). In theory, these circulating cells or progenitors could traffic to injured or inflamed tissue and at some low frequency incorporate into the injured tissues. This model suggests that in the absence of tissue

injury, bone marrow-derived progenitor populations would be found at low frequencies in the circulation, and would not traffic to the healthy, steady-state tissue, which is consistent with the experimental and clinical studies. If this model of recruitment of progenitors to injured tissue is correct, we would predict that progenitors would be observed at increased levels in the circulation of patients with heart or lung injury, and at lower or undetectable levels in patients without such injury. The goal of this study was to determine whether hematopoietic, mesenchymal, or epithelial progenitors are mobilized into the circulation of patients with severe cardiac and/or respiratory failure.

This study evaluated the blood of patients who required extracorporeal membrane oxygenation (ECMO) for life support (28–30), because they represent a clinical model for the most severe acute lung and/or heart injury, yet the condition is potentially reversible and blood samples are available for study. Our results demonstrate that multiple progenitor cell populations are present in the circulation of patients on ECMO at higher levels than controls. This study was not designed to determine the source of these circulating progenitors or whether they contribute to tissue repair. However, it does suggest that progenitor cell mobilization occurs in response to respiratory and cardiac injury, which may be the first step in the process by which nonresident progenitor cells are recruited to injured tissues. Some of the results of these studies have been previously reported in the form of an abstract (31).

## METHODS

### Blood Sample Collection and Processing

The study protocol was reviewed and approved by the Committee on Clinical Investigations at Children's Hospital Los Angeles and the Institutional Review Board at University of California, Los Angeles with waiver of informed consent and Health Insurance Portability and Accountability Act research authorization for the use of existing deidentified specimens. Waste blood from the clinical lab was obtained from intensive care unit (ICU) patients with only age recorded. The blood from discarded ECMO circuits was collected in blood bags with anticoagulant CPD-A1. The clinical data collected were: total hours on ECMO, number of hours each circuit was used, whether it was the first or subsequent circuit, diagnosis, patient age, and survival outcome. Umbilical cord blood was collected in anticoagulant citrate dextran (Sigma-Aldrich, St. Louis, MO). Fresh (< 4 h) whole blood units were obtained from the blood bank at Children's Hospital Los Angeles with informed consents from normal adult volunteers. Mononuclear cells were recovered after density gradient centrifugation on Ficoll-Paque Plus (Amersham Biosciences, Uppsala, Sweden) for culture studies.

### Hematopoietic Colony Assay

Clonogenic hematopoietic progenitor colony forming unit (CFU) assays were performed using manufacturer's recommendations (Methocult GF H4434; StemCell Technologies [SCT], Vancouver, Canada). Each sample was plated in triplicate at multiple cell concentrations ranging from  $5 \times 10^3$  to  $5 \times 10^5$  cells per ml. Plates were examined at 12 to 14 days and dilutions with approximately 30 to 50 colonies per plate counted. Each colony was recorded as erythroid (BFU-E), myeloid (CFU-GM), or mixed (CFU-GEMM). The sum of triplicate CFUs was averaged at each concentration and the data were normalized and reported as the CFU per  $10^5$  mononuclear cells (MNC).

### Mesenchymal and Epithelial Cell Culture

Mesenchymal progenitors or multipotent mesenchymal stromal cells, hereafter referred to as MSC following the guidelines of the International Society for Cellular Therapy (ISCT) (32, 33), were cultured following published methods (34). The first six samples were plated in Mesencult (SCT) and subsequent samples in MSCGM (Cambrex, Walkersville, MD). Nonadherent cells were washed out the following

day, and the medium was changed at 7 days and then twice weekly thereafter. MSC colonies were visible after 10 to 14 days and counted. MSC were expanded *in vitro* and the population doubling was calculated at each passage and summed to yield the cumulative population doubling (34). Human bone marrow MSC were obtained from the Tulane Center for Gene Therapy and served as positive controls.

Epithelial cultures were initiated as described above for MSC and maintained with weekly feeding with half conditioned MSC medium and half fresh MSCGM. Cells detached with Accutase (Millipore, Billerica, MA) were transferred to collagen- or fibronectin-coated plastic chamber slides for immunofluorescence studies.

### Flow Cytometry

To evaluate CD34<sup>+</sup> cell frequency in whole blood leukocytes, 100  $\mu$ l of whole blood from ECMO or cord blood were blocked with 1% intravenous immunoglobulin (IVIG), before addition of the phycoerythrin-conjugated-anti-CD34 antibody or isotype control. After 30 minutes of incubation, the red cells were lysed with Facslyse; cells were then pelleted, washed with phosphate-buffered saline (PBS), resuspended in PBS, and lysed a second time. The final cell pellet was resuspended in PBS and analyzed in a FACS Calibur (Becton-Dickinson, Franklin Lakes, NJ).

MSC cultures were harvested with Accutase, washed, and resuspended in PBS. For direct staining, cell suspensions were blocked with 1% human immunoglobulin followed by addition of primary antibodies fluorescently conjugated. For indirect staining, the same steps were used, followed by addition of conjugated secondary antibodies. The samples were washed in PBS and analyzed in a FACS Calibur. The antibody and technical details are provided in the online supplement.

### Immunofluorescence and Histological Analysis

Cells cultured on chamber slides were fixed with cold methanol, air dried, and processed immediately or stored at  $-80^\circ\text{C}$ . The cells were washed, blocked, and incubated with primary and secondary antibodies each for 1 hour at room temperature, followed by application of mounting media with 4',6-diamidino-2-phenylindole and a coverslip. The slides were examined under a fluorescent microscope and photographed. Details of the antibodies, staining, acquisition methods, and equipment are provided in the online supplement.

For histochemical staining and analysis, all samples were fixed in 10% formalin, paraffin embedded, and sectioned as described in the online supplement.

### Mesenchymal Differentiation Studies

Chondrogenic differentiation was performed in pellet cultures as described (35). Histological sections were stained with Safranin O or Alcian blue. For adipogenic differentiation, MSC were grown to 80% confluence and then the medium was replaced with Adipogenic Induction medium (SCT) or adipogenic conditioning medium for 2 to 4 weeks until lipid droplets were visible as described (35). Cells were fixed and stained with Oil red O stain. For osteogenic differentiation, MSC were grown to confluence and switched to Osteogenic Induction Medium (SCT) following supplier's protocols. At 3 weeks the cells were fixed and stained for calcium deposition using Alizarin red S stain.

### Statistical Analysis

The distribution of continuous variables was first tested for normality. For normally distributed variables, equality of the means was tested by the *t* test. For all other continuous variables, equality of distribution was tested by the Mann-Whitney *U* test. Results were reported as means  $\pm$  SD except for CD34<sup>+</sup> cells and hematopoietic CFU assays, which were reported as means  $\pm$  SEM. Median and range were given when the data did not follow a normal distribution. For categorical variables, the distribution or proportions of patients between groups were compared by the Fisher exact test. Univariate logistic regressions were used to evaluate the associations between duration of ECMO support and frequency of hematopoietic colonies or mesenchymal colonies. Correlation coefficients were determined using simple linear regression. *P* less than 0.05 was considered significant and all hypothesis testing was two-sided. Statistical analysis was performed using STATA for Windows (version 8.0, College Station, TX).

TABLE 1. SUMMARY OF THE AGE AND DIAGNOSES OF THE STUDY POPULATION

| Neonatal (N = 27) (1–26 d)            | Pediatric (N = 16) (3 mo–17 yr)                        | Adults (N = 4) (21–78 yr)                    |
|---------------------------------------|--|--|
| 6 Diaphragmatic hernia (1VSD)         | 3 Heart transplant rejection                           | 2 Heart transplant rejection                 |
| 4 PPHN                                | 2 Cardiomyopathy                                       | 1 Heart transplant + coronary artery disease |
| 3 Meconium aspiration                 | 2 Myocarditis  | 1 Myocardial infarct                         |
| 3 Hypoplastic left heart              | 2 Meningitis   |  |
| 3 Sepsis + PPHN                       | 1 TAPVR repair   |  |
| 1 Alveolar capillary dysplasia + PPHN | 1 Liver transplant + cardiomyopathy                    |  |
| 1 CCAM + PPHN                         | 1 Anomalous coronary artery                            |  |
| 1 Truncus arteriosus                  | 1 Ebstein anomaly status post Fontan                   |  |
| 1 TAPVR                               | 1 Tetralogy of Fallot status post repair               |  |
| 1 PPHN with multisystem organ failure | 1 Pulmonary atresia + ventricular septal defect repair |  |
| 1 Critical AS + PS                    | 1 Single ventricle                                     |  |
| 1 ASD, AS, PS                         |  |  |
| 1 AV canal, interrupted aortic arch   |  |  |

Definition of abbreviations: AS = aortic stenosis; ASD = atrial septal defect; AV = canal, atrioventricular canal; PPHN = persistent pulmonary hypertension of the newborn; CCAM = congenital cystic adenomatoid malformation of the lung; PS = pulmonary stenosis; TAPVR = total anomalous malformation pulmonary venous return; VSD = ventricular septal defect.

## RESULTS

### Patient and Blood Sample Characteristics

Sixty blood samples from 49 patients on ECMO and 51 control samples were obtained between December 2004 and February 2009. Blood from a single ECMO circuit was obtained from 42 patients and multiple circuits (two to five) were obtained from 7 patients. Two ECMO samples were clotted or hemolyzed and were discarded. Thus, this study evaluated 58 samples from 47 patients undergoing ECMO. The mean length of time on ECMO was  $138 \pm 120$  hours (range: 13–720 h). The patients' ages ranged from 1 day to 78 years (median of 15 d). The patient population consisted of 27 neonates (less than 28 d), 16 pediatric patients (7 wk to 17 yr), and 4 adult (21 to 78 yr) patients who required ECMO for respiratory and/or cardiac failure. The patients placed on ECMO for respiratory diagnoses were all neonates. The patients placed on ECMO for cardiac support included neonates, pediatric patients, and adults, although all had respiratory dysfunction as well. All patients placed on ECMO support regardless of indication were on mechanical ventilation. One patient required cardiac support but had sufficient lung function and was placed on a ventricular assist mechanical device. Further details of the patients' primary diagnoses are shown in Table 1. Fifteen cord blood samples and six normal adult volunteer blood samples were obtained and analyzed for hematopoietic, mesenchymal, and/or epithelial progenitors. Hematopoietic progenitor assays were also performed on waste blood samples of patients not on ECMO. Our

analysis included samples from 16 neonatal intensive care unit (NICU) patients and 14 pediatric and cardiothoracic ICU patients of pediatric age (PICU), which had sufficient mononuclear cells to perform the hematopoietic CFU assay.

The mean blood volume of ECMO samples was  $288 \pm 134$  ml (range: 27–744 ml). Sample processing was initiated between 0.25 and 27 hours (mean 5.9 h) from circuit disconnection. Mean cord blood samples were lower in volume,  $59 \pm 27$  ml (range: 15–100 ml,  $P < 0.001$ ) and were processed between 2 and 26 hours (mean 11.8 h) after collection. The mean mononuclear cell recovery per milliliter was lower in ECMO than cord blood ( $1.46 \times 10^6 \pm 1.23$  vs.  $2.77 \times 10^6 \pm 2.04$ ). However, due to the larger volume of blood, the total mononuclear cell yield was higher for ECMO blood samples ( $3.90 \pm 2.81 \times 10^8$  cells) than for cord blood samples ( $1.40 \pm 0.99 \times 10^8$  cells,  $P < 0.001$ ). The mean adult volunteer blood samples volume was larger than ECMO samples, at  $432 \pm 41$  ml ( $P < 0.001$ ) with a similar MNC yield of  $1.65 \pm 0.82 \times 10^6$ /ml and a higher total MNC yield of  $6.99 \pm 3.19 \times 10^8$  (Table 2).

### Hematopoietic Progenitor Frequency Is Increased in ECMO Blood Samples

Hematopoietic progenitor CFU assays were performed on 41 ECMO blood samples. The controls for these studies were: 15 umbilical cord blood samples, 16 NICU blood samples, 14 PICU blood samples, and 6 normal adult volunteers. Three cord blood samples had too many CFU to count accurately; there-

TABLE 2. SUMMARY OF BLOOD SAMPLE CHARACTERISTICS

|               | ECMO (N = 58)   | Normal Adult (N = 6)           | Cord Blood (N = 15)                                       | P Value ECMO vs. Adult | P Value ECMO vs. CB     |
|---------------|---|--------------------------------|---|------------------------|-------------------------|
| Volume, ml    | $288 \pm 134$   | $432 \pm 41^*$                 | $59 \pm 27^*$   | 0.0002                 | $1.509 \times 10^{-16}$ |
| MNC/ml        | $1.46 \pm 1.22 \times 10^6$                             | $1.65 \pm 0.82 \times 10^6$    | $2.77 \pm 2.04 \times 10^6^*$                             | NS                     | 0.03                    |
| CFU/ml        | $23 \pm 6$  | $3 \pm 2^*$                    | $20 \pm 7$  | 0.001                  | NS                      |
| MNC yield     | $3.90 \pm 2.8 \times 10^8$                              | $6.99 \pm 3.19 \times 10^{8*}$ | $1.40 \pm 0.99 \times 10^{8*}$                            | 0.05                   | $7.51 \times 10^{-7}$   |
| MSC + samples | 18/58   | 0/6                            | 3/15  | NS                     | NS                      |
| MSC colony    | 1 per $2 \times 10^8$ MNC<br>(0.24 to $5 \times 10^8$ ) | None                           | 1 per $0.7 \times 10^8$ MNC<br>(0.48 to $1 \times 10^8$ ) |                        | NS                      |

Definition of abbreviations: CB = cord blood; CFU = colony-forming units; ECMO = extracorporeal membrane oxygenation; MNC = mononuclear cells; MSC = mesenchymal stromal cells.

Data presented are mean  $\pm$  SD except for CFU, which is mean  $\pm$  standard error of the mean. The frequency of MSC colony is given as mean and range.

\* Significant difference at  $P \leq 0.05$  using *t* test.

fore, the results of only 12 cord blood CFU are reported. The mean frequency of hematopoietic progenitors (CFU) from the ECMO group as a whole ( $204 \pm 35$  CFU per  $10^5$  MNC, or  $23 \pm 6$  CFU/ml) was not different from cord blood ( $214 \pm 34$  CFU per  $10^5$  MNC or  $20 \pm 7$  CFU/ml blood), but was higher than normal adult volunteers ( $6 \pm 1$  CFU/ $10^5$  MNC or  $3 \pm 2$  CFU/ml blood,  $P = 0.0012$ ). Because we only had CFU data on two adult ECMO samples, we could not assess whether the difference between ECMO and adult controls was significant. The adult ECMO samples were only used for assessment of mesenchymal and epithelial progenitor cells in this study.

We next analyzed the pediatric and neonatal ECMO samples in comparison to age-matched controls from the NICU and PICU (Figure 1A). As described in Figure 1A the median frequency of CFU in the neonatal ECMO group (256 CFU per  $10^5$  MNC) was statistically higher than the neonatal ICU control group (56 CFU per  $10^5$  MNC,  $P = 0.002$ ). We also determined that the median frequency of hematopoietic progenitors in the pediatric ECMO samples (78 CFU per  $10^5$  MNC) was higher than PICU group (7 CFU per  $10^5$  MNC,  $P = 0.001$ ). There were no significant differences between the median frequency of CFU from the neonatal ECMO and the cord blood groups (256 vs. 200 CFU per  $10^5$  MNC).

Comparison of the frequencies of each of the three types of hematopoietic progenitors—myeloid (CFU-GM), erythroid (BFU-E), and multipotent myelo-erythroid (CFU-GEMM)—are shown in Figure 1B for the neonatal and pediatric ECMO and control groups. The frequency of BFU-E and CFU-GM were increased in patients on ECMO compared with age-matched controls. CFU-GEMM were observed in blood from patients on ECMO and NICU control blood but not in pediatric controls. These data demonstrate that blood samples from patients undergoing ECMO have higher frequencies of circulating hematopoietic progenitors than age-matched controls.

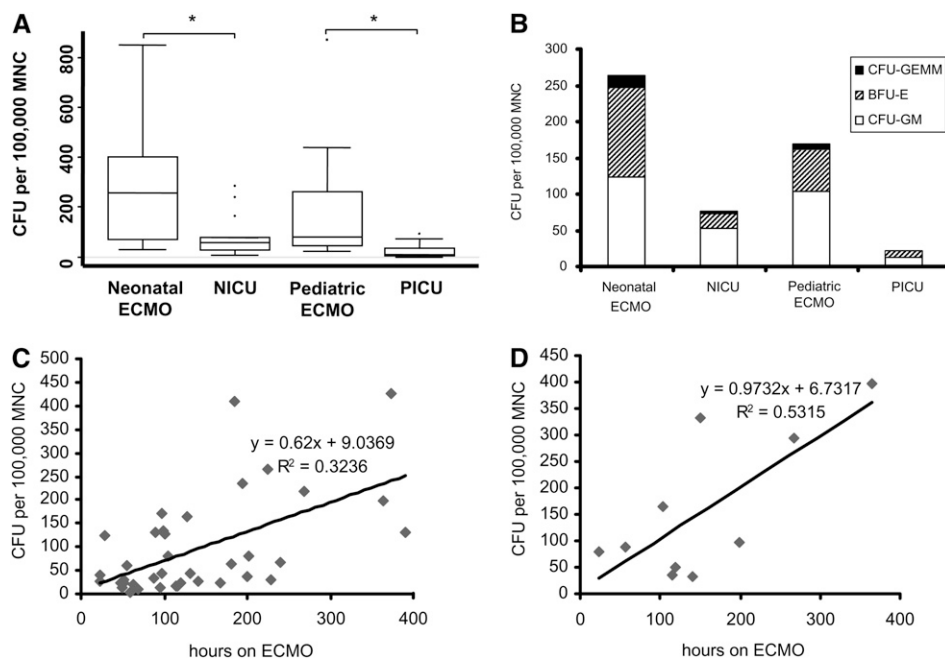
A positive correlation was observed between the frequency of CFU and the duration of ECMO support up to 400 hours for all patients regardless of the number of circuit changes ( $R^2 =$

0.32,  $P < 0.001$ ; Figure 1C). In the subset of patients undergoing ECMO for which we had access to multiple sequential ECMO circuits, the frequency of CFU was significantly increased with longer duration of ECMO support ( $R^2 = 0.53$ ,  $P < 0.05$ ; Figure 1D). There was no correlation between the frequency of CFU hematopoietic progenitors and the time from sample collection to processing (data not shown). There was also no correlation between the hematopoietic CFU frequency in the ECMO population and survival outcome.

We subjected a unit of leukocyte-reduced irradiated red blood cells comparable to those given to patients undergoing ECMO to Ficoll separation and erythrocyte cell lysis and were unable to recover MNC to culture. By the American Association of Blood Banks definition leukocyte-reduced blood products contain less than  $5 \times 10^6$  leukocytes per unit. For patients undergoing ECMO, blood products are also irradiated with 25 to 50 Gy to induce DNA breaks so that residual leukocytes are unable to proliferate. This high dose of irradiation prevents cell proliferation and transfusion-associated graft versus host disease (36).

### Flow Cytometry Analysis for CD34<sup>+</sup> Cells in Whole Blood

The frequency of CD34<sup>+</sup> cells from ECMO and cord blood were similar, with  $0.1486 \pm 0.0288\%$  ( $n = 15$ ) and  $0.1318 \pm 0.0299\%$  ( $n = 11$ ), respectively. The percentage of CD34<sup>+</sup> cells did not correlate directly with CFU. However, these analyses were done on different population of cells (whole blood for CD34 analysis and MNC for CFU) and cannot be directly compared. Interestingly, of the 15 ECMO samples for which we had CD34<sup>+</sup> data, 4 samples gave rise to MSC and epithelial colonies. These four samples were among the five highest in CD34<sup>+</sup> frequency (range 0.22–0.28%). However, because in theory CD34<sup>+</sup> cells could have originated from either the irradiated blood donor or the ECMO recipient patient, we focused our analysis on the functional colony assay, because irradiated cells would not be able to divide and give rise to a colony, as described above.



**Figure 1.** Number of hematopoietic colonies in the blood of patients on extracorporeal membrane oxygenation (ECMO) and control patients. (A) Data presented are median, 25th, and 75th percentile with 95% confidence interval (CI). The dots represent data points above the 95% CI. The data represented are comparisons of neonatal and pediatric patients on ECMO to patients not on ECMO from the neonatal intensive care unit (NICU) or pediatric ICU (PICU) of similar age. The median frequency of colony-forming units (CFU) was higher in the neonatal ECMO compared with NICU group ( $P = 0.002$ ) and pediatric ECMO compared with PICU group ( $P = 0.001$ ). \*Indicates statistical significance between bracketed groups ( $P < 0.05$ ). (B) Mean values of the different subsets of CFU for the neonatal and pediatric groups of patients. (C) A positive linear correlation was observed between the CFU frequency and duration of ECMO support in hours for all ECMO samples ( $r = 0.5688$ ,  $P < 0.001$ ). (D) A positive

linear correlation was observed between the CFU frequency and duration of ECMO support in patients with multiple samples analyzed,  $r = 0.7280$ ,  $P < 0.05$ .

## Mesenchymal Colonies Were Recovered from ECMO

### Blood Samples

MSC were identified in 18 of 58 ECMO blood samples, 3 of 15 cord blood samples, and 0 of 6 normal adult control samples evaluated. ECMO MSC grew in discrete colonies of fusiform spindle-shaped cells (Figure 2A), whereas cord blood MSC had discrete colonies as well as cells scattered over the surface of the culture dish (not shown). Colonies were evident by 2 weeks after plating and were counted. One ECMO sample gave rise to 35 MSC colonies, whereas other samples produced between 1 and 7 MSC colonies. The first cell harvest (i.e., first trypsinization) for each sample yielded a range of  $1 \times 10^5$  to  $6 \times 10^6$  total cells with between 30,000 and 480,000 cells per colony. The mean frequency of MSC was one colony per  $2 \times 10^8$  mononuclear cells plated (range of 0.24 to  $5.35 \times 10^8$  MNC) or one colony of MSC per 96 ml blood (range 12 to 337 ml) in ECMO samples. This frequency was similar to that from cord blood with an average of one MSC colony per  $0.7 \times 10^8$  MNC plated (range 0.48 to  $1 \times 10^8$  MNC) or one colony of MSC per 43 ml blood (range 14–80 ml). A summary of blood sample characteristics and MSC recovery is presented in Table 2. MSC from ECMO and cord blood samples were expanded *in vitro* for a minimum of 5 and maximum of 17 passages, with the exception of two samples which did not regrow after the first trypsinization and replating. An additional MSC sample was discarded at passage two due to contamination.

The total population doubling for MSC cultures was  $10 \pm 5.8$  over the culture period after the first trypsinization. The doubling time was approximately 2 days for the first two to three passages. However, as seen in other studies (34), the rate of cell division decreased at later passages as the cells became larger and took on a more polygonal shape. One-third of the ECMO MSC cultures stopped growing after 2 months, whereas two-thirds of the MSC cultures continued to proliferate for 3 to 4 months and up to 20 population doublings from the initial harvest.

### Factors Influencing MSC Recovery

Given the variability in the detection of MSC among the ECMO samples, we performed multiple sets of comparisons and statistical analyses to determine what factors influenced the recovery of MSC. The most consistent difference between the samples with MSC and those without MSC was the time from disconnection of the ECMO circuit and sample collection to the processing of the blood samples in the laboratory. Specifically, 17 of 39 samples (43.5%) processed within 4 hours of collection gave rise to MSC, whereas only 1 of 19 samples (5.2%) processed at more than 4 hours after sample collection gave rise to MSC colonies ( $P = 0.002$ ; Fisher exact test). The patient's age did not influence MSC recovery, as MSC were isolated from neonatal and pediatric patients undergoing ECMO at similar frequencies. Specifically, samples from 11 of 27 (41%) neonatal

patients and 6 of 16 (37%) pediatric patients produced MSC. The general indication for ECMO support did not influence recovery of MSC as colonies were obtained from 11 patients with primary cardiac diagnoses and 7 with primary respiratory diagnoses. One adult cardiac ECMO patient produced both MSC and epithelial cells.

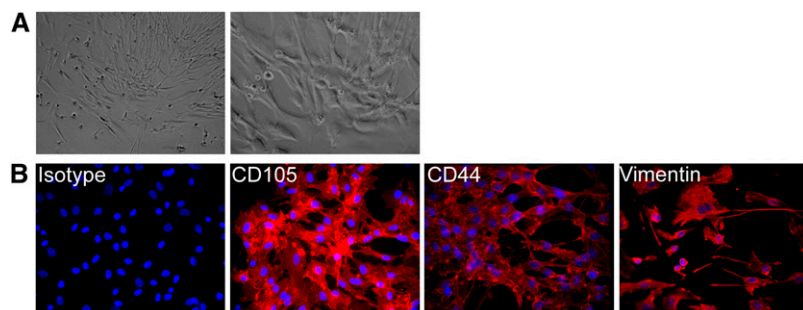
Of the seven patients from whom two or more circuits were obtained, three patients had MSC recovery from the first or second circuit, whereas MSC were not recovered from any patient at subsequent circuits, suggesting that the mobilization of MSC occurs early in the course of ECMO support. The total time on ECMO support for the samples that gave rise to MSC was 88 to 374 hours, with a median of 99 hours. There was no significant correlation between duration of ECMO support and number of MSC colonies recovered from the ECMO circuit blood.

MSC colonies were isolated from 18 patients undergoing ECMO; 11 survived and 7 died (61% average survival). Of the 29 patients from whom no MSC were isolated, 18 survived and 11 died (62% average survival). Therefore, the recovery of MSC from the peripheral blood of patients undergoing ECMO was not associated with improved survival. No MSC were isolated from the six normal adult blood units evaluated, despite optimal processing in less than 4 hours and the availability of larger blood volumes with higher mononuclear cell yield (see Table 2).

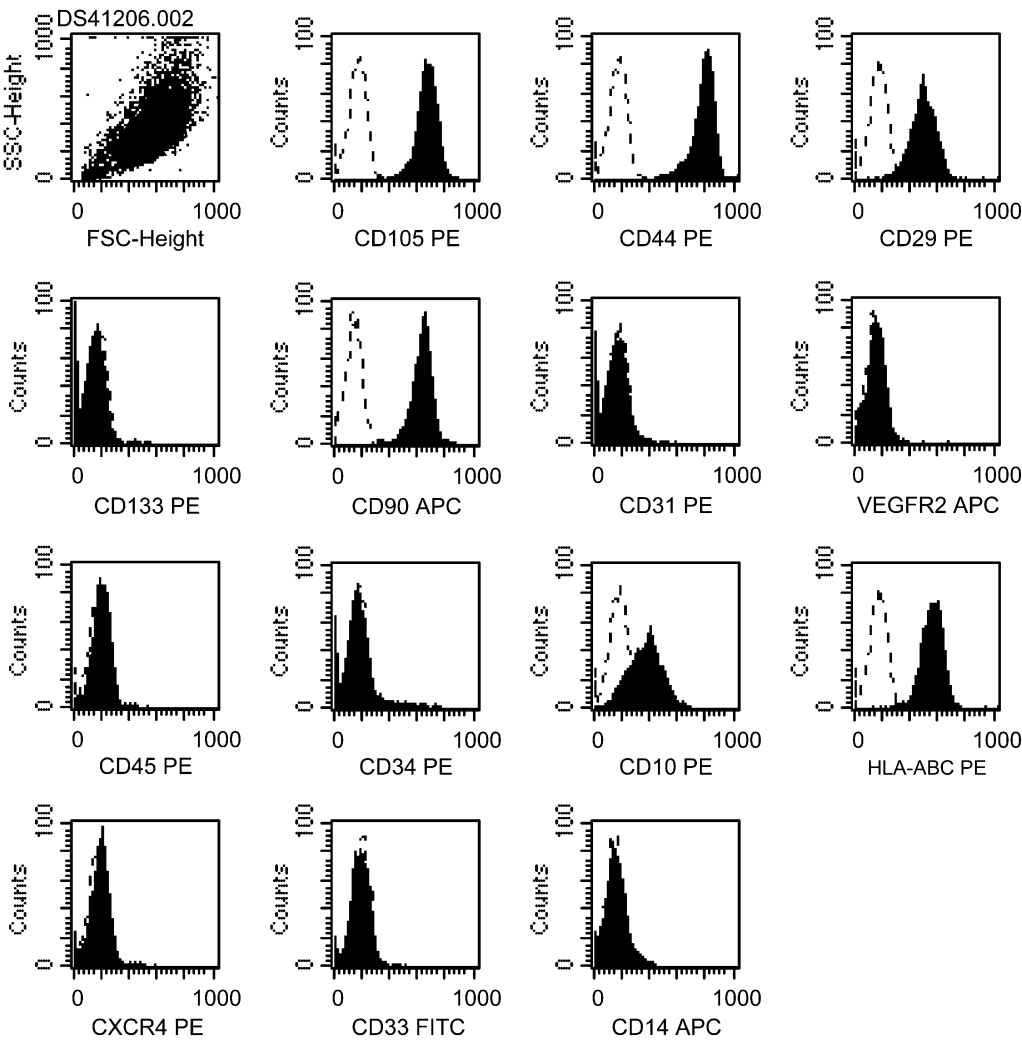
### Immunophenotypic Analysis of Cultured MSC

We evaluated the expression of a series of MSC and blood markers using immunofluorescence microscopic and flow cytometry analyses. These analyses determined that the ECMO-derived MSC expressed many markers characteristic of bone marrow- and cord blood-derived MSC (33, 37). We first evaluated the expression of MSC markers in candidate cells with a fusiform morphology transferred to chamber slides. A representative immunofluorescence panel is shown in Figure 2 (lower panel) and demonstrates that the fusiform-shaped cells are positive for CD105, CD44, and vimentin and negative for CD45 (Figure 2 and data not shown).

We next evaluated the cells for a wide variety of cell surface markers using immunostaining followed by flow cytometry analysis. These studies determined that the ECMO-derived MSC have similar characteristics to cord blood- and bone marrow-derived MSC. For example, more than 95% of the cells in every ECMO MSC culture expressed CD44, CD90, HLA-A,B,C, and CD105 at passages higher than three. The ECMO-derived MSC samples had variable expression of CD29 (32–96%), CD14 (0–23%), and CD31 (0–6%). There was a low and variable level of the hematopoietic marker CD45 (0.3–6%) at early passages; however, it was less than 1% at passages greater than three, indicating that hematopoietic cells were gone by this time. CD34 was negative in all samples by passage



**Figure 2.** Morphologic and immunofluorescence analysis of extracorporeal membrane oxygenation (ECMO) blood-derived mesenchymal stromal cells (MSC). (A) Phase contrast photomicrographs of an MSC colony of fusiform spindle-shaped cells at low (left,  $\times 40$ ) and high (right,  $\times 200$ ) magnification. (B) Immunofluorescence analysis of cultured MSC from ECMO blood ( $\times 200$ ). ECMO MSC were immunoreactive for CD105, CD44, and vimentin Cy3 conjugates shown in red. Nuclei were stained with 4',6-diamidino-2-phenylindole (blue).



**Figure 3.** Representative flow cytometry analysis of cultured mesenchymal progenitors isolated from a patient on extracorporeal membrane oxygenation at passage five. Data with isotype control antibody are shown in dotted line.

three, except two that had up to 23% positive at passages as high as seven. Similar to MSC from other sources, ECMO-derived MSC at passages greater than three were consistently negative for SSEA1, CD33, CD45, CXCR4, CD133, and VEGFR2. Flow cytometry analysis of a representative ECMO MSC sample at passage five is shown in Figure 3, and a summary of the cell surface phenotype is presented in Table 3.

**Differentiation Analysis of Cultured MSC**

The immunophenotype of MSC has commonly been used to characterize MSC; however, they are best defined by their capacity to differentiate into multiple mesenchymal lineages. The next set of experiments evaluated the potential of ECMO-derived MSC to undergo osteogenic, adipogenic, and chondrogenic differentiation. Similar to bone marrow- and cord blood-derived MSC, all evaluated ECMO-derived MSC samples were successfully differentiated into all three lineages. Interestingly, it took longer for the ECMO-derived MSC and cord blood MSC to differentiate into adipocytes (3–4 wk) as compared with bone marrow MSC ( $\leq 2$  wk). Representative histochemical analysis is shown for adipogenic (Figure 4A), osteogenic (Figure 4B), and chondrogenic (Figures 4C and 4D) differentiation conditions. These results indicate that the peripheral blood-derived MSC isolated from the ECMO circuits of critically ill patients with cardiorespiratory failure are comparable to those MSC identified in bone marrow and umbilical cord blood.

**Epithelial Colonies Were Recovered from ECMO Blood Samples**

Colonies of highly adherent cells with cobblestone morphology were observed in 6 of the first 47 ECMO samples (Figure 5A). Four of the six ECMO samples with epithelial colonies also had MSC colonies, whereas two samples produced only epithelial

**TABLE 3. FLOW CYTOMETRY ANALYSIS OF CELL SURFACE MARKERS ON MESENCHYMAL STROMAL CELLS**

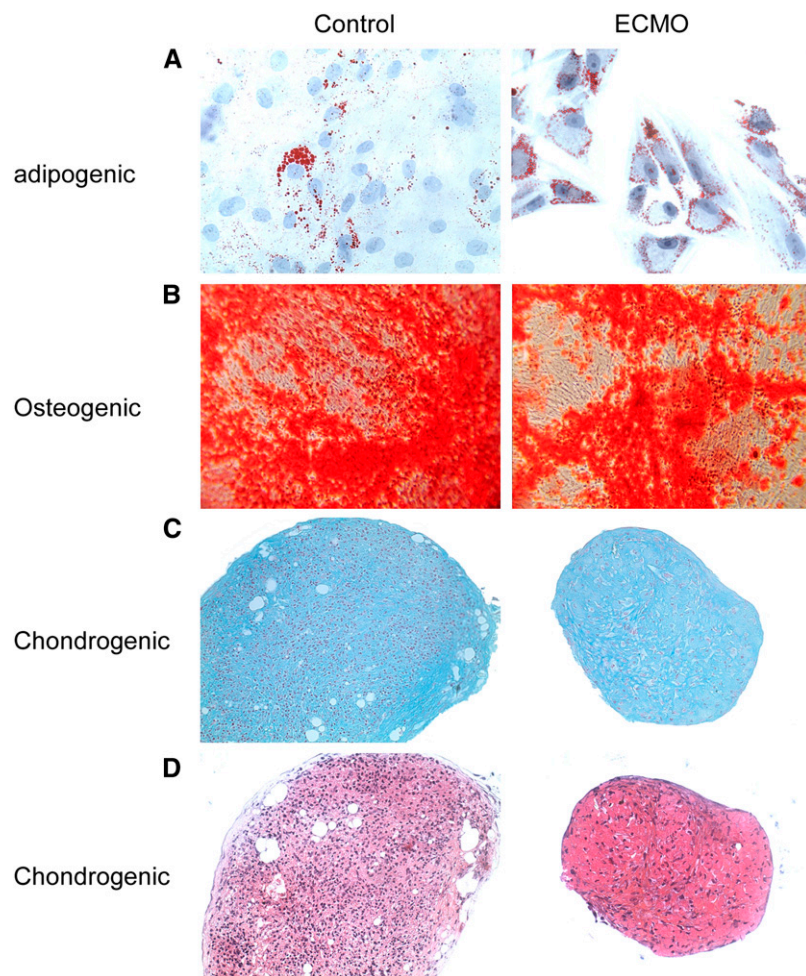
| Marker  | BM MSC | CB MSC | ECMO MSC (N)  |
|---------|--------|--------|---------------|
| CD45    | Neg    | Neg    | Neg* (12)     |
| CD105   | +++    | +++    | +++ (12)      |
| CD44    | +++    | +++    | +++ (11)      |
| HLA-ABC | +++    | +++    | +++ (12)      |
| CD34    | Neg    | Neg    | Variable (10) |
| CD29    | +++    | +++    | ++ (11)       |
| CD33    | NE     | Neg    | Neg (10)      |
| CD14    | Neg    | Neg    | Variable (10) |
| CXCR4   | Neg    | Neg    | Neg (12)      |
| CD90    | +++    | +++    | +++ (5)       |
| CD133   | Neg    | Neg    | Neg (4)       |

*Definition of abbreviations:* BM = bone marrow; CB = cord blood; ECMO = extracorporeal membrane oxygenation; NE = not evaluated; MSC = mesenchymal stromal cells; Neg = negative.

+++ : 97–100%; ++ : 32–96%; Variable: 0–23%.

\* For MSC at passages greater than three.





**Figure 4.** Histological analysis of tri-lineage differentiation of mesenchymal stromal cells (MSC). (A) Adipogenic differentiation extracorporeal membrane oxygenation (ECMO) patient blood-derived MSC and control (bone marrow). Intracytoplasmic fat globules stained with Oil red O ( $\times 400$ ). (B) Osteogenic differentiation of ECMO blood-derived MSC or control (cord blood) MSC with Alizarin red staining of calcium deposits ( $\times 40$ ). Adjacent sections from chondrogenic differentiation pellet cultures from ECMO patient-derived MSC and control (bone marrow) were histochemically stained with (C) Alcian blue and (D) Safranin O ( $\times 200$ ).

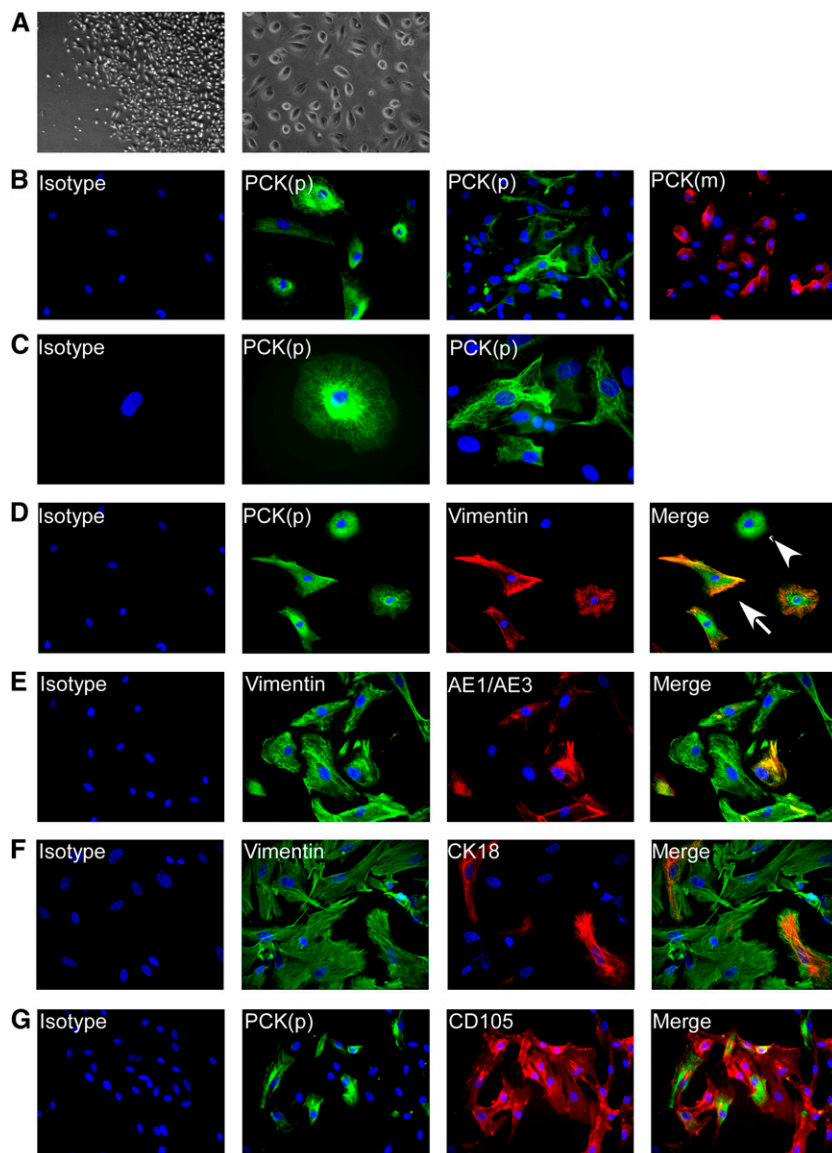
colonies. The epithelial colonies were first noted at 2 weeks in culture, had a limited expansion capacity compared with MSC, and had a life span of approximately 2 months. They were highly adherent and did not reattach to tissue culture-treated plastic after trypsinization of the primary colonies or plates. Initial experiments evaluated a variety of subculturing methods, substrates, and culture media. We determined that harvesting cells with Accutase and replating on collagen- or fibronectin-coated dishes produced the optimal reattachment to plastic chamber slides. Although we compared a variety of commercially available specialized media to the MSC-GM medium in which we first observed the cells, we were unable to identify a medium to facilitate the growth of these cells (see online supplement for details). However, we did note that weekly half-media changes with MSC-conditioned medium were superior to fresh media changes, and/or that plates with mixed epithelial and MSC cells had enhanced growth and survival. Although the significance of this observation is unclear, it may suggest that the MSC were producing a factor that enhanced the growth of the epithelial cells, and/or that a labile growth factor was reduced in the conditioned MSC-GM medium previously cultured at  $37^{\circ}\text{C}$  for 1 week. Epithelial colonies were not observed in cord blood ( $n = 15$ ) or normal adult blood ( $n = 6$ ) cultures.

Initial immunophenotyping on the epithelial colonies determined that the cells were CD45 negative and pancytokeratin positive using several different pancytokeratin antibodies (Figures 5B and 5C and data not shown). In some cultures, there was variable staining of pancytokeratin among the different

epithelial cells in the culture. This may result from different levels and/or types of cytokeratin expression in these differentiated cells, as each of the different pancytokeratin antibodies we used in this study only recognize a few of the specific cytokeratins. Specifically, mouse monoclonal pancytokeratin antibody from Sigma recognizes cytokeratins 1, 5, 6, and 8. The Vector Labs mouse monoclonal cytokeratin AE1/AE3 recognizes cytokeratins 1 to 6, 8, 10, 14, 15, 16, and 19. The Dako Rabbit polyclonal pancytokeratin wide spectrum recognizes cytokeratins 4 to 6, 8, 13, and 16. Thus, we are confident that the epithelial cells in our cultures expressed cytokeratins; however, it is unclear which specific types and whether the types were consistent within all the epithelial cells within a culture. Further analysis would be required to distinguish this.

The final 11 ECMO blood samples in our studies focused on the culture and characterization of these epithelial colonies. Two of the samples from the 11 patients undergoing ECMO had mixed cultures with both MSC and epithelial cells (one adult and one neonate). Immunofluorescence analysis of the mixed cells transferred to collagen-coated chamber slides identified two populations of pancytokeratin-positive cells. To further characterize these cells, we transferred the cells from these mixed cultures to collagen-coated chamber slides and performed a series of double immunofluorescence analyses using a variety of epithelial and mesenchymal markers.

One population of cells had an ovoid morphology similar to those originally observed to be pancytokeratin positive (Figures 5C and 5D, *arrowhead*). These cells were confirmed cytokeratin positive using a second pancytokeratin antibody (cytokeratin



**Figure 5.** Immunostaining and histological analysis of epithelial colonies. (A) Phase contrast photomicrographs of a cobblestone epithelial-like colony at low (left,  $\times 40$ ) and high magnification (right,  $\times 200$ ). (B–G) Immunofluorescence analysis with the antibodies shown on each photomicrograph (details of each antibody shown in online supplement). (B) Pancytokeratin immunopositive cells from three different patients using either rabbit polyclonal pancytokeratin antibodies (PCKp) with a FITC-conjugated secondary antibody (green) or mouse monoclonal pancytokeratin antibody (PCKm) with a Cy3-conjugated secondary antibody (red). The middle two pictures with cytokeratin-positive cells of various shapes were from an adult (middle left) and a neonatal patient (middle right) who had mixed cultures of mesenchymal stromal cells (MSC) and epithelial cells. The right panel shows an example of ovoid cell shape from a patient who had only epithelial colonies and no MSC on the dish. (C) Higher magnification of pancytokeratin-positive cells from the same patients shown directly above in B. D–G are double-immunostained samples and isotype controls. (D) The arrow-head points to a cytokeratin-positive ovoid cell (green PCKp). The arrow points to a fusiform-shaped cell positive for both pancytokeratin and vimentin from an adult patient on extracorporeal membrane oxygenation (yellow/orange). E–G show some double-positive cells from a neonatal patient expressing both mesenchymal markers (Vimentin or CD105) and epithelial markers (cytokeratin AE1/AE3 and cytokeratin 18 [CK18]) in a yellow/orange color. Original magnifications: B, D–G,  $\times 200$ ; C,  $\times 400$ .

AE1/AE3), as well as positive for cytokeratin 18 (Figures 5E and 5F). These ovoid cells were negative for CD45, EpCam, E-Cadherin, Aquaporin 5, Cytokeratin 5, and Cytokeratin 6a by immunofluorescence microscopy (data not shown).

The second population of pancytokeratin-positive cells had polygonal or fusiform morphologies similar to mesenchymal cells and was also identified in the mixed cultures (Figure 5D, arrow; Figures 5E–5G). After transfer to collagen-coated chamber slides these cells were consistently positive for mesenchymal markers CD44, CD105, and vimentin. Double immunostaining for both mesenchymal and epithelial markers showed that a subset of these cells was also positive for pancytokeratin and/or cytokeratin 18 (Figures 5D–5G and data not shown). These cells were distinct from the classical MSC described above and shown in Figure 2, which was pancytokeratin negative. These polygonal epithelial-mesenchymal cells were negative for CD45, EpCam, E-Cadherin, Aquaporin 5, Cytokeratin 5, and Cytokeratin 6a, and may be the result of mesenchymal–epithelial transition, or vice versa. A total of eight ECMO samples had epithelial colonies isolated from the ECMO circuit blood: one adult, four pediatric, and three neonatal ECMO samples. Epithelial colonies were only observed in ECMO circuit blood samples collected during the first 5 days of ECMO support. The patients with circulating epithelial pro-

genitors were on ECMO support for 44 to 127 hours (median 98 h). Of those eight patients, six had both MSC and epithelial colonies recovered from their blood. Survival was lower in patients with epithelial cell mobilization compared with patients without circulating epithelial cell recovery (3 out of 8 or 38% compared with 28 out of 39 or 72%). However, this trend did not reach statistical significance ( $P = 0.075$ ), possibly because of the low numbers of patients.

## DISCUSSION

We hypothesized that progenitor cell populations would be mobilized into the circulation in response to severe tissue injury. Our study evaluated whether progenitor cell populations were mobilized into the circulation in patients with severe respiratory and/or cardiac injuries placed on extracorporeal life support. ECMO support is invasive and labor intensive, reserved for moribund patients or *in extremis* patients with an estimated mortality risk of 80% or greater with maximal conventional medical therapy (28–30).

Our studies demonstrated that hematopoietic, mesenchymal, and epithelial progenitors were mobilized into the blood of patients placed on ECMO for cardiorespiratory support. Several



other studies have shown mobilization of hematopoietic and/or endothelial progenitors into the circulation in patients with acute myocardial infarction (38), congestive heart failure (39), or acute lung injury and acute respiratory distress syndrome (ARDS) (40). It was suggested that mobilization occurred in response to increased serum levels of endogenous chemokines or growth factors including G-CSF and SDF1 $\alpha$  (38, 41). Several interleukins and hematopoietic cytokines, such as IL-6 and IL-8, are also elevated in the blood of patients on ECMO (42, 43) and patients with ARDS (44, 45). Further, a study by Wiedermann and colleagues demonstrated higher concentrations of G-CSF and IL-8 in the bronchoalveolar fluid than serum of patients with ARDS establishing a concentration gradient that may direct cell migration from the circulation (46). Thus, our first set of studies evaluated the frequency of circulating hematopoietic progenitors in samples from patients undergoing ECMO and ICU patients. However, as patients undergoing ECMO are transfused daily with leukocyte-reduced and irradiated blood products, we used a clonogenic, proliferation-based assay rather than cell surface marker expression of CD34 to calculate progenitor frequency. This ensured that we were evaluating patient hematopoietic progenitors and not residual ones remaining in the irradiated transfused blood products, which would not be able to proliferate (36). Our data demonstrated that the peripheral blood of patients undergoing ECMO had significantly higher frequencies of hematopoietic progenitors than age-matched patients not on ECMO from the NICU, PICU, or normal adult controls.

We also evaluated the frequency of the specific hematopoietic progenitors anticipating that the highest level of hematopoietic progenitors would be for the myeloid lineages given that most patients undergoing ECMO have acute lung injury with sepsis and/or pneumonia. Surprisingly, the highest level of hematopoietic progenitors in the ECMO samples were erythroid, and not myeloid as found in controls. For a newborn infant, the volume in the ECMO circuit is approximately twice the infant's blood volume resulting in a 1:3 dilution of the nucleated cells and platelets at the initiation of ECMO and at each subsequent ECMO circuit change. Although patients undergoing ECMO have a relative dilutional neutropenia compared with their pre-ECMO state (47), their hemoglobin is kept in the normal range with leukocyte-reduced and irradiated packed red blood cell transfusions (36). We speculate that the increase in overall hematopoietic progenitor number may be a physiological response to hemodilution and/or tissue injury/hypoxia signals through the HIF-1 $\alpha$  signaling pathway (48). A positive correlation between the higher frequency of hematopoietic progenitors and increased duration of ECMO support suggests that the mobilization was ECMO related.

Mesenchymal progenitors were isolated from the peripheral blood of one-third of the patients placed on ECMO. During steady state, MSC have been found in the bone marrow, umbilical cord blood, and solid tissues (49). MSC are rarely, if ever, observed in the steady-state peripheral blood, but can be mobilized by growth factor treatment (50, 51). Others have described cells with a cell surface phenotype consistent with MSC in the circulation after tissue injury such as acute myocardial infarction or severe burns (52, 53). The frequency of MSC was relatively low in our study, with between 1 and 35 colonies recovered per ECMO sample of approximately  $10^9$  mononuclear cells. The frequency of circulating MSC was lower in our studies than in these other studies, which used flow cytometry for cell surface phenotype to quantify MSC. However, the cell surface phenotype is not definitive and overestimates the number of functional MSC (32, 33). Thus, the level of mobilization and frequency of MSC observed in other studies is not directly comparable to the frequency observed in our study.

MSC were recovered from 20% of the cord blood samples processed in our study. However, there is a variation in the ability to detect MSC in cord blood with some studies not observing MSC (54) to other studies identifying MSC in 29% of samples (34). These published studies highlight the critical factors for isolating MSC from cord blood of a minimum volume of 33 ml and processing within 15 hours from collection. Our yield of 20% seems to be in between these reports, likely because of the variable sample size (15–100 ml) we received for analysis, as these represented only a fraction of the total cord blood volume, and the time to sample processing. The relative frequency of MSC colony observed in our study (one colony per  $4.8 \times 10^7$  to  $10^8$  MNC) is in line with the published results from Javed and coworkers (49) ( $0.6 \pm 0.4$  colonies/ $10^8$  MNC) in cord blood.

It was interesting that of all of the statistical comparisons to identify the sample characteristics that produced MSC, the requirement for blood sample processing within 4 hours of collection was the only significant parameter. Thus, given that one-third of our samples were processed at greater than 4 hours, our studies have likely underestimated the incidence or frequency of MSC mobilization in patients placed on ECMO support. The critical requirement for rapid processing is a novel finding that must be taken into account in the evaluation of past studies and setting up future clinical studies.

In other studies, fibrocytes that contribute to lung fibrosis in immune-deficient mouse models have been found circulating in patients with lung fibrosis (55–58). Fibrocytes were identified by the expression of CD34, CD45, CXCR4, and collagen type I. Fibrocytes also have limited expansion capacity. The MSC identified in our studies are consistent with an MSC phenotype (negative for CD45, CXCR4, and CD34) and could be expanded for many population doublings. Thus, demonstrating that the cells identified in our study are circulating MSC and not fibrocytes.

Our studies also demonstrated that ECMO samples gave rise to epithelial colonies with CD45-negative, cytokeratin-positive cells (hereafter referred to as epithelial progenitors). These epithelial progenitor cells were circulating in the blood of patients on ECMO, but not normal or umbilical cord blood controls. This is, to our knowledge, the first report of circulating epithelial progenitors recovered from the peripheral blood of patients with heart and/or lung injury. Circulating epithelial cells that can contribute to the repair of tracheal epithelium have been identified in mice as CD45 positive and CK5 double positive (8), although given the differences in species, injuries, and experimental design it is unclear whether the epithelial progenitors observed in our study are the human equivalent to those identified by Gomperts and colleagues in mice (8).

In other samples, we observed mixed cultures, which had cells with epithelial and others with mesenchymal morphology. We considered that these were two independent populations of cells arising from independent progenitors; however, we cannot exclude the possibility that these two populations arose from a single progenitor. On transfer of these mixed cultures onto chamber slides for immunostaining analysis, we determined that there were cells expressing exclusively epithelial markers and others coexpressing mesenchymal and epithelial markers. However, because these mesenchymal-epithelial cells were observed after 1 month in culture and a transfer to collagen, it is unclear whether these epithelial-mesenchymal cells were present in the original ECMO blood samples or developed *in vitro* as a culture artifact or differentiation event. Other studies have generated cytokeratin-positive cells *in vitro* through the culture of blood mononuclear cells or MSC with airway epithelial cells, growth factor treatment, or specialized media (9, 59–61). In the study by Paunescu, the bone marrow MSCs continued to express

mesenchymal markers after induction of cytokeratin, thus demonstrating a mesenchymal-to-epithelial transition (61). It is possible that the mesenchymal-epithelial cells observed in our studies arose from a similar mesenchymal-to-epithelial transition. Further studies would be required to clarify this issue.

An interesting finding of our study is the temporal mobilization of the various progenitor populations. Our data suggest that the epithelial and mesenchymal progenitors are mobilized early after the injury and placement on ECMO. Specifically, epithelial progenitors were only observed in circuits collected during the first 5 days of ECMO support, and MSC within the first 2 weeks of ECMO support. In contrast, hematopoietic progenitor mobilization increased with time on ECMO. One limitation of our study is that we obtained the waste blood from the ECMO circuit at random intervals during ECMO, either when technical complications required a circuit change or after the patient was disconnected. The majority of the samples were at the end of an ECMO run when the patient had recovered heart or lung function. Therefore, early mobilization events after acute lung or heart injury could have been missed because of the lack of early samples. Thus, it is likely that our observations are an underestimation of the true physiologic progenitor mobilization events in this patient population.

Our studies clearly demonstrate the mobilization of multiple progenitor populations into the circulation of patients with severe respiratory and/or cardiac injury placed on ECMO life support. However, the mechanism of progenitor mobilization in these studies is unclear, although it was likely contributed to by several mechanisms. One possible mechanism is that progenitor mobilization occurred in response to the mediators released from the acutely injured organs. For example, many cytokines, including IL-6, IL-8, and TNF- $\alpha$  (20, 21, 23), are elevated in the blood and/or tracheal fluid of patients with lung injury (44–46) and/or placed on cardiopulmonary bypass or ECMO support (42, 43, 62–64).

Other factors that likely contribute to the mobilization of progenitors in patients undergoing ECMO include blood-for-ign surface interactions between the blood and ECMO circuit and/or physiologic response to hemodilution that results from expanding the circulating blood volume with a corresponding decrease in concentration of circulating nucleated cells. Our study could not distinguish between these mechanisms, because we did not have blood samples from patients before the initiation of ECMO life support for comparison. The findings of mesenchymal or epithelial progenitors in the blood were not significantly associated with improved survival outcomes in our study, although a trend toward lower survival outcome was observed for patients in whom epithelial progenitors were recovered.

Our studies describe the mobilization of hematopoietic, mesenchymal, and epithelial progenitors into the blood of patients with cardiac and/or respiratory injury placed on ECMO for life support. The tissue of origin for these mobilized progenitors is unknown. It is likely that the hematopoietic progenitors originated in the bone marrow or other hematopoietic organs. However, the origin of the MSC and epithelial progenitors is less clear as they could be mobilized from the injured tissues, bone marrow, or other tissues throughout the body. One possibility is that these MSC were mobilized in response to the severe cardiac or lung injury, and could theoretically home to the injured tissue. In support of this theory, Hennrick and colleagues identified MSC in the tracheal fluid in a subset of premature infants with respiratory distress placed on mechanical ventilation (65). The patients from which MSC were recovered had higher levels of the cytokine MCP-1 in their tracheal fluid, compared with those that did not have MSC, suggesting that the MSC

migrated in response to MCP-1 or other mediators in the injured tissue. In support of this hypothesis, the patient MSC migrated *in vitro* to recombinant MCP-1 and tracheal fluid from these patients (65). Future studies will be required to determine whether the circulating progenitors identified in our study can home to the area of tissue injury and support, or contribute to tissue repair in patients placed on ECMO.

**Conflict of Interest Statement:** K.C.T.B. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. D.S. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. X.W. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. B.H. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. P.F. received \$1,001–\$5,000 from Ikaria in lecture fees and \$50,001–\$100,000 from Philippe Friedlich, MD, Inc. as an expert witness. C.L. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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