Isolation of amniotic stem cell lines with potential for therapy

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Stem cells capable of differentiating to multiple lineages may be valuable for therapy. We report the isolation of human and rodent amniotic fluid–derived stem (AFS) cells that express embryonic and adult stem cell markers. Undifferentiated AFS cells expand extensively without feeders, double in 36 h and are not tumorigenic. Lines maintained for over 250 population doublings retained long telomeres and a normal karyotype. AFS cells are broadly multipotent. Clonal human lines verified by retroviral marking were induced to differentiate into cell types representing each embryonic germ layer, including cells of adipogenic, osteogenic, myogenic, endothelial, neuronal and hepatic lineages. Examples of differentiated cells derived from human AFS cells and displaying specialized functions include neuronal lineage cells secreting the neurotransmitter L-glutamate or expressing G-protein-gated inwardly rectifying potassium channels, hepatic lineage cells producing urea, and osteogenic lineage cells forming tissue-engineered bone.

Amniotic fluid is known to contain multiple cell types derived from the developing fetus[1,2]. Cells within this heterogeneous population can give rise to diverse differentiated cells including those of adipose, muscle, bone and neuronal lineages[3–6]. We now describe lines of broadly multipotent AFS cells, and use retroviral marking to verify that clonal human AFS cells can give rise to adipogenic, osteogenic, myogenic, endothelial, neurogenic and hepatic lineages, inclusive of all embryonic germ layers. In this respect, they meet a commonly accepted criterion for pluripotent stem cells, without implying that they can generate every adult tissue.

Neurons, hepatocytes and osteoblasts are among the cell types for which improved stem cell sources may open up novel therapeutic applications. For each of these examples we show that AFS cells can yield differentiated cells that express lineage-specific markers and acquire characteristic functions in vitro. In addition, we present initial studies indicating that AFS cells induced toward particular lineages can generate specialized cells after implantation in vivo. We show that AFS cells directed to neural lineage differentiation by exposure to nerve growth factor (NGF) are able to widely engraft the developing mouse brain in a manner similar to that observed previously for neural stem cells[7]. In addition, we document the formation of tissue-engineered bone from printed constructs of osteogenically differentiated human AFS cells in immune-deficient mice.

RESULTS

Clonal stem cell lines from amniotic fluid

Approximately 1% of the cells in cultures of human amniocentesis specimens obtained for prenatal genetic diagnosis express the surface antigen c-Kit (CD117), the receptor for stem cell factor[8] (**Supplementary Table 1** online). We used immunoselection with magnetic microspheres to isolate the c-Kit–positive population from many amniocentesis specimens and found that these cells can be readily expanded in culture as stable lines, termed AFS cells. We have routinely established clonal AFS cell lines with a typical doubling time of about 36 h and no need for feeder layers. Sub-confluent cells show no evidence of spontaneous differentiation. However, under specific inducing conditions AFS cells are able to give rise to lineages representative of the three embryonic germ layers.

We used flow cytometry to assess markers expressed by human AFS cells (**Supplementary Fig. 1** and **Supplementary Methods** online). Five clonal lines gave similar results. The cells were positive for Class I major histocompatibility (MHC) antigens (HLA-ABC), and some were weakly positive for MHC Class II (HLA-DR). The AFS cells were negative for markers of the hematopoietic lineage (CD45) and of hematopoietic stem cells (CD34, CD133). However, they stained positively for a number of surface markers characteristic of mesenchymal and/or neural stem cells, but not embryonic stem (ES) cells, including CD29, CD44 (hyaluronan receptor), CD73, CD90 and CD105 (endoglin)[5,9,10]. Human AFS cells also were positive for stage-specific embryonic antigen (SSEA)-4 (ref. 11), a marker expressed by ES cells but generally not by adult stem cells. The AFS cells did not express other surface markers characteristic of ES[12,13] and embryonic germ (EG) cells[14], SSEA-3 and Tra-1-81. Some lines were weakly positive for Tra-1-60. Over 90% of the cells expressed the transcription factor Oct4, which has been associated with the maintenance of the undifferentiated state and the pluripotency of ES and EG cells[15].

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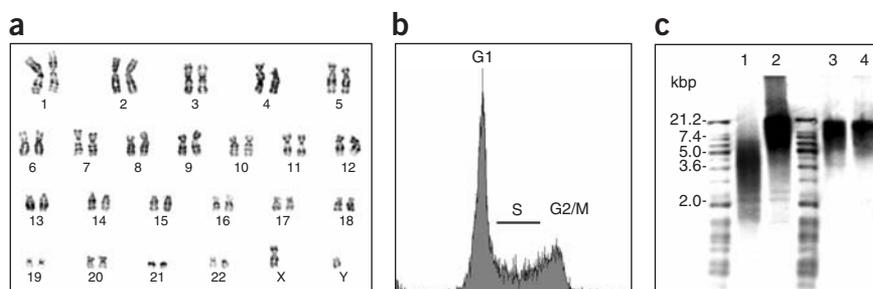
Figure 1 Clonal human AFS cells have a normal karyotype and retain long telomeres. (**a**) Giemsa band karyogram showing chromosomes of late passage (>250 p.d.) cells. (**b**) Flow cytometry of late passage cells showing DNA stained with propidium iodide. G1 and G2/M indicate 2n and 4n cellular DNA content, respectively. S indicates cells undergoing DNA synthesis, intermediate in DNA content between 2n and 4n. (**c**) Conserved telomere length of AFS cells between early passage (20 p.d., lane 3) and late passage (250 p.d., lane 4). Short length (lane 1) and high length (lane 2) telomere standards are provided in the assay kit. Marker lengths are indicated. p.d., population doubling.

We also isolated AFS lines by immunoselection of cells expressing c-Kit from the amniotic fluid of mice and rats. The rodent AFS cells closely resemble the human AFS cells in their growth properties and capacity for *in vitro* differentiation (data not shown). Murine AFS cells, like those from human amniotic fluid, express both embryonic and adult stem cell markers (**Supplementary Fig. 2** online).

Whereas normal somatic stem cells are nontumorigenic, ES cells grow as teratocarcinomas when implanted *in vivo*[12,16–18]. None of four human AFS cell lines tested, including late-passage cells, formed tumors in severe combined immunodeficient (SCID)/Beige mice (CB17/lcr.Cg-*Prkdc*^{scid}*Lyst*^{bg}/Crl). Moreover, karyotypes of eleven human lines from pregnancies in which the fetus was male revealed one X and one Y chromosome and a normal diploid complement of autosomes (**Fig. 1a**). There were no obvious chromosomal rearrangements as judged by Giemsa banding. The AFS cells, even after expansion to 250 population doublings (p.d.), showed a homogeneous, diploid DNA content in the G1 (prereplicative) phase of the cell cycle (**Fig. 1b**). The G1 and G2 cell-cycle checkpoints appeared intact. Analysis of terminal restriction fragments[19] showed that the average length of telomeres of human AFS cells stayed constant, ~20 kbp, between early (20 p.d.) and late passage (250 p.d.) cells (**Fig. 1c**).

Cloned AFS cells are broadly multipotent

We tested lines obtained from nineteen different amniocentesis donors and found that AFS cells were able to differentiate along adipogenic, osteogenic, myogenic, endothelial, neurogenic and hepatic pathways[20].
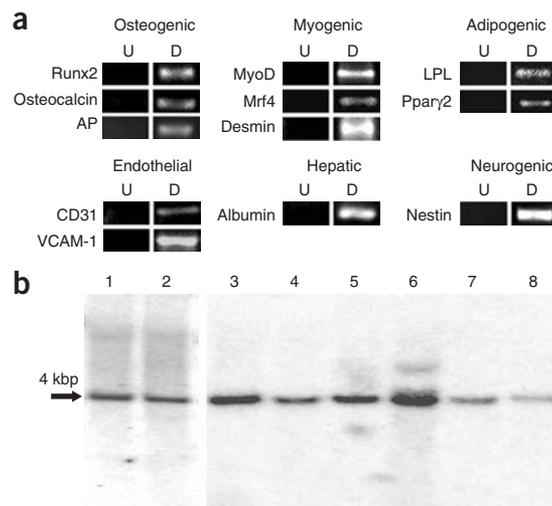
The induced differentiation to multiple fates could be documented by expression of mRNAs for lineage-specific genes (**Fig. 2a**)

Multilineage differentiation was characteristic of AFS cells cloned by limiting dilution. To rigorously confirm that cloned AFS cells and their differentiated derivatives descended from a single cell, we used marking with a retroviral vector. Because retroviral DNA will insert into almost any chromosomal region[21], the presence of a provirus at a unique genomic site can identify a clonal population descended from the cell in which an integration event occurred. We infected a presumptively clonal AFS line with a vector encoding green fluorescent protein (CMMP-eGFP) and identified candidate GFP-positive subclones. One contained a single GFP provirus, identified by a unique 4-kbp *Bam*H1 junction fragment between viral and host sequences in a Southern blot of genomic DNA (**Fig. 2b**). The cells were recloned by limiting dilution, and the resulting second round subclones again contained the signature 4-kbp *Bam*H1 junction fragment. We found that these subclones displayed the same capacity for differentiation along six lineage pathways as the original line. Furthermore, each population of differentiated cells contained the marker provirus, documented by the 4-kbp junction fragment (**Fig. 2b**). This finding excludes the possibility that the putatively cloned populations were actually oligoclonal, comprising distinct progenitors for different lineages. We conclude that the AFS cells are indeed broad-spectrum multipotent (that is, pluripotent) stem cells.

Function of differentiated cells derived from AFS cells

In addition to showing that induced stem cells express lineage-specific markers, it is important to confirm that they can give rise to cells with sufficient specialized function to have potential therapeutic utility. We assessed functionality with at least one lineage corresponding to each embryonic germ layer.

Neuronal lineage differentiation (ectoderm) was induced in a two-stage system. In a first culture phase, over 80% of the initial AFS cell population became positive for nestin, a marker first defined in neural stem cells[22] (**Fig. 3a**). Under conditions expected to bias for

Figure 2 Clonal human AFS cells are broadly multipotent. Clonality of AFS cells and differentiated cells derived from was verified by retroviral marking. (**a**) RT-PCR analysis of mRNAs for lineages indicated using a retrovirally marked second round subclone of AFS cells. U: Control undifferentiated cells. D: cells maintained under conditions to promote osteogenic (8 d), myogenic (8 d), adipogenic (16 d), endothelial (8 d), hepatic (45 d), neurogenic (8 d) differentiation. (**b**) Southern blot analysis of inserted GFP retroviral DNA in differentiated cells from (**a**); arrow indicates 4-kbp junction fragment in *Bam*H1 digested DNA from undifferentiated cells of a GFP positive subclone of AFS cells (lane 1) and the second round subclone (lane 2) used for transcript analysis after differentiation under adipogenic (lane 3), endothelial (lane 4), hepatic (lane 5), osteogenic (lane 6), myogenic (lane 7), and neurogenic (lane 8) conditions.
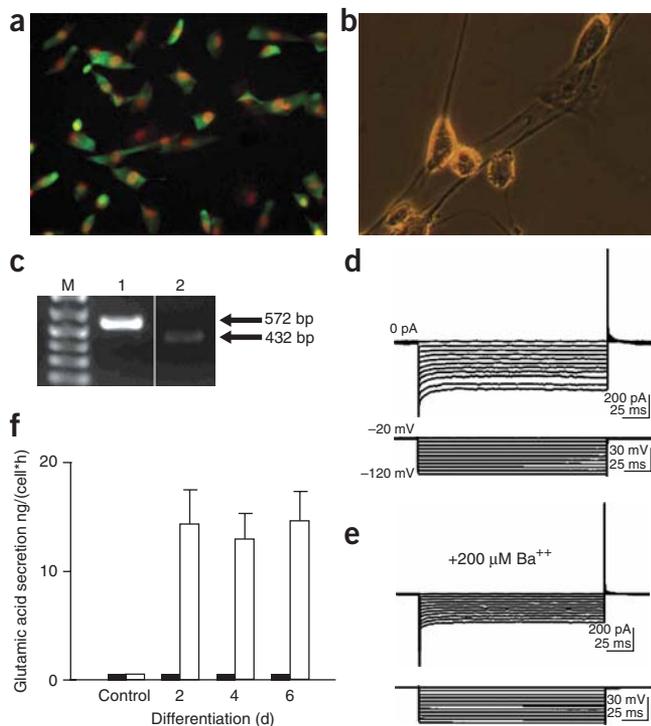
Figure 3 Neurogenic differentiation of human AFS cells in culture. (**a**) Immunofluorescence staining for nestin after 8 d in first stage neurogenic differentiation. (**b**) Phase contrast micrograph showing cells after second stage of neurogenic differentiation under conditions biasing for production of dopaminergic neurons – individual cells with pyramidal morphology, as shown, were assessed for potassium channels by voltage clamping. (**c**) RT-PCR showing expression of glyceraldehyde-3-phosphate dehydrogenase (lane 1) and GIRK2 (lane 2) after second stage of neurogenic differentiation. M indicates size markers. Sizes of PCR products are shown. (**d,e**) Whole cell voltage clamping on individual cells as in **b** was carried out as described in Methods. High potassium bath (**d**). High potassium bath + 200 μM BaCl$_2$ (**e**). (**f**) Secretion of neurotransmitter glutamic acid in response to potassium ions in cells after neurogenic differentiation in the presence of NGF (open bars) and after maintenance of undifferentiated cells in standard growth medium (closed bars).

throughout life and from which resident neural progenitors readily migrate into and integrate within cerebral parenchyma[7]. The grafted cells dispersed throughout the host mouse brains and survived efficiently for at least 2 months. Within the first month human cells already were present in a variety of brain regions including periventricular areas, the hippocampus and the olfactory bulb, where they integrated seamlessly and appeared morphologically indistinguishable from surrounding murine cells (**Fig. 4**). Neither deformation of the host brain nor any neoplastic process was evident. The pattern of migration of the transplanted cells was not random; they populated the same areas in all of the engrafted mice. However, the number of engrafted human cells was higher in the brains of the twitcher mutants (∼70% of injected cells) than in the wild-type recipients (∼30%).

We observed induction of markers characteristic of the hepatocyte lineage (endoderm) in AFS cells cultured for up to 45 d in a multi-step system. Proteins expressed by hepatic differentiated AFS cells included albumin, alpha-fetoprotein, hepatocyte nuclear factor 4 (HNF4), hepatocyte growth factor receptor (c-Met) and the multidrug
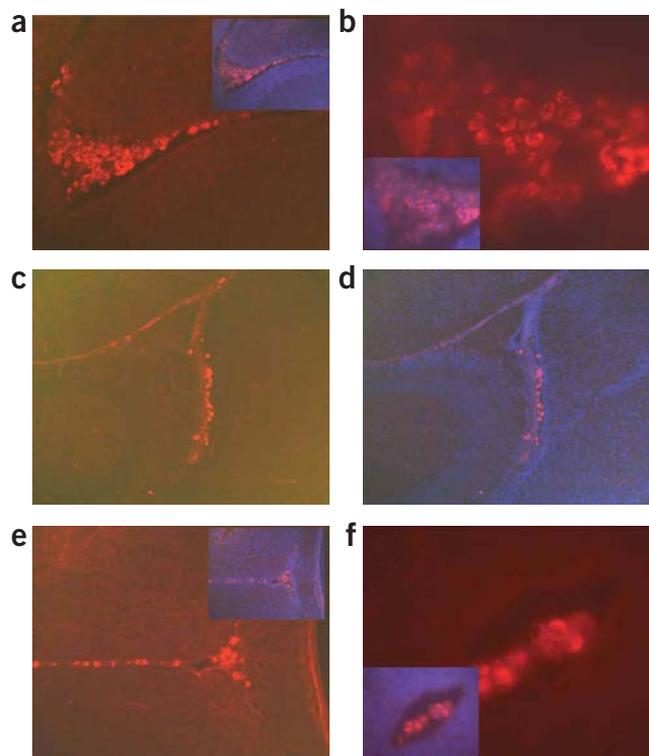
dopaminergic neurons[23], a fraction of these cells assumed a distinct pyramidal morphology (**Fig. 3b**). Transcript analysis showed expression of the *GIRK2* gene, encoding a member of the G-protein-gated inwardly rectifying potassium (GIRK) channel family, a marker of dopaminergic neurons[24] (**Fig. 3c**). Voltage clamping of individual cells revealed a barium-sensitive potassium channel consistent with the GIRK2 channel (**Fig. 3d,e**). Furthermore, after application of another neurogenic induction protocol using NGF, AFS cells acquired the ability to secrete the excitatory neurotransmitter L-glutamate in response to stimulation by potassium ions (**Fig. 3f**).

The ability to participate in the development of the central nervous system provided another test of neural lineage differentiation of AFS cells. After induction in neurogenic medium, human AFS cells were injected into the lateral cerebral ventricles of the brains of newborn mice, both wild-type and homozygous twitcher (C57BL/6J-Galc^{twi}) mutants, as done previously with neural stem cells[25]. The twitcher mice are deficient in the lysosomal enzyme galactocerebrosidase and undergo extensive neurodegeneration and neurological deterioration, initiating with dysfunction of oligodendrocytes, similar to that seen in the genetic disease Krabbe globoid leukodystrophy[26]. Implantation into the lateral ventricles affords the cells access to the subventricular zone, a secondary germinal zone in the cerebrum that persists

Figure 4 Engraftment of neurogenically differentiated human AFS cells in mouse brain. Differentiation was induced by incubation with NGF and cells were injected into brains of newborn mice as described. Samples shown were obtained 1 month after injection into twitcher mice (wild type and *twi* mutant recipients gave qualitatively similar results). Red staining with antibody to a 65-kDa mitochondrial protein (perinuclear localization) reveals human cells. Blue staining (DAPI: superimposed image in **d** and insets of **a,b,e,f**) shows cell nuclei. (**a**) Lateral ventricle. (**b**) Higher magnification view of lateral ventricle. (**c**) Periventricular area and hippocampus. (**d**) Same field as **c**, with DAPI staining superimposed. (**e**) Third ventricle. (**f**) Olfactory bulb.
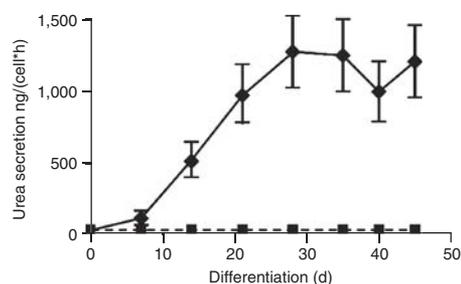
Figure 5 Urea secretion by human AFS cells after hepatogenic *in vitro* differentiation. Urea secretion was assessed from undifferentiated human AFS cells maintained in normal growth medium (rectangles) or after hepatogenic differentiation (diamonds).

resistance membrane transporter MDR1[20]. Furthermore, hepatic lineage cells obtained by differentiation of AFS cells were able to secrete urea, a characteristic liver-specific function (**Fig. 5**).

Differentiation of AFS cells in osteogenic medium *in vitro* yielded functional osteoblasts (mesoderm), which produced mineralized calcium (**Fig. 6a** and **Supplementary Fig. 3** online). The cells also expressed and secreted alkaline phosphatase, a surface marker of osteoblasts (**Supplementary Fig. 3**)[27]. As a 'proof of concept' of the utility of AFS cells for tissue engineering, we asked whether the cells could contribute to bone formation *in vivo*. For this purpose we embedded human AFS cells in an alginate/collagen scaffold by thermal inkjet printing[28,29]. Printed cell/scaffold constructs cultured in osteogenic inductive medium for 45 d stained intensely for mineralized calcium with Alizarin red S and showed nodules consistent with bone formation (**Supplementary Fig. 3**).

For the *in vivo* assessment, printed human AFS cell/scaffold constructs were prepared, incubated for 1 week in osteogenic medium and implanted subcutaneously in immunodeficient mice. An unseeded control scaffold also was implanted in each mouse. After 8 weeks constructs were recovered and analyzed histologically using von Kossa's stain (**Fig. 6b,c**). Highly mineralized tissue was observed from the implanted cell-seeded scaffolds but not from the implanted unseeded scaffolds. After 18 weeks the generation of hard tissue within the printed constructs was assessed by micro CT scanning of the recipient mice (**Fig. 6d–f** and **Supplementary Video** online). At sites of implantation of the scaffolds containing AFS cells, blocks of bone-like material were observed with density somewhat greater than that of

mouse femoral bone. Control scaffolds lacking AFS cells did not promote the formation of bony tissue.

DISCUSSION

We have demonstrated that stem cells can be obtained routinely from human amniotic fluid, using backup cells from amniocentesis specimens that would otherwise be discarded. The AFS cells grow easily in culture and appear phenotypically and genetically stable. They are capable of extensive self-renewal, a defining property of stem cells. The absence of senescence and maintenance of long telomeres for over 250 p.d. far exceeds the typical 'Hayflick limit' of about 50 p.d. for many post-embryonic cells, which generally is attributed to the progressive shortening of telomeres[30].

The consistent presence of a Y chromosome in lines derived from cases in which the amniocentesis donor carried a male child implies that AFS cells originate in the developing fetus. The surface marker profile of AFS cells and their expression of the transcription factor Oct4 suggests that they represent an intermediate stage between pluripotent ES cells[12,16,17] and lineage-restricted adult stem cells. Unlike ES cells, AFS cells do not form tumors *in vivo*. A low risk of tumorigenicity would be advantageous for eventual therapeutic applications.

Potential sources of AFS cells in the developing fetus are diverse[31]. CD117 (c-Kit), the surface marker used for immunoselection of AFS cells, plays an important role in gametogenesis, melanogenesis and hematopoiesis[32,33]. This receptor protein is present on human ES cells[34], primordial germ cells and many somatic stem cells, including, but not limited to, those of the neural crest[35,36]. Despite sharing expression of c-Kit, AFS cells appear clearly distinct from ES cells, germline stem cells and certain adult stem cell populations, such as hematopoietic stem cells, on the basis of differences both in a variety of cell surface markers and in gene expression patterns assessed by transcriptional profiling[37]. Thus, the role of AFS cells in ontogeny is not yet clear.

AFS cells can serve as precursors to a broad spectrum of differentiated cell types. We used retroviral marking of AFS cell clones to rigorously assess their multipotent character. Cells from a marked clone were induced to differentiate along six distinct lineages (adipogenic, osteogenic, myogenic, endothelial, neurogenic and hepatic). DNA of the resulting specialized cells contained a unique marker junction fragment between proviral and cellular sequences in the genome. In addition to the markers and cell types documented here, we have demonstrated that human AFS cells of the same clone can be induced to express markers characteristic of cardiac muscle, including cardiac myosin, troponin I and troponin T, and of pancreatic beta-cells, including Pax6, neurogenin D and insulin (data not shown). Human AFS cells can therefore yield differentiated cells corresponding to each of the three embryonic germ layers. The full
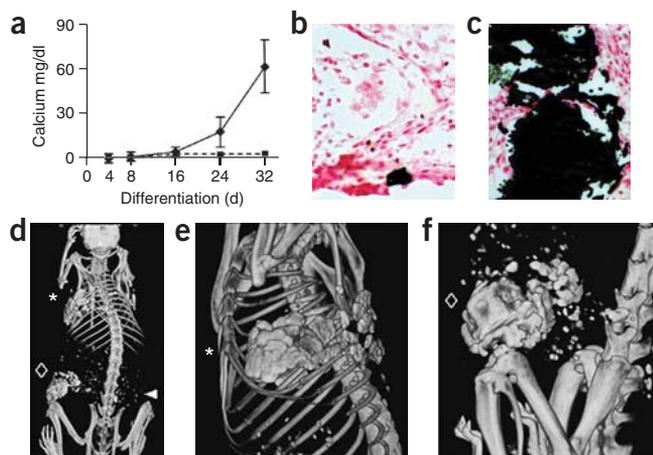

Figure 6 Tissue engineered bone from human AFS cells. (**a**) Calcium deposition of human AFS cells maintained in osteogenic differentiation medium *in vitro* was quantified by measuring calcium-cresolophthalein complex levels (closed line) and compared to that of undifferentiated (broken line) AFS cells. (**b**) Staining by method of von Kossa of control printed, unseeded alginate/collagen scaffold recovered 8 weeks after implantation in *nu/nu* mouse. (**c**) Staining by method of von Kossa of printed scaffold of AFS cells in alginate/collagen scaffold recovered 8 weeks after implantation; black staining indicates strong mineralization. (**d–f**) Micro CT scan of mouse 18 weeks after implantation of printed constructs. ◄ region of implantation of control scaffold without AFS cells; *, ◇ scaffolds seeded with AFS cells. Close-up views of seeded scaffolds indicated by symbols (**e,f**). Movie file of Micro CT scan is available in Supplementary Information (**Supplementary Video**).

range of adult somatic cells to which AFS cells can give rise remains to be determined.

Most current strategies in cell therapy and tissue engineering use cells obtained from biopsies of patients' tissues. However, for some applications autologous cells from an appropriate tissue cannot be readily obtained or expanded in culture. Stem cell lines that can be propagated easily, maintain genetic stability, and can be induced to differentiate into desired specialized cells may offer an alternative source[38].

AFS cells are able to differentiate along adipogenic, osteogenic, myogenic, endothelial, neurogenic and hepatic pathways. We show the acquisition of lineage-specific functionality by AFS cells differentiated *in vitro* toward neurons, osteoblasts and hepatocytes. For some cell types, acquisition of a full terminally differentiated phenotype is difficult to achieve in culture. The expression of characteristic functions may demand multistage protocols, as exemplified here by the expression of GIRK channels after neurogenic differentiation. Similarly, we observed urea secretion after hepatogenic differentiation. This represents a liver-specific function that requires the coordinated expression of several enzymes and specific mitochondrial amino acid transporters[39].

Another approach is to initiate differentiation *in vitro* and/or to use a regulatory gene to bias lineage-specific differentiation, and then allow cells to complete their development and acquisition of specialized functions *in vivo*. We find that neurogenically induced human AFS cells are capable of migration and integration into the parenchyma of the mouse brain, particularly in the twitcher disease model in which endogenous cells undergo degeneration, creating a 'cellular vacuum'[25]. In this study we did not assess in detail the phenotypes of the implanted human cells. However, the pattern of incorporation and morphologies of cells derived from the AFS cells appeared similar to those obtained previously in the same animal model after implantation of murine neural progenitor and stem-like cells[7]. In that case the donor-derived cells were identified as astrocytes and oligodendrocytes.

We also find that human AFS cells, seeded in a printed scaffold and exposed for one week to osteogenic-inducing medium, can form bone over a period of several months *in vivo* after subcutaneous implantation. After the induction period in culture, the cells expressed several genes characteristic of the osteoblast lineage, as shown by RT-PCR and secreted alkaline phosphatase, but did not yet show extensive deposition of calcium. By 2 months after implantation the constructs had undergone extensive mineralization, and after 4–5 months analysis by micro CT scanning confirmed the production of high-density tissue-engineered bone.

We conclude that AFS cells are pluripotent stem cells capable of giving rise to multiple lineages including representatives of all three embryonic germ layers. AFS cells hold potential for a variety of therapeutic applications. They are obtained from routine clinical amniocentesis specimens. We have isolated similar stem cell populations from prenatal chorionic villus biopsies and from placental biopsies obtained after full-term pregnancies. In the future, banking of these stem cells may provide a convenient source both for autologous therapy in later life and for matching of histocompatible donor cells with recipients.

METHODS

Isolation of AFS cells. Confluent back-up human amniocentesis cultures were received from the clinical cytogenetics laboratory, and cells were harvested by trypsinization and either expanded once or immediately subjected to immunoselection. AFS cells were grown in α-MEM medium (Gibco, Invitrogen) containing 15% ES-FBS, 1% glutamine and 1% penicillin/streptomycin

(Gibco), supplemented with 18% Chang B and 2% Chang C (Irvine Scientific) at 37 °C with 5% CO_2 atmosphere. For derivation of the murine AFS cell line for which the antigenic profile is shown (**Supplementary Fig. 2**), amniotic fluid was collected from a pregnant C57BL/6J mouse at embryonic day 11.5 d under light microscopy using a 30-gauge needle, and the recovered cells were cultured in 24-well dishes. After expansion to confluence (5–7 d), a single-cell suspension was prepared by gentle trypsinization. For immunoselection of c-Kit–positive human or murine cells from single-cell suspensions, the cells were incubated with a rabbit polyclonal antibody to CD117 (c-Kit), specific for the protein's extracellular domain (amino acids 23–322) (Santa Cruz Biotechnology). The CD117-positive cells were purified by incubation with magnetic Goat Anti-Rabbit IgG MicroBeads and selection on a Mini-MACS apparatus (Miltenyi Biotec) following the protocol recommended by the manufacturer. Human cells also were selected with monoclonal anti-CD117 directly conjugated to MicroBeads (Miltenyi Biotec). AFS cells were subcultured routinely at a dilution of 1:4 to 1:8 and not permitted to expand beyond ∼70% of confluence. Clonal AFS cell lines were generated by the limiting dilution method in 96-well plates.

Telomere length assay. Determination of the lengths of terminal restriction fragments was carried out using the TeloTAGGG Telomere Length Assay kit (Roche Molecular), according to the manufacturer's protocol.

Absence of tumor formation. Cells of each of four independent human AFS cell lines were injected into the rear leg muscles of 4-week-old male SCID/Beige mice (CB17/lcr.Cg-*Prkdc*^{scid}*Lyst*^{bg}/Crl) (Charles River Laboratories). For each line eight mice were injected with $3–8 \times 10^6$ cells per animal. Three months after injection the mice were killed and the injected muscles were subjected to histological examination. No tumors were observed.

Retroviral marking. Cells were infected with CMMP-eGFP[40] obtained from the Harvard Gene Therapy Initiative (Harvard Medical School). Cell clones that expressed GFP were obtained by limiting dilution and expanded. Clonality was confirmed by Southern blot analysis with a GFP probe. Genomic DNA was obtained from 1×10^7 cells by phenol-chloroform extraction. 10 mg of DNA was digested overnight at 37 °C with *Bam*HI and subjected to electrophoresis for 3 h in a 1% agarose gel. The separated fragments were denatured with alkali, transferred onto membranes (Zeta-Probe GT, Bio-Rad) and incubated with a ^{32}P-labeled GFP DNA probe overnight at 65 °C in Rapid-hyb buffer (Amersham Biosciences). The hybridized blots were washed according to the manufacturer's instructions. Bound probe was visualized by autoradiography.

Differentiation of AFS cells in culture. Cells were induced to differentiate in culture under the conditions described below. RNA was then extracted for RT-PCR analysis to confirm lineage-specific gene expression.

Adipogenic. Cells were seeded at a density of 3,000 cells/cm^2 and were cultured in DMEM low-glucose medium with 10% FBS, antibiotics (Pen/Strep, Gibco/BRL), and adipogenic supplements (1 μM dexamethasone, 1 mM 3-isobutyl-1-methylxanthine, 10 μg/ml insulin, 60 μM indomethacin (Sigma-Aldrich))[41].

Osteogenic. Cells were seeded at a density of 3,000 cells/cm^2 and were cultured in DMEM low-glucose medium with 10% FBS (FBS, Gibco/BRL), Pen/Strep and osteogenic supplements (100 nM dexamethasone, 10 mM beta-glycerophosphate (Sigma-Aldrich), 0.05 mM ascorbic acid-2-phosphate (Wako Chemicals))[41].

Myogenic. Cells were seeded at a density of 3,000 cells/cm^2 on plastic plates precoated with Matrigel (Collaborative Biomedical Products; incubation for 1 h at 37 °C at 1 mg/ml in DMEM) in DMEM low-glucose formulation containing 10% horse serum (Gibco/BRL), 0.5% chick embryo extract (Gibco/BRL) and Pen/Strep[42]. Twelve hours after seeding, 3 μM 5-aza-2′-deoxycytidine (5-azaC; Sigma-Aldrich) was added to the culture medium for 24 h. Incubation then continued in complete medium lacking 5-azaC, with medium changes every 3 d[43].

Endothelial. Cells were seeded at 3,000 cells/cm^2 on plastic plates precoated with gelatin and maintained in culture for 1 month in endothelial cell

medium-2 (EG-M™-2, Clonetics; Cambrex Bioproducts) supplemented with 10% FBS, and Pen/Strep. Recombinant human bFGF (StemCell Technologies) was added at intervals of 2 d at 2 ng/ml.

Neurogenic. Cells were seeded at a concentration of 3,000 cells/cm^2 on tissue culture plastic plates and cultured in DMEM low-glucose medium, Pen/Strep, supplemented with 2% DMSO, 200 μM butylated hydroxyanisole (BHA, Sigma-Aldrich)[44] and NGF (25 ng/ml). After 2 d the cells were returned to AFS growth medium lacking DMSO and BHA but still containing NGF. Fresh NGF was added at intervals of 2 d. For a two-stage induction procedure designed to yield dopaminergic cells, the AFS cells were first seeded on plates coated with fibronectin (1 μg/ml) and incubated in DMEM/F12 medium supplemented with N2 and 10 ng/ml bFGF for 8 d. Fresh bFGF was added every second day. Under these conditions over 80% of cells showed expression of nestin. The cells then were transferred to conditions biasing to production of dopaminergic neurons[23].

Hepatic. Cells were seeded at a density of 5,000 cells/cm^2 on plastic plates coated with Matrigel. They were expanded in AFS grown medium for 3 d to achieve a semi-confluent density. The medium was then changed to DMEM low-glucose formulation containing 15% FBS, 300 μM monothioglycerol (Sigma-Aldrich), 20 ng/ml hepatocyte growth factor (Sigma-Aldrich), 10 ng/ml oncostatin M (Sigma-Aldrich), 10^{-7} M dexamethasone (Sigma-Aldrich), 100 ng/ml FGF4 (Peprotech), 1× ITS (insulin, transferrin, selenium; Roche) and Pen/Strep. The cells were maintained in this differentiation medium for 2 weeks, with medium changes every third day. They were then harvested using trypsin, and plated into a collagen sandwich gel (0.11 mg/cm^2 for both the lower and upper layers)[45,46]. The hepatogenic cultures were maintained for up to 45 total days.

RT-PCR. Analysis of mRNA expression by reverse transcription (RT)-PCR was carried out using standard protocols. Primer sequences, PCR conditions and DNA fragment sizes are available on request. Transcripts encoding the following proteins were assessed for the specified lineages. Osteogenic: runx2, osteocalcin, alkaline phosphatase (AP). Myogenic: MyoD, Mrf4, desmin. Adipogenic: lipoprotein lipase (LPL), peroxisome proliferator activated receptor (Pparγ). Endothelial: CD31, vascular cell adhesion molecule-1 (VCAM-1). Hepatic: albumin. Neurogenic: nestin.

Nestin immunofluorescence. Cells were fixed with 4% paraformaldehyde in PBS and incubated with rabbit antiserum against nestin (Abcam) followed by FITC-labeled anti-rabbit IgG prepared in goats (Vector).

Whole-cell voltage clamp recording for GIRK channel. Cells were induced using the two-stage procedure designed to bias for dopaminergic neurons. Methods for voltage clamp recording from individual cells were similar to those described previously[47]. Coverslips (11 mm) containing cultured neuronal morphology cells derived from human AFS cells were transferred to a Zeiss Axioskop2 microscope with DIC and phase-contrast optics. During recording, cells were perfused with a standard bathing medium (140 mM NaCl, 5 mM KCl, 1.5 mM CaCl$_2$, 1 mM MgCl$_2$, 10 mM HEPES, pH 7.2, 37 °C). Whole-cell patch clamp electrodes (1- to 2-μm tip diameter, 2–5 MΩ) were pulled from borosilicate glass and filled with intracellular recording solution (100 mM KCH$_3$SO$_3$, 40 mM KCl, 0.2 mM EGTA, 0.02 mM CaCl$_2$, 1 mM MgCl$_2$, 2 mM ATP, 300 μM GTP, 10 mM HEPES buffer) for voltage clamp measures. Putative pyramidal neurons were visualized by phase contrast optics at 10–20× and recording electrodes placed next to the target while slight pressure was applied to the pipette contents. Appropriate gigaohm seals were achieved and the cell-attached patch was ruptured and the cells assessed for recording using an AxoClamp 2A amplifier. Voltage clamp recordings and command voltage steps were controlled by a Digidata 1320 controller connected to the AxoClamp. Recordings are analyzed using pClamp software (Axon Instruments). Compensation for junction potentials, series resistance (5-10 MΩ), leakage, and capacitance (10–30 pF) used amplifier controls, as well as subtraction within the pClamp program. After the seal stabilized, the bathing medium was changed to a 'high-K' solution suitable for recording the GIRK inward rectifier potassium channel (44 mM NaCl, 96 mM KCl, 1.5 mM CaCl$_2$, 1 mM MgCl$_2$, 10 mM HEPES, 1.0 μM TTX, pH 7.2, 37 °C). After 2 min in the bath, the

resting membrane potential rose to approximately –20 mV owing to the increased extracellular potassium. From this baseline, GIRK was evoked using transient (150 ms) hyperpolarizing voltage steps (–10 to –100 mV). After recording the inward currents, barium (BaCl$_2$ 200 μM) was added to the bath to block GIRK-specific inward current. A reduction in the amplitude of currents evoked by the same hyperpolarizing voltage step protocol in the presence of barium indicated the presence of GIRK-mediated potassium current.

L-glutamate secretion. Cells were treated with 50 mM KCl and extracellular L-glutamic acid was measured using the Amplex Red Glutamic Acid/Glutamate Oxidase Assay Kit (Molecular Probes, Invitrogen) following the manufacturer's recommendations.

AFS cell implantation in mouse brain. Human AFS cells were cultured in NGF-containing neurogenic induction medium. Thirteen wild-type CD-1 mice and twenty twitcher mice were transplanted as neonates (P0, P1 or P2) as described[25]. Homozygous twitcher pups were obtained by mating of heterozygous C57BL/6J-Galc^{twi} breeders (initially obtained from Jackson Laboratories). Briefly, cryoanesthetized pups received an intracerebroventricular injection of 5×10^4 cells in 2 μl of Hank's Balanced Salt Solution, containing trypan blue to facilitate injection, per ventricle. The lateral ventricles were visualized by transillumination of the head. Pups were returned to maternal care until weaning. Injected mice were sacrificed at intervals up to 2 months after transplantation and perfused with 4% paraformaldehyde. Cryopreserved brain tissue embedded in OCT (TissueTek, Miles) was serially sliced into 20-μm thick sections. Engrafted cells were detected by indirect immunofluorescence using a human-specific monoclonal antibody to a 65-kDa mitochondrial protein (MAB1273; Chemicon). The secondary anti-mouse IgG antibody was conjugated with either Texas Red or rhodamine. Nuclei of all cells were visualized by DAPI staining.

Urea production. Cells were differentiated to the hepatic lineage as described. The cells were incubated with NH$_4$Cl and ornithine, and a urea nitrogen assay kit (Sigma-Aldrich) was used following the manufacturer's instructions.

Printed scaffolds for bone differentiation. Printing of scaffolds and cells was carried out with an HP DeskJet 550C printer modified as previously described[28,29]. For three-dimensional structures, a z-axis module with a controlled elevator chamber was added to the modified printers. AFS cells were suspended in CaCl$_2$ solution at $2–3 \times 10^6$ cells/ml. Scaffold/cell constructs (rectangular sheet, $8 \times 6 \times 4$ mm) were fabricated by layer-by-layer printing of the AFS cells into an alginate/collagen composite gel. These samples were cultured in osteogenic differentiation medium for 1 week before implantation. Cell-free scaffolds incubated in this medium were prepared as a control.

Implantation of printed bone constructs. Experiments were performed according to ACUC protocols at Wake Forest University Health Sciences. Printed constructs with and without AFS cells were implanted subcutaneously into outbred athymic nude (nu/nu) mice (Charles River Laboratories). Three constructs with cells and one control construct lacking cells were implanted per mouse. After 8 weeks some implants were retrieved and analyzed by von Kossa's staining to visualize the mineralized tissues. The printed samples were fixed in 10% formalin and embedded in paraffin. Sections of 5 μm were prepared and stained with 5% AgNO$_3$ and counterstained with nuclear fast red solution. After 18 weeks implanted mice were scanned using a microCT scanner (MicroCAT).

Note: Supplementary information is available on the Nature Biotechnology website.

AUTHOR CONTRIBUTIONS
P.D.C., G.B. and M.M.S., cell isolation and *in vitro* differentiation; T.X. and J.J.Y., *in vivo* bone engineering; C.C.S. and S.S., neuronal differentiation; L.P.,

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