In Vivo Bone Formation by Progeny of Human Embryonic Stem Cells

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The derivation of osteogenic cells from human embryonic stem cells (hESCs) or from induced pluripotent stem cells for bone regeneration would be a welcome alternative to the use of adult stem cells. In an attempt to promote hESC osteogenic differentiation, cells of the HSF-6 line were cultured in differentiating conditions in vitro for prolonged periods of time ranging from 7 to 14.5 weeks, followed by in vivo transplantation into immunocompromised mice in conjunction with hydroxyapatite/tricalcium phosphate ceramic powder. Twelve different medium compositions were tested, along with a number of other variables in culture parameters. In differentiating conditions, HSF-6-derived cells demonstrated an array of diverse phenotypes reminiscent of multiple tissues, but after a few passages, acquired a more uniform, fibroblast-like morphology. Eight to 16 weeks post-transplantation, a group of transplants revealed the formation of histologically proven bone of human origin, including broad areas of multiple intertwining trabeculae, which represents by far the most extensive in vivo bone formation by the hESC-derived cells described to date. Knockout-Dulbecco's modified Eagle's medium-based media with fetal bovine serum, dexamethasone, and ascorbate promoted more frequent bone formation, while media based on α -modified minimum essential medium promoted teratoma formation in 12- to 20-week-old transplants. Transcription levels of pluripotency-related (*octamer binding protein 4*, *Nanog*), osteogenesis-related (*collagen type I*, *Runx2*, *alkaline phosphatase*, and *bone sialoprotein*), and chondrogenesis-related (*collagen types II and X*, and *aggrecan*) genes were not predictive of either bone or teratoma formation. The most extensive bone was formed by the strains that, following 4 passages in monolayer conditions, were cultured for 23 to 25 extra days on the surface of hydroxyapatite/tricalcium phosphate particles, suggesting that coculturing of hESC-derived cells with osteoconductive material may increase their osteogenic potential. While none of the conditions tested in this study, and elsewhere, ensured consistent bone formation by hESC-derived cells, our results may elucidate further directions toward the construction of bone on the basis of hESCs or an individual's own induced pluripotent stem cells.

Introduction

THE UTILITY OF pluripotent stem cells, such as human embryonic stem cells (hESCs), as a source of specific cell population is currently limited by poor understanding of the requirements for differentiation into various cell types [1]. Moreover, any therapeutic use of hESCs requires the creation of techniques that would reliably purge the hESC-derived populations of cells with tumorigenic potential, in addition to dealing with donor/recipient compatibility issues.

In the field of bone biology, it has been relatively easy to achieve in vitro osteogenesis by hESC progeny [2–10]. However, it has been convincingly demonstrated that osteogenic markers and phenotypes forced upon cells by artificial in vitro stimuli may have little relevance to the genuine differentiation potential of these cells in physiological conditions, that is, in vivo [11–18]. On the other hand, attempts

to receive in vivo bone formation by hESC progeny achieved, until recently, little more than small mineralized deposits, reminiscent of dystrophic calcification, with vague histology (no evidence of osteoblasts and osteocytes) and no proof of tissue origin [5,19,20]. Only recently was formation in vivo of small bone nodules by hESC descendants convincingly demonstrated [3]. However, even this innovative article lacked critical information regarding several aspects of their in vivo model: frequency or reliability of bone formation, bone development beyond the early 6-week time point, tumorigenic potential of hESC-derived strains, and the like.

In the current report, hESC-derived cells were cultured for prolonged periods of time in differentiating conditions, in either Knockout (KO)-Dulbecco's modified Eagle's medium (DMEM)-based or α -modified minimum essential medium (α MEM)-based media that included fetal bovine serum (FBS) or related products, with or without osteogenic supplements

or medium conditioned by osteogenic cells, along with a number of other variations in culture parameters. Eight to 16 weeks after *in vivo* transplantation, some of the strains formed histologically proven bone of human origin, considerably more extensive than hESC-based bone observed in earlier studies; other strains formed teratoma-like tumors in 12- to 20-week-old transplants. Both cultivation and transplantation conditions that promote *in vivo* osteogenesis versus tumor formation are analyzed.

Materials and Methods

Preparation of mouse embryonic fibroblasts

Embryos from 13.5-day-pregnant CF1 female mice were minced, after removal of heads and visceral organs, into small pieces and digested with 0.05% Trypsin-ethylenediaminetetraacetic acid (EDTA) (Invitrogen, Grand Island, NY; Cat. no. 25300) for 20 min at 37°C. The resulting suspensions were plated into T-175 flasks (BD Biosciences, Bedford, MA; Cat. no. 353112) in mouse embryonic fibroblast (MEF) medium: 88% KO-DMEM (Cat. no. 10829; Invitrogen), 10% heat-inactivated FBS (FBS-HyCl; HyClone, Logan, UT; Cat. no. 16000), 1% nonessential amino acids (NEAA; Cat. no. 11140), and 1% L-Glutamine (Cat. no. 25030; both Invitrogen), and cultured at 37°C. The ensuing MEFs were passaged, upon approaching confluence, with Trypsin-EDTA and split at a 1:5 ratio; the second, third, and fourth passages were performed in a similar way. MEFs of the fourth passage were irradiated with 65 Gy using a ¹³⁷Cs irradiator. Irradiated MEFs of the fourth passage and nonirradiated MEFs of the first, second, and third passages were aliquoted, frozen in a 1:1 mixture of MEF medium and 2×freezing medium (60% KO-DMEM, 20% FBS, and 20% dimethyl sulphoxide [Sigma Aldrich Inc., St. Louis, MO; Cat. no. D2650]), and stored in liquid nitrogen.

Cultures of undifferentiated hESCs

HSF-6, a governmentally approved line of hESCs, was purchased from the University of San Francisco (San Francisco, CA) at passage 35. In preparation for plating HSF-6 cells, 6-well plates (Nunc, Roskilde, Denmark; Cat. no. 140675) were covered with 0.1% gelatin solution (Sigma, St. Louis, MO; Cat. no. G1890), and irradiated fourth-passage MEFs were plated at 2×10^5 cells per well in 3 mL of MEF medium. After 2 days, the wells were washed with phosphate-buffered saline (Invitrogen; Cat. no. 10010), and HSF-6 cell colonies were plated over the subconfluent MEFs in 2.5 mL per well of hESC medium: 78.5% DMEM/F12 (Cat. no. 11330), 20% KO serum replacement (KOSR; Cat. no. 10828; both Invitrogen), 1% NEAA, 0.5% L-Glutamine, β -mercaptoethanol (3.5 μ L per 500 mL; Sigma; Cat. no. M7522), and 5 ng/mL human recombinant basic fibroblast growth factor (bFGF; Invitrogen; Cat. no. 13256-029). The cultures were maintained at 37°C, 5% CO₂ in air, 100% humidity, with daily medium replacements. The cultures were watched daily, and negative selection was routinely performed: HSF-6 colonies demonstrating signs of differentiation were encircled from beneath and removed with a Pasteur pipette.

Passages were performed every 5 to 7 days. The cultures were washed with phosphate-buffered saline and treated with 1 mg/mL solution of collagenase type IV (Invitrogen; Cat. no. 17104-019) for 20–40 min at 37°C. Then, depending on

the degree of detachment, the HSF-6 colonies were either washed off with a pipette or scraped with a cell scraper (Corning, Inc., Corning, NY; Cat. no. 3010). The detached colonies were allowed to sediment twice for 10 min each in 15 mL centrifuge tubes (Corning, Inc.; Cat. no. 430766) to wash off collagenase; after that, the colonies were broken into smaller pieces by triturating them several times using a pipette with a 1 mL tip pressed against the bottom of the tube. The resulting small colony pieces presented a spectrum of sizes but mostly consisted of several hundreds of cells. According to the widely accepted techniques used in this study, the colonies were never digested to form single-cell suspensions. The pieces were plated into fresh MEF-covered 6-well plates; the split ratio depended on the number and the size of initial colonies, aiming at generating several dozen (ideally, 30 to 50) small colonies per well. HSF-6 cells were frozen in 1:1 mixture of hESC medium and 2×freezing medium and stored in liquid nitrogen.

Cultures of HSF-6 cells in differentiating conditions

Undifferentiated HSF-6 cells at passage 60 (Experiment 1), 62 (Experiment 2), and 58 (Experiment 3) were used to generate differentiating cultures, an approach broadly based on the technique described by Olivier et al. [9]. First, HSF-6 colonies (normally, a few dozen per well) were allowed to grow in the MEF-covered 6-well plates for longer periods of time than is advisable for maintaining their undifferentiated status: for 9 days (Experiment 1), 6 days (Experiment 2), and 20 days (Experiment 3) after the previous passage. The colonies, together with the remaining MEFs, were then thoroughly scrapped with a 200 μ L tip attached to the pipette, leaving very few cells behind; the resulting fragments of variable sizes were pipetted without trituration, and allowed to sediment twice to wash off the original medium. The ensuing suspensions were transferred as follows: in Experiment 1, cells from either 1 well (Groups A through I) or 3 wells (Groups J and K) per T-25 flask (Nunc); in Experiment 2, cells from 2 wells per T-75 flask (Corning, Inc.; Cat. no. 430641); in Experiment 3, cells from one well per T-75 flask. The cultures generated in this fashion were designated "passage 0." The cultures were maintained at 37°C, either at 8% CO₂, according to Olivier et al. [9] (Experiments 1 and 2), or at conventional 5% CO₂ (Experiment 3).

In 3 differentiation experiments, 3 different sets of culture media were employed (Table 1); various medium compositions used in this study were named with consecutive capital letters, in alphabetical order. Unlike hESC medium, all these media included either serum or a related blood product; all media also contained 2 mM GlutaMAX™ (Cat. no. 35050) and a combination of 100 U/mL penicillin and 100 μ g/mL streptomycin sulfate (Cat. no. 15140, both Invitrogen). Each concoction employed for the differentiating cultures was based either on the literature [2–10] or on our own experience with osteogenic cell cultures. Altogether, 12 medium compositions were used to generate 16 hESC-derived cell strains (Table 1).

In Experiment 1, KO-DMEM originally employed for MEF cultures was used for hESC differentiation; to it, 1% NEAA, 2 mM GlutaMAX, and a combination of 100 U/mL penicillin and 100 μ g/mL streptomycin sulfate were added. Most of these compositions included 10% FBS: either heat-inactivated

TABLE 1. LONG-TERM CULTURES OF HSF-6-DERIVED CELLS IN DIFFERENTIATING CONDITIONS: CULTURE MEDIA, INTERVALS BETWEEN THE CONSECUTIVE PASSAGES, AND IN VIVO TRANSPLANTATION RESULTS

Exp No.	Culture medium	Pass	Pass	Pass	Pass	Bone score			
		1	2	3	4	8 weeks	12 weeks	16 weeks	20 weeks
1	A. KO-DMEM + 10% FBS-HyCl	41 days	12 days	6 days	8 days	1	0	0	ND
	B. KO-DMEM + 10% FBS-HyCl + D/A	45 days	7 days	3 days	4 days	1	0	1	ND
	Same, carpet (25 days)					0	0	0	ND
	C. KO-DMEM + 5% hPL		dead at the passage 0						
	D. KO-DMEM + 10% FBS-HyCl		dead at the passage 0						
	E. KO-DMEM + 10% FBS-HyCl + D/A		dead at the passage 0						
	F. KO-DMEM + 10% FBS-VB	40 days	6 days	6 days	8 days	1	0	ND	ND
	G. KO-DMEM + 10% FBS-VB + 1% ITS ⁺		dead at the passage 0						
	H. KO-DMEM + 10% FBS-VB + D/A	40 days	6 days	3 days	3 days	0	0	0	ND
	Same, carpet (25 days)					1	0	2	ND
	I. KO-DMEM + 10% FBS-VB + D/A + 1% ITS ⁺	34 days	14 days	7 days	5 days	1	0	ND	ND
	Same, carpet (41 days)					ND	0	0	ND
	J. KO-DMEM + 5% hPL	32 days		dead at the passage 1					
	K. KO-DMEM + 5% hPL + D/A + 1% ITS ⁺	32 days	7 days	6 days	4 days	0	0	0	ND
	Same, carpet (28 days)					0	0	0	ND
2	L. α MED + 20% FBS-VB + D/A	45 days	4 days	6 days		0	0	1	0
	Same, carpet (22 days)					0	0	0	0
	M. α MED + 20% FBS-VB + D/A	62 days	4 days	4 days		0	1	0	0
	Same, carpet (22 days)					0	0	0	0
	N. α MED + 20% FBS-VB + D/A	69 days	4 days	4 days		0	0	0	0
	Same, carpet (15 days)				ND	0	0	0	0
	O. α MED + 20% FBS-VB	45 days	4 days	6 days		0	1	0	0
	P. α MED + 20% FBS-VB	62 days	4 days	4 days		0	0	0	0
3	Same, carpet (22 days)					0	0	1	0
	Q. α MED + 20% FBS-VB	69 days	4 days	4 days		0	0	0	0
	Same, carpet (15 days)					0	0	0	0
	R. 50% (KO-DMEM + KOSR); 50% CM					0	0	0	ND
	Same, carpet (23 days)					0	2	0	0
	S. 50% (α MED + 20% FBS-VB + D/A); 50% CM + D/A					0	0	0	ND
	Same, carpet (23 days)	53 days	6 days	4 days	4 days	0	0	0	0
	T. 50% (KO-DMEM + KOSR + bFGF); 50% CM					0	0	0	ND
	U. 50% (α MED + 20% FBS-VB); 50% CM					0	0	0	ND
	Same, carpet (23 days)					0	0	0	0

Passages from 1 to 4: time intervals, in days, since the previous passage.

For media components' abbreviations, see Materials and Methods section.

Same, carpet (25 days): the same medium as above, but cells of the latest passage were cultured for additional 25 days on HA/TCP particles (carpet cultures, see Materials and Methods section).

Transplantation results: 8 weeks, 12 weeks, 16 weeks, 20 weeks, times post-transplantation, in weeks, when the transplants were harvested; 0, no bone formation; bold 1 and 2, definitive, histologically proven bone of scores 1 and 2, respectively; numbers in dark gray shadows: the transplants featuring teratoma formation.

ND, not done.

FBS used for MEF cultures (FBS-HyCl) or nonheat-inactivated FBS preselected for human bone marrow stromal cell (hBMSC) cultures (FBS-VB; Valley Biomedical, Winchester, VA; Cat. no. BS3033). To other media, 5% human platelet

lysate (hPL), kindly provided by Ms. Vicky Fellowes (Cell Processing Section, Department of Transfusion Medicine, NIH), was added instead of FBS. To prepare hPL, human platelets were purified following apheresis and added, at

1.5×10^9 /mL, to pooled, citrated apheresis plasma, together with 40 U/mL of heparin. Platelets were then destroyed by freezing and thawing, and debris was removed by centrifugation and filtration. To some of these media, 1% ITS⁺ (BD Biosciences; Cat. no. 354352) and/or the combination of 10^{-8} M dexamethasone (Dex; Sigma; Cat. No. 3 D-4902) and 10^{-4} M L-ascorbic acid phosphate magnesium salt n-hydrate (AscP; Wako Chemicals USA, Inc., Richmond, VA; Cat. no. 013-12061) were also added (Table 1).

In Experiment 2, media routinely used for hBMSC cultures were used: α MEM (Invitrogen; Cat. no. 12571) with 20% FBS-VB, with 2 mM GlutaMAX and a combination of 100 U/mL penicillin and 100 μ g/mL streptomycin sulfate, with or without Dex/AscP (hBMSC medium).

In Experiment 3, either hESC medium (KO-DMEM with 20% KOSR and NEAA, with or without bFGF) or hBMSC medium (α MEM with 20% FBS-VB, with or without Dex/AscP), both with 2 mM GlutaMAX and a combination of 100 U/mL penicillin and 100 μ g/mL streptomycin sulfate, was used at 50%. The remaining 50% consisted of hBMSC medium conditioned for 3 days by subconfluent cultures of hBMSCs of the third or fourth passage. Strains of hBMSCs used for such conditioning had been generated from pieces of bone and bone marrow received as surgical waste from patients undergoing reconstructive surgery, according to an institutionally approved protocol [21,22]. The conditioned medium (CM) was filtered through the Millex-GV, polyvinylidene fluoride (PVDF) 0.22 μ m, a syringe-driven filter unit (Millipore Corporation, Bedford, MA; Cat. no. SLGV033RS), before its use for the hESC-derived cell cultures.

Medium replacements in the passage 0 cultures, as well as in the later passage cultures, were performed usually twice a week, or more frequently when required by medium pH. To create culture conditions promoting mesenchymal differentiation [9], cells were kept in the passage 0 cultures long after

reaching confluence. Consequently, the first passage was performed at the following intervals (Table 1): 32 to 45 days (Experiment 1), 45 to 69 days (Experiment 2), or 53 days (Experiment 3) after the passage 0. The cultures were washed with Hanks' balanced salt solution (Invitrogen; Cat. no. 14170) and treated with 2 portions of a mixture of collagenase type IV (0.5 mg/mL) and dispase (1 mg/mL; Invitrogen; Cat. no. 17105-041) in Hanks' balanced salt solution at 37°C: first for 45 min, then for another 15 min. Warm 0.25% (1 $\times$) trypsin without EDTA (Invitrogen; Cat. no. 15050) was then added on the top of the remaining cells for 1 h at 37°C. In a few cases, cells still attached following the enzymatic treatments were detached with a cell scraper. All portions of detached cells were combined, pipetted, pelleted for 6 min at 1,000 rpm, and plated into either a T-75 flask (Experiment 1) or a T-175 flask (BD Biosciences; Cat. no. 353112; Experiments 2 and 3), into the same medium as their corresponding passage 0 culture. The second, third, and fourth passages were performed 3 to 14 days after the previous passage (Table 1) with 2 portions of warm Trypsin + EDTA, 5 min each, at 37°C. Both portions of detached cells were combined in cold culture medium, pipetted, pelleted, and resuspended, and live cells were counted with the SPotlite Hematocytometer (American Scientific Products, McGaw Park, IL; Cat. no. B3175) using Trypan Blue exclusion technique (0.4% Trypan Blue Solution; Sigma; Cat. no. T8154). After each passage, either the entire harvest of each particular strain or just a portion of it (Table 2) was plated into a T-175 flask for further propagation, using the same medium as for the generation and earlier cultivation of this strain. For 4 strains (B, H, N, and P), cell viability was determined at the last passages before their in vivo transplantation (strains B and H, passage 4; strains N and P, passage 3) by counting, in quadruplicate, the numbers of live and dead cells. In addition, attachment capabilities of these 4 strains were studied: 22 h after the cells had been plated into their last

TABLE 2. HSF-6-DERIVED STRAIN YIELDS AT THE CONSECUTIVE PASSAGES IN DIFFERENTIATING CONDITIONS

Exp No.	Culture medium	Numbers of cells harvested, $\times 10^6$			
		Pass 1	Pass 2	Pass 3	Pass 4
1	A. KO-DMEM + 10% FBS-HyCl	0.3	3.3	7.0 (1.5)	30.8
	B. KO-DMEM + 10% FBS-HyCl + D/A	1.4	6.0	12.4 (2.0)	93.0
	F. KO-DMEM + 10% FBS-VB	1.7	2.6	9.2 (1.5)	24.5
	H. KO-DMEM + 10% FBS-VB + D/A	0.8	2.7	10.1 (2.5)	54.5
	I. KO-DMEM + 10% FBS-VB + D/A + 1% ITS ⁺	0.3	0.4	3.2 (2.0)	6.2
	K. KO-DMEM + 5% hPL + D/A + 1% ITS ⁺	1.5	2.0	9.0 (1.5)	37.8
2	L. α MEM + 20% FBS-VB + D/A	11.7	15.7 (2.0)	140.5	ND
	M. α MEM + 20% FBS-VB + D/A	23.0 (4.0)	115.6 (2.0)	1,161.8	
	N. α MEM + 20% FBS-VB + D/A	23.0 (2.0)	86.3 (1.5)	817.0	
	O. α MEM + 20% FBS-VB	12.2	15.0 (2.0)	159.7	
	P. α MEM + 20% FBS-VB	13.8 (4.0)	76.2 (2.0)	670.6	
	Q. α MEM + 20% FBS-VB	15.6 (2.0)	60.8 (1.5)	753.9	
3	R. 50% (KO-DMEM + KOSR); 50% CM	NC (1/3)	6.6 (1.5)	14.1 (2.0)	47.2
	S. 50% (α MEM + 20% FBS-VB + D/A); 50% CM + D/A	(1/4)	56.0 (1.5)	552.5 (1.5)	4,714.6
	T. 50% (KO-DMEM + KOSR + bFGF); 50% CM	(1/3)	12.9 (1.5)	47.3 (2.0)	201.0
	U. 50% (α MEM + 20% FBS-VB); 50% CM	(1/3)	40.8 (1.5)	315.5 (1.5)	1,977.1

Prospective cell yields at passages 2 to 4 were calculated based on the proportions of total cell harvests that had been plated for further expansion (shown in parentheses, in millions, or in shares of the total harvests in Experiment 3, passage 1). If no parentheses are shown, all harvested cells were plated for further expansion.

NC, not counted; ND, not done.

passage cultures, unattached cells were removed and pelleted, and their numbers were counted, in quadruplicate.

For each strain, cells of the last passage (passage 3 or 4, depending on the experiment; see Table 1) were used for in vivo transplantation using a technique described below. In addition to this technique, most strains were transplanted using the carpet culture technique, based on the novel cultivation and transplantation approach originally developed for hBMSCs (Mankani, Kuznetsov, Geron Robey, article in preparation). From 1.0×10^6 to 1.5×10^6 HSF-6-derived cells of the third or fourth passage were seeded into a 35-mm bacteriological, nontissue culture-treated dish (Fisher Scientific, Pittsburgh, PA; Cat. no. 08757100A) on the top of a layer of an osteoconductive material, hydroxyapatite/tricalcium phosphate (HA/TCP) ceramic powder (particle size, 0.5–1 mm; Zimmer, Warsaw, IN; Cat. no. 97-1109-231) in the same medium as their corresponding monolayer cultures. As much as 300 mg of HA/TCP particles was used to evenly cover the bottom of a 35-mm dish with a single layer of the vehicle. After the cells had adhered to the particles, medium replacements were performed twice a week. Fifteen to 41 days later (Table 1), when the cells and the particles formed cohesive layers (carpets), the latter were used for in vivo transplantation.

Both undifferentiated and differentiating HSF-6 cultures were observed, and pictures were taken, using a Nikon Diaphot inverted microscope (Nikon Instruments Inc., Melville, NY) equipped with a Retiga 1300 camera (Quantitative Imaging Corp., Surrey, Canada; Cat. no. 01-RET-1300-CLR-12-C).

Analysis of karyotypes of undifferentiated HSF-6 cells and of differentiated HSF-6-derived strains

Karyotypes of undifferentiated HSF-6 cells and of 2 HSF-6-derived strains at their last passages before transplantation were analyzed. Fragments of HSF-6 colonies from passage 79 were plated into 2 MEF-covered T-25 flasks with nonvented caps (Invitrogen; Cat. no. 353081), at 3 wells and 1.5 wells per flask, in hESC medium. Cells of strain H at the passage 4 and of strain N at the passage 3 were plated into 2 T-25 flasks each, at 2×10^5 and 5×10^5 cells per flask, in their corresponding media. Two days later, when the colony fragments demonstrated good attachment and growth, and cells of both HSF-6-derived strains reached between 50% and 75% confluence, the cultures were sent to Cell Line Genetics (Madison, WI), where karyotypes of 20 cells per culture type were studied using the G-banding technique.

In vivo transplantation of undifferentiated HSF-6 colonies and differentiated HSF-6-derived strains

In this article, in vivo transplantation techniques originally developed for hBMSCs [21,22] were employed for transplantation of both undifferentiated HSF-6 cells and HSF-6-derived differentiated cell strains. Briefly, undifferentiated HSF-6 colonies of passage 62 were detached with collagenase, and either triturated or left unbroken. The colonies from one well were mixed with 40 mg of HA/TCP powder in a single 1.8 mL CryoTube tube (Nunc; Cat. no. 375418) containing 1 mL of the hESC medium. Differentiated HSF-6-derived cells grown under various culture conditions (Table 1) were mixed with 40 mg HA/TCP at the following densities: Experiment 1,

$1.5\text{--}2.0 \times 10^6$; Experiment 2, 2.5×10^6 ; and Experiment 3, $1.4\text{--}2.1 \times 10^6$ cells per tube in 1 mL of the medium the cells had been cultured in. The colonies and their fragments, or differentiated cells, were allowed to attach to the particles for 90 min at 37°C with slow rotation (25 rpm). The particles with attached cells, together with unattached cells or colony fragments, were then collected by brief centrifugation (1,000 rpm, 1 min), thus minimizing the loss of unattached material; the cell-particle mixtures were then transplanted in vivo. After such procedures, >80% of cells had been shown earlier to be either directly attached to HA/TCP particles or otherwise included into the transplant [23]. The carpet cultures that represented cohesive sheets of HA/TCP particles bonded by cells were cut into 4 to 6 equal pieces each, and the pieces were used for in vivo transplantation. The transplants were placed through the central dorsal skin incision into subcutaneous pockets in the backs of the 8–15-week-old immunodeficient female beige homozygous mice (bg-nu/nu-xid; Harlan Sprague Dawley, Indianapolis, IN). One transplant was placed into each pocket, with up to 6 transplants per animal, and the incision was closed with the 9-mm steel wound clips (Roboz Surgical Instrument Co., Inc., Rockville, MD; Cat. no. RS-9262). Surgery was performed under isoflurane anesthesia in accordance to the specifications of an institutionally approved small animal protocol. The transplants were harvested 8, 12, 16, and 20 weeks post-transplantation, fixed with fresh 4% phosphate-buffered formalin (Sigma; Cat. no. H-6148), and demineralized with 10% EDTA, pH 8.0 (Quality Biologicals, Inc., Gaithersburg, MD; Cat. no. 351-027-101). The transplants (disc-shaped structures 0.7–1.0 mm thick) were then cut into 2 halves along the largest axes and embedded in paraffin with their cut surfaces toward the knife. Serial 6- μ m-thick sections were prepared in such a way that most areas of the transplant were represented on the slides. The sections were deparaffinized, hydrated, and stained with hematoxylin and eosin. The stained sections were examined histologically, and pictures were taken using a Zeiss Axioplan 2 microscope equipped with an AxioCam HRc and AxioCam MR monochrome cameras (all Carl Zeiss, Inc., Thornwood, NY). The extent of bone, if any, within each transplant was scored on a semiquantitative, exponential scale from 0 to 4 in a manner similar to that described previously for the transplants of human BMSCs (of both adult and pediatric origin) [21]:

- score 0, no bone formation;
- score 1, minimal bone formation, just a single or a few bone trabeculae, in one or in a few sections;
- score 2, weak bone formation, bone is in several parts of several sections, but it occupies only a small portion of each section;
- score 3, moderate bone formation, bone occupies a significant portion but less than one half of most sections;
- score 4, abundant bone formation, bone occupies greater than one half of each section.

When the bone scores reported on this scale have been compared to histomorphometric measurements of tissue sections, a high correlation was previously observed between the bone score and the square root of the fraction of bone area to total transplant area ($r=0.973$) [23]. This scale measures relative abundance of bone versus nonbone tissues in the transplants, so despite the fact that transplants of

hESC-derived cells demonstrated broader tissue repertoire than transplants of BMSCs, the scale can be applied to the former, as well as to the latter. At the same time, bone formation in transplants of hESC-derived cells was less abundant, so only scores 0, 1, and 2 were applicable.

Analysis of the origin of new bone

To determine the origin of cells forming new bone, in situ hybridization for human-specific *alu* repetitive DNA sequences was performed. Deparaffinized sections of the transplants were treated for 15 min with 3% hydrogen peroxide (Fisher Scientific, Fair Lawn, NJ; Cat. no. H325-500) followed by consecutive applications of 0.1% solution of Proteinase K (InnoGenex, San Ramon, CA; Cat. no. BS-1420-06) for 10 to 30 min and of 1% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA; Cat. no. 15710) for 10 min. Hybridization, detection, and staining were then performed using the Rembrandt® Series In Situ Hybridization and Detection kit (Invitrogen; Cat. no. A001K.9905) following the manufacturer's recommendations. Sections of human and mouse bone were used as positive and negative controls, respectively. The stained sections were mounted, without counterstaining, in the VectaMount™ AQ aqueous mounting medium (Vector Laboratories, Inc., Burlingame, CA; Cat. no. H-5501) for microscopic examination.

Reverse transcription-mediated polymerase chain reaction and quantitative real-time polymerase chain reaction

For both reverse transcription (RT)-polymerase chain reaction (PCR) and quantitative real-time (Q-RT) PCR, RNA was extracted from undifferentiated HSF-6 cells of passage 66 and from 4 differentiated, HSF-6-derived strains: strains B and H at passage 4 and strains N and P at passage 3. For the HSF-6-derived strains, these were the last passages before either transplantation or initiation of carpet cultures. A strain of hBMSCs shown to form abundant bone upon in vivo transplantation that had been generated from a 1-year-old male with polydactyly was used as a positive control for expression of genes associated with osteogenesis and chondrogenesis; this strain had been generated in medium with Dex/AscP and used at passage 3. Total cellular RNA was extracted using RNeasy® MiniKit (Qiagen, Valencia, CA; Cat. no. 74104) following the manufacturer's instructions. On-column DNA digestion was performed using the RNase-free DNase set (Qiagen; Cat. no. 79254), and 0.4 µg of RNA was used for cDNA synthesis by the reverse transcription.

RT-PCR was performed to determine expression of genes regulating pluripotency: *octamer binding protein 4* (*Oct4*) and *Nanog*. cDNA was synthesized using SuperScript III First Strand Synthesis (Invitrogen; Cat. no. 18080-051) according to the manufacturer's instructions. Forward and reverse primer sequences, amplicon sizes, and GenBank access numbers are listed in Table 3. *Glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) was included as a housekeeping gene. PCR was performed using Platinum Tag DNA polymerase (Invitrogen; Cat. no. 10966-018) on the GeneAmp® PCR System 9700 cyclor (Applied Biosystems, Inc., Foster City, CA). After a single incubation at 94°C for 2 min to completely denature the template and activate the enzyme,

samples were run at the following settings: denaturation at 94°C for 30 s; annealing at 54.0°C for 30 s, followed by extension at 72°C for 60 s, for a total of 35 (*GAPDH*) or 30 cycles (*Oct4* and *Nanog*); and final elongation at 72°C for 7 min. PCR products were analyzed by electrophoresis on 6% TBE gels (Invitrogen; Cat. no. EC62652BOX) stained with the SYBR® Safe DNA gel stain (Invitrogen; Cat. no. S33102). A 100 bp DNA ladder, Trailt™ (Invitrogen; Cat. no. 10488-058) was applied to determine the sizes of DNA bands. The gels were imaged with the EpiChem³ Darkroom equipped with the 3UV™ Transilluminator (both UVP, Upland, CA).

Q-RT-PCR was performed to compare the expression levels of genes associated with osteogenic differentiation (*collagen type I*, *Runx2*, *alkaline phosphatase* [ALP], and *bone sialoprotein* [BSP]), of those associated with chondrogenic differentiation (*collagen types II* and *X*, and *aggrecan*), and of markers of 2 germ layers not represented by the above-mentioned genes (ectoderm—*keratin* and *neurofilament*; endoderm—*α1-antitrypsin* and *α fetoprotein*). For Q-RT-PCR, 3 independent sets of total cellular RNA, and 3 corresponding sets of cDNA, were generated from each cell strain under analysis. cDNA was synthesized using iScript cDNA (BIO-RAD, Hercules, CA; Cat. no. 170-8890). Primers for PCR amplification of cDNA were designed using Beacon Designer 6 software (Premier Biosoft International, Paolo Alto, CA) employing parameters set for real-time PCR with SYBR Green Design, for a Tm of 62°C ± 2°C, with an amplicon size of 75–150 bp (Table 3). Real-time PCR was performed to quantify expression of mRNA using a MyiQ™ Single Color Real-Time PCR Detection System (BIO-RAD) with iQ SYBR Green Supermix (BIO-RAD; Cat. no. 170-8882). For each primer set, melting curves were examined, and products were analyzed by electrophoresis, to assure that a single peak and a single band were produced, respectively. Q-RT-PCR results, received in triplicate and expressed as cycle threshold (CT) values, were normalized to the levels of *GAPDH*, generating δCT values; levels of relative expression were calculated as $2^{-\delta CT}$.

Statistical analysis

Analysis of variance and post-test comparison were performed using the Bonferroni multiple comparison test (InStat; GraphPad, San Diego, CA). Differences were considered statistically significant at $P < 0.05$. In Fig. 6, each bar represents mean ± standard error of mean (SEM); values of statistical significance are shown in the legend to the figure.

Results

Undifferentiated HSF-6 cells in vitro and in vivo

Undifferentiated HSF-6 cells were cultured in the conventional hESC conditions, in hESC medium, on top of MEF feeder cell layer, until passage 81. After each passage, rapidly growing colonies developed that were comprised of hundreds, and later of thousands of small, tightly packed cells with a high nuclear/cytoplasmic ratio (Fig. 1a–d). Most colonies demonstrated strictly delineated, sharp borders and absence of internal structuralization, consistent with their undifferentiated nature. Colonies showing signs of differentiation were routinely removed in the process of negative selection. Of many factors important for HSF-6 cultivation,

TABLE 3. PRIMERS USED FOR POLYMERASE CHAIN REACTION

<i>Gene</i>	<i>GenBank access No.</i>	<i>Forward primer, base pairs</i>	<i>Reverse primer, base pairs</i>	<i>cDNA, bp</i>
RT-PCR				
<i>Nanog</i>	NM_024865.2	ttccttctccatggatctg 377–396	tctgctggaggctgaggtat 589–570	213
<i>Oct4</i>	NM_203289.3	agtgagaggcaacctggaga 518–537	gccggttacagaaccacact 642–623	125
Q-RT-PCR				
<i>Col type I alpha 1</i>	NM_000088.3	tggcaaagaaggcggcaaagg 2829–2849	agcaccagcaggaccatcagc 2961–2941	133
<i>Runx2</i>	NM_004348.3	cccaggcagttcccaagcatttc 1475–1497	ggtagtgagtggtggcgacatac 1613–1590	139
<i>ALP</i>	NM_000478.3	gcaccgccaccgcctacc 592–609	ccacagattcccagcgtccttg 741–719	150
<i>BSP</i>	NM_004967.3	agaggaggaggaagaaggagagac 326–349	gccagtggttagcagaaagtg 437–415	112
<i>Col type 2 alpha 1</i>	NM_033150.2	gccccagaggtgacaaaggagag 3312–3334	aaggaccagcaggaccagaagc 3452–3431	141
<i>Aggrekan</i>	NM_001135.2	ggcatttcagcgggttccttctcc 2400–2422	cagcagttgtctctcttctacgg 2535–2512	136
<i>Col type X alpha 1</i>	NM_000493.3	ggacaacagggaaccacaggag 571–592	tggacgaccaggagcaccatc 693–671	123
<i>Keratin 14</i>	NM_000526.4	agatgattggcagcgtggaggag 1185–1207	ccagcaggcggcggtagg 1319–1302	135
<i>Keratin 17</i>	NM_000422.2	ttggcagcgtggaggagcag 1158–1177	ccagcaggcggcggtagg 1286–1269	129
<i>Neurofilament</i>	NM_021076.3	gcccaattcttctcgcttccag 1318–1341	ggtctctctgtctgttctccac 1463–1440	146
<i>α Feto-protein</i>	NM_001134.1	gacccgaactttccaagccataac 740–763	gcagacaatccagcacatctctc 882–859	143
<i>α-1 antitrypsin</i>	NM_001002236.2	ggagaggaagcacatcacccattg 2729–2752	agcaacaggcacaagaagtgcagg 2830–2807	102
<i>GAPDH</i>	NM_002046.3	gcctcccgcttcgctctctg 15–34	cgttgactccgaccttcaccttc 130–107	116

Q-RT-PCR, quantitative-real-time-polymerase chain reaction.

the most crucial were the proper density of the MEF layer, the intensity of trituration at the time of passage, and the extreme temperature sensitivity of undifferentiated HSF-6 cells.

Twenty metaphases of HSF-6 cells of passage 79 were examined using G-banding. Nineteen cells demonstrated an apparently normal female karyotype (Fig. 1e), and one cell showed loss of chromosome 13, which is considered an insignificant, nonclonal aberration. These results are consistent with a normal female karyotype (Cell Line Genetics).

When undifferentiated HSF-6 colonies had been transplanted in vivo, either following trituration or avoiding it, 12- to 20-week old transplants developed multiple tumors, apparently teratomas. The tumors varied in size from macroscopic (pea-size) to microscopic and included several tissue types, such as diverse epithelial layers and cords (Fig. 1g), and nodules of undifferentiated mesenchymal condensation-type cells, the latter comprising the bulk of the tumors (Fig. 1f–h). Between the tumors and the vehicle particles, a variety of nondescriptive tissues developed (not shown); no bone formation was observed in these transplants, either within or outside the tumors, at any time post-transplantation.

Before transferring HSF-6 cells into differentiating conditions, HSF-6 colonies were allowed to grow in the MEF-covered 6-well plates for several more days than is advisable for maintaining their undifferentiated status (see Materials

and Methods section). During this additional time, the colonies became very large, each colony consisting of tens of thousands of tightly packed cells, some colonies coalescing with each other; at this stage, roughly 3×10^5 to 10×10^5 HSF-6 cells could be harvested from each well.

Growth of HSF-6-derived cells in long-term cultures in differentiating conditions

Our approach to in vitro differentiation of hESC-derived cells was roughly based on the assumption formulated by Olivier et al. [9] that emphasized the need of prolonged in vitro cultivation and starvation for hESC mesenchymal differentiation. While having implemented the long cultivation part of the approach, we were unable to follow their starvation regimen of weekly or even every other week medium replacements, due to rapid cell proliferation and consequent medium acidification that took place in our cultures. After having lost 5 HSF-6-derived strains, C, D, E, G, and J, from Experiment 1 (see Table 1) due to apparent medium over-acidification, we switched to a more conventional medium replacement schedule: twice a week, or more often, as dictated by the medium pH. From the remaining cultures, 16 strains were generated using 12 different medium compositions: A, B, F, H, I, K, L through N, O through Q, R, S, T, and U (Table 1).

FIG. 1. Cultures of undifferentiated HSF-6 cells, their karyotypic analysis, and tissues formed by them upon in vivo transplantation. **(a–d)** Colonies of HSF-6 cells in passage 59 cultures. Each colony consists of hundreds to thousands small, tightly packed cells with high nuclear/cytoplasmic ratio. Note strictly delineated, sharp borders of the colonies and absence of internal structuralization, demonstrating the undifferentiated nature of the colonies. The colonies lay on/among the irradiated mouse embryonic fibroblasts. Live cultures; **(a, b)** 4 $\times$; **(c, d)** 10 $\times$. **(e)** HSF-6 cell of passage 79 demonstrating normal female karyotype. **(f–h)** The transplants of undifferentiated HSF-6 cells: **(f)** 12-week-old; **(g, h)** 16-week-old. v, vehicle; m, mesenchymal-type cell condensation; e, epithelial tissue. H&E; **(f, g)** 10 $\times$; **(h)** 20 $\times$. H&E, hematoxylin and eosin. Color images available online at www.liebertonline.com/scd.

In all 3 experiments, most HSF-6 colony fragments plated at passage 0 in the various media formulations, attached to the plastic surfaces; in a few days, they were well spread and exhibited areas of outgrowth. These patches, or islands, of attached cells grew rapidly and soon developed large spreading cellular fields and areas consisting of multilayers. The time when most of the passage 0 cultures reached con-

fluence varied broadly between the experiments and constituted, in Experiment 1, 10 days, in Experiment 2, 28 days, and in Experiment 3, 17 days after plating. After the passage 0 cultures had reached confluence, they were left undisturbed, with 2 or more medium replacements per week, for additional periods of time. Passage 1 was performed between 32 and 45 days (Experiment 1), between 45 and 69

days (Experiment 2), and at 53 days (Experiment 3) after plating (Table 1). During this additional time before passage 1, the passage 0 cultures became superconfluent, with broad areas of dense multilayers.

The cell morphologies within the passage 0 cultures were extremely diverse (Fig. 2a–g) revealing epithelial- (Fig. 2b, g), mesenchymal- (Fig. 2d), and neural-like (Fig. 2c) cell areas, among other, less definitive phenotypes, as well as complex structures formed on the top of underlying layers (Fig. 2a, e). In many cases, different parts of a culture displayed different morphologies, revealing a variety of differentiations within a single culture. There were no obvious correlations between the types of media used in the cultures and the cell morphologies developed by the 0 passage strains. With consecutive passages, cell morphology gradually became more uniform, such that by passage 3, most strains displayed predominantly fibroblastic, yet somewhat distinctive, morphologies (Fig. 2h–j).

HSF-6-derived cells proliferated far more rapidly and generated significantly higher cell yields in α MED-based media than in media based on KO-DMEM (Table 2), despite the fact that in the passage 0 cultures, initial confluence

was reached later by strains cultured in α MED-based than in KO-DMEM-based media. Among other ingredients, Dex/AsCP seemed to further stimulate cell proliferation (Table 2). Comparing cell yield numbers provided in Table 2 with time intervals between the consecutive passages (Table 1), one can see that different strains demonstrated drastically different rates of proliferation and that, indeed, in several α MED-based media, cell proliferation was extremely intense: starting with 1 or 2 wells containing a few dozen undifferentiated HSF-6 colonies, or roughly one million cells, cell numbers could reach billions in just 3 or 4 passages (Table 2, strains M, S, U).

All analyzed HSF-6-derived strains demonstrated high viability and high attachment to culture plastic. When cells of 4 strains were detached at their last passage before transplantation, dead cells in strains B (passage 4), H (passage 4), N (passage 3), and P (passage 3) constituted (mean $\pm$ SEM): $2.54\% \pm 0.90\%$, $1.89\% \pm 0.93\%$, $1.59\% \pm 0.25\%$, and $1.01\% \pm 0.64\%$, respectively, of total cell numbers. Twenty-two hours after these 4 cell populations had been plated into the new flasks, unattached cells in strains B, H, N, and P constituted (mean $\pm$ SEM) $1.24\% \pm 0.39\%$, $2.49\% \pm 0.28\%$, $0.58\% \pm 0.20\%$, and $1.85\% \pm 0.36\%$, respectively, of total plated cell numbers.

FIG. 2. Cultures of HSF-6-derived cells in conditions promoting differentiation. (a–g) Monolayer cultures, passage 0: (a) strain B, 41 days; (b) strain C, 20 days; (c) strain F, 34 days; (d) strain H, 34 days; (e) strain I, 34 days; (f) strain M, 38 days; (g) strain P, 38 days. (h–j) Monolayer cultures, passage 3: (h) strain A, 4 days; (i) strain K, 2 days; (j) strain R, 3 days. (k) Strain H, passage 4, growing on the hydroxyapatite/tricalcium phosphate particles (carpet culture) 23 days. Note strong cell membranes (arrow) covering hydroxyapatite/tricalcium phosphate vehicle particles (black). Strains A, B, C, F, H, I, and K belong to Experiment 1; strains M and P, to Experiment 2; and strain R, to Experiment 3. Live cultures; (a, d, f, k) 4 $\times$; (b, c, e, g, h, i, j) 10 $\times$. (l) Strain H cell of passage 4 demonstrating normal female karyotype.

Twenty metaphases each of 2 HSF-6-derived strains, strain H at passage 4 and of strain N at passage 3, were examined using G-banding. In strain H, 19 cells demonstrated an apparently normal female karyotype (Fig. 2l), and one cell showed a nonclonal chromosome aberration 46,XX,del(3)(p10), which most likely represented a technical artifact. In strain N, all 20 cells demonstrated an apparently normal female karyotype (not shown). These results are consistent with a normal female karyotype of strains H and N (Cell Line Genetics).

In an attempt to increase the osteogenic potential of HSF-6-derived strains, long-term cultures of the third- or fourth-passage cells on HA/TCP particles were also generated (carpet cultures). When cells were added to bacteriological, nontissue culture-treated dishes containing HA/TCP particles, most cells adhered to the latter. After 2 to 3 weeks of cultivation in such conditions, cell proliferation resulted in the formation of rigid sheets of cells and HA/TCP particles (Fig. 2k), such that each culture formed a disc-shaped cohe-

sive structure, strong enough to be lifted with forceps and cut with scissors.

In vivo transplants of differentiated HSF-6-derived cells

Histologically proven bone was formed in a number of transplants of differentiated HSF-6-derived cells; definitive bone was found in at least one transplant of 10 out of 16 strains (Table 1, Fig. 3a–f). The most extensive bone of score 2 was formed in a 16-week-old transplant of strain H, grown as a carpet (Fig. 3a, b, f), and in a 12-week-old transplant of strain R, grown as a carpet (Fig. 3c). In both transplants, bone was observed in most sections and in multiple areas of each section. It featured numerous trabeculae deposited on the surfaces of ceramic particles and containing numerous embedded osteocytes, as well as occasional osteoblasts located at the bone forming surface (Fig. 3b). The new bone exhibited intense green fluorescence under ultraviolet light, consistent

FIG. 3. Transplants of differentiated HSF-6-derived cells in immunocompromised mice. (a–f) Transplant areas featuring bone formation: (a, b, f) bone of score 2 in different areas of 16-week-old transplant of strain H, carpet; (c) bone of score 2 in 12-week-old transplant of strain R, carpet; (d) bone of score 1 in 16-week-old transplant of strain L; (e) bone of score 1 in 8-week-old transplant of strain H, carpet; (g) human bone (positive control for *alu*); (h) mouse bone (negative control for *alu*); (i) nondescriptive tissue formed in 12-week-old transplant of strain B, carpet; (j) mesenchymal-type cell condensation formed in 16-week-old transplant of strain Q, carpet; (k) mesenchymal-type cell condensations formed in 20-week-old transplant of strain M; (l) cartilage formed in 20-week-old transplant of strain S, carpet. Strains B and H belong to Experiment 1; strains L, M, and Q, to Experiment 2; and strains R and S, to Experiment 3. v, vehicle; b, bone; m, mesenchymal-type cell condensation; mc, mesenchymal-type cell condensation showing signs of chondrogenic differentiation; c, cartilage; black arrows, osteocytes; yellow arrows, osteoblasts. (a–e, i–l) H&E, (f–h) staining for *alu*; (a, i, l) 10×; (c, d, f–h, j, k) 20×; (b, e) 40×. Color images available online at www.liebertonline.com/scd.

FIG. 4. Characterization of score 2 bone formed in the 16-week-old transplant of strain H, carpet. **(a)** Under ultraviolet light, new bone demonstrates intense green fluorescence consistent with highly mineralized bone matrix. **(b)** Under polarized light, collagen bundles can be observed in bone matrix that are organized in parallel patterns consistent with the lamellar structure of new bone. b, bone; H&E; **(a)** 20 $\times$; **(b)** 40 $\times$. Color images available online at www.liebertonline.com/scd.

with well-mineralized matrix (Fig. 4a); under polarized light, it demonstrated collagen bundles organized in parallel patterns, indicative of its lamellar structure (Fig. 4b). In several other transplants from Experiments 1 and 2, bone of score 1 was observed (Table 1, Figs. 3d, e and 5). This bone was found only in several sections of a transplant and usually in a single area of a section, and was represented by a few trabeculae or by a solitary trabecula. In all transplants, the new bone was formed on the surface of HA/TCP particles; the areas of the new bone were surrounded by fibrous tissue.

The osteocytes of the new bone invariably stained positively for *alu* (Fig. 3f; g, human bone, positive control; h,

mouse bone, negative control) demonstrating its human origin. While some culture conditions, such as those employed in cultures of strains B and H (KO-DMEM with 10% FBS of either lot, with Dex/AscP) seemed to promote bone formation more often than others (Table 1), not a single culture condition ensured consistent bone formation in each transplant of a particular strain (Fig. 5). Cells from 13 strains were transplanted using both regular transplantation technique and carpet technique. Out of these strains, 3 (H, P, and R) formed bone only in carpet transplants but not in regular ones, 4 strains (B, I, L, and M) formed bone only in regular but not in carpet transplants, 6 strains formed no bone in any transplants, and

FIG. 5. Bone and teratoma formation in the transplants of HSF-6-derived strains. Cells of each strain were transplanted either using regular technique only (strains A, F, and O) or using both regular and carpet techniques. From 2 to 4 transplants were harvested per strain/per technique. Each vertical rectangular represents a single transplant. The bars represent, from bottom to top: 8-, 12-, 16-, and 20-week-old transplants of a strain/technique. The numbers in the bars represent the bone scores of the transplants. # signs mark the transplants featuring teratoma formation.

not a single strain formed bone in both regular and carpet transplants (Table 1, Fig. 5). The most extensive bone of score 2, however, was formed only in carpet transplants of strains H and R (Table 1; Fig. 5).

Most transplants negative for bone formation demonstrated areas of nondescriptive tissues around HA/TCP particles; an example of such growth is shown in Fig. 3i. In addition, numerous 12- to 20-week-old transplants in Experiment 2 and a single transplant in Experiment 1 demonstrated nodules composed of mesenchymal condensation-type cells, very similar to the areas prevalent in tumors formed by undifferentiated HSF-6 cells (Fig. 3j). Contrary to the latter tumors, however, all teratoma-like nodules formed by differentiated HSF-6-derived cells were microscopic, and some of them exhibited more differentiated morphology, occasionally featuring cartilage-like lacunae (Fig. 3k), whereas in some transplants, areas of more differentiated, metachromatic cartilaginous tissue were formed (Fig. 3l). The incidence of teratoma formation in the transplants of HSF-6-derived strains is shown in Table 1 (numbers in dark gray shadows) and in Fig. 5 (# sign). Several transplants in Experiment 2 featured both teratoma-like nodules and bone tissue (Table 1, Fig. 5). Not a single teratoma-like nodule was formed in Experiment 3 where culture medium included 50% of medium conditioned by hBMSCs; also, no teratoma-like nodules were formed in any transplants harvested at the earliest time point, 8 weeks post-transplantation (Table 1, Fig. 5).

RT-PCR and Q-RT-PCR analysis

The gene expression analysis of the following 6 strains was performed: undifferentiated HSF-6 cells; 4 HSF-6-derived differentiated strains at their last passages before either in vivo transplantation or initiation of carpet cultures; a strain of hBMSCs that had been cultured in the medium with Dex/AscP and had demonstrated exuberant in vivo osteogenesis. The 4 HSF-6-derived strains were chosen because they demonstrated contrasting in vivo differentiation patterns. Strain B formed bone in 2 out of 3 regular transplants but not in carpet transplants. Strain H formed bone in 2 out of 3 carpet transplants, and in one of these transplants, the most extensive bone of score 2 was observed; however, this strain formed no bone in regular transplants. Strains B and H showed no evidence of teratoma formation. To the contrary, strain N formed no bone in any transplants, and teratomas in 3 out of six 12- to 20-week-old transplants. Strain P formed bone in just 1 carpet transplant, and was the most consistent of all strains in terms of teratoma formation: it formed teratomas in all six 12- to 20-week-old transplants.

Markers of pluripotency, *Oct4* and *Nanog*, were highly expressed in undifferentiated HSF-6 cells but negative in all other strains (Fig. 6A). *Collagen type I*, which is a marker of most connective tissues, as well as of bone, was absent from undifferentiated HSF-6 cells, notably positive in all 4 HSF-6-derived strains (it was somewhat higher in strain B than in 3 other strains, but the difference was short of statistical significance), and expressed at a significantly higher level by hBMSCs (Fig. 6B). *Runx2*, an early marker of osteogenic commitment, was negative in undifferentiated HSF-6 cells, weakly positive in all HSF-6-derived strains, and moderately positive in hBMSCs, though the differences between the latter 5 strains were statistically insignificant (Fig. 6C). An

early marker of osteogenesis, *ALP*, was expressed only in undifferentiated HSF-6 cells and in hBMSCs, with all 4 HSF-6-derived strains negative for this marker (Fig. 6D). A late osteogenic marker, *BSP*, was moderately expressed only by hBMSCs (Fig. 6E). Thus, no significant differences in expression of 4 osteogenic markers were found between 2 osteogenic strains, B and H, and a nonosteogenic strain, N.

A widely accepted marker of chondrogenesis, *collagen type II*, was weakly expressed by all 6 strains analyzed, with slightly higher expression by undifferentiated HSF-6 cells (Fig. 6F). Another chondrogenic marker, *aggrecan*, was weakly expressed only by hBMSCs (Fig. 6G). *Collagen type X*, a marker of late-stage chondrocyte hypertrophy, was very weakly expressed by all 6 strains, with higher expression demonstrated by hBMSCs (Fig. 6H). Thus, all chondrogenic markers studied were expressed at low levels by all strains analyzed (see Y axes presenting gene expression levels relative to GAPDH), with no differences between 4 HSF-6-derived strains.

None of the 6 strains studied demonstrated any expression of endoderm markers, $\alpha 1$ -antitrypsin and α -fetoprotein (not shown). A marker of ectoderm, *keratin-14*, was expressed by neither HSF-6 cells nor hBMSCs but was expressed by all HSF-6-derived strains, and the levels of expression by strains B and H were higher than by strains N and P (Fig. 6I). This pattern of expression was mimicked closely by a related ectoderm marker, *keratin-17* (not shown). *Neurofilament*, another marker of ectoderm, was weakly expressed by all 6 strains, but significantly stronger by strains H and P than by strain B and BMSCs (Fig. 6J).

Discussion

The derivation of osteogenic cells from hESCs would be a significant advance from several perspectives. Not only would genetically manipulated, hESC-derived osteogenic cells serve as a continuous source of cells to study bone diseases, but also an infinite resource of osteogenic precursors for bone regeneration could be generated provided histocompatibility issues could be overcome. Even if hESCs are never used in therapeutic tissue engineering and regenerative medicine, the findings and discoveries made using hESC models will most likely apply to an individual's own induced pluripotent stem (IPS) cells [24], even though the 2 different pluripotent cell types may not be totally identical. In order for these prospects to become a reality, however, a 2-fold challenge must be overcome: (1) how to compel hESCs or IPS cells to reproducibly differentiate along the osteogenic pathway, and (2) how to reliably eliminate cells capable of teratoma formation from constructs destined for in vivo implantation. None of these problems has been solved, and no studies to date promise an easy solution.

The most achievable of these goals has been in vitro osteogenesis by hESC progeny. Upregulation of osteogenic markers and formation of mineralized nodules by hESC-derived cells, either following embryoid body formation or by avoiding this step, has been achieved by several groups using 2 major approaches: culturing hESC descendants in medium with FBS plus osteogenic supplements and/or co-culturing them with human or mouse osteogenic cells [2–10]. However, it was shown previously that patterns of osteogenic differentiation observed in artificial conditions in vitro usually do

FIG. 6. Gene transcription analysis comparing expression of genes regulating pluripotency (*octamer binding protein 4* and *Nanog*), genes associated with osteogenic (*collagen type I*, *Runx2*, *ALP*, and *BSP*), and chondrogenic (*collagens type II* and *type X*, *aggrecan*) differentiation, and markers of ectoderm (*keratin 14*, *neurofilament*) in cultures of undifferentiated HSF-6 cells, differentiated HSF-6-derived strains, and hBMSCs. **(A)** Results of reverse transcription (RT)-polymerase chain reaction. Lane 1, undifferentiated HSF-6 cells at passage 66; lane 2, HSF-6-derived strain B that formed the most extensive bone in carpet transplants and no teratomas, at passage 4; lane 3, HSF-6-derived strain H that formed no bone and a few teratomas, at passage 3; lane 4, HSF-6-derived strain N that formed no bone and a few teratomas, at passage 3; lane 5, HSF-6-derived strain P that formed abundant teratomas, at passage 3; lane 6, hBMSCs generated in medium with dexamethasone/L-ascorbic acid phosphate magnesium salt n-hydrate at passage 3 (positive control for genes associated with osteogenesis and chondrogenesis). See Table 3 for primers and amplicon sizes. **(B–J)** Results of quantitative-real time (Q-RT)-polymerase chain reaction comparing gene expression by the 6 strains listed above. Y axes show gene expression relative to *glyceraldehyde-3-phosphate-dehydrogenase*, mean $\pm$ standard error of mean. **(B) Collagen type I.** Differences between strains B, H, N, and P are statistically insignificant. BMSCs versus strains H and N, $P < 0.05$, versus strain P, $P < 0.01$. **(C) Runx2.** Differences between strains B, H, N, P, and hBMSCs are statistically insignificant. Undifferentiated HSF-6 cells versus all other groups, $P < 0.001$. **(D) ALP.** Differences between strains B, H, N, and P are statistically insignificant. Undifferentiated HSF-6 cells versus strain B, $P < 0.01$, versus strains H, N, and P, $P < 0.001$; BMSCs versus strains B, H, N, and P, $P < 0.001$. **(E) BSP.** Differences between strains B, H, N, and P are statistically insignificant. BMSCs versus strains B, H, N, and P, $P < 0.001$. **(F) Collagen type II.** Undifferentiated HSF-6 cells versus strain P, $P < 0.05$; differences between all other groups are statistically insignificant. **(G) Aggrecan.** Differences between strains B, H, N, and P are statistically insignificant. BMSCs versus strains B and H, $P < 0.01$, versus strain N, $P < 0.05$. **(H) Collagen type X.** Differences between strains B, H, N, and P are statistically insignificant. BMSCs versus strains B and P, $P < 0.01$, versus strain H, $P < 0.001$. **(I) Keratin 14.** Strain B versus strain N and BMSCs, $P < 0.001$; strain B versus strain P, $P < 0.01$; strain H versus strain N, $P < 0.05$; strain H versus BMSCs, $P < 0.01$. **(J) Neurofilament.** Strain B versus strains H and P, $P < 0.05$; BMSCs versus strains H and P, $P < 0.01$. hBMSC, human bone marrow stromal cell. ALP, alkaline phosphatase; BSP, bone sialoprotein.

not correlate with, and cannot foretell, the way that the same cells differentiate upon in vivo transplantation. The degree of osteogenic differentiation of rat and human BMSCs in vitro inversely correlated with the amount of bone formed by these

cells in vivo [12,13]. Several human and rat BMSC populations that demonstrated strong osteogenesis in vitro formed no bone following in vivo transplantation [14,15]. On the contrary, rabbit BMSC strains that displayed adipocytic differentiation

in vitro formed good bone in diffusion chambers in vivo [11]. Human muscle fibroblasts were able to undergo osteogenic differentiation in vitro but not in vivo [17]. More generally, many human fibroblast-like cell strains derived from various organs that have never shown the ability to form bone in vivo, were able to undergo osteogenesis in vitro [16,18]. Taken together, these data strongly suggest that markers and/or phenotypes forced upon cells by artificial in vitro stimuli have little relevance to the genuine differentiation potential of these cells. In addition, in vitro assays are not useful in revealing the tumorigenic potential of hESC-derived cells.

Conversely, the use of transplantation assays under defined experimental conditions [21,22,25,26] has become a valuable standard for delineating the osteogenic potential of cell populations. Not only does it analyze the physiological osteogenic function in vivo, but it also provides the most convincing result, the formation of histologically proven bone tissue [27]. These assays are more physiologically relevant than commonly used in vitro differentiation assays. Having said this, obviously, studies in immunocompromised mice are only a start and cannot fully predict how hESC-derived cells will behave in a human environment. In the current study, using the in vivo transplantation assay, histologically proven bone of human origin was formed 8, 12, and 16 weeks post-transplantation by hESC-derived strains generated through several regimes of in vitro differentiation; this bone constituted the first demonstration of in vivo osteogenic capacity of HSF-6-derived cells. Bone of score 2 formed in the transplants of strains H and R was considerably more extensive than any bone, formed in vivo by several hESC lines, described thus far [3,5,19,20]. The new bone demonstrated numerous osteocytes embedded in lamellar, well-mineralized matrix, as can be judged by parallel organization of collagen bundles revealed under polarized light, and by intense green fluorescence revealed under ultraviolet light; such fluorescence was shown earlier to selectively distinguish mineralized bone in sections stained with eosin-containing dyes [28,29]. These results imply that long-term cultivation of hESC-derived strains in differentiating conditions employed in this study, including several weeks in postconfluent, multilayered cultures, followed by multiple passages and transplantation in HA/TCP vehicles, may, indeed, lead to the creation of clinically relevant bone tissue by nudging a subset of hESC descendants toward the osteogenic pathway.

Among multiple medium compositions tested, no single composition was found to ensure consistent bone formation by HSF-6-derived strains. The same was true for other variable culture parameters, such as total time in monolayer culture, time intervals between consecutive passages, and percentage of carbon dioxide in the gas phase of the cultures. Yet, some concoctions, such as KO-DMEM + FBS + Dex/AsCP (media B and H), promoted bone formation more often than others. Most individual components tested, such as hPL, KOSR, bFGF, ITS⁺, as well as medium conditioned by the osteogenic cells, hBMSCs, failed to promote in vivo bone formation by HSF-6-derived cells. The choice of FBS lot and the use of heat-inactivated versus not inactivated FBS did not seem to make any difference either. At this point, it is not clear why KO-DMEM-based media are more favorable to osteogenic differentiation of HSF-6-derived strains than α MEM-

based media; it is possible that lower cell proliferation rates observed in KO-DMEM-based media may support a better differentiation process. As far as the stimulating effect of Dex/AsCP is concerned, these osteogenic supplements have been long known to accelerate in vitro osteogenic differentiation of cells already committed to osteogenesis. More recently, Dex, either alone or in combination with AsCP, was shown to stimulate in vivo bone formation by cells with low osteogenic potential, as well as by osteogenic cells transplanted with less than optimal vehicles [30–33]. Since Dex alone was shown to decrease collagen synthesis [34], AsCP may have increased bone formation by stimulating the secretion of extracellular matrix proteins [35].

While the use of carpet cultures failed to increase overall incidence of bone formation, the most extensive bone of score 2 was formed by 2 strains exclusively employing the carpet culture technique; in standard, noncarpet, transplants, far less extensive bone of score 1 was formed, at best. To generate carpet cultures, strains H and R, following 4 passages, and 52 to 67 days, respectively, in monolayer cultures, were additionally cultured for 25 and 23 days on the surfaces of HA/TCP particles before transplantation. Whereas the carpet technique employed in this article is a novel one, a number of earlier studies, in an attempt to increase bone formation, used co-cultivation of osteogenic cells with various scaffolds as a prelude to in vivo transplantation, not just in an experimental [30–32,36], but also in a clinical settings [37,38]. Our data suggest that co-culturing some HSF-6-derived strains with HA/TCP increases their bone-forming potential and may propel hESC-derived cells toward osteogenesis. In addition, cartilage formation was observed in a few carpet transplants, probably due to the fact that the carpet cultures are rather dense, and most likely, the interior is less exposed to nutrients and oxygen than the periphery.

More generally, HA/TCP particles were used in this study because it had been convincingly demonstrated that among various groups of scaffolds, calcium phosphate ceramics, and, in particular, synthetic, biphasic calcium phosphates (consisting of various proportions of HA and TCP), are by far superior in promoting in vivo bone formation by osteogenic cell populations [22,39–41]. For supporting bone formation, several characteristics of HA/TCP vehicles are important, such as biocompatibility, Ca/P ratio, surface roughness, macroporosity, and interconnectivity of pores [42–44]. Apparently, HA/TCP particles manufactured by Zimmer and used in this study possess the right combination of qualities: they promote extensive bone formation by both human and mouse BMSCs [22,23,45]. At the same time, HA/TCP scaffolds are thought to be mostly osteoconductive rather than osteoinductive [44,46–48]; that is, they promote bone formation by cells already put through a differentiative scheme (primed to osteogenesis), such as BMSCs, but do not induce bone formation by nonosteogenic cells, such as skin fibroblasts [22]. This is most likely the reason why undifferentiated HSF-6 cells did not form bone when transplanted alongside HA/TCP. Yet, some HSF-6-derived strains that showed no progress along the osteogenic line (were *ALP*- and *BSP*-negative) formed bone when transplanted with HA/TCP. This suggests that HA/TCP may be slightly osteoinductive, depending on the stage of cell differentiation, and can nudge toward bone formation cells pre-committed to osteogenesis. Moreover, for some HSF-6-derived strains, such as strain H, a

longer contact with HA/TCP brought about by carpet cultures may further increase the osteoinductive influence. Taken together, our results suggest that the use of HA/TCP scaffolds, possibly in conjunction with the carpet technique, and further refinement of the culture conditions may improve osteogenic differentiation by hESC-derived cells.

In an attempt to identify early indicators of subsequent bone formation, we compared the transcription levels of several genes associated with osteogenesis in 4 HSF-6-derived strains, 3 of which demonstrated contrasting patterns of bone formation. Strain B formed bone in 2 out of 3 regular transplants but not in carpet transplants. Strain H formed bone in 2 out of 3 carpet transplants, and in one of these transplants, the most extensive bone of score 2 was observed; this strain, however, formed no bone in regular transplants. Strain N formed no bone in any transplants and was, thus, chosen as a negative control for bone formation. Expression of 4 widely accepted osteogenic markers was compared in these strains, as well as in undifferentiated HSF-6 cells and in strongly osteogenic hBMSCs.

Collagen type I is widely distributed in most connective tissues and is also the most abundant bone protein expressed during the osteoblast proliferation and extracellular matrix biosynthesis [49,50]. *Collagen type I* was expressed by strains B, H, N, and P (at similar levels by 3 latter strains, and at slightly higher level by strain B), and, at significantly higher level, by hBMSCs. The transcription factor Runx2 induces osteogenic commitment by promoting expression of osteoblast-specific genes, and is an accepted early marker of osteogenic commitment [48,51,52]. *Runx2* was expressed at low, and similar, levels by strains B, H, N, and P and, at slightly higher level, by hBMSCs. ALP is the most commonly used marker of osteogenic differentiation downstream of Runx2; it provides inorganic phosphate to promote mineralization, and also facilitates mineralization by hydrolyzing its inhibitor [49,53,54]. Among the strains studied, *ALP* was expressed by undifferentiated hESCs (in agreement with some previous observations [3,10,55] and contrary to other [2]), as well as by hBMSCs; it was indistinguishable in all of HSF-6-derived strains, osteogenic or not. *BSP* is a protein found almost exclusively in mineralized tissues where it is important for nucleation of HA crystal formation; as such, *BSP* is considered a very late marker of osteogenic differentiation [56,57]. Among the strains tested, *BSP* was expressed only by osteogenically committed hBMSCs. Taken together, these 4 markers of osteogenesis, *Coll I*, *Runx2*, *ALP*, and *BSP*, failed to distinguish between the osteogenic and non-osteogenic HSF-6-derived strains. Recently, similar findings were reported for osteogenic and nonosteogenic strains of hBMSCs: no positive correlation was found between the osteogenic potential of the strains and expression of *Runx2*, *ALP*, *collagen type I*, *osteopontin*, and *BSP* [58]. Apparently, many of our HSF-6-derived strains contained cells committed to mesenchymal differentiation (expressed *collagen type I*) and primed for osteogenesis (expressed *Runx2*). However, even the best bone-forming strains, B and H, showed no further progression along the osteogenic line of differentiation, and did not spontaneously achieve the stages of osteogenic maturation where *ALP* and *BSP* are expressed. To proceed toward osteogenesis, these strains required an additional nudge accomplished by the slight osteoinductive effect of HA/TCP.

Chondrogenic differentiation is closely related to, and sometimes precedes, osteogenesis. The following 3 markers were thus included into our gene expression assay: collagen type II and aggrecan, the most abundant cartilage proteins considered classical markers of chondrogenesis, and collagen type X, which is a marker of late-stage chondrocyte hypertrophy associated with endochondral ossification [59–62]. All of these markers were expressed at either low or very low levels by the strains under study, and none of them could distinguish between the osteogenic and nonosteogenic HSF-6-derived strains.

Previously, a small number of publications described in vivo bone formation by cells, derived from several hESC lines, that had been either pretreated with osteogenic supplements [5,20] or co-cultured with bone-derived cells [19]. Four to 11 weeks post-transplantation, small, mineralized, von Kossa-positive deposits were observed that were designated “bone” despite absence [5,20] or very poor bone histology [19], and no proof of donor origin. In the most convincing demonstration of bone formation by hESC descendants published to date [3], osteogenic differentiation was induced by culturing BG01-derived cells in medium with osteogenic supplements followed by infection with a GFP-carrying retroviral vector and selection of cells positive for GFP and ALP. The hESC-derived strain thus generated was transplanted into calvarial defects of immunocompromised mice where 6 weeks later, small nodules of histologically convincing bone were observed. Unfortunately, Arpornmaeklong and co-authors [3] did not report whether bone nodules were formed in all recipients, in some, or just in a single one, suggesting that, in their hands, bone formation was sporadic at best, as it was in our study. In that article, the new bone was formed only in the periphery of the circular calvarial defect, close to the recipient’s bone surrounding the defect. While human origin of at least some osteocytes was shown by immunostaining, the authors concluded that “human cells participate in the regeneration of bone,” leaving room for parallel bone formation by the osteoblasts of recipient origin. In this regard, transplantation into a heterotopic site where transplanted cells would not be influenced by host skeletal tissues seems to better suit the purpose of analyzing the true differentiation potential of cells under study.

Before the initiation of differentiation, HSF-6 cells demonstrated a typical morphology of undifferentiated hESC colonies, showed high expression of markers of pluripotency, *Oct4* and *Nanog* [63], and had a normal karyotype that featured no aneuploidy often developed by cultured hESCs [64]; together with teratoma formation, these features proved the undifferentiated nature of HSF-6 cells [55,63]. In differentiating conditions, during prolonged cultivation in various media, HSF-6-derived strains underwent substantial morphological changes. At passage 0, they demonstrated an array of diverse phenotypes, reminiscent of multiple tissue types, as well as the formation of multilayered sheets and complex structures. By passage 3, all strains acquired more uniform, fibroblast-like, yet somewhat variable, morphology. Despite the wide variety of phenotypes observed in the strains at passage 0 and, to a lesser extent, at passage 1, no obvious correlation was observed between the cell morphologies of the strains and the frequencies of either bone or teratoma formation by these strains following several passages and

in vivo transplantation. All HSF-6-derived strains analyzed at passages 3 and 4 demonstrated high viability and high attachment capability; no significant chromosomal aberrations occurred in these strains over the extended culture period in differentiating conditions.

For the future therapeutic use of hESCs or IPS cells, prevention of tumor formation represents a goal at least as important as the primary goal of achieving a desired, in our case, osteogenic, differentiation. Teratomas, or tumors consisting of a range of differentiated somatic tissues of all 3 germ layers, are formed by hESCs implanted into immunocompromised mice in vivo [55,65,66]. While diploid, low-passage hESCs form mostly benign teratomas, culture-adapted hESCs can switch to formation of highly malignant teratocarcinomas [55], an extremely alarming prospect, potentially jeopardizing the entire field of hESC/IPS-based therapy. The formation of teratomas was observed in all transplantation sites tested, and also by hESCs both injected without any vehicle and transplanted in conjunction with various scaffolds [67–70]. For the latter reason, we did not investigate new transplantation conditions and used our standard technique that employs HA/TCP-based scaffolds, for transplantation of both undifferentiated HSF-6 cells and differentiated HSF-6-derived strains. Using this approach, teratomas consisting of multiple tissues, but mostly of mesenchymal-type cell condensations, were routinely observed in 12- to 20-week-old transplants of undifferentiated HSF-6 cells.

It was suggested earlier that to eradicate teratoma formation by hESC-derived cells, period of their cultivation in differentiating conditions should be >2 weeks, thus allowing them to differentiate beyond the pluripotent phenotype [69]. We cultured HSF-6-derived strains in various differentiating conditions for much longer periods, ranging from 7 to 14.5 weeks. Yet, teratoma-like nodules were formed 12 to 20 weeks post-transplantation by some, but not by other strains of HSF-6 descendants, suggesting that contamination with less differentiated cells persisted in some, but not in all, of the strains. Molecular analysis demonstrated that neither strains B and H, that formed no teratomas, nor strains N and P, that gave rise to either less frequent or ample teratomas, respectively, expressed *Oct4* and *Nanog*; all these strains, thus, lost their undifferentiated status [63]. Apparently, the expression of the pluripotency markers was not a prerequisite for teratoma formation. Markers of osteogenic and chondrogenic differentiation analyzed in this study were not predictive of teratoma formation either, as could be expected. In an attempt to further characterize HSF-6-derived strains and to find molecular signs predicting tumor formation, the expression of embryonic germ layer markers [65] was analyzed. For endoderm, it was α -fetoprotein, which is synthesized by the yolk sac visceral endoderm and by fetal liver, and is highly expressed by teratocarcinomas and liver tumors [71,72], and α -1-antitrypsin, a serpin protease inhibitor [73]. Neither of these genes was expressed by any of the 6 strains studied. For ectoderm, we analyzed the expression of 2 keratins that are filament-forming proteins of epithelial cells: keratin 14 of stratified epithelium and keratin 17 of squamous epithelium [74], and of neurofilament, the cytoskeletal component of myelinated axons from central and peripheral nervous system [75]. While *neurofilament* was expressed at very low levels by all strains studied, *keratins* were absent from both HSF-6 cells and hBMSCs but were considerably, and variably, expressed

by 4 HSF-6-derived strains: their expression was significantly higher in nontumorigenic strains B and H than in teratoma-forming strains N and P. This result may be interpreted as an indication that our HSF-6-derived strains, which are of nonclonal origin, represent mixed cell populations and, in addition to cells exhibiting mesodermal differentiation, can include cells with markers of ectoderm. Surprisingly, the strains that contained higher numbers of ectodermal, keratin-expressing cells demonstrated no tumorigenicity, contrary to those strains that included fewer ectodermic cells, but, apparently, more cells of undifferentiated nature bearing some other, as yet unknown, markers. Obviously, this hypothesis is highly speculative and should be further corroborated by broader and more rigorous data. More generally speaking, it would be very important, indeed, to positively identify those genes whose transcript levels could pick out the hESC-derived strains capable of forming tumors. Without being able to directly eliminate tumorigenic cells from hESC-derived populations, it still may be possible to develop culture conditions unfavorable for proliferation and survival of these cells. Certainly, the effectiveness of such “cleansing” should be supported by repeated in vivo transplantation experiments before even considering therapeutic use of these cell populations. At the moment, for absence of a better way, this has been the approach employed in this article. In our experiments, all teratomas, with a single exception, were formed by the strains generated in the α MEM-based media, the same media that promoted the fastest cell proliferation rates and the highest cell yields. Only a single case of a teratoma-like nodule was observed among all transplants of strains generated in media based on KO-DMEM, the media that, quite coincidentally, promoted much slower cell proliferation. Interestingly, no teratomas were formed in Experiment 3 where 50% of all media represented medium conditioned by hBMSCs. Even strains S and U that were generated in entirely α MEM-based media, and that demonstrated very high proliferation rates, formed no teratomas. This observation suggests that media conditioned by hBMSCs expedite hESC differentiation and inhibit teratoma formation. It cannot be ruled out, however, that a longer cultivation of HSF-6 colonies on the MEF layers before the passage 0 (20 days in Experiment 3 vs. 6 days in Experiment 2 and 9 days in Experiment 1) could by itself have decreased the tumorigenic potential of the strains. Previously, no teratomas were observed in 6-week-old transplants of hESC-derived cells [3]. Similarly, our 8-week-old transplants demonstrated no teratoma formation; all tumors were observed in the transplants harvested between 12 and 20 weeks post-transplantation. Apparently, to reliably rule out tumorigenic potential of hESC descendants, and, in particular, to make these observations clinically relevant, the transplants should be allowed to stay in vivo for longer periods of time. This corresponds to the course taken by others who routinely screened recipient animals for teratomas 3 to 4 months after hESC injection [76].

Recently, a method for consistent in vivo bone formation by mouse ESC-derived cells was designed, based on their cultivation on ceramic scaffolds in chondrogenic medium, followed by in vivo implantation of the constructs [77]. While such an approach did not work for hESCs, this achievement gives hope that a comparable accomplishment with hESCs may be within our reach. The sequence of treatments that can drive dependable osteogenic differentiation of hESCs or IPS

cells in vivo still needs to be developed. The current article, not unlike all other studies published to date, has not achieved consistent bone formation by hESC progeny. Yet, it presents the most extensive in vivo bone formation by hESC-derived cells attained thus far; it would be important to verify whether similar results could be achieved by applying our methodology to other strains of hESC, as well as of IPS cells. This approach, based on a relatively simple, although lengthy, technique, without complex steps such as viral infection or cell selection, seems to be a promising avenue for further pursuit of hESC- and IPS-based bone formation. We hope that this article elucidates ways, both to be followed and to be avoided, in the hunt for this important goal.

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Author Disclosure Statement

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