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Differentiation of hES Cells to a Parathyroid-Like Phenotype

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Iatrogenic hypoparathyroidism is the most common complication of cervical endocrine surgery. Current management is limited and palliative. As the molecular steps in parathyroid development have been defined, they may be replicable in vitro, with a goal of cellular replacement therapy. Human embryonic stem cell (hESC) lines were investigated as a model for parathyroid regeneration in vitro. BG01 was selected as a model based on expression of genes of interest in embryoid bodies (EBs). Established strategies for mouse embryonic stem cell differentiation into definitive endoderm were modified and extended to maximize the expression of definitive markers of parathyroid development. The optimal approach included the use of Activin A at 100 ng/mL with BG01 cells grown on murine embryonic fibroblasts for 5 days under conditions of increasing serum concentration. After 5 days, the cells were allowed to mature further in tissue culture without murine fibroblasts but with continuous Activin A. Our strategy produced differentiated cell cultures that expressed intermediate markers of endoderm and parathyroid development (CXCR4, EYA1, Six1, and Pax1), as well as markers of committed parathyroid precursors or developed parathyroid glands (glial cell missing-2 [Gcm2], CCL21, calcium sensing receptor [CaSR], and parathyroid hormone [PTH]). We further characterized the cells by testing conditioned medium from various time points in our differentiation scheme for the presence of PTH. We found that by keeping the cells in culture 2 weeks after the withdrawal of Activin A, the cells were able to produce PTH. Further in vivo work will be needed to demonstrate proper functionality of the cells developed in this way.

Introduction

In humans, the parathyroid glands develop in the third and fourth pharyngeal pouches in contiguity with the developing thymus (third pharyngeal pouch) and parafollicular cells of the thyroid (fourth pharyngeal pouch) [1]. In the last 10 years, a great deal has been learned about the molecular events in the development of the parathyroid glands and the thymus gland, mainly using mouse models.

In murine parathyroid development, positioning of precursor cells occurs at about day 10 in the third pouch endoderm [2]. Modern molecular tools have allowed dissection of the parathyroid organogenesis to separate thymus development from parathyroid gland development (Fig. 1). Much of this work has been done in the process of studying the development of the thymus [3], demonstrating the parallel development of the parathyroid glands [2,4,5]. Thus, parathyroid development can be followed using well-characterized markers. Parathyroid precursor development is associated with expression of four specific transcription factors in the pharyngeal endoderm: Hoxa3, Pax1, Eya1, and Pax9. At this phase, the same transcription factors are also expressed in the cells that subsequently become thymus. The second phase of organogenesis, initiation, which includes the overt development of the conjoined thymus and parathyroid rudiment, occurs at about day 11. There is no known difference in transcription factor expression between these two eventual organs at this stage of development, although one end of the rudiment subsequently becomes parathyroid while the other becomes thymus. This thymus and parathyroid primordium includes expression of Six1 and Pbx1. Engineered murine mutants that lack either of these transcription factors do not develop normal parathyroid glands [6,7].

The outgrowth and patterning phase of parathyroid development results in regional differences in the growing rudiment. This includes the development of separate transcription factor pathways between the two areas which occurs at about day 12. It appears that sonic hedgehog has an effect on the patterning. Sonic hedgehog is a secreted glycoprotein expressed during development that binds to specific cell surface receptors and activates them. Sonic hedgehog,

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Bmp-4, and noggin act in a complex and complementary fashion to affect the outgrowth and patterning of the parathyroid precursor separate from the thymic precursor cells [4,8]. By murine embryonic day 13.5, the parathyroid cell mass and thymus cell mass are separate. They express different patterns of transcription factors. The thymic cells express Foxn1, which is not expressed in the parathyroid cell mass. Similarly, the parathyroid cells are the only cells in the embryo that express Gcm2 (glial cell missing-2 homolog, also known as GcmB), which makes this an excellent, specific marker for parathyroid differentiation [2]. The final differentiation phase of parathyroid development is specified by the expression of the transcription factor Gcm2, as well as the mature parathyroid functional components calcium sensing receptor (CaSR) and parathyroid hormone (PTH). *Gcm2* expression continues into adulthood only in the cells that produce parathyroid hormone and have simultaneous expression of the calcium sensing receptor controlling PTH production. Although the expression of these genes is both spatially and temporally correlated with parathyroid differentiation (Fig. 1), only *Gcm2* has been assigned a functional role in parathyroid development [5] and is a specific marker for parathyroid glands, even in the adult. The expression of the rest of the genes serves as markers of the third pharyngeal pouch differentiation.

In spite of this advanced understanding of the in vivo process of organogenesis, there is little known to date about methods for re-creating this process using less differentiated cells as a starting point. There are some data utilizing a variety of agents to try to recreate an endoderm phenotype and, particularly, a definitive endoderm that ultimately forms an adult organ rather than a part of the extra-embryonic tissues of development [9–14]. The definitive endoderm is initially formed during gastrulation, at which time progenitor cells migrate through the primitive streak and emerge either as definitive endoderm or as mesoderm. Attempts to recapitulate this endoderm differentiation process have included the use of factors that activate pathways used by nodal and transforming growth factor beta (TGF-β) [10].

Investigators have used mouse embryonic stem cells cultured with and without serum and with Activin A (a TGF-β-like molecule) in order to promote the formation of cells with endoderm potential [14]. These investigators have been able to generate definitive endoderm and mesoderm using Activin-A stimulation. Similar studies in human embryonic stem cell (hESC) lines support the concept that undifferentiated cells can be promoted to differentiate through an intermediate meso-endoderm phase and to express genes consistent with definitive (not visceral) endoderm [10]. The next steps in the promotion of definitive endoderm cells to specific pharyngeal endoderm phenotype have not been demonstrated in any published literature.

Our goal is to define conditions that can induce an undifferentiated cell population to develop into parathyroid cells, to use for cellular replacement therapy. Parathyroid glands are optimal for cellular replacement therapy because: (1) each parathyroid cell contains the complete function of the organ, and the total number of cells needed is low; (2) no

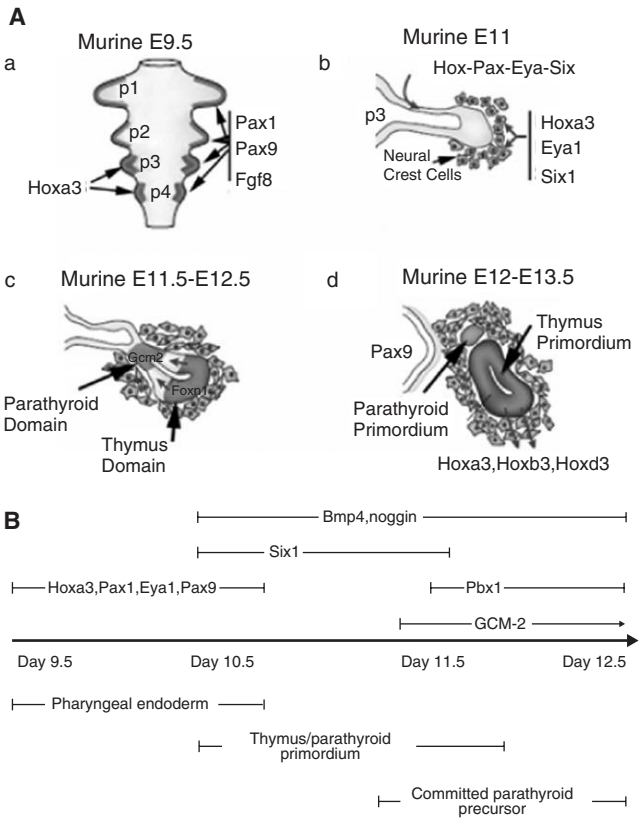

FIG. 1. (**A**) Parathyroid development. (a) Positioning of the eventual parathyroid cells in third pouch endoderm. At mouse embryonic Day 9.5, pharyngeal pouches have formed, and the cells that will become parathyroid cells are positioned in the third pouch (p3). In humans, parathyroid cells form from both the third and fourth pouches; however, there is no parathyroid development from the fourth pouch in mice. (b) Initiation of thymus and parathyroid cells. A distinct identity as a separate group of cells occurs at about embryonic Day 11 in the mouse. These are the only mouse cells that have this pattern of transcription factor expression (Hoxa3 and Eya1 expression also occurs in adjacent neural crest cells). (c) Growth and patterning. Expansion and regionalization of the thymus/parathyroid primordial cells occurs from Days 11.5 to 12.5 in the mouse embryo. The ventral portion of the cluster expresses Bmp4 and forms the thymus domain, while the dorsal portion expresses Noggin and becomes the parathyroid domain. Individual cells commit to either a GCM2-expressing parathyroid fate or a Foxn1-expressing thymus fate. (d) Separation of parathyroid rudiment from thymus. From Days 12 to 13.5, the parathyroid rudiment expands and separates from the thymus rudiment. Parathyroid-committed cells continue to be the only cells that express GCM2; other molecular changes are unknown, except that Noggin expression stops after separation. (Figure modified with permission from Blackburn and Manley [2].) (**B**) Timeline of gene expression for parathyroid development.

architectural arrangement of parathyroid cells is needed to support or enhance the function of the organ; and (3) transplantation of autologous parathyroid cells has been proven to reconstitute normal parathyroid function. We have used hESC lines as a model system to test culture conditions that promote this. These initial studies demonstrate that we can induce the expression of markers of both parathyroid development and of mature parathyroid glands (CaSR, PTH, Gcm2, and CCL21). However, to be clinically applicable, this process must be replicated in an endogenous adult stem cell population to create cells for replacement of parathyroid function. The process defined here to differentiate embryonic stem cells to a parathyroid-like phenotype is one step along that pathway.

Materials and Methods

Cell culture

Undifferentiated hESC lines were cultured under standard conditions. The lines were maintained on a feeder layer of irradiated mouse embryo fibroblasts (MEFs) at 4×10^5 cells/60 mm^2 dish. The MEFs were supplied by the Michigan Center for Human Embryonic Stem Cell Research Core. The culture medium was hESC medium (DMEM/F12 [Invitrogen, Carlsbad, CA], 20% Knockout Serum replacer [KOSR] [Invitrogen], nonessential amino acids [Invitrogen], 2 mM L-glutamine [Invitrogen], 0.1 mM β-mercaptoethanol [Sigma, St. Louis, MO], and 4 ng/mL basic fibroblast growth factor [bFGF, Invitrogen]). Cultures were fed daily, and manually passaged every 3–4 days using pulled glass pipettes. For some experiments, cells were passaged using enzymatic methods (TrypLE Select, Invitrogen). For experiments using growth on gelatin-covered plates, 0.1% gelatin was used to coat the plates (Sigma).

Differentiation protocols

Embryoid body formation. The presidentially approved hES cells BG01 were allowed to differentiate in the Michigan Center for Human Embryonic Stem Cell Research Core into embryoid bodies (EBs) by maintaining the cultures in hESC medium without MEFs or bFGF in nontissue culture plates. RNA was isolated at days 1, 3, 5, 7, 9, 12, and 14. For longer-term evaluation of the embryoid body differentiation, cells were maintained without MEFs in culture for up to 5 weeks, and harvested at 1-week interval from weeks 2–5 for RT-PCR analysis.

Differentiation in high-dose Activin A. BG01 cell differentiation into EBs was also performed at University of Michigan hESC core. Briefly, BG01 cells were transferred to uncoated Petri dishes after mechanically removing them from the MEFs plate, and grown in DMEM-based medium without bFGF, in the presence or absence of Activin A (R&D Systems, Foster City, CA) 10 μg/mL and 20% KOSR. BG01 EBs were grown in 20% KOSR and RNA was isolated at days 1, 3, 5, 7, and 9 for RT-PCR analysis.

Definitive endoderm differentiation. BG01 cells were cultured on MEFs in RPMI supplemented with 100 ng/mL Activin A, glutamax and varying concentrations of fetal bovine serum (FBS) per the protocol described by D'Amour [10]. FBS concentrations were 0% for the initial 24 h, 0.2% for the second 24 h, and 2.0% for subsequent days of differentiation. Cells were harvested at days 1, 2, 3, and 5 for RNA isolation. For a schematic of the protocols used, see Figure 2.

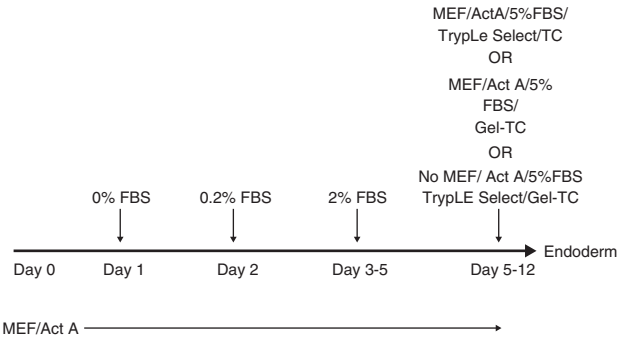

FIG. 2. Experimental design. Timeline schematic of the differentiation protocols used to obtain endoderm. Different culture conditions were used for the cells starting at Day 5, but the resulting cells all expressed the same markers, so the uncoated tissue culture plates were used for all subsequent experiments.

Extended endoderm differentiation. BG01 cells were cultured on MEFs in RPMI with Activin A per the D'Amour protocol for 5 days, followed by dispersion using TrypLE Select and re-plating on tissue culture plates containing Activin A and MEFs (MEF/ActA/TC) or gelatin, MEFs, and Activin A (MEF/ActA/Gel) with 5% FBS for 7 days, before harvesting. Control cultures included cells maintained on MEFs with continued Activin A exposure for 12 days (MEF/ActA/12d). Some cultures were then fed in differentiation medium with 5% FBS without Activin A for 1 or 2 weeks post day 12 of Activin A exposure. At 1- or 2-week post exposure, RNA was isolated for RT-PCR analysis. For a schematic representation of the best differentiation conditions (Bingham protocol), see Figure 3.

PCR

Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA) and cDNA synthesis was performed with ReactionReady First Strand cDNA synthesis kit (SuperArray, Frederick, MD) per the manufacturers' instructions. PCR were performed using PCR primer sets spanning exon

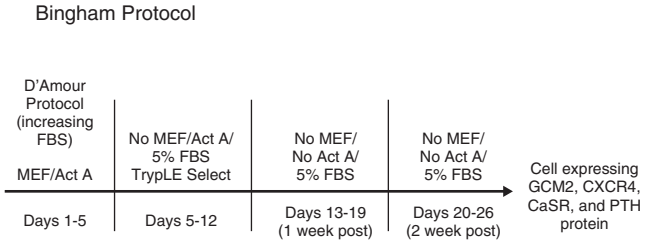

FIG. 3. Final differentiation protocol. Schematic representation of the protocol that ultimately resulted in cells that had parathyroid markers and expressed parathyroid hormone (PTH) (Bingham protocol).

Table 1. Primer Pairs Used for RT-PCR

Gene	*Full name*	*Super Array® catalog number*	*Accession no.*
FoxA2	Forkhead box A2	PPH00976A	NM_021784
Sox17	SRY (sex determining region Y)-box 17	PPH02451A	NM_0022454
CXCR4	Chemokine (c-x-c motif) receptor 4	PPH00621	NM_003467
Eya1	Eyes absent homolog 1	PPH10542A	NM_000503
Six1	SIX homeobox 1	PPH09458A	NM_005982
Hox3A	Homeobox 3A	PPH14990	NM_030661
Pax1	Paired box 1	PPH06915A	NM_006192
Noggin	Noggin	PPH01926A	NM_005450
Bmp-4	Bone morphogenic protein 4	PPH00546A	NM_130851
Gcm2	Glial cells missing homolog 2	PPH02433A	NM_004752
CaSR	Calcium sensing receptor	PPH02127	NM_000388
CCL21	Chemokine (C-C motif) ligand 21	PPH00541B	NM_002989
PTH	Parathyroid hormone	PPH09061A	NM_00315

splicing sites (Superarray), GAPD internal standard primers, and PCR mix (Superarray). The catalog and accession numbers for the primer pairs are listed in Table 1. The RT-PCR products were separated on 2% agarose gels, stained with ethidium bromide, and visualized under UV. Gel images were documented with the BioRad ChemiDoc EQ system. Human parathyroid adenoma cDNA was used as the positive control for PCR. PCR protocol was one cycle at 95°C for 15 min, then 30 cycles of 15 s at 95°C, 30 s at 55°C, and 30 s at 72°C with a final extension of 7 min at 72°C.

Immunofluorescence

Cells were treated as stated earlier, removed from culture using TrypLE Select, and spun onto slides using a Cytospin 4 (ThermoShandon [Thermo Scientific, Madison, WI]). Cells were fixed in 2% paraformaldehyde at room temperature for 15 min and blocked in goat serum at room temperature for 30 min. Cells to be stained with CXCR4 or Sox17 were permeabilized with 0.1% Triton X-100 for 10 min at room temperature. Anti-CXCR4 (Cat# sc-53534; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-CaSR (Cat# sc-47741; Santa Cruz Biotech), and anti-Sox17 (Cat# sc-2009; Santa Cruz Biotech) were incubated with the cells overnight at 4°C. Cells were then exposed to appropriate secondary antibodies (goat anti-rabbit IgG-FITC or goat anti-mouse IgG-FITC (Cat# sc-2012; sc-2010, Santa Cruz Biotech) for 30 min at room temperature. Cells were covered in glass using Prolong Gold antifade with Dapi (Molecular Probes, Eugene, OR) and stored in the dark at 4°C until analysis. Markers were visualized using a Leitz Laborlux S fluorescent microscope (W. Nusbaum, Inc., Chicago, IL).

PTH ELISA

At each time point along the differentiation scheme (Fig. 2), conditioned medium was collected and stored at −80°C until use. Conditioned medium was thawed on ice, spun at 1000 rpm for 5 min. Supernatant was used in a commercial PTH ELISA kit (Cat# DSL-10–8000; Beckman Coulter, Inc., Fullerton, CA). Biologically replicated samples were each tested in duplicate.

Results

Embryoid body differentiation

BG01 hES cells were cultured without MEFs in standard hESC medium and formed EBs. EBs expressed endoderm markers Eya1 and Six1 without specific stimulation (Fig. 4A). There was no evidence of significant expression of later endoderm or parathyroid markers, including Gcm2. However, EBs from cultured for 14–35 days expressed Pax1, HoxA3, Eya1, and Six1 (Fig. 4B). Based on these results, BG01 cells did not appear to have a block or defect in the potential to develop into endoderm.

Since Activin A was previously used to differentiate hESC cells into endoderm, we incubated our BG01 EBs with various concentrations of Activin A to identify differentiation effects mediated by Activin A. BG01 EBs in low levels of KOSR (1%) were sustained poorly. Expression of early pharyngeal endoderm development markers suggests that the cells do not express markers of parathyroid development under these conditions.

Definitive endoderm differentiation

To further differentiate BG01 cells toward parathyroid-like cells, we cultured undifferentiated BG01 cells for 5 days on MEFs in low-dose Activin A and increasing doses of FBS as per the protocol of D'Amour [10]. As the cells remained in culture, they developed changes in cellular structure and began expressing markers of endoderm development. Microscopically, cells changed from the typical hES appearance to grow as monolayers of cuboidal cells without cytoplasmic projections (Fig. 5A, panels a–d). As previously described, the cells develop expression of FoxA2 and Sox17 in response to the Activin A and serum stimuli (Fig. 5B; Table 2). The cells do not express more downstream markers of parathyroid gland development.

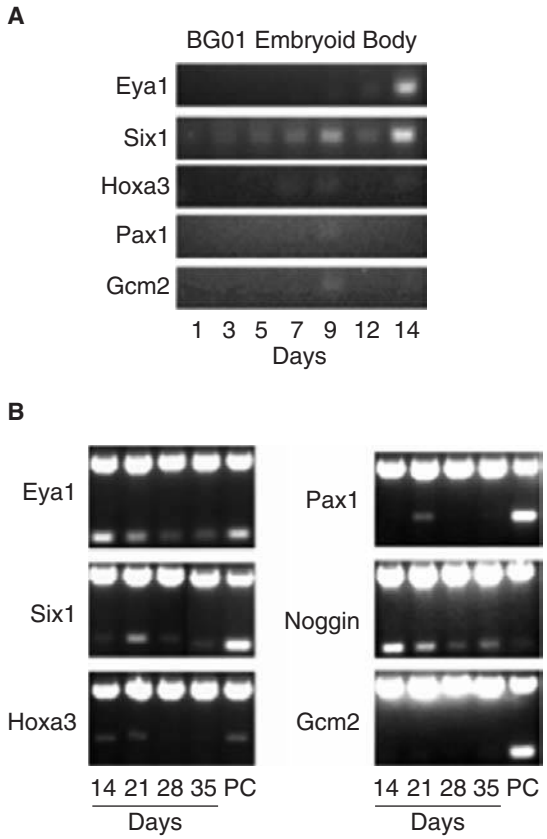

FIG. 4. Expression of endoderm markers in hES cell-derived embryoid bodies (EBs). (**A**) Expression of Hoxa3, Pax1, Eya1, Six1, and Gcm2 in spontaneously differentiated EBs from BG01 hES cell lines for up to 14 days. BG01 shows some expression of Six1 in particular, indicating that there is no block in the pathways to endoderm development in this line. BG01 was thus selected for further experiments. (**B**) Longer-term culture of BG01-derived EBs, up to 5 weeks, showed no evidence of further spontaneous development to endoderm or expression of parathyroid markers (Gcm2). PC is the positive control mRNA isolated from a parathyroid adenoma.

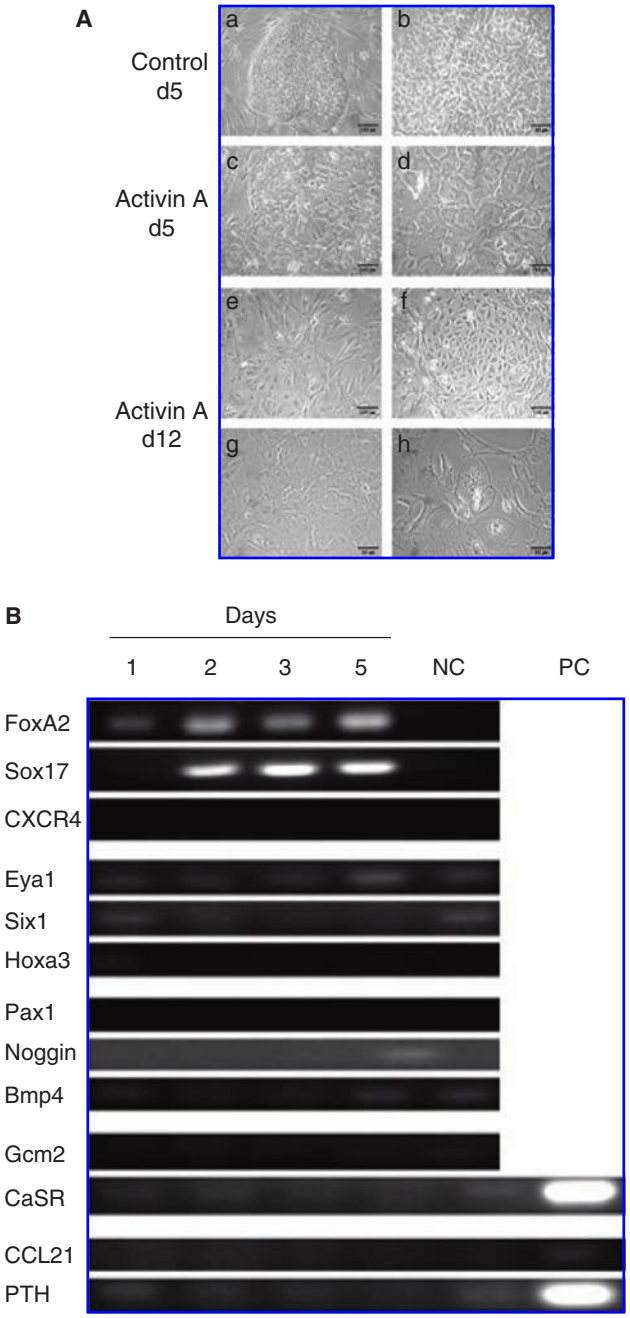

FIG. 5. BG01 cell differentiation with low-dose Activin A per the D'Amour protocol [10]. (**A**) Phase contrast images of cells under various culture conditions. Panels a–b: Control cells (no Activin A). Panels c–d: Cells cultured for 5 days in Activin A. Panels e–h: Cells cultured for 12 days in Activin A. (**B**) RT-PCR expression of markers at Days 1–5 in Activin A culture. With exposure of BG01 cells to Activin A 100 ng/mL and increasing FBS concentration over a 5-day period, the cells developed expression of markers associated with definitive endoderm. These include FoxA2 and Sox17, each of which increase over baseline. In addition, there is some induction of markers that appear in parathyroid development, including Eya1, BMP4, and noggin. There does not appear to be significant induction of definitive markers of parathyroid development, such as the calcium sensing receptor (CaSR), CCL21, Gcm2, or parathyroid hormone (PTH).

All BG01 cells that were treated with the 5-day D'Amour protocol expressed CXCR4 whether they were exposed to Activin A while on MEFs or, subsequent to EB differentiation, in tissue culture dishes (Fig. 6; Table 3). The cells continued to grow as a monolayer of cuboidal cells (Fig. 5A; panels e–h). In addition, the timing of Activin A exposure and differentiation status of the cells when exposed to Activin A did not have any effect on the development of FoxA2, Sox17, Eya1, Bmp4, or noggin expression. Therefore, cells allowed to differentiate for 5 days in Activin A expressed more markers of endoderm than EBs.

Parathyroid-like cell differentiation

BG01 cells that were cultured per the D'Amour protocol for 5 days and then allowed to further differentiate for 7 more

Table 2. Summary of Low-Dose Activin A Stimulation of BG01 (D'Amour Protocol)

	24 h	*48 h*	*72 h*	*5 days*	*5 days control (no Activin A)*	*Positive control (parathyroid adenoma)*
Endoderm markers						
FoxA2	+	++	++	++		
Sox17		+++	+++	+++		
CXCR4						
Thymus/parathyroid primordium markers						
Eya1	+	+	+	++	+	
Six1	+				+	
Hoxa3	+					
Pax1						
Noggin				+		
Bmp4	+	+	+	++	++	
Committed parathyroid precursor cells or parathyroid gland						
Gcm2	+	+			+	
CaSR	+	+			+	+++++
CCL21						+
PTH	+				+	+++++

days (12 day cells) on tissue culture plates or gelatin-exhibited marker expression characteristic of definitive endoderm, thymus/parathyroid primordium (Hoxa3, Pax1, Six1), and parathyroid precursor cells (Gcm2, CCL21, CaSR, PTH), compared to cells maintained on MEFs with only Activin A exposure or to cells that were allowed to differentiate to EBs prior to Activin A exposure (Fig. 6A; Table 3). There did not appear to be a substantial difference in whether the extended differentiation was allowed to occur on tissue culture plates or gelatin. This procedure is hereafter referred to as the Bingham protocol.

Further differentiated BG01 cells (12d cells) had some markers of parathyroid cells, namely GCM2, CaSR, CXCR4, and PTH by RT-PCR. To determine if the 12 day cells also

Table 3. Summary of Low-Dose Activin A Followed by Extended Tissue Culture Differentiation

	MEF/ActA/12d (Bingham Protocol)	*MEF/ActA/Gel*	*MEF/ActA/TC*	*EB/ActA/FBS2*	*EB/ActA/FBS5*
Endoderm markers					
FoxA2					
Sox17	+	+	+		
CXCR4	++++	++++	++++	++++	++++
Thymus/parathyroid primordium markers					
Eya1	+++	+++	+++	+++	+++
Six1	+	++	+++	+	+
Hoxa3		+	+		
Pax1	+	+	++	+	+
Noggin	++++	++++	++++	++++	++++
Bmp4	++	++	++	++	++
Committed parathyroid precursor cells or parathyroid gland					
Gcm2		++	++	+	+
CaSR		++	+++	+	
CCL21		+	+		
PTH		++	++	+	+

Abbreviations: MEF, mouse embryo fibroblast; ActA, Activin A; 12d, 12 days of Bingham protocol; Gel, gelatin-coated tissue culture plates; TC, uncoated tissue culture plates; EB, embryoid bodies; FBS2, 2% fetal bovine serum; FBS5, 5% fetal bovine serum.

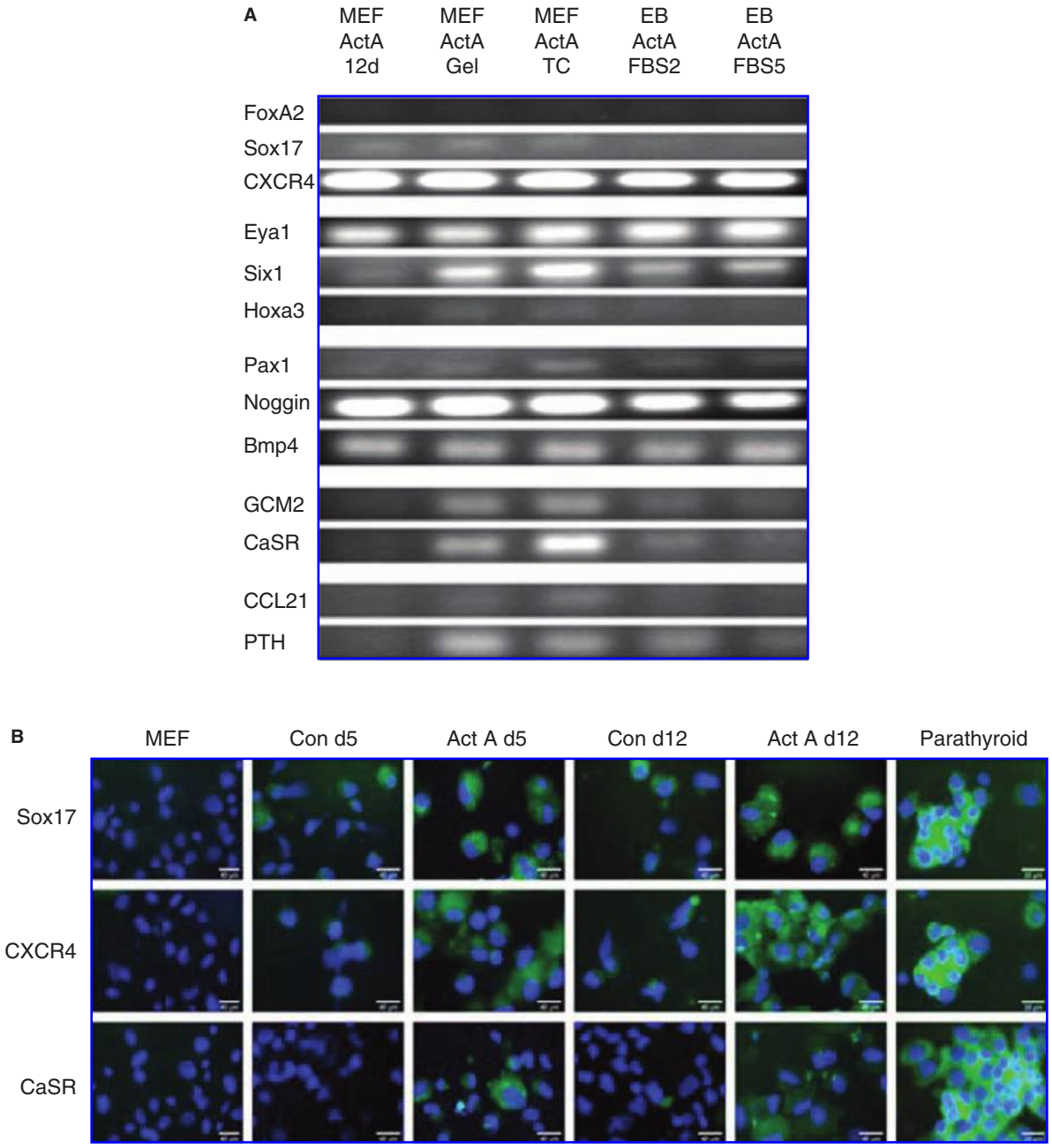

FIG. 6. Extended differentiation of BG01 cells after Bingham protocol. (**A**) Expression of markers under various culture conditions. MEF/ActA/TC group was cultured on MEFs in RPMI with Activin A per the D'Amour protocol for 5 days, followed by dispersion using TrypLE Select and re-plating on tissue culture plates with 5% FBS for 7 days; the MEF/ActA/Gel group was the same, but was plated on gelatin for the last 7 days. The MEF/ActA/12d treatment was maintained on MEFs with Activin A exposure throughout. The EB/ActA/FBS2 group was allowed to differentiate into EBs on tissue culture plates for 5 days before dispersion with TrypLE Select and growth on gelatin while being treated with Activin A 100 ng/mL and FBS 2% for 7 days; the EB/ActA/FBS5 was the same but maintained in FBS 5%. Initial exposure to Activin A while cultured on MEFs, followed by extended differentiation on either gelatin or a tissue culture plate, appears to enhance the appearance of downstream parathyroid development markers. (**B**) Immunofluorescence for Sox17 (top row), an endoderm marker; CXCR4 (middle row), an epithelial cell marker; and CaSR (bottom row), calcium sensing receptor, in cells that were cultured under various conditions. Abbreviations: MEF, mouse embryo fibroblasts; Con, control, no Activin A exposure; Act A d5, 5 days in Activin A under D'Amour differentiation conditions; Act A d12, 12 days in Activin A under Bingham protocol conditions. The bar is 40 μm. Note that most of the cells stain positive for all three markers, indicating that the cells are similar to those that become functional parathyroid. (*Continued*)

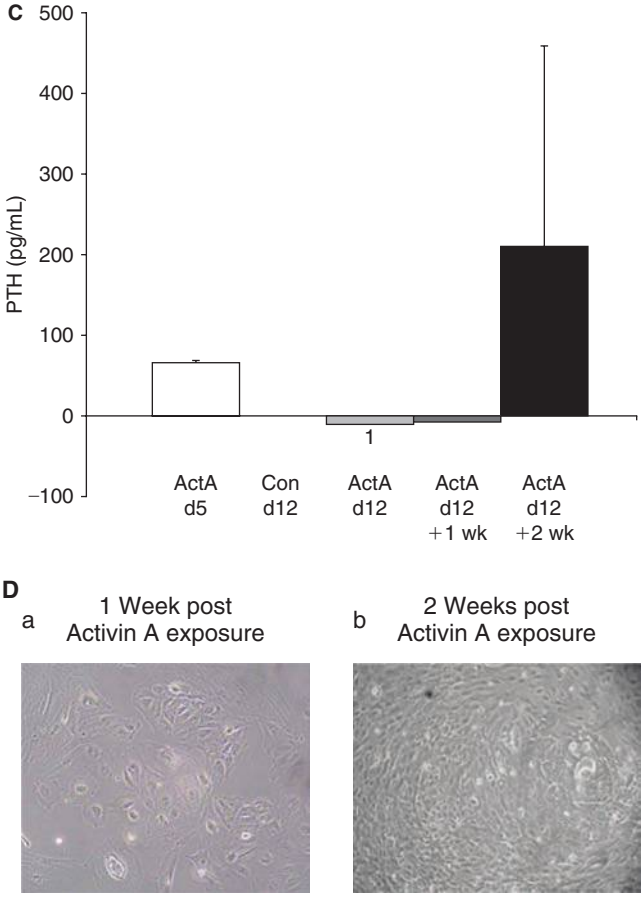

FIG. 6. (*Continued*) (**C**) PTH ELISA. Conditioned medium was taken each step of differentiation from biological replicates. The conditioned medium was tested in duplicate for the expression of PTH in a commercial PTH ELISA. Note that the 12-day cells did not express any PTH. Abbreviations: Act A, Activin A; d5, 5 days in differentiation culture (D'Amour protocol); d12, 12 days in D'Amour differentiation culture; Con, control cells (no differentiation); +1 wk or +2 wk, cells cultured for 1 or 2 week after day 12, no Activin A present. (**D**) Phase contrast images of cells that were in culture past 12 days. Panel a: Cells that were in Activin A differentiation for 12 days (Bingham protocol) and kept for 1-week post-removal of Activin A. Panel b: Cells that were in Activin A differentiation for 12 days (Bingham protocol) and kept for 2-week post-removal of Activin A.

had protein expression of the parathyroid markers, we performed immunofluorescence (IF) for CXCR4, CaSR, and Sox17 (an earlier endoderm stage marker). We could not perform IF for GCM2 as no antibody is available either commercially or privately. We also determined PTH expression by PTH ELISA. We found that CXCR4, CaSR, and Sox17 were all expressed in the 12 day cells (Fig. 6B). However, the 12 day cells had little to no PTH as determined by ELISA (Fig. 6C).

Since the cells differentiated by the Bingham protocol did not express PTH, we cultured the 12 day cells in 12 day medium minus the Activin A for an additional 1 or 2 weeks. At 1 week, the cells had a very flat, vaculated appearance (Fig. 6D; panel a) and little to no PTH expression by ELISA (Fig. 6C). At 2 weeks, the cells had a more uniform cuboidal appearance (Fig. 6D; panel b) and PTH expression (Fig. 6C).

Taken together, the data presented here indicate that this approach can use hESC cells, differentiate them through the endoderm stage, and make cells that express markers of parathyroid tissue. To our knowledge, this is the first description of in vitro differentiation into parathyroid-like tissue.

Discussion

Loss of parathyroid gland function (hypoparathyroidism) is the most frequent permanent complication of thyroid and parathyroid surgery, and is also common after ablative laryngeal operations. The four parathyroid glands are small and delicate structures that share their blood supply with the thyroid gland. Although autografting damaged parathyroids is widely recognized as the best approach to preventing hypoparathyroidism, the rate of permanent hypoparathyroidism after thyroid surgery is 1–15% [15]. Hypoparathyroidism results in chronic hypocalcemia and low-turnover bone disease that is poorly managed by currently available replacement methods. There are currently many thousands of people living with this iatrogenic condition.

For several reasons, diseased parathyroids are optimal for cellular replacement therapy. The normal parathyroid cell detects serum calcium concentration through calcium-sensing receptors expressed on the cell surface [16]. The signal transduction apparatus of the cell modulates the release of intracellularly produced PTH into the circulation based on serum calcium levels and not on any trophic hormonal signals or permissive microenvironment. The parathyroid cell needs only access to the circulation to provide the complete function of the organ. The total weight of parathyroid tissue in normal people is 150–200 mg; re-implantation of far smaller amounts (<50 mg) of parathyroid tissue typically results in normal parathyroid function [1]. In their normal state, parathyroid glands have no ducts (as do exocrine organs), follicles (as does the thyroid gland for thyroxine storage), or portal circulation (as do the hypothalamus and hypophysis). There is no need to reconstitute any type of structural arrangement to have parathyroid function from engineered or autografted cells. The cellular collections that develop from autografted parathyroid cells have the same bland appearance as normal parathyroid glands, without specific architectural arrangement, though the autografted tissue typically has less interposed adipose cells than a normal parathyroid gland.

Parathyroid cells are routinely autografted into heterotopic locations during the course of surgical therapy for thyroid or parathyroid diseases [17–19]. The only requirement is access to the microcirculation that develops spontaneously, and the glands can function in any site, though commonly placed in the musculature of the forearm or the presternal subcutaneous tissue. In preparation for tissue transplant, the parathyroid tissue is mechanically disrupted into small bits of tissue that can live from diffused nutrients at the transplant site until neovascularization occurs. The parathyroid

fragments are then placed into tissue and protected from direct trauma during wound healing. Resumption of measurable, normal parathyroid function occurs in 6–10 weeks for patients who otherwise have no endogenous parathyroid function. The rate of complete normalization of parathyroid function after autograft of fresh parathyroid tissue is 70–95%. If the tissue is cryopreserved before autografting, the complete function rate is 50–80% [1]. The autografted parathyroid tissue responds normally to changes in serum calcium concentration. Thus, the parathyroid gland is the optimal organ for replacement therapy. Attempts to utilize cellular replacement have been successful for hypoparathyroid patients either who have genetically identical parathyroid tissue available [20], or who are immunosuppressed to avoid rejection of a separate organ allograft (typically kidney) and thus tolerate a parathyroid allograft [19,21,22]. Allograft without immunosuppression has not been successful over the long term because of immune-mediated rejection [23]. Thus, the need exists for an optimal cellular replacement therapy not subject to immune rejection.

In the current studies, we have attempted to modify existing strategies for differentiating hES cells in vitro into definitive endoderm and, subsequently, into parathyroid glands. We have selected BG01 cells as a model system based upon their ability to express some markers of endoderm development with undirected differentiation into EBs, suggesting that the cell line has not accumulated defects that may limit its ability to differentiate into endoderm. We used a differentiation strategy described by D'Amour [10] to direct the cells toward an endoderm lineage. By adapting additional conditions (Bingham protocol), we have defined an extended differentiation strategy that induces the expression of markers of definitive parathyroid cells.

Significant work remains to be done in order to settle on an optimal strategy for the development of parathyroid cells in vitro. Ongoing studies are evaluating the expression of the proteins in the cell culture; however, ultimately it will be important for the cells to have parathyroid function with calcium-responsive secretion of parathyroid hormone.

Ultimately, use of these differentiation techniques for patient care will require the identification of an endogenous stem cell population that can be used as a starting point for differentiation and subsequent parathyroid autograft after in vitro differentiation. This could avoid the pitfall of immune rejection that limits the use of parathyroid allograft currently. The optimal cell population for use is not yet determined; however, use of hESC provides an important and readily available model system to investigate the differentiation strategy.

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