Differentiation of precursors into parathyroid-like cells for treatment of hypoparathyroidism

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Background. Hypoparathyroidism is the most frequent permanent complication of thyroid surgery. Our hypothesis is that human precursor cells in culture can be differentiated into parathyroid cells and used to reconstitute function. Human embryonic stem cells (hESCs) are a stable model to study differentiation into parathyroid-like cells. In prior work, the BG01-hESC line was stimulated to form parathyroid-like cells. This cell line is no longer available, however, and additional studies were needed to confirm and extend prior observations.

Methods. Increasing concentrations of fetal bovine serum and timed exposure to Activin A were used to differentiate H1-hESC into parathyroid-like cells. The potential benefit of Sonic hedgehog exposure on parathyroid-like cell development also was evaluated by serial alterations of culture conditions. Calcium-sensing receptor (CaSR), GCM2, and PTH expression (RT-PCR) and PTH protein secretion (ELISA) were used as markers of differentiated cells.

Results. We successfully modified our prior protocol to generate cells that express CaSR, GCM2, and PTH RNA from undifferentiated H1-hESC. The cells also secreted PTH.

Conclusion. We replicated parathyroid differentiation using H1-hESC cells. Our data advance the project toward in vitro differentiation of precursor cells isolated from individual patients for autotransplantation. (Surgery 2010;148:1186-90.)

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Because the parathyroid glands and their blood supply can be difficult to identify and preserve in situ, loss of parathyroid gland function (hypoparathyroidism) is the most frequent, permanent complication of thyroid and parathyroid surgery. Thus, even though prevention of hypoparathyroidism by autografting damaged parathyroids is widely recognized as the best approach, the rate of permanent hypoparathyroidism after thyroid surgery is nearly 14% in population-based series. The chronic, detrimental effects of hypoparathyroidism on bone, teeth, skin, and nails are well documented. Hypoparathyroidism results in chronic hypocalcaemia and low-turnover bone disease that can be palliated by multiple daily doses of vitamin D analogues and calcium. The condition, however, is poorly managed by currently available replacement methods.

Replacement of parathyroid hormone (PTH) itself is available with teriparatide (Forteo; Eli Lilly and Company, Indianapolis, IN), a 1-34 N-terminal protein fragment of PTH; however, the serum half-life of synthetic PTH is less than 5 minutes, providing ineffective replacement therapy.

We began our parathyroid replacement studies by using human embryonic stem cells (hESCs) as a stable model system. We induced BG01-hESCs to differentiate into cells that expressed the parathyroid markers PTH, calcium-sensing receptor (CaSR), CXCR4, and GCM2. The differentiated cells also secreted intact PTH as determined by enzyme-linked immunosorbent assay (ELISA). Concurrent to our development of differentiating procedures, the BG01-hES cell line was determined to not have the appropriate informed consent approval, and our university recommended ending research that relied on these cells. Testing with BG01 cells was therefore terminated after the publication of our article. Here we present data with another hESC line (H1 from WiCell) that validates our original data with BG01 cells and extends those observations to include in vitro effects of Sonic hedgehog exposure. Our ability to differentiate 2 independent hESC cell lines into
parathyroid-like cells using our published protocol strongly suggests our approach will be useful to produce parathyroid cells from individual patient progenitors and allows for continued use of a stable hESC model for additional research.

**MATERIALS AND METHODS**

**Cell culture.** Undifferentiated cells were cultured as we previously described. Briefly, hESC lines were maintained on feeder layers of mouse embryo fibroblasts (MEFs) (Global Stem, Inc., Rockville, MD) on 0.1% gelatin. Cells were cultured in hESC culture medium (DMEM/F12; 20% Knockout Serum Replacer [KOSR]; Invitrogen, Carlsbad, CA), nonessential amino acids, 2-mmol/L L-glutamine, 0.1-mmol/L β-mercaptoethanol, and 4-ng/mL basic fibroblast growth factor (bFGF). Cultures were fed daily and mechanically passed every 3–4 days.

**Differentiation protocols.** H1 cells were differentiated using the Bingham Protocol (Fig 1). Briefly, cells were kept on MEFs and were fed Activin A and increasing amounts of fetal bovine serum (FBS) for 5 days. Cells were cultured on or off the MEF feeder layer and were fed 5% FBS and Activin A ± Sonic hedgehog (Shh) for an additional 7 days. Cells were then kept in culture for an additional 2 weeks ± Activin A ± Shh. Cells were tested for marker expression and PTH secretion at each major time point in the protocol.

**PCR.** Total RNA was isolated using TRIzol reagent, and cDNA synthesis was performed using the ReactionReady First Strand cDNA synthesis kit (SuperArray, Frederick, MD). PCR reactions were performed using primer sets we have previously published that span exon splicing sites. GAPD internal standards were used in each reaction. The PCR protocol was 1 cycle at 95°C for 15 minutes, then 30 cycles of 15 seconds at 95°C, 30 seconds at 55°C, and 30 seconds at 72°C with a final extension of 7 minutes at 72°C.

**ELISAs.** At each major time point of the differentiation protocol, conditioned media were collected and stored at −80°C until use. Media were used in 2 different commercial PTH ELISA kits (Cat #DSL-10-8000, Diagnostic Systems Laboratories, Brea, CA; and Cat#PT019T, Calbiotech, Spring Valley, CA) since the DSL kit became difficult to obtain. TSH, Thyroxine (freeT4), and calcitonin from conditioned media also were assayed by ELISA (Cat # TS-045T; Calbiotech; Cat# F4107T; Calbiotech, Cat# 40-056-205003; and GenWay Biotech, respectively). The cell number was not obtained as the conditioned media were taken from cells used in the next step of the differentiation process. All samples were tested in triplicate.

**RESULTS**

Previously, we were able to differentiate BG01-hESCs cells into parathyroid-like cells that expressed parathyroid markers GCM2, CaSR, and PTH and that also secreted PTH. As our ultimate goal is to use cells from an individual, hypoparathyroid patient and differentiate them into parathyroid cells for replacement, we wanted to show that we could differentiate more than 1 cell line. Thus, we determined whether the Bingham protocol could also differentiate the H1 hESC into PTH-producing parathyroid-like cells.

H1 hESCs were subjected to the Bingham protocol ± the differentiation factor Shh. Cellular RNA was isolated at all of the major time points of the differentiation protocol and characterized by RT-PCR to determine the expression of parathyroid markers. Differentiated H1 cells expressed all of the parathyroid markers (GCM2, CaSR, CXCR4, and PTH) at 1 week posttreatment with Activin A (Fig 2), 1 week earlier than the BG01 cells did. The addition of Shh caused the cells to express more of the markers at the same time point.

Media from the differentiated cells cultured both on and off MEFs were tested in commercial PTH ELISAs for i-PTH secretion. The most secreted PTH (Fig 3) was observed at the same time point as PTH peaked in the PCR analysis (Fig 2), 2 weeks after Activin A treatment. The addition of Shh caused PTH to be released earlier, at 1 week post–Activin A/Shh treatment. The earlier release of PTH after treatment with Shh also was observed previously with the BG01 cells (data not shown). More PTH was secreted when the cells were cultured throughout the differentiation protocol with MEFs (Fig 4). The cells had the same appearance as the in vitro differentiated BG01 cells did.

As we used general differentiation factors, it also is conceivable that the cells are producing factors specific to other endocrine organs produced from pharyngeal endoderm, although we used assessment of parathyroid-specific transcription factors to guide the development of the protocol. Thus, we performed ELISAs for TSH, T4, and calcitonin. The differentiated cells did not produce TSH, T4, or calcitonin (data not shown).

**DISCUSSION**

Mechanical damage to the parathyroids is most often a result of operations on the thyroid. The nature of hypoparathyroidism along with the simplicity of the parathyroid glands makes hypoparathyroidism an ideal candidate for treatment by cellular replacement.
To begin to understand how to differentiate cells into parathyroid cells in vitro, we treated BG01 hESC with a combination of increasing FBS and timed Activin A exposure. The differentiated cells expressed the parathyroid markers CaSR, GCM2, and PTH, and they secreted PTH. Our overall objective, however, is to isolate precursor cells from patients, differentiate them in vitro, and return the differentiated cells as an autograft. Thus, it is important to know that we could differentiate more than 1 cell line. To this end, we repeated our experiments with the H1 hESC. The results presented here show that our differentiation protocol has the potential to be used to differentiate cells originating from individual patients into PTH-secreting cells.

In current clinical practice, if parathyroid tissue is damaged during thyroid surgery, it is devascularized and mechanically disrupted into small bits of tissue that can survive on diffused nutrients at the autograft site until neovascularization occurs. Resumption of measurable, normal parathyroid function occurs in 6–10 weeks postgrafting for patients who otherwise have no endogenous parathyroid function. The rate of complete normalization of parathyroid function after fresh autograft of parathyroid tissue is >90% and is approximately 50% using cryo-preserved tissue, and the grafted parathyroid tissue responds normally to changes in serum calcium concentration. Unfortunately, unless damage to the parathyroids is recognized in the operating room, autografting is not possible.

Many challenges of organ replacement therapy are avoided during parathyroid replacement because of the nature of the parathyroid glands. Parathyroid glands are optimal for cellular replacement therapy because: (1) each parathyroid cell...
Parathyroid cells release PTH in response to changing serum calcium concentrations to balance appropriate serum levels of ionized calcium, with solid form calcium salts used for bone structure. A phenotype of parathyroid cells in culture is their ability to release PTH in response varying calcium levels. Our differentiated cells released only modest amounts of PTH and were grown in a medium that had moderate levels of calcium. If our cells are truly responsive to calcium concentrations, then we would expect to observe only modest levels of PTH secreted at those levels of calcium, which is what we observed. Future studies will incorporate calcium concentration-dependent PTH analysis.

Because general differentiation factors were used, another possibility is that the cells producing PTH also may produce other factors specific to the endocrine organs derived from the pharyngeal endoderm, such as thyroid hormone or thymus markers. Production of these factors, particularly of thyroid hormone, could prove therapeutic for other diseases. The thyroid markers, TSH, T4, and calcitonin were not produced by our cells, however, indicating that the cells were not differentiated into thyroid. This specificity of parathyroid-like cell development also validates our approach of optimizing the appearance of parathyroid-specific transcription factors in the evolution of the Bingham differentiation protocol.

The data presented here bring us closer to autologous cellular replacement therapy for hypoparathyroidism. Work is ongoing to assess whether the cells can compensate for PTH deficiency in an animal model.

REFERENCES

DISCUSSION
Dr Bradford Mitchell (Morgantown, WV): I assume the assay that you are using is intact PTH?

Dr Kathleen M. Woods Ignatoski (Ann Arbor, MI): Yes.

Dr Bradford Mitchell (Morgantown, WV): You are showing RT-PCR and mRNA and what looks to me like...
constitutive secretion of PTH. Have you looked at it in varying concentrations of calcium because otherwise this would not be a great model. If it is not responding to calcium, it would be a great concern.

Dr Kathleen M. Woods Ignatoski (Ann Arbor, MI): We started looking at calcium with the BGO-1 cells. And before we could optimize the conditions, we were told we could no longer use the BGO-1 cells, and at the same time, we were trying to switch and use another cell line, the H-1s. We got the H-1s up and going, and then we decided we should just go for it and try the thymus because we received similar results.

So, right now we are just using thymus. And the next step is to see whether we have calcium regulation, but we have not done that yet.

Dr Bradford Mitchell (Morgantown, WV): And the next suggestion would be to look at a calcium-sensing receptor stain to see that not only is it in the cell but that also it is expressed appropriately on the surface.

Dr Kathleen M. Woods Ignatoski (Ann Arbor, MI): We actually just got a thymus the other day before I left. And we had that slide prepared, but we just have not been able to do that yet.

Dr Mark Cohen (Kansas City, KS): I have a question about 1 of the statements you made about parathyroid and immunogenicity lacking of autografts. And so the question I have is, are you planning to take thymic tissue from the same patient and then deliver it to that patient later after the development? Or are you taking thymic tissue from an unknown source and then delivering it?

Dr Kathleen M. Woods Ignatoski (Ann Arbor, MI): No, our ultimate goal is to take it from the same patient.

Dr Jennifer Rosen (Boston, MA): A few questions: The first is, have you thought about using calcium-sensing receptor to do live-cell sorting in your thymus?

And the second question is, have you tried taking parathyroid tissue from patients and growing it in different concentrations of calcium, using that as, say, a growth factor for the cells?

Dr Kathleen M. Woods Ignatoski (Ann Arbor, MI): For your first question, we are thinking about it. We don’t want to flow sort. We are worried about if the flow sorting would damage the cells in some way. So, we are trying to figure out a way to affinity-purifying the cells using calcium-sensing receptor and magnetic beads? And we have only tried that once. We did get a population of cells out, but then we have to figure out how to grow them from there.

Regarding your second question, using conditioned media as a growth factor, that was very intriguing. We have not done that. We would have to get parathyroid tissue to do it, but we have not done it. It would be interesting.

Dr James Lee (New York, NY): The 2 questions I have, number 1, have you thought about looking at fat-derived stem cells? It seems like it would be an easier source of cells to harvest, but also many people have had a lot of good results de-differentiating them and then taking them down the neuroendocrine line.

The second question, as always with stem cells, what is the durability of these stem cells? How many passages can you get from these cells?

Dr Kathleen M. Woods Ignatoski (Ann Arbor, MI): Let me go back to the second question first. The durability is, what we saw there was at a week, at the end, when we pulled out the thymus, that was only a week we received expression of PTH. A month later, we did not have any expression of PTH, so we need to figure out the cell biology to keep the PTH expression.

Regarding your second question on fat-derived stem cells, that is our alternative step in our grant to use. We decided to go with the thymus because it was from the same primordium. We thought it would be easier to pull them out from there, but we could go back and use fat-derived stem cells.

Dr Herb Chen (Madison, WI): I will take the privilege of the last question, and it has to do with sonic hedgehog signaling. Did you look at downstream markers like Lee 1 when you introduced the Sonic hedgehog? And is the whole process of differentiation dependent on, like, did you treat with an inhibitor like cyclopamine?

Dr Kathleen M. Woods Ignatoski (Ann Arbor, MI): We have not looked at any of the downstream markers. Our whole goal was to try and drive the cells to produce PTH and then also produce PTH in a calcium-sensing manner. And we have not looked to see how any of those drugs were acting. We just were trying to manipulate the cells. So we have not done any of the signaling.