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Generation of Thyrocytes from Embryonic Stem Cells

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1. Introduction

The thyroid gland is one of the largest endocrine glands in the body. It is found on the anterior of the neck and is composed of two connected, cone-like lobes. The thyroid inherits its name from the Greek word for "shield", which the related thyroid cartilage resembles. It is the only endocrine organ in the body that can absorb iodine and synthesize the thyroid hormones triiodothyronine (T_3) and thyroxine (T_4), which regulate homeostasis and metabolism and affect the growth and function of the entire body. The thyroid gland also produces another hormone called calcitonin, which regulates calcium homeostasis. The thyroid hormones and calcitonin are produced by two distinct cell types in the thyroid gland. Thyroid follicular cells (also known as thyrocytes) produce the thyroid hormones. They are derived from the endoderm of the pharyngeal floor. Parafollicular cells, derived from neural crest cells within the ultimobranchial bodies of the fourth pharyngeal arch, synthesize calcitonin. Almost 70% of the thyroid gland is composed of thyrocytes arranged in follicles; parafollicular cells are scattered throughout the interfollicular space (Di Lauro, 2003; Di Lauro et al., 1995).

Many thyroid diseases are caused by the abnormal proliferation and differentiation of thyroid cells. These abnormalities can lead to developmental defects and benign and malignant tumor formation. Most commonly, the thyroid gland is either overactive—a condition called hyperthyroidism—or underactive—a condition called hypothyroidism. Congenital hypothyroidism occurs in about one of every 4,000 births, making it the most frequent endocrine disorder in newborns. 85 percent of cases are caused by a disturbance in the thyroid gland's organogenesis that causes the gland to be absent, hypoplastic or located in an unusual position. In the remaining 15 percent of cases, congenital hypothyroidism is caused by defects in thyroid hormone synthesis (Kopp, 2002; LaFranchi, 1999; Van Vliet, 2003).

The function of the thyroid gland is controlled by the hypothalamus and the pituitary gland (Di Lauro, 2003). Although most scientists agree on the causes of disorders involving aberrations of the hypothalamus-pituitary-thyroid axis, the cellular and molecular mechanisms leading to thyroid developmental abnormalities remain largely elusive. Many of the early studies documenting the complex process of thyroid gland development were performed in animals with genetically engineered mutations (Mansouri et al., 1999). Although these sophisticated mouse models have furthered our understanding of the

genetic and cellular events that lead to thyroid development *in vivo* (De Felice et al., 1998; Macchia et al., 1998; Parlato et al., 2004; Postiglione et al., 2002; Zannini et al., 1997), their utility is limited by the fact that many important mutations cause embryonic lethality. As a result, cellular models have been generated to screen for genes involved in various aspects of thyroid cell physiology. Important studies have been carried out with established thyroid cell lines (FRTL-5 and PC C13) (Ambesi-Impiombato and Coon, 1979; Zimmermann-Belsing et al., 1998) and primary cultures from fetal or adult thyroids. However, despite their experimental utility, primary and immortalized thyroid cells are themselves not without limitations. Primary thyroid cultures are often contaminated with other cell types. They are also unstable and have a limited life span in culture. And although the widely used FRTL-5 cell line, which originated from a normal rat thyroid, can be propagated indefinitely and retain most of the features of differentiated thyrocytes, FRTL-5 cells are incapable of forming follicles in culture. Furthermore, some FRTL-5 cell clones develop into tumors when injected into nude mice (Ossendorp et al., 1990). Finally, these cell lines are limited in their ability to model human development and provide few clues about the developmental aspects of thyroid disease.

It is only recently that embryonic stem (ES) cells have offered the possibility to establish a robust cell-based model for examining the genetic and epigenetic mechanisms of human disorders from a developmental biology perspective. Originally isolated from the inner cell mass of a blastocyst-stage embryo, ES cells can replicate for long periods of time *in vitro*. They are pluripotent, which means that they can differentiate into all derivatives of the three primary germ layers: ectoderm, mesoderm, and endoderm (Evans and Kaufman, 1981; Martin, 1981; Thomson et al., 1998). Under defined culture conditions, ES cells can be instructed to form cellular aggregates known as embryoid bodies. The differentiation of these embryoid bodies, to a limited extent, recapitulates embryonic development (Keller, 1995): the cellular aggregates first appear as hollow balls, called cystic embryoid bodies, and next form internal structures such as a yolk sac and cardiomyocytes. They are also able to differentiate into the more than 220 cell types found in the human body.

The availability of human ES cells and the ability to access cell populations representing the earliest events of embryogenesis offer an unprecedented opportunity to model human development. Analysis of human ES cells derived from patients with specific genetic disorders will provide novel insights into the disease process and should prove useful for identifying new therapeutic targets. Ultimately, the ability to genetically engineer stem cells may allow clinicians to test the effects of new drugs and to develop clinically relevant screening assays that would not otherwise be possible.

2. Embryonic thyroid gland development

Some of the first insights into thyroid organogenesis came from histological analyses of the developing thyroid glands. The thyroid gland develops from the foramen cecum, the endoderm located in midline of the floor of the pharynx between the first and second pharyngeal pouches. The morphogenesis of the thyroid gland, like that of many endoderm-derived organs, begins with the recruitment of a specific group of cells to the thyroid fate. At human embryonic day 20 (E20), and mouse E8, the median thyroid bud appears as a thickening in the floor of the pharynx. One of the earliest markers defining the thyrocyte lineage is the transcription factor paired box gene 8 (Pax8). Mutations in this gene have been associated with thyroid dysgenesis, thyroid follicular carcinoma and thyroid adenoma

(Macchia et al., 1998). Thyroid transcription factor-1 (TTF-1; also known as Nkx2.1, T/EBP (thyroid-specific-enhancer-binding protein) or TITF1) and thyroid transcription factor-2 (TTF-2; or FoxE1) are also important (Di Lauro, 2003; Di Lauro et al., 1995; Kopp, 2002; Zhang et al., 2006). Expression of these transcription factors in the thyroid gland begins with thyroid morphogenesis and is essential for the development of thyrocytes.

At about E50 in humans and E13.5 in mice, the thyroid diverticulum starts its migration from the pharyngeal floor to its definitive pretracheal position. By E60 (E14 in mouse), the thyrocyte precursors express thyroid stimulating hormone receptor (TSHR). By E70 (E15.5 in mouse), thyroid follicular organization appears with the expression of multiple thyroid-specific proteins essential for thyroid hormone biosynthesis, including sodium-iodide symporter (which transports iodide into thyroid cells), thyroglobulin (the precursor of thyroid hormones), and thyroid peroxidase (the enzyme responsible for thyroglobulin iodination). The organogenesis of the thyroid gland is often disturbed in humans, which leads to a variety of clinical conditions including agenesis, ectopy and hypoplasia. All of these conditions are collectively called thyroid dysgenesis. Currently, one of the biggest challenges in thyroid research is the identification of the key molecules that regulate thyroid development. Dissecting the genetic and signaling pathways involved might facilitate the understanding of the crucial roles of various molecules in these developmental processes.

3. Differentiation of mouse ES cells to thyrocyte lineage

Previous biological tools used to study thyroid development were limited to human thyroid cell lines, mouse xenograft models and organ cultures. However, the recent availability of a range of ES cell lines has allowed us to begin to define key molecular and cellular characteristics of thyrocyte-fated populations. Unique molecular markers including early-stage transcription factors, structural proteins and metabolic regulators facilitate the distinction between undifferentiated ES cells and those that have committed to the thyrocyte lineage.

These molecular markers are particularly important because the thyrocyte lineage is one of the least recognizable cell types in a culture of differentiating ES cells. Unlike cardiovascular lineage cells, which exhibit characteristic, spontaneous, and rhythmic contractions, identification of the thyrocyte lineage by conventional screening is very challenging. One thyrocyte identification strategy relies on an ES cell reporter line in which enhanced green fluorescent protein (GFP) cDNA has been targeted to a specific cell surface protein. This approach has allowed us to monitor thyrocytes in differentiating cultures and to isolate cellular representatives of early developmental stages. Another strategy mimics embryonic development by exposing ES cells and their derivatives to growth factors and hormones that they would normally encounter *in vivo*. One such factor is thyroid stimulating hormone (TSH or thyrotropin). TSH is synthesized and secreted by the pituitary gland to regulate the development and function of the thyroid gland. Previous studies have shown that the binding of TSH to TSHR is an important trophic stimulus of the thyroid gland (Davies et al., 2005). Furthermore, the thyroid gland is much smaller than normal in mutant mouse lines with nonfunctional TSH or TSHR (e.g. $Tshr^{hyt}Tshr^{hyt}$, *TSHR-KO*, $pit^{dw}pit^{dw}$) (Marians et al., 2002; Pichurin et al., 2004; Postiglione et al., 2002). Histological analysis has indicated that thyroids from these mice have fewer follicles and more non-follicle-associated interstitial cells than do wild-type thyroids.

In 2003, my laboratory developed a protocol to convert mouse CCE ES cells into thyrocyte-like cells (Lin et al., 2003). In our protocol, ES cells are first grown in standard media for six days as embryoid bodies and then for an additional 11 days in the presence of TSH. We first observed thyrocyte-like cells after 17 days of differentiation. Detailed analysis of these cells reveals that they have some of the properties of thyrocytes: They express the thyroid markers Pax8, sodium-iodide symporter, thyroglobulin, and thyroid peroxidase as well as functional TSHR (Lin and Davies, 2006; Lin et al., 2003). However, this strategy produced only variable and transient thyrocyte-like cells, which were neither pure nor present in sufficient quantities for additional functional studies.

3.1 TSH-dependent differentiation of thyrocytes from mouse ES cells

We next used a mouse ES cell line with a GFP cDNA targeted to the *TSHR* locus to investigate the role of TSHR in thyroid development *in vitro*. We found that the appearance of GFP-positive cells was dependent on the formation of embryoid bodies from undifferentiated ES cells. In our protocol, we first treat ES cell-derived embryoid bodies with TSH for three days under serum-free conditions to direct the differentiation of the embryoid bodies into the pre-thyrocyte stage. Next, the embryoid bodies are dissociated and a fluorescence-activated cell sorter is used to generate a pure sample of TSHR-expressing cells (based on their GFP expression). Upon reaggregation on Matrigel, GFP-positive cells can be further induced by TSH to differentiate into mature thyrocytes after a period of 21 days (Arufe et al., 2006).

Mouse ES cell-derived thyrocytes arising from this approach are phenotypically similar to thyroid follicles derived from primary tissue cultures (Arufe et al., 2006). The thyrocytes are organized into one or more neofollicle-like clusters that express follicular thyroid markers and they have a functional sodium-iodide symporter (Arufe et al., 2006) as depicted in Fig. 1 and Fig. 2. This observation is of particular interest because cells from immortalized thyroid cell lines such as FRTL-5 cannot form follicles when grown as monolayer in culture. These studies demonstrate that the early development of a mouse thyrocyte is clearly regulated by TSH.

Similarily, Jiang *et al* have demonstrated that, under the influence of TSH and insulin, cells of the mouse E14 ES cell line begin to co-express TSHR, sodium-iodide symporter, thyroid peroxidase and thyroglobulin mRNA (Jiang et al., 2010). Although the ultrastructural features of these cells (identified with electron microscopy) are similar to those of adult thyrocytes, the cells had no signs of secretory vesicles. This may explain why these thyrocytes fail to produce thyroid hormones and suggests that conditions must be further optimized to allow for *in vitro* differentiation of ES cells into functional thyrocytes.

3.2 TSH-independent induction of thyrocytes from mouse ES cells

Recently, the Davies laboratory demonstrated the TSH-independent induction of thyroid endoderm from ES cells by activin A. Activin A is a member of the TGFβ superfamily, which has been shown to be critical to the regulation of endoderm formation *in vitro* and *in vivo* (D'Amour et al., 2005; Kubo et al., 2004; Mfopou et al., 2007; Ninomiya et al., 1999; Rippon et al., 2006). Adding activin A to cultures of embryoid bodies markedly increased endodermal markers, including Gata4, CXCR4 and Foxa2 and α-fetoprotein. In fact, a small population of cells expressing Pax8, TSHR and sodim-iodide symporter can be generated with activin A alone (Ma et al., 2009). Although these findings parallel those of several other reports suggesting that thyroid cell development can occur in the absence of TSH (Marians

et al., 2002; Valentine et al., 1994), this strategy failed to sustain a stably differntiated thyrocyte phenotype.

3.3 Insulin and insulin-like growth factor-1 are critical to mouse thyrocyte maturation

Our discovery of the importance of step-wise exposure of ES cells to a synchronized sequence of factors has allowed us to learn more about thyroid differentiation *in vitro*. We have recently used the step-wise administration of activin A, TSH, insulin and insulin-like growth factor-1 to successfully generate mature, thyroglobulin-expressing thyrocytes from pluripotent ES cells. First, activin A and TSH were used to induce the differentiation of definitive endoderm and thyrocyte progenitor cells expressing *Sox17, Foxa2*, and *TSHR*. These progenitor cells were then converted into cellular aggregates that, in the presence of insulin and insulin-like growth factor-1, further differentiated into thyroglobulin-expressing thyrocytes (Arufe et al., 2009). These data show that the addition of insulin and insulin-like growth factor-1 to the late stages of embryoid body culture enable the long-term propagation and differentiation of mature thyrocytes.

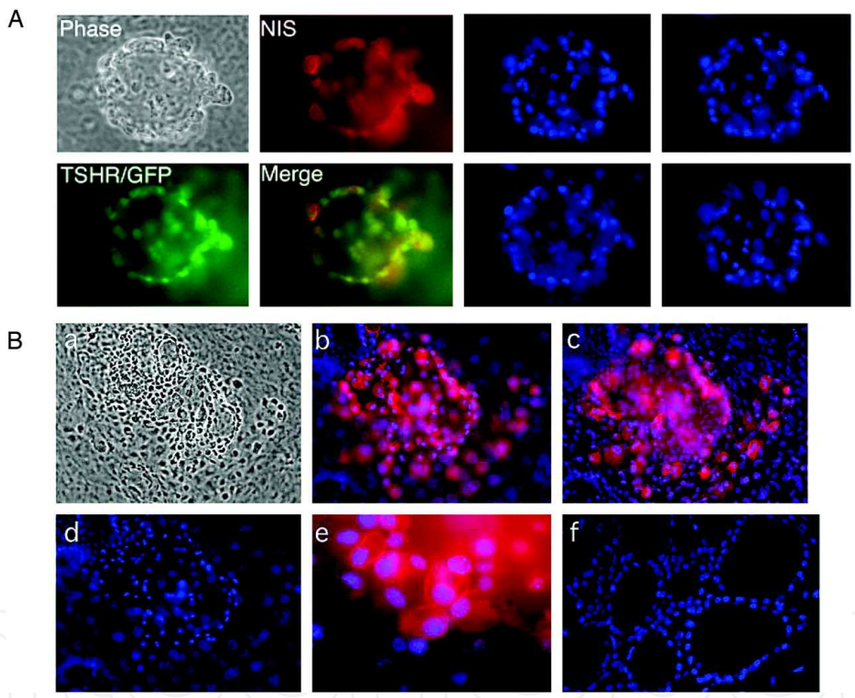

Fig. 1. Thyroid follicle-like clusters derived from mouse *TSHR*$^{+/-}$ ES cells after 21 days of differentiation visualized microscopically (*phase*) or after staining with an antibody to sodium-iodide symporter (*red*). A. TSHR expression is indicated by the green GFP signal. An overlaid image shows the colocalization of sodium-iodide symporter with TSHR (*yellow*). B. Immunofluorescent images of several thyroid follicle-like clusters. a. Phase contrast exposure. b and c, Immunofluorescent staining demonstrating the expression of sodium iodide symporter. Note that a rim of cells several layers thick adhered closely to the sodium iodide symporter-positive cells. d. Nuclear DAPI staining. e. High magnification of anti-sodium iodide symporter immunofluorescence, demonstrating the expression of sodium iodide symporter protein in the plasma membrane. f. Nuclear DAPI staining of native mouse thyroid tissue (from Arufe et al. (2006))

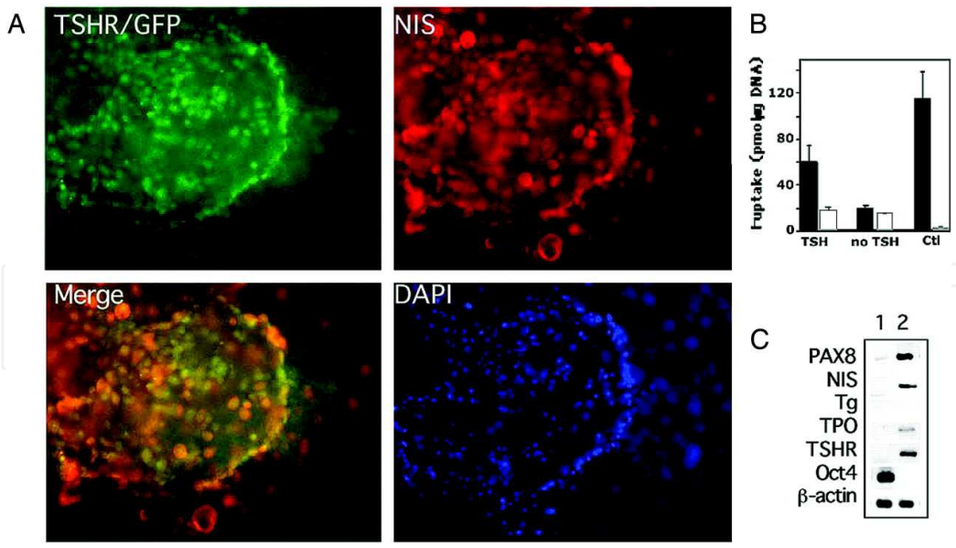

Fig. 2. Thyroid potential of mouse ES cell-derived thyrocytes. A. Several clusters of GFP+ sodium iodide symporter+ cells derived from *TSHR*$^{+/-}$ ES cells after staining with an antibody to sodium iodide symporter (*red*). In merging, some areas showed overlay (*yellow*). *Blue* indicates nuclear DAPI staining. B. Cells treated with TSH showed I^- uptake activity whereas cells maintained in the absence of TSH did not. C. Gene expression analysis by RT-PCR shows differentiation of thyrocytes from ES cells. RNA was isolated from undifferentiated ES cells (lane 1) and thyrocytes grown for 21 days (lane 2) (from Arufe et al. (2006))

4. Generation of thyrocyte progenitors from human ES cells

We are currently using the HES2 human ES cell line to develop a human model of thyroid cell differentiation. Undifferentiated HES2 cells stain strongly for ES-alkaline phosphatase, a non-tissue-specific isozyme precursor expressed at high levels in undifferentiated mouse and human ES cells. They also express the pluripotent stem markers *Nanog, Rex1* and *Oct4*. Together, the expression profiles for these human ES cells contribute to their "stemness" phenotype.

We used combinations of growth factors as well as enrichment through cell surface receptors to design a step-wise differentiation protocol for human ES cells similar to that used in mouse ES cells. This included the formation of embryoid bodies, the induction of endoderm, and the specification of thyroid endoderm. We cultured day 4 embryoid bodies, which express TSHR, in the presence of various combinations of TSH and activin A. RT-PCR analysis confirmed that the endoderm markers *Foxa2* and *Sox17* were strongly expressed by day 4 in cell cultures treated with activin A, but were not expressed in the non-treated cultures. By day 7, *Foxa2* and S*ox17* genes, indicative of endoderm development, were up-regulated in cell cultures treated with activin A. In contrast, treatment with activin A did not significantly alter the expression of the mesoderm marker *c-fms* — the receptor for macrophage colony-stimulating factor — or *Gata1*, a zinc finger transcription factor necessary for hemopoisis (Bang and Goulding, 1996; Simon, 1993; Simon et al., 1992; Yamane et al., 1997). These data suggest that activin A up-regulates endodermal gene expression in the embryoid bodies without inducing mesoderm development. After seven

total days of embryoid body development, we found that the expression of *TSHR* and *TTF-1* was also significantly up-regulated in TSH-treated cell cultures (Thomas & Lin unpublished observations). Since *TSHR* and *TTF-1* are nearly undetectable in the absence of TSH, the high levels of expression in these cells indicate that the endoderm has undergone specification to a thyrocyte fate. We are currently optimzing this protocol in an attempt to generate mature thyrocytes from these human ES cells.

5. Concluding remarks

The first ES cell lines were isolated from mouse blastocysts in 1981. Now, almost three decades later, the world's first human clinical trial of ES cell-based therapy in patients with acute spinal cord injury has been approved by the U.S. Food and Drug Administration. Stem cell technology has revolutionized modern biology and medicine and provides us with unique opportunities to explore the molecular mechanisms that control basic biological and disease processes. The studies described in this review represent an important step in the establishment of a new model to investigate key regulators and events of thyrocyte development. They also outline how to generate a viable thyrocyte from mouse ES cells. Although the molecular triggers for directing thyrocyte progenitors toward mature human thyrocytes remain unknown, our investigation of human ES cell differentiation will no doubt provide an attractive model with which to study human thyroid disease. Such cell-based models of disease are important. They could forward our understanding of the molecular basis of thyroid development and aid in the elucidation of the underlying pathology of thyroid disease and the screening for potential therapeutic agents.

Human ES cell lines were first reported in 1998. Since that time, various laboratories have derived a spectrum of genetically diverse human ES cell lines. Although most of the lines expressed similar profiles of genes and surface marker antigens characteristics of human ES cells, there were intrinsic genetic variations. Furthermore, the slow growth of human ES cells may allow the accumulation of genetic changes during prolonged passage. It is therefore important to clearly identify the characteristics of specific human ES cell lines — including clonability, chromosome stability, the expression of a variety of molecular and biochemical markers, and the ability to differentiate into various types of cells and tissues and to form teratomas in immunocompromised animals — before using these lines to screen new drug compounds and develop innovative cell therapies for diseases.

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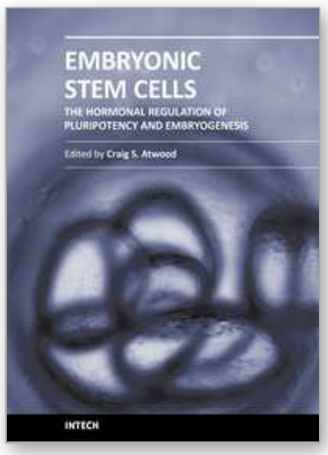

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