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# The effect of thyroid hormone-dependent dermal fibroblast proliferation: an investigation of connective tissue growth factor and proliferative cell nuclear antigen

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BOSTON UNIVERSITY  
SCHOOL OF MEDICINE

Thesis

**THE EFFECT OF THYROID HORMONE-DEPENDENT DERMAL FIBROBLAST  
PROLIFERATION: AN INVESTIGATION OF CONNECTIVE TISSUE GROWTH  
FACTOR AND PROLIFERATIVE CELL NUCLEAR ANTIGEN**

by

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B.S., York University, 2009

Submitted in partial fulfillment of the  
requirements for the degree of  
Master of Arts

2013

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**ABSTRACT**

Thyroid hormone has significant impact on skin homeostasis and cutaneous wound healing. Previous research has demonstrated both *in vitro* proliferation of keratinocytes and fibroblasts and *in vivo* stimulation of epidermal and dermal layers in response to triiodothyronine (T<sub>3</sub>) administration. However, the physiological mechanism of action involving T<sub>3</sub> signaling and the specific intermediate factors of T<sub>3</sub>-induced cell proliferation are poorly understood. Currently, there is no working model of T<sub>3</sub>-dependent dermal fibroblast proliferation. In order to gain a more complete understanding of thyroid hormone regulation in wound healing, two known proliferative growth factors, connective tissue growth factor (CTGF) and proliferative cell nuclear antigen (PCNA), were chosen as potential mediators of T<sub>3</sub>-stimulated fibroblast proliferation. *In vitro* dermal fibroblast cultures were dosed with one of three experimental T<sub>3</sub> concentrations (10<sup>-9</sup> M, 10<sup>-8</sup> M and 10<sup>-7</sup> M) and western blot analysis was

conducted to determine whether CTGF and PCNA expression are regulated by  $T_3$  stimulation. The results indicated no significant change in CTGF or PCNA expression dependent on  $T_3$  concentration. The implications of the findings were addressed and suggestions for future research directions have been proposed. It is still unclear which growth factors are involved in  $T_3$ -regulated fibroblast proliferation. Once these mediators are identified, it will be possible to construct a mechanism of action to integrate the findings and ultimately develop a complete understanding of cutaneous physiology.

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## ABBREVIATIONS

CTGF	Connective Tissue Growth Factor
D1	Type 1 Deiodinase
D2	Type 2 Deiodinase
D3	Type 3 Deiodinase
ECM	Extracellular Matrix
FBS	Fetal Bovine Serum
FGF-2	Fibroblast Growth Factor 2
GH	Growth Hormone
HPTA	Hypothalamic-Pituitary-Thyroid Axis
HSPG	Heparin Sulfate Proteoglycans
IP	Intraperitoneal
K1	Keratin 1
K10	Keratin 10
KDa	Kilo Daltons
KO	Knockout
MAPK	Mitogen Activated Protein Kinase
PCNA	Proliferating Cell Nuclear Antigen
PI3K/Akt	Phosphoinositide 3-Kinase (PI3K/Akt)
PKC	Protein Kinase C
PMN	Polymorphonuclear Cells

rT3	Reverse Triiodothyronine
T3	Triiodothyronine
T4	Thyroxine
TBG	Thyroxine Binding Globulin
TH	Thyroid Hormone
TG	Thyroglobulin
TGF- $\beta$	Transforming Growth Factor Beta
TPA	12-O-tetradecanolyphorbol-13-acetate
TR	Thyroid Hormone Nuclear Receptor
TRE	Thyroid Hormone Response Element
TRH	Thyrotropin-releasing hormone
TSH	Thyroid Stimulating Hormone
VEGF	Vascular Endothelial Growth Factor
WT	Wild Type

## INTRODUCTION

### *Thyroid Hormone Overview*

Thyroid hormones (TH) are multipurpose systemic regulators of fetal and juvenile development and play a major role in adult metabolic homeostasis. Most tissues and organs respond to TH and consequently its cumulative effects may be measured by systemic oxygen consumption and metabolic rate (*Werner & Ingbar's the Thyroid*, 2005; Yen, 2001). The diversity of cell types modulated by TH activity implicates its importance. Some of the major systems affected by TH include the heart, muscle, liver, bone, brain, and skin (Yen, 2001). The variety of anabolic and catabolic properties of TH action further illustrate its importance in metabolic regulation and homeostasis. **Table 1** provides an overview of the complexity of systemic TH action.

The thyroid gland, located in the anterior neck, produces TH, derived from the amino acid tyrosine. Biosynthesis of TH occurs exclusively in the thyroid gland and requires dietary iodine. Large amounts of iodine can be stored within the gland, allowing for the accumulation of a three-month reserve (Brent, 2012; Yen, 2001). Thyroglobulin (TG) is synthesized within in the follicular cells of the thyroid gland and contains tyrosine ring residues within its amino acid sequence. TG is secreted into an extracellular storage compartment, the colloid space, where iodination of tyrosine occurs followed by tyrosine ring coupling, TH formation and subsequent TH storage (**Figure 1**).

Table 1. Overview of thyroid hormone systemic effects.

<u>Target system</u>	<u>Physiological Function</u>
Sympathetic adrenergic system	<ul style="list-style-type: none"> <li>↑ <math>\beta</math>-adrenergic receptor activity</li> <li>↑ <math>\beta</math>-receptors in heart, liver, muscle, adipocytes</li> </ul>
Energy expenditure	<ul style="list-style-type: none"> <li>↑ <math>\text{Na}^+/\text{K}^+</math>-ATPase expression</li> <li>↑ thermogenesis, heat dissipation</li> <li>↑ adaptation to cold climate</li> </ul>
Oxygen consumption	<ul style="list-style-type: none"> <li>↑ number mitochondria in most tissues</li> </ul>
Heart	<ul style="list-style-type: none"> <li>↑ contractility (positive inotropic effect)</li> <li>↑ sarcoplasmic <math>\text{Ca}^{2+}</math>-ATPase expression</li> <li>↑ activity and number of <math>\beta</math>-adrenergic receptors</li> </ul>
Skeletal muscle	<ul style="list-style-type: none"> <li>↑ protein catabolism and glycogenolysis</li> <li>Muscle fiber type switch from slow to fast</li> </ul>
GI tract	<ul style="list-style-type: none"> <li>maintains normal gut motility</li> <li>↑ glucose absorption</li> </ul>
Cholesterol	<ul style="list-style-type: none"> <li>↑ cholesterol synthesis</li> <li>↑ LDL clearance (by ↑ hepatic LDL receptors)</li> </ul>
Skeletal effects	<ul style="list-style-type: none"> <li>promotes bone maturation, advances bone age</li> <li>promotes bone growth (permissive for IGF-1 and maintains normal GH gene expression)</li> <li>↑ bone turnover in adults</li> </ul>
Plasma hormones	<ul style="list-style-type: none"> <li>maintains normal half-lives of hormones and drugs</li> </ul>
Brain/nervous system	<ul style="list-style-type: none"> <li>maintenance of normal mental/emotional function and normal reflexes, including respiration</li> </ul>

Figure taken from Thyroid Physiology presentation by Dr. Seaton, 2012. Boston University School of Medicine.

The major secretory product of the thyroid gland is tetraiodothyronine (thyroxine or  $T_4$ ), at a rate of 80-100  $\mu\text{g}/\text{day}$ .  $T_4$  is primarily a prohormone that may be converted into the biologically active triiodothyronine ( $T_3$ ) or its inactive isoform reverse triiodothyronine ( $rT_3$ ) by a family of selenium deiodinase

enzymes. Type 1 deiodinase (D1) and type 2 deiodinase (D2) convert  $T_4$  into  $T_3$  and may also degrade  $T_3$  during periods of elevated TH in serum (Bianco & Kim, 2006; Bianco et al., 2002). D1 is activate in liver and kidney while D2 functions in the pituitary, brain and adipose tissue (Huang et al., 2011; Yen, 2001). Type 3 deiodinase (D3) is primarily active in skin and is responsible for the conversion of  $T_4$  into  $rT_3$ , a major regulatory step in TH activity (Huang et al., 2011; Yen, 2001). Peeters et al., (2013) have recently demonstrated that D3 is critical for normal functioning of the hypothalamus-pituitary-thyroid axis. In D3 knockout (KO) mice, follicular cells in the thyroid become unresponsive to thyroid stimulating hormone (TSH) and consequently the mice develop hypothyroidism. As such, D3 may be the most important deiodinase regulating TH homeostasis (Barca-Mayo et al., 2011; Peeters et al., 2013).

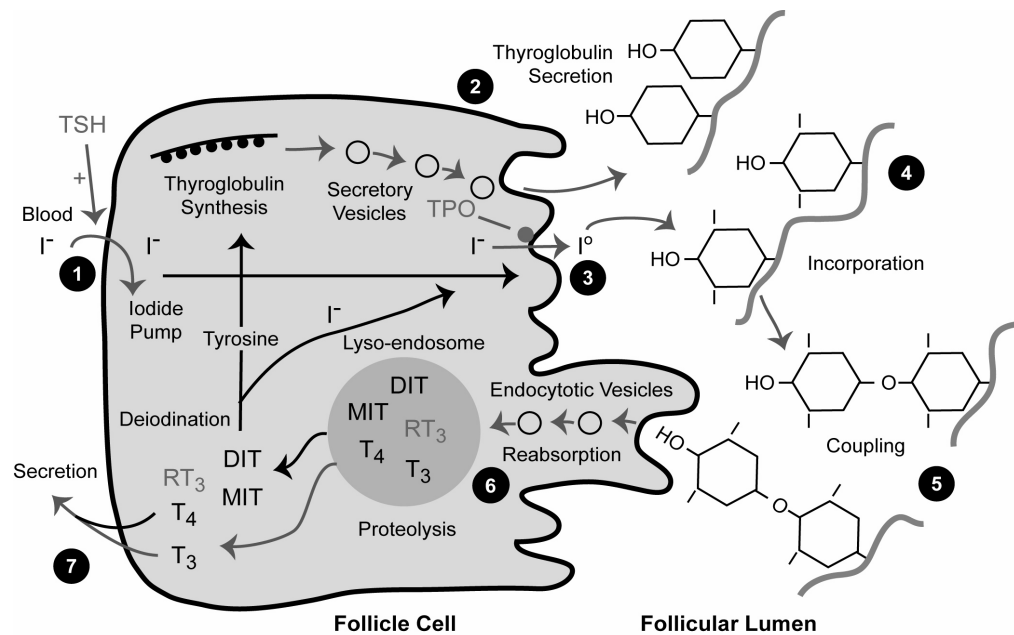


Figure 1. Thyroid hormone biosynthesis. Figure taken from Thyroid Physiology presentation by Dr. Seaton, 2012. Boston University School of Medicine.

T<sub>4</sub> demonstrates a higher degree of molecular stability, as evidenced by its 7-day half-life compared to the 1-day half-life of T<sub>3</sub>. As such, T<sub>4</sub> is utilized as a TH reservoir in plasma (Brent, 2012; *Werner & Ingbar's the Thyroid*, 2005; Yen, 2001). The majority of circulating T<sub>4</sub> and T<sub>3</sub> are bound to the plasma proteins thyroxine binding globulin (TBG), albumin and transthyretin to prevent TH destruction, while a miniscule amount of unbound TH determines biological activity. Extrathyroidal deiodinase activity in the liver accounts for 80% of circulating T<sub>3</sub> derived from the T<sub>4</sub> precursor. Although T<sub>4</sub> is 40 times more abundant in plasma than T<sub>3</sub>, 90% of thyroid hormone nuclear receptors (TR) in target cells bind preferentially to T<sub>3</sub> to execute biological activity (*Werner & Ingbar's the Thyroid*, 2005).

Circulating plasma TH levels are tightly controlled by negative feedback inhibition involving the hypothalamic-pituitary-thyroid axis (HPTA). Thyrotropin-releasing hormone (TRH) is synthesized and secreted by the median eminence and arcuate nucleus within the hypothalamus. TRH travels through the hypophyseal portal system to the anterior pituitary, where it binds to TRH receptors along the cell membrane of thyrotrope cells, which subsequently triggers exocytosis of secretory granules containing thyroid stimulating hormone (TSH). TSH enters systemic circulation and binds to TSH receptors along the follicular cells of the thyroid gland, inducing the release of T<sub>4</sub> and T<sub>3</sub> (Oetting & Yen, 2007). The classic negative feedback loop model applies to the HPTA, where end products, T<sub>4</sub> and T<sub>3</sub>, inhibit production of earlier hormones in the axis,

TRH and TSH. TH exerts inhibitory effects on both the hypothalamus and pituitary by binding to nuclear receptors within these tissues and suppressing the expression of TRH and TSH, respectively (**Figure 2**) (Brent, 2012; *Werner & Ingbar's the Thyroid*, 2005). Several additional hormones also regulate the HPTA. Glucocorticoids inhibit TRH synthesis, decrease TRH receptor affinity to TRH and inhibit deiodinase conversion of  $T_4$  into  $T_3$  thus decreasing TH biological activity. Dopamine and somatostatin inhibit thyrotropes from synthesizing and secreting TSH (*Werner & Ingbar's the Thyroid*, 2005).

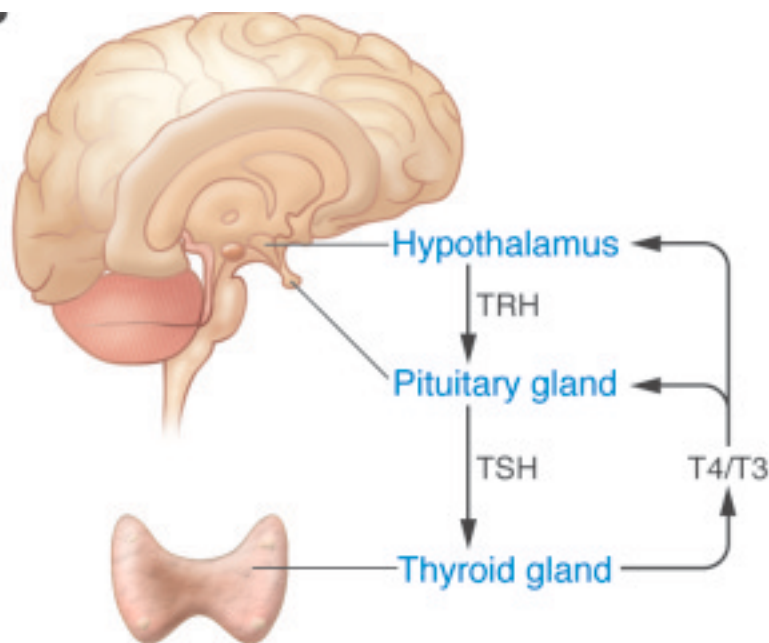


Figure 2. Hypothalamic pituitary thyroid axis. Negative feedback regulation from end products T4/T3 to earlier products TRH and TSH. Figure taken from Brent, 2012.

Thyroid hormones primarily function through a genomic mechanism of action within target cells. THs enter cells through high-affinity plasma membrane

carriers, a regulatory step in TH activity (Yen, 2001). Intracellular D2 converts  $T_4$  into  $T_3$  and any excess  $T_3$  is subsequently inactivated into  $rT_3$  by  $D_3$ . In the nucleus,  $T_3$  binds with thyroid hormone nuclear receptors (TR), which belong to the retinoid/thyroid/vitamin D superfamily of nuclear receptors (Oetting & Yen, 2007; Yen, 2001). Three TR isoforms (TR- $\alpha$ 1, - $\alpha$ 2 and - $\beta$ ) have been identified. TRs possess multiple binding domains for the regulation of TH activity. One TR domain is bound to a DNA sequence uniquely related to TH gene expression, known as a thyroid hormone response element (TRE). In the absence of  $T_3$ , TR is bound to a corepressor protein that inhibits modification of the TRE. However, when  $T_3$  is present, TRs heterodimerize, bind with a coactivator, a retinoid X receptor (RXR) and together with the TRE, mediate TH regulated genes (**Figure 3**) (Brent, 2012). The number of TRs within a cell determines the degree of biological activity in response to  $T_3$  stimulation. Several tissues, including the liver and heart, contain large quantities of TRs and thus express large changes in protein expression when stimulated by  $T_3$ .

There is also recent evidence that TH displays non-genomic effects in some target cells. In these cells, there is a rapid onset of action in response to TH stimulation that involves signaling cascades, independent of TRs and gene expression (Yen, 2001).  $T_3$  has been implicated in  $Ca^{2+}$ -ATPase,  $Na^+/K^+$ -ATPase and  $Na^+/H^+$ -ATPase activation in addition to mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K/Akt) pathways (Aranda et al., 2009; Kress, Samarut, & Plateroti, 2009; Lin et al., 2012).  $T_3$  also enhances glucose

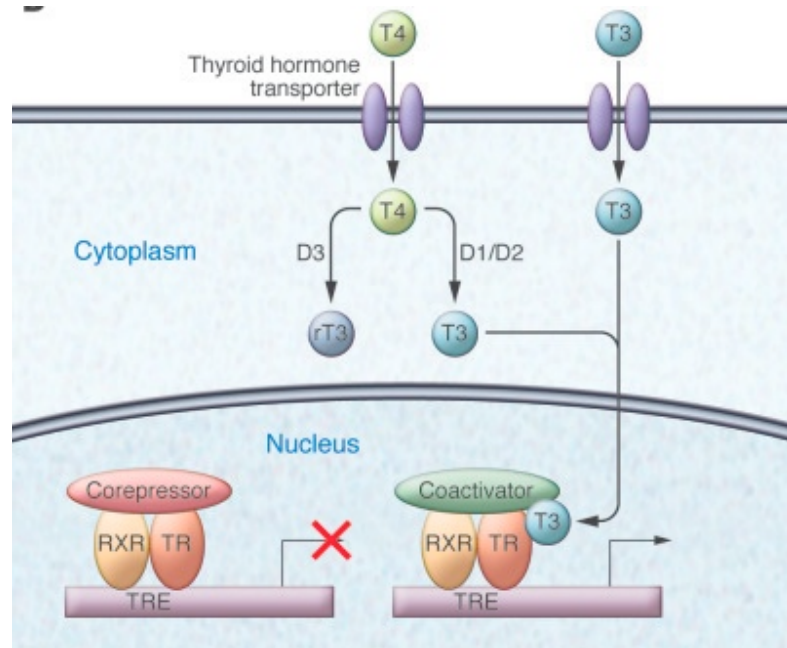


Figure 3. Thyroid hormone action on target cell. T3 enters the nucleus and binds to the TR-coactivator-RXR-TRE complex to activate DNA translation and protein expression. Figure taken from Brent, 2012.

absorption into cells via adenylyl cyclase activation, suggesting it can mediate other cell membrane transporters (Aguayo-Mazzucato et al., 2013). There is further evidence that T<sub>3</sub> mediates mitochondrial activity (Chocron et al., 2012; Kvetny et al., 2009; Sterling & Brenner, 1995; Sterling, 1991), affects cytoskeleton polymerization (Farwell, DiBenedetto, & Leonard, 1993; Zamoner et al., 2007) and potentiates the effects of cytokines in host defenses (Huang et al., 2012; Ilias et al., 2007; Lin, Thacore & Davis, 1994). This culminating research provides an alternative view of the role of TH and suggests TH can act both genomically and non-genomically in target tissues. Further research is necessary to understand the full impact of TH biological activity. However, insights from

thyroid-related pathology have served as a template for identifying TH target tissues and discovering the underlying TH-dependent physiology.

As noted earlier, TH is necessary for normal human development. Children in TH-deficient states present with perturbations in bone maturation and brain development, regardless of normal growth hormone (GH) levels. Thyroid dysfunction is more common in females, likely as a consequence of higher rates of autoimmune disease in women. In addition to autoimmunity, several other factors cause thyroidopathy including iodine deficiency, radiation exposure, pituitary dysfunction and thyroid cancer (Gessl, Lemmens-Gruber, & Kautzky-Willer, 2012).

Hyperthyroidism, also known as thyrotoxicosis, is the result of elevated serum TH. The primary cause of thyrotoxicosis is Graves' Disease, an autoimmune disorder which produces TSH receptor-stimulating antibodies that mimic TSH, bind with TSH receptors on the thyroid gland and cause TH hypersecretion (Eckstein et al., 2009; *Werner & Ingbar's the Thyroid*, 2005). The classical symptom of Graves' Disease is exophthalmos, the accumulation of mucopolysaccharides in the orbital cavity leading to protrusive eyes. Systemically, elevated serum TH leads to overstimulation of beta-adrenergic receptors and promotes tachycardia, hyperdefecation, increased basal metabolic rate and generalized anxiety (Gessl et al., 2012; Safer, 2011).

Hypothyroidism is characterized by reduced levels of serum TH caused by insufficient TSH stimulation resulting from pituitary dysfunction or by diminished

TH secretion of the thyroid gland. Hashimoto's Thyroiditis is a leading cause of hypothyroidism in the United States, whereby autoantibodies target follicular cell proteins, disrupting normal functioning of the thyroid gland (Lee & Hasteh, 2009; *Werner & Ingbar's the Thyroid*, 2005). Initially patients present with enlarged thyroids, clinically defined as goiters, that result from inflammatory responses in autoimmunity. In late-stage thyroiditis, the thyroid gland atrophies. Diminished serum TH causes bradycardia, lethargy, reduced metabolic rate, cold intolerance and memory impairment (Safer, 2011). Extreme fluxes in TH levels can cause life-threatening emergencies including thyroid storm (hyperthyroidism) or myxedema coma (hypothyroidism) (Gessl et al., 2012).

Of particular interest are the skin manifestations resulting from thyroidopathy. Hyperthyroid dermatopathy includes hyperhidrosis, hyperpigmentation and erythma, all resultant from an increased basal metabolic rate. Skin appears smooth and thin, often associated with alopecia (Safer, 2011). Hypothyroid cutaneous symptoms are characterized by pale skin, xerosis and myxedema, the accumulation of glycosaminoglycans leading to edematous states (Safer, 2011). Clinical observations of TH-dependent skin are often complicated by the fact that most thyroidopathy is autoimmune in nature and therefore represents a confounding etiology of symptoms.

### ***The Integument System***

The skin serves as a protective barrier, shielding the host from the external environment. It is an essential nonspecific defense against infection and plays a vital role in maintaining fluid volume and electrolyte balance (McLafferty, Hendry, & Alistair, 2012). Skin is comprised of three distinct layers. The epidermis is the outermost layer, composed of sheet-like configurations of keratinocytes undergoing continuous differentiation beginning from basal stem cells along the basement membrane. The epidermis protects against UV radiation, fluid loss and contains pro-inflammatory cytokines for rapid pathogenic defense. Beneath the basement membrane exists the dermis, composed primarily of resident fibroblasts, extracellular matrix (ECM) and a rich vasculature. The dermis is crucial to maintaining homeostasis and is largely responsible for wound repair following tissue damage. The innermost layer is the hypodermis containing adipocytes for thermoregulation and fat reserves for energy utilization (McLafferty et al., 2012).

Following tissue injury, three phases of wound healing reconstruct the damaged region and attempt to restore integument to its original state: inflammation, proliferation and tissue remodeling (Baum & Arpey, 2005; Werner & Grose, 2003). The process does not occur in discrete stages, but instead works as a coordinated mechanism involving several cell types, cytokines, growth factors, structural proteins and enzymes. The immediate response to tissue damage is vasoconstriction and clot formation via fibrin and platelet

aggregation. This action minimizes excessive hemorrhagic loss, creates a barrier against invading pathogens and releases cytokines involved in the reparation process (Werner & Grose, 2003).

Subsequent inflammatory responses begin within hours of tissue damage and include hyperemia, via histamine and leukotrienes C4 and D4, and increased permeability of local capillaries, via complement C3a and C5a, to facilitate diapedesis of neutrophils (Demidova-Rice, Hamblin, & Herman, 2012). Neutrophils are the first cell type to invade the wound site, largely in the form of polymorphonuclear cells (PMNs), and are responsible for apoptosis and phagocytosis of damaged cells and surrounding debris. Complement C3a and C5a, thrombin and transforming growth factor beta (TGF- $\beta$ ) also act as chemokines to recruit monocytes and lymphocytes to the wound site. Once monocytes enter interstitial space they transform into macrophages and continue the degradation of necrotic tissue. Macrophages also release fibroblast growth factor 2 (FGF-2), TGF- $\beta$  and connective tissue growth factor (CTGF) that induce myofibroblast differentiation, migration and stimulate resident fibroblasts in the dermis to synthesize structural components of the ECM. Vascular endothelial growth factor (VEGF) and angiopoietin stimulate neoangiogenesis, which serves to oxygenate and nourish the migrating and proliferating cells (**Table 2**) (Baum & Arpey, 2005; Demidova-Rice et al., 2012).

Table 2. Wound healing: cells, growth factors and time intervals.

<i>Approximate Timing</i>	<i>Associated Visible Changes</i>	<i>Process</i>	<i>Cells</i>	<i>Cell Adhesion Molecules</i>	<i>ECM Components</i>	<i>Key Growth Factors and Enzymes</i>	
24–48 hours	Eschar sloughing  Neoepidermis	Reepithelialization	Keratinocytes	$\beta_1$ integrins	Provisional ECM Collagen Fibronectin Vitronectin Tenascin E	MMP/TIMP FGF-2 FGF-7 FGF-10 GM-CSF Nitric oxide GRO- $\alpha$ /CXCR-1	TGF- $\beta$ NGF HGF HB-EGF IL-6 Leptin sAPP
Days 4–7	Granulation tissue	Angiogenesis	Endothelial cells	$\alpha_v\beta_3$ integrin	Provisional ECM Collagen GAG Proteoglycan	MMP/TIMP VEGF FGF Angiopoietin TGF- $\beta$ PDGF	
Days 3–21		Net collagen deposition	Fibroblasts	Integrins			EGF IGF-1
Day 2–several weeks		Growth factor production	Macrophages			FGF-2  S1P	CTGF Cyr61

CTGF = connective tissue growth factor; Cyr61 = cysteine-rich 61; ECM = extracellular matrix; EGF = epidermal growth factor; FGF = fibroblast growth factor; GM-CSF = granulocyte-macrophage colony-stimulating factor; GAG = glycosaminoglycan; GRO = growth-related oncogene; HB-EGF = heparin-binding epidermal growth factor; HGF = hepatocyte growth factor; IGF = insulin-like growth factor; IL = interleukin; MMP = matrix metalloproteinase; NGF = nerve growth factor; PDGF = platelet-derived growth factor; sAPP = secretory domain of  $\beta$ -amyloid precursor protein; S1P = sphingosine-1-phosphate; TGF = transforming growth factor; TIMP = tissue inhibitor of metalloproteinase; VEGF = vascular endothelial growth factor.

The procession of wound healing involves coordinated action from many cell types, cytokines, growth factors and enzymes. Table taken from Baum & Arpey, 2005.

The recruitment of fibroblasts, macrophages and leukocytes, the deposition of ECM and the induction of angiogenesis defines granulation tissue and commences the transition into the proliferative phase of wound healing (Baum & Arpey, 2005). The proliferative phase begins 24 hours after tissue injury and may persist for days to weeks depending on wound severity. Re-epithelialization of keratinocytes, involving migration, proliferation and cell differentiation, is also characteristic of the proliferative phase. Tissue remodeling involves wound contraction via myofibroblast activity to reduce the surface area

of the site of injury and diminish scar tissue deposition. The remodeling phase also replaces ECM type III collagen with type I collagen for enhanced tissue durability (**Figure 4**) (Werner & Grose, 2003).

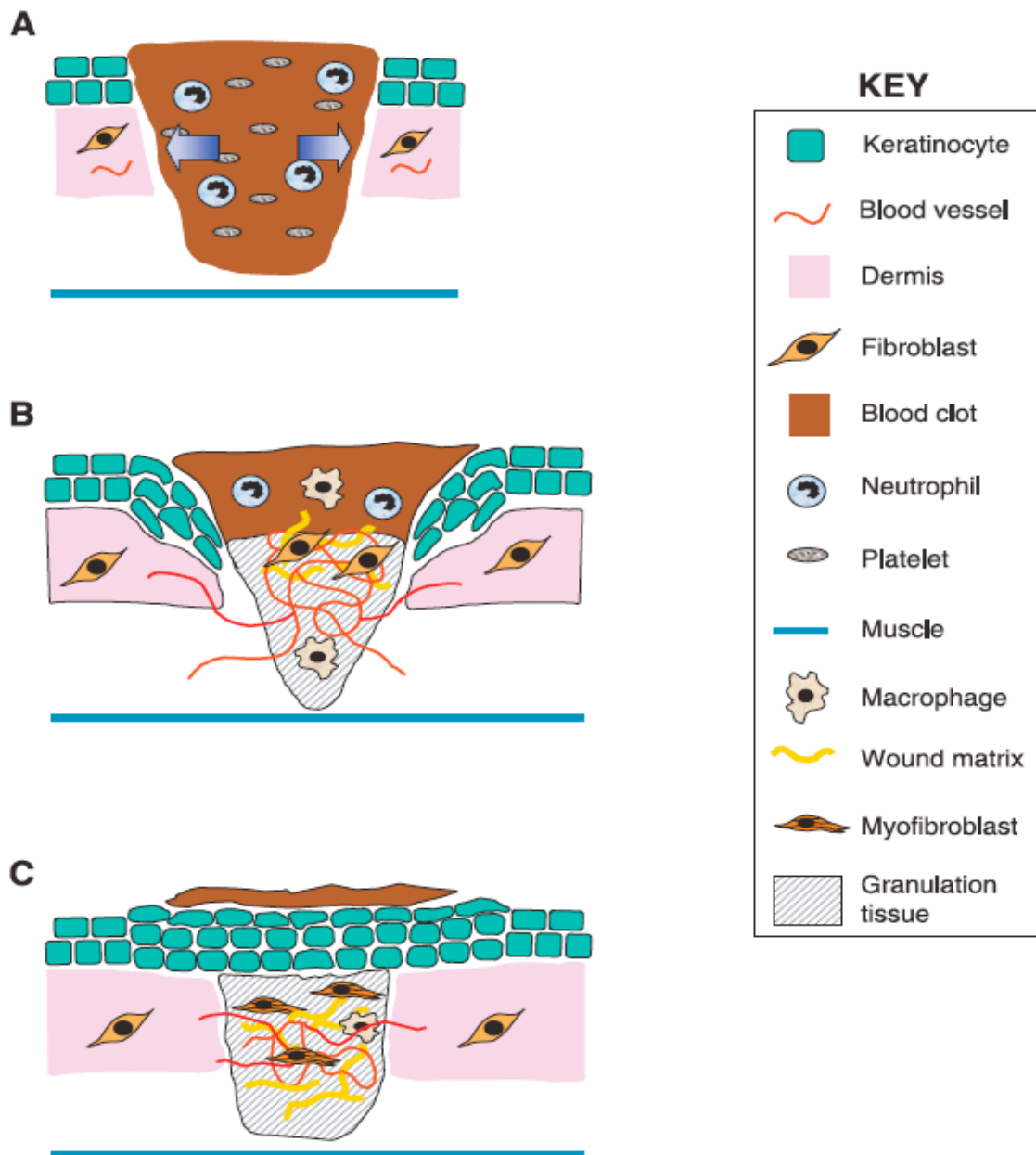


Figure 4. Stages of cutaneous wound repair. A: Inflammation. Blood clot formation and neutrophil invasion. B: Proliferation. Macrophage phagocytosis, epithelial migration, angiogenesis and cell proliferation. C: Tissue Remodeling.

Myofibroblast contraction and collagen deposition. Figure taken from Werner & Grosse, 2003.

Of particular interest is the role of CTGF in the proliferative phase of wound healing. CTGF is released by platelets, macrophages and fibroblasts (Blalock et al., 2012; Grotendorst & Duncan, 2005; Igarashi et al., 1993). It belongs to the CNN family of proteins, which share 38 homologous cysteine residues and four structural binding domains (Baum & Arpey, 2005; Seher et al., 2011; Werner & Grose, 2003). The numerous binding domains enable CTGF to interact with numerous cytokines, membrane receptors and proteoglycans, contributing to a large display of biological activity. CTGF is regarded as a *multifunctional matrixcellular protein* (Leask & Abraham, 2003). TGF- $\beta$  induces CTGF mRNA synthesis in dermal fibroblasts uniquely during wound healing (Chujo et al., 2005; Igarashi et al., 1993; Mori et al., 1999) via the Protein Kinase C (PKC) and ras/MEK/ERK pathway (Chen et al., 2004; Leask & Abraham, 2003). CTGF promotes fibroblast proliferation, myofibroblast differentiation, induces ECM remodeling and functions as a downstream regulator of TGF- $\beta$  by increasing the affinity of TGF- $\beta$  type II receptors to their ligand. The most prominent function of CTGF is the promotion of fibroblast cell adhesion during the proliferative phase of wound healing by binding directly to heparin sulfate proteoglycans (HSPG) and stimulating the interaction between integrins and fibronectin (**Figure 5**) (Chen et al., 2004; Leask & Abraham, 2003). Furthermore, CTGF overexpression is constitutively found in dermal fibrotic scar tissue and is

believed to play a critical role in fibrotic disease (Igarashi et al., 1993; Seher et al., 2011; Werner & Grose, 2003).

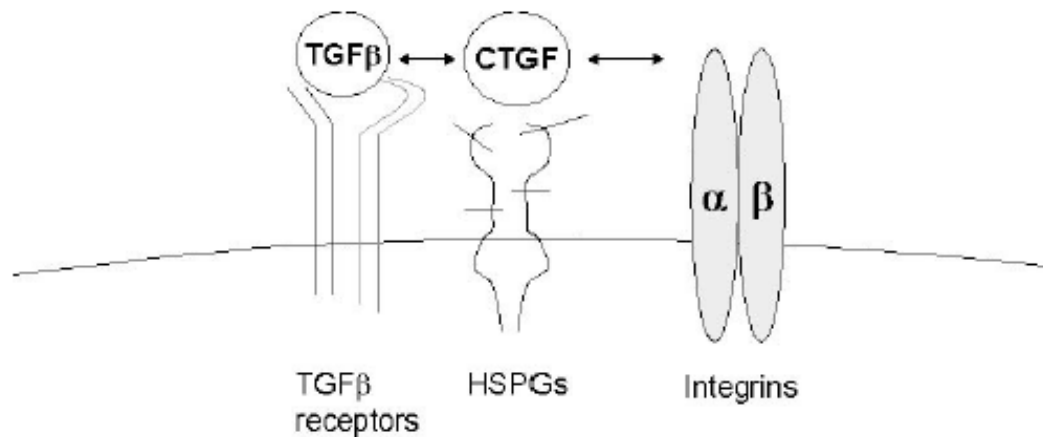


Figure 5. Proposed mechanism of connective tissue growth factor (CTGF) cell binding. Figure taken from Leask & Abraham, 2003.

Another key element of interest in the proliferative phase of wound healing is the role of proliferating cell nuclear antigen (PCNA) in fibroblasts. PCNA is a homotrimeric ring-shaped protein found in all eukaryotic cells and is a marker of proliferation (Scovassi & Prosperi, 2006). It is synthesized in S and early G1 phases of the cell cycle and is essential for DNA replication and repair, active in DNA polymerase processivity and an important contributor of cell cycle control (Chiara et al., 2012; Hergott & Kalnins, 1991; Scovassi & Prosperi, 2006). It is also a critical regulator of cell proliferation and apoptosis during acute inflammation (Chiara et al., 2012) and is induced by oxidative DNA damage and cell destruction (Savio et al., 1998). Post-translational ubiquitination of PCNA can occur via three different mechanisms and regulators, all modifying the same

lysine residue (Hoegel et al., 2002), which suggests PCNA serves alternative functions dependent on the needs of the cell. During the proliferative phase of wound healing, PCNA is involved in DNA replication and cell division of dermal fibroblasts and keratinocytes (Onuma, Mastui, & Morohashi, 2001).

### ***Thyroid Hormone Action in Wound Repair***

TH acts directly on skin through TRs found throughout the epidermis and dermis. All TR isoforms (TR- $\alpha$ 1, - $\alpha$ 2 and - $\beta$ ) have been identified in skin cells (Ahsan et al., 1998), including keratinocytes, fibroblasts, vascular endothelial cells, smooth muscle cells and multiple cell types devoted to hair follicles. D3 (T<sub>4</sub> into rT<sub>3</sub>) and D2 (T<sub>4</sub> into T<sub>3</sub>) activity has been reported in skin (Safer et al., 2003; Schröder-van der Elst et al., 1998), with D3 the most prominent deiodinase (Villar et al., 2000). Deiodinase activity is critical to TH regulation and the homeostasis and physiology of skin. Contreras-Jurado et al., (2011) have recently demonstrated that TRs, in addition to TH, are important regulators of the inflammatory response during wound healing. In comparison to wild-type (WT) mice, TR knockout (KO) mice displayed augmented inflammatory responses after administration of 12-O-tetradecanolyphorbol-13-acetate (TPA), a pro-inflammatory agent, demonstrating that TR is necessary for skin homeostasis. The authors discovered the TR KO mice had increased p65/NF- $\kappa$ B and STAT3 pathway activation causing overexpression of pro-inflammatory cytokines and chemokines. TR KO mice also demonstrated reduced keratinocyte proliferation,

attributed to decreased expression of cyclin D1 (Contreras-Jurado et al., 2011). This recent evidence concludes that both TH and TR are necessary for normal skin metabolism and homeostasis.

*In vitro* studies have also provided support that TH stimulates keratinocyte and fibroblast proliferation. Safer et al., (2004) have shown that T<sub>3</sub> stimulates expression of keratin genes, promoting wound healing. Keratins 1 (K1) and 10 (K10) trigger epidermal differentiation, while K6a, K16 and K17 are responsible for epidermal proliferation and production of intermediate filaments. Keratin gene KO mice demonstrated diminished epidermal thickness and delayed wound repair (Safer, Crawford, & Holick, 2004; Wojcik, Bundman, & Roop, 2000). TH also inhibits production of collagen, fibronectin and hyaluronic acid in fibroblasts, suggesting it is involved in controlling fibrosis during wound healing (De Rycker, Vandalem, & Hennen, 1984; Murata et al., 1987; Smith et al., 1982). Interestingly, when keratinocytes and fibroblasts were cocultured, T<sub>3</sub> produced divergent effects in contrast to cells cultured separately. When cultured in isolation, keratinocyte and fibroblast proliferation both followed a T<sub>3</sub> dose-dependent trend, with maximal effect at 0.5 nM T<sub>3</sub> for keratinocytes and 0.1 nM T<sub>3</sub> for fibroblasts (Safer et al., 2003). However, when keratinocytes were cocultured with fibroblasts, T<sub>3</sub> administration inhibited keratinocyte proliferation (Safer et al., 2003). The greatest inhibition of keratinocytes occurred when fibroblasts were maximally stimulated at 0.1 nM of T<sub>3</sub>. The authors suggested that T<sub>3</sub> promoted inhibitory signals (not yet identified) in fibroblasts to block

keratinocyte proliferation.

In order to understand the effect of TH on cocultured cells, consideration of *in vivo* studies is necessary. Topical application of exogenous T<sub>3</sub> in mice has been shown to stimulate epidermal proliferation, dermal thickening, hair growth (Safer et al., 2001) and to accelerate wound healing (Safer, Crawford, & Holick, 2005). Untreated hypothyroid mice have delayed wound healing in comparison to hypothyroid mice treated with intraperitoneal (IP) T<sub>3</sub>. After IP T<sub>3</sub> administration was discontinued, healing rates reverted to original values (**Figure 6**) (Safer et al., 2004). Another interesting finding concerns the divergent effects of topically and systemically (IP) administered T<sub>3</sub> in mice. Safer et al., (2003) discovered that topical application of T<sub>3</sub> produced a dose-dependent rise in epidermal and dermal thickness and proliferation, while IP T<sub>3</sub> administration caused the converse effect of epidermal thinning. Furthermore, topical T<sub>3</sub> application had no direct effect on serum T<sub>3</sub> levels whereas IP T<sub>3</sub> administration caused significant fluxes in systemic TH homeostasis, including cutaneous metabolism. The results suggest that TH possesses both anabolic and catabolic properties in cutaneous target tissue and the authors surmise that skin deiodinases may exert a neutralizing effect in the event of local or systemic thyrotoxicosis.

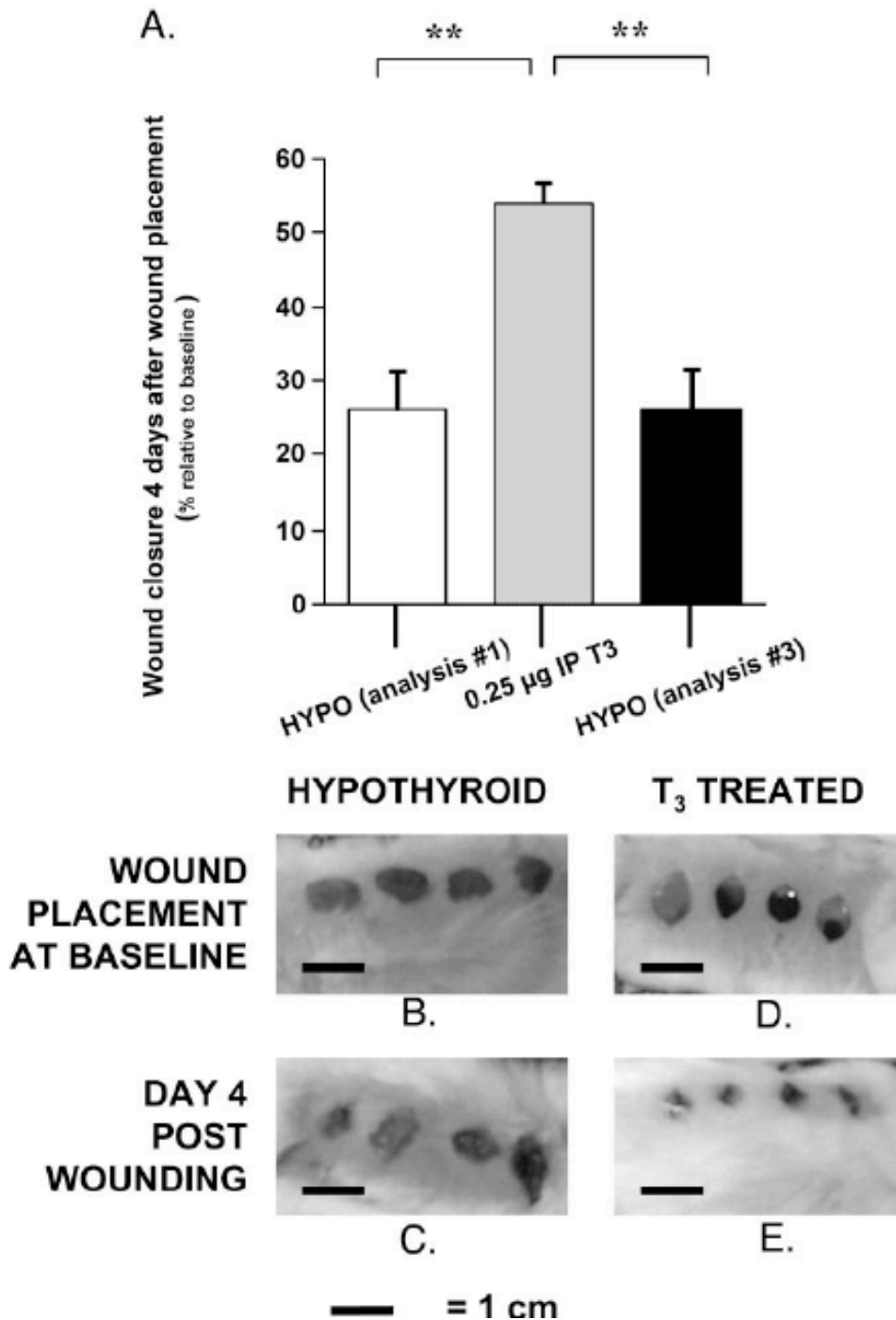


Figure 6. Thyroid hormone dependent wound healing. A: In hypothyroid mice wound healing lagged significantly behind healing in mice treated with IP T<sub>3</sub>. B: Hypothyroid mouse with baseline wounds. C: Hypothyroid mouse on day 4. D: IP T<sub>3</sub>-treated mouse at baseline. E: IP T<sub>3</sub>-treated mouse on day 4. Figure taken from Safer et al., (2004).

### **Specific Aims**

As demonstrated in previous research,  $T_3$  administration to *in vivo* mice models and *in vitro* cell cultures significantly increases the rate of cutaneous wound healing. However, the physiological mechanism of action involving  $T_3$  signaling and the specific intermediate factors of  $T_3$ -induced cell proliferation are poorly understood. Currently, there is no working model of  $T_3$ -dependent dermal fibroblast proliferation.

The goal of the present study is to investigate potential growth factors involved in dermal fibroblast proliferation in response to  $T_3$  stimulation. Specifically,

- (1) Determine whether connective tissue growth factor (CTGF) expression is regulated by  $T_3$  concentration.
- (2) Determine whether proliferating cell nuclear antigen (PCNA) expression is regulated by  $T_3$  concentration.
- (3) Propose a theoretical mechanism of action for thyroid hormone-dependent fibroblast proliferation involving CTGF and PCNA.

The study will conduct western blot analyses of *in vitro* dermal fibroblast cultures after administration of selected  $T_3$  concentrations. The ultimate goal is a better understanding of  $T_3$  function in wound repair that can be exploited for clinical application in patient populations who present with impaired wound healing capability.

## METHODS

### *Cell Culturing*

Human dermal fibroblasts were cultured from primary skin samples donated from discarded tissue following neonatal circumcision at Boston Medical Center. Growth media was produced using Dulbecco's-modified Eagle's medium (DMEM) (Cat#12430-054, Gibco Life Technologies), 5% fetal bovine serum (FBS) (Cat# SH30088.03, HyClone ThermoScientific) and 1% amphotericin B antibiotic (Cat# 17-745E, Lonza). Fibroblasts were isolated from the tissue samples, nourished with growth media and incubated at 37°C in 5% CO<sub>2</sub> until 80% confluence was achieved.

### *Cell Passing*

Growth media was removed from the fibroblast stock plate, then 3 mL of ethylenediaminetetraacetic acid (EDTA) solution was added, swirled for 10 seconds and removed. EDTA solution was made from 100 mg EDTA stock powder (Cat# AM9260G, Invitrogen) and 500 mL phosphate buffered saline (PBS). 3 mL of trypsin (Cat# T4549-100ML, Sigma-Aldrich Co.) was added to the stock plate and incubated for 10 minutes. Next, 3 mL of media (DMEM, FBS, antibiotic) was added to the stock plate to neutralize trypsin. The fibroblast solution was dislodged from the stock plate using a 10 mL pipet then added to a 14 mL tube and centrifuged for 5 minutes. The supernatant was removed from

the centrifuge tube and the fibroblast pellet was resuspended with 1 mL of media per experimental plate. Each experimental plate had 9 mL of media and 1 mL of resuspended fibroblast solution added. Plates were incubated for 3-5 days. Media was replaced every 3 days until 80% confluence was achieved.

### *Dosing*

Media was removed from each experimental plate and washed with 3 mL of PBS, repeated twice. Next, 10 mL of DMEM was added to each plate. T<sub>3</sub> stock solutions were prepared by combining 6.5 mg of T<sub>3</sub> powder (Cat# 06B262013, ICN Pharmaceuticals), 40 µL of 5 M NaOH and 10 mL of distilled H<sub>2</sub>O. T<sub>3</sub> dilutions were prepared as follows: 10 µL of 10<sup>-3</sup> M T<sub>3</sub> stock solution was added to a plate containing only 10 mL DMEM, forming a 10<sup>-6</sup> M T<sub>3</sub> solution. To produce the 10<sup>-7</sup> M T<sub>3</sub> experimental plate, 1000 µL of the 10<sup>-6</sup> M T<sub>3</sub> solution was added to the first experimental plate. To produce the 10<sup>-8</sup> M T<sub>3</sub> experimental plate, 100 µL of the 10<sup>-6</sup> M T<sub>3</sub> solution was added to the second experimental plate. To produce the 10<sup>-9</sup> M T<sub>3</sub> experimental plate, 10 µL of the 10<sup>-6</sup> M T<sub>3</sub> solution was added to the third experimental plate. A fourth experimental plate was left as a control, without addition of T<sub>3</sub>. Each experimental plate then received 100 µL of double-stripped bovine serum (DSS). DSS was made from 500 mL of FBS, 25 g of resin (Cat# 142-2822, Bio-Rad Laboratories) and 15 g of activated charcoal (Cat# 242276, Sigma-Aldrich Co.). Plates were incubated overnight (12-18 hours).

### *Cell Lysis*

Experimental plates were washed with 3 mL of EDTA. EDTA was then removed and 3 mL of trypsin was added. The plates were incubated for 10 minutes. To neutralize the trypsin, 3 mL of media (without antibiotic) was added. The experimental plates were scrapped and pipetted to dislodge the fibroblasts. With a 10 mL pipet, each plate of fibroblast solution ( $10^{-7}$  M,  $10^{-8}$  M,  $10^{-9}$  M and 0 M) was added to a 15 mL tube and centrifuged for 5 minutes at 1200 revolutions per minute (RPM). The supernatant was then removed. Each tube with remaining pellet was resuspended with 250  $\mu$ L of lysis buffer solution and placed on ice for 20 minutes. The lysis buffer solution was made by combining one tablet of Protease Inhibitor Cocktail (Cat# 11836153001, Roche Diagnostics) to 10 mL of RIPA buffer (Cat# R0278-500ML, Sigma-Aldrich Co.) and shaking the lysis buffer solution for 10 minutes. Each tube containing the resuspended pellet and lysis buffer solution was centrifuged for 10 minutes at 14000 RPM at 4°C. The supernatant from each tube was transferred into a 1.5 mL labeled Eppendorf tube and stored at -80°C.

### **Western Blotting**

#### *Buffers*

50 mL of SDS running buffer stock solution (20X) (Cat# NP0002, Invitrogen) was added to 950 mL of distilled H<sub>2</sub>O to form 1X running buffer solution. Transfer buffer stock solution (10X) was made by combining 30.3 g of

tris amino methane (Tris Base) (Cat# 69L-9053, Fisher Scientific), 144 g of glycine (Cat# 521M-12294, Fisher Scientific) and filling distilled H<sub>2</sub>O to 1 L and shaking to dissolve the solution. Functional transfer buffer (1X) was made from 100 mL of 10X transfer buffer stock, 200 mL of methanol (Cat# 1700L6808, Fisher Scientific) and filling to 1 L with distilled H<sub>2</sub>O. Buffer solutions were kept refrigerated.

### *Gel Electrophoresis*

Eppendorf tubes containing experimental T<sub>3</sub> solutions (10<sup>-7</sup> M, 10<sup>-8</sup> M, 10<sup>-9</sup> M and 0 M) were defrosted at ambient temperature for 10 minutes. 13 µL from each experimental solution was added to a newly labeled eppendorf containing 5 µL of lithium dodecyl sulfate (LDS) (Cat# NP0007, Invitrogen) and 2 µL of dithiothreitol (DDT)/sample reducing agent (Cat# NP0009, Invitrogen). Samples were then placed in a 70°C water bath for 10 minutes.

The Bio-Rad Mini-Gel Box Electrotransfer kit was assembled according to the Bio-Rad instructional protocol, using NuPAGE Bis-Tris gels (Cat# NP0322, Invitrogen). Approximately 950 mL of 1X running buffer solution was poured into the electrotransfer gel box, overflowing into all chambers. 500 µL of NuPAGE antioxidant (Cat# NP0005, Invitrogen) was added to the middle chamber of the box. The first well of the gel was loaded with 15 µL of standard ladder (Cat# LC5925, Invitrogen) and the remaining wells were loaded with 15 µL of the samples. Gel electrophoresis was run at 176 Volts for 35-40 minutes.

### *Membrane Transfer*

Following gel electrophoresis, the gel was transferred to a nitrocellulose membrane (Cat# LC2001, Novex Life Technologies) using the transferring cassette apparatus, according to the Bio-Rad instructional protocol. Approximately 950 mL of 1X transfer buffer was poured into the transfer box and the transfer was run at 220 mA for 50 minutes.

### *Membrane Blocking and Antibody Incubation*

Following membrane transfer, the nitrocellulose membrane was placed in a blotting box with 2 mL of 5% bovine serum albumin (BSA) blocking solution. The blocking solution was made from 5 mL of BSA powder (Cat# SH40098.03, HyClone ThermoScientific) and 100 mL of Tris-Buffered Saline and Tween 20 (TBST). The membrane was blocked at ambient temperature for 60 minutes then the blocking solution was removed. The primary antibodies of interest, anti-PCNA (Cat# sc-7907, Santa Cruz Biotechnology) and anti-CTGF (Cat# H0001490-M01, ABNOVA) along with the control antibody  $\beta$ -Actin (Cat# 8H10D10, Cell Signaling Technology) were combined into a 1:1000 dilution solution of 6 mL BSA and 6  $\mu$ L of primary antibodies and added to the blotting box. The primary antibody solution was incubated overnight at 4°C. Next, the primary antibody solution was removed from the blotting box and the nitrocellulose membrane was washed with 1X TBST and placed on the vortex machine for 5 minutes, repeated twice. The secondary antibody solution containing anti-rabbit at a 1:1000 dilution (6 mL BSA

and 6  $\mu$ L anti-rabbit) and anti-mouse at a 1:5000 dilution (6 mL BSA and 1.2  $\mu$ L anti-mouse) was added to the blotting box and placed on the vortex machine at ambient temperature for 60 minutes. The secondary antibody solution was then removed and the membrane was washed with 1X TBST and placed on the vortex machine for 5 minutes, repeated twice.

#### *Membrane Exposure*

2 mL of enhanced chemiluminescence (ECL) substrate A and 2 mL of ECL substrate B (Cat# WP20005, Invitrogen) were added to the blotting box containing the nitrocellulose membrane for 5 minutes. In a dark room the membrane was placed into a developing cassette and exposed to chemiluminescence photographic film (Cat# 28906835, GE Healthcare) for 2-minute and 8-minute intervals.

## RESULTS

Human dermal fibroblasts were experimentally dosed *in vitro* with one of four  $T_3$  concentrations ( $10^{-7}$  M,  $10^{-8}$  M,  $10^{-9}$  M and 0 M) and subjected to western blot analysis. Two growth factor proteins, CTGF and PCNA, were qualitatively analyzed for  $T_3$ -dependent expression. Both CTGF and PCNA gene expression was detected.

### *CTGF expression in fibroblasts was unchanged by $T_3$*

CTGF expression was measured with respect to the control protein  $\beta$ -actin. CTGF gene expression was detected in all experimental  $T_3$  conditions (0 M,  $10^{-9}$  M,  $10^{-8}$  M,  $10^{-7}$  M) in equal amounts (**Figure 7**). There was no evidence of change in CTGF expression resulting from  $T_3$  concentration (**Figure 8**). There was no measureable difference in nitrocellulose membranes exposed to photographic development at 2-minute or 8-minute intervals.

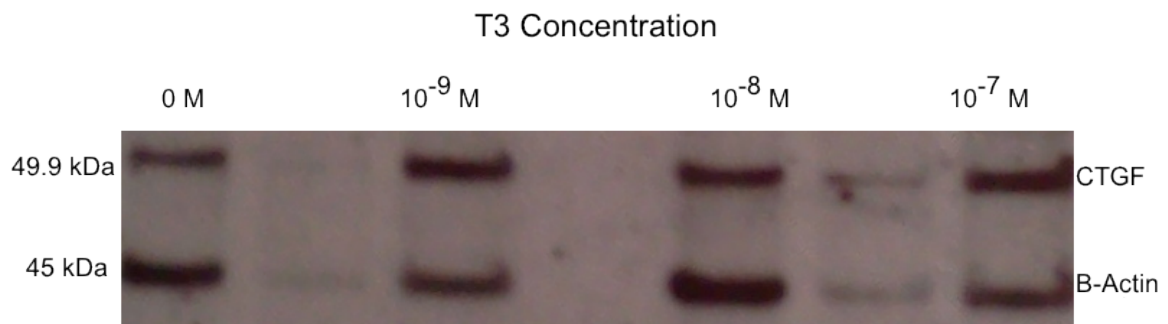


Figure 7. Western blot of connective tissue growth factor (CTGF) from human dermal fibroblasts. Top bands represent CTGF, lower bands represent  $\beta$ -actin. Lane 1 through 4 represents increasing  $T_3$  concentration (0 M,  $10^{-9}$  M,  $10^{-8}$  M,  $10^{-7}$  M). Measurement scale in kilo Daltons (kDa).

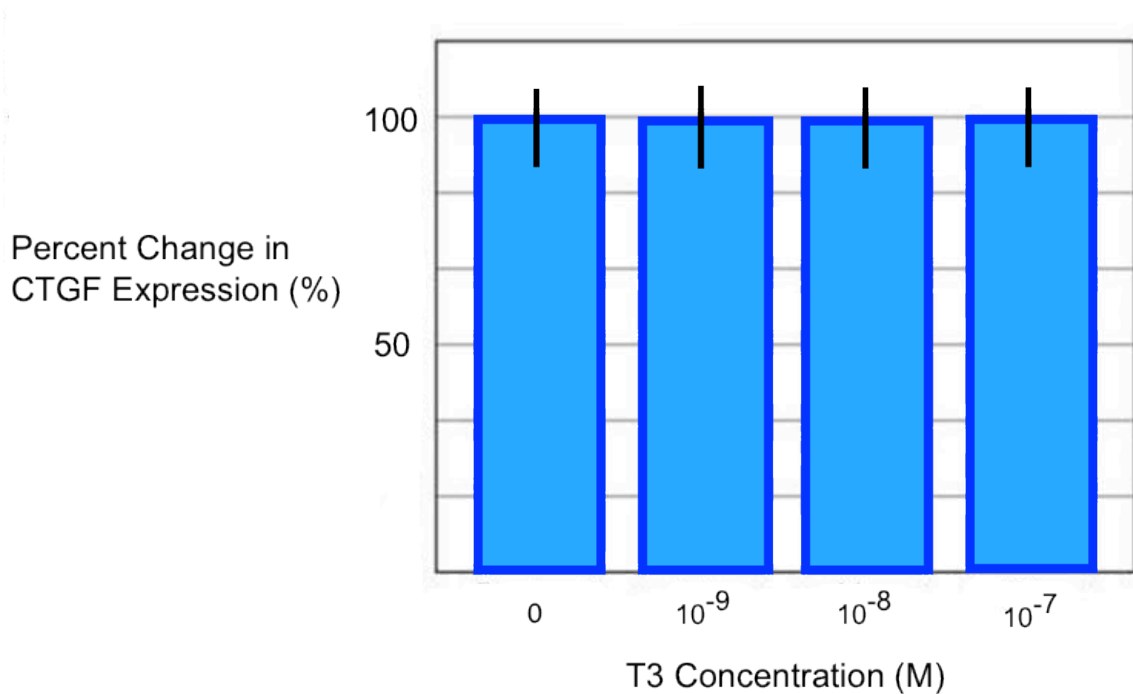


Figure 8. Percent change in connective tissue growth factor (CTGF) expression with  $T_3$  concentration. Increasing  $T_3$  concentration in fibroblast cultures had no effect on overall CTGF expression. Each experimental  $T_3$  condition (0 M,  $10^{-9}$  M,  $10^{-8}$  M,  $10^{-7}$  M) expressed the same amount of CTGF.

*PCNA expression in fibroblasts was unchanged by  $T_3$*

PCNA expression was measured with respect to the control protein  $\beta$ -actin. PCNA gene expression was detected in all experimental  $T_3$  conditions (0 M,  $10^{-9}$  M,  $10^{-8}$  M,  $10^{-7}$  M) in equal amounts (**Figure 9**). There was no evidence of change in PCNA expression resulting from  $T_3$  concentration (**Figure 10**). There was no measureable difference in nitrocellulose membranes exposed to photographic development at 2-minute or 8-minute intervals.

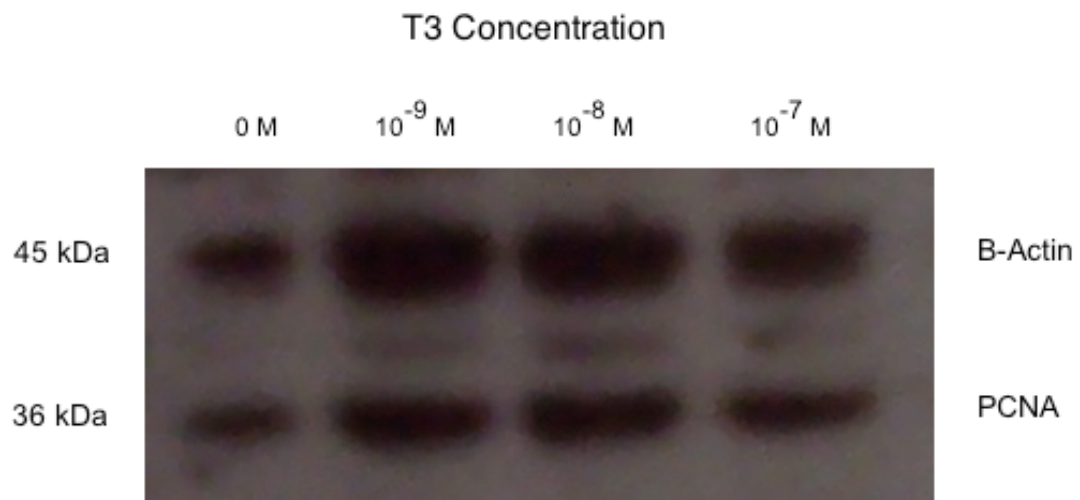


Figure 9. Western blot of proliferative cell nuclear antigen (PCNA) from human dermal fibroblasts. Top bands represent  $\beta$ -actin, lower bands represent PCNA. Lane 1 through 4 represents increasing  $T_3$  concentration (0 M,  $10^{-9}$  M,  $10^{-8}$  M,  $10^{-7}$  M). Measurement scale in kilo Daltons (kDa).

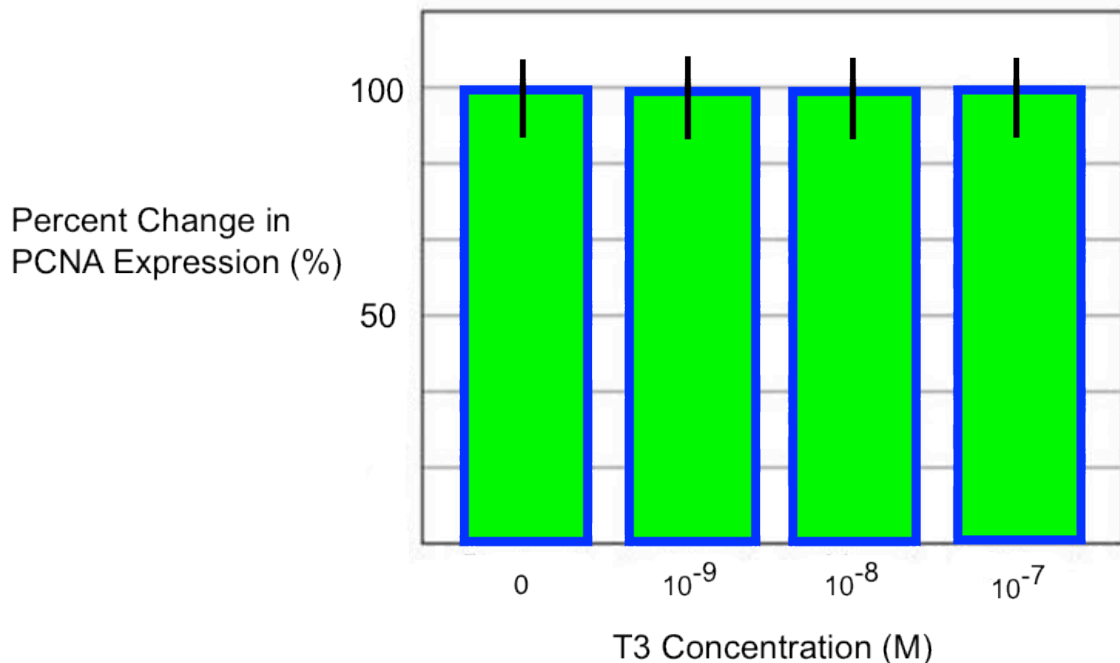


Figure 10. Percent change in PCNA expression with  $T_3$  concentration. Increasing  $T_3$  concentration in fibroblast cultures had no effect on overall PCNA expression. Each experimental  $T_3$  condition (0 M,  $10^{-9}$  M,  $10^{-8}$  M,  $10^{-7}$  M) expressed the same amount of PCNA.

## **DISCUSSION**

Thyroid hormones (TH) are active in most tissues and organ systems. Thyroid hormone nuclear receptors (TR) are found in numerous cell types including hepatocytes, osteocytes, myocytes and neurons. Consequently the effect of TH may be measured by systemic oxygen consumption and metabolic rate. TH is essential for normal growth, development and homeostasis in humans. Therefore a complete understanding of the role that TH plays in human physiology will help elucidate the underlying pathology of many thyroid-related disorders.

The physiological mechanism of action involving TH and cutaneous wound healing is still poorly understood. Mounting evidence suggests that TH and TRs play an integral role in skin homeostasis, inflammatory responses to cell damage and the proliferative and migratory events in the reparatory process (Ahsan et al., 1998; Contreras-Jurado et al., 2011; Safer et al., 2003, 2005). The current study attempted to identify potential growth factors involved in the mediation and propagation of dermal fibroblast proliferation. Once the key regulatory components in TH-dependent wound healing are identified, a theoretical mechanism of action may be proposed that will integrate the findings and establish groundwork for future research. The current study evaluated connective tissue growth factor (CTGF) and proliferative cell nuclear antigen

(PCNA) as possible growth factors involved in TH-dependent fibroblast proliferation.

CTGF is a *multifunctional matrixcellular protein* (Leask & Abraham, 2003) and a downstream regulator of TGF- $\beta$ . Previous research demonstrates that CTGF stimulates fibroblast proliferation and cell adhesion, ECM remodeling and is a major regulator of fibrotic scar tissue (Igarashi et al., 1993; Seher et al., 2011; Werner & Grose, 2003). Consequently, CTGF was an appealing candidate for TH-dependent fibroblast proliferation. Similarly, PCNA is a fundamental indicator of cell proliferation (Scovassi & Prospero, 2006). It is present in all eukaryotic cells and has been shown to regulate DNA synthesis and repair following cell damage. As such, PCNA is also a logical growth factor to study in TH-dependent cutaneous wound healing and fibroblast proliferation.

Western blot analysis was performed as a preliminary means of identifying the proteins upregulated by dermal fibroblasts following *in vitro* addition of T<sub>3</sub> to four experimental plates with varying concentrations of T<sub>3</sub>. Western blotting was conducted using CTGF and PCNA antibodies. The results demonstrate that both CTGF and PCNA gene expression were present in fibroblast cultures. However, neither CTGF nor PCNA expression was altered by TH activation. While there is evidence that CTGF and PCNA are present during fibroblast proliferation, it is not likely that there is direct regulation via T<sub>3</sub> mechanisms.

CTGF expression was measured in four experimental conditions. Fibroblasts were dosed with 10<sup>-7</sup> M, 10<sup>-8</sup> M, 10<sup>-9</sup> M and 0 M of T<sub>3</sub>. CTGF was

qualitatively measured in comparison to  $\beta$ -actin, which served as the control parameter. While CTGF expression was observed in each  $T_3$  condition (**Figure 7**), there was no apparent change in intensity. Each condition displayed the same amount of CTGF protein relative to  $\beta$ -actin. CTGF expression was also observed in the control condition that lacked  $T_3$ , suggesting that CTGF does not require  $T_3$  stimulation during fibroblast proliferation. One potential explanation may clarify these results. It is possible that  $T_3$  concentrations were insufficient to demonstrate any effect on CTGF production. Previous research (Safer et al., 2003) has demonstrated that  $T_3$  stimulates fibroblast proliferation with maximal effect at 0.5 nM of  $T_3$ . The current study used  $T_3$  concentrations above, below and inclusive of that range without significant effect. Perhaps if the  $T_3$  concentration were augmented to  $10^{-6}$  M an observable change in CTGF expression would occur. Since CTGF is largely expressed in fibrotic diseases, it is likely that a greater concentration of  $T_3$  would be necessary to induce fibroblast production of CTGF and therefore increasing  $T_3$  to  $10^{-6}$  M would be warranted.

PCNA expression was measured in four experimental conditions. Fibroblasts were dosed with  $10^{-7}$  M,  $10^{-8}$  M,  $10^{-9}$  M and 0 M of  $T_3$ . PCNA was qualitatively measured in comparison to  $\beta$ -actin, which served as the control parameter. While PCNA expression was observed in each  $T_3$  condition (**Figure 9**), there was no apparent change in intensity. Each condition displayed the same amount of PCNA protein relative to  $\beta$ -actin. PCNA expression was also observed in the control condition that lacked  $T_3$ , suggesting that PCNA does not require  $T_3$

stimulation during fibroblast proliferation. One possible explanation may delineate these results. Since PCNA is indicative of cell proliferation (Scovassi & Prosperi, 2006) it is possible that the fibroblasts cultured to 80% confluence were overly stable and had already achieved maturity. In other words, the majority of fibroblasts were not in a proliferative state. This would support the observation that PCNA expression was unchanged regardless of  $T_3$  concentration. Future research involving  $T_3$ -dependent PCNA expression should consider using *in vitro* fibroblasts at 50-70% confluence to determine whether any measureable change would occur.

CTGF and PCNA were studied with the intention of proposing a mechanism of action for TH-dependent fibroblast proliferation. Since the results have demonstrated no qualitative difference in CTGF or PCNA expression, it was not possible to hypothesize about a possible intracellular biological mechanism. It is still unclear whether  $T_3$  induces fibroblast proliferative growth factors via genomic or non-genomic signals. Traditionally,  $T_3$  binds to thyroid hormone nuclear receptors (TR) within target cell nuclei and promote thyroid hormone response elements (TRE) to induce DNA expression. However, recent evidence suggests that  $T_3$  also stimulates non-genomic cellular responses, including activation of cell membrane receptors and cytoplasmic signaling cascades (Aranda et al., 2009; Kress et al., 2009; Lin et al., 2012). It is therefore possible that  $T_3$  exerts influence on cutaneous wound healing by either genomic or non-genomic mechanisms. Furthermore, the possibility that a combination of genomic

and non-genomic factors regulate  $T_3$ -dependent wound healing should also be considered.

The current study was not intended to encapsulate a complete rendering of fibroblast activity during the proliferative phase of wound healing, but rather it was meant to serve as an introductory sampling of possible growth factors involved in the regulatory process. Nonetheless, one key limitation became apparent post-facto. The growth media used during fibroblast *in vitro* cell culturing contained fetal bovine serum (FBS) to supplement growth factors present *in vivo*. Previous research has shown that FBS contains endogenous levels of  $T_3$  (Moeller et al., 2009) and thus could have inadvertently stimulated fibroblasts to produce TH-dependent growth factors in the control group (0 M of  $T_3$ ). In order to subjugate this possibility, double-stripped serum (DSS) was added to the experimental plates during  $T_3$  fibroblast dosing to minimize extraneous influence of growth factors, cytokines and other lipophilic compounds. However, there remains a marginal chance that FBS in the original growth media influenced fibroblast growth and subsequent secretory products. Future attempts should restrict the use of FBS and use instead a  $T_3$ -free serum in the growth media.

Furthermore, there are two additional factors that may not contribute significantly to the results but should nevertheless be considered. The first concerns membrane exposure times during photographic development. The current study chose 2-minute and 8-minute exposure intervals with the intent of

comparing the results of brief (2-minute) and long (8-minute) exposures. The results demonstrated no change in intensity dependent on photographic exposure. Although the chosen time intervals for the brief and long conditions were presumably disparate, it is worth considering increasing the long exposure interval to 12 or 15 minutes in the event that subtle but significant differences become apparent.

The second constraint reflects the qualitative nature of the current analysis, which relies heavily on subjective characterizations of intensity gradients. For a thorough and objective analysis, future studies should incorporate the use of quantifiable software, for instance the use of ImageJ Technology, to differentiate among intensity bands in western blot analyses.

Future directions should also consider the effects of *in vitro* cocultured keratinocytes and fibroblasts with regard to TH-dependent growth factors. Previous research by Safer et al., (2003) has demonstrated that T<sub>3</sub> inhibits keratinocyte proliferation in cocultured fibroblast samples. The authors suggested that T<sub>3</sub> induced fibroblasts to produce inhibitory proliferative signals directed at keratinocytes. However, these proposed inhibitory signals were not investigated. By replicating the current study with cocultured samples, it may be possible to discover these inhibitory growth factors that act in an autocrine or paracrine regulatory mechanism.

Understanding the role of TH in wound healing is critical to developing strategies to treat thyroid-related skin perturbations and aid patient populations

who present with impaired wound healing capability. The current study focused on identifying the growth factors responsible for TH stimulated fibroblast proliferation. At this time, it appears as though CTGF and PCNA may not be directly involved in the TH-dependent mechanism of action. Continued research should investigate other possible proliferative growth factors with the intent of ultimately discovering the underlying TH-dependent skin physiology.

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