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The effects of topical thyroid hormone application on growth factor expression in human fibroblasts

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Thesis

THE EFFECTS OF TOPICAL THYROID HORMONE APPLICATION ON
GROWTH FACTOR EXPRESSION IN HUMAN FIBROBLASTS

by

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THE EFFECTS OF TOPICAL THYROID HORMONE APPLICATION ON GROWTH FACTOR EXPRESSION IN HUMAN FIBROBLASTS

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ABSTRACT

It has long been known that thyroid hormones (TH) are essential for normal growth, development and cell metabolism. Studies have also shown that topical TH application can increase epidermal cell proliferation and accelerate the process of cutaneous wound healing. Despite these promising results, few researchers have examined the mechanisms and mediators through which TH may exert its effects on skin cells. In this study, human fibroblasts were stimulated with increasing triiodothyronine (T₃) concentrations. A western blot was then conducted on the stimulated cell samples to determine whether two key growth factors – epidermal growth factor (EGF) and fibroblast growth factor (FGF-7) – that have been implicated in wound healing were upregulated due to topical T₃ stimulation. It was found that while EGF (indirectly detected via the presence of the epidermal growth factor receptor (EGFR)) trended towards being upregulated at higher T₃ concentrations, no such effect was observed with FGF-7. It was
therefore determined that the EGF pathway may be an important mechanism through which TH exerts its effects on human fibroblasts and the process of cutaneous wound healing.
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ABBREVIATIONS

D1 – Type I Deiodinases
D2 – Type II Deiodinases
D3 – Type III Deiodinases
DIT – Diiodotyrosine
EGF – Epidermal Growth Factor
EGFR – Epidermal Growth Factor Receptor
ERK – Extracellular – Regulated Signal Kinase
FGF – Fibroblast Growth Factor
FGFR – Fibroblast Growth Factor Receptor
FSH – Follicle Stimulating Hormone
hCG – Human Chorionic Gonadotropin
HB-EGF – Heparin-binding EGF-like Growth Factor
IGF – Insulin – Like Growth Factor
IL – 1 – Interleukin – 1
KGF – Keratinocyte Growth Factor (Fibroblast Growth Factor – 7)
KGFR – Keratinocyte Growth Factor Receptor
LH – Luteinizing Hormone
MAPK – Mitogen Activated Protein Kinase
MEK – MAPK/ERK Kinase
MIT – Monoiodotyrosine
PDGF – Plasma Derived Growth Factor
PLCγ – Phospholipase Cγ
PI3K/Akt – Phosphatidylinositol-3-OH Kinase
PMNs – Polymorphonuclear Leukocytes (neutrophils)
RTK – Receptor Tyrosine Kinase
RT-PCR – Real-Time Polymerase Chain Reaction
STAT – Signal Transducers and Activators of Transcription
rT₃ – Reverse Triiodothyronine
T₃ – Triiodothyronine
T₄ – Tetraiodothyronine
TGF – α– Transforming Growth Factor Alpha
TBG – Thyroxine-Binding Globulin
TG – Thyroglobulin
TH – Thyroid Hormones
TPO – Thyroid Peroxidase
TNF – α – Tumor Necrosis Factor Alpha
TR – Thyroid Hormone Receptors
TRE – Thyroid Hormone Response Elements
TRH – Thyrotropin Releasing Hormone
TSH – Thyroid Stimulating Hormone
INTRODUCTION

Thyroid hormone biosynthesis

It has long been known that thyroid hormones (TH) are key players in growth, development and metabolism. Their effects in the body are ubiquitous and wide-ranging, with target organs found in the cardiovascular, neural, skin, and muscular systems (Wong and Schaffer, 2008). Despite its pervasive nature, thyroid hormone’s effects on and mechanism of action in cutaneous wound healing have not been extensively researched.

The synthesis of thyroid hormone occurs in the thyroid gland, a butterfly-shaped organ located near the trachea (Molina, 2010). The follicular cells of the thyroid gland are responsible for the synthesis of thyroid hormone, and are bordered by thyroglobulin (TG) and glycoprotein containing cells known as the colloid (Barrett et al., 2012). When the follicular cells are in the process of synthesizing thyroid hormone, they appear smaller, as opposed to their larger appearance during states of inactivity (Barrett et al., 2012).

The thyroid hormone (TH) itself is derived from the amino acid tyrosine, and its production and subsequent release in the body is tightly regulated via the Hypothalamic-Pituitary-Thyroid negative feedback axis (Molina, 2010). Iodine is a key constituent for thyroid hormone biosynthesis, and its transport across thyrocytes is controlled through a system of membrane transporters (Barrett et al., 2012). The synthesis of thyroid hormone includes several steps and requires
the presence of thyroid peroxidase (TPO) (Fig. 1) TPO is responsible for the oxidation of iodide into iodine (Fig.1).

![Diagram of thyroid hormone biosynthesis]

**Figure 1. Biosynthesis of thyroid hormone (TH).** Iodide is activated by thyroid peroxidase (TPO) to iodine, and it is added to a tyrosine ring to yield monoiodotyrosine (MIT), and diiodotyrosine (DIT). A small amount of MIT and DIT combine to yield triiodothyronine ($T_3$), while the majority of DIT molecules combine to yield tetraiodothyronine ($T_4$). Figure downloaded from Molina, 2010 at http://www.accessmedicine.com/content.aspx?aID=6169456.
While the tetraiodothyronine (T₄) version of the hormone is synthesized in larger amounts, it is the triiodothyronine (T₃) variant that has greater biological activity (Molina, 2010). The thyroid also synthesizes a small amount of the biologically inactive compound reverse triiodothyronine (rT₃), although much of the rT₃ and T₃ are obtained from interconversion from T₄ at the tissue level (Fig. 2) (Molina, 2010).

Figure 2. An overview of the secretion and interconversion of thyroid hormones (TH). The numbers refer to average daily amounts for an adult human. The interconversion occurs mostly at the tissue level with the help of deiodinases. Type I deiodinases (D1) and Type II deiodinases (D2) convert tetraiodothyronine (T₄) to triiodothyronine (T₃), while T₄ is converted to reverse triiodothyronine (rT₃) via the actions of Type III deiodinases (D3). Figure taken from Barrett et al., 2012 at http://www.accessmedicine.com/content.aspx?aID=56262695.
A unique feature of thyroid hormone (TH) physiology is that the human body has a reserve of TH that is capable of sustaining normal bodily functions for a period of approximately two months (Barrett et al., 2012). Additionally, TH bound to proteins in the circulatory system also acts as a more immediate reserve for the body, as only the unbound hormones can exert their biological effects on cells (Barrett et al., 2012). The major protein carriers for thyroid hormones in the blood stream appear to be albumin, thyroxine-binding globulin (TBG), and transthyretin (Barrett et al., 2012).

In order to convert the large amount of T₄ produced by the thyroid gland into the biologically active T₃ and the inactive rT₃, enzymatic molecules known as deiodinases are required. There are three different types of deiodinases that have been identified in the body, and all contain the trace element selenocysteine, which is essential for the enzymatic activity of the deiodinases (Molina, 2010).

Type I deiodinases (D1) – present mostly in the liver, kidney and thyroid – convert T₄ to T₃ (Molina, 2010). Type II deiodinases (D2) also convert T₄ to T₃, and are normally found in the pituitary gland, cardiovascular and skeletal muscle system, brain and thyroid (Molina, 2010). D2 is also known to play an essential role in the pituitary gland as part of the Hypothalamic-Pituitary-Thyroid negative feedback axis (Molina, 2010). Type III deiodinases (D3) convert T₄ to rT₃, and have been found in the placenta, brain and skin (Molina, 2010).
Thyroid hormone regulation

The rate of production of thyroid hormones (TH) is tightly regulated via the Hypothalamic-Pituitary-Thyroid negative feedback axis (Fig.3). In essence, the hypothalamus secretes an entity known as the thyrotropin releasing hormone (TRH), which directly stimulates the thyrotropes in the anterior pituitary gland to release thyroid stimulating hormone (TSH). The TSH travels in the circulatory system to reach the thyroid gland, where it stimulates the thyroid follicles to produce the thyroid hormones T4, T3 and rT3 (Barrett et al., 2012).

Much of the negative feedback depends on the restriction of TSH production, effectively shutting down the communication between the anterior pituitary and thyroid gland (Molina, 2010). High amounts of unbound T4 and T3 act to inhibit the release of TSH from the anterior pituitary, as do certain other signaling molecules released during physiological states of stress (Barrett et al., 2012).

Structurally, TSH consists of an α-subunit (identical to the α-subunit found in the follicle stimulating hormone (FSH), luteinizing hormone (LH), and the human chorionic gonadotropin hormone (hCG)), and a distinct β-subunit which accounts for its physiologic activity (Sathananthan et al., 2008). TSH acts on the thyroid via a G-protein – linked receptor to stimulate the production of thyroid hormones in the follicular cells (Sathananthan et al., 2008).

It is interesting to note that in the absence of the pituitary gland and endogenous TSH production, the thyroid gland starts to degenerate (Barrett et
al., 2012). In such a scenario, the biological activity of the thyroid gland can be restored via the exogenous administration of TSH (Barrett et al., 2012).

Figure 3. The Hypothalamic-Pituitary-Thyroid negative feedback axis. Thyrotropin releasing hormone (TRH) produced by the hypothalamus stimulates the anterior pituitary gland to release thyroid stimulating hormone (TSH). TSH stimulates the thyroid gland to produce the thyroid hormones (TH), tetraiodothyronine (T\textsubscript{4}) and triiodothyronine (T\textsubscript{3}). High levels of free T\textsubscript{4} and T\textsubscript{3} subsequently act as negative feedback regulators to inhibit the release of TSH from the thyrotropes in the anterior pituitary. Figure downloaded from Molina, 2010 at http://www.accessmedicine.com/content.aspx?aID=6169456.
Thyroid Hormone mechanism of action

Given the lipophilicity of the thyroid hormones (TH), their physiological effects are mediated intracellularly, and the hormone is thought to enter the cell via plasma membrane carrier proteins (Molina, 2010) (Fig.4). Once inside the cells, the hormone binds to TH specific nuclear receptors and Thyroid Hormone Response Elements (TRE), and thus affects the transcriptional regulation of select genes (Molina, 2010) (Fig.4). Thyroid hormone receptors have been identified in a variety of tissues including skin fibroblasts, hair follicles, and epidermal keratinocytes (Safer et al., 2001).

Figure 4. The biological effects of thyroid hormone are regulated intracellularly. Plasma membrane receptors allow tetraiodothyronine (T$_4$) and triiodothyronine (T$_3$) to enter the cell, and type I deiodinases (D1) convert T$_4$ to T$_3$ in the cell membrane. T$_3$ then mediates its effects in the nucleus by binding to TH nuclear receptors and Thyroid Hormone Response Elements (TRE), thereby producing the desired gene product. Figure downloaded from Molina, 2010 at http://www.accessmedicine.com/content.aspx?aID=6169456.
Thyroid hormone and wound healing

Prior to the demonstration that the addition of thyroid hormone (TH) to human skin cultures increased epidermal cell proliferation, TH’s cutaneous effects have long been observed in thyroid related pathologies (Safer et al., 2003). When there is a lack of TH synthesis in the body leading to hypothyroidism, the epidermis is noticeably dry; while in states of high TH synthesis leading to hyperthyroidism, the epidermis is observed to be thinner than normal (Safer, 2012). The presence of thyroid hormone receptors (TR) in the skin has also been demonstrated, as has the expression of deiodinases (Safer, 2012). Despite these findings that indicate that TH has an effect on skin cells and their proliferation, very little data exists examining whether TH could be used in a topical manner to accelerate cutaneous wound healing.

A few studies conducted by Safer et al. have demonstrated that topical thyroid hormone application can increase epidermal cell growth and thus accelerate wound healing in mice (Fig. 5) (Safer et al., 2004; and Safer et al., 2005). Using Real-Time Polymerase Chain Reaction (RT-PCR), the investigators determined that keratinocyte genes in the epidermis were upregulated in skin cells stimulated by thyroid hormone when compared with cells that were not stimulated (Safer et al., 2004). Keratin is an important constituent of the epidermis, and accelerating its expression can aid in improving wound healing (Safer et al., 2004).
Additionally, Safer et al. have also shown *in vivo* that daily topical application of thyroid hormone on the skin of wounded mice resulted in a 58% increase in wound healing when compared to wounded mice that were not given any topical thyroid hormone (Fig. 5) (Safer et al., 2005). Their data indicated that epidermal keratin 6 protein expression increased *in vivo* with increasing thyroid hormone (TH) concentration (Safer et al., 2005). Epidermal keratin 6 is an important wound repair protein and its upregulation by TH indicates that topical TH application can potentially be used in cases of slow healing wounds (Safer et al., 2005).
Figure 5. The effect of topical thyroid hormone application on wound healing in mice. A and B indicate wounds at the start and finish of the experimental period, respectively. The wounds to the left of each panel received no thyroid hormone, while the ones pictured to the right received a daily dose of triiodothyronine (T₃). C indicated that there was significant reduction in the wounds treated by T₃ at the end of the study. Figure taken from Safer, et al. 2005.
An overview of wound healing

The efficient and proper closure of cutaneous wounds is essential to maintaining the body’s microenvironment. As the largest organ in the body, the skin serves an essential role in preventing the entry of environmental pathological agents into cells. The improper or slow healing of cutaneous wounds can lead to the dysfunction of key bodily process and result in severe morbidity and even mortality (Singer and Clark, 1999). In 2009, Sen et al. estimated that approximately US$25 billion is spent every year in the United States due to complications arising from slow healing wounds (Sen et al., 2009). This figure is only expected to rise as conditions that impair cutaneous wound healing – such as diabetes – increase in prevalence (Sen et al., 2009). There is thus a critical need to find inexpensive and novel therapies that can accelerate the process of wound healing.

In order to comprehend the events and mediators involved in cutaneous wound healing, it is essential to have a basic understanding of the structure of the skin. The skin is composed of three major layers known as the epidermis, the dermis, and the subcutaneous adipose tissue (Mihm et al., 2012). The major cell type found in the epidermis is the keratinocyte, while an extracellular matrix consisting of collagen and other elastic fibers forms a large portion of the dermis (Mihm et al., 2012). There are also several glands and hair follicles that can be found entrenched into the three cutaneous tissues (Mihm et al., 2012).
The process of cutaneous wound healing itself is a complicated and multi-step process that involves the coordinated efforts of several biological mediators and cell types (Fig. 6). The end goal is to restore the proper functioning and structure of the skin, and to close off the injured area to pathological agents found in the environment. Cutaneous wound healing can broadly be divided into three key phases known as the inflammatory phase, the granulation tissue formation phase, and the tissue remodeling phase (Fig. 6) (Singer and Clark, 1999). While the phases do begin in a sequential order, they also overlap and there are no clear temporal markers separating one phase from another (Fig. 6) (Singer and Clark, 1999).

The inflammatory phase begins immediately following tissue injury. A fibrin clot is formed to curb any bleeding in the area, and the clot also serves as a provisional mesh framework that cells can later use to move into the wounded region (Barbul, 2010). A fundamental hallmark of the inflammatory phase is the presence of polymorphonuclear leukocytes (PMNs) – better known as neutrophils – in the wounded area (Fig. 7) (Barbul, 2010). The neutrophils act as a first line of defense and function in bacterial phagocytosis and the removal of dysfunctional cell remains (Barbul, 2010).

The platelets present in the blood clot also release several chemical mediators – such as epidermal growth factor (EGF), transforming growth factor beta (TGF-β), and platelet-derived growth factor (PDGF) – that serve to recruit fibroblasts and monocyte derived macrophages to the wound (Fig. 7) (Barbul,
Macrophages are essential for proper bacterial clearance, and the release of chemical mediators and growth factors needed to recruit cells (e.g. endothelial cells and fibroblasts) that are vital for proper healing to occur (Fig. 7) (Barbul, 2010).

**Figure 6. The phases of cutaneous wound healing.** The top panel serves to indicate the overlap between the different phases of wound healing, with the timescale beginning in units of days followed by months. The middle panel shows the key cells involved in each phase, while the bottom panel shows the major structural proteins deposited in each phase of healing. Figure downloaded from Barbul, 2010 at http://www.accessmedicine.com/content.aspx?aID=5013857.
The granulation tissue formation or proliferation phase of cutaneous wound healing overlaps with the tail end of the inflammation phase, and has been observed to occur approximately 4 days after injury (Fig. 6) (Barbul, 2010). The key cell types involved in this phase are the fibroblast cells – which are needed for the formation of the extracellular matrix – and endothelial cells, which are essential to the process of angiogenesis (Barbul, 2010). Both fibroblast and endothelial cells are recruited to the wound site by growth factors and cytokines secreted by the cells in the inflammatory phase (Barbul, 2010). The matrix synthesized by the activated fibroblasts consists mainly of collagen types I and III, and proteoglycans, and it is needed for subsequent wound closure and scar formation (Barbul, 2010).

The final phase of wound healing – known as the extracellular matrix remodeling phase – consists of amending the previously laid down granulation tissue collagen matrix to form a more permanent scar (Singer and Clark, 1999). This phase overlaps with the granulation tissue formation phase and can last from a period of days to months (Fig. 6) (Singer and Clark, 1999). Remodeling of the previously laid down ECM takes place due to the actions of enzymatic matrix metalloproteinases secreted by macrophages and fibroblasts (Singer and Clark, 1999). Matrix metalloproteinases break down collagen fibers, and the final product of remodeling represents an equilibrium between proper collagen degradation and collagen production (Barbul, 2010). The proliferation of epithelial cells to replace those lost to injury also continues in this phase of cutaneous
wound healing, and EGF, PDGF, and keratinocyte growth factor (KGF) have been thought to play an important role in this process (Barbul, 2010).

Figure 7. Key mediators in cutaneous wound healing as observed 3 days after injury. The inflammatory phase of wound healing involves several cytokines and growth factors that work in a coordinated fashion to repair a wounded area. Shown above are the functional manifestations of the fibroblast growth factor (FGF), insulin-like growth factor (IGF), keratinocyte growth factor (KGF), platelet-derived growth factor (PDGF), transforming growth factor (TGF), and vascular endothelial growth factor (VEGF). Figure taken from Singer and Clark, 1999.
Growth factors and thyroid hormone induced wound healing

Effective cutaneous wound healing involves a highly choreographed sequence of growth factor activation (Fig. 7). There are several growth factors that have been identified in wound healing, and each one plays a specific role in repairing the wound and restoring the integrity of the skin (Table 1). It is therefore not unreasonable to postulate that thyroid hormones (TH) may exert their effects on wound healing by upregulating or mediating the effects of some of these growth factors, and for the purposes of this study, we will be focusing on the epidermal growth factor (EGF), and the keratinocyte growth factor (KGF) (Fisher et al., 1982).

*Epidermal growth factor (EGF)*

In 1983, Hoath et al. conducted a study to examine the effects of TH application on EGF concentration in neonatal mouse skin (Hoath et al., 1983). EGF is normally secreted by platelets, macrophages, and fibroblasts cells, and plays a critical role in promoting epithelial cell proliferation and migration in tissues following injury (Yu et al., 2010).

Hoath et al. found that daily injections of T₄ and T₃ resulted in a dose-dependent increase in EGF concentrations in the neonatal mouse skin samples, indicating that TH effects on skin could be mediated via the EGF pathway (Hoath et al., 1983). The stimulation of the mouse skin samples with 0.2 µg and 0.1 µg of T₃ resulted in EGF concentrations rising from 152 ± 11 pg/mg protein in the
control population to 408 ± 57 pg/mg protein (for 0.2 µg T<sub>3</sub> application) and 575 ± 61 pg/mg protein (for 1.0 µg T<sub>3</sub> application) in the EGF treated population (Hoath et al., 1983). The researchers also demonstrated that the biologically inactive rT<sub>3</sub> had no noticeable effect on EGF concentration in the neonatal mouse skin samples (Hoath et al., 1983).

Additionally, in a randomized and double-blind clinical trial, Brown et al. showed that topical application of EGF on wounded human skin resulted in a decrease in the time required for the skin to heal by up to 50 percent by one day, and heal by up to 100 percent by 1.5 days (Brown et al., 1989). Despite data in support of EGF’s potential role in accelerating wound healing, there has been a dearth of data examining its use in therapeutic applications.

EGF is part of a family of proteins that includes transforming growth factor alpha (TGF-α), and the heparin-binding EGF-like growth factor (HB-EGF) (Yu et al., 2010). TGF-α and HB-EGF are also important in wound healing and are primarily secreted by keratinocyte cells (Yu et al., 2010). All three ligands exert their effects on cells by binding to the receptor tyrosine kinase (RTK) epidermal growth factor receptor (EGFR).

EGFR is a 170-kDa transmembrane glycoprotein, and it is known to activate several signaling cascades that mediate the cutaneous wound healing effects of the EGFR ligands (Fig. 8) (Koppikar and Grandis, 2008). EGFR is comprised of an extracellular ligand binding domain, a transmembrane domain, and a catalytic cytoplasmic domain (Fig. 8) (Eswarakumar et al., 2005). In this study we will
measure EGFR upregulation as a proxy for EGF production by TH stimulated fibroblasts.

**Table 1. Growth factors involved in cutaneous wound healing.**

<table>
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<tr>
<th>Growth Factor</th>
<th>Production</th>
<th>Known Effects</th>
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<tr>
<td>1. Epidermal Growth Factor (EGF)</td>
<td>Platelets, macrophages, fibroblasts</td>
<td>Stimulates fibroblasts to secrete collagenase to degrade the matrix during the remodeling phase. Stimulates keratinocyte and fibroblast proliferation. May reduce healing time when applied topically.</td>
</tr>
<tr>
<td>2. Transforming Growth Factor</td>
<td>Platelets, macrophages, lymphocytes, hepatocytes</td>
<td>TGF-a: Mitogenic and chemotactic for keratinocytes and fibroblasts TGF-b1 and TGF-b2: Promotes angiogenesis, up-regulates collagen production and inhibits degradation, promotes chemoaattraction of inflammatory cells. TGF-b3 (antagonist to TGF-b1 and b2): Has been found in high levels in fetal scarless wound healing and has promoted scarless healing in adults experimentally when TGF-b1 and TGF-b2 are suppressed.</td>
</tr>
<tr>
<td>3. Vascular Endothelial Growth Factor (VEGF)</td>
<td>Endothelial cells</td>
<td>Promotes angiogenesis during tissue hypoxia.</td>
</tr>
<tr>
<td>4. Fibroblast Growth Factor (FGF)</td>
<td>Macrophages, mast cells, T-lymphocytes</td>
<td>Promotes angiogenesis, granulation, and epithelialization via endothelial cell, fibroblast, and keratinocyte migration, respectively.</td>
</tr>
<tr>
<td>8. Keratinocyte growth factor</td>
<td>Fibroblasts</td>
<td>Stimulates keratinocyte migration, differentiation, and proliferation.</td>
</tr>
</tbody>
</table>

The table represents only a partial list of the growth factors implicated in wound healing. Figure downloaded and amended from Gabriel et al., 2011 at http://emedicine.medscape.com/article/1298196-overview#aw2aab6b4.
Figure 8. The effects of ligand binding on the epidermal growth factor receptor (EGFR). Shown above are the major signaling cascades affected by the activation of EGFR, namely the mitogen-activated protein kinase (MAPK) pathway, the phosphatidylinositol-3-OH kinase (PI3K/Akt) pathway, the phospholipase Cγ (PLCγ) pathway, and the signal transducers and activators of transcription (STAT) pathways. Figure downloaded from Koppikar and Grandis, 2008 at http://link.springer.com.ezproxy.bu.edu/chapter/10.1007/978-1-59745-356-1_6.
**Keratinocyte growth factor (KGF)**

KGF, also known as fibroblast growth factor-7 (FGF-7), is a 28 kDa protein that is part of a family of growth factors that are involved in stimulating the proliferation and differentiation of a variety of cell types (Werner, 1998). KGF in particular acts on epithelial cells, and in the process of wound healing it is secreted by fibroblasts stimulated by cytokines and growth factors released by platelets, neutrophils, and macrophages (Fig. 9) (Werner, 1998).

There are four known RTK based fibroblast growth factor receptors (FGFR), and they are generally referred to as FGFR1, FGFR2, FGFR3, and FGFR4 (Werner, 1998). KGF is known to only bind to the IIIb isoform of the FGFR2 receptor (Werner, 1998). For successful binding to occur between the fibroblast growth factors and their receptors, heparin sulphate oligosaccharides and cycsteine-rich FGF-binding proteins are needed (Werner, 1998).

There have been several studies describing the essential role that KGF plays during cutaneous wound healing. It is know that the IIIb isoform of the FGFR2 receptor is present in the epidermal layer of skin (Werner, 1998). In 1992, Werner et al. demonstrated that there was a 160-fold increase in KGF mRNA immediately following a cutaneous wound, indicating that dermally secreted KGF might play an essential role in the proliferation of keratinocytes in the epidermis during the granulation tissue formation and ECM remodeling phase of wound healing (Werner et al., 1992).
Additionally, it was found that the exogenous application of KGF on cutaneous wounds in pigs caused an increase in epithelial cell proliferation, and the PDGF secreted by platelets was essential in stimulating fibroblasts to secrete KGF in response to a cutaneous wound (Werner, 1998). There have also been in vivo studies conducted in mice that have demonstrated that the lack of KGF leads to decreased keratinocyte proliferation and angiogenesis (Peng et al., 2011). Given such data, it is vital that we understand whether KGF secretion by fibroblasts can be upregulated by the topical application of TH, so that we can have an inexpensive method with which we can stimulate KGF production in fibroblasts and accelerate wound healing.

![Figure 9](image.png)

**Figure 9.** The release of keratinocyte growth factor (KGF) from fibroblasts is mediated by cytokines released by macrophages, platelets and polymorphonuclear leukocytes (PMNs). The major stimulators for KGF release from fibroblasts include platelet-derived growth factor (PDGF), transforming growth factor alpha (TGF-α), tumor necrosis factor alpha (TNF–α), and interleukin-1 (IL–1). Glucocorticoids are known to inhibit the release of KGF. Figure taken from Werner, 1998.
Objectives

The cutaneous effects of TH have long been observed in cases of thyroid dysfunction, and recent studies have also demonstrated the potential therapeutic role topical TH application may play in accelerating cutaneous wound healing (Safer et al., 2005, and Safer et al, 2004). Despite the promising data observed in these studies, few researchers have attempted to characterize the mechanisms through which TH may exert its effects on wound closure.

The goal of this study is to determine the effects of TH stimulation on growth factors released by fibroblasts cells. In particular, we will examine whether EGF and KGF secretion is upregulated in a dose-dependent manner when fibroblasts are stimulated with increasing amounts of TH. Both EGF and KGF are secreted by fibroblasts when they are stimulated by cytokines released by platelets, macrophages, and neutrophils during wound healing, and they are critical to epidermal proliferation (Fig.7 and Fig. 9). Given the essential nature of their roles in skin regeneration, it is plausible that TH could exert its effects on cutaneous wound healing by upregulating the expression of EGF and KGF.

We will conduct western blots on fibroblast cells stimulated with increasing concentrations of T₃, in order to ascertain EGF (indirectly measured via the expression of EGFR) and KGF upregulation. If the expression of EGF or KGF is enhanced in TH stimulated fibroblasts, we can have a better idea of the biological pathways though which TH mediates its effects on wound healing.
MATERIALS AND METHODS

Cell Culture

Human dermal fibroblasts were used in all experimental procedures, and the fibroblasts were grown in a monolayer to approximately 80% confluence before use. Overgrown cells were not used in the experiments. Sterile petri dishes were used to grow the cells and all cell handling took place under a sterile cell culture approved hood.

Cell culture media was prepared using high glucose HEPES Dulbecco’s Modified Eagles Medium (DMEM), 5% charcoal stripped Fetal Bovine Serum (FBS), and 1% antibiotic. Before passage or removal from the petri dish, cells were washed with Ethylenediaminetetraacetic acid (EDTA) and dislodged from the petri dish using cell culture grade trypsin. In order to enable the cells to come off easily from the plates, they were incubated with pre-warmed Trypsin at 37° C for no more than 5 – 10 minutes. The media was changed roughly once every 3 days or as necessary, and the cells were grown at 37° C and 5% CO₂.

Cells were maintained and used in experimental procedures for no more than 3 – 4 weeks at a time. This was done to avoid complication of the data due to anomalies resulting from older cells, and to also prevent any kind of contamination from clouding the experimental outcome. In order to pass and maintain the cell line, a 1/0 dilution of cells to media was used.
Dosing the fibroblasts

A 1mM T<sub>3</sub> stock solution was prepared under sterile conditions by adding 6.5 mg of T<sub>3</sub> (Sigma-Aldrich, catalog number – T67407) to 40ul of 5M NaOH, and then diluting the solution with distilled water to give a final stock volume of 10ml. The stock solution was refrigerated at 4° C and was kept protected from light. The solution was made fresh for every new batch of fibroblasts.

When the cells had reached the desired confluency and were ready to be dosed with T<sub>3</sub>, the 1mM T<sub>3</sub> stock solution was diluted in DMEM to yield the following T<sub>3</sub> concentrations: 10<sup>-7</sup>M T<sub>3</sub> and 10<sup>-8</sup> M T<sub>3</sub>. A serial dilution of the 1mM T<sub>3</sub> stock solution was conducted to yield the T<sub>3</sub> concentrations, and the total volume in the petri dishes was maintained at 10ml.

The fibroblasts were dosed with the varying T<sub>3</sub> concentrations approximately 16 – 18 hours before they were lysed. Additionally, one plate of the cells was not dosed and was used as an experimental control.

Cell lysis

Lysis buffer was made by adding 1 protease inhibitor cocktail tablet (Pierce Biotechnology Ltd., catalog number – 78410) to 10ml of Radioimmunoprecipitation (RIPA) lysis buffer (Santa Cruz Biotechnology Inc., catalog number – sc-24948). The buffer was kept on ice when used immediately or stored at -4° C for future use.
Fibroblasts stimulated with T₃ were washed with EDTA and removed from the plate using trypsin and a sterile cell scraper. The cells were spun down at 1200 RPM for 5 minutes or until a visible cell pellet was formed. The supernatant was removed and 250ul of lysis buffer was added to each tube. Cells were resuspended in the lysis buffer and placed on ice for approximately 15 – 20 minutes. The cell suspension was subsequently spun down for 10 minutes at 4°C, and the supernatant was collected and stored at -80°C until experimental use.

**Western Blot solutions**

NuPAGE® MES SDS 20X running buffer (Invitrogen Life Technologies, catalog number – NP0002) was diluted to 1X for use. 10X transfer buffer stock was prepared by adding 30.3g of Tris Base and 114g of glycine to 1L of distilled water. A 1X stock of the transfer buffer was prepared on the day of the experiment by adding 100ml of 10X transfer buffer stock to 200ml of methanol, and bringing the volume up to 1L with distilled water.

An Amersham ECL detection solution (GE Healthcare, catalog number – RPN2132) was used to detect the proteins after the western blotting procedure. Detection solution was assembled immediately prior to membrane exposure as per kit instructions.
Western Blot antibodies

The primary antibodies used in the experimental procedures included a 28 kDa rabbit polyclonal IgG FGF-7 (H-73) antibody (Santa Cruz Biotechnology Inc., catalog number – sc-7882), a 170 kDa rabbit polyclonal IgG EGFR (1005) antibody (Santa Cruz Biotechnology Inc., catalog number – sc-03), and a 45 kDa mouse monoclonal β-Actin antibody (Cell Signaling Technology, catalog number – 8H10D10). The FGF-7 and EGFR primary antibodies were diluted 1:500 in 5% BSA/Tris-Buffered Saline and Tween-20 (TBST), while the β-Actin antibody was diluted 1:1000 in 5% BSA/TBST.

The secondary antibodies used for detection were the horseradish-peroxidase-conjugated goat anti-rabbit IgG antibody (Sigma-Aldrich, catalog number – A6154), and the horseradish-peroxidase-conjugated goat anti-mouse IgG antibody (Sigma-Aldrich, catalog number – A4416). Both secondary antibodies were diluted 1:1000 in 1% BSA/TBST.

Western Blot procedure

Cell samples were prepared according to standard western blotting protocol. The frozen lysed fibroblasts were defrosted at room temperature. 13ul of the cell sample was mixed with 2ul of NuPAGE® 10X DTT/Sample reducing agent (Invitrogen Life Technologies, catalog number – NP0004), 5ul of NuPAGE® 4X LDS sample buffer (Invitrogen Life Technologies, catalog number – NP0007), and this mixture was heated at 70°C for 10 minutes. Following this, NuPAGE®
Novex 4-12% Bis-Tris Gels (Invitrogen Life Technologies, catalog number – NP0322BOX) were loaded with 15ul of the treated cell samples, and 15ul of SeeBlue® Plus2 Pre-Stained Standard ladder (Invitrogen Life Technologies, catalog number – LC5925). The gels were run at 170V for approximately 35 – 40 minutes.

Nitrocellulose membranes (Invitrogen Life Technologies, catalog number – LC2001) were used to set up the transfer cassettes, and the transfer was run at 220mA for 50 minutes. The membrane was then removed from the transfer chamber and blocked for 1 hour in a 5% BSA/TBST blocking solution, which was made by adding 5mL of powdered BSA to 1X TBST.

Primary antibodies diluted in 5% BSA/TBST were added after the blocking period, and the membrane was allowed to incubate overnight at 4°C on a shaker. The membrane was washed three times with 5% BSA/TBST before the addition of the secondary antibody, which was diluted in 1% BSA/TBST, prepared by adding 10ml of 5% BSA/TBST to 50ml of TBST. The membrane was shaken in the secondary antibody mixture for 1 hour at room temperature.

Prior to the addition of the ECL detection solution, the secondary antibody was removed and the membrane was washed three times with 5% BSA/TBST. Hyperfilm ECL (GE Healthcare, catalog number – 28906835) was used to visualize the membrane after treatment with the ECL detection solution.
Results

The present study was conducted with the intention of understanding the growth factors and signalling pathways through which TH mediates its effects on cutaneous wound healing. Given the multiplicity of growth factors and cytokines implicated in wound healing, we decided to focus on EGF and KGF. Both EGF and KGF are strongly upregulated during injury and cutaneous wound healing, and function in the proliferation of epidermal cells and skin restoration. It is therefore plausible that TH functions in accelerating cutaneous wound healing via the EGF or KGF signalling pathway.

Human fibroblast cells were stimulated with $10^{-7}$ M T$_3$ and $10^{-8}$ M T$_3$. There was also a control set of fibroblast cells that were not stimulated by T$_3$. The cells were incubated with the exogenous TH for approximately 18 hours, after which they were lysed and a western blot was conducted to examine whether the addition of TH resulted in the upregulation of EGF (as measured by the presence of EGFR) or KGF. β – actin was used as a loading control during the experiments.

As evidenced in Fig. 10 and Fig. 11, fibroblasts stimulated with TH trended towards upregulated levels of EGF but not KGF, although statistical significance was not observed due to the small sample size. The control cells showed a small amount of endogenous EGF expression, this being consistent with established knowledge that fibroblasts secrete EGF (Fig. 10). Addition of exogenous T$_3$ resulted in moderately increased expression of EGF, with the $10^{-7}$ M T$_3$
stimulated cells displaying stronger EGF expression when compared to the control cells with no added T₃, and the cells stimulated with a lower (10⁻⁸ M) concentration of T₃ (Fig. 10). Moreover, since the β–actin expression levels remained consistent across all cell samples, we can be confident in the conclusion that stimulation with T₃ does trend towards upregulating EGF expression in fibroblasts (Fig. 10).

KGF expression did not appear to be altered by exogenous T₃ stimulation of the fibroblasts (Fig. 11). As expected, a baseline level of KGF was expressed by all fibroblast samples, and β–actin was again used as a loading control and was found to be constant across all the cells tested (Fig. 11).
Figure 10. Triiodothyronine (T₃) stimulation of fibroblasts increases epidermal growth factor (EGF) expression. A. EGF expression trended towards increasing at higher T₃ concentrations, as observed by comparison of band intensities. B. Representative blot. Lane 1 depicts unstimulated fibroblasts, lane 2 represents fibroblasts stimulated with 10⁻⁸ M T₃, and lane 3 represents fibroblast stimulated with 10⁻⁷ M T₃. As observed in lane 3, the expression of EGFR (170 kDa) appears to be upregulated at higher T₃ concentrations. The β–actin loading control can be observed at 42 kDa.
Figure 11. Triiodothyronine (T₃) stimulation of fibroblasts does not appear to impact keratinocyte growth factor (KGF) production. A. KGF expression did not seem to be affected by T₃, as observed by comparison of band intensities. B. Representative blot of fibroblast cells stimulated with T₃. Lane 1 represents unstimulated fibroblasts, lane 2 represents fibroblasts stimulated with 10⁻⁸ M T₃, and lane 3 represents fibroblast stimulated with 10⁻⁷ M T₃. The amount of KGF (28 kDa) across all fibroblast cell samples did not exceed the base levels seen in lane 1. The β – actin loading control can be observed at 42 kDa.
Discussion

Wound healing is a tightly controlled process that involves the concerted efforts of several growth factors and cytokines. These chemical mediators function in bacterial clearance, epidermal cell proliferation, and subsequent wound contraction and closure. Improper or slow healing wounds can cause significant disruptions of cellular processes, and they represent a major drain on healthcare resources (Sen et al., 2009). There is therefore a need for novel and inexpensive therapies that can accelerate the process of wound healing.

Safer et al. have conducted several studies evaluating the use of topically applied TH in accelerating wound healing (Safer et al., 2005, Safer et al., 2004). While the pathways through which TH mediates its effect were not extensively evaluated, the data is clear in its indication that TH applied exogenously can accelerate the closure of wounds in vivo (Safer et al., 2005). The therapies used currently in hastening cutaneous wound closure are expensive since they involve the isolation and purification of protein mediators (Safer et al., 2005). TH can therefore be used as an economical means through which we can activate the pathways involved in wound closure and skin regeneration (Safer et al., 2005).

In this study we set out to evaluate whether the effects of TH on cutaneous wound healing are mediated via growth factors – specifically EGF and KGF. Both EGF and KGF are produced by dermal fibroblast cells and are significantly upregulated during injury. Their major function is keratinocyte cell proliferation and migration, and they are essential to the restoration of the epidermis following
a cutaneous wound. Lack of either EGF or KGR has been known to result in impaired wound healing (Repertinger et al., 2004 and Werner, 1998).

After conducting western blots on lysed control fibroblast cells and fibroblasts stimulated with $10^{-7}$ M $T_3$ and $10^{-8}$ M $T_3$, it was determined that while EGF production in fibroblasts appeared to be enhanced by the addition of topical $T_3$, no such induction effect was observed for KGF (Fig. 10 and Fig. 11). The fibroblasts stimulated with the highest amount of $T_3$ used in this experiment ($10^{-7}$ M $T_3$) trended towards higher EGF expression when compared to control cells (no added $T_3$), and fibroblasts stimulated with a lower amount of $T_3$ ($10^{-8}$ M $T_3$).

This result is not entirely unexpected and confirms the data published by Hoath et al. (Hoath et al., 1983). Their studies indicated that the exogenous addition of TH to the skin of neonatal mice resulted in a surge of EGF expression in the tissue (Hoath et al., 1983). The researchers went on to postulate that it is likely that TH mediates its cutaneous effects via the major signaling pathways activated when EGF binds to its receptor (Fig. 8), either by increasing the expression of EGFR or by increasing the affinity of binding between EGF and EGFR (Hoath et al., 1983). While our study did not attempt to differentiate between the two scenarios described in Hoath et al., future experiments with a larger sample size can be conducted in order to isolate the exact mechanism through which TH upregulates the functional manifestations of EGF.

Our study also found that KGF expression levels were not upregulated in fibroblast cells stimulated by $T_3$. KGF levels appeared to have remained the
same in stimulated and unstimulated fibroblasts, leading us to postulate that T₃ does not mediate its effects on wound healing via KGF – dependent pathways.

This result is significant in that it allows us to understand better how T₃ functions in wound healing. KGF is essential to keratinocyte migration, proliferation, and differentiation; and its absence is known to result in improperly healed wounds (Werner et al., 1992). It is possible that the large induction of EGF by T₃ is sufficient to result in successful wound closure, although this must be furthered evaluated by conducting both in vitro and in vivo studies in samples devoid of KGF but stimulated with increasing T₃ concentrations.

Additionally, in a study conducted by Brauchle et al. it was demonstrated that several serum growth factors expressed during wound healing could upregulate the expression of KGF in fibroblast cells (Brauchle et al., 1994). One of these growth factors was EGF, although it was found to not be as potent a stimulator of KGF expression as PDGF (Brauchle et al., 1994). It can then be hypothesized that even though T₃ may not directly upregulate KGF expression in fibroblasts, it could function in an indirect manner by upregulating EGF, which in turn could stimulate KGF production. In this manner stimulation of fibroblasts with T₃ can result in the functional effects of both EGF and KGF.

There is also the possibility that the amount of T₃ that was used to stimulate the cells in our study was insignificant in rendering the upregulation of KGF expression. Future repetitions of this experiment should ideally include a larger sample size and a broader T₃ concentration range to obtain statistical
significance, although this may be problematic as large amounts of T3 could potentially result in cell toxicity and death.

In conclusion, the present study has shown that exogenous addition of T3 to human fibroblast cells appears to stimulate the production of EGF, but not KGF. This information is critical to expanding our understanding of how T3 functions in accelerating wound healing, and can be applied in support of developing novel and inexpensive therapies that use T3 in the treatment of slow healing wounds.
References


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EDUCATION

Boston University, Boston, MA
Master of Arts in Medical Sciences, 2013 (expected)

Wellesley College, Wellesley, MA
Bachelor of Arts in Biological Chemistry, 2009

RESEARCH EXPERIENCE

Children’s Hospital Boston, Boston, MA – July 2009 – May 2011
Research Assistant
• Looked after general lab operations and reagent supplies
• Maintained the experimental cell lines and prepared them for experiments
• Primarily studied the pneumococcal pilus with the goal of understanding its role and method of regulation in the bacterium, Streptococcus pneumoniae

WORK EXPERIENCE

Peer Resource Advisor
• Led resume and internship search workshops in dormitories. Edit student resumes and cover letters in individual counseling
• Advised students in the use of center’s resources and career counseling services. Promote center’s services to students

Supplemental Instructor in Biology
• Organized sessions on improving academic performance. Focused on strengthening problem-solving and study skills
COMMUNITY SERVICE EXPERIENCE

St. Francis House, Boston, MA – June 2007 – August 2007
Summer Intern
• Facilitated the daily workings of the in-house Boston Healthcare for the Homeless clinic by assisting the healthcare providers in communicating with patients and collecting and analyzing patients’ medical records
• Collaborated with course instructors in the Moving Ahead Program to administer computer skills training to the homeless

Nrityanjali Academy, Secunderabad, India – June 2006 – August 2006
Summer Intern
• Assisted outreach staff of the NGO in disseminating HIV/AIDS related information to urban slum populations
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LEADERSHIP EXPERIENCE

Sexual Health Educator (SHE)
• Organized campus-wide events on women’s health issues.
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Resident Assistant
• Executive paraprofessional in student residence hall
• Acted as a peer resource and mentor for hall residents
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SKILLS

Languages: Fluent in English. Proficient in Hindi.
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