

CLK1 and its Effects on Skin Stem Cell Differentiation

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ABSTRACT

The primary objective of this study was to determine the effects of CDC-like kinase 1 (CLK1) on the differentiation of a telogen skin stem cell line as a follow-up study to Dr. Lyle's previous work on the differentiation of hair follicle cells. CLK1 knockdown strains were created using shRNA, then differentiated via several induction pathways. Cells lacking CLK1 differentiated along the sebocyte differentiation pathway similarly to the controls, but displayed no epidermal differentiation. These results suggest that CLK1 is necessary for epidermal differentiation, but plays no role in the differentiation of sebocytes.

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BACKGROUND

Adult Stem Cell Theory

It is generally known that some tissues contain adult stem cells that have the ability to replenish the tissue by differentiating into different cell types. When a stem cell divides, it produces one new stem cell and one transit-amplifying (TA) cell (Roh et al., 2010). The new daughter stem cell maintains the stem cell line, while the TA cell has the multi-potency to develop into various cell types characteristic of that tissue to help maintain tissues whose cells need replacement (Roh et al., 2004). TA cells receive signals from the surrounding cells and environment to determine their differentiation pathway, a process which can be replicated *in vitro* (Clarke & Fuller, 2006). The TA cells then undergo a series of cell divisions before becoming fully differentiated (**Figure 1**).

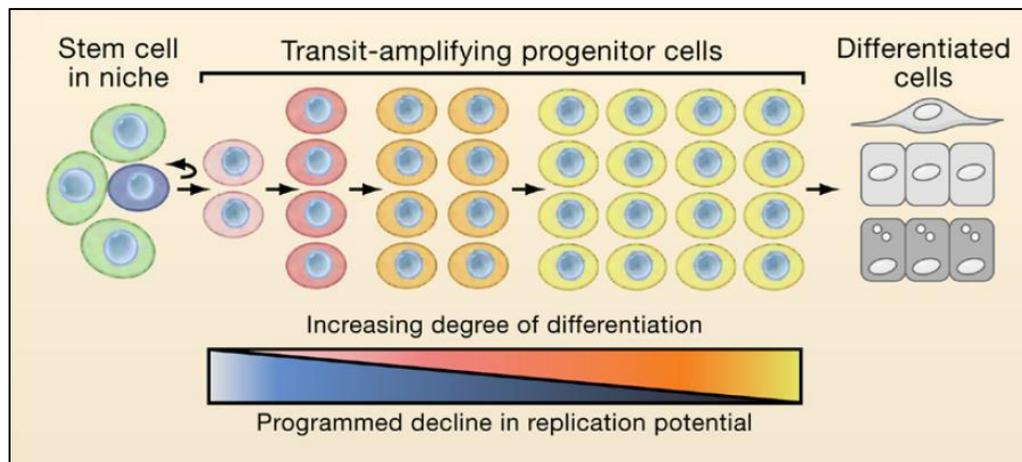


Figure 1: Diagram of Stem Cell Differentiation. Stem cells (dark blue, left) receive signals from the surrounding niche (green) and divide to create new stem cells (reverse arrow) and transit amplifying (TA) cells. TA cells undergo a series of divisions prior to becoming fully differentiated. (Clarke & Fuller, 2006)

Since stem cells have nearly unlimited self-renewal abilities, it is necessary for tissues to strictly regulate their divisions to prevent indefinite replication and growth. Most tissues contain a very small number of adult stem cells that give rise to many TA cells to supply the tissue with

the cells it requires for turnover (Clarke & Fuller, 2006). TA cells go through limited cell divisions, thus the stem cells are the only long-lived cells within the tissue (Clarke & Fuller, 2006). In this way, mutation accumulation is minimized, and the regulation of growth is maintained (Clarke & Fuller, 2006).

Stem Cells of the Human Skin

Human Skin Growth and Biology

While adult stem cells are likely important in all human organs, they are especially important in the skin. As the largest organ of the human body, the skin is composed of various cell types, and it requires constant maintenance to remain healthy. Human skin is comprised of three main layers: the epidermis, dermis, and subcutaneous tissue (**Figure 2**).

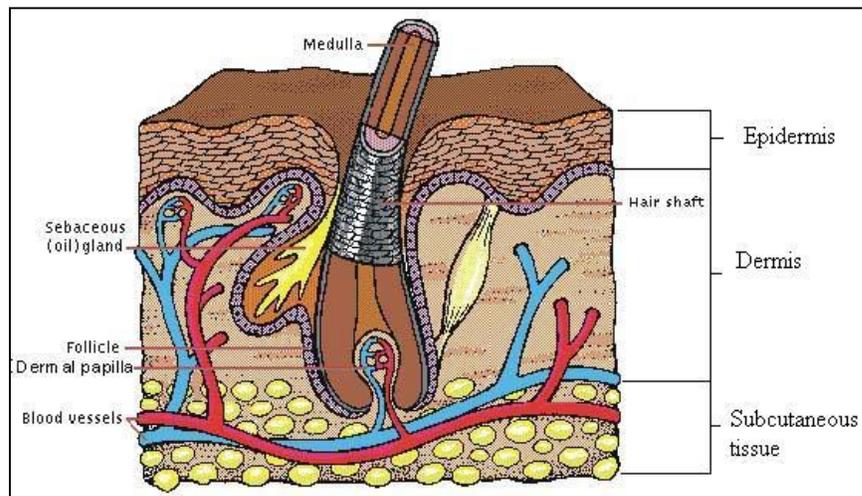


Figure 2. Schematic of the Skin Layers and Hair Follicle Structure. The skin is composed of three main layers (see the right side). Hair follicles reside in the two outermost layers. (Natural Hair, 2009)

Interspersed hair follicles traverse both the epidermis and dermis and are the location of our skin stem cell of interest, the telogen stem cell. These stem cells are located in the bulge region of the hair follicle (**Figure 3**) (Roh et al., 2010). Telogen stem cell differentiation is

controlled by three hair growth stages: anagen (growth), catagen (regression), and telogen (rest) (Roh et al., 2008). At anagen onset, the stem cells in the bulge are activated to proliferate, generating the lower follicle and promoting hair growth (Roh et al., 2008). This phase lasts as long as the life of the individual hair, which can be up to eight years on the human scalp. Then during catagen, the lower follicle regresses until there is no follicle below the bulge region (Roh et al., 2008). Telogen is the period of rest where there is no hair growth in the follicle until the next anagen cycle. Thus, telogen stem cells are cells in the stage of rest within the follicle bulge (Roh et al., 2008).

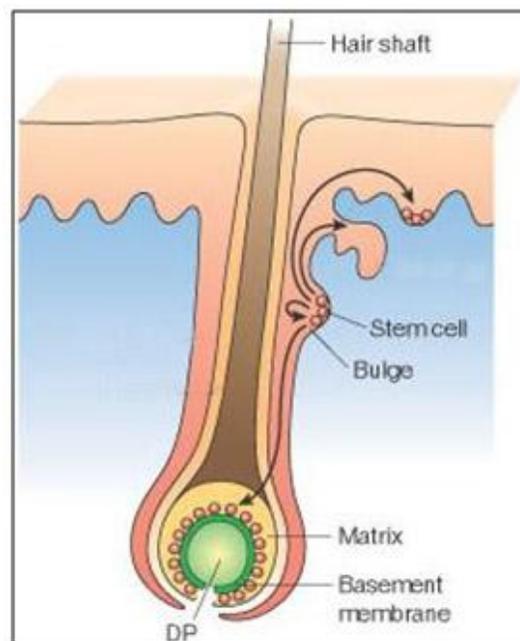


Figure 3. The Location of Skin Stem Cells. Schematic of a hair follicle showing the bulge region (right side) which contains adult stem cells. The stem cells produce TA cells that move vertically up or down the shaft to eventually replace other cells in the tissue. (Spradling et al., 2001)

Properties of Primary Skin Stem Cells

Roh et al. have isolated and characterized primary skin stem cells based on their biochemical properties (2005). As with all adult stem cells, it was shown that the division of a skin stem cell yields a daughter stem cell to maintain the stem cell line and a TA cell which will

differentiate into other skin cells (Roh et al., 2005). The skin stem cells are biochemically and structurally undifferentiated, slow-cycling, and can renew without limits (Roh et al., 2005). Their location in the hair follicle bulge is similar to other known stem cell niches in the human body in that the stem cells are tightly adhered to membrane in a well-protected cavity (Roh et al., 2005).

Telogen stem cells are multi-potent and can differentiate into different types of skin cells including: hair, sebocyte, and epidermal cells (Roh et al., 2004). These cells give rise to the lower hair follicle, hair shaft, sebaceous (sweat) glands, and the outer epidermis respectively (Roh et al., 2004). *In vitro*, these cells can be induced to differentiate by culture components, or by cell signaling between dermal papilla cells and telogen stem cells, both of which were utilized in this MQP study (Roh et al., 2004).

Roh et al. showed that keratin 15 (K15) and integrin β 1 are markers for stem cells of the bulge area, and thus, they are often referred to as keratinocytes (2005). Alternatively, TA cells express no K15 and much lower levels of β 1 integrin than the primary stem cells (Roh et al., 2005).

Immortalized Skin Stem Cell Line Tel-E6E7

Cell Line Creation

In order to study the properties and behaviors of skin stem cells, an immortalized cell line was created by the Lyle lab and Cecillia Roh of Beth Israel Medical Center of Boston (Roh et al., 2008). Explained briefly, human telogen skin stem cells were extracted from adult scalp skin by chemical digestion and physical dissection (Roh et al., 2008). The line was immortalized using a retroviral vector, then continually passaged to eliminate the possibility of cancerous cells (Roh et

al., 2008). The cell line was named Tel-E6E7, and will be referred to as such throughout this report (Roh et al., 2008).

Similarities of Tel-E6E7 to Primary Skin Stem Cells

Roh et al. continued their study by comparing the properties the created Tel-E6E7 cells to those of primary skin stem cells (2008). Immunofluorescence assays and Western blots were used to determine that the Tel-E6E7 cells express K15 and β 1 integrin, the same markers as primary skin stem cells (**Figure 4**) (Roh et al., 2008).

It was also determined by differentiation assays that the formation of three different cell lineages was possible for the Tel-E6E7 cells, indicating that they possess the same multi-potency as primary skin stem cells (Roh et al., 2008). Additional assays measuring Tel-E6E7 cell migration and adhesion illustrated slow movement and high adhesion, showing further similarities to primary skin stem cells (Roh et al., 2008).

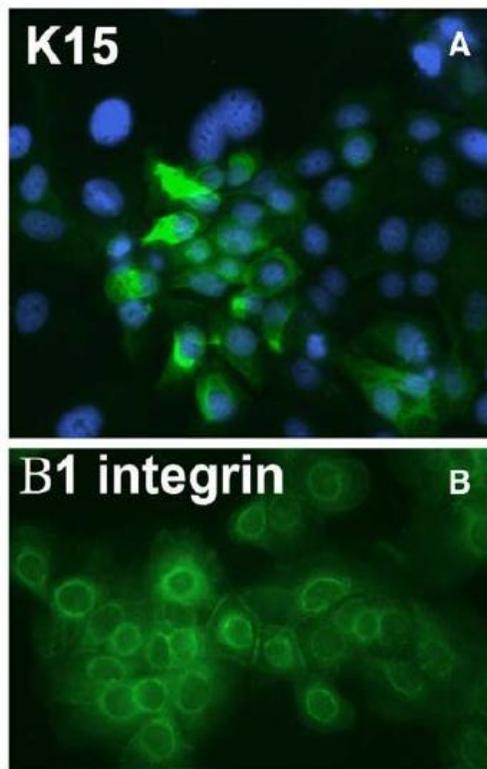


Figure 4. Expression of Skin Stem Cell Markers in Immortal Tel-E6E7 Cells. Tel-E6E7 cells exhibit positive staining (green) for K15 (**A**) and β 1 integrin (**B**) by IFA. (Roh et al., 2008)

CDC-Like Kinases and CLK1

It is generally understood that cellular development is partially regulated by various protein kinases. CDC-like kinase (CLK) is a protein kinase with dual specificity; it can phosphorylate both serine/threonine and tyrosine residues (Roh et al., 2010). CLKs are part of a protein kinase family which contains a conserved sequence at the C-terminus and the signature

amino acid motif 'EHLAMMERILG' (Hanes et al., 1994). This family is termed the LAMMER family and consists of CLK1-4, which differ mainly in their N-terminal regions (Nayler et al., 1997).

CLK1 was one of the first dual specificity kinases discovered (Menegay et al., 2000). CLK1 autophosphorylates serine, threonine, and tyrosine residues, and phosphorylates other substrate's serine and threonine residues (Ben-David et al., 1991). The conserved C-terminus region contains the catalytic domain of the molecule (**Figure 5**). The N-terminal domain includes a "functional nuclear localization signal which may direct CLK1 into the nucleus" and may also bind to substrate (Duncan et al., 1995; Menegay et al., 2000). CLK1 can be negatively regulated by its own N-terminal domain, which when truncated increases enzymatic activity (Menegay et al., 2000). The N-terminus was also shown to contain a regulatory region, including receptors for epidermal growth factor (EGF), as well as other growth factors (Menegay et al., 2000). Additionally, through its interaction with SR-proteins, CLK1 regulates its own pre-mRNA splicing (Duncan et al., 1997).

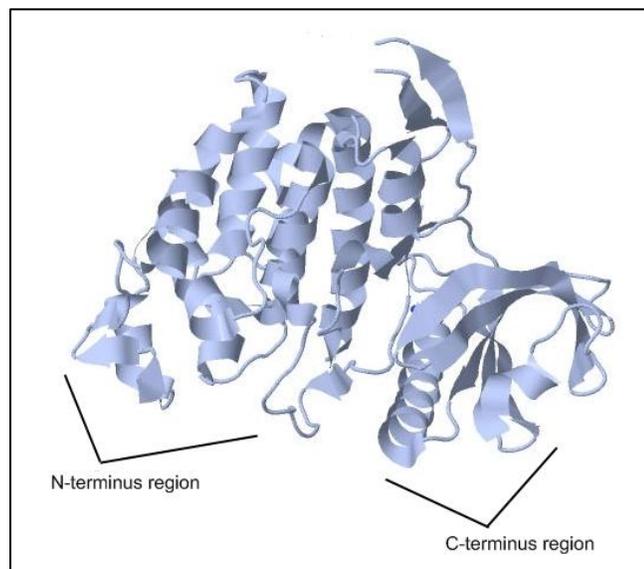


Figure 5. Structure of CLK1. The C-terminus of CLK1 contains the protein's catalytic site and the N-terminus contains substrate-binding and localization sites. (Debrecezeni et al., 2005)

Interaction of CLK1 with SR-Proteins

The primary mechanism through which CLK1 may be affecting stem cell differentiation is by phosphorylating the serine group of serine- and arginine-rich (SR) proteins. SR proteins are stored in the nuclear speckles of the nucleoplasm and are partly responsible for the choice of splicing sites during pre-mRNA splicing (Duncan et al., 1997; Misteli et al., 1998). Once phosphorylated by CLK1, the SR protein is capable of movement within the nucleoplasm and can bind to nearby large nuclear ribonucleoproteins involved in splicing (Duncan et al., 1997). Here, the SR proteins bind to small “exonic splicing enhancers / silencers” (Voet et al., 2008). These domains are approximately 5-10 nucleotides long, are located in the exons, and their binding can shift the 5’ splice site (Zahler et al., 1992; Voet et al., 2008). Past research has shown that the selection of these splice sites is done in a SR protein concentration-dependent manner (Zahler et al., 1993). Tissue-specific expression of SR proteins suggests that each SR protein is designed for regulating concentrations of proteins necessary for tissue upkeep (Voet et al., 2008).

SR Protein Structure

Originally all SR proteins were identified as containing a conserved phosphorylated epitope region recognized by monoclonal antibody mAb104, but now SR protein structure is far better understood (Roth et al., 1990). SR proteins have 2 domains, one or two highly conserved RNA-recognition motifs (RRM, **Figure 6**) and a SR domain (Voet et al., 2008). The RRM(s) bind(s) to RNA within the spliceosome, and the SR domain mediates the protein-protein interactions (Voet et al., 2008). Within the SR domain, there is also a serine residue which can be phosphorylated, activating the SR protein. SR proteins are highly conserved in all cells, and

in a study comparing six cell types across different species, all contained SR proteins of approximately 20, 30, 40, 55, and 75 kDa in size (Zahler et al., 1992).

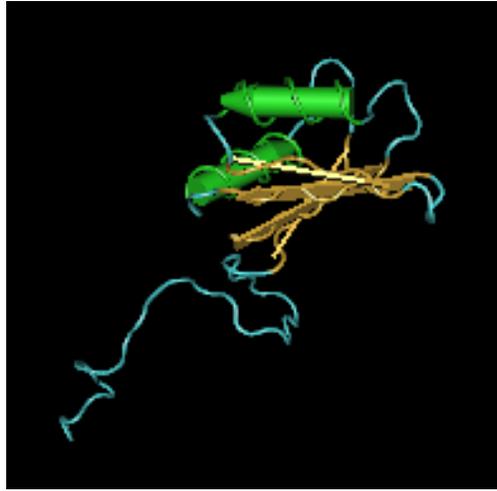


Figure 6. RNA Recognition Motifs of Protein SRp20. The domain shown is the RNA-binding region of the SRp20 protein (the SR domain is not shown). (Hargous et al., 2006)

SRp20

SR protein 20 (also known as Splicing Factor arginine-serine rich 3, SFRS3) is of interest to this MQP study as it is specifically phosphorylated by CLK1 (Roh et al., 2010). This relationship may be important to the regulation of mRNAs responsible for the differentiation of epithelial stem cells (Roh et al., 2010).

SRp20 has only one RRM and is 20 kDa in size (Zahler et al., 1992). Although all SR proteins are highly conserved in the animal kingdom, SRp20 is an especially highly conserved SR protein (Zahler et al., 1992; Zahler et al., 1993). For example, past studies have shown SRp20's amino acid structure to be completely identical to that of mouse SRp20 (Ayane et al., 1991). More recent investigations have shown that SRp20 self-regulates its own pre-mRNA splicing, and that SR protein ASF/SF2 inhibits this process, thus inhibiting the production of SRp20 (Jumaa & Nielsen, 1997). This is significant because ASF/SF2 has been shown to

interact with CLK1 and is over-expressed in some tumor cells (Menegay et al., 2000; Karni et al., 2007).

Potential Role of CLK1 and SR Proteins in Cancer Cell Proliferation

The SFRS1 gene which codes for ASF/SF2 has been determined to be a proto-oncogene (Karni et al., 2007). Mutated ASF/SF2 has the potential to alternatively splice pre-mRNA into non-functional or oncogenic forms, which is critical as this SR protein controls the splicing of various tumor suppressor genes, kinases, and kinase receptors (Karni et al., 2007). CLK1 could have a possible role in some cancers due to this relationship between CLK1, SRp20, and ASF/SF2. Additionally CLK1 could also be related to some cancers due to its interactions with other SR proteins. Many protein kinases other than CLK1 have been targeted for cancer therapy research in the past due to their interactions in important signal transduction cascades, and several inhibitors for these kinases have been approved by the FDA for cancer treatment (Levitzki, 2003).

Role of CLK1 and SR Proteins in Stem Cell Differentiation

Past investigations have suggested that cdc-like kinases are crucial for cellular development, as experiments involving mutated CLK in *Drosophila* resulted in severe developmental and differentiation defects (Yun et al., 1994; Yun et al., 2000). Another study showed that CLK1 expression caused PC12 cells to undergo neuronal differentiation (Myers et al., 1994; Menegay et al., 2000). These studies all indicate that CLK1 activity plays an important role in cellular differentiation, although the role may differ depending on the tissue.

CLK1 and Telogen Stem Cells

In previous experiments in Dr. Lyle's lab, the CLK1 gene was found to be up-regulated in the stem cells of human hair follicles (Roh et al., 2010). The Lyle lab hypothesized that CLK1 had an integral role in the process of stem cell differentiation, maintaining an undifferentiated state of the telogen stem cells. In order to determine the effects of CLK1 on the differentiation of telogen stem cells, preliminary research was performed using constitutively active and dominant negative strains of Tel-E6E7 for CLK1. The study showed that constitutively active CLK1 inhibited the differentiation of telogen stem cells into hair follicle cells when induced by co-culture with dermal papillae (DP) cells (Roh et al., 2010). The dominant negative CLK1 strain of telogen stem cells showed uninhibited differentiation of telogen stem cells into hair follicle cells in the presence of differentiation signals (Roh et al., 2010). Thus, the preliminary data indicates that CLK1 may act to maintain the undifferentiated state of telogen stem cells. In order to validate the dominant negative CLK1 results and to eliminate any potentially off-target effects of the dominant negative kinase, E6E7 strains that do not contain any functional CLK1 need to be developed and studied. Additionally, the effects of CLK1 on other telogen stem cell differentiation pathways need to be investigated.

PROJECT PURPOSE

As mentioned previously, earlier studies in the Lyle lab using a dominant negative CLK1 Tel-E6E7 strain need validation by studying clones without endogenous CLK1 in order to eliminate the potential for off-target effects during hair follicle differentiation. Additionally, the effects of CLK1 on other Tel-E6E7 differentiation pathways need to be investigated. To do this, several CLK1 knockdown clones will be created to perform differentiation experiments. Our hypothesis is that these differentiation experiments will yield results indicating that CLK1 maintains telogen stem cell lineage, and inhibits differentiation into sebocyte and epidermal cells.

METHODOLOGY

Cell Culture

Culture Media

The following table shows each of the media used for culturing and their components.

Table 1: Summary of All Culture Media and Their Uses		
<i>Media</i>	<i>Components</i>	<i>Use:</i>
Keratinocyte Medium (KCM)	<ul style="list-style-type: none"> • Base medium: DMEM (Dulbecco's Modified Eagle Medium) and Hams F12 (3:1, Gibco) • 10% FBS (Fetal Bovine Serum) • 24 mg/L adenine • 0.1 nM cholera toxin • 0.4 µg/mL hydrocortisone • 5 µg/mL T / 2nM T3 • 6.5 µg/mL insulin • 1% Pen/Strep • 0.5 mg/L fungizone/amphotericin B • pH 7.2 	Creation KCM+EGF medium, culture of Tel-E6E7 cells prior to epidermal differentiation
KCM+EGF Medium	<ul style="list-style-type: none"> • Base medium: KCM • 10nM EGF (epidermal growth factor) 	Culture of all Tel-E6E7 cells (including controls and clones)
J2 Medium	<ul style="list-style-type: none"> • Base medium: High-glucose DMEM (Gibco) • 10% FBS • 1% Pen/Strep 	Culture of 3T3-J2 cells
J2-Conditioned Medium	<ul style="list-style-type: none"> • Base medium: J2 • Co-cultured with 3T3-J2 cells for 2-4 days, then collected and filter sterilized 	Culture of all Tel-E6E7 (including controls and clones)
Sebocyte Medium	<ul style="list-style-type: none"> • Base medium: DMEM and Hams F12 (1:1, Gibco) • 6% FBS • 2% human serum • 1% Pen/Strep • 10nM EGF 	Culture of Tel-E6E7 cells prior to induction of sebocyte differentiation
Sebocyte Differentiation Medium	<ul style="list-style-type: none"> • Base medium: Sebocyte Medium • 10⁻⁴ M arachidonic acid 	Induction of sebocyte differentiation in Tel-E6E7 cells
Keratinocyte Growth Medium (KGM)	<ul style="list-style-type: none"> • PCT Epidermal Keratinocyte Medium, calcium-free (CellnTEC, #Cnt-07CF) 	Culture of Tel-E6E7 cells prior to induction of epidermal differentiation
Epidermal Differentiation Medium	<ul style="list-style-type: none"> • Base medium: KGM • 1.5 mM calcium chloride 	Induction of epidermal differentiation in Tel-E6E7 cells
Freezing Medium	<ul style="list-style-type: none"> • 80% FBS • 20% DMSO (dimethyl sulfoxide) 	Freezing of cells not in culture

Telogen E6/E7 Cell Line Creation and Culture

The Tel-E6E7 cell line used in this project was provided by the Cancer Biology Department of UMass Medical School. This line was created as previously described in Roh et al. 2008. Skin from human adult scalps was digested to collect the hair follicles and isolate the telogen skin stem cells from the bulge region. The follicles were dissected, and the stem cells were isolated, plated, and further cultured in keratinocyte medium (KCM) supplemented with epidermal growth factor (EGF) (Roh et al., 2004; Roh et al., 2008). The primary cell line was immortalized by transduction with a retroviral vector (LXSN-16E6E7), selected, and cultured to thirty passages in KCM. The Tel-E6E7 cells remained stable for twelve months of the continuous passages and did not form independent colonies in soft agar assays, suggesting that the immortalization did not yield cancerous cell properties.

This line was cultured in medium containing 50% KCM-EGF and 50% J2-conditioned medium or 100% KCM-EGF with a feeder layer of 3T3-J2 cells on 10cm plates.

3T3-J2 Cell Line

A 3T3-J2 cell line was used as a feeder cell layer and in co-culture for the making of J2-conditioned medium. These cells were provided by the Cancer Biology Department at UMass Medical School. Prior to use as a feeder layer, the cells were treated with a mitomycin C mixture (15 µg/mL mitomycin C in DMEM without serum) for two hours to inhibit cell proliferation. The cells were then washed, treated with trypsin, counted, and frozen at -80°C until needed.

Trypsin Stock

10x stock trypsin was diluted 1:5 with Hanks Balance Salt Solution to give the working solution of 2x trypsin. Stock versene was mixed 1:1 with 2x trypsin to yield a working solution of 1x trypsin with versene for use with the 3T3-J2 cells.

Culture Passaging

All cell lines were passaged by the same method. Cells were washed with 1x PBS (phosphate buffer sulfate) and 2x trypsin was used to dislodge the adhered cells from the bottom of the plate. Serum-containing medium was added once cells had lifted, and the cells were then transferred to a conical tube. The suspension was spun at 200G for 5 minutes, then re-plated at the desired dilution on a new plate.

Freezing Cells

To preserve cells when not in culture, cells were frozen at -80°C . Plated cells were washed with 1x PBS, treated with trypsin, and neutralized with medium as for cell passage. Following centrifugation, the cells were resuspended in medium. Freezing medium was added to the cell suspension and the entire mixture was transferred to a cryotube and placed in a cooler in the -80°C freezer.

Thawing Cells

When it was necessary to thaw frozen cells, the cryotubes were placed in a 50mL conical of 70% ethanol and then in a 37°C water bath for several seconds until thawed. The suspension was washed with 5 mL PBS, spun at 200G for 5 minutes. The cells were resuspended in 1 mL medium and plated.

shRNA Knockdown of CLK1

In order to create a cell line that will express minimal amounts of CLK1 protein, shRNA techniques were used to create a knock down. RNA interference (RNAi) is a method of regulating gene expression which results in the down-regulation of gene expression due to the

binding of short RNA sequences to complementary mRNA molecules (Voet et al., 2008). Small hairpin RNAs (shRNAs) contain complementary regions separated by a small ‘loop’ that causes the transcript to fold back on itself forming a ‘short hairpin’ (McIntyre & Fanning, 2006). Although, this formation is very similar to those formed by naturally occurring microRNAs, shRNAs can be specifically designed to inactivate expression of target genes (McIntyre & Fanning, 2006).

The immortalized Tel-E6E7 cell line was transfected with three different shRNA clones targeting CLK1, created by and obtained from UMass Medical School shRNA Core. Polybrene (4 mg/mL) was mixed with dilutions of the viral vectors (1:2, 1:10, and 1:20) then applied to Tel-E6E7 cells on 6-well plates. Transfected cells were selected using Puromycin until a non-transfected control showed 100% death. Following selection, cells were fed twice weekly as described above. In order to confirm protein knockdown, cellular lysates were collected, denatured, and analyzed by Western blots using an anti-CLK1 antibody (Abgen, AP7529).

Sebocyte Differentiation Assay

Tel-E6E7 cells were cultured on 6 cm plates on cover slips in 50% KCM+EGF and 50% J2-conditioned media until approximately 70% confluent. The medium was then changed to sebocyte medium. After 24 hours, differentiation was induced by adding sebocyte differentiation medium containing 10^{-4} M arachidonic acid to half the wells. Three days later, cover slips were removed and fixed. Half of the slips were washed twice with propylene glycol twice for five minutes each, stained with 0.7% Oil Red O in propylene glycol for seven minutes, and then washed once with 85% propylene glycol in distilled water. These were then viewed with a light microscope. The remaining slips were subjected to immunofluorescence assays for Keratin7

(Dako, Clone OV-TL, #M7018), a differentiation marker. Cell pellets were also lysed and analyzed by Western Blots for Keratin 7.

Epidermal Differentiation Assay

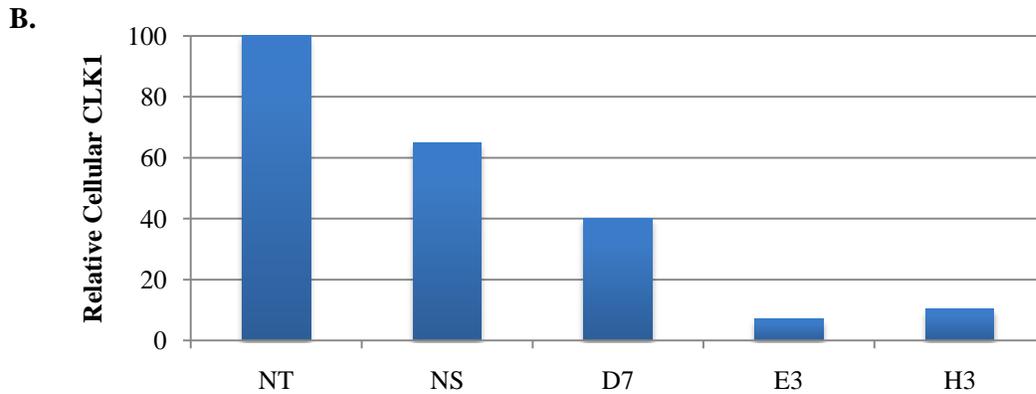
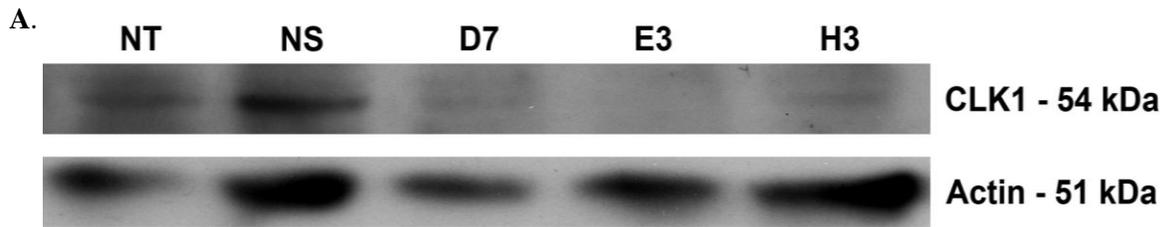
Tel-E6E7 cells were cultured on 6-well plates on cover slips in 50% KCM+EGF and 50% J2-conditioned medium until approximately 50% confluent. The medium was then changed to KCM with no EGF supplement. Twenty-four hours later, the medium was changed to KGM. After 24-hour incubation, differentiation was induced in half the wells with epidermal differentiation medium containing 1.5 mM calcium chloride. Three days later, cover slips were removed, fixed, and subjected to immunofluorescence assays for Keratin 1 (Santa Cruz Biotech, N-20 sc-17091), an epidermal cell marker.

RESULTS

The goal of this study was to create Tel-E6E7 knockdown clones lacking CLK1 and determine their ability to differentiate along sebocyte and epidermal pathways.

CLK1 Knockdown

To knockdown CLK1 mRNA, it was first necessary to transfect Tel-E6E7 cells with viral vectors encoding shRNAs targeted against the coding region of the CLK1 gene. The three resulting clones each showed a considerable knockdown of CLK1 protein on a Western blot (**Figure 7, Panel A**). A quantification of the Western blot was done by normalizing the band intensity for each clone to that of the actin load marker and the non-transfected control (**Figure 7, Panels B and C**). This analysis showed that clones E3 and H3 provided the best knockdowns, with a 14-fold and 10-fold reduction of CLK1, respectively. Clone D7 showed only a 2.5-fold knockdown. The variation of CLK1 knockdown among the clones is likely due to the different affinities for shRNAs binding their mRNA targets, which could result from some binding sites existing in mRNA secondary structures less accessible to the shRNAs, or in the knockdown of a protein by numerous mechanisms.



C.

Clone Information				
Clone Name	Open Biosystems UMass Core Catalog Number	Clone ID	Location	CLK1 Reduction Normalized to Non-Transfected Control
D7	VGH5518-100989144	V3LHS_344840	172_0850-D -7	2.5-fold
E3	VGH5518-100987865	V3LHS_344838	172_0837-E -3	14-fold
H3	VGH5518-100993244	V3LHS_344837	172_0893-H -3	10-fold

Figure 7. CLK1 Knockdown. (A) Following transfection of Tel-E6E7 cells with shRNAs and selection, cell lysates were collected and analyzed by Western blots for CLK1. (B) Western blot quantification data was normalized to the non-transfected (NT) control cell line. This blot shows that all three shRNA experimental clones (D7, E3, H3) yielded a knockdown of CLK1 protein; clones E3 and H3 showed the best knockdowns of 14- and 10-fold, respectively. (C) Table provides information regarding the viral vectors used to make each clone. The normalized knockdown reductions of CLK1 are also shown.

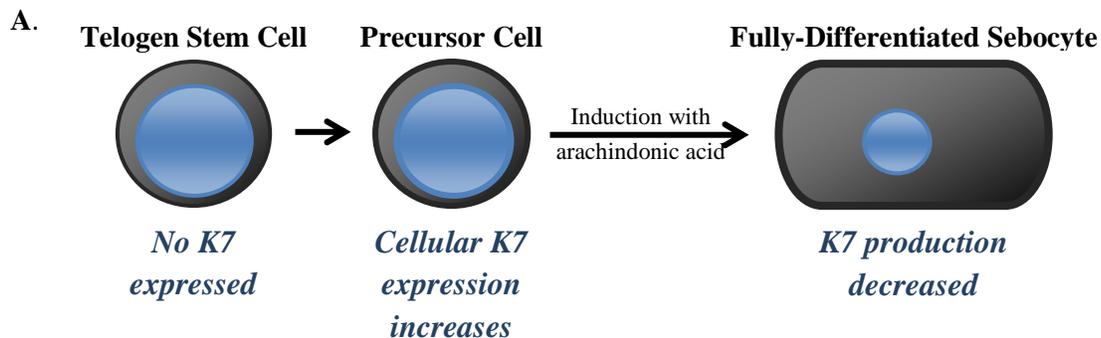
(NT – non-transfected control; NS – non-silencing control; D7, E3, H3 – three different transfected clones)

Sebocyte Differentiation Assay

Tel-E6E7 cells were cultured in sebocyte medium, and then half were induced to differentiate with arachidonic acid. The resulting cells were analyzed by keratin 7 (K7) immunofluorescence assays, Oil Red O staining, and a K7 Western blot (**Figure 8**).

In the immunofluorescence assays, the cells were stained for keratin 7 (green), which is known to be up-regulated in sebocyte precursor differentiated cells and down-regulated in fully-differentiated cells (**Figure 8, Panel A**). The data show that keratin 7 (K7) is highly concentrated in the nuclear region of the non-induced precursor cells (**Figure 8, Panel B, non-induced**). In contrast, in induced cells residual K7 disperses throughout the differentiated cell (**Figure 8, Panel B, induced**). As shown in the assay photographs of the induced cells, K7 intensity is reduced and more widespread independent of the knockdown status, suggesting that CLK1 is not required for sebocyte differentiation.

These observations are supported by the Oil Red O stains of the same cells (**Figure 8, Panel B, right columns**). Round, red droplets indicating the presence fatty lipids of sebaceous glands are seen in all induced control and knock down cell lines, but not in non-induced cells. The K7 Western blot of cellular lysates from the assayed cells also shows no difference in the differentiation pattern between the knockdown clones and the controls (**Figure 8, Panels C and D**). These results suggest that the absence of CLK1 is not important for sebocyte differentiation.



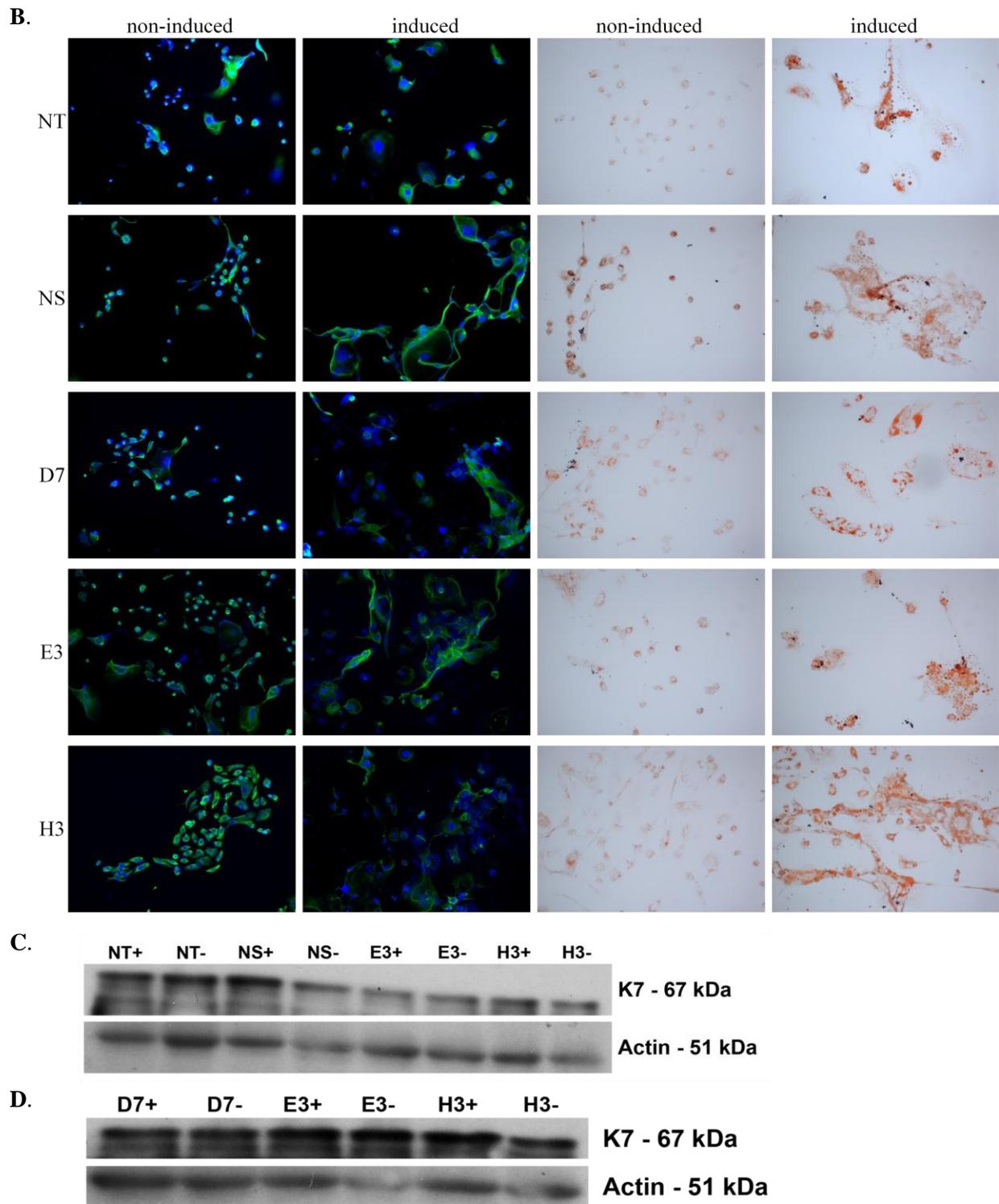


Figure 8. Sebocyte Differentiation Assay. (A) Panel shows the distribution of keratin 7 during sebocyte differentiation. (B) Immunofluorescence staining for keratin 7 (FITC, green) and cell nuclei (Dapi, blue). When cells are introduced to sebocyte media (left-most non-induced panels), they enter a precursor differentiation state in which they express higher levels keratin 7 (K7) concentrated near the nucleus. Following induction with arachidonic acid (induced), K7 production is decreased and residual protein is dispersed within the cell. The two right columns show Oil Red O staining for fatty lipids, a marker of differentiated sebocytes. Larger red droplets as seen in the right-most, induced panels. These assays show equal sebocyte differentiation in both the controls and the CLK1 knockdown clone. (C and D) Western blots of cellular lysates shows no difference in K7 expression levels between the controls and the knockdown clones. Panel C compares the best knockdown clones to the two controls, while Panel D shows another blot comparing all the knockdown clones. (NT – non-transfected control; NS – non-silencing control; D7, E3, H3 – three different transfected clones; + induced with arachidonic acid; - not induced)

Epidermal Differentiation Assay

Tel-E6E7 cells were cultured in KGM medium, and then half were induced to differentiate with 1.5 mM calcium chloride. The resulting cells were subjected to keratin-1 (K1) immunofluorescence assays (**Figure 9**). In the immunofluorescence assays, the cells were stained for keratin-1 (green), an epidermal cell marker. The only cells to show the presence of K1 (differentiation) were the controls expressing normal levels of CLK1 (**Figure 9**, upper right two panels). Consistently among the three experimental CLK1 knockdown clones, no K1 was observed (**Figure 9**).

These results suggest that knockdown of CLK1 significantly reduces epidermal differentiation in telogen stem cells; therefore, CLK1 must be necessary for the cells to differentiate along this pathway.

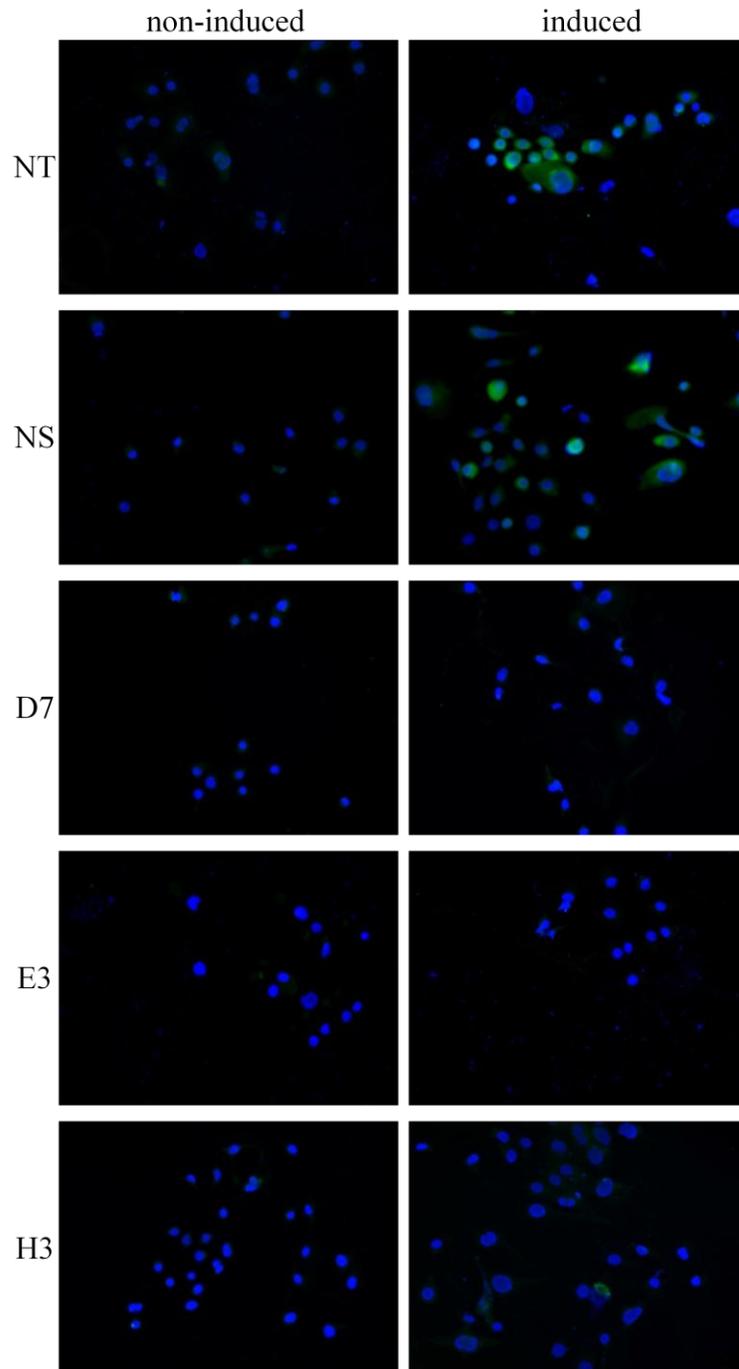


Figure 9. Epidermal Differentiation Assay. Immunofluorescence staining for keratin 1 (FITC, green) and cell nuclei (Dapi, blue). This assay clearly shows that the knockdown clones lacking CLK1 (D7, E3, H3) do not express keratin 1, the marker of epidermal differentiation. This indicates that these knockdown cells were unable to differentiate by the epidermal pathway.

DISCUSSION

After the successful creation of the Tel-E6E7 CLK1 knockdown clones by shRNA transfections, these clones were used in differentiation assays to test their abilities to enter the epidermal and sebocyte pathways. CLK1 did not appear to play a role in sebocyte differentiation, but did appear to be necessary in epidermal differentiation. These results differed from the previous Lyle lab experiments which showed that CLK1 maintained telogen stem cells and inhibited differentiation into hair follicle cells (Roh et al., 2010). Thus, we concluded that CLK1 plays a different role in each differentiation pathway of Tel-E6E7.

Future experiments using the CLK1 knockdown clones should include hair follicle cell differentiation in order to validate the CLK1 dominant-negative data obtained by the previous study. This experiment, along with the experiments using the sebocyte and epidermal pathways, should be run in triplicate to ensure the reproducibility of the data. With the data from these experiments, Dr. Stephen Lyle will hopefully be able to publish his paper and proceed to study the effects of CLK1 on other applications.

Further investigations of CLK1 could include a potential relationship between CLK1 and skin cancers. Many protein kinases other than CLK1 have been targeted for cancer therapy research in the past due to their interactions in important signal transduction cascades, and several inhibitors for these kinases have been approved by the FDA for cancer treatment (Levitzi, 2003). CLK1 especially could be a potential therapeutic target due to its involvement in the selection of pre-mRNA splice sites by its phosphorylation and subsequent activation of SR proteins (Duncan et al., 1997). Some SR proteins, such as ASF/SF2, have been discovered to have oncogenic properties when mutated or rendered non-functional (Karni et al., 2007). Furthermore, CLK1 and the SR protein of this study, SRp20, have both been previously shown

to interact with ASF/SF2 which is over-expressed in some tumor cells (Jumaa & Nielsen, 1997; Menegay et al., 2000; Karni et al., 2007). Thus, the relationship between CLK1 and skin cancers should be considered for investigation.

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