ABILITY OF DOUBLE-STRANDED RNA TO PROMOTE REPROGRAMMING IN KERATINOCYTES

by
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Abstract

The ability to change site-specific skin identity could have numerous potential advantages in the study of skin regenerative medicine. In human skin, volar and non-volar regions are formed during development and are maintained throughout our lifetime. Previous studies demonstrated that this site-specific skin identity is regulated by dermal fibroblasts underlying different epidermal regions. However, the mechanism by which this regulation is maintained remains unclear. Here, we investigate the potential for reprogramming skin identity using double-stranded RNA analog (poly(I:C)) in co-cultures of keratinocytes and fibroblasts. To examine this, cells were cultured in the presence or absence of poly(I:C) and specific genes including *KERATIN9* and *WNT7b* were analyzed by qRT-PCR to characterize reprogrammed skin identity. Initial experiments confirmed that *KRT9* is intrinsically expressed in keratinocytes in the absence of fibroblasts, although its expression is induced by fibroblasts. We found that poly(I:C) treatment of solo-cultures stimulates *KRT9* mRNA expression over an extended period of time. In addition, *KRT9* was preferentially induced in co-culture with volar fibroblasts over non-volar fibroblasts. Significantly, we identified that poly(I:C) induces the WNT/β-catenin signaling pathway and elevates *KRT9* expression, a novel mechanism by which poly(I:C) may modulate skin identity. These results establish poly(I:C) treatment as a viable method for *KRT9* induction. Collectively, the present study provides the undiscovered effects of poly(I:C) and suggests the possibility of clinical relevance to reprogram skin identity at the stump site of amputees.

Acknowledgements

I would like to thank Dr. Luis Garza, who has been my mentor in research for nearly five years, for all of the opportunities and guidance he has given me. I need to thank my colleagues in my lab, Dongwon Kim, Amadeus Zhu, Zulfiquer Hossain and Tabetha Ratliff for all of their assistance over this last year. And of course, I must thank my family, without their support I most certainly would not be where I am today.

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Introduction

The regulation of tissue identity is a major topic in skin regenerative medicine. The potential to change skin tissue identity, for example, to revert scars to original tissue, could be extremely beneficial in the treatment of various skin diseases and ailments. The ability to reprogram skin tissue identity could have numerous clinical applications, not the least of which is amputee stump site problems and prosthetic use. Current prosthetic design has certainly advanced; however, amputees still deal with skin-breakdown at the stump site. Several studies have found that amputees report a high incidence of issues such as redness, abrasion, and folliculitis (inflammation of the hair follicles) (Meulenbelt *et al*., 2011). In fact, forty-eight percent of Vietnam veterans still have problems at the stump site nearly forty years after amputation. These difficulties lead to a reduction in prosthetic use as well as a reduction in continuous-walking distance (Yang *et al.*, 2012). Our goal is to improve the quality of life for amputees. This may be achieved by changing the stump site skin from non-volar (non-palmoplantar; dorsal) to volar (palmoplantar; ventral) skin.

Human skin consists of two layers: the epidermis, the outermost layer, and the dermis, which contains fibroblasts. The epidermis is divided into several strata (from top to bottom): stratum corneum, stratum granulosum, stratum spinosum and stratum basale (Farris). Each sub-layer is made up of differentiating keratinocytes that migrate upward from the stratum basale. As they migrate, the cells begin to change shape and lose their cytoplasm, which is replaced with keratin. Once the keratinized cells (corneocytes) reach the stratum corneum, they slough off, in a process termed desquamation (McGrath).

Volar skin is found on the palms of the hands and soles of the feet. This skin is characterized by a thick epidermis and lack of hair follicles or pigmentation. Volar epidermis is comprised of a thicker stratum corneum as well as a fifth epidermal layer, the stratum lucidum (Farris), designed to help the palms and soles handle friction. Volar keratinocytes express *KERATIN9* (*KRT9*), which is responsible for the structural resiliency of volar skin. *KRT9* is the only gene found throughout the skin that is accepted as being limited to the suprabasal layers of the epidermis on the palms and soles (Knapp *et al.*, 1986; Yamaguchi *et al.*, 1999). This exclusivity makes it a valuable marker for palmoplantar skin. In fact, mutations in *KRT9* can result in hyperkeratosis (increased production of keratins), a condition seen in skin diseases like epidermolytic palmoplantar keratoderma (EPPK) (Reis *et al.*, 1994). Patients who have EPPK display thickened epidermis but only at the palms and soles (Reis *et al.*, 1994), further confirming the site-specific nature of *KRT9*.

Initial experiments in our laboratory attempted to induce *KRT9* expression in human non-volar keratinocytes through co-culture with volar dermal fibroblasts. A co-culture system is appropriate based on the knowledge that site-specific fibroblasts are important for programming proliferation and differentiation in keratinocytes (El-Ghalbzouri *et al.,* 2002). Our initial co-culture experiments, though successful, yielded only moderate levels of *KRT9* induction and have been difficult to reproduce (Kim *et al*., unpublished data).

Polyinosinic:Polycytidylic acid (poly(I:C)) is a synthetic double-stranded RNA (dsRNA) that is structurally identical to the dsRNA released from damaged cells upon skin wounding. It has been reported that poly(I:C) recognizes the Toll-like receptor-3

(TLR3) pathway and activates its downstream signaling (Alexopoulou *et al.* 2001). This activation prevents normal keratinocyte differentiation and promotes the acquisition of stem cell features in keratinocytes (Nelson *et al.*, 2015, in revision). The activation of this pathway appears to be important in complete cutaneous wound healing, including regeneration of hair follicles, as opposed to the formation of fibrotic scar tissue that is usually seen in healed wounds (Nelson *et al.*, 2015, in revision). Recently, it was shown that poly(I:C) treatment of keratinocytes leads to a partial epithelial-to-mesenchymal (EMT) transition and up-regulation of keratinocyte stem cell genes such as Tumor protein p63 (TAp63), Chromobox homolog 4 (CBX4), and KRT15 as well as WNT genes (Nelson *et al.*, 2015, in revision). Keratinocytes that have been treated with poly(I:C) for as little as 24 hours lose their characteristic cuboidal shape and take on a long, spindly morphology (Fig. 1), resembling fibroblasts or migratory keratinocytes seen during healing (Nelson *et al.*, 2015, in revision).

We hypothesized that treatment with poly(I:C) may prime keratinocytes to be more receptive to signaling from fibroblasts. If so, poly(I:C) treatment of non-volar keratinocytes prior to co-culture with volar fibroblasts could result in greater induction of *KERATIN9* than has previously been achieved.

Results

KRT9 is expressed in non-volar keratinocytes in the absence of fibroblasts

Recently, Kim *et al.* found that non-volar keratinocytes have an innate ability to induce *KRT9* in the absence of any fibroblasts (unpublished data). They induced *KRT9* expression in non-volar keratinocytes cultured at high density in keratinocyte basal media (KBM), without growth factors to stimulate differentiation (unpublished data). That result is confirmed under a different set of experimental conditions in this study (Fig. 2B). In this study, the keratinocytes were again cultured at high density, but in KGM (KBM with defined growth factors; insulin, epidermal growth factor, transferrin, hydrocortisone, bovine pituitary extract, and epinephrine), which promotes proliferation rather than differentiation. Even under diverse conditions, the keratinocytes alone show higher *KRT9* levels compared to co-cultures of keratinocytes and both non-volar (scalp) and volar (sole) fibroblasts (Fig. 2B). We conclude that non-volar keratinocytes can be induced to express *KRT9* when cultured at high density, even in the presence of growth factors.

Poly(I:C) treatment of non-volar keratinocytes stimulates KRT9 expression over time

Previously our lab identified that poly (I:C) stimulates hair regeneration in wounded mouse skins by regulating EMT-related genes such as vimentin and E-cadherin (Nelson *et al.*, 2015, in revision). Thus, we speculate whether poly (I:C) modulates site-specific skin identity. To examine this, keratinocytes were plated at high density and treated with poly (I:C) in time-course experiments (Fig. 3A). Cells were harvested every day for 5 days (Fig. 3A) and RNA samples were used to measure levels of *KRT9*. Since

KRT9 is exclusively expressed in suprabasal layers of volar epidermis (Knapp *et al.*, 1986; Yamaguchi *et al.*, 1999), keratinocytes on Day +3 (D3), which express extremely low levels of *KRT9* (Fig. 3B), represent undifferentiated cells. By comparison, untreated keratinocytes express *KRT9* mRNA from D4 and its levels have decreased by D7 (Fig. 3B), suggesting that keratinocytes are undergoing differentiation and some terminally differentiated keratinocytes are undergoing apoptotic process.

Although levels of *KRT9* mRNA were not significantly different between the untreated and the treated cells during early culture periods, poly(I:C)-treated keratinocytes continued to produce *KRT9* from D6 and the highest levels of *KRT9* are seen on D7 (Fig. 3B). Considering the possibility of apoptosis without poly(I:C) on D7, our data suggest that poly (I:C) maintains cell survival, resulting in a sustained amount of *KRT9* mRNA. In addition, these results strongly support our previous and current experiments demonstrating that non-volar keratinocytes are able to produce *KRT9* in the absence of fibroblasts (Fig. 2B).

KRT9 expression is induced in co-cultures of non-volar keratinocytes and volar fibroblasts

Non-volar keratinocytes were cultured in the presence of non-volar and volar fibroblasts (Fig. 2A) from several areas on the body; including scalp, dorsal foot, and ventral foot (sole). *KRT9* expression was induced in the presence of scalp fibroblasts, but was more highly induced in co-cultures with sole fibroblasts compared to pre-confluent keratinocyte control (Fig. 2B). In both cases, *KRT9* expression was less than in cultures of keratinocytes alone, suggesting fibroblasts may have an inhibitive effect on *KRT9* induction. To assess a possible inhibitive influence, keratinocytes and sole fibroblasts

were co-cultured at ratios of 10:1, 1:1 and 1:5 keratinocytes:fibroblasts (Fig. 4A). For every condition, 75,000 keratinocytes were used and fibroblasts were added accordingly. In looking at the data in Figure 4B, there is a clear pattern of decreasing *KRT9* mRNA expression as the number of fibroblasts increases.

Poly(I:C) treatment of non-volar keratinocyte and fibroblast co-cultures induces KRT9 expression

The co-culture system was next adapted in order to investigate what effect poly(I:C) treatment might have on *KRT9* induction in co-culture. As in the poly(I:C) treatment protocol, cells were plated on Day -2 (D-2) prior to treatment (Fig. 5A). However, in this case, keratinocytes and fibroblasts were plated together, at a 10:1 ratio of keratinocytes to fibroblasts, and then both treated with poly(I:C) on D0 (Fig. 5A). The 10:1 ratio was chosen based on the results in Figure 4B, which showed that in the case of a co-culture, a small fibroblast number is warranted. The co-culture experiment then continued as laid out in Figure 5A.

Combined results of each high cell count (Table 1) repeat indicate that poly(I:C)-treated keratinocyte-sole-fibroblast co-cultures express a greater amount of *KRT9* mRNA over untreated co-cultures (Fig. 5B). However, the combined results also show that poly(I:C)-treated keratinocyte-foot-fibroblast co-cultures expressed a large amount of *KRT9* mRNA as well (Fig. 5B). It is interesting to note that after poly(I:C) treatment, *KRT9* expression was greater in both co-cultures than in keratinocytes cultured alone (Fig. 5B), unlike the initial co-cultures, where keratinocytes alone showed the best induction (Fig. 2B). Under low cell count (Table 1) conditions (Fig. 5C), induction was

not as robust, but poly(I:C)-treated keratinocyte-sole-fibroblast co-cultures expressed the most *KRT9*.

Poly(I:C) treatment of non-volar keratinocyte and fibroblast co-cultures induces WNT7b expression

Kim *et al.* recently found that canonical WNT/β-catenin signaling is important in the induction of *KRT9* in keratinocytes (unpublished results). They treated keratinocytes with DKK1, an inhibitor of the canonical WNT pathway (Glinka *et al.* 1998), and found that *KRT9* mRNA expression was decreased by nearly half (Kim *et al.,* unpublished results). Based on this information, we speculated that poly(I:C) may induce *KRT9* through a WNT/β-catenin signaling mechanism. We tested this hypothesis by probing for WNT mRNA expression in co-culture, in this case *WNT7B* (important in hair follicle formation (Kandyba & Kobielak, 2014)). The cDNA generated from the high cell count (Table 1) co-cultures discussed above was used to probe for *WNT7b* by qRT-PCR. The results show a significant increase in *WNT7b* mRNA expression in poly(I:C)-treated keratinocyte-sole-fibroblast co-cultures (Fig. 6), suggesting that WNT ligands may function via autocrine as well as paracrine manners.

Effect of DKK1 treatment on poly(I:C)-treated non-volar keratinocytes

Since we found that poly (I:C) induces WNT ligand expression in co-culture, we attempted to inhibit WNT signaling using DKK1, an inhibitor of canonical *WNT* signaling (Glinka *et al.*, 1998). Keratinocytes were treated with DKK1 (100ng/ml) with poly(I:C) and a time course experiment was carried out as described in Figure 7A. Cells treated with both DKK1 and poly(I:C) were designated +DKK1/+PIC and cells treated

only with poly(I:C) were designated –DKK1/+PIC. Samples were collected at the time of plating (pre-confluent keratinocytes), and on Day 3 (D3), D6 and D7. These days were chosen because the greatest disparity in *KRT9* expression was seen between these time points in the original time course (Fig. 3B). Both *KRT9* and *WNT7b* mRNA expression was decreased in the presence of DKK1 with poly(I:C) at the early time point, D3, which was the date of re-plating (Fig. 7B and C). However, *KRT9* induction was greater in the +DKK1/+PIC cultures than in the -DKK1/+PIC cultures at the late time points (Fig. 7B). *WNT7b* induction appears to have ceased on or before D6 (Fig. 7C).

Discussion

Skin identity throughout the human body is highly site-specific and tightly regulated (Compton *et al.*, 1998). This is well established through structural and functional analysis of the skin on our hands and feet. Non-volar skin makes up the dorsal regions of our hands and feet and is no different from the skin on the rest of the body. Our palms and soles however, consist of volar skin, which is thicker and more resistant to damage and irritation. The mechanisms that specify skin identity are not well understood. A deeper understanding of how skin identity is regulated and maintained could potentially lead to numerous clinical applications. We aim to reprogram non-volar skin at amputated limb stump sites to take on volar identity. Successful reprogramming at this site could relieve skin diseases caused by prosthetic use.

Volar skin is characterized by the presence of *KERATIN9*, a keratin not widely expressed in other skin types (Knapp *et al.*, 1986; Yamaguchi *et al.*, 1999). Yamaguchi *et al*. showed that *KRT9* mRNA expression could be induced in non-volar keratinocytes by co-culture with volar fibroblasts, a result that we have reproduced, though induction was generally moderate. In this study, we sought to enhance *KRT9* induction through pre-treatment of keratinocytes with poly(I:C). Colleagues in my laboratory have found that poly(I:C) treatment of keratinocytes results in a partial epithelial-to-mesenchymal transition of the cells as well as upregulation of keratinocyte stem cell genes (Nelson *et al.*, 2015, in revision). It is our hypothesis that these poly(I:C)-treated cells may be better primed to follow differentiation signals from fibroblasts.

As a starting point, normal co-culture experiments with no poly(I:C) treatment were carried out in order to ensure that we were able to repeat the previously reported

results. We found that co-culture of keratinocytes with volar (sole) fibroblasts resulted in greater *KRT9* induction than co-cultures with non-volar (scalp) fibroblasts. We also report the interesting result that non-volar keratinocytes, in the absence of fibroblasts, express *KRT9* quite robustly. This clearly demonstrates that non-volar keratinocytes have an innate ability to express *KRT9* and is a result that has been reproduced separately by many individuals in this laboratory (unpublished data). Further, these results suggest to us that non-volar fibroblasts may inhibit the expression of *KRT9* in non-volar skin, rather than producing a signal to activate non-volar identity.

To investigate what effect poly(I:C) treatment might have on innate *KRT9* expression in keratinocytes, a solo-culture time course experiment was carried out, in which untreated and treated keratinocytes were collected for several successive days. At the early time points, *KRT9* expression is relatively similar. But, whereas *KRT9* expression began to drop off in untreated cells at later time points, treated cells continued to express *KRT9*. Poly(I:C) treatment appears to not only have enhanced *KRT9* mRNA expression, but also extended the duration of *KRT9* mRNA synthesis. This result supports our hypothesis that poly(I:C) treatment augments *KRT9* expression and raises some new questions for future exploration. What is the mechanism of action of poly(I:C) in *KRT9* induction? Does poly(I:C) treatment impact epigenetic mechanism in keratinocytes, as implied by the prolonged period of mRNA synthesis? And now that we have evidence that poly(I:C) treatment does induce *KRT9*, the most immediate question becomes what effect might we see after treating co-cultures of keratinocytes and fibroblasts?

In *in vivo* conditions, keratinocytes are always in the presence of fibroblasts; so using keratinocytes alone for skin reprogramming would likely be unsuccessful. Early studies attempted to growth epidermal grafts transplanted to areas of full-thickness (down to muscle layer) wounds. While these wounds are able to heal, the newly healed skin is unable to stop the surrounding margins of the wound from encroaching on the site, and so it is eventually abolished (Billingham & Silvers, 1963). Other studies tested the ability of isolated epidermis to maintain active growth and found that the epidermis quickly degraded in the absence of the dermis (Briggaman & Wheeler, 1968).

Given this knowledge, we chose to go back to the co-culture system and adapt the protocol to include a poly(I:C) treatment step. This involved optimizing culture conditions, concentration of poly(I:C) treatment, cell number and density for plating, and appropriate cell harvesting dates. Kim *et al*. have shown that *KRT9* is best induced when cells are plated at high density and cultured in KBM media to support differentiation (unpublished results). However, another colleague in our laboratory has found that poly(I:C) treatment works best when cells are plated at low density and are cultured in KGM media to support proliferation (Zhu, unpublished data). To reconcile these two protocols, an experiment was done wherein the cells were plated at high density and treated with poly(I:C) in KGM media. Unfortunately, these cells did not survive past the first day after treatment (unpublished data) and it was concluded that high-density plating was not conducive to poly(I:C) treatment. As a successful poly(I:C) treatment was deemed most necessary for a positive outcome in this experiment, we chose to use the second described culture conditions.

It was also important to decide exactly when the fibroblasts would be added to the cultures. In previous experiments, Kim *et al*. mixed the keratinocytes and fibroblasts together at the beginning of the experiment and plated the cells in that way in order to ensure correct cell counts (unpublished results). With the addition of the treatment step, this would mean waiting several days before adding the fibroblasts as the poly(I:C) treated cells cannot be trypsinized for at least two days after treatment. As this was undesirable, we took a chance and decided to plate the keratinocytes and fibroblasts together and treat all of the cells with poly(I:C). As fibroblasts themselves do not produce keratins, we could be certain that the measured *KRT9* expression was synthesized by the keratinocytes.

The new poly(I:C) treatment co-culture protocol has been successful in inducing *KRT9* expression. The co-culture experiments were done under low and high cell count conditions (as described in Table 1). In both cases, poly(I:C) treatment has resulted in greater induction of *KRT9* in keratinocyte-fibroblast co-cultures. While we expected that sole-fibroblasts co-cultures would show the greatest induction after treatment, we found that foot-fibroblasts co-cultures also express a large amount of *KRT9* under some conditions. This indicates to us that poly(I:C) may be acting to overcome site-specific regulation. The increased induction, for both sole and foot fibroblasts, was most significant under high cell count conditions. However, even under low cell count conditions, induction was higher in poly(I:C)-treated co-cultures than in untreated co-cultures. Of great interest was the fact that in several of the co-culture experiments, the poly(I:C)-treated co-cultures showed greater *KRT9* induction than the keratinocytes alone, either treated or untreated. This is the opposite of what is seen in the original co-

culture experiments as mentioned earlier. All of this leads us to believe that poly(I:C) must be doing something to allow the keratinocytes and fibroblasts to interact in a new way. At this point, we do not know what that exactly that something is, but we can certainly speculate.

Poly(I:C) is a dsRNA agonist for the TLR3 damage-sensing pathway (Alexopoulou *et al.* 2001). Activation of the TLR3 pathway by dsRNA promotes the acquisition of stem cell features in keratinocytes as well as the induction of *WNT* genes (Nelson *et al.*, 2015, in revision). We know based on our own results that *WNT7b* mRNA expression is highly induced after poly(I:C) treatment. When considered together with the finding by Kim *et al.* that WNTs can activate *KRT9* (unpublished results), an argument could be made that poly(I:C) is acting through the WNT/β-catenin pathway to turn on *KRT9*. As a preliminary test of this hypothesis, we chose to carry out a time course experiment with the addition of a DKK1 treatment. We expected that treatment with DKK1 would result in decreased expression of both *KRT9* and *WNT7b.* Our results found that both *KRT9* and *WNT7B* mRNA expression was decreased on Day 3 by treatment with DKK1, implying inhibition by DKK1. However, *KRT9* mRNA expression was greater in the +DKK1/+PIC cultures than in the -DKK1/+PIC cultures at Day 6 and Day 7. While the *WNT7b* result is promising, the *KRT9* finding is unexpected and conflicts with our other results indicating that poly(I:C) treatment increases *KRT9*. We wonder if this might indicate that past a certain point, for example six days after poly (I:C) treatment, a different set of secreted factors is responsible for *KRT9* induction. This is plausible given the significant changes in morphology seen after poly(I:C) treatment. However, it is still too early in the exploration of this question to be drawing conclusions.

More time is needed for repeating this experiment and optimizing DKK1 treatment conditions before anything can be said with certainty. In continuing this work, we will also explore the effect of poly(I:C) on skin regeneration using an *in vivo* model.

In summary, this work develops an improved method by which *KRT9* induction can be upregulated in *in vitro* co-culture. These findings demonstrate that poly(I:C) treatment of keratinocytes results in enhanced *KRT9* induction not only in the presence of volar fibroblasts but in the presence of non-volar fibroblasts as well. This strongly supports our hypothesis that poly(I:C) treatment modulates site-specific skin identity. We expect the present research will provide a clinical method by which we may achieve our goal of reprogramming skin at the stump site of an amputee.

Methods

Isolation of human foreskin keratinocyte isolation

Human primary foreskin keratinocytes were isolated from newborn circumcision foreskin with parent's permission. All instruments were kept in 70% ethanol (EtOH) when not in use and rinsed in PBS before use. To make dispase solution, 0.04g of dispase II (Sigma-Aldrich, St.-Louis, MO) was dissolved in 10ml PBS, drawn up into a syringe and filtered into a 15ml conical tube.

To begin isolation, foreskins were cut so that they lay flat, and were then transferred to a 50 ml conical tube containing 25ml of 70% EtOH, followed with vigorous shaking to remove any contaminants. Once all foreskins were cut, the conical tube was shaken for 60 seconds. To remove remaining 70% EtOH, foreskins were transferred to a 50 ml conical tube containing 25 ml of PBS-A (PBS + 1X antibiotic-antimycotic (antibiotics) (Life Technologies, Grand Island, NY)) and which was vigorously shaken for 60 seconds. The foreskins were then trimmed by removing fat tissues using scissors and washed twice with 70% EtOH and PBS-A. Finally, the foreskins were cut into thin strips (about 0.5 cm x 2 cm) and incubated in 0.4% dispase II (Sigma) solution at 4 °C overnight.

The following day, epidermis layers were separated from the dermis and collected by spinning down in PBS for 1 minute at 2000 rpm. After aspirating off the PBS, epidermis layers were incubated in 10 ml of pre-warmed trypsin-EDTA (Life Technologies, Frederick, MD) for 5 minutes with gentle shaking. Next, the trypsin solution was carefully poured onto a mesh filter with 70 µm poresize placed over a new conical tube containing 10 ml of trypsin neutralizing solution (TNS) (Life Technologies)

and cell suspension was centrifuged for 5 minutes at 2000 rpm. The supernatant was aspirated off and cells were re-suspended in 5 ml of keratinocyte growth media (KGM-GOLD) (Lonza, Walkersville, MD). The cells were counted using the Countess Automated Cell Counter (Life Technologies) and plated at minimum $2x10^6$ cells cells per 10 cm plate.

Keratinocyte culture

Primary human foreskin keratinocytes (HFKs) were cultured in KGM-GOLD (Lonza); media was replaced every two days. Volar (sole) and non-volar (scalp and foot) fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) (Corning, Manassas, VA) with 10% fetal bovine serum (FBS) (Gemini, West Sacramento, CA) and 1X antibiotic-antimycotic (Life Technologies). Media was replaced every three days. Cells were kept at pre-confluent state to prevent differentiation prior to use in co-culture experiments.

Co-culture experiments

HFKs and sole, scalp or foot fibroblasts were mixed together at a 10:1 keratinocyte:fibroblast ratio and seeded onto 6-well plates (Day -2). After incubation for 2 day, cells in three wells were treated with poly(I:C) (20μg/ml) in KGM-GOLD (Day 0); the remaining three wells were left untreated. The next day (Day +1), cells were washed with PBS to remove poly(I:C) and incubated with fresh KGM-GOLD for two additional days; except in the case of time course experiments, where incubation length was extended up to seven days. To analyze gene expression, cells were harvested and used for RNA isolation. For comparison, keratinocytes harvested on Day -2 served as a pre-confluent control. For co-culture experiments, keratinocytes alone were always included

and were treated with poly(I:C) to compare gene expression to that of the co-culture samples. As poly(I:C) can be somewhat cytotoxic, a larger number of cells were plated for poly(I:C)-treatment than for non-treated cells. Two variations of cell counts were used, termed high cell count and low cell count. Table 1 contains the keratinocyte and fibroblast cell counts plated for each variation.

RNA Isolation and Quantitative real-time PCR (qRT-PCR)

RNA was isolated for HFKs and fibroblasts with RNeasy Mini Kit (Qiagen, Valencia, CA) and treated with DNase I (Qiagen) to eliminate genomic DNA. Concentration of the isolated RNA was analyzed with a NanoDrop2000c (Thermo Scientific, West Palm Beach, FL). Reverse transcriptase reactions were carried out as described in the protocol for the high-capacity RNA to cDNA kit (Life Technologies). Following this, qRT-PCR was performed for genes of interest using Taqman probes and Fast Start Universal Probe Master (Roche, Indianapolis, IN). Relative expression of mRNAs was analyzed by the C_T (threshold of cycle) value of target genes and quantified by normalizing to ribosomal protein large P0 (*RPLP0*) (housekeeping gene) using the $\Delta\Delta C_T$ method (Livak *et al.*, 2001). P values were determined using a two-tailed t-test; a p value of 0.05 was accepted for statistical significance.

Figures

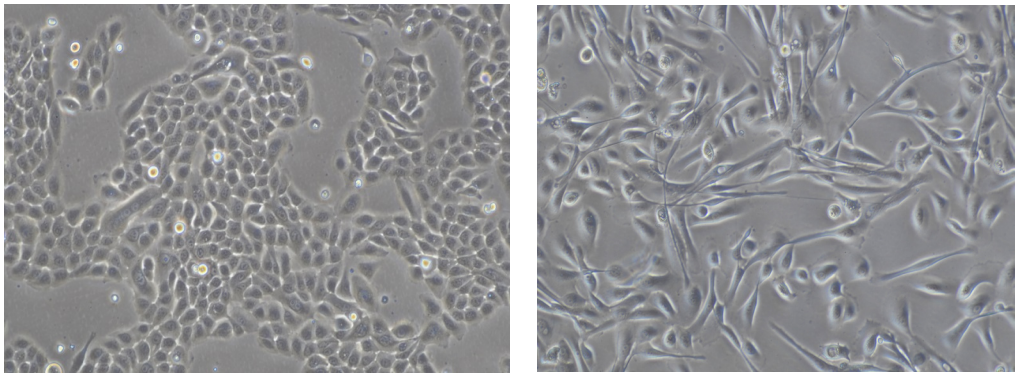

Figure 1. Keratinocyte morphology in presence of poly(I:C). Left panel: Normal, untreated keratinocytes. Right panel: Keratinocytes after 24-hour PIC treatment.

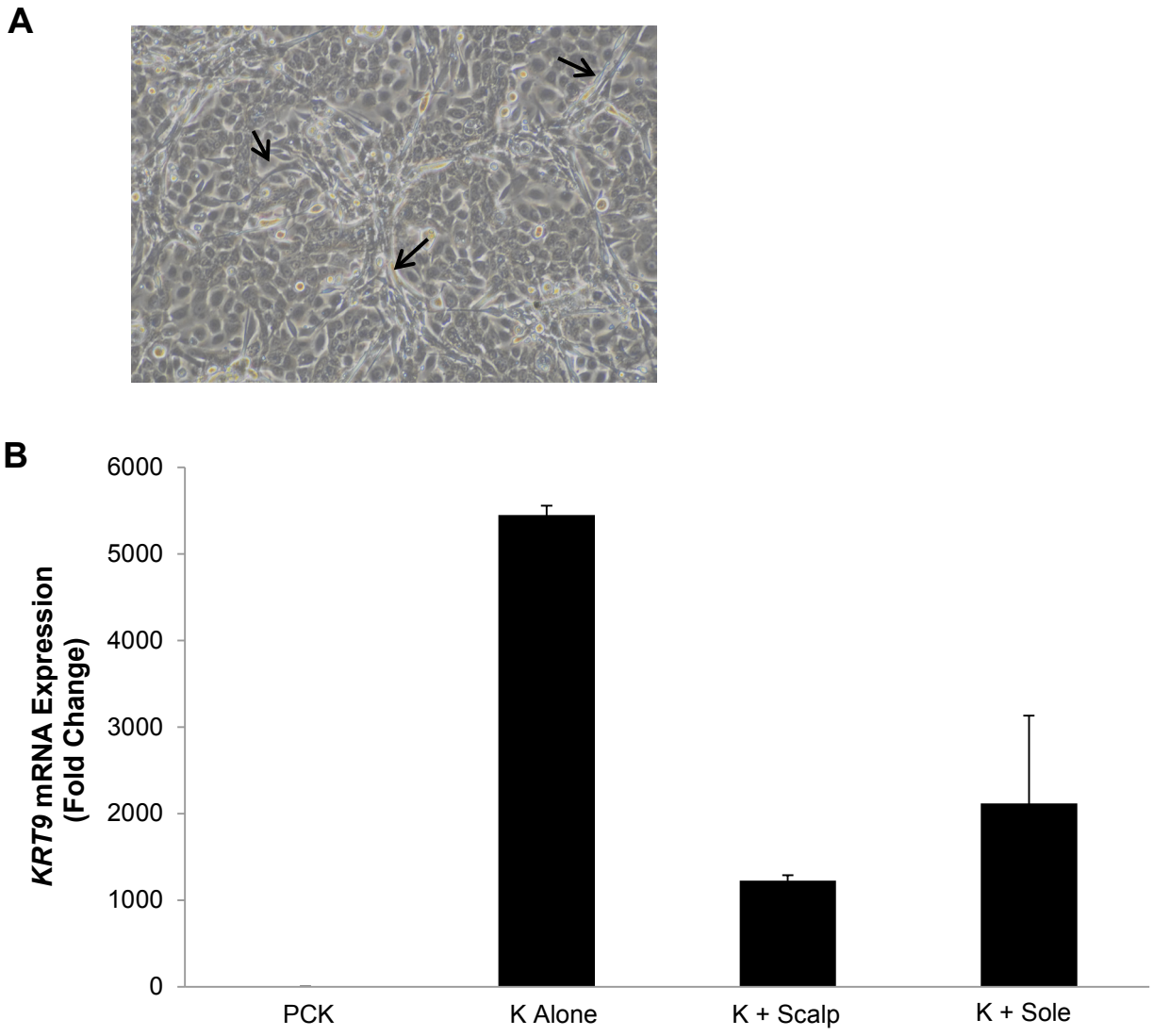

Figure 2. Induction of *KRT9* expression in keratinocytes and fibroblast co-cultures. (A) Keratinocytes cultured with fibroblasts; arrows denote fibroblasts. **(B)** *KRT9* mRNA expression in untreated keratinocytes alone and keratinocyte co-cultures. PCK= pre-confluent keratinocytes, K = keratinocytes.

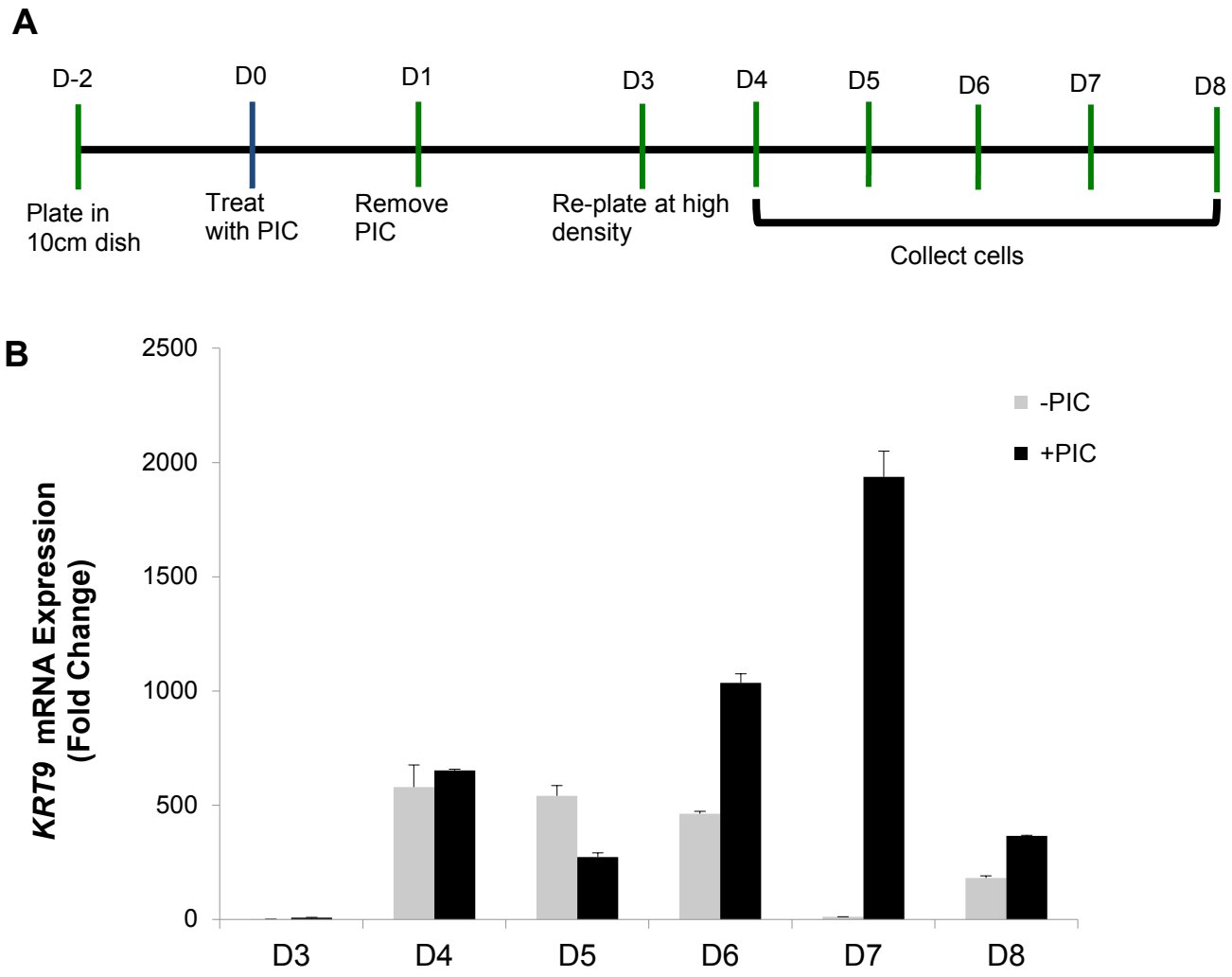

Figure 3. **Time Course of *KRT9* mRNA expression in keratinocytes after PIC treatment**. **(A)** Timeline for PIC-treatment keratinocyte solo-culture experiment; D3= Day 3. **(B)** *KRT9* mRNA expression with and without PIC treatment over the course of one week. PIC= poly(I:C).

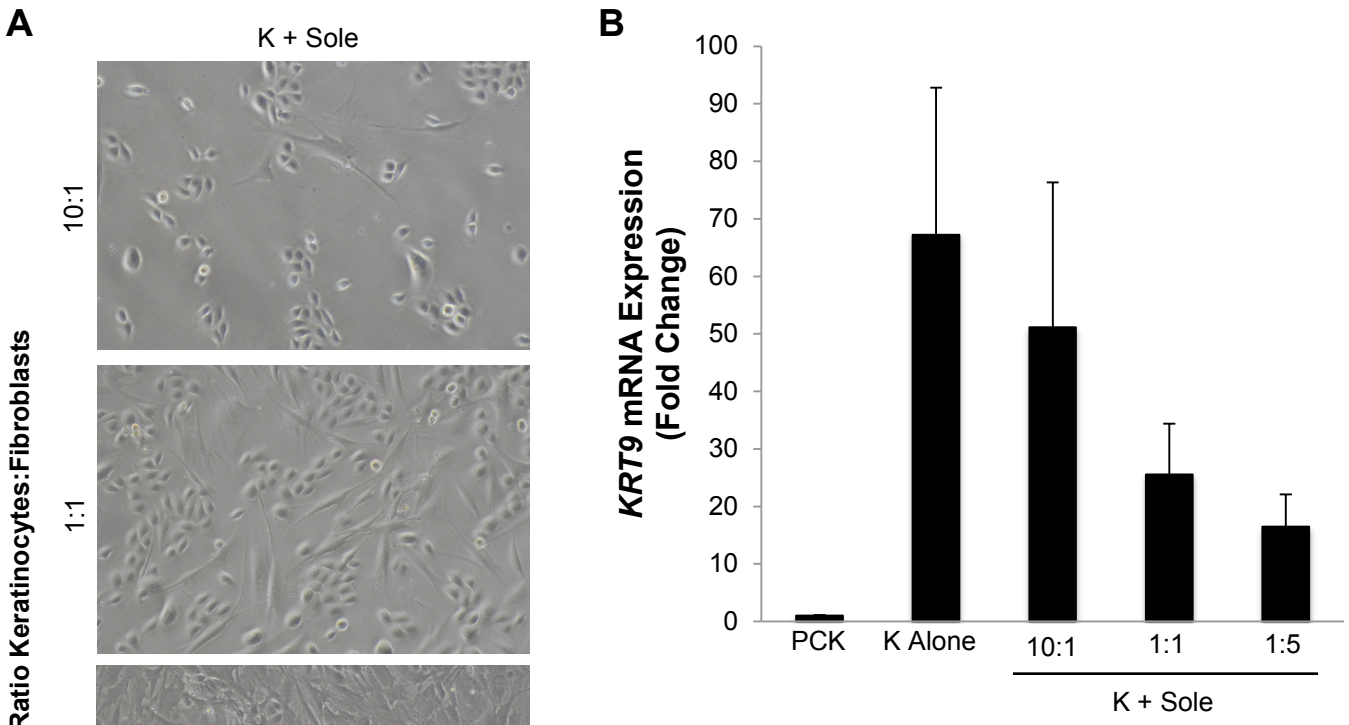

Figure 4. Effect of increased fibroblast number on *KRT9* mRNA expression in co-culture. **(A)** Keratinocytes Keratinocytes cultured with sole fibroblasts at ratios: 10:1, 1:1, and 1:5 keratinocytes:fibroblasts. **(B)** *KRT9* mRNA expression in keratinocytes alone and in co-culture for each ratio tested. PCK= pre-confluent keratinocytes, K = keratinocytes.

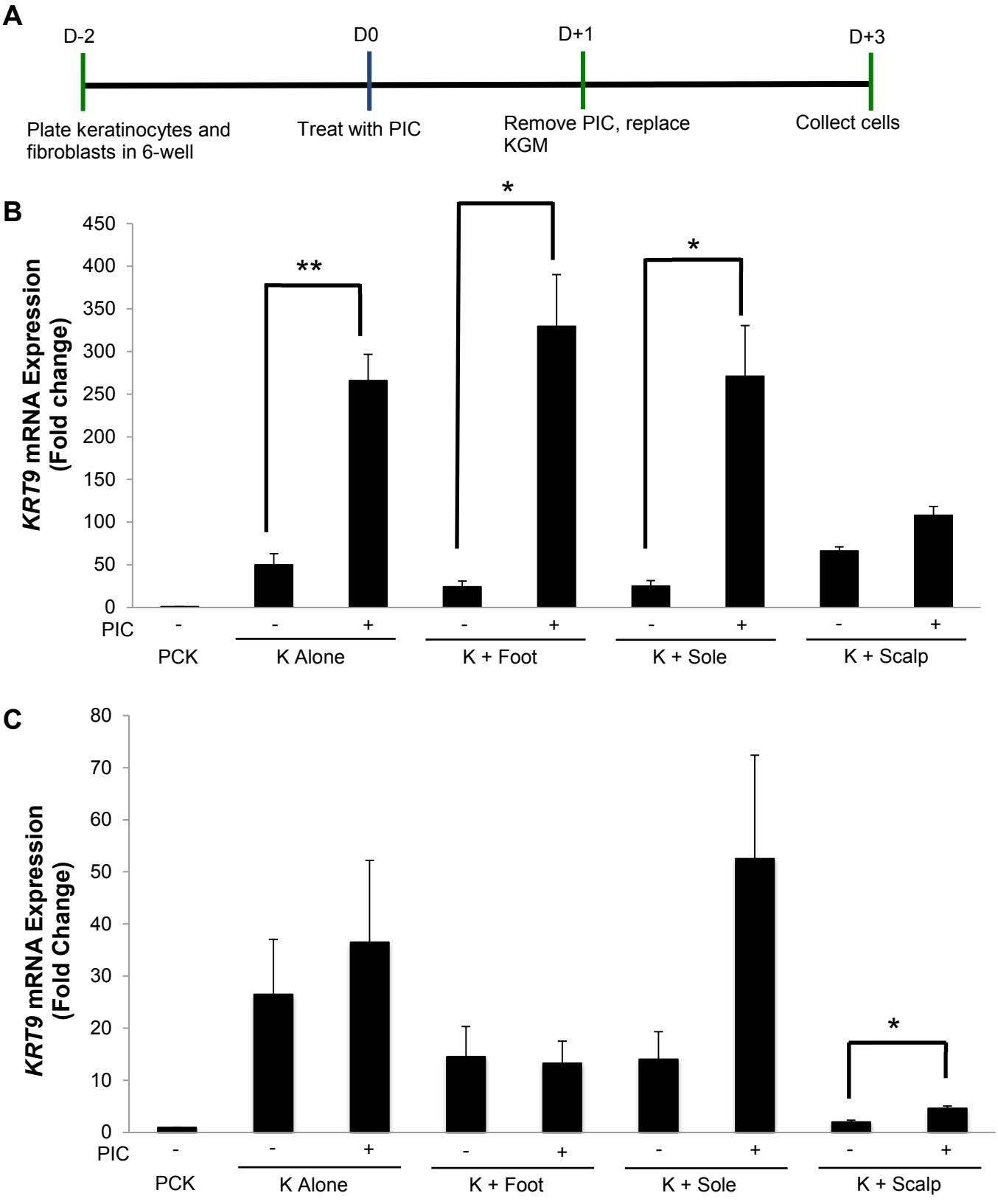

Figure 5. *KRT9* mRNA expression after PIC treatment in co-culture. (A) Timeline for PIC-treatment co-culture experiments; D-2= Day -2. **(B)** *KRT9* mRNA expression with PIC treatment; high cell count (see Table 1); n=5. **(C)** *KRT9* mRNA expression with PIC treatment; low cell count (see Table 1); n=6. *p<0.05, **p=0.0006; PIC= poly(I:C), PCK= pre-confluent keratinocytes, K = keratinocytes.

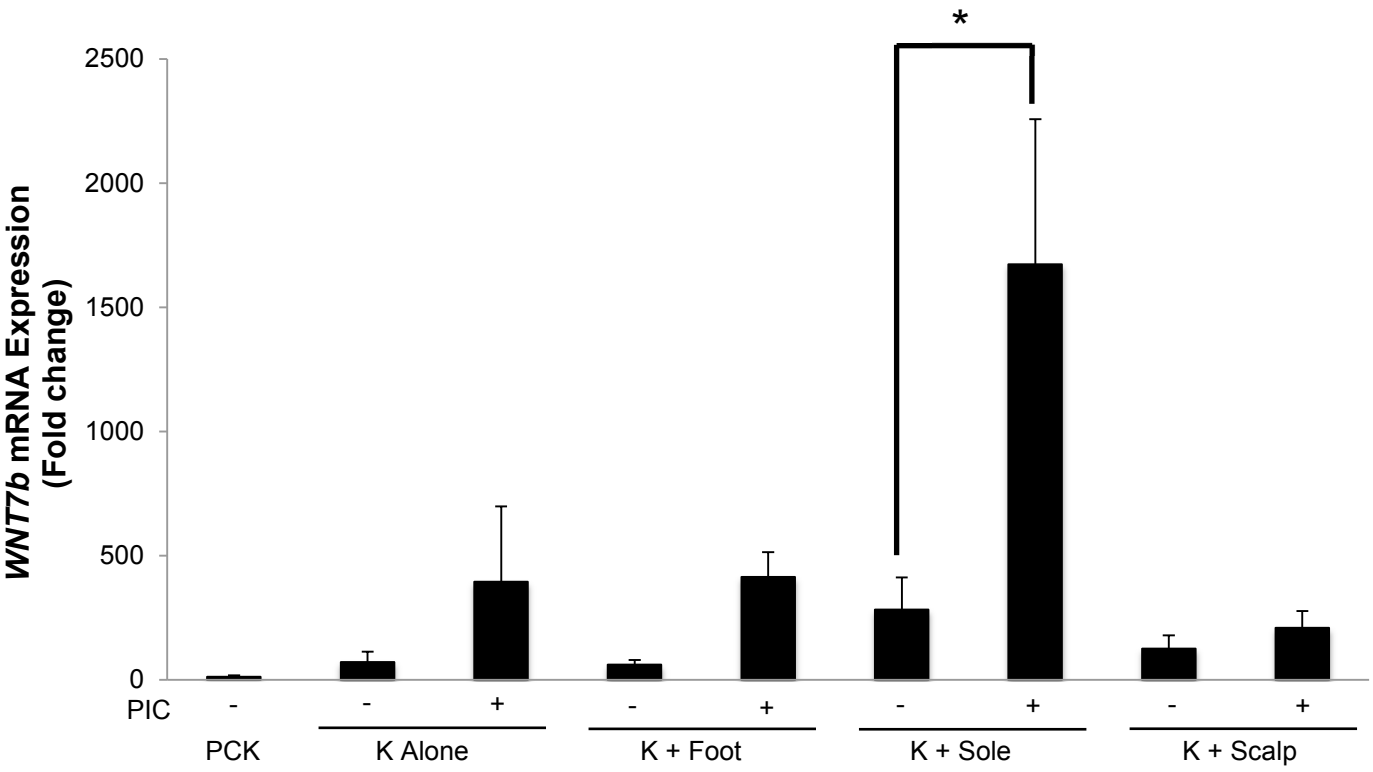

Figure 6. *WNT7b* mRNA expression after PIC treatment in co-culture. *WNT7b* mRNA expression with PIC treatment; high cell count (see Table 1); n=5. *p<0.05; PIC= poly(I:C), PCK= pre-confluent keratinocytes, K = keratinocytes.

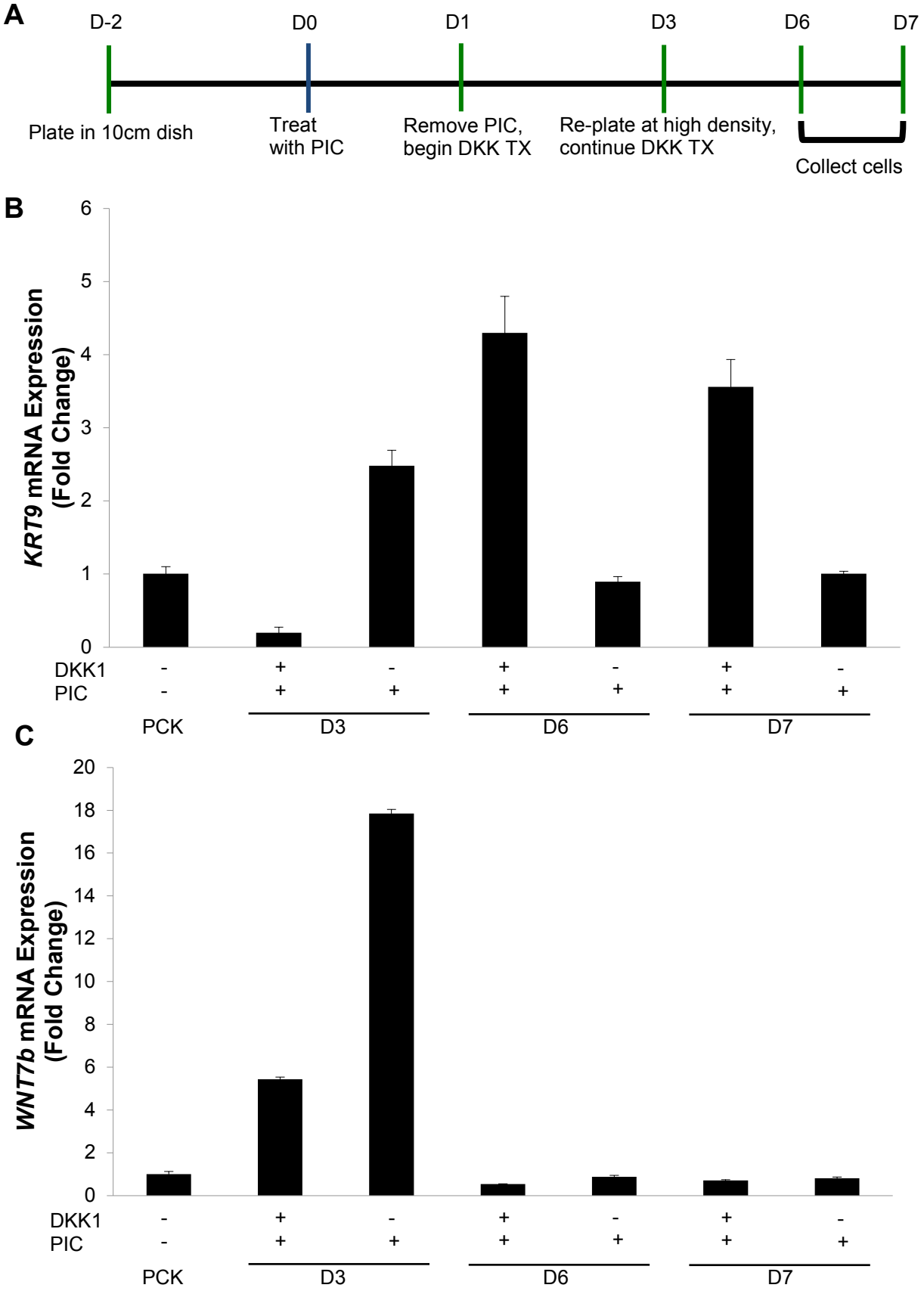

Figure 7. Effect of DKK1 and PIC treatment on *KRT9* and *WNT7b* mRNA expression. **(A)** Timeline for DKK1 and PIC-treatment experiment. **(B)** *KRT9* mRNA expression with PIC treatment +/- DKK1; low cell count (see Table 1). **(C)** *WNT7b* mRNA expression with PIC treatment +/- DKK1; low cell count (see Table 1); n=3 for D6 & D7. PIC= poly(I:C).

Tables

Table 1. Cell Number for PIC Treatment in Solo-culture and Co-culture

	Poly(I:C) (PIC)	Keratinocytes (K)	Fibroblasts (Sole, Scalp, Foot)
High Cell Count	+PIC	750,000	75,000
	-PIC	250,000	25,000
Low Cell Count	+PIC	75,000	7500
	-PIC	25,000	2500

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Curriculum Vitae
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EDUCATION

Johns Hopkins University, Baltimore, MD
Masters of Science in Molecular and Cellular Biology — August 2014-May 2015
Bachelor of Science in Molecular and Cellular Biology — August 2010-May 2014
GPA: 3.3, Science GPA: 3.17

Pine Crest Preparatory School, Fort Lauderdale, FL — August 2006-May 2010
High School Diploma
GPA: 4.4, Cum Laude

RESEARCH EXPERIENCE

Student Researcher — February 2011-June 2015
Johns Hopkins University, Department of Dermatology, Baltimore, MD

Assisted in study of the role of prostaglandin D2 (PGD2) in hair follicle neogenesis (regrowth) after wounding in mice
Successfully bred PTGDR$^{-/-}$ and GPR44$^{-/-}$ double knockout mouse
Assisted in study of role of interleukin-6 (IL-6) and signal transducer and activator of transcription 3 (STAT3) in hair follicle neogenesis after wounding in mice
Assisting in study of role of keratin 9 (K9) in promoting palmoplantar skin cell identity and the possible use of K9 to induce this cell identity in cells of different origin
Techniques utilized include: DNA extraction, PCR and Gel Electrophoresis, Research animal husbandry, Wound induced hair follicle neogenesis (WIHN) assay, Confocal laser scanning microscopy, Protein Isolation and Assay, Western blot, Immunohistochemistry, Human Keratinocyte and Fibroblast Isolation, Cell Culture

MEDICALLY RELATED EXPERIENCE

Medical Shadowing — March 2014-May 2014
Baltimore Washington Medical Center, Baltimore, MD
Dr. Gayatri Nimmagadda, M.D.

3 hours/week
Observe patient follow-up appointments to discuss patient progress and future treatments
Learn about new and current chemotherapy and radiation treatments
Observe breast and abdomen exams
Observe hospital consultations and exams

Medical Assistant, Resnik Skin Institute, Miami, FL — May 2011-August 2011

Took patient histories
Assisted in various medical and cosmetic procedures including biopsy collection, surgeries, BOTOX® and other filler injections
Explained aftercare procedures
Submitted skin samples and blood for lab testing
Completed patient call-backs
Scheduled patient appointments and follow-up appointments
Submitted prescriptions
Completed medical chart notes

COMMUNITY SERVICE EXPERIENCE

Supporting Hospitals Abroad with Resources and Equipment Volunteer — March-December 2014
Johns Hopkins Hospital, Baltimore, MD

1 hour/week, must volunteer at least 3 hours/month
Sort and organize unused medical equipment from operation rooms
Box, count and label equipment for shipment
Attend monthly general body meetings

Health Leads Advocate August 2011-December 2013
Johns Hopkins University and Bayview Medical Center, Children's Medical Practice, Baltimore, MD
Assist families in connecting with local and government resources
Resources include: food stamps (SNAP), food pantry location, medical insurance, childcare, utilities and energy assistance, specialty doctor appointments, and many more
Preformed client intake and input information into electronic database
Attended weekly Reflection Session or Follow-Up Support Session with group leadership
Made follow-up phone calls and emails to clients to discuss progress of case and next steps

WORK EXPERIENCE

Student Assistant January 2013-May 2015
Service Desk, Milton S. Eisenhower Library, Johns Hopkins University, Baltimore, MD
Patron interaction
Book, DVD, VHS, Computer disc checkout
Provide general library information and assistance with catalog searches
Locate and process requested items
Assist in booking and use of audiovisual viewing rooms
Processing new arrivals (Books, DVD, VHS, computer disc, microfilm, microform)
Processing course reserve lists from professors
Setting up microform scanners for patrons

LEADERSHIP EXPERIENCE

Health Leads Program Coordinator August 2012-May 2013
Johns Hopkins University and Bayview Medical Center Children's Medical Practice, Baltimore, MD
Planned weekly Reflection Session (RS) and Follow-Up Support Session (FUSS) programs
Arrange room reservations for weekly RS or FUSS meetings
Organized volunteer semester shift schedules and tracked volunteer hours and attendance
Assisted with new volunteer recruitment and training
Liaised with clinic/hospital staff regarding volunteer clearances and desk efficiency
Implemented use of new screening tool to identify patients who may benefit from our resources

AWARDS & HONORS

Florida Junior Academy Science, Finalist, Senior Medical Section, *An Expression Profile of Three Cortisol Enzymes in Porcine Skin* 2010
Dean's List, Johns Hopkins University Spring 2013, Fall 2013

JOURNAL ARTICLES & ABSTRACTS

Vukelic S, Stojadinovic O, Pastar I, Rabach M, Krzyzanowska A, Lebrun E, Davis SC, Resnik S, Bream H, Tomic-Canic M. Coritsol synthesis in epidermis is induced by IL-1 and tissue injury. J Biol Chem. 2011; 286(12):10265-75

Neslon AM, Reddy Sk, Hossain MZ, Katseff AS, Ratliff TS, Zhu A, Chang E, Kim D, Resnik SR, Page C, Kim D, Whittam AJ, Miller L, Garza L. TLR3 Activation Links Tissue Damage to Epimorphic Regeneration in the Skin. Manuscript In Revision. 2015.

POSTERS & PRESENTATIONS

Resnik SR. Levels of Fluoride in Tap Water versus Bottled Water. Presented at the Junior Science, Engineering and Humanities Symposium, Gainesville, Florida, February 3-5, 2008

Resnik SR. An Expression Profile of Three Cortisol Enzymes in Porcine Skin. Presented at the Florida Junior Acadmey of Science, Indian River State College, Florida, March 20, 2010

Nelson A, Katseff A, Resnik S, Garza L. Interleukin 6 promotes adult de novo hair follicle organogenesis

through STAT3 phosphorylation. Presented at 2013 International Investigative Dermatology Meeting, Edinburgh, Scotland, May 8-11, 2013.

Neslon AM, Katseff AS, Ratliff TS, Resnik SR, Whittam AJ, Miller L, Garza L. Double stranded RNA and TLR3 initiates hair follicle neogenesis after wounding. Presented at Society for Investigative Dermatology 2014 Annual Meeting, Albuquerque, New Mexico, May 9, 2014.

SKILLS:

Computer skills: MS Excel, Word and Powerpoint, Adobe Photoshop, ImageJ
Language skills: Proficiency in Spanish