How did you begin working in this area?

I got my first permanent job, following my post doc, as a bench scientist with the skin biology group of the Pharmaceutical Research Institute of Johnson & Johnson. I was involved in basic research of hair growth and epidermal differentiation. We found that a delicate balance of endogenous serine proteases and their inhibitors is essential for skin homeostasis. Minor changes to this balance could induce apoptosis and terminal differentiation of the epidermis, could delay hair growth, or could initiate pre-acne lesions (1-2). The study was so exciting that it was shocking for us to learn that our group would be moved to the Consumer Company of Johnson & Johnson. With baby shampoo and dental floss in mind, all of us, a group of molecular and cellular biologists, were worried for our future.

It was a very positive surprise to learn that skin care products actually “work”, and provide real benefits to the consumers, and that their activity is based on mechanistic understanding of biological processes. It was also surprising to find out that I would be involved in pigmentation studies. Yes, I had heard the word “melanocyte” before, but I knew nothing about pigment cell biology. With no electronic access to literature (remember those days?) I had to learn the basics by reading in the library and attending a pigmentation conference. I was fortunate to find a PASPCR meeting, and I was more than pleased to see how warmly I was welcomed, and how friendly the other attendees were to a very naive newcomer. They made me “feel at home” from the first moment. This was my first sign that I was going to like pigmentation.

How did you start?

Armed with basic concepts of pigment cell research, I was to identify safe and effective agents that modulate skin pigmentation. To my surprise, my highest business-related priority was to identify new skin lightening agents. Coming from Israel, I could not imagine anyone who does not want to tan…

The big question was how to start. Together with Stan Shapiro, we decided on a three-pillar approach. First, we should do “something different”, and not follow the classic approaches. At that time, depigmentation studies were aimed at either inhibiting tyrosinase, or at identifying melanocyte-specific cytotoxic agents. We decided to look at other targets and mechanisms. Second, we wanted to employ keratinocyte-melanocyte interactions. At that time, the primary model system used was melanocyte monolayer cultures. We reasoned that because melanocytes in a culture touch each other, they exchange non-physiological signals, resulting in non-physiological artifacts. Within the skin, of
course, melanocytes do not touch other melanocytes. They interact with keratinocytes, receive signals from keratinocytes and provide keratinocytes with melanin-loaded melanosomes. Based on this hypothesis we decided to employ keratinocyte-melanocyte cultures in all our studies. Our third "how to start" point was to expand on our skin-related serine protease knowledge. If serine proteases are important for hair growth, epidermal differentiation and acne, why not in pigmentation?

**What have you found so far?**

The most important point that we found is that the keratinocytes are important players in the regulation of skin pigmentation.

Using synthetic trypsin inhibitors as preliminary research tools we were able to reduce pigment production in keratinocyte-melanocyte cultures (3-4). That was a good start, confirming the validity of the third pillar of our approach. We were less happy with our second pillar, as keratinocyte-melanocyte co-cultures took much more time and effort than we had expected. With limited resources, we chose to move to melanocyte cell line cultures. Surprisingly, we could not reproduce the trypsin inhibitors depigmenting effect, which was very reproducible in the co-culture system. This was the first clue that the keratinocytes are important. Creating co-cultures with no keratinocyte-melanocyte direct contact, we lost the depigmenting effect of the serine protease inhibitors again. We concluded that trypsin inhibition is not affecting either the melanocyte directly, or a pigmentary soluble factor, and that a keratinocyte-melanocyte contact is required for trypsin inhibitors to affect pigmentation (3-4). Meanwhile, we were able to induce skin lightening in a dark skinned Yucatan swine with our synthetic inhibitors, and were all excited about the validation of our approach (3-4).

Studies for mechanistic understanding of trypsin inhibition in pigmentation were initiated. We were fascinated to find that only in the presence of keratinocytes, trypsin inhibitors reduced TRP-1 and increased TRP-2 (DCT) expression in the melanocytes. We hypothesized that reduced TRP-1 levels would lead to reduced Tyrosinase half-life, and that increased TRP-2 levels would enhance the production of brown vs. black eumelanin, both processes leading to a lighter skin color. With no effect in melanocytes monocultures, and no effect on tyrosinase expression levels within the co-cultures, we showed that serine protease inhibitors could affect melanogenic gene expression, but only when keratinocyte-melanocyte contact is established. Searching for that keratinocyte-melanocyte contact-interaction that affects melanogenic gene expression, we could only think of melanosome transfer. Our next set of studies used keratinocytes alone, with either isolated melanosomes or with similar size fluorescent beads, to show that trypsin inhibitors reduce melanosome phagocytosis by keratinocytes (5). Similarly, we showed that keratinocyte phagocytosis of *E. coli* particles is inhibited by trypsin inhibitors. We documented effects on keratinocyte cytoskeletal organization and keratinocytes morphology that correlated with their phagocytic ability (5). We concluded that the regulation of skin pigmentation is mediated, in part, by the keratinocytes phagocytic activity.
Reducing melanosome phagocytosis by the keratinocyte resulted in reduced pigment production by the melanocyte. As simple as a “supply and demand” process. However, we did not understand the kinetics that was observed. Some serine protease inhibitors-induced events were “too fast” to be mediated by proteolytic digestion or by its inhibition. Some observed events were so fast, as if we were affecting a signaling process. We needed to further explore the molecular mechanism of trypsin inhibition in melanosome phagocytosis.

The keratinocyte receptor PAR-2 regulates skin color

Historically, it was only a few years after three homologs of the thrombin receptor were identified, making a group of four unique receptors that are activated by a serine protease cleavage. They were renamed PARs (protease-activated receptors), when PAR-1, 3 and 4 are activated by thrombin cleavage, and PAR-2 is activated by trypsin cleavage. The serine protease cleavage of PARs creates new N-termini sequences, which serve as tethered ligands to activate the receptors. PARs are also activated by soluble peptides that represent the unique sequences exposed upon the serine protease cleavage of each PAR. Keratinocytes were found to express both PAR-1 and PAR-2. We hypothesized that melanosome phagocytosis by keratinocytes might be mediated by a PAR, to possibly link our signaling-like kinetics with a protease effect. Using each PAR-specific activating peptide, and specific thrombin and trypsin inhibitors, PAR-2 was identified as a keratinocyte phagocytic receptor (1-5). SLIGRL, the PAR-2-specific activating peptide, enhanced keratinocyte phagocytosis in culture, and enhanced pigment deposition in vitro and in human skin. Altogether, we showed reduced pigment deposition in human skins when PAR-2 activation was inhibited, and visible tanning when PAR-2 was activated (2, see Fig. 1).

Modified from Seiberg, PCR 14:236 (2001)
What are the implications of this discovery? How did Industry benefit from this basic research?

We initiated studies to identify safe and effective skin lightening agents, with consumer needs in mind. The synthetic trypsin inhibitors that induced skin lightening were indeed safe and effective, but would not serve our consumers needs, as such molecules would be considered drugs. We needed to take our new biological understanding and use it to create consumer products for skin lightening. With natural extracts for skin care in mind, we looked for safe natural sources of serine protease inhibitors. We were delighted to find numerous, naturally occurring trypsin inhibitors, in many edible plant families. Our favorite was STI (soybean trypsin inhibitor), which we found very effective in inhibiting keratinocyte phagocytosis and pigment deposition. BBI (Bowman-Birk inhibitor), a trypsin and chymotrypsin inhibitor from Soy, was also effective in inducing skin lightening. Therefore, we evaluated the potential of a non-denatured extract of the whole soybean in inducing skin lightening.

Scientifically, it was a great success. Non-denatured soybean extracts induced skin lightening (6). We worried that STI and BBI would not penetrate the skin, but, to our surprise, the whole soybean extract enabled the delivery of active STI and BBI through the stratum corneum. We thought that we are “done”, only to find that every other commercialization aspect of this scientific success was a nightmare. My colleague Jue-Chen Liu and her team were to develop a product from a non-denatured bean extract. That was a whole new concept. Having proteins as active agents, Jue-Chen faced numerous challenges, from the sterilization of the extract without causing protein denaturation, through formulating a mixture of molecules that had the tendency to lose activity. Jue-Chen needed to deliver active proteins through the stratum corneum, and, not to forget, to maintain cosmetic aesthetics. (Molecular biologists do not care if their active agent is smelly, and neglect to remember that consumers of skin care products do care…) The implementation of scientific knowledge in product development was fascinating. I learnt so much during that time!!! Our team supported the product development process by testing formulated extracts for activity. It might sound boring, but when it is your “baby” it is most exciting to see that a formulation “works”, and to witness the positive results of the first clinical study. Our non-denatured Soy products are now available in many of our brands, they “work”, and we are very proud of them.

We had shown that Soy reduces skin pigmentation, and prevents or reduces UVB-induced tanning (6). Then we started wondering if Soy would also affect hair pigmentation, or, in experimental terms, if we could turn a black mouse into a white one. While inducing gray hair has not been on our business goals, reducing the visibility of unwanted hair was something that would benefit our consumers. A study was initiated and failed; the mice grew black hair. Interestingly, it was a very different type of hair, looking shorter, softer, and more “neat” and organized. To our surprise, we found that non-denatured Soy
extracts, STI and BBI, delay hair follicle growth and differentiation, resulting in shorter, thinner, and less pigmented hair shafts (7). We could explain this effect, in part, by inhibiting proteases involved in hair growth and cycling, and by the phyto-estrogenic effects of the Soybean extracts (7). Topical daily application of Soy reduced men’s facial hair growth and hair dimensions, and made women’s legs hair growth slower and less visible. Following double blind, placebo controlled clinical studies by our colleagues, another series of products was launched, with hair minimization (“shave less often”) benefits. These products are out only because initially we wanted to make white mice....

As we continued with hair studies, we noticed (and quantified) that Soy-treated skins were firmer, and more elastic, relative to control or vehicle treated skins. An Elastin staining revealed an increase in dermal elastin fibers in Soy-treated skins relative to controls (8). Studies were initiated to understand this effect, identifying enhancement of the elastin gene promoter and inhibition of elastases by the Soybean extracts (8). Starting with pigmentation, we now had an active extract that provided other skin care benefits, including the most desired “anti-aging” effects.

The ethnic connection
Skin color is the most notable difference among ethnic skins, and differences in pigmenatry processes among ethnic skins are of great interest to the pigment cell research community. The pattern of melanosome distribution within the epidermis is skin color-dependent. Ray Boissy and his colleagues showed that in vitro, this melanosome transfer pattern is regulated by the ethnic origin of the keratinocytes, not the melanocytes (9). Therefore, we hypothesized that PAR-2 may play a role in the modulation of pigmentation in a skin-type dependent manner. Work by Glynis Scott and colleagues showed that PAR-2 is UVB-inducible (10). Interestingly, untreated controls of different ethnic origins, presented in this study, suggested a higher level of PAR-2 protein in darker skins. Connie Lin initiated ethnic skin studies, and found, together with team members, that the PAR-2 pathway is differentially expressed in ethnic skins, and correlates with pigment deposition. Protein levels of PAR-2 and trypsin (a natural PAR-2 activator within skin), as well as the enzymatic activation of PAR-2 within the skin, were enhanced in darker skins (11). Microsphere phagocytosis was found to be more efficient in keratinocytes derived from highly pigmented skins, and PAR-2 induced phagocytosis resulted in more efficient microsphere ingestion in dark skin-derived keratinocytes (11). These results demonstrate not only that PAR-2 expression and activity correlate with skin color, but also that ethnic skin phenotypes are not only melanocyte-related.

Connie followed up on this observation with a DNA chip analysis of keratinocytes from dark and light skins, and identified many ethnic differences within keratinocytes, that are not directly related to pigment production or distribution. From pigmentation and the PAR-2 pathway, a new research direction was emerged, for the understanding of the ethnic keratinocyte. We do not know where this direction will lead us, but we are sure that our future findings would be translated into improved skin care products that target different skin
types. One of Connie’s interesting candidates is the cathepsin L2 gene, which is involved in epidermal desquamation. Connie and Nannan Chen showed that L2 levels are significantly reduced in keratinocytes of darker skins (12). We hypothesize that reduced L2 proteolytic activity within the stratum corneum of darker skins is involved, at least in part, in the creation of the “ashy skin phenotype”, when light reflectance from dead stratum corneum cells of dark skins results in a dull, ashy look.

**Soy and skin cancer prevention**

We started our Soy journey with the inhibition of PAR-2 activation by two soybean-derived protease inhibitors, STI and BBI. BBI is a known cancer-suppressive agent, that is effective in different species, in many organ systems and tissue types, and when given via several different routes of administration (13). Interestingly, BBI has never been evaluated in UVB-induced skin cancer models, and STI has not been evaluated as a possible cancer prevention agent at all. Together with Stan Shapiro, and with Allan Conney from Rutgers and his team, we found that STI, BBI, and the non-denatured soybean extracts, reduced skin tumor formation and progression in high risk hairless mice pretreated with low doses of UVB for a long time (14). Following denaturation, the soybean extract had no effect on skin tumor development or progression. Pretreatment of swine skin with the non-denatured soy extract reduced or eliminated UVB-induced DNA damage, with no sunscreen protection by the extract (14). Ongoing studies for mechanistic understanding revealed multiple pathways that are affected by the Soy extract. These include enhancement of pChk-1 activation, enabling longer repair time before replication, moderate anti-oxidative effects, and reduced UVB-induced COX-2 expression and activation (studies in progress).

Post-inflammatory hyper-pigmentation is of major concern to darker skinned individuals. Identifying the preventive, COX-2 inhibitory activity of Soy, brought us back to skin pigmentation studies. Is part of the depigmenting activity of this non-denatured soy extract depends on its anti-inflammatory, COX-2 inhibitory activity? Would daily treatment with non-denatured Soy reduce the incidence of post-inflammatory hyper-pigmentation? Preliminary studies by our team show promising results. Clearly, these studies could add to the enhancement of human health and quality of life.

**What is next?**

Basic research is so appealing to many of us, because it takes us into unknown territories and gets us excited about new learning. It might be surprising to many academics, but applied research is as challenging and exciting, and sometimes as unpredictable, as basic research. Only the questions are different. Starting with melanogenesis and the basics of pigmentation, we found ourselves studying not only keratinocytes of different ethnic origins but also the possibility of skin cancer prevention. A focused approach and tuning to the needs of our consumers led us to skin lightening products. Scientific curiosity and
a “hair whitening” experiment directed us to new learning (and products) of skin elasticity and hair growth. We are fortunate to combine both basic and applied research, and to enjoy the excitement of both scientific curiosity and focused problem solving. We continue studies of ethnic keratinocytes and of post-inflammatory hyper pigmentation, while other team members expand our knowledge on skin elasticity and hair growth. We expect results from these studies to tell us “what’s next”.

Of course, none of these excitements would have happened without my many colleagues and collaborators within J&J and the academic community. I would like to thank everyone who contributed to these studies, who were engaged in inspiring discussions, and who contributed to the wonderful environment of collaborative research.

References


