FINAL REPORT
2010.01.01. – 2011.03.31.

ROLE AND REGULATION OF THE ENDOCANNABINOID SYSTEM IN THE HUMAN SEBACEOUS GLAND

I. AIM OF THE PROJECT

In the current, highly focused, basic research project performed by an expert, multidisciplinary, international research team, we have systematically and mechanistically investigated the **role and regulation of the endocannabinoid system in the biology of the human sebaceous gland**.

II. BACKGROUND

The **sebaceous gland (SG)** is one of the key adnexal components of the pilosebaceous unit of the human skin. Due recent research efforts, it has become evident that the SG is not only a mere "passive" cutaneous "adnexum" to establish the physico-chemical barrier function of the skin against constant environmental challenges (by producing the oil-enriched sebum), but it rather functions as **an "active" neuro-immuno-endocrine cutaneous organ**. Moreover, it was also proposed that the proper execution and regulation of the neuro-immuno-endocrine mechanisms in the SG take a central position in the establishment of the physiological human skin homeostasis. No wonder therefore that pathological alterations in sebaceous lipid synthesis and related immuno-endocrine functions very often lead to the development of such high-incidence clinical conditions as e.g. acne vulgaris, seborrhea, atopic eczema, etc.

Intriguingly, various lipid mediators and signaling molecules of the emerging **endocannabinoid system (ECS) have lately been identified in the skin**. Indeed, several human skin cell compartments produce prototypic endocannabinoids such as anandamide (N-arachidonoylethanolamine, AEA) and 2-arachidonoylglycerol (2-AG) as well as they express enzymes involved in the synthesis and metabolism of these lipid mediators. Moreover, G-protein coupled metabotropic cannabinoid receptors (CB), i.e. CB_1 and CB_2, were identified both *in situ* and *in vitro* on numerous skin cell populations such as e.g. epidermal keratinocytes, keratinocyte subpopulations of hair follicles, and sebaceous gland-derived sebocytes.

Recent studies have furthermore suggested that the **cutaneous ECS is functional and implicated it in various biological processes**. For example, by focusing on the human pilosebaceous unit, we have shown that the locally produced AEA, via the activation of CB_1, inhibited *in vitro* hair shaft elongation and induced apoptosis-driven premature catagen regression [Telek et al, 2007]. Intriguingly – using human SG-derived immortalized, non-malignant SZ95 sebocytes which establish one of the best available cell culture systems to model the biology of the human SG – we have furthermore presented that prototypic **endocannabinoids** are present in SZ95 sebocytes and, most probably via the stimulation of CB_2-coupled intracellular signaling pathways, dose-dependently **induce lipid production** and (chiefly apoptosis-driven) cell death. In these cells, endocannabinoids also activated the mitogen-activated protein kinase (MAPK) system and up-regulated the expression of key genes involved in lipid synthesis (e.g. peroxisome proliferator-activated receptor, PPAR, isoforms and some of their target genes). Of further importance, we finally found that

sebocytes with "silenced" CB_2 exhibited significantly suppressed basal lipid production [Dobrosi et al, 2008].

III. HYPOTHESIS

Taken together, our previous results have suggested that **human sebocytes utilize an autocrine/paracrine, endogenously** (and most probably constitutively) **active**, CB_2-mediated **endocannabinoid signaling system** for regulating lipid production and cell death. According to this, **it was proposed** that the constitutively active ECS in sebocytes may fundamentally be involved in the physiological regulation of human SG biology. Along these lines, it was furthermore **hypothesized** that possible alterations in the activity and/or regulation of the ECS may contribute to the pathogenesis of such SG-coupled skin disorders (e.g. acne vulgaris, seborrhea, dry skin, eczema) which are characterized by pathologically altered lipid synthesis and related immuno-endocrine functions.

A validity of the above hypothesis was investigated during this project by defining the role ECS in the immuno-endocrine functions of the human SG as well as the regulation of the ECS by various sebaceous factors.

IV. TASKS AND RESULTS

Since we lack appropriate animal model systems compatible to the human SG, the majority of the below research efforts was carried out on *in vitro* cultured human SG-derived immortalized, non-malignant SZ95 sebocytes (Task 1). In addition, to translate the result obtained on *in vitro* cultured SZ95 sebocytes to the *in vivo* conditions, healthy and diseased human skin samples were also investigated (Task 2). In the below sections, we introduce the specific Tasks and present our experimental results obtained during the OTKA NNF project.

Task 1. To determine the expression, regulation, and functional roles of the ECS in the human SG – Experiments on *in vitro* cultured human SG-derived immortalized SZ95 sebocytes

Task 1.a. Functional expression of members of the ECS in human SZ95 sebocytes

First, we investigated the molecular expression of elements of the ECS such as (i) receptors: metabotropic CB_1 and CB_2; ionotropic transient receptor potential vanilloid (TRPV) channels; and (ii) enzymes involved in the synthesis (NAPE-PLD, DAGL) or degradation (FAAH, MAGL) of the endocannabinoids. Using complementary static imaging techniques (immunocytochemistry methods followed by confocal microscopy, flow cytometry), Western blotting, and quantitative "real-time" Q-PCR, **we have successfully identified these molecules in SZ95 sebocytes**. Moreover, we have further confirmed that **the sebaceous ECS is indeed functional** since pharmacological inhibition as well as RNAi-mediated gene silencing of the enzymes profoundly altered the production of endocannabinoids (e.g. AEA, 2-AG), as measured by mass spectrometry.

To further assess the functional existence of the ECS in the SG, we have also started to investigate the effects of various phytocannabinoids, derived from the marijuana plant *Cannabis sativa*. Intriguingly, we found that **Cannabidiol** (CBD), a non-psychoactive phytocannabinoid – in contrast to the lipogenic effects of the endocannabinoids – did not affect basal lipid synthesis of the sebocytes. Of further importance, CBD markedly **abrogated the effects of endocannabinoids as well as other lipogenic factors (e.g. arachidonic acid, testosterone, linoleic acid) to stimulate lipid synthesis**.

We then investigated the involvement of various signaling mechanisms in the sebostatic action of CBD. Inhibition of CB_1 or CB_2 did not prevent the effect of CBD. However, the decrease of the extracellular Ca^{2+} concentration ($[Ca^{2+}]_e$) markedly suppressed the action of CBD to inhibit lipid synthesis. In numerous cellular systems, CBD was shown to modulate Ca^{2+}-permeable TRP channels. Among these, we have identified the expression of TRPV1-V4 and TRPA1 on SZ95 sebocytes. Indeed, CDB increased intracellular $[Ca^{2+}]_i$ which was prevented by ruthenium red, a non-specific inhibitor of TRP channels. Of further importance, RNAi-mediated silencing of **TRPV4** (but not of the other TRP channels) fully abrogated the action of CBD to suppress lipid synthesis. In addition, the specific TRPV4 agonist GSK1016790A fully mimicked the sebostatic action of CBD, again arguing for the involvement of TRPV4 in mediating the action of the phytocannabinoid (**Fig. 1.**).

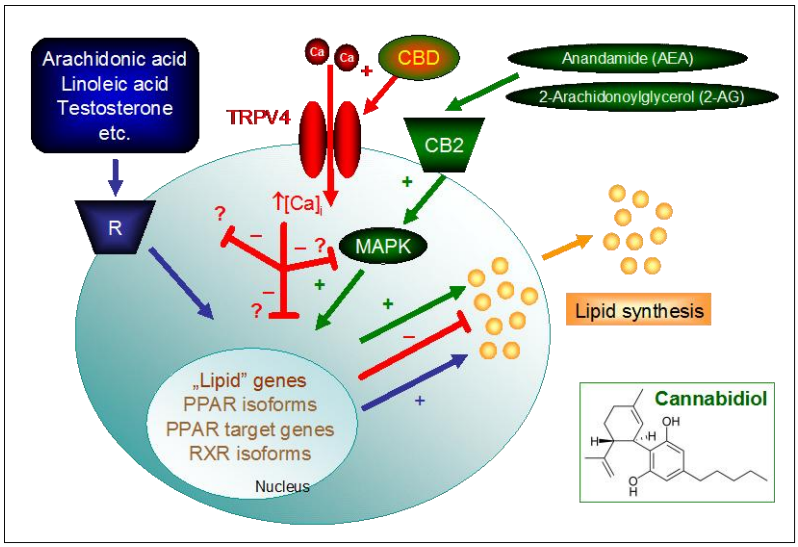

Fig. 1. *Effects of endocannabinoids and cannabidiol on lipid synthesis of human SZ95 sebocytes*
Endocannabinoids, via the stimulation of the CB_2-MAPK-PPAR isoforms pathway, stimulate sebaceous lipid synthesis. In contrast, cannabidiol (CBD) inhibits sebaceous lipogenesis (induced by various factors) by stimulating the ion channel TRPV4 and increasing $[Ca^{2+}]_i$.

In a new collaborative effort, initiated with the Laboratory of Prof. Mauro Picardo (Cutaneous Physiopathology, San Gallicano Dermatological Institute, IRCCS, Rome, Italy) a leading expert in the field of functional lipidomics, we have also started to investigate whether the sebostatic action of CBD to quantitatively suppress sebaceous lipid production is also realized in a "qualitative" manner; namely, we measured the effect of CBD on the level of individual lipids of the sebum produced by SZ965 sebocytes. Of great importance, we found that the "pro-acne agent" arachidonic acid, as expected, markedly elevated the levels of various free fatty acids (20:4, 22:4, 18:3), cholesterol ester, squalene, and certain acylglycerol derivatives, which effects were fully **reversed by the application of CBD**.

We have also started to study the putative **"down-stream" signaling mechanisms** that may mediate the cellular effect of the TRPV4-mediated elevation of $[Ca^{2+}]_i$ induced by CBD. We have tested antagonists of various Ca^{2+}-dependent signal transduction mechanisms (e.g. protein kinase C isoforms, calcineurin/protein phosphatase 2B) but we were unable to modulate the cellular action of CBD. We have therefore launched a series of MicroArray study to reveal global gene expression changes induced by the phytocannabinoid; the analysis of these large amounts of data is under progress.

Nevertheless, these above (quite intriguing) data introduce CBD as a molecule with a significant, "universal", and both quantitative and qualitative sebostatic function and a novel mechanism of action (i.e. activation of TRPV4). Our findings may therefore encourage one to systematically explore whether CBD can be exploited in the management of such common

skin disorders (e.g. acne vulgaris, seborrhea) which are characterized by pathologically elevated lipid (sebum) production of the SGs.

Task 1.b. Regulation of expression of elements of the ECS by endogenous mechanisms in human SZ95 sebocytes

The aim of this Task was to reveal whether **endogenous immuno-endocrine mechanisms**, which were shown to play fundamental roles in SG biology, **modulate the sebaceous ECS**. In our studies, we investigated (i) components of the CRH-ACTH-cortisol axis and other POMC-derived peptides (α-MSH, β-endorphin); (ii) sexual steroids (estrogens, androgens); and (iii) pro- and anti-inflammatory mediators such as interleukins (e.g. IL-1β, IL-4, IL-6) and other cytokines (e.g. TNFα).

Table 1. Effects of various hormones and mediators on the expressions of elements of the ECS in SZ95 sebocytes

Treatment with	*Endocannabinoid (HPLC/MS)*		*Quantitative "real-time" Q-PCR*			
	AEA	*2-AG*	*CB_1*	*CB_2*	*NAPE-PLD*	*FAAH*
CRH (a.v.)	↑	↑	NE	no change	↑	no change
ACTH (a.v.)	↑	↑	NE	no change	no change	no change
Cortisol	↓	↓	NE	↓	↓	↑
α-MSH (a.v.)	↑	↑	NE	no change	no change	no change
β-endorphin (a.v.)	↑	↑	NE	no change	no change	no change
Testosterone (a.v.)	↑	↑	NE	↑	↑	↑
Estradiol	↓	↓	NE	↓	↓	↑
IL-1β	↑	↑	NE	↑	no change	no change
IL-4	no change	↓	NE	no change	no change	no change
IL-6	no change	no change	NE	no change	no change	no change
TNFα	no change	↓	NE	no change	no change	no change
Transforming growth factor-β2	↓	↓	NE	no change	no change	no change
Substance P (a.v.)	no change	no change	NE	no change	no change	no change
Prolactin (a.v.)	↑	↑	NE	no change	no change	no change

↑ or ↓: significant ($p<0.05$) up- or down-regulation. a.v.: these mediators were found to stimulate sebaceous lipid synthesis and/or were implicated in the pathogenesis of acne vulgaris. NE: not expressed

Intriguingly, we found that most hormones (i.e. **CRH, ACTH, α-MSH, β-endorphin, testosterone, prolactin**), which were previously shown to stimulate sebaceous lipid synthesis and/or were implicated in the pathogenesis of acne [reviewed in Tóth et al., 2011]; as well as the pro-inflammatory **IL-1β stimulated the production of the lipogenic endocannabinoids**. In addition, some of them also up-regulated the expression of CB_2 receptor which, as we have shown before [Dobrosi et al., 2008], mediates the lipid synthesis-promoting action of AEA and 2-AG. Conversely, the anti-lipogenic **cortisol and estradiol suppressed endocannabinoid production and CB_2 expression levels**. Of further importance, those hormones which activate nuclear receptors (and hence modulate gene transcription) also modified the expressions of NAPE-PLD and FAAH. Finally, the other cytokines investigated and the neuropeptide substance P (which was also found to stimulate lipid synthesis) did not really affect the ECS. Collectively, these data (summarized in **Table 1.**) suggest that the sebaceous ECS in under the control of numerous immuno-endocrine mechanisms.

Task 1.c. Involvement of the ECS in cellular functions of endogenous immuno-endocrine mechanisms in human SZ95 sebocytes

We have also investigated the **putative involvement of the sebaceous ECS in the cellular effects of the above hormones and cytokines**. For this, we first defined the exact effects of the above agents on various sebocyte functions such as lipid synthesis and cell death/survival.

As expected, all of the above "lipogenic" hormones and mediators (i.e. CRH, ACTH, α-MSH, β-endorphin, testosterone, substance P, prolactin) stimulated the lipid synthesis of SZ95 sebocytes (Nile Red-based fluorimetry). It should be noted, however, that their lipogenic effects were much less (cca. 20-40 %) than the degree of lipid synthesis induced by endocannabinoids (especially after short-term, 24-48 hrs treatment). Moreover, using a series of fluorimetric assays, we have also shown that most of these hormones decreased the viability of the cells, which effect is characterized by an induction of a chiefly apoptosis-driven cell death. The other hormones and cytokines (listed in **Table 1.**) did not modulate cellular lipid synthesis and survival.

To reveal whether the ECS may act as a "down-stream" target pathway of these lipogenic hormones, we also investigated how the activation or inhibition of ECS activity may affect their cellular functions. For this, we employed (i) pharmacological "inhibition" protocols (i.e. various antagonists); and (ii) molecular biology "inhibition" protocols (i.e. RNAi of CB_2 receptors and/or enzymes involved in the synthesis of endocannabinoids). The "ECS-suppressed" cells were then challenged by the lipogenic hormones and the cellular responses were evaluated.

Based on our functional experiments, the achieved results could be classified into 3 groups:

- The lipogenic and apoptosis-inducing effects of testosterone and β-endorphin (which, as shown in **Table 1.**, up-regulated endocannabinoid levels) were significantly, yet only partially (by cca. 40-50 %), prevented by AM630 (specific antagonist of CB2) or the RNAi-mediated silencing of CB_2. These data suggest that the **ECS plays a key role in mediating their actions**.
- In contrast, the cellular actions of CRH, ACTH, α-MSH, and prolactin, which also elevated endocannabinoid production (**Table 1.**), were intriguingly not modified by inhibition or down-regulation of CB_2. These rather unexpected findings argue for that these agents induce cellular lipid synthesis independently from the ECS, most probably by acting via distinct signal transduction mechanisms. To test this hypothesis, we investigated the co-administration of these hormones with the endocannabinoids and with inhibitors of enzymes involved in endocannabinoid metabolism. We found that these "activation protocols" exerted **additive lipogenic effects** to the actions of these hormones suggesting that their effects to induce endocannabinoid synthesis was rather an "accidental" event.
- Finally, the effects of substance P (which did not modify endocannabinoid levels) were also not affected by these interventions.

Task 1.d. Regulation of immuno-endocrine functions of SZ95 sebocytes by the ECS

We have also defined the possible regulation of the above hormones and cytokines and the related functions by the ECS **("the other way around" regulation**). For this, first SZ95 sebocytes were treated by AEA and 2-AG, harvested, and then subjected to (i) Human Cytokine Array (which allow the simultaneous detection of 36 cytokines typically involved in inflammatory processes, R&D systems); and (ii) Quantitative "real-time" Q-PCR analysis of the cytokines and enzymes involved in their synthesis.

We found that both endocannabinoid markedly elevated the expression (Q-PCR) and production (array) of the pro-inflammatory and "pro-acne" IL-1β, IL-4, IL-6, and IL-8. Interestingly, 2-AG (but not AEA) also stimulated the synthesis of the rather anti-inflammatory IL-10. Of further importance, both endocannabinoids markedly induced the gene expressions of 5-lipoxygenase and cyclooxygenase-1 and -2, key enzymes involved in the production of such inflammatory mediators as leukotrienes and prostaglandins. In good accord with these findings, endocannabinoids elevated the production of key leukotrienes and prostaglandins (e.g. LTB_4, PGE_2). These data clearly suggest that endocannabinoids are indeed involved in the regulation of sebaceous inflammatory processes.

Task 2. To define the expression patterns of elements of the ECS in the SG of human skin as well as their alterations in various human SG diseases

In parallel to the above experiments performed on cultured human sebocytes, we have also investigated the *in situ* expression of elements of the ECS in the SG on healthy human skin samples as well as on tissues obtained from patients with various SG skin disorders. Along with healthy human skin samples, tissues of obtained from patients with SG diseases were also studied.

Task 2.a. Sample collection

We have selected numerous histological sections among samples of the histological bank of the Department of Dermatology and Pathology of the University. Moreover, we have collected numerous human skin samples (obtained mostly from dermatosurgery surplus material); these samples were either processed for routine histology (for immunohistochemical determinations) or total RNA was isolated (for quantitative "real-time" Q-PCR assessment).

Task 2.b. Determination of expression patterns of elements of the ECS in the SG of healthy and diseased skin

In parallel to sample collection, we have performed the identification of expression of ECS elements in the SG. We first employed immunohistochemistry on histological sections to identify the *in situ* protein expression of ECS members. We have successfully optimized the techniques (both for fluorescence and light microscopy visualization) for the detection of members of the ECS. Using immunohistochemistry, we found that all members of the ECS (i.e. CB receptors, enzymes) are expressed in human sebaceous glands *in situ* (**Fig 2.**), similar to those found in the cultured SZ95 sebocytes (see under Task 1.a.)

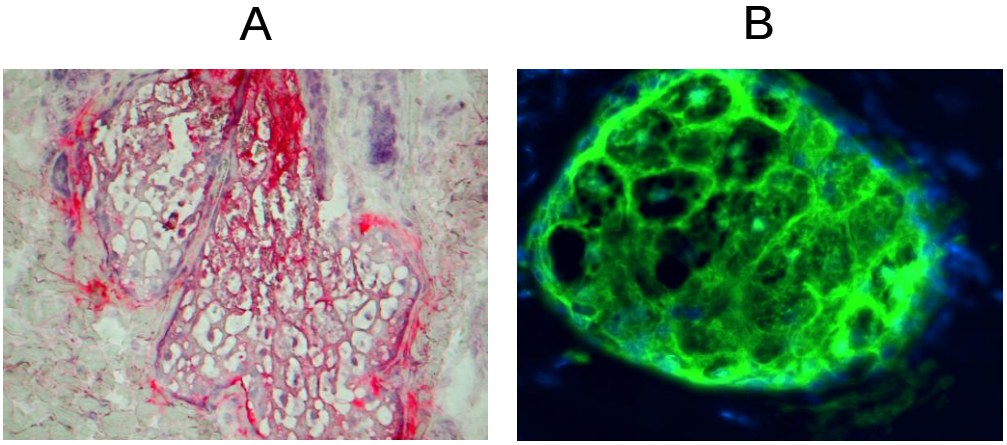

Fig. 2. *Immunohistochemical labeling of FAAH (A, alkaline phosphatase) and CB2 (B, FITC) in human SG in situ*

In addition, we have also established the methods of detecting specific mRNA transcripts of the ECS elements. For these experiments, whole SG samples were dissected from human skin samples and snap-frozen. Total RNA was then isolated and quantitative "real-time" Q-PCR experiments were performed using commercially available TaqMan probes. Using this technique, we have identified the specific mRNA transcripts of all members of the ECS, similar to the protein data detailed above.

In addition, we compared the expression levels of ECS members in samples of healthy individuals and of patients suffering from acne vulgaris. Of great importance, our quantitative "real-time" Q-PCR data suggest that the expression of CB_2 and NAPE-PLD (similar to the levels of 5-lipoxygenase, cyclooxygenase-2, and IL-6, used as "positive" controls for SGs in acne vulgaris skin) [Zouboulis et al., 2006] markedly increased in the diseased samples. In our ongoing experiments, we investigate the expression of the ECS members in rosacea and dry skin samples (atopic eczema).

Additional experimental data

Although it was not planned in the original Project, we also had the chance to investigate the role of the ECS in the regulation of human epidermal functions (i.e. proliferation, apoptosis, and intracellular Ca^{2+} homeostasis). Briefly, we found that, similar to human SG-derived sebocytes, the prototypic endocannabinoid AEA exhibited a profound role in the regulation of growth and survival of human epidermal keratinocytes. Of great importance, we have also shown that AEA initiated the onset of a novel signal transduction "chain-of-event" which involved the sequential activation of the metabotropic CB1 receptor $\rightarrow$ the ionotropic cannabinoid receptor TRPV1 $\rightarrow$ and the influx of Ca^{2+}.

In addition, in close collaboration with our strategic collaboration partners (Laboratory of Prof. Paus), we are also involved in the exploration of the role of the ECS and related TRPV channels in the control of human hair growth. As part of these experiments, we have shown that the activation of the ionotropic cannabinoid receptor TRPV3 ion channel inhibited hair shaft elongation and induced premature catagen regression of the hair follicle.

It appears, therefore, that on different human skin cell types, different ECS receptors and signaling pathways are involved in the regulation of key cutaneous functions.

V. DISSEMINATION AND PUBLICATIONS

Only those publications are listed below which (i) are closely related to the project; (ii) contain data obtained in experiments sponsored by the project grant support; and (iii) literally mention(ed) the reference number of the project grant support. (Details of other publications can be found at www.pubmed.com, search word: biro t)

Publications of the Project

i) Full papers

1. *Tóth B.I., Dobrosi N., Dajnoki A., Czifra G., Oláh A., Szöllősi A.G., Juhász I., Sugawara K., Paus R., Bíró T.* (2011): Endocannabinoids Modulate Human Epidermal Keratinocytes Proliferation and Survival via the Sequential Activation of Cannabinoid Receptor-1 and Transient Receptor Potential Vanilloid-1. *J. Invest. Dermatol. (**Epub ahead of print**)*, DOI 10.1038/jid.2010.421, **IF: 5,543 – A copy submitted to OTKA**

2. *Borbíró I., Lisztes E., Tóth B.I., Czifra G., Oláh A., Szöllősi A.G., Szentandrássy N., Nánási P.P., Péter Z., Paus R., Kovács L., Bíró T.* (2010): Activation of Transient Receptor Potential Vanilloid-3 Inhibits Human Hair Growth. *J. Invest. Dermatol. (**accepted for publication**)* **IF: 5,543 – A copy submitted to OTKA**
3. *Oláh A., Tóth B.I., Czifra G., Szöllősi A.G., Borbíró I., Sugawara K., Camera E., Picardo M., Zouboulis C.C., Paus R., Bíró T.* (2010): Cannabidiol Inhibits Sebogenesis via TRPV4. *Nat. Med. (**submitted for publication**)* **IF: 27,136**

ii) Reviews
1. *Tóth B.I., Oláh A., Szöllősi A.G., Czifra G., Bíró T.* (2010): „Sebocytes' Makeup": Novel Mechanisms and Concepts in the Physiology of the Human Sebaceous Glands. *Pflüg. Arch. Eur. J. Phy. (**Epub ahead of print**)* DOI 10.1007/s00424-011-0941-6 **IF: 3,695 – A copy submitted to OTKA**
2. *Moran M.M., McAlexander M.A., **Bíró T.**, Szallasi A.* (2010): TRP Channels as Therapeutic Targets. *Nat. Rev. Drug Discov. (**submitted for publication**)* **IF: 29,059**

Ph.D. Disssertation of the Project
1. *Borbíró I.*. (2011): Új mechanizmusok a humán hajnövekedés biológiai folyamatainak szabályozásában. *Ph.D Dissertation, Debrecen, 2011 (Date of defense: April 21, 2011)* **– A copy submitted to OTKA**
2. *Dobrosi N.* (2011): Az endocannabinoid rendszer szerepe humán bőr ederetű sejtek biológiai folyamatainak szabályozásában. *Ph.D Dissertation, Debrecen, 2011 (Date of defense: April 29, 2011)* **– A copy submitted to OTKA**

Citable Abstracts of the Project
1. *Oláh A., Tóth I.B., Szöllősi A.G., Czifra G., Sugawara K., Zouboulis C.C., Paus R., Bíró T.* (2010): Endo- and phytocannabinoids differentially regulate biology of human epithelial skin cells. *J. Invest. Dermatol.* **130**:S107 Suppl 1

Conference Presentations of the Project
i) International conferences
1. *Oláh A., Tóth I.B., Szöllősi A.G., Czifra G., Sugawara K., Zouboulis C.C., Paus R., Bíró T.* (2010): Endo- and phytocannabinoids differentially regulate biology of human epithelial skin cells. Annual Meeting of the Society for Investigative Dermatology, Atlanta, GA, USA
2. *Bíró T.* (2010): Thermo-TRP channels as key regulators of the human pilosebaceous unit. European Neuropeptide Club Meeting, Pécs, Hungary – **Invited Lecture**
3. *Bíró T.* (2010): (Endo)cannabinoid control of human sebaceous gland functions. 2nd Skin Physiology International Meeting, Vichy, France – **Invited Lecture**
4. *Bíró T.* (2010): Thermo-TRP channels as key regulators of the human skin biology. "TRP Channels and Sensory Biology" topical workshop, Alicante, Spain – **Invited Lecture**

ii) National conferences
1. *Bíró T.* (2010): Az endokannabinoid rendszer a humán bőrben. Magyar Élettani Társaság Vándorgyűlése, Szeged – **Invited Lecture**
2. *Oláh A., Jenei Á., Tóth Balázs, Bíró Tamás* (2010): A hőérzékeny TRP csatornák szerepe a humán faggyúmirigysejtek biológiájában – Fókuszban a TRPV3. Magyar Élettani Társaság

Vándorgyűlése, Szeged
3. *Paragh L., Oláh A., Papp J., Pöstényi Z., Zákány N., Sós K.E., Tóth I.B., Bíró T.* (2010): Új receptor a Cannabidiol célmolekulái között? A TRPV4 szerepe a Cannabidiol celluláris hatásainak kialakításában. Magyar Élettani Társaság Vándorgyűlése, Szeged
4. *Rab T., Oláh A., Czifra G., Pöstényi Z., Mócsai G., Bíró T.* (2010): Az endokannabinoid rendszer vizsgálata humán immortalizált SZ95 sebocyta sejtvonalon. Magyar Élettani Társaság Vándorgyűlése, Szeged