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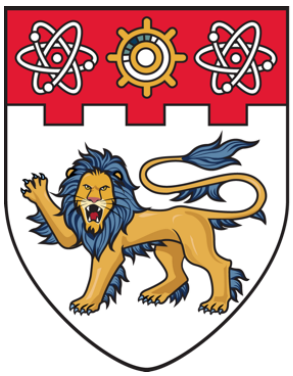
Junnarkar, Seetanshu Bharat

2019

Junnarkar, S. B. (2019). The delta opioid receptor influences circadian rhythms in human N/TERT-1 keratinocytes through the β-arrestin pathway. Doctoral thesis, Nanyang Technological University, Singapore.

https://hdl.handle.net/10356/104426

https://doi.org/10.32657/10220/49501



NANYANG TECHNOLOGICAL UNIVERSITY
SINGAPORE

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A thesis submitted to the Nanyang Technological University in partial fulfilment of the requirement for the degree of Doctor of Philosophy

2019

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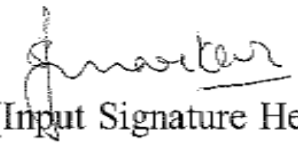

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Professor Tan Nguan Soon Andrew

Acknowledgements

In humility I dedicate this thesis to my Gurus Swami Niranjananand Saraswati and Swami Satsangananda Saraswati and my parents Mr Bharat and Mrs Rekha Junnarkar. My Gurus and my parents have been both my source of inspiration and the guiding principles of my life and for that I am eternally grateful to them.

With immense pleasure and with a deep sense of gratitude I thank my thesis supervisors Professor Walter Wahli, Professor Tan Nguan Soon Andrew, Professor Paul Bigliardi, Professor Mei Qi- Bigliardi and Professor Ray Dunn, without their guidance and support this academic endeavour wouldn't have come to realization. In the same breath I'd also like to thank Professor Birgit Lane for her guidance during the course of my doctoral studies and for giving me the opportunity to pursue my project at the Institute of Medical Biology (IMB), A-STAR.

I'd also like to take this opportunity to thank Dr Christine Neumann, Dr Yuri Hebert Dancik, Ms Ong Li Ming Sheena, Dr Graham Wright and his team from the IMB Microscopy Unit (IMU), Ivy low and other members of the FACS facility at the Singapore Immunology Network (SIgN) and the School of Biological Sciences Nanyang Technological University (NTU SBS) and Lee Kong Chian School Of Medicine (LKCSoM) for their much appreciated contributions to my doctoral studies. I'd like to specially thank Ms May Chong from NTU SBS for her time and patience.

My colleagues Alicia Yap, Cheryl Leong, Akanksha Pant, Brent, Dr Sriram Gopu, Lauren Sargisson, Shermaine Eng, Jennica Tan, Dr Khek Chian, Dr Tong San, Dr Jamie Francis Trott, Rauzan, Stephen Wearne and Dr Kim Goh have been very kind and generous with their advice and resources and for that I am grateful.

I’d also like to thank some of my friends that I met in Singapore during the course of my PhD Mr Bernard Lim, Yuan Li, Luis Cobar, Maria Ramos, Cindy, James Chen, Belle, Mohammed Ali, Ricardo Ancona, Sathiya Priya, Taniya Pandey and the many others for their encouragement and time.

I’d also like to acknowledge and thank my teachers Professor Veena Rambhal, Professor Usha Ashtekar, Dr Varsha Singh, Dr Milind Patole and Mrs Kashmira Hegde for their guidance during my undergraduate and post graduate years of study. Their faith in my potential gave me a reason to believe in myself.

This acknowledgment would be incomplete without the mention of my loving sister Saudamini, my grandparents, aunts, uncles, cousins, my lovely nieces Ananya and Diya, my friends Dr Farrokh Wadia, Mrs Statira Wadia, Shirin Wadia, Malcum Marolia, Pooja Bhandari, Batul Hafizji, Shubhi Kaushik, Zainab Kapasi, Akansha Shah, Ketki Wandre, Rahil Mirchandani, Shushank, Kritika and Aruna Suratkal, Ashley Braganza, Karan Singh, Ryan Sequeira, Tarini and Sarita Challa, Khodadad and Rivka Irani and many others who I equally love. I thank them from the bottom of my heart for their love and good wishes which have seen me through the years.

Contents

Summary

The circadian rhythmicity of critical skin cell functions related to homeostasis, regeneration and aging has recently been highlighted. In this study, we suggest an important link between cutaneous opioid receptor (OPr) activity and circadian rhythmicity. We find that activation of the Delta-opioid receptor (DOPr) by its endogenous agonist Met-enkephalin in N/TERT-1 keratinocytes results in a phase shift in the expression of the core clock gene *Per2* and in the nuclear localization of the DOPr - β-arrestin 1 complex. Furthermore, DOPr activation enhances and induces a phase shift in the rhythmic binding of β-arrestin1 to the *Per2* promoter. Coupled with this finding, β-arrestin1 was found to regulate the transcription of its target genes, including *Per2,* by facilitating histone 4 acetylation. Taken together, we propose that activation of DOPr leads to a phase shift in *Per2* expression via β-arrestin1 facilitated chromatin remodelling. We believe our results have potential implications in wound healing, DNA repair and chronopharmacology.

Introduction

Skin: Anatomy, homeostasis and Sensory properties

Anatomy: Epidermis, Dermis and Subcutaneous fat layer

Skin is the largest organ of the body and primarily provides protection. Broadly defined, skin consists of three layers, the outermost layer being the epidermis, the middle dermis and the innermost subcutaneous fat layer (Bolognia, Cooper, & Glusac, 2008; Fitzpatrick, 1993). The dermal layer of skin is vascularized by blood vessels and innervated by sensory fibres. These blood vessel and sensory fibres are associated with the various appendages of skin and influence the homeostatic and protective properties of skin (Figure 1).

Epidermis

The protective functions of skin against major environmental stresses such as water loss and microorganism infection rely in part on the epidermis (Blanpain C & Fuchs E 2009). Various appendages of the epidermis such as hair follicles, sweat glands, scales and feathers are generated by the epidermis (Sawyer, R. H., Rogers, L., Washington, L., Glenn, T. C. & Knapp, L. W. 2005). Thermal regulation, protection from environmental radiation, camouflage, reproductive behaviour and issuance of social status are some of the functions that are served by these appendages (Liu S, Zhang H & Duan E 2013). Tissue homeostasis has been defined as the physiological process that maintains a constant number of cells in renewing organs. Maintenance of tissue homeostasis and repair following injuries is facilitated by stem cells (SCs) that are located in SC niches within these organs that undergo constant renewal such as the skin (Blanpain C, Horsley V & Fuchs E 2007). Recent studies have paid much attention to the dynamics governing the SC cornucopias (Plikus MV et al., 2015).

The skin barrier functions to protect the animal from the time of its birth until its death and hence it must be established before the animal leaves the confines of the womb (Sotiropoulou PA & Blanpain C. 2012). In context to this it has been shown that the skin epidermis in mice arises from the embryonic ectoderm post gastrulation at about embryonic day 9.5. The subsequent stratification of the epidermis and the initiation of hair follicle (HF) morphogenesis is brought about by the population of this epidermal layer by mesenchymal cells (Hardy MH 1992; Botchkarev VA et al., 1999). Upon stratification, the innermost basal layer of the epidermis and the mesenchyme together generate the basement membrane which is primarily made up of matrix proteins and growth factors and also serves at the platform on which the mature epidermal layer will rest (Noramly S & Morgna BA 1998; Gat U 1998; Dasgupta R & Fuchs E 1999).

Stratification of the epidermis starts at about embryonic day 12.5 to embryonic day 15.5. During this process it has been observed that the cells occasionally divide suprabasally and subsequently undergo the differentiation program (Beck B & Blanpain C 2012). The differentiation program is a discrete set of transcriptional stages that leads the cells committed for differentiation from the basal layer of the epidermis to the skin surface via intermediate stages. Cells at different stages of differentiation form specific layers known as the spinous layers, granular layers and finally dead, flattened stratum corneum cells. As the cells differentiate the existing cells are pushed superficially away from the basal layer (Sotiropoulou PA & Blanpain C. 2012).

Upon maturation of the epidermis, homeostatic regulation governs the epidermis via the periodic execution of the differentiation program by the basal cells. This differentiation program involves the outward movement of the keratinocytes in a columnar fashion and the eventual terminal differentiation and sloughing off of the keratinocytes. The first step in the execution of this 2-3 week long differentiation programme is the switching off of the expression of genes encoding for Keratin 5 and Keratin 14 and the switching on of genes encoding for Keratin 1 and Keratin 10 to form an even more robust IF network that is interlinked with desmosomes (Fuchs E & Green H 1980). These

cytoskeleton changes strengthen cell-cell junctions and provide resistance against mechanical stress (Coloumbe PA & Wong P 2004; Kim S et al., 2006).

The cells of the spinous layer differentiate to form the granular layer. They produce electron-dense keratohyalin granules packed with the protein profilaggrin, which, when processed, bundles keratin intermediate filaments even more to generate large macrofibrillar cables. Furthermore cells of the granular layer express additional structural proteins known as cornified envelope proteins, which are rich in glutamine and lysine residues, and are synthesized and subsequently deposited beneath the plasma membrane of the granular cells. These proteins become enzymatically crosslinked, which results in an indestructible proteinaceous sac. Specifically, the cells become permeabilized to calcium, they activate transglutaminase, generating γ-glutamyl ε-lysine crosslinks to create an indestructible proteinaceous sac to hold the keratin macrofibrils (Coloumbe PA & Wong P 2004; Kim S et al., 2006). This sac serves as a scaffold for specialized lipid bilayers that are extruded from intracellular lamellar granules into the extra-cellular space between squames (dead flattened stratum corneum cells), thereby waterproofing the skin surface. The final steps of terminal differentiation involve the destruction of cellular organelles including the nucleus, and the extrusion of lipid bilayers, packaged in lamellar granules, onto the scaffold of the cornified envelope. When terminal differentiation is complete, the squames exist as dead cellular ghosts that are sandwiched by lipids on the outside and filled with an indestructible fibrous mass of keratins that is encased by the cornified envelope. Although squamous cells are eventually shed from the skin surface and are replaced by differentiating cells from below, they serve as the barrier that keeps harmful microbes out and essential body fluid in (Blanpain C & Fuchs E 2009).

The molecular mechanisms that orchestrate skin differentiation remain poorly understood. Studies based on mice have contributed significantly to identifying the pathways and transcriptional factors that are essential for proper epidermal stratification and acquisition of the skin barrier function.

Gene regulation can be controlled at multiple levels. These include regulation of mRNA synthesis (i.e. transcription), the stability of mRNA product, the rate at which mRNA is translated and the stability of the protein product. The most common mode of regulation is transcriptional regulation.

Every gene includes sequences designed to control the rate of transcription (i.e. the rate of RNA synthesis). These sequences can be divided into two classes, sequences that control the rate of basal transcription and sequences that modulate basal transcription and mediate the responses to stimuli.

The sequences that control basal transcription are common to most genes and include the TATA box sequence and the INR (initiator) sequence. These sequences are bound to by a set of ubiquitous proteins that are used by nearly all promoters to initiate and maintain RNA transcription. These proteins include the TATA binding protein, RNA polymerase II (Pol II) and other transcription factors including TFIIA, TFIIB, TFIIE, TFIIF, TFIIH and transcriptional activators. The function of this region of DNA is to ensure that RNA synthesis is initiated at the appropriate position along the DNA sequence. In the absence of other transcriptional regulators these proteins drive transcription at a low but detectable rate.

Regulated expression brings into play another set of DNA sequences. The process of activation and suppression of transcription is controlled by a diverse family of proteins known as transcription factors. Transcription factors mediate the final steps in the relay of information from the cell surface to the nucleus and the gene. In its simplest form this is accomplished by binding of the transcription factor to a DNA sequence (i.e. a cis-acting element, a silencer or enhancer) that is usually located upstream and adjacent to the sequence that encodes the gene. The elements are called cis-acting because they are linked, on the same DNA strand, to the gene that they regulate. A silencer is a DNA sequence that mediates a reduction in transcriptional rate, whereas an enhancer is a DNA sequence that mediates an increase in transcriptional rate. These sequences are most often located between 50 and 5000 bp upstream of the binding sites for the basal transcriptional proteins. However, they

sometimes are also located in the introns of the gene they control. Binding of the transcription factor to these sites facilitates gene activation or suppression.

Transcription factor proteins usually contain several functional domains. At a minimum, they contain a domain that binds to DNA and a domain that regulates transcription (Kadonega et al., 1988). They also contain a domain that regulates their activity (i.e. via phosphorylation sites or a ligand binding site) (Jackson et al., 1990). The DNA binding domain interacts with specific DNA sequences usually 8-20 bp in length, to associate the transcription factor with the target gene. Once the transcription factor associates, the transcriptional regulatory domain functions to activate (or suppress) transcription (Jackson et al., 1990). Generally these domains are functionally and physically separable.

Finally, members of different transcription factor families can bind to closely juxtaposed binding sites and participate as multimeric assemblies to regulate gene expression (Wu 1994). This "combinatorial" regulation differentially activates transcription based on the particular transcription factor binding sites present in the target gene, whether the transcription factor is in an active or inactive form and the abundance of each factor.

In addition to driving basal cell transcription, transcription factors also integrate signals that are relayed from the cell surface via signal transduction pathways. In keratinocytes these pathways regulate cell differentiation and proliferation (Rubin & Rice 1988; Rosental et al., 1991; Choo et al., 1993; Dlugoz and Yuspa 1994). Thus in many cases, the regulation of gene expression by differentiation regulating agents has been a focus of study (Brown et al., 1994; Lu et al., 1994; Welter et al 1994; Fischer et al., 1996).

microRNAs (miRNAs) provide an additional layer of complexity to the transcriptional regulatory switches by downregulating the expression of their target genes. miRNAs seem to function in the fine-tuning of the signalling transcription factor circuitry, which prompts a basal epidermal SC to terminally differentiate. miR-203 is an example of a miRNA that regulate keratinocyte

differentiation. Overexpression of this miRNA in the basal layer keratinocytes leads to their premature differentiation and a reduction in their proliferative potential. Knockdown of miR-203 leads to decreased cell proliferation.

Histone modifications have emerged as epigenetic regulators of epidermal differentiation. Histone methylation has been found to be crucial for epidermal differentiation and stratification (Leboeuf et al., 2010)

Notch signalling pathway	- The canonical Notch signalling pathway plays an important role in basal cell to spinous cell transition. - RBPJ is the DNA binding protein that forms a bipartite transcription factor with the Notch intracellular domain to relay active Notch signalling to the nucleus. The conditional knockdown of RBPJ effectively blocks the differentiation of basal layer keratinocytes into spinous layer cells. - Excessive Notch signalling converts basal cells into spinous cells.
Mitogen-Activated Protein Kinase (MAPK) pathway	- The p38 MAPK pathway, ERK1/2 MAPK pathway and JNK signalling pathway controlling diverse cellular behaviours, including cell proliferation, differentiation and apoptosis. - MAPK signalling pathways integrate and mediate various signals and play a major role in regulating keratinocyte differentiation and the function of skin barrier. - The ERK1/2 signalling pathway has been shown to control keratinocyte differentiation; low ERK1/2 activity could induce keratinocyte differentiation and apoptosis. - Epidermal differentiation would be enhanced when JNK is inhibited. - Inhibition of MAPK decreases the expression of filaggrin.
Nuclear Factor- κB (NF-κB) pathway	- NF-kappaB is constitutively expressed in a resting state in both human cultured keratinocytes and the epidermis. - The NF-kappaB subunits, p50, p65, RelB, and c-Rel (but not p52), were detected in keratinocytes and in normal epidermis at mRNA and protein levels. The four subunits were expressed in a cytoplasmic (rather than a nuclear) pattern in both basal and suprabasal keratinocytes.

	- NF-κB subunits have been shown to translocate from the cytoplasm to the nucleus in cells within the differentiating suprabasal layers of stratified epithelium. - NF-κB strongly inhibits epithelial cell death. - Premature apoptotic cell death occurs in the absence of normal NF-κB function in stratified epithelium (Wu et al., 1993).
p63 pathway	-The basal to spinous switch is one of the most important events in the keratinocyte differentiation programme. This event is regulated by p63. - Knockout studies have shown p63 to induce epidermal stratification and differentiation in mouse embryos. - Gain and loss of function studies have shown p63 to play an important role in maintenance of the renewal potential of different epithelial stem cells.
Activin protein 1 (AP1) family	- AP1 transcription factor is a target of the protein kinase C signal transduction pathway (Cano and Mahadevan, 1995). - Extracellular stimuli stimulate phospholipase C, which converts membrane localized phosphatidylinositol to inositol trisphosphate and 1,2 diacylglycerol. The 1,2 diacylglycerol, in turn, activates various protein kinase C isoforms (Nishizuka, 1992) and protein kinase activates signalling in the mitogen activated protein kinase cascade that ultimately results in the phosphorylation of AP1 transcription factors (Cano and Mahadevan, 1995). - Phorobol esters, such as 12-0-tetradecanoylphorbol-13-acetate (TPA0 are well known enhancers of keratinocyte differentiation and operate via activation of this pathway. - AP1 factors function as homo and heterodimers of jun (c-jun, junb, jun D) and foc (Fra-1, Fra-2, c-fos, fosB) family members (Cohen et al., 1989; Ransone and Verma 1990) that bind to DNA sequence 5'-GTGAGTCAG-3'.- - Involved in regulating cell proliferation, cell differentiation and cell death (BassetSeguin et al., 1990; Smeyen et al., 1993; Eckert et al., 1997) . - AP1 plays a role in gene regulation for the following reason: a. AP1 consensus DNA binding sites are present in many keratinocyte genes.

	b. AP1 binding sites have been shown to mediate calcium and phorbol ester dependent responses for several epidermal genes. c. AP1 factors are expressed in a differentiation dependent manner in cultured keratinocytes and in-vivo epidermis. UV light and tissue trauma regulate AP1 activity. d. Keratinocytes utilize signal transduction pathways that lead to activation of AP1 factors.
Activin protein 2 (AP2) family	- AP2 transcription factor includes a carboxy-terminal DNA binding domain and an amino terminal proline rich segment that is required for transcriptional activation (Williams and Tjian, 1991). - AP2 binds a homodimer of two individual AP2 proteins to a GC-rich consensus AP2 DNA binding site (5'-GN_4GGG-3') (Williams and Tjian, 1991). - AP2 is a transcriptional activator of human Involucrin gene expression (Kachinskas et al., 1994). - Mutation of AP2 binding site in the promoter of the Keratin14 gene results in a loss of transcriptional activity (Leask et al., 1991). - AP2 is an activator of gene transcription in the developing epidermis as it has been found to localize in epidermal cell lineages (Leask et al., 1991).
SP1	- Sp1 is a zinc finger-containing, sequence-specific DNA binding protein, originally isolated from HeLa cells (Kadonga et al., 1986). - Binds to GC box consensus sequence 5'-GGGCGG-3'. - Contains four domain A, B,C, and D. - Sp1 cooperates with ets factor binding site (EBS) to activate expression of the transglutaminase type 3 (TG3) promoter (Lee et al., 1996). - Similarly SP1 binds to the promoters of Involucrin, keratin3 and SPRR2A genes and induces their expression (Eckert RL et al.,1997).
Ets factor	- There are 20 different proteins that contain the "ets domain" and are constituents of the ets family of transcription factors. - The ets domain facilitates the binding of the ets transcription factors to DNA (Macleod et al., 1992). - The effects of ets factors on gene expression are variable, in some cases resulting in activation and in other contexts in suppression

	of gene expression. This appears to result from the finding that ets proteins interact with non-Sp1 transcription factors that bind other nearby cis-acting elements, and because the DNA sequence surrounding the ets consensus element influences the ets factor ability to bind (Maroulakou et al., 1994). - Ets binding sites have been shown to be functional regulators of the TG3 (Lee et al., 1996), Involucrin and SPRR2A genes (Fischer et al., 1996).
POU domain	- POU proteins bind as monomers and regulate gene transcription via the octamer binding motif 5'- ATGCAAAT-3'. - POU factors including Oct11 (Skn-1a/Epoc-1) and Oct 6, have been localized in the epidermis and some octamer binding proteins are preferentially expressed in specific epidermal layers (Agarwal and Sato 1991; Yukawa et al., 1993; Andersen et al., 1993; Faus et al., 1994). - Functional studies indicate that several POU domain proteins (Oct-1, Oct-2, Brn4, SCIP, Skn1a, and Skn1i) suppress expression of the Involucrin promoter in keratinocytes (Welter et al., 1996). -POU domain factors suppress the K5 promoter (Faus et al., 1995). - Skn-1a POU domain factor activates the Keratin 10 promoter (Andersen et al., 1993)
CCAAT/ enhancer-binding protein (C/EBP)	- These are leucine zipper domain proteins that form homo- and heterodimers (Mcknight, 1992). - C/EBP family members bind to the C/EBP response element in the proximal regulatory region of the involucrin promoter. - C/EBP β has been detected in the nuclei of cultured keratinocytes (Wang et al., 1996) and induces Involucrin expression.
Other transcription factors	- Vitamin D response elements have been identified in the 3' end of the Keratin 1 gene and these elements appear to modulate the calcium-dependent increase in Keratin 1 expression. Calcium-dependent increase in Keratin 1 expression is also mediated via an adjacent AP1 site (Huff et al., 1993; Lu et al., 1994). -VDR activation suppresses calcium induced differentiation (Bo L et al., 1993) - Binding sites for IFNϒ-activated STAT factor are present in the K17 gene and may mediate

	the increase in K17 expression that is observed during tissue inflammation (Jiang et al., 1994).
Interferon Regulatory factor (IRF6)	- Knockdown studies have shown IRF6 to regulate keratinocyte differentiation and proliferation (Ingraham RC et al., 2006).
Grainyhead-like 3 (GRHL3)	- Expressed in the differentiated suprabasal layers. - Regulates gene expression of structural components associated with the stratum corneum, extracellular lipid composition and cell adhesion in the granular layer. - Induces transglutaminase1 gene expression. Transglutaminase1 is important for cross-linking the structural components of the superficial epidermis.
Kruppel-like factor 4 (Klf4)	- Klf4 belongs is a zinc finger-type transcription factor. - Knockdown results in perinatal death as a result of dehydration. - Regulates skin barrier function and differentiation marker expression (Segre et al., 1999). - Ectopic expression of Klf4 in the basal layer leads to premature barrier formation, accelerated differentiation and reduced proliferation (Jaubert et al., 2003).

Table1: Signalling pathways in the epidermis that regulate epidermal homeostasis.

Dermis

The embryonic mesoderm derived dermal component of skin mainly contains extracellular matrix (ECM) composed of collagen, elastic fibres and glycosaminoglycans (Lopez-Ojeda W & Oakley AM 2018). Collagen strengthens skin by forming fibres with high tensile strength and stability via crosslinking and self-aggregation (Green EM et al., 2014). The extracellular matrix is secreted by fibroblasts which are the main cell type present in the dermis.

The dermis is highly vascularised by superficial and deep plexus, which are connected by straight collaterals. The superficial plexus sends thin blood vessels towards the outer layers thus nourishing the epidermis (A. T. Slominski et al., 2012)

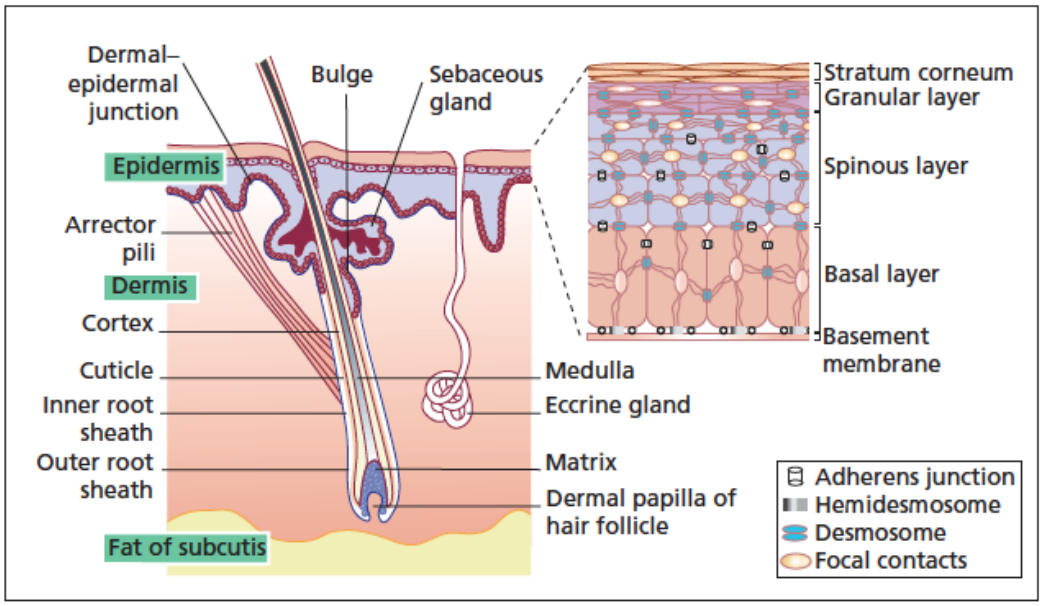


Figure 1- Anatomy of skin: Skin consists of 3 main layers, the outermost being the epidermis, the middle dermis and the innermost subcutaneous fat layer or hypodermis. The epidermis is stratified and consists of an outermost cornified layer followed by a granular layer, spinous layer, a stem cell rich basal layer and finally basement membrane. A major component of the dermal layer is collagen which is secreted by the fibroblasts. The dermal layer is highly vascularized and is innervated by nerve fibres. The hair follicles, sebaceous glands and sweat glands are important constituents of the dermal layer. The innermost subcutaneous fat layer is made up fat lobules and provides insulation. (McGrath et al., 2008)

This vasculature is usually found to be intertwined with the appendages of skin (Corder, Castro, Bruchas, & Scherrer, 2018). The appendages of skin that are of epidermal origin include hair follicles, sweat glands and sebaceous glands. On account of their proximity to hair follicles sebaceous glands lubricates and consequently protects hair by secreting a protective lipid substance. Sweat production is a common thermoregulatory mechanism and is generated by sweat glands. These glands are coiled eccrine glands located in the subcutis and traverse the dermis and pour their secretions over the epidermis (Sawyer, R. H., Rogers, L., Washington, L., Glenn, T. C. & Knapp, L. W. 2005).

Subcutaneous Fat layer

The highly vascularized subcutaneous fat tissue layer is made up of distinct fat-lobules. This distinction is a result of the separation of the fat lobules by fibrous septae. This fat layer serves to isolate, cushion and provide energy to skin (Slominski & Wortsman, 2000).

Skin innervation

Lastly, skin is innervated by sensory nerve fibres and also expresses a variety of sensory receptors. These include pain sensing nociceptors, itch sensing prurireceptors, temperature sensitive thermoreceptors; and touch sensitive mechanoreceptors (Zimmermann et al., 2014). Epidermis is innervated by unmyelinated C-fibers with still largely unknown function. In addition to the role on epidermal skin barrier and immune system, the keratinocytes have a sensory function and they express a variety of neuropeptides and neuro-receptors, such as the opioid receptors (Lumpkin & Caterina, 2007).

Epidermal Homeostasis: Stem cell populations, Regeneration and Ageing

The epidermis on account of being the most superficial layer of the skin, is constantly exposed to insults and shedding of the cornified layer, consequently it has a high turnover. The epidermis also gives rise to the previously described appendages of skin. Hence the maintenance of skin homeostasis and barrier functions takes the form of maintaining an equilibrium between cell-loss, cell division and repair (Solanas & Benitah, 2013).

The stem cell populations

Major contributors to the maintenance of epidermal barrier functions are the Epidermal Stem Cells (EpSCs). Murine based skin studies have identified three stem cell populations one of which is localized to the non-cycling portion of the hair follicle referred to as the bulge and contributes solely to hair follicle cycling (Fuchs, 2009; Jaks et al., 2008; R. J. Morris et al., 2004; Tumbar et al., 2004). Another population is localized at the place where the epidermis and the hair follicle meet. These stem cells contribute to maintaining epidermal integrity and regeneration of the sebaceous glands. Yet another population of basal interfollicular EpSCs, contribute to daily epidermal homeostasis (Clayton et al., 2007; Fuchs & Horsley, 2008; Janich et al., 2011). Disruption of skin homeostasis due to stressors such as wounding can elicit recruitment of all three stem cell populations to produce

the epidermis, sebaceous glands and hair follicles (Fuchs & Horsley, 2008; Levy, Lindon, Zheng, Harfe, & Morgan, 2007; Snippert & Clevers, 2011).

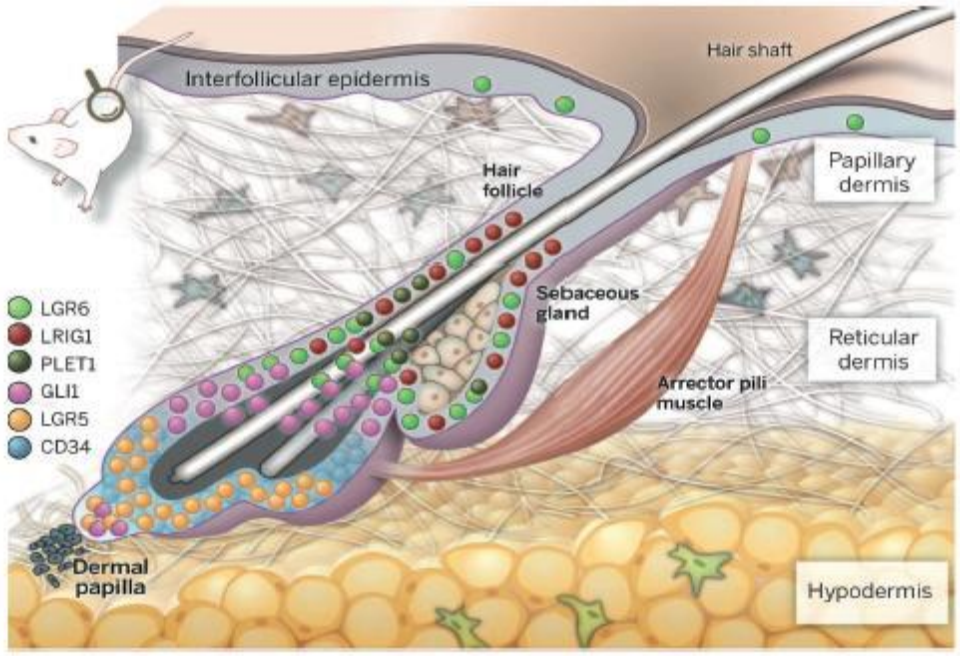


Figure 2- Stem cell niches and heterogeneity in stem cell markers: Cells expressing different stem cell markers are localized to different regions in skin. LGR6 is present in the basal layer of the epidermis. LGR6 and LRIG1 are localized to the hair follicle. PLET1, GLI1, LGR5 and CD34 are mainly found in the dermal papilla region of the hair follicle (Watt, 2014).

Resident stem cells in the basal layer of the epidermis adhere to the basement membrane which separates the epidermis from the dermis and consists of a laminin 5 rich ECM, by expressing α3β1 and α6β4 integrins. Proliferation of basal epidermal cells is ensured by dermal fibroblasts which secrete factors such as fibroblast growth factors (FGF)-7, FGF-10, insulin growth factor (IGF), epidermal growth factor (EGF) ligands and transforming growth (TGF)-β (Hsu, Li, & Fuchs, 2014a).Upon observing the trajectory followed by basal epidermal stem cells by label retaining studies it has become apparent that these cells give rise to transit amplifying cells, which are short-lived progenitors that undergo several rounds of division. Following which they migrate upwards and undergo a differentiation program and serve to amplify the keratinocyte population (Potten, Al-Barwari, Hume, & Searle, 1977; Potten & Loeffler, 1987). Additionally the basal stem cell population also consists of a reservoir of quiescent basal cells that don't behave as committed progenitors. This

quiescent basal cell population comprises of two sub-populations of cells in the IFE and hair follicles which play a role in regeneration. One sub-population consists of cells expressing enhanced levels of transcription factors such as NOTCH3, grainyhead-like-3 and some members of SPPR (sphingosine-1-phosphate receptor) family that regulate keratinocyte differentiation. These cells also express lower levels of stem cell markers such as α6 and β1 integrin and are referred to as committed progenitors. While the other population expresses higher levels of stem cell markers. Quiescent stem cell population exhibit lower rates of proliferation than the committed progenitors (Mascre et al., 2012).

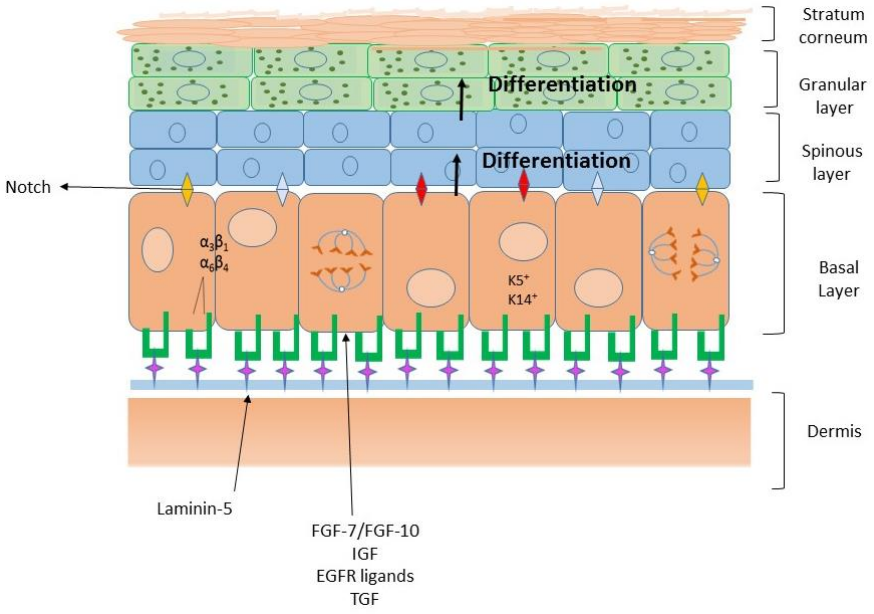


Figure 3 - The differentiation program: The epidermis maintains a single basal layer of proliferative cells that adhere to an underlying basement membrane that is rich in ECM and growth factors. Periodically, these basal cells extricate themselves from the cell cycle and undergo differentiation. The process of differentiation involves the transition of these cells from the spinous layer, to the granular layer and finally the stratum corneum. At each stage of differentiation the cells express specific keratins. Basal undifferentiated cells express keratin 5 and keratin 14 whereas spinous layer cells exhibit keratin 1 and keration 10 and finally granular layer cells exhibit keratin 1 and keratin 10. Upon terminal differentiation they move outwards and are eventually shed from the skin surface (Hsu et al., 2014a).

Cycles of growth (anagen), degeneration (catagen) and rest (telogen) are characteristic features of hair follicles (HFs). As mentioned previously, HFs consists of stem cell populations and murine studies suggest that there are two stem cell populations in HFs both of which can proliferation. One of these two populations have a molecular signatures that are similar to quiescent cells in the basal

layer of the epidermis and are localized to the bulge (Bu-SCs) and the other primed population is localized to the hair germ just below the bulge (Greco et al., 2009). These populations are responsible for initiating hair growth (Hsu, Pasolli, & Fuchs, 2011; R. J. Morris, 2004; R. J. Morris et al., 2004; Tumbar et al., 2004) and do not give rise to differentiated cells (Hsu, Li, & Fuchs, 2014b)

Regeneration

From transplantation and skin grafting studies it is now apparent that significant contributions to the regenerative and homeostatic properties of skin come from stem cells and their niches (Barrandon, 1998). Recent studies have shown tissue regeneration to be affected by signals that are released from heterologous niche cells and from the downstream progeny of stem cells by influencing the timing of stem cell activity (Potten, Saffhill, & Maibach, 1987). To explain for the loss of cells during normal cell turnover in skin different theories have been proposed. One of which is based on morphological and proliferation studies, according to this theory the skin IFE is subdivided into discrete “epidermal-proliferation units” (EPUs) which consists of slow cycling stem-cells together with around 10 transit amplifying cell progeny that undergo terminal differentiation after a definite number of cell divisions (Potten, 1981; Potten & Loeffler, 1987; Potten, Wichmann, Loeffler, Dobek, & Major, 1982).The concept of EPUs is reinforced by clonal marking of IFE cells using retroviruses (Ghazizadeh & Taichman, 2001, 2005; Kolodka, Garlick, & Taichman, 1998; Mackenzie, 1997) or mutagens (Ro & Rannala, 2004, 2005), which have shown distinct columns of IFE cells with relatively large life spans to cover the epidermis from the basal layer to the outermost cornified layer. Another theory endorses the idea that the skin is supported by a single equipotent, committed progenitor cell population in which random cell fate decisions bring about a balance between proliferation and differentiation (Clayton et al., 2007; Doupe, Klein, Simons, & Jones, 2010). It has been proven that stem cells contribute directly to skin regeneration and repair under conditions of wounding, whereas committed progenitor cells make a lesser contribution (Blanpain & Fuchs, 2009; Potten & Booth, 2002). Nonetheless, basal keratinocytes are also capable of regenerating the epidermis upon

transplantation, indicating the existence of plasticity between stem cells and their early progeny (A. Li, Pouliot, Redvers, & Kaur, 2004).

Ageing

Dermal and epidermal thinning, decrease in epidermal proliferative capacity, loss of dermal elasticity and consequently wrinkling, greying, thinning and loss of hair are observations made in context of ageing. Surprisingly, studies on ageing in mice have shown no change in stem cell numbers in hair follicles instead the telogen (rest) phase of the hair cycle becomes resistant to activation (Keyes et al., 2013), the aged epidermis maintains a constant number of bulge stem cells (Doles, Storer, Cozzuto, Roma, & Keyes, 2012; Giangreco, Qin, Pintar, & Watt, 2008; Stern & Bickenbach, 2007) but sebaceous glands and hair follicles undergo a significant change in morphology (Eaglstein, 2001). In humans a decrease in proliferation and colony forming capacity (Barrandon & Green, 1987)) and a reduction of stem cells and stem cell markers (Giangreco et al., 2008; Jones & Watt, 1993; Legg, Jensen, Broad, Leigh, & Watt, 2003; Stern & Bickenbach, 2007; Toyoshima et al., 2012) have been reported. This observation has been supported by the fact that when the Wnt signalling pathway which maintains skin homeostasis by inducing the activation of β-catenin and the mammalian target of rapamycin (mTOR) pathway becomes aberrant it initially stimulates growth of hair follicles, subsequently senescence sets in and finally the epidermal stem cell compartment and hair is lost. Ageing is also accompanied by an increased susceptibility to cancer. To this end it has been shown that β-catenin signalling contributes to tumour formation and the mTOR pathway suppresses tumour formation (Castilho, Squarize, Chodosh, Williams, & Gutkind, 2009).

Sensory properties of skin and its role in homeostasis

The common embryonic ectodermal origin of skin and the central nervous system (CNS) may serve to explain the observed similarities between the two (A. T. Slominski et al., 2012). The skin and the CNS share common mediators. Furthermore, the cutaneous opioidergic (Bigliardi-Qi et al., 2006; Bigliardi, Bigliardi-Qi, Buechner, & Rufli, 1998; Bigliardi, Tobin, Gaveriaux-Ruff, & Bigliardi-Qi, 2009)

and cannabinnoidergic (Biro, Toth, Hasko, Paus, & Pacher, 2009) systems have been shown to play a role in the maintenance of skin homeostasis.

Skin is extensively innervated by somatosensory and autonomic nerve fibres (Bigliardi-Qi, Lipp, Sumanovski, Buechner, & Bigliardi, 2005; Bigliardi et al., 2004; Bolognia et al., 2008; Fitzpatrick, 1993; Joachim et al., 2007; Siemionow, Gharb, & Rampazzo, 2011; A. Slominski & Wortsman, 2000; Weedon, Malo, Brooks, & Williamson, 2010; Yosipovitch et al., 2004). The cell bodies (perikarya) of the nerve fibres that innervate the skin in the region of the face and neck resides in the trigeminal ganglia and those that innervate the skin in other parts of the body resides in the dorsal root ganglia (DRG).

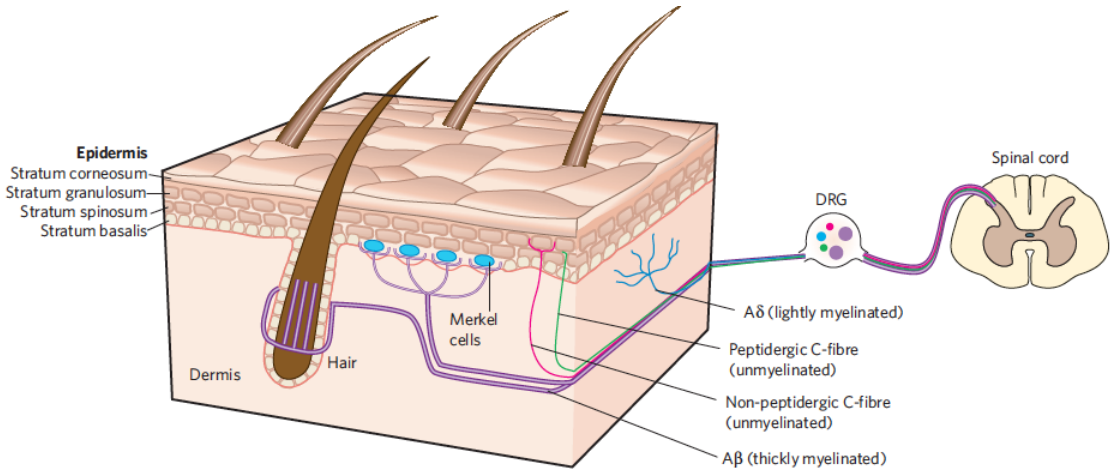


Figure 4 - Sensory Properties of Skin: Skin is innervated by somatosensory and autonomic nerve fibres. The cell bodies of these nerve fibres reside either in the trigeminal ganglia or the dorsal root ganglia. Various stimuli trigger the release of neuro transmitters which results in the conduction of impulses via nerve fibres to appropriate centres in the brain via the spinal cord. Appropriate responses are then transmitted via similar efferent pathways (Lumpkin & Caterina, 2007).

According to one theory, various kinds of stimuli activate their cognate receptors on peripheral nerve endings which results in the conduction of impulses via the afferent sensory fibres this results in the secretion of neurotransmitters such as substance P and CGRP at the site of stimulus. A newer theory postulates the existence of a keratinocyte-nerve unit and the keratinocytes are the sensory cell with many sensory receptors (opsin, olfactory, taste) and the opioid receptors in skin cells are

modulate and gate the signal that goes from the keratinocyte directly to the unmyelinated epidermal C-fiber.(Bigliardi et al., 2009; Krishnan-Kutty et al., 2017; P. P. Toh et al., 2016)

Upon transferring the stimuli to the perikarya, further conduction of the impulse to the dorsal spinal cord neurons via synapses is mediated through the sensory axons. The DCN (dorsal column nuclei) or LCN (lateral column nuclei) serve as ascending routes for sensory cutaneous inputs. These inputs are then transmitted to the thalamus. The thalamus is connected to the hypothalamic paraventricular nucleus (PVN). The significance of this connection lies in the fact that the PVN regulates the function of the pituitary gland, which influences the homeostasis of the organism via the endocrine system, including the HPA stress axis.

Cutaneous nerve innervation consists of a plexus of fibres in the reticular layer of the dermis, a superficial plexus in the papillary layer and intraepidermal nerve fibres (Bolognia et al., 2008; Legat & Wolf, 2009; McArthur, Stocks, Hauer, Cornblath, & Griffin, 1998; A. Slominski & Wortsman, 2000). Intraepidermal nerve terminals are found to be associated with Merkel cells, cold receptors and mechanoreceptors in the basal layer of the epidermis. The nerve fibres that traverse the dermis extend and terminate in the epidermis with or without branching in all the layers of the epidermis including stratum corneum (Waller et al., 2011). Autonomic nerves innervate arterioles, glomus bodies, hair erector muscles, and apocrine and eccrine glands in the skin.

Although cutaneous nerve innervation, more specifically the C-fibres that penetrate the epidermis, may seem as primary sensors for environmental factors such as temperature, humidity amongst other stresses, the sparseness of nerve fibres in the epidermis and the extreme sensitivity exhibited by skin contradicts this notion (L. Wang, Hilliges, Jernberg, Wiegleb-Edstrom, & Johansson, 1990). Skin can detect patterns on a much smaller scale than expected if nerve terminals were the only sensors (Loomis & Collins, 1978). Novel co-culture experiments from our lab have shown that peripheral nerve fibres directly connect to the keratinocytes and that information is conducted from the keratinocytes to the nerve fibres (Krishnan-Kutty et al., 2017). Therefore keratinocytes have

been suggested to be at the forefront of skin surface perception. Studies have shown keratinocytes to express receptors which are sensitive to and activated by temperature, mechanical stress, osmotic pressure and chemical stimuli. Additionally, a variety of endocrinological receptors and their ligands as well as other sensory elements of the CNS have been found to be localized in skin. They play a vital role in skin homeostasis (Denda, Ashida, Inoue, & Kumazawa, 2001; Denda, Fujiwara, & Hibino, 2006; Koizumi et al., 2004; A. Slominski, 2005; A. Slominski & Wortsman, 2000). It has thus been hypothesized that activation of the CNS elements in the epidermis results in transduction of impulses to the CNS via C-fibre terminals and these transduced signals could then influence the overall body homeostasis (Denda et al., 2006).

Introduction to opioids

Opioids have been used for palliative care since time immemorial. A Sumerian papyrus from *circa* 1500 BC prescribes a cure for children made of poppy plants, Homer describes the use of poppy extracts for treatment of wounded in the Trojan war (Pratt, 1994) and by the eighth century it was extensively used and abused in Asia and Europe (Watkins & Mayer, 1982). The role of endogenous and exogenous opioids and their cognate receptors in pain relief has been extensively studied and put to use (Y. Feng et al., 2012). Primary contributions to the field of opioids have come from studies in the CNS. Initially, the precise location of morphine-induced analgesia was identified in the primate brain (Pert & Yaksh, 1974, 1975). Thereafter it was found that stimulation of certain areas in the brain particularly the periaqueductal grey caused analgesia (Mayer & Liebeskind, 1974; Mayer, Wolfle, Akil, Carder, & Liebeskind, 1971), which could be blocked by treatment with opioid antagonist naloxone (Akil, Mayer, & Liebeskind, 1976).The DOPr was discovered through studies which aimed to compare the effects of morphine and the enkephalins on the electrically induced contractions in the guinea pig ileum and mouse vas deferens. The study showed morphine to have greater effects on the contractions of the ileum and the enkephalins had stronger effects on the contraction of the vas deferens hence suggesting the existence of two different types of receptors (Hughes, 1975; Hughes, Kosterlitz, & Leslie, 1975; Hughes, Smith, Morgan, & Fothergill, 1975). This also resulted in the naming of the predominant opioid receptors of the vas deferens as “delta opioid receptors” (Lord, Waterfield, Hughes, & Kosterlitz, 1977). The Mu and kappa opioid receptors were identified by their differential in vivo effects as result of treatments with morphine and ketocyclazocine respectively (Gilbert & Martin, 1976; Martin, Eades, Thompson, Huppler, & Gilbert, 1976). Similar studies led to the identification and isolation of endogenous opioids, beginning with Methionine-enkephalin (Metenk) and Leucine-enkephalin (Leuenk) (Hughes, Smith, Kosterlitz, et al., 1975) followed by β-endorphin (Belluzzi & Stein, 1977; C. H. Li & Chung, 1976)(and lastly dynorphin (Goldstein, Tachibana, Lowney, Hunkapiller, & Hood, 1979). Further studies discovered the precursors to these peptides. It was found that pro-opiomelanocortin (POMC) is the precursor

protein to adrenocorticotropic hormone (ACTH), melanocyte-stimulating hormone (MSH), lipotropin, corticotropin-like intermediate lobe peptide (CLIP) and β-endorphin (β-END) whereas pro-enkephalin (PENK) yields enkephalins (ENK), mainly Metenk and Leuenk and finally proteolytic cleavage of prodynorphin (PDYN) results in the formation of dynorphin A (DYNA) and B (DYNB) (Y. Feng et al., 2012; Hollt, 1992; A. Slominski, 2005).

In summary, the effects of the opioid compounds are mediated through activation of opioid receptors (OPr). These OPrs are part of the seven-transmembrane family of G protein-coupled receptors (GPCRs). Depending on the biological effects elicited by opioid analogues, three classes of the Gi/Go/Gq coupled opioid metabotropic receptor families have been identified: mu (μ for Morphine, MOPr), delta (δ, for vas *d*eferens, DOPr) and kappa (κ, for ketocyclazocine, KOPr) (Martin *et al.*, 1976; Lord *et al.*, 1977). In addition, an orphan opioid-like nociceptin receptor (NOPr) was also characterized (Borsodi & Toth, 1995; B. B. Feng, Wang, & Zhai, 2013; Ribeiro, Kennedy, Smith, Stohler, & Zubieta, 2005; A. Slominski et al., 2013; J. Traynor, 1989; J. R. Traynor & Elliott, 1993; Zaki et al., 1996).

It is noteworthy, that none of the endogenous opioid peptides is highly selective or specific to one particular type of OPr (Zadina, Hackler, Ge, & Kastin, 1997). This is due to three major factors:

1. The ligand-binding domain of opioid receptors interacts with a tyrosine residue at the N-terminal of peptide ligands, which is common to all the opioid peptides.

2. Several similarities in the primary structures as well as the signal transduction pathways of MOPr, DOPr and KOPr exist.

3. Opioid receptors and non-opioid receptors are able to form homomeric and heteromeric complexes and this ability may modulate the response of opioid receptors to binding of opioid ligands (Ananthan, 2006; Barry & Zuo, 2005; Y. M. Feng et al., 2013; Lamberts, Rosenthal, Jutkiewicz,

& Traynor, 2018; Law & Loh, 1999; D. A. Pasternak et al., 2004; G. W. Pasternak, 2004; Waldhoer, Bartlett, & Whistler, 2004)

Nonetheless, studies have shown that β-END binds to MOPr with highest affinity followed by DOPr and least for KOPr. Leuenk/Metenk exhibit highest affinity for DOPr and less affinity for MOPr. DYN has highest affinity for KOPr followed by MOPr (Jordan, Cvejic, & Devi, 2000; Tominaga, Ogawa, & Takamori, 2007).

Stress, HPA axis & endogenous opioids- Systemic and skin homeostasis

Role of the HPA axis in stress

Stress may be viewed as a two component entity comprising of a stressor and stress response. A stimulus, internal or external to an individual that assumes the form of a real or perceived threat to an individual's homeostasis is defined as a stressor. The subsequent interaction between the stress and the organism is referred to as a stress-response (Drolet et al., 2001). This typically involves the induction of the hypothalamic-pituitary-adrenal axis (HPA). Briefly, stress induces the production of Corticotropin Releasing Factor (CRF) in the paraventricular nucleus (PVN) (Chrousos, 1995; Chrousos & Gold 1992; Owens & Nemeroff, 1991). Thereafter, CRF binds to CRF1 type receptors in the pituitary gland (da Costa, Ma, Ingram, Lightman, & Aguilera, 2001; Hillhouse & Grammatopoulos, 2006; Perrin & Vale, 1999) increasing the production and secretion of POMC derived peptides, which are then released into systemic circulation (Hillhouse & Grammatopoulos, 2006; Pritchard & White, 2007; Smith & Funder, 1988). ACTH then goes on to interact with the melanocortin 2 receptor (MC2-R) on the adrenal cortex, stimulating the production and secretion of cortisol in humans or corticosterone in rodents. These corticosteroids mobilize energy reserves, buffer tissue damage and suppress the immune system, counteracting the effect of the stressors. Inhibition of CRF and POMC secretion and production is mediated through corticosteroids by well-studied feedback mechanisms. The various elements of the HPA axis are regulated by proteins that maybe produced in the brain

and peripheral tissues such as cells of the immune system (Besedovsky & Rey, 2007; Blalock & Smith, 2007; Chesnokova & Melmed, 2002).

Role of opioids in systemic homeostasis

As mentioned above, the perception of stress in the CNS induces the expression of POMC, which produces the stress hormone ACTH as well as the opioid peptide β-END (Guillemin et al., 1977). Opioid peptides are also known to be produced in the periphery by the DRG neurons, immune cells and keratinocytes which inhibits $Ca2^{+}$ and Na^{+} channels, hence preventing depolarisation under conditions of pain. This action of the opioids serves to block firing of neurons (C. Stein, 2018). Deep brain stimulation, pain and the MOPr agonist morphine share similar transduction pathways, in which signals emanating from the CNS descend through the dorsolateral funiculus and inhibit nociceptive neurons located in either the DRG or trigeminal nucleus caudalis (Watkins & Mayer, 1982). Opioids are believed to act on the afferent part of pain known as the "pain-matrix" since they dull the intensity and sharpness of pain. Pain relief or analgesia take place on several levels of the neuraxis including the central grey and midbrain structures of the rat brain(Mayer et al., 1971; Reynolds, 1969), the telencephalon such as the thalamus (Albe-Fessard, Berkley, Kruger, Ralston, & Willis, 1985) and central nucleus of the amydgala (Manning, 1998).

Endogenous opioids and their receptors are expressed throughout the central, peripheral and autonomic nervous systems as well as in various endocrine tissues and target organs (Drolet et al., 2001). In the brain, enkephalin-synthesizing neurons are widespread and complex and some are found to be associated with stress related regions of the CNS such as the PVN via ENK-ergic innervation or have ENK-perikarya, but some are also associated with autonomic regulation. It has been suggested that the ENK-ergic system may play a role in maintaining homeostasis by influencing the HPA axis and the autonomic nervous system (Howlett & Rees, 1986; Katoh, Nabeshima, & Kameyama, 1990; Katoh, Nabeshima, Ukai, & Kameyama, 1992; Przewlocki, Machelska, & Przewlocka, 1993; Szekely, 1990). Consistent with this idea, ENK and other opioids are capable of

modifying the synthesis and release of hypothalamic releasing agents such as CRF (Borsook & Hyman, 1995; Szekely, 1990). Induction of physical stress in the form of osmotic stress, intraperitoneal injections of hypertonic saline and psychogenic stress in the form of acute and chronic immobilization in Wistar rats were found to induce ENK expression (Drolet & Rivest, 2001).

Hence, the evident and apparent involvement of the ENK-ergic system suggests that opioids act by terminating stress responses (McCubbin, 1993). In concurrence, it was found that deletion of DOPr in mice resulted in higher anxiety levels (Filliol et al., 2000). Blocking of OPr activity by naloxone resulted in an increase in HPA responses in chronically stressed animals as compared to their controls (degli Uberti et al., 1995).

In conclusion, ENK in the central, peripheral and autonomic nervous system represents a modulatory mechanism by which an organism may adapt to stress by balancing the effect of stress-response with the harmful effects that accompany prolonged exposure to stress. Consistent with this notion was a study, which showed an injection of an enkephalin analogue in the intra central amydgala (intra-CeA), attenuates cold restraint induced gastric mucosal lesions in rats while intra CeA naloxone potentiated restraint induced gastric pathology (Ray & Henke, 1990; Ray & Ray, 1998).

Role of Opioids in skin homeostasis:

The systemic application of opioids in cases of acute pain, chronic itch and wounding requires balancing pain relief and the side effects of opioid administration such as nausea, vomiting, mental clouding, constipation and sedation, dose-dependent sleep/wake cycle disruption and alterations in hormone levels (Dimsdale, Norman, DeJardin, & Wallace, 2007; Shaw, Lavigne, Mayer, & Choiniere, 2005; C. Stein, 2003). Taking this point into consideration, it appears that local opioid application would help circumvent the adverse effects of systemic opioid application. A rational basis for this as a solution is the analgesic ability of local opioid application to induce opioid receptors outside the CNS on peripheral neurons, tissues and cells such as keratinocytes, fibroblasts and melanocytes (Bigliardi-Qi, Sumanovski, Buchner, Rufli, & Bigliardi, 2004; Bigliardi et al., 1998; Rachinger-Adam,

Conzen, & Azad, 2011; A. T. Slominski et al., 2013; C. Stein, 1993, 2003; C. Stein, Hassan, Lehrberger, Giefing, & Yassouridis, 1993). Further supporting this idea are studies showing that local opioid application does indeed reduce the adverse effects of systemic opioid application (Cerchietti et al., 2002; Flock, 2003; LeBon, Zeppetella, & Higginson, 2009; Platzer, Likar, Stein, Beubler, & Sittl, 2005).

Role of endogenous opioid ligands and receptors in skin homeostasis.

As mentioned before, POMC is the precursor for β-END. The presence of POMC in skin was first detected by a study in mice and hamster melanomas (A. Slominski, 1991). Further studies showed that mouse skin (A. Slominski, Paus, & Mazurkiewicz, 1992) and human skin (A. Slominski, Wortsman, et al., 1993) are positive for the β-END peptide.

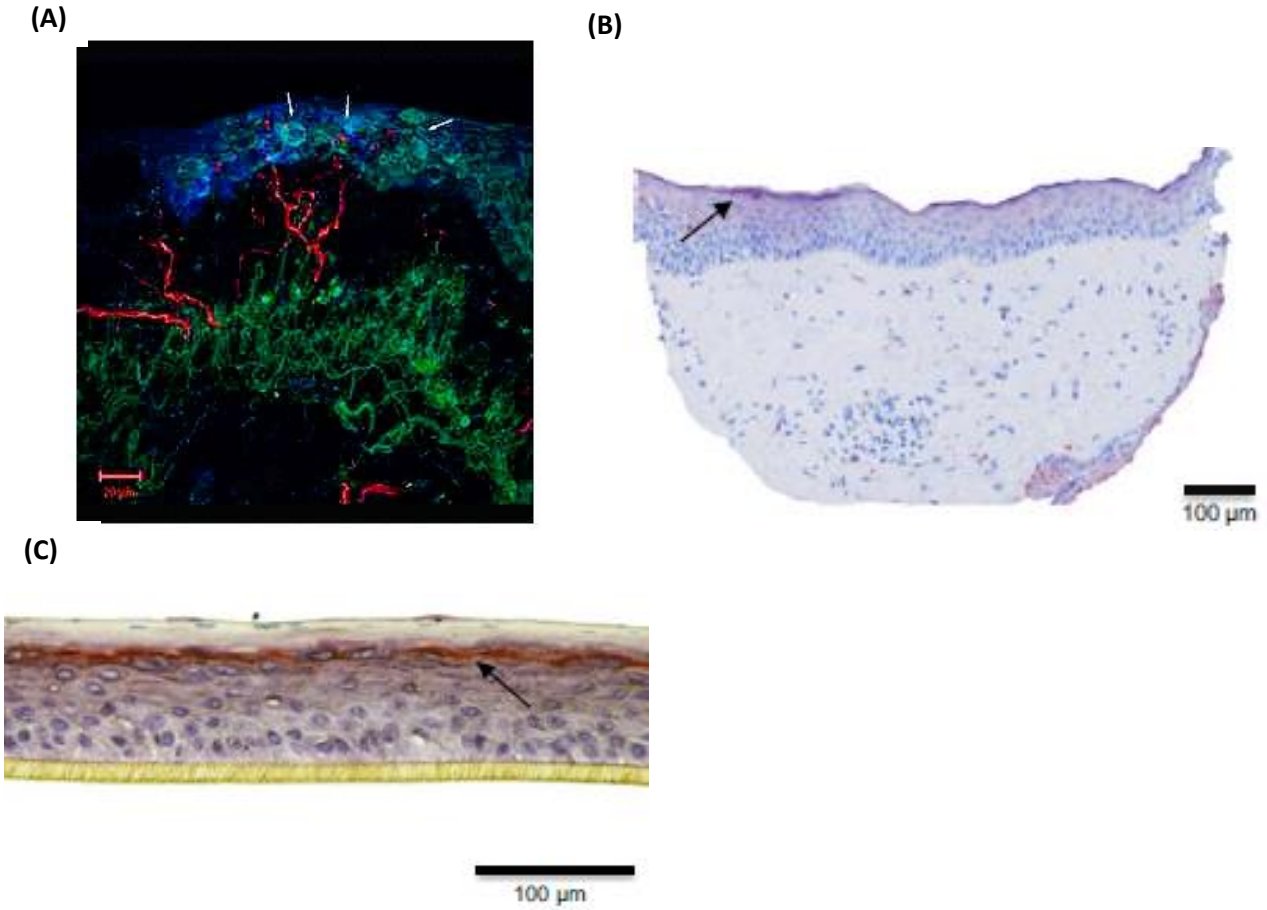


Figure 5 - Localisation of opioid receptors and their ligands in skin. (A) Immunostaining of the of µ-opiate receptor (green), β-endorphin (blue) and PGP 9.5 (red) in 50 µm cryostat sections of normal human skin (breast) showed higher expression of Beta endorphin was expressed in keratinocytes that clustered around nerve endings . (B) The arrows point to keratinocytes expressing Beta endorphin. Likewise the black arrow shows DOPr to be constitutively expressed in the granular layer

cell membrane in normal human skin explants and (C) in normal reconstructed human epidermis (RHE) (Bigliardi-Qi et al., 2004; Chajra et al., 2015).

Subsequently it was found that skin cells, both in normal and pathological conditions, possess the ability to transcribe and translate the POMC gene (Nissen & Kragballe, 1997; A. Slominski, 2005; A. Slominski, Malarkey, Wortsman, Asa, & Carlson, 2000; A. Slominski et al., 1992; A. Slominski, Wortsman, et al., 1993; Zagon et al., 1996). Furthermore, the epidermis dermis adnexa exhibits the presence of POMC derived peptides. POMC is also released by cutaneous nerve endings (A. Slominski, Paus, & Wortsman, 1993; Tobin & Kauser, 2005a, 2005b; Wintzen & Gilchrest, 1996). UV radiation induces the expression of β-END in skin. This observation has been used to explain the addictive behaviour observed in beach goers (Fallahzadeh & Namazi, 2009; Fell, Robinson, Mao, Woolf, & Fisher, 2014).

β-END is capable of stimulating keratinocyte migration in vitro (Bigliardi-Qi et al., 2004; Bigliardi et al., 1998; Tominaga et al., 2007), epidermal and follicular melanogenesis (Kauser, Thody, Schallreuter, Gummer, & Tobin, 2004) and also has a role in hair growth, wound healing and cellular differentiation (Bigliardi et al., 2009; Schmelz & Paus, 2007). Modulation of the number of dendritic processes of hair follicle melanocytes was also found to be regulated by - β-END (Kauser et al., 2004; Kauser, Thody, Schallreuter, Gummer, & Tobin, 2005)

In situ hybridization and immunostaining based studies showed spatial and temporal pattern in expression of PENK in normal human skin. PENK expression is localized to the epidermis, specifically to the stratum spinosum and stratum granulosum, and outer root sheath keratinocytes of hair follicles, myoepithelial cells of eccrine glands and some fibroblasts of the follicular dermal sheath and follicular papilla of skin. In the case of skin pathologies, the specific spatial and temporal expression of PENK shown in normal condition is lost (A. T. Slominski et al., 2011; Zagon et al., 1996). The same study also found that UVB and LPS, which are common environmental stressors, induce PENK expression in keratinocytes in a dose dependent manner (A. T. Slominski et al., 2011).

Likewise, dynorphins are expressed in human skin cells and cutaneous nerve fibres (Grando et al., 1995; Hassan, Pzewlocki, Herz, & Stein, 1992; Salemi et al., 2005; Taneda et al., 2011; Tominaga et al., 2007). Expression of dynorphins is upregulated in psoriasis and it regulates pilomotor activity and nociception (Gibbins, 1992; Tominaga et al., 2007).

Skin not only expresses opioid peptides but also their receptors (Bigliardi et al., 1998; Salemi et al., 2005; Tachibana & Nawa, 2005). Furthermore, cells of ectodermal origin such as keratinocytes and melanocytes express OPrs, however at a much lower level than neurons (Bigliardi et al., 1998). In skin OPrs modulate skin homeostasis, angiogenesis and wound healing (Bigliardi PL et al., 2015; Bigliardi PL et al., 2003).Finally, the contribution of OPr targeted therapies could potentially be 2-fold since evidence suggests that OPr agonists influence the inflammatory and proliferation phase of wound healing and also ameliorate pain as has been described in the previous section (Wang Y et al., 2017).

DOPr is present in the granular, suprabasal and to a lesser extent in the basal layer keratinocytes of the epidermis. Melanocytes, dermal fibroblasts, immune cells (dendritic cells), epidermal and dermal nerve endings were also found to express DOPr (Bigliardi, Dancik, Neumann, & Bigliardi-Qi, 2016). Recent studies have confirmed the involvement of the DOPr in skin homeostasis (Bigliardi-Qi et al., 2006; Neumann, Bigliardi-Qi, Widmann, & Bigliardi, 2015). DOPr activation plays a role in maintaining skin barrier functions and epidermal integrity by modulating cell-cell adhesion during wound healing (Bigliardi-Qi et al., 2006; Neumann et al., 2015). It influences expression of desmogleins, which are proteins found in desmosomes. Desmosomes are cell-cell junctional complexes, which connect cells via intermediate filaments (IF) (Green & Simpson, 2007). Overexpression of DOPr reduced the expression of desmoglein 1/4, whereas in a knockout mouse model of DOPr increased expression of desmoglein 1/4 was observed. Activation of DOPr also results in a rearrangement of the desmogliens at cell-cell junctions. Typically desmogleins appear as long linear arrays, emanating from the cell borders, whereas activation of DOPr resulted punctate

expression (Neumann et al., 2015). DOPr mediated ERK1/2 signalling delays Keratin 10 expression in an in vitro model, furthermore it also dysregulates expression of differentiation markers such as involucrin, loricrin and filaggrin. These proteins are involved in the generation of the cornified envelope, which is crucial in maintaining skin barrier function. The regulation of POU2F3 expression by DOPr activation might link keratin 10, involucrin and filaggrin expression changes, as these genes are targets of this transcription factor. DOPr knockout mice have a thinner epidermis, further emphasising the role of DOPr and hence the opioid system in skin homeostasis (Bigliardi et al., 2016; Neumann et al., 2015). Further evidence for the role of the DOPr in epidermal homeostasis has come from a study involving Rubixyl which is a DOPr peptide that is derived from the large subunit of spinach d-ribulose 1,5 bisphosphate carboxylase/oxygenase. Chajra et al., 2015 have shown this peptide to repair damaged skin by decreasing TEWL, increasing hydration and decreasing wrinkle depth at the periocular and perilabial area. Through their studies they have also shown DOPr expression to be downregulated under inflammatory condition which could then be rescued by the Rubixyl peptide. Furthermore they have shown altered expressions of genes and proteins which are markers of epidermal integrity and barrier function properties to be restored by Rubixyl treatment (Chajra et al., 2015).

MOPr is expressed by keratinocytes of the stratum basale and the upper more differentiated layers of the epidermis, melanocytes, dermal fibroblasts, nerve endings in the dermal and epidermal layers, hair bulbs, follicular dermal papilla and the outer root sheath of hair and the sebaceous glands and the immune cells (dendritic cells) of skin and is regulated in inflammatory diseases, such as chronic atopic eczema, psoriasis, skin cancers and in chronic non-healing wounds in humans. Topically applied Naltrexone increases the MOPr expression in epidermal keratinocytes in patients with chronic atopic dermatitis and reduces significantly the chronic pruritus (Bigliardi-Qi, Bigliardi, Buchner, & Rufli, 1999; Bigliardi-Qi, Bigliardi, Eberle, Buchner, & Rufli, 2000; Bigliardi-Qi et al., 2005; Bigliardi-Qi et al., 2004; Bigliardi et al., 1998; Bigliardi, Buchner, Rufli, & Bigliardi-Qi, 2002; Bigliardi et al., 2016; Bigliardi, Sumanovski, Buchner, Rufli, & Bigliardi-Qi, 2003). The expression of MOPr was

stronger than DOPr in keratinocytes but the reverse was true in case of mesenchyme-derived fibroblasts (Bigliardi et al., 2009). The latter observation was in keeping with a previous study, which showed that PENK has a stronger expression in fibroblasts as compared to the other compartments in skin (A. T. Slominski et al., 2011).

A recent study sought to explain the role of MOPr in wound healing. This study showed MOPr expression to be enhanced in wound margins. More specifically, MOPr expression co-localizes with vasculature in wounds and wound margins. In this study wounding enhanced setting β-END secretion and this was then shown to induce cell proliferation. Consistent with this finding, activation of the MOPr by morphine led to faster wound healing and downregulation of secretion of pain related neuropeptide substance P in control mice when compared with MOPr knockout mice. The role of MOPr in wound healing was further validated using MOPr selective and non-selective antagonist both of which inhibited cell proliferation induced by wounded keratinocytes conditioned medium. Finally, the study conclusively showed MOPr to regulate wound healing during the proliferative phase of wound healing (Y. Wang et al., 2017).

KOPr is expressed in keratinocytes, melanocytes, dermal fibroblasts, epidermal and dermal nerve endings and the resident immune cells in skin (Bigliardi et al., 2016; Bigliardi et al., 2009; Salemi et al., 2005) and it is involved in chronic pruritus which is associated with skin conditions like atopic eczema.

In summary, one could conclude that OPr-induced signalling can modulate various aspects of skin homeostasis by regulating the expression of various proteins associated with the epidermal processes of cell differentiation, migration, cytokine expression, wound healing and scar formation (Bigliardi et al., 2009; A. Slominski et al., 2003).

Mechanism of action of the opioid system

Several studies exemplify the idea that antinocicepive activities of the OPrs can be realized by the activation of OPrs outside the CNS (Salemi et al., 2005). OPrs are members of the GPCR family and

their inhibitory activities are mediated by the activation of Gi (inhibitory), G0 and Gq proteins, which downregulates adenylyl cyclase activity leading to inhibition of protein phosphorylation (Chizhmakov et al., 2005). OPr activation has also been shown to modify ion-channel activity by inhibiting Ca2+ channels and simultaneously activating K^+ inward rectifying channels (Jordan et al., 2000) which eventually results in the cessation of the sensation of pain by blocking communication between two sensory cells.

Just as is the case with most GPCRs, once activated the OPrs are phosphorylated by GRKs which results in the recruitment of Beta arrestins (βarr) which in turn recruits the machinery necessary for internalization of the receptor hence desensitizing the cell to further ligand based signals. Apart from playing a vital role in desensitizing the cell to exogenous signals, the βarr have now come to be recognized as signalling molecules which recruit important signalling molecules like the MAPKs the βarr-GPCR complexes. The MAPKs modulate several activities such as cell proliferation, differentiation, apoptosis, transcription factor regulation, ion channel regulation, neurotransporter regulation, and protein scaffolding (Raman, Chen, & Cobb, 2007). MAPKs can regulate these effects over either short or long temporal domains to affect intra- and extracellular functions. All the opioid receptor subtypes stimulate phosphorylation of ERK 1/2, as well as JNK and p38 (Al-Hasani & Bruchas, 2011; Bruchas, Macey, Lowe, & Chavkin, 2006; Eisinger & Ammer, 2008; Macey, Lowe, & Chavkin, 2006; Neumann et al., 2015).

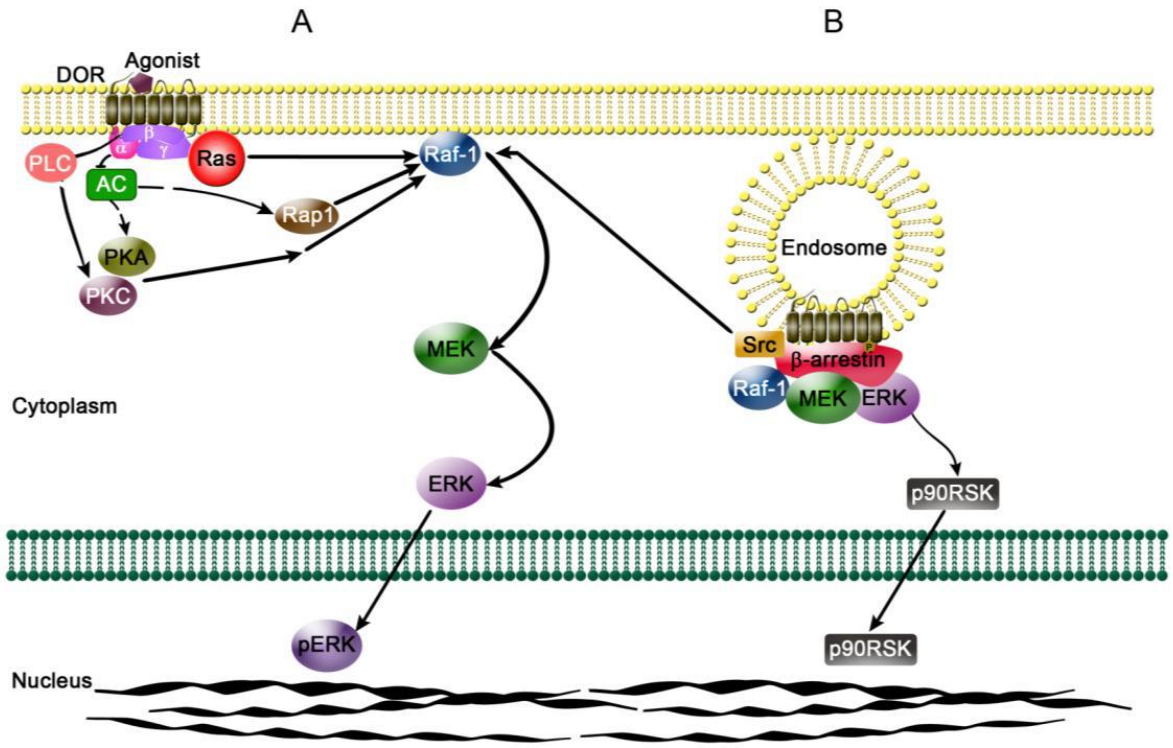


Figure 6 – The activated DOPr pathway: (A) Binding of the agonist to the DOPr modifies the helical arrangement of the receptor by rearranging the positions of the transmembrane domains 3, 6 and 7 thus resulting a change from inactive to active state (Decaillot et al., 2003). This rearrangement leads to the exchange of GDP for GTP on the Gα subunit and the subsequent hydrolysis of the Gαβ/γ into Gα and Gβ/γ subunits. Through unclear mechanisms $G\alpha_i$ mediated inhibition of adenylyl cyclase's activity to convert AMP into cAMP results in lower levels of cAMP. This reduction enhances PKA activity which in-turn facilitates the interaction between Rap1 and B-Raf eventually leading to the activation of ERK (Dugan et al., 1999). The Gβ/γ subunit mediates the release of intracellular calcium stores and thus the stimulation of Ca2+/calmodulin-dependent kinase by initiating phosphoinositide hydrolysis and IP3 receptor activation. This also results in the activation of Rap1 (Belcheva & Coscia, 2002; Belcheva, Haas, Tan, Heaton, & Coscia, 2002; Kinashi, 2005; Sahyoun, McDonald, Farrell, & Lapetina, 1991) in a PKC dependent manner which also results in the phosphorylation of ERK. Thereafter ERK phosphorylates its substrates such as transcription factors in the nucleus and influences gene expression and thus regulates the physiology of the cell.

(B) Termination of G-protein signalling: Another consequence of DOPr activation is the GPCR kinases (GRKs) mediated phosphorylation at the Serine 363, Threonine 361 and Threonine 358 residues of the agonist bound receptor (Claing, Laporte, Caron, & Lefkowitz, 2002; Guo et al., 2000). This phosphorylation facilitates the binding of βarr 1 and 2 to the C-terminal tail and third intracellular loop of the receptor and thus commences the processes of desensitization (Cen, Xiong, Ma, & Pei, 2001; Cen, Yu, et al., 2001). Subsequently the β- arrestins recruit clathrin and form a clathrin adaptor complex AP2 which drives receptor internalization and formation of endosomes which directs the receptor back to the cell membrane via a process of recycling or for degradation via the lysososmal pathway (Claing et al., 2002). The β- arrestins also leads to the initiation of arrestin-mediated signalling pathways. To this end, it has been shown that βarr2 acts as a scaffold that binds Raf, MEK and ERK which eventually leads to the activation of ERK 1 and ERK 2 (Lefkowitz & Shenoy, 2005). This activated ERK1/2 then phosphorylates p90RSK and triggers another signalling cascade.

However it isn't clear how the endogenous ligands may mediate their analgesic and anti-inflammatory effects in the periphery, but a widely accepted idea is that opioids produced by skin act in an autocrine and/or paracrine manner on their cognate receptors. Through canonical signalling pathways, cellular function and gene expression are influenced. Opioid production in skin may also exert its physiological effects by entry into systemic circulation via dermal vasculature or interaction with cutaneous nerve fibres (Bigliardi et al., 2009; Borzsei et al., 2008; A. Slominski & Wortsman, 2000; A. Slominski, Wortsman, Luger, Paus, & Solomon, 2000). This interaction results in nervous signals being transduced to the dorsal root ganglion (DRG), which might then synaptically inhibit the actions of pro-pain/inflammatory neuropeptides (Borzsei et al., 2008; A. Slominski & Wortsman, 2000; Tobin, 2006). Furthermore, these signals could be transferred to the thalamus, hypothalamus, paraventricular and arcuate nuclei and amydgala via the nucleus of the solitary tract (NTS) (Armario, 2010; Przewlocki, 2004; Przewlocki & Przewlocka, 2005; A. Slominski, 2005; A. Slominski & Wortsman, 2000). This mechanism of signal transduction may also serve to explain how skin produced opioids enhance the reward system and thus addiction.

Finally, evidence for the role of OPrs in maintaining homeostasis also comes from studies which show that OPrs exhibit constitutive activity even in the presence of stress by triggering signalling cascades in an agonist-independent manner (Corder, Castro, Bruchas, & Scherrer, 2018; Corder et al., 2013; Polter et al., 2017; Yao et al., 2016).

Regulation of DOPr expression and signalling mechanisms

From studies mostly based on the central nervous system it is now apparent that DOPr mediated effects are dependent on various factors ranging from genetic variability to activation induced intracellular mediators which includes transcriptional regulation and post-translational events . Inter-individual differences in response to DOPr targeted drugs has been linked to variations in sequences within the *Oprd1* gene (Lotsch & Geisslinger, 2011). Differences in terms of DOPr

mediated analgesia (Kim et al., 2006), DOPr maturation in the Golgi apparatus and activation induced internalization have been reported as a result of variations the DOPr gene sequence (Leskela, Markkanen, Alahuhta, Tuusa, & Petaja-Repo, 2009).

At the transcriptional level DOPr mRNA and protein expression was found to be enhanced in the skin of patients afflicted with fibromyalgia (Salemi et al., 2005; Salemi et al., 2007) and in patients with hypertrophic scars (Cheng, Liu, Fu, Sheng, & Li, 2008). Likewise, conditions of pain and inflammation also have an effect on DOPr mRNA expression (Narita, Kaneko, et al., 2006; Narita, Kuzumaki, Miyatake, et al., 2006; Narita, Kuzumaki, Narita, Kaneko, Hareyama, et al., 2006; Narita, Kuzumaki, Narita, Kaneko, Tamai, et al., 2006; Narita, Miyoshi, Narita, & Suzuki, 2007). DOPr expression has also been found to be modulated epigenetically by DNA methylation and a set of transcription factors such as STAT6, Ikaroa, Sp1/Sp3, E-twenty six 1, upstream stimulatory factor (USF) and AP2 (L. N. Wei & Loh, 2011).

Post translational regulation that relates to the genesis of the DOPr protein and its maturation, exerts its effects firstly in the endoplasmic reticulum and the sarcoendoplasmic reticulum in a calcium and ATP dependent manner (Tuusa, Leskela, & Petaja-Repo, 2010) and secondly in the Golgi apparatus by promoting the formation of DOPr-MOPr heterodimers through the involvement of the transport protein 4 RTP4 as opposed to the generation of DOPr monomers (Decaillot, Rozenfeld, Gupta, & Devi, 2008).

The fourth level of regulation has been observed at the cell surface where the mature receptor is expressed. There are have several reports of various GPCRs forming heterodimers or large heteromers (Massotte, 2010) and likewise similar observations have been made in context to DOPrs. DOPr has been shown to interact with MOPr and KOPr (Gupta et al., 2010; Kabli et al., 2010; van Rijn, Whistler, & Waldhoer, 2010), CB1 cannabinoid receptors (Bushlin, Rozenfeld, & Devi, 2010) and

alpha2-adrenergic receptors (van Rijn et al., 2010), chemokine receptors (Parenty, Appelbe, & Milligan, 2008; Pello et al., 2008).

Upon activation of the DOPr intracellular effectors regulate the receptor density and activity by both signalling and trafficking processes. As mentioned previously activation of DOPr results in the stimulation of the inhibitory heterotrimeric Gi/o proteins, which regulates ion channels (Williams et al., 2001). Activation of the DOPr also results in the phosphorylation of the receptor by GRK which results in the internalization of the receptor via the beta arrestin pathway. This internalization results in the redistribution of the receptor (Cahill, Holdridge, & Morinville, 2007; Ritter & Hall, 2009) and also elicits the epigenetic regulation of its target genes (Kang et al., 2005).

As for the redistribution of the DOPr it has been reported that upon activation the receptor is targeted to the lysosome for degradation (Pradhan et al., 2009). This phenomena has an impact on the receptor expression at cell surface.

Epigenetic modifications mediated by the activated DOPr are based on the βarr1 mediated internalization of the SNC-80 activated receptor. It has been reported that the activation of the receptor leads to the internalization of the receptor. Subsequently βarr1 undergoes translocation to the nucleus. Thereafter it binds to CREB and acts as a scaffold for histone acetyltransferase p300 which consequently acetylating histone 4 (H4) in the promoter of two genes that regulate cell proliferation *c-fos* and *p27/kip1* thus increasing their expression. The authors of this paper also show that this increase leads to reduced proliferation of cancerous glial cells (Kang et al., 2005).

The circadian rhythm

Diurnal differences and seasonal variations in the environment are a natural outcome of the Earth's rotation about its axis and its revolution around the sun. As a consequence, all organisms, from cyanobacteria to humans, show periodic differences in behaviour and physiology. These differences facilitate survival in terms of escaping the threat of predators or to simply meet the metabolic demands of the body. Over decades, the rhythmic regulation of organismal physiology has been studied extensively. Cycles with a period of approximately 24 hours are considered to be circadian. The term "circadian" was coined by Franz Halberg and has been derived from the Latin words circa meaning "about" and dies "a day" (Halberg F et al., 2003). Furthermore, Franz Halberg had chanced upon the role of circadian rhythms in systemic physiology while developing a bioassay for cortisone. The readouts of this assay were measured in terms of eosinophil counts. He found a variability in eosinophil numbers in C3H mice with a low or high incidence of breast cancer. He then went on to make 3 conclusions which effectively laid the foundation of chronobiological studies and his future work. The first being that eosinophil counts vary as a function of the day time, the second that the timing of high and low eosinophil counts depended on the timing of light exposure and feeding cycle and, lastly, that mice that were born blind exhibited rhythmicity but with a period that significantly differs from 24 hours (Cornelissen G 2008). Further scientific pursuits based on the mouse liver made it apparent that phospholipid, RNA, DNA activity exhibited rhythmicity along a 24 hour cycle. These observations made it apparent that this circadian cycle would eventually finds its application in the treatment of cancer. To this end Franz Halberg successfully cured L1210 leukemia by timing its treatment with ara-C. Likewise Halberg F and Gupta BD were able to double the two-year disease-free survival rate of patients with peri-oral tumors by regulating the timing of radiation therapy (Cornelissen G 2008). Finally, Franz Halberg is regarded as the father of modern chronobiology and its undisputed leader (Pauly JE & Scheving LE 1987).

Jürgen Walther Ludwig Aschoff, Erwin Bünningand and Colin Pittendrigh are also considered to be the founders of the field of Chronobiology. In a landmark study by Aschoff et al. (1960), the sleep-wake cycle of a German volunteer was observed under controlled conditions which subsequently lead them to conclude that the circadian nature of biological processes was a result of an endogenous time keeping system and not a mere consequence of the diurnal variation in light exposure. Their studies also proved light to be a potent Zeitgeber. The term "Zeitgeber" (from German, meaning "time-giver") was coined by Aschoff in 1960, which referred to external time cues that synchronized endogenous oscillators to the environmental cycle (Aschoff, 1960).

In 1960, Ronald J. Konopka and Seymour Benzer undertook a genetic screen in Drosophila melanogaster and isolated mutants that had altered circadian rhythms. He isolated three mutants, one that abolished, another that shortened and yet another that lengthened the circadian rhythms. These mutations were mapped to the same gene, which was named the Period gene (Per) in 1971 (Konopka & Benzer, 1971).

Subsequently, in 1985 Martin Ralph chanced upon a mutant Syrian hamster (Mesocricetus auratus) that exhibited differences in its locomotor activity as compared to its littermates. In that this mutant exhibited an endogenous free-running period of 22 hours compared to 23.5 hours, at that point in time this was the shortest reported circadian period for the said species. From genetic experiments it was concluded that this aberrant phenotype was heritable (Ralph & Menaker, 1988). This mutation was designated as tau (after the circadian symbol for period length) and the mutant hamsters were referred to as tau mutants. Further studies on the tau mutant provided four of the primary seminal contributions to chronobiology. First, the pivotal pacemaker for the circadian clock resides in the suprachiasmatic nucleus (SCN) of the hypothalamus (Ralph & Block, 1990; Ralph, Foster, Davis, & Menaker, 1990). Second, the circadian period of the entire animal/tissue was an average of periods of individual SCN clock cells (C. Liu, Weaver, Jin, et al., 1997; C. Liu, Weaver, Strogatz, & Reppert, 1997). Third, signals in various forms emanating from the SCN drive circadian

rhythms (Silver et al., 1996) and fourth, oscillators reside in the mammalian retina, retinal ganglion cells, Müller cells and retinal pigmental epithelium (RPE) cells and these oscillators are independent of the central pacemaker (Tosini & Menaker, 1996, 1998a, 1998b).

Using a comparative genomics approach called positional syntenic cloning, the Takahashi laboratory later demonstrated that the tau mutation manifests itself in a single nucleotide change in the gene encoding casein kinase I epsilon (CK1ε) (Lowrey et al., 2000). CKIε is a serine/threonine protein kinase and has been shown to phosphorylate Period.

Thereafter, the search for circadian rhythm mutants was extended to mice. Genetic screens similar to those in drosophila found some mice with a period lengthening phenotype in that they showed wheel running activity four hours later than their siblings. These mutants were called Clock mutants. Genetic mapping experiments coupled with phenotype rescue experiments, in which fragments of chromosome 5 DNA were injected into the mice embryos (Antoch et al., 1997), found that the Clock gene is localized to chromosome 5 in mice. The gene is 1000 base pairs in size and consists of 24 exons. The mutant mice have an adenine to thymine transversion in the Clock gene, which caused skipping of a single exon in the Clock protein (King et al., 1997). Further studies showed that Clock heterodimerizes with another circadian factor called aryl hydrocarbon receptor nuclear translocator-like (Arntl or *Bmal1*) and transactivates *Per* and Cryptochrome (CRY) genes (Gekakis et al., 1998).

One of the first studies to illustrate the importance of circadian rhythms in humans was done with patients suffering from Advanced Sleep Phase Syndrome (ASPS), which was found to be caused by a mutation in the PERIOD 2 (*PER2*) gene (K. L. Toh et al., 2001).

Luciferase reporter constructs for *Bmal1* and *Per2* were established to study gene expression in real time. For example studies in Rat-1 fibroblasts showed that clock genes in single fibroblasts exhibit robust oscillations with an amplitude which was similar to that in the tissue. These oscillations were independent of the neighbouring cells and also exhibited variability in their circadian periods. Intercellular oscillator coupling similar to that in the SCN was recapitulated in fibroblasts; however in

dissociated cells cultures there is a loss of synchrony (Welsh, Takahashi, & Kay, 2010; Welsh, Yoo, Liu, Takahashi, & Kay, 2004).

The functional relevance of peripheral clocks was further emphasised by studies in pancreatic cells. The growth and survival of beta cells was found to be regulated by their intrinsic circadian clock and showed that the circadian clock plays a vital role in the onset of diabetes mellitus (Marcheva et al., 2010).

The Circadian Circuit

A well-received and proven paradigm of mammalian circadian biology endorses the idea of a hierarchical arrangement, in which the Suprachiasmatic Nucleus (SCN) of the hypothalamus acts as a core pacemaker thereby synchronizing the circadian molecular oscillators in the organs of the periphery via nerve and blood dependent signals that may emanate either directly or indirectly from the SCN. Conventional Calcium (Ca2+), cyclic adenosine monophosphate (cAMP) and neuropeptide signalling facilitates the coupling of oscillators between SCN cells (Bollinger & Schibler, 2014; Harmar, 2003; O'Neill & Reddy, 2012).

The notable differences between the mechanism of synchronization in the SCN and in peripheral organs arises from the fact that in SCN neurons the phase is controlled by light-dark cycles perceived by the retina whereas in peripheral organs the phase of the oscillators are adjusted by zeitgebers such as hormones and temperature (Mohawk et al., 2012). A recent discovery attests to the existence of peropsin in keratinocytes (P. P. Toh et al., 2016). Peropsin is a blue light sensitive protein which is also found in the retina and is believed to be vital for the transduction light signals received by the eye to the SCN (Bailey & Cassone, 2004). This may serve to explain how light synchronizes peripheral organs such as skin.

At the molecular level there are no observed differences in the circadian clock of SCN neurons and peripheral cells (Balsalobre, Damiola, & Schibler, 1998). In keeping with the paradigm of the circadian clock, the core of the circadian clock is governed by an autoregulatory gene expression

feedback (refer to Figure 6). The loop is driven by two inducers CLOCK and *BMAL1* and two repressing transcription factors Period 1/2 and Cryptochrome 1/2. Briefly, CLOCK and *BMAL1* heterodimerize and activate transcription of PER 1/2 and CRY 1/2 genes, as well as other clock-controlled genes (CCGs). The E-box (CACGTG) elements in the promoter region of the PER, CRY and CCGs genes are thereby target binding site of CLOCK/*BMAL1*. Once expressed and active, the PER and CRY proteins undergo heterodimerization and repress their own transcription by suppressing CLOCK/*BMAL1* activity. REV-ERBα (also NR1D1, nuclear receptor subfamily 1 group D member 1) is among the genes activated by CLOCK/*BMAL1* and its induction results in the generation of a ligand-sensitive transcription factor that represses the expression of the core clock gene *BMAL1* and additional clock target genes. The RAR related orphan receptor (ROR) family of transcriptional activators likely competes with the REV-ERB family of repressors for the same binding sites, adding further robustness to the clock (Abraham et al., 2010).

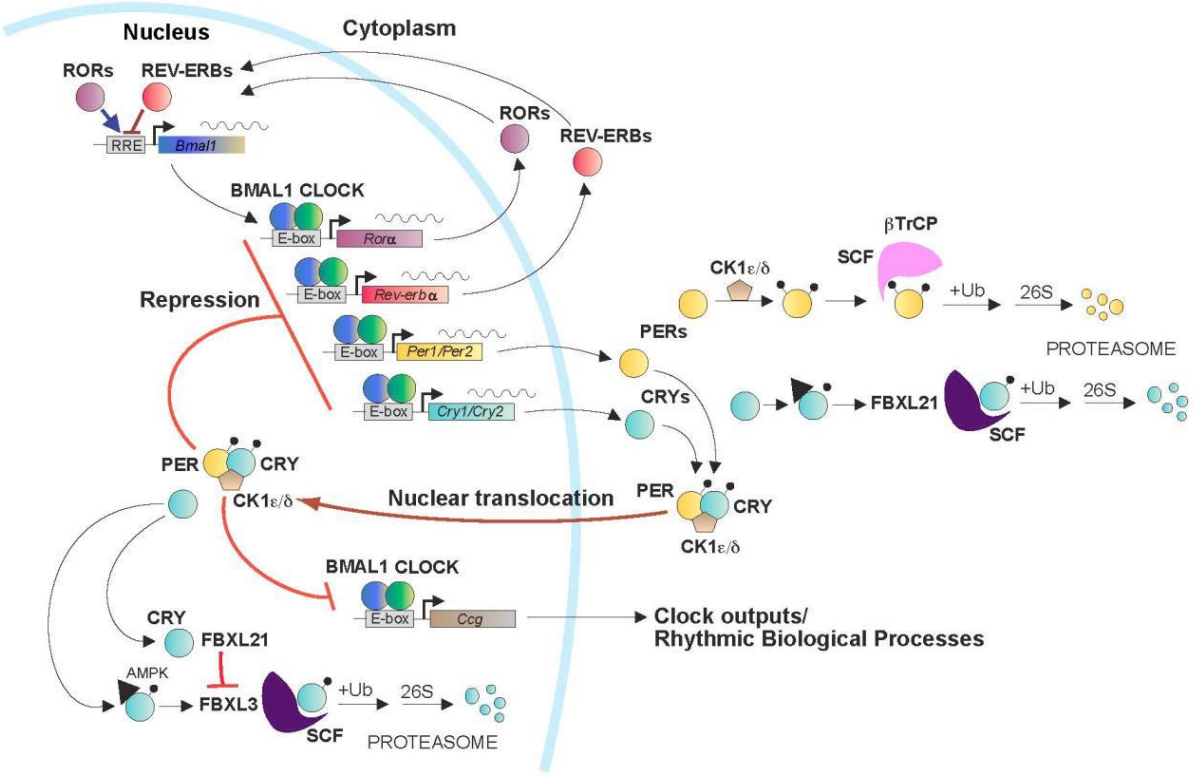


Figure 7 - Molecular circuitry of the circadian clock in mammals: The feedback loops induce the transcription and repression of clock genes. The positive feedback loop is governed by *BMAL1*/CLOCK, while the negative feedback loop is governed by PER1/2 and *CRY1*/2. Regulation of

these core clock elements is achieved through post translational modification including phosphorylation by CK1ε/δ, AMPK and ubiquitination. 26S protein complexes, RREs, RORs and CCGs also regulate the molecular mechanisms of the clock (Yoo et al., 2013).

Other feedback loops of the circadian clock machinery include several transcription factors, which are expressed with distinct rhythms, such as the proline- and acid-rich basic region leucine zipper (PAR-bZIP) family members DBP (D site of albumin promoter-binding protein), TEF (thyrotroph embryonic factor) and HLF (hepatic leukemia factor), the basic leucine zipper (bZip) protein E4BP4 and the basic helix-loop-helix proteins (bHLH) DEC1 and DEC2 (BHLHE40, BHLHE41). All these proteins are transcriptional targets of the CLOCK/*BMAL1* heterodimer (Gachon, 2007; Lowrey & Takahashi, 2004; J. S. Takahashi, Shimomura, & Kumar, 2008). The PAR-bZip family members play vital roles in neurotransmitter metabolism, xenobiotic detoxification and other immune functions (Gachon, 2007). DEC2 interacts with *BMAL1* or competes for E-box binding motifs in the promoter region of the PER1 gene and consequently represses the CLOCK/*BMAL1* mediated transactivation of PER1 (Dardente & Cermakian, 2007; Dardente, Fortier, Martineau, & Cermakian, 2007).

Although for decades the PER proteins have been described as the main negative regulators of the clock. A recent study has described the function of *CRY1* as the primary repressor of the transcriptional-translation feedback loop (TTFL). It has been suggested that *CRY1* binds to the CLOCK/*BMAL1* heterodimer, which is bound to the promoter regions of its target genes and inhibits the transcription of these target genes. The same study also showed that *PER2*, on binding to the CLOCK/*BMAL1* heterodimer, in presence of *CRY1*, displaces the promoter bound CLOCK/*BMAL1* heterodimer. However in the absence of *CRY1*, *PER2* has no effect on CLOCK/*BMAL1*-mediated transcription. The study of Ye et al. (2014) underscores the complex interaction of numerous factors during clock gene regulation (Ye et al., 2014).

Genetic studies on circadian rhythms

Traditionally observations made in circadian biology in context to physical and chemical stimuli were based on locomotor activities of experimental animals. Of late, with the help of bioluminescent

reporter constructs and other molecular techniques, the discovery of cell-autonomous circadian clocks and their constituent oscillators have been possible. A few relevant findings based on the central nervous system and peripheral organs have been listed below.

Behavioural observation

The Clock gene knockout mice showed no apparent circadian phenotype apart from having periodicities that were on average 20 minutes shorter than that of wild-type mice (R. Debruyne & Barriel, 2006). This was because neuronal PAS domain protein 2 (Npas2) was found to compensate for Clock by binding to *Bmal1* providing the same functions that the Clock/*Bmal1* heterodimer performed (J. P. DeBruyne, Weaver, & Reppert, 2007). Subsequent studies showed that *Bmal1* knockout mice exhibited arrhythmicity in circadian activity and aged prematurely as compared to control mice. Constitutive expression of *Bmal1* in *Bmal1* knockout mice or cells was found to restore circadian rhythmicity (A. C. Liu et al., 2008; McDearmon et al., 2006), indicating that *Bmal1* mRNA cycling is not necessary for circadian rhythm generation. These studies in effect substantiated a role for the positive regulators of the transcriptional feedback loop that governs the cell autonomous circadian clock.

Upon probing for the role of the negative regulators such as the Per and Cry genes of the transcriptional feedback loop it became apparent that Per1 knockout mice maintain their rhythmicity but with a periodicity which is shorter by 0.5 hours to one hour whereas *Per2* knockout mice have a period that is 1.5 hours shorter than the control mice for several days before they experience arrhythmicity. Hence, *Per2* seems to be essential for maintaining rhythmicity. Knockout of both Per1 and *Per2* renders the mice arrhythmic (Bae et al., 2001; Zheng et al., 2001). Knockout of *Cry1* and *Cry2* have opposite effects on circadian behaviour. *Cry1* knockout animals have a periodicity that is shortened by one hour whereas the *Cry2* knockout mice have a one hour longer free running period (Thresher et al., 1998; van der Horst et al., 1999; Vitaterna et al., 1999). *Cry1* and *Cry2* double knockout mice experience complete loss of behavioural and molecular rhythmicity. The

various combinations of *Per* and *Cry* knockout mice have led to the conclusion that Per1 is a non-allelic suppressor of *Cry1* and *Cry2* is a non-allelic suppressor of *Per2*. Constitutively, expressing the *Per2*, *Cry1* and *Cry2* genes in the central pacemaker or the SCN of transgenic mice induces arrhythmicity in the locomotor activity of the mice in a conditional and reversible manner (Chen et al., 2009).

Cell and tissue based observations

The previous studies assessed the role of a global knockout of the core components of the circadian clock on the rhythmic locomotor activities of the sentient beings. The results of these studies implied that rhythmic locomotor activity is a readout of the rhythmic activities of the molecular components of the circadian clock such as *Bmal1* and *Per2* at the cellular level. With the advent of robust molecular techniques such as luciferase driven promoter constructs and similar bioluminescent recording techniques it became possible to identify the exact contributions of these oscillators to physiology and the rhythmicity of other core components of the molecular clock at the cellular level.

Surprisingly, it was found there was a correlation between the differences seen in locomotor activities and core clock gene expressions at the cellular level. Similar to the roles played in maintaining rhythmic circadian locomotor activity Per1, Per2 and Cry1 were found to be essential in maintaining rhythmic circadian oscillations of core clock gene expressions in dissociated fibroblasts (S. Liu, Cai, Sothern, Guan, & Chan, 2007). Consistent with the behavioural phenotype of *Cry2* null mice, *Cry2*-/- fibroblast cells were found to be rhythmic in clock gene expressions albeit with a slightly longer period compared to individual wild-type cells.

Likewise, it was shown that *Bmal1* null mutants experience an immediate loss of circadian behaviour upon transfer to constant conditions (Bunger et al., 2000). In uncoupled SCN explants *Bmal1*-/- mutants showed rhythmic *Per2* expression (H. W. Ko et al., 2010), but single dispersed *Bmal1*-/- SCN neurons are arrhythmic, hence it was thought that the *Per2* oscillations observed in *Bmal1*-/- SCN explants was stochastic. These studies and studies based on Tetrodoxin (TTX), which is a chemical

that desynchronizes SCN neurons by selectively and reversibly blocking voltage-dependent Na+ channels (Yamaguchi et al., 2003) it became apparent that the seemingly stochastic rhythmic expression of *Per2* in *Bmal1*-/- SCN explants was a result of intercellular coupling between the neurons of the SCN (C. H. Ko et al., 2010).

However, single *Cry1*-/- and Per1-/- SCN neurons exhibited arrhythmicity in gene expression, which is in contrast to *Cry1*-/- and Per1-/- SCN tissue explants which don't express arrhythmicity. This study rationalized this observation by demonstrating that this difference in rhythmicity is a result of the prevalent intercellular coupling in the SCN neurons (A. C. Liu, D. K. Welsh, et al., 2007).

Circadian rhythms in skin and skin homeostasis

Several observations have hinted at the involvement of various components of the circadian apparatus in the physiology of skin. Skin and its appendages form the primary interface between an organism and its environment. In most cases, the environment and its posse of insults exhibit diurnal variations hence skin in response displays diurnal changes in its physiological processes. The development of diurnal variation in skin function as a result of fluctuations in the external environment is bolstered by the observation that skin development proceeds normally in clock-mutated mice (Kondratov, Kondratova, Lee, et al., 2006; Kondratov, Shamanna, Kondratova, Gorbacheva, & Antoch, 2006; K. K. Lin et al., 2009; Plikus et al., 2013) and maturation of the circadian clock occurs postnatally (Ansari, Agathagelidis, Lee, Korf, & von Gall, 2009; Kovacikova, Sladek, Bendova, Illnerova, & Sumova, 2006; Sladek et al., 2007). In keeping with this observation, skin functions are under circadian control both extrinsically, via signals from the SCN and also via an intrinsic molecular clock (Al-Nuaimi et al., 2014; Plikus et al., 2013). Approximately 1400 genes in mouse skin have been shown to display circadian rhythmicity, further suggesting circadian rhythms to dominate skin function (Geyfman & Andersen, 2009). Several features of skin such as the ability to track stem cell populations, tissue regeneration, ageing, cell proliferation and cell metabolism have made it an apt model to study the impact of circadian rhythms on tissue homeostasis. Additionally,

different skin cell types possess an intrinsic clock with an intrinsic period (S. A. Brown et al., 2005; Zanello, Jackson, & Holick, 2000), which lends plasticity to skin homeostasis.

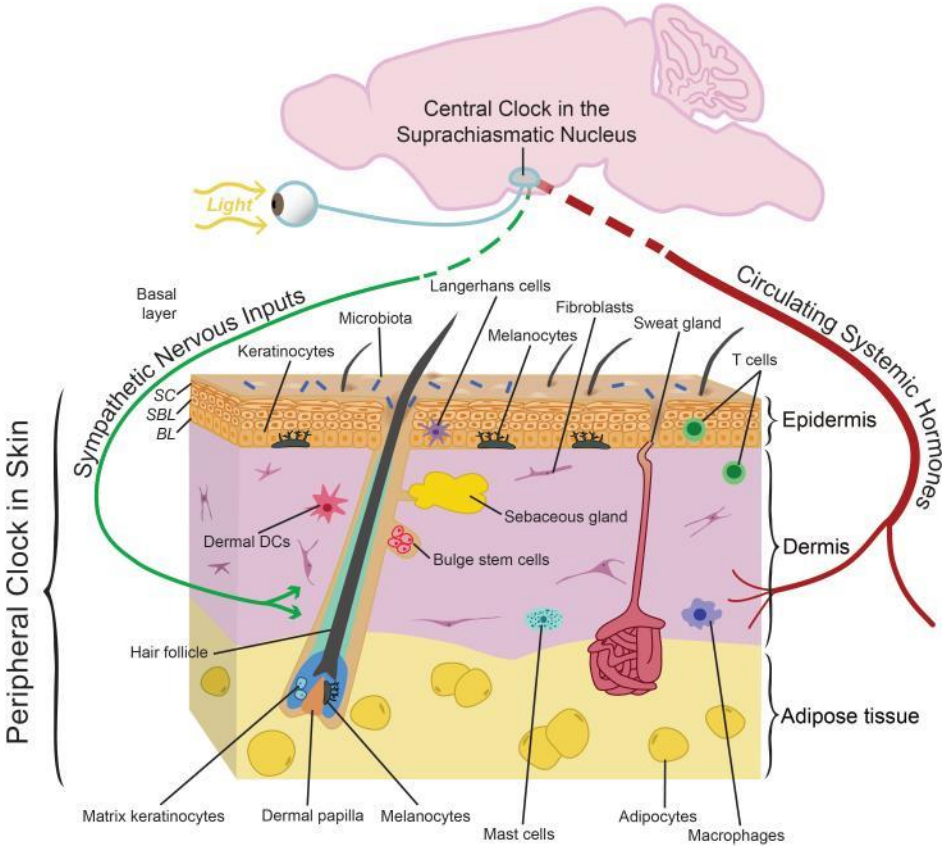


Figure 8 – Mechanism of synchronization of skin: It has been shown that light upon striking the retina conveys signals to the circadian clock in the suprachiasmatic nucleus (SCN) of the brain via the retinohypothalamic tract (RHT). This results in a trigger of the sympathetic nervous system which is then conveyed as a rhythmic input to skin via cutaneous nerve fibres. Signals from the SCN are also transmitted to the pituitary gland via the hypothalamus by polarizing and depolarizing events. This results in secretion of hormones which target and activate their target cells in skin in a rhythmic manner. Thus skin function is synchronized via both circulating systemic hormones and sympathetic inputs (Plikus et al., 2015).

Interfollicular Epidermal Clock

Progenitors and stem cells of the basal compartment of the epidermis play a pivotal role in maintaining epidermal integrity. Studies have shown that cell cycle events such as DNA replication and repair in stem cell/progenitor cells undergo diurnal variation and this variation is regulated by intrinsic and robust circadian activity in these cells (Gaddameedhi & Sancar, 2011; Gaddameedhi, Selby, Kaufmann, Smart, & Sancar, 2011; Geyfman, Kumar, et al., 2012; Janich et al., 2013; Kumar, Andersen, & Takahashi, 2013; K. K. Lin et al., 2009). This pattern of circadian clock activity is also

observed in the upper epithelial compartments of the hair follicle where stem cells that contribute to maintenance and repair reside (Geyfman, Gordon, Paus, & Andersen, 2012; Jensen et al., 2009; Plikus, 2012; Plikus et al., 2012; Snippert & Clevers, 2011).

Amongst some of the many physiological parameters of the suprabasal layer, skin pH, stratum corneum capacitance and transepidermal water loss are found to have circadian rhythmicity in their function (Geyfman & Andersen, 2009; Le Fur et al., 2001; Reinberg & Touitou, 1996; Yosipovitch et al., 1998). Transepidermal water loss from the epidermal compartment of skin has been found to be regulated by Auaporin 3 (Aqp3) which is a membrane transporter for water and glycerol. This receptor's expression is positively regulated by a CLOCK-*Bmal1* heterodimer induced transcription factor known as D-box binding PAR bZIP transcription factor (DBP), in both mouse and human skin. This observation is further supported by the finding that knockout of CLOCK in mice decreases stratum corneum hydration (Hara-Chikuma & Verkman, 2008a, 2008b, 2008c). Like DBP, *Per2* too is under the control of the CLOCK-*Bmal1* heterodimer. From studies on human epidermal stem cells it was reported that the previously described epidermal differentiation program which has a significant role to play in maintaining epidermal integrity was found to be under circadian clock control, more specifically *Per2*. It was reported that induction of differentiation in keratinocytes as a result of stress signals such as TGF-beta was dependent on the phase of *Per2* expression. The same study also showed that overexpression of *Per2* lead to a higher expression of differentiation markers. (Janich et al., 2013).

Hair Follicle Clock

Hair follicles harbour stem cells and progenitor cells which also contribute significantly to skin homeostasis. Recent studies strongly suggest the involvement of circadian elements in the physiology of these cell populations. Although the hair follicle cycle lasts more than 24 hours, yet each of the transition phases shows distinct circadian properties. It was seen that during the telogen and the transition from telogen to anagen, the hair germ progenitors that lie below the isthumus of

the hair follicle exhibit robust circadian gene expression (K. K. Lin et al., 2009). However, bulge stem cells exhibit a patchy expression of circadian genes (Janich et al., 2011; Plikus et al., 2013). On complete expansion of the hair lineage during anagen, core clock (Al-Nuaimi et al., 2014; Plikus et al., 2013) and clock output genes (K. K. Lin et al., 2009) were found to be highly expressed in the epithelial hair matrix cells, located at the very base of the hair follicle. Likewise dermal papilla fibroblasts exhibit strong circadian gene expression (Plikus et al., 2013).

Transcriptomic studies have shown the expression of only 6% of clock regulated genes to overlap between telogen and anagen hair, suggesting that different tissues or different physiological states of a tissue may activate specific circadian programs or vice-versa (Yan & Owens, 2008). To gain an insight into the circadian programs regulating hair follicle cycling, human hair follicles were cultured in vitro. One of the first conclusion made was that they possess robust circadian rhythms and knockdown of their core clock genes prolonged their growth and induced hyperpigmentation of the hair follicles.

Further support for the role of clock genes in regulating hair follicle activity has come from target gene promoter driven bioluminescent and core clock gene knockdown studies. Global knockdown of *Bmal1* in mice was found to delay the expansion of hair germ and the initiation of anagen (K. K. Lin et al., 2009). A similar knockdown of *Cry1*/*Cry2* disrupted the daily mitotic rhythms of hair matrix cells (Plikus et al., 2013). Comparative genome studies on two distinct *Per2* promoter driven bioluminescent BuSC populations revealed that cells with higher-reporter expression, express higher levels of mediators belonging to canonical WNT and TGF-β signaling pathways. These pathways have been implicated in stem cell activation (Janich P et al., 2011). This study involving the *Per2* promoter driven bioluminescent BuSC populations also observed that the number of cells belonging to the population of cells that express lower levels of the bioluminescent reporter decreased during the progress of the hair follicle cycle to the active phase. However, deletion of *Bmal1* in keratinocytes did not bring about any observable difference in BuSc activation (Geyfman, Kumar, et al., 2012). This

observation is contrary to what would be expected if BuSC activation was under circadian control since deletion of *Bmal1* normally renders cells arrhythmic. Nonetheless from these studies it is apparent that circadian genes are involved in stem cell activities in hair follicles. This fact coupled with the observation that proliferation of hair follicle progenitors and stem cells are different from the progenitor/stem cells in the basal layer of the epidermis led to the discovery that circadian proteins govern the mitotic activities of the hair matrix, by gating the G2/M cell cycle checkpoint in a rhythmic manner (Plikus et al., 2013).

Immune cells

Immune cells which form an important component of the epidermis are also known to possess robust circadian rhythms and it is believed that the immune system may have diurnal variations in its ability to counter infections (Scheiermann, Kunisaki, & Frenette, 2013). Transcriptomic studies on mouse skin have shown that many immune related genes are under the influence of the circadian clock (Geyfman, Kumar, et al., 2012). The intrinsic circadian clock of mast cells in skin was found to regulate the cutaneous anaphylactic reactions as well as IgE-mediated degranulation. This same study also demonstrated that delayed-type skin allergic reactions in mice, which is similar to human allergic contact dermatitis, is exacerbated in Clock gene mutated mice (Takita et al., 2013). Evidence supporting the interdependence of circadian rhythms and immunity has been come from studies on pinealectomised hamsters which exhibit a loss in both rhythmic melatonin signals and rhythmic turnover of dendritic cells in skin and which consequently compromised the cutaneous antigen-specific delayed-type hypersensitivity reactions (Prendergast et al., 2013).

Melanocytes

The sunscreen properties of skin are attributed to the melanin producing cells in skin which are known as melanocytes. Given the diurnal variation in sun exposure it comes as no surprise that melanocytes have a robust circadian clock (Lengyel, Battyani, Szekeres, Csernus, & Nagy, 2013; Sandu et al., 2012; Zanello et al., 2000). Consequently circadian variations in the pigment producing function of these cells could prove detrimental to skin homeostasis. To this effect it was shown that

hyper-pigmentation of hair as a result of the knockdown of *Bmal1* is the result of an altered melanocyte function. This effect was attributed to an increase in TRYP1/2 and tyrosinase expression and activity as well as enhanced melanocyte dendricity (Hardman et al., 2015).

Epidermal adaptation to stressors exhibiting diurnal variation

As mentioned previously epidermal progenitors contribute significantly to epidermal homeostasis and hence have received much attention. Consequently it was reported that epidermal progenitors in humans undergo diurnal variations in proliferation (Bullough, 1949; Fortuyn-van Leyden, 1917) and that they also go through the DNA synthesis (S-phase) of the cell cycle during the time of maximum solar exposure. A seemingly plausible argument for these observations could be that these cells have adapted their physiology to minimize the overlap of ultraviolet radiation (UV) sensitive cell cycle phases and genotoxic factor generating oxidative metabolism (Johnson, 2010; Khapre, Samsa, & Kondratov, 2010; Masri, Cervantes, & Sassone-Corsi, 2013). Support for this argument comes from murine skin studies which have shown the highest number of epidermal progenitors to be in the S-phase during the night, which is in contrast to the situation in human epidermal progenitors (P. J. Brown, Dove, Tuffnell, & Ford, 1992; W. R. Brown, 1991). This observation also suggests that skin function in part relies on an intact circadian clock in skin (Gaddameedhi & Sancar, 2011; Gaddameedhi et al., 2011; Geyfman, Kumar, et al., 2012; Plikus et al., 2013). This suggestion was subsequently validated by experiments wherein loss of *Bmal1* in keratinocytes resulted in a higher and seemingly constant proportion of cells in the S-phase. This indicates that *Bmal1* a core clock protein acts to suppress the cells from entering the S-phase during the day (Geyfman, Kumar, et al., 2012). Furthermore and consistent with this finding was that xeroderma pigmentosum group A (XPA), was found to cycle antiphasic with S-phase progression, with the nadir in expression observed in the night in mouse skin (Gaddameedhi et al., 2011). XPA serves to excise lesions induced in DNA by UV (Ikehata & Ono, 2011). Hence, if the activity of XPA were to be induced during the S-phase it would stall the process of DNA synthesis and hence enhance genomic instability which may then lead to increased errors in DNA replication

(Gaddameedhi et al., 2011). In keeping with the above described interplay between cellular clock machinery and cell cycle activity, studies in the liver have shown components of the cell cycle such as Wee1 and G2/M components to be key targets of the circadian clock (Matsuo et al., 2003). This suggests that the apparent rhythmicity observed in Wee1 expression in the mouse skin (Geyfman, Gordon, et al., 2012; K. K. Lin et al., 2009) might be a result of it being a target of the cutaneous circadian clock. Other targets of the circadian clock include NONO, which controls G1/S cell cycle transition by regulating the expression of proteins that control cell cycle progression such as p16INK4a, p21, cyclin D1, Myc (Khapre et al., 2010; Maier & Kramer, 2013) and KLF9. KLF9 is a transcription factor which exhibits circadian rhythmicity in its expression and also regulates cell proliferation in the epidermis (Sporl et al., 2012).

Given the entwined nature of cell cycle progression, diurnal variations in skin functions and the circadian molecular components in skin an idea was conceived which questioned the existence of circadian rhythmicity in the sensitivity of skin to UVB induced DNA damage. Subsequently studies gave credence to this idea and UVB treatment at night was found to induce higher DNA damage and consequently higher number of skin tumours in the mouse skin (Gaddameedhi et al., 2011). These diurnal differences in skin sensitivity were obliterated in *Bmal1*, *Cry1* and *Cry2* mutated mice, suggesting the sensitivity of skin to be controlled by core molecular circadian machinery. The time of day dependent cell proliferation, sensitivity of the human epidermal layer to UVB and possibly DNA repair is opposite to that of mice, which also serves to explain the high incidence of skin cancer as a result of sun-exposure in human subjects (Jablonski & Chaplin, 2010).

Skin Circadian rhythms play a role in ageing

Decrease in cell proliferation, reduction in repair capability and an increase in cellular senescence are some of the key features of ageing. Ageing is also characterised by aberrant expression of core molecular components of the circadian clock machinery. Furthermore, the link between ageing and circadian rhythms has come from studies on cellular senescence, which is characterized by an

irreversible replicative block (Campisi, J. 2013; van Deursen et al., 2014). Genetic studies have shown that the loss of *Bmal1* in mice induces an aged phenotype, which includes premature skin ageing, delayed hair regrowth, thinning of the cutaneous fat layer (Kondratov, Kondratova, Gorbacheva, Vykhovanets, & Antoch, 2006; Kondratov, Kondratova, Lee, et al., 2006; Kondratov, Shamanna, et al., 2006; K. K. Lin et al., 2009) and deficiency in wound closure (Kowalska et al., 2013). From these studies it is now apparent that *Bmal1* counters tissue senescence by negatively regulating the generation of reactive oxygen species and the mTOR pathway (Iglesias-Bartolome & Gutkind, 2012; Khapre et al., 2014).

Role for circadian proteins in wound healing and cancer

Wound-healing studies have substantiated a role for components of the circadian molecular machinery in skin regeneration. Wound healing involves the generation of granulation tissue (scar) this stage is achieved by the induction of senescence in myofibroblasts (Jun & Lau, 2010a, 2010b; Kowalska et al., 2013). Myofibroblasts in wounded tissue initially undergoes a proliferative burst and then enters a state of replicative senescence; this step serves to keep cell over-proliferation in check. This process of senescence is initiated by CCN1 adhesive protein signalling and executed via the activation of both p53-p21 and p16Ink4a–pRb senescence pathways (Jun & Lau, 2010a). Upon acquiring the senescent phenotype, myofibroblasts secrete multiple cytokines and matrix metalloproteinases, which help with formation of a mature scar. For the replicative block to occur it is necessary that the p16Ink4a cell cycle inhibitor is activated post CCN1 signalling (Jun & Lau, 2010b) and this activation is mediated by NONO, a transcription factor that serves as a link between the circadian clock and the induction of replicative senescence. NONO is a binding factor of core clock protein *Per2* (Kowalska et al., 2013; Maier & Kramer, 2013) and it cycles similar to *Per2*. NONO and Per1/2 double mutant mice exhibit a loss in rhythmicity in p16Ink4a expression and scars in these mutants display overproliferation.

In a certain sense cancer and wound healing represent two sides of the same coin. They are both characterized by migration of cells. In the case of wounds migration of keratinocytes and fibroblasts ensures the closure of wounds and in the case of cancer at a particular stage known as metastasis cancerous cells start to migrate to different parts of the body eventually leading to organ failure. Circadian rhythms have recently been found to govern the migration of fibroblasts and keratinocytes. This study has suggested that actin polymerisation, which drives fibroblast and keratinocyte motility by forming lamellipodia and filopodia at the leading edge of the cell has a circadian rhythm to its dynamic activity. They have shown that migration and adhesion of the two aforementioned cell types peaks at the same time as the peak in *Per2* expression. Efficiency of wound healing was found to be highest in wounds made in the active phase. By using inhibitors this study also conclusively shows that actin dynamics are an output of the circadian clock and that actin dynamics doesn't have an effect on the cycling of clock genes (Hoyle et al., 2017). This study has also served as a rationale for dissecting the crosstalk between the DOPr and core components of circadian clock machinery since overexpression of the DOPr in keratinocytes led to a higher number of filopodia and DOPr activation in these keratinocytes also led to their faster migration (Bigliardi, Neumann, Teo, Pant, & Bigliardi-Qi, 2015)

Opioids and Circadian Rhythms

Chronotherapy, amongst other aspects of skin biology, is an underexplored yet promising area of skin biology. In general, the aim of chronotherapy is to coordinate drug administration with circadian rhythms to maximize the therapeutic effect of the drug and to minimize the side effect of drug treatment. The therapeutic efficacy of drug treatment in part relies on the metabolism of drugs by metabolic enzymes. Metabolic enzymes in skin exhibit circadian rhythmicity (Geyfman, Kumar, et al., 2012) hence drug treatments may be coordinated to ensure that pathways responsible for metabolizing the drug or the drug- targets are at their nadir and/or when the drug- targets are expressed at the highest. This principle has been tested and it was found that hair loss as a result of

radiation therapy is time- of -day dependent (Plikus et al., 2013). However, successful devising of chronotherapies can only be achieved on a comprehensive understanding of the aetiology of a condition or a disease.

Diseases are often associated with pain and stressed states, consequently opioids are administered since they play a role in modulating stress, more importantly they suppress the expression of stress related HPA axis elements at the systemic level (Drolet & Rivest, 2001). A multitude of studies also show elements of the HPA axis to be regulated by the molecular elements of circadian rhythms and vice versa. Similarly studies also show opioid treatments is able to influence circadian rhythms of systemic constituents such as hormones, hence suggesting that opioids could influence circadian rhythms possibly through a crosstalk between opioid induced pathways and HPA axis pathways in a time dependent manner. Furthermore, peripheral circadian oscillators mirror the individual chronotype i.e. the circadian period length and phase (Akashi et al., 2010; S. A. Brown et al., 2005). Hence, skin cell circadian rhythms could be used as readouts of the inherent chronotype of the individual and opioid based therapies may then be designed to counter pain and stress states.

Evidence for the involvement of circadian elements in stressed states comes from studies based on mice mutated for core clock components. For example it has been shown that the HPA-axis function is severely altered in several of the clock mutant mouse models. For instance, Per1 and *Per2* are found to regulate corticosterone levels (Dallmann, Touma, Palme, Albrecht, & Steinlechner, 2006) Likewise, glucocorticoid production is found to be impaired in *Bmal1* knockout mice (Arntl-deficient mice) (Leliavski, Shostak, Husse, & Oster, 2014). Glucocorticoids are well studied and are known to modulate metabolic pathways and more importantly to entrain the circadian rhythmic physiology of various organs by phase shifting the expression of several core clock genes in peripheral organs including the liver, kidneys and heart (Balsalobre et al., 1998). Dexamethasone, a glucocorticoid, was found to synchronize MEF fibroblasts by strongly inducing *Per2* expression (Cheon, Park, Cho, & Kim, 2013). Furthermore, acute and chronic physical stress elevate Per1 mRNA levels in mouse

peripheral tissues (Yamamoto et al., 2005) and forced swim tests elevate mPer1 expression in the PVN (Takahashi *et al.*, 2001). These studies strongly suggest the existence of a crosstalk between the stress-induced HPA axis and clock genes (Perreau-Lenz & Spanagel, 2008).

A role for opioids in cessation of HPA axis related stress responses come from studies on Alcohol Use Disorders (AUDs) and Substance Use Disorders (SUDs), which includes the abuse of opioids. In general AUDs and SUDs induce the expression of stress axis elements such as systemic glucocorticoid levels are elevated (Glavas, Ellis, Yu, & Weinberg, 2007; Loosen et al., 1993; Loosen, Chambliss, Pavlou, & Orth, 1991; Sarkar, 2012; Wong & Schumann, 2012) and diurnal rhythms of secretion of ACTH are altered (S. X. Li, Shi, et al., 2009; H. Liu et al., 2009; Shi et al., 2009). In case of heroin addicts it is observed that upon abstinence for 30 days, their cortisol levels are elevated and ACTH rhythms are altered (S. X. Li, Liu, Jiang, & Lu, 2009; Shi et al., 2009). In cases of stress such as these, treatment with opioids was found to normalize endocrine rhythms (Brennan, 2013).

Lastly, given that skin possesses an HPA axis homologue and that stress could perturb skin metabolism and thus homeostasis, we speculate that chronotherapies involving opioids in keeping with their anti-stress properties could restore skin homeostasis**.**

Epigenetics: A link between DOPr and circadian rhythms

Epigenetics literally means “in addition to changes in gene sequences”. It is now recognized as a collection of processes that alter gene activity without changing the DNA sequence. These changes in some cases are known to be heritable (Weinhold, 2006). In order to elaborate further on the role of epigenetic changes it would be necessary to discuss certain features relating to the genetic information of a cell.

The genetic information of a cell is stored in the nucleus. In its most basic form this information is stored as DNA. DNA in eukaryotic cells is double stranded and consists of four basic nucleotides which pair as Adenosine and Thymine (A-T) and Cytosine and Guanine (G-C). Specialized

combinations of DNA sequences contribute to the formation of genes which ensure the survival of an organism since they encode for vital molecules such as enzymes, cytoskeleton elements amongst others. This double stranded DNA is condensed by winding around proteins known as histones collectively these components are referred to as chromatin. The condensed chromatin is uncondensed from time to time to facilitate the binding of molecular machinery that transcribes the gene sequence by a process of transcription which results in generation of RNA. RNA is subsequently translated into a protein by a process of translation (Luger, Dechassa, & Tremethick, 2012)

This change in chromatin architecture which facilitates the activation and transcription of genes is made possible by a host of covalent modifications at the various histones that the DNA is wound around. These modifications are known as post translational modifications (PTMs) and are associated with activation and repression of transcription. In the case of histones PTMs take on the form of phosphorylation/ dephosphorylation which is addition/removal of phosphate molecules to/from histones, or ubiquitination which is addition of ubiquitin groups to histones, or sumoylation which is addition of small ubiquitin like molecules to histones, or methylation which is addition of methyl groups to histones or finally acetylation/deacetylation which is addition/removal of acetyl molecules to/from histones. Furthermore these covalent modifications that alter the degree of chromatin condensation have become known as the “histone code”. For example, deacetylation, dephosphorylation or methylation of histone lysine residues promotes a transcription-inhibitory nucleosome conformation, whereas acetylation of lysine or phosphorylation of serine residues induces a transcription-permissive nucleosome conformation (Jenuwein & Allis, 2001; Strahl & Allis, 2000).

An important experiment that implicated chromatin remodelling as a possible circadian regulatory mechanism was an experiment in mice demonstrating that light pulses during the subjective night promote phosphorylation of the ser10 residue of histone H3 (Crosio, Cermakian, Allis, & Sassone-Corsi, 2000). Subsequently it was demonstrated that rhythmic acetylation of PER1 and *PER2*

promoters at lys9 of histone H3 is mediated by CLOCK and NPAS2 heterodimers which recruit histone acetyl-transferases (HATs) to the PER1 promoter (Curtis et al., 2004; Etchegaray, Lee, Wade, & Reppert, 2003). CLOCK by virtue of its histone acetyl transferase (HAT) activity towards lysine residues of histone H3 and H4 has been shown to play a role in rhythmic acetylation of histones at clock-controlled genes, while activating transcription with its partner *BMAL1* (Doi, Hirayama, & Sassone-Corsi, 2006).

Therapeutic regulation of epigenetic changes such as acetylation of histones is now a common phenomenon in clinics to treat conditions such as cancer. This advancement in cancer therapy has come about because of two main reasons the first being that it has been widely observed that acetylation homeostasis undergoes disruption in cancerous cells and second that acetylation of histones is a reversible process. Several Histone deacetylases (HDACs) such as Vorinostat (SAHA) and Belinostat amongst others have been approved by the Food and Drug administration (FDA) for cancer therapy (Li Yixuan & Seto Edward 2016). Regulation of acetylation of histones by cell receptors such as G protein (heterotrimeric guanine nucleotide-binding protein)-coupled receptors (GPCRs), also known as 7 transmembrane helical (7TM) receptors has gained momentum since these receptors continue to remain a major source of new pharmaceuticals Pain and addiction are commonly encountered phenomena in the clinic and hence are the focus of extensive research efforts in academia, government and pharma. In most cases these clinical conditions are countered by administering opioids which bind to their cognate GPCRs known as OPrs. As mentioned previously there are 3 main kinds of OPr they are MOPr, DOPr and KOPr. Most of the information on epigenetic changes mediated by the OPrs pertains to MOPr but there is some evidence as to the involvement of DOPr.

Morphine a MOPr agonist, is reduces histone methylation (Sun et al., 2012) and induce histone H3 acetylation in the nucleus accumbens (Sheng, Lv, Wang, Zhou, & Hui, 2011) and amygdala (Y. Wang et al., 2015) , two brain regions involved in the reward control. As mentioned previously epigenetic

modifications are an inheritable change in gene expression and in keeping with this fact, studies in animals have conclusively shown offspring's of a morphine exposed mother and a drug-naïve father, to have enhanced locomotor activity (E. M. Byrnes, 2005). However only male offspring were shown to develop an enhanced morphine tolerance (J. J. Byrnes, Babb, Scanlan, & Byrnes, 2011) and female offspring exhibited anxiety-like behaviour (J. J. Byrnes et al., 2011).

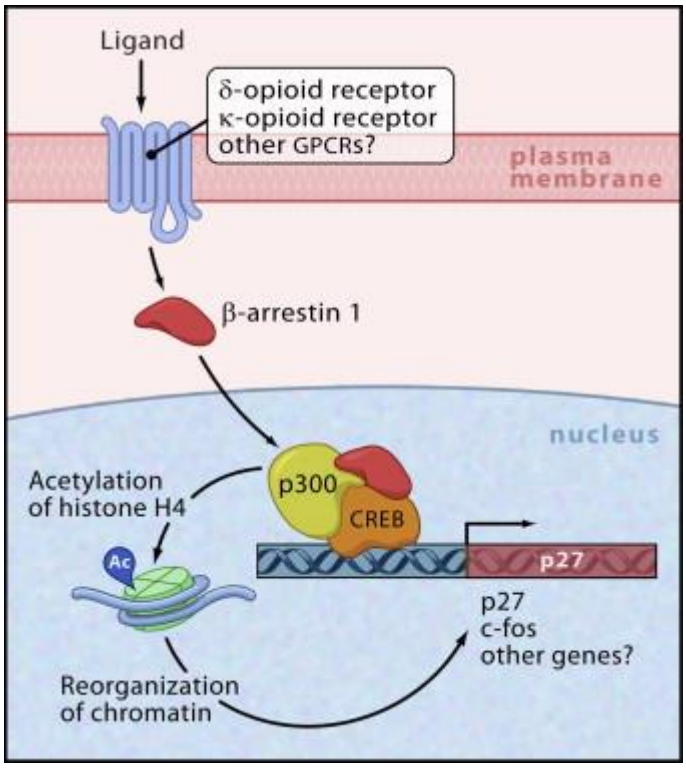


Figure 9 - Activation of the Delta Opioid Receptor (DOPr) results in epigenetic changes: Activation of the DOPr leads to internalization of the receptor via the recruitment of βarr1. βarr1 then translocates to the nucleus and binds to CREB hence bringing about the acetylation of histone H4 in the promoter of its target genes such as *c-fos* and *p27* (Beaulieu & Caron, 2005).

Activation of the DOPr in DOPr/βarr1 overexpressing HEK 293 FT cells was found to induce H4 acetylation, a transcription permissive conformation in the promoter region of target genes like c-Fos and p27, both of which negatively regulate cell proliferation. The same study also showed activation of DOPr to reduce proliferation of cancerous glial cells (Kang et al., 2005).

Taken together it is apparent that core components of the circadian molecular circuit are regulated by epigenetic mechanisms, which in turn could be regulated by OPrs. This potential of OPrs to regulate circadian rhythms could then be harnessed in a clinical setting.

Chronopharmacology of opioids

Chronopharmacology studies the outcome of scheduling drug administration and likewise, chronopathology makes connections between an illness and rhythmic physiological activities such as the rhythmic cardiac and respiratory patterns amongst others. Alterations in rhythmicity are a characteristic associated with several pathological states. Likewise, the intensity and perception of pain, often associated with pathologies, has been shown to exhibit rhythmicity. Consistent with this finding is that circadian changes also impacts the perceived effects of pain relievers**.**

In 1967 it was demonstrated that morphine, an agonist of the MOPr, induces analgesia and its efficacy showed circadian variation (R. W. Morris & Lutsch, 1967). To further characterize this diurnal variation in morphine-induced analgesia studies using mice demonstrated that the analgesic effect was highest three hours prior to midnight when nocturnal animals are most active, whereas the minimum analgesic effect occurred at about three hours past noon when animals are at rest (Bernstein, 1977). This finding was attributed to the cyclical expression of MOPr. A higher level of MOPr was found to be expressed at 21:00 h than at 09:00 h (Bernstein, 1977). It was concluded that the cyclical nature of morphine-induced-analgesia stemmed from the diurnal variation in MOPr expression.

The SCN is believed to be a master-pacemaker and regulates the rhythmic expression of endocrine receptors and their ligands including cortisol and β-END. Studies in human volunteers have shown that shortly after waking, a sharp 38–75% (average 50%) increase in the blood level of cortisol occurs in about 77% of healthy adults (Wust, Federenko, Hellhammer, & Kirschbaum, 2000). Similarly, a study in 6 healthy volunteers demonstrated, β-END undergoes diurnal variations. A peak in

expression was found to occur at 8:00h and trough values at 20:00 h (Petraglia et al., 1983). A recent study in healthy human volunteers using fentanyl (also a MOPr agonist) showed diurnal variations in its analgesic property. Fentanyl induced maximum pain relief at 17:30 h and least pain relief in the early morning at five-thirty. In the case of fentanyl, it has been shown to modify the daily pacemaker possibly by acting directly on SCN electrical activity and control of *Per* genes (Vansteensel et al., 2005). It has been speculated that this variation in the analgesic properties of fentanyl and morphine could be due to a direct effect of these opioids on pathways shared by the circadian and opioid system or a secondary influence due to diurnal variations in hormones or endogenous opioid peptides that affect the pain and/or anodyne response to analgesics.

Ever since the discovery of the DOPrs in the hamster SCN, much attention has been focussed on the function of enkephalins in modulating the circadian rhythms (Meijer et al., 2000; Vansteensel et al., 2005). The DOPr has been shown to modulate wheel running activity in hamsters and hence has an ability to modulate circadian rhythms. The DOPr, unlike other opioid receptors, does not undergo circadian variations in expression. Hence making it an attractive target for therapy since effects mediated through this receptor is more likely to generate reproducible results when compared to other opioid receptor subtypes (Pacesova et al., 2015).

Delta opioid receptor in therapy

The DOPr is expressed in the various areas of the brain and its expression has been shown to be modulated in various neurodegenerative diseases. The DOPr is also expressed in cardiomyocytes, kidney cells and submucosal and myentric neurons. Their expression has been found to upregulated under conditions of stress and hence it has proposed that these receptors play a protective role by maintaining homeostasis. There have also been studies which have shown the functional activity of the DOPr to increase under conditions of stress.

As mentioned previously stress induces the secretion of hormones such as CRH and endogenous enkephalins (Amir, Galina, Blair, Brown, & Amit, 1980; Kalivas, Stanley, & Prange, 1987) which

suggests that DOPr is involved in the feedback stress response. To test this hypothesis mice exposed to stress were treated with DPDPE, the results of this test confirmed that DOPr activation brings about cessation of the stress response (Hebb, Poulin, Roach, Zacharko, & Drolet, 2005). Similar studies involving foot-shock stress (Margolis, Mitchell, Hjelmstad, & Fields, 2011), forced swim test (Commons, 2003), sleep deprivation (Fadda, Tortorella, & Fratta, 1991) and lastly genetic deletion of the DOPr or its endogenous ligands (Filliol et al., 2000; Konig et al., 1996) have attested to the involvement of DOPr in maintaining systemic homeostasis.

On account of their anti-stress effects, low abuse liability (Negus, Gatch, Mello, Zhang, & Rice, 1998) and lack of physical dependence (Brandt, Furness, Mello, Rice, & Negus, 2001) drugs targeting the DOPr have entered clinical trials. These clinical studies have yielded mixed results. Amongst several DOPr targeting drugs AZD2327 is a drug which has anxiolytic and anti-depressive properties is in phase II clinical trials. Another drug known as TRK-851 has hinted at its ability to cure persistent cough (Nagase & Fujii, 2011; Sakami et al., 2008).

Finally, given the role that circadian rhythms have in opioid receptor expression and function and the potential effects of the delta opioid receptor in alleviating stressed states it does seem rational to devise therapies based on the circadian rhythms of the individual to enhance the DOPr mediated effects.

Aim of thesis

Skin is the largest organ of the body and serves as an interface between the internal and external environment of the organism. The external environment is subject to diurnal variations which arise from the planet's rotation. In anticipation of these diurnal changes and their corresponding insults skin just like various other organs has incorporated a robust circadian rhythm in its physiology. Although robustness in skin's circadian physiology is achieved in part from signals emanating from the central nervous system and endocrine glands via clock resetting mechanisms, various receptors present on the different constituents of skin lends plasticity to its circadian functions by virtue of their sensory properties thus facilitating effective responses to environmental cues.

G protein coupled receptors (GPCRs) which are generally present on the cell membrane and are known to sense the environment are commonly used as targets for clinical therapy. Opioid receptors (OPrs) are a subset of GPCRs which are targeted for therapy under conditions of inflammation, pain and wounding. Activation of the OPrs in the CNS has been found to modulate circadian rhythms. These receptors except the delta opioid receptor (DOPr) have also been found to exhibit a diurnal variation in expression thus making the DOPr an attractive target for therapy

Furthermore from unpublished microarray data from our group we have found that the activation of the DOPr in keratinocytes leads to the regulation of core circadian genes. In an attempt to harness the potential of the DOPr in a clinical setting we directed this study to gain an insight into the molecular events that leads to the regulation of circadian rhythms by the DOPr in keratinocytes.

Material and methods

Cell Culture

Human skin keratinocyte cell line N/TERT-1 was obtained from and cultured as described by the Rheinwald Laboratory (Dickson et al., 2000; Rheinwald et al., 2002). These cells are derived from neonatal foreskin and immortalized by transfection to express telomerase reverse transcriptase (TERT). They were cultured in keratinocyte-serum free medium (K-SFM) supplemented with 0.2 ng/ml Epidermal Growth Factor (EGF) and 25 µg/ml Bovine Pituitary Extract (BPE) and an adjusted Ca^{2+} concentration of 0.4 mM at 37 °C and 5% CO_2. Upon reaching 50% confluence cells were subcultured using TrypLE™ Express. Trypsinisation was stopped by removing the enzyme solution after centrifugation and approximately 1×10^4 cells per T-75 flask were carried as stock. Cells were subcultured every seven days and medium was changed every 2-3 days**.**

Primary Human Keratinocytes (PHKs) were obtained from the Skin Cell Bank. The Institute of Medical Biology (IMB) maintains and supports a skin cell bank of human cells collected for ethically approved research.

Treatments

Keratinocytes were seeded in 6-well plates and used at 70% confluence for all experiments. On the day of the experiment cells were treated with 1 µM Dexamethasone (Sigma D4902) for one hour. Thereafter the media was replaced with K-SFM, BPE- and EGF-free (for the control group) or were treated with 100 nM Metenkephalin (Metenk) or 10 µM Naltrindole (NTI) or 100 nM Metenk + 10 µM NTI in K-SFM (BPE- and EGF-free). The cells were kept under constant dark conditions in the incubator throughout the course of this experiment.

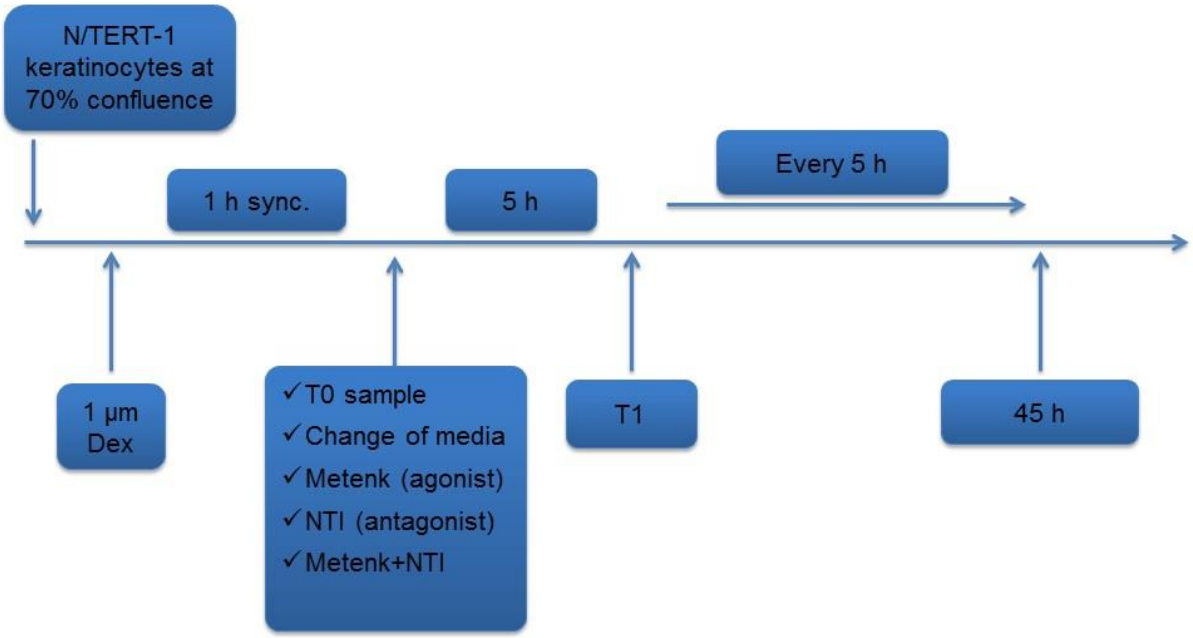


Figure 10 - Treatments: N/TERT-1 keratinocytes were seeded in 6 well plates and upon reaching 70% confluence they were synchronized by incubating them with 1µM Dexamethasone for 1h. Thereafter the T0 sample was collected in 300µl RLT lysis buffer (Cat No./ID: 79216, Qiagen), and for the other samples the medium was replaced with a medium containing the agonist 100nM Metenkephalin (Metenk), or the antagonist 10µM Naltrindole (NTI), or a combination 100nM Metenk+ 10µM NTI or the control medium, and samples were collected every 5h for the next 45h.

RNA isolation and Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted using RNeasy Kit (Qiagen) according to manufacturer's protocol. Quality and concentration of the RNA in the samples were verified using a NanoDrop spectrophotometer. Total RNA (1 µg) was reverse transcribed into cDNA in a 20 µl reaction using PrimeScript RT Reagent Kit (Takara, Japan). The resulting cDNA was diluted to obtain a concentration of 20 ng/µl cDNA.

Quantitative Real Time Polymerase Chain Reaction (qPCR)

For the quantification of gene expression, 100 ng of cDNA template per reaction was amplified using SYBR green master mix and specific Quantitect primers from QIAGEN for *BMAL1*, *PER2*, *DEC2, DBP and TEF*. To test for the presence of DOPr expression the following primers were use Forward: ACGTGCTTGTCATGTTCGGCATCGT , Reverse : ATGGTGAGCGTGAAGATGCTGGTGA : Gene expression was normalized based on the values of the expression of RPL13A used as reference (Forward primer 5'- CTC AAG GTC GTG CGT CTG AA- 3' and reverse primer 5'-TGG CTG TCA CTG CCT GGT ACT-3'). Quantification was performed using the comparative $2^{-\Delta\Delta CT}$ Method.

For ChIP experiments that were designed to test for the binding of βarr1 and DOPr to acetylated Histone 4 and CREB in the *Per2* promoter. The following target sequence was identified with the help of the UCSC genome browser. This sequence aligns with the promoter region of the human *Per2* gene.

5'-AGCCCCGGGAGGCCTGCATGCTGTTCACACACTCAGTCAGGTGGCCCCTCCTCCTGTTCC

TCTGACATTGACACCTCGACACACTCCCCGCCCCCTCTCCCTAACACATACACACACAAATGCCAAGCAAACCC

AGGCCCGGCTGCTGCGCCCTGCACACACCCAAAGGCTCTTTTGTTTCTTCCCTCCCATTGACGTCAATGGGGAG

CTCCATTGTTCTGGAAACAAGAGTAAACAGAC

AGCTCATCCACACCTTACCGAGATTCTTCTTCATGCTTTT- 3'

The primers for this sequence were as follows Forward: ATCTGCATACATGAGGGGCG

Reverse: GGAACCGACGAGGTGAACAT and the product lengths were 114 base pairs. The primers hybridize and amplify the region highlighted in green. This target DNA sequence in the promoter region of the Per2 gene was chosen because it contained a canonical CREB protein binding sequence which is highlighted in red.

DNA construct

The plasmid used for N/TERT-1 experiments was a lentiviral expression clone with pEZ-Lv122 vector backbone including the DOPr-GFP open reading frame (NCBI entry U10504) purchased from

GeneCopoeia (no. EX-A1155-Lv122; Rockville, MD). pReciever-Lv127 vector backbone including the β-arrestin 1 – CFP open reading frame (NCBI Accession number NM_004041) was purchased from GeneCopoeia (no. EX-V0563-LV127; Rockville, MD). SMARTvector lentiviral Human ARRB1 catalogue number V3SH11240-225279371 with a hCMV promoter was purchase from Dharmacon. The following oligonucleotides for the DOPr knockdown were used forward:

5'-CGCGTCCCCTGCTCTCCATCGACTACTATTCAAGAGATAGTAGTCGATGGAGA GCATTTTTGGAA- 3' and reverse:

5'-TTCCAAAAATGCTCTCCATCGACTACTATCTCTTGAATAGTAGTCGATGGAGAGCAGGGGA-3'. These oligonucleotide sequences were phosphorylated and inserted into dephosphorylated pLVTHM plasmids using a T4 ligase. Refer to Figure 11 for construct information.

Lentivirus

For production of recombinant lentiviral particles, HEK 293Ta cells were transfected with 1.5 µg of purified DOPr plasmid and 1 µg of each human lentiviral packaging vectors psPax and pMD2.G (Yang et al., 2004) using Qiagen Effectene Transfection Reagent (Qiagen, Singapore), according to the manufacturer's instructions. Two days after transfection, virus-containing medium was harvested and concentrated by ultracentrifugation at 22,000 rpm at 4 °C for 2 hours 16 minutes in a Beckman Coulter JS-24.38 rotor. Cells were infected at 30% confluence with a multiplicity of infection of approximately 10, in the presence of 10 µg ml^{-1} Polybrene (Millipore, Singapore). After 24 hours, the viral particle–containing medium was replaced with fresh medium, and cells were cultured for at least 48 hours before use.

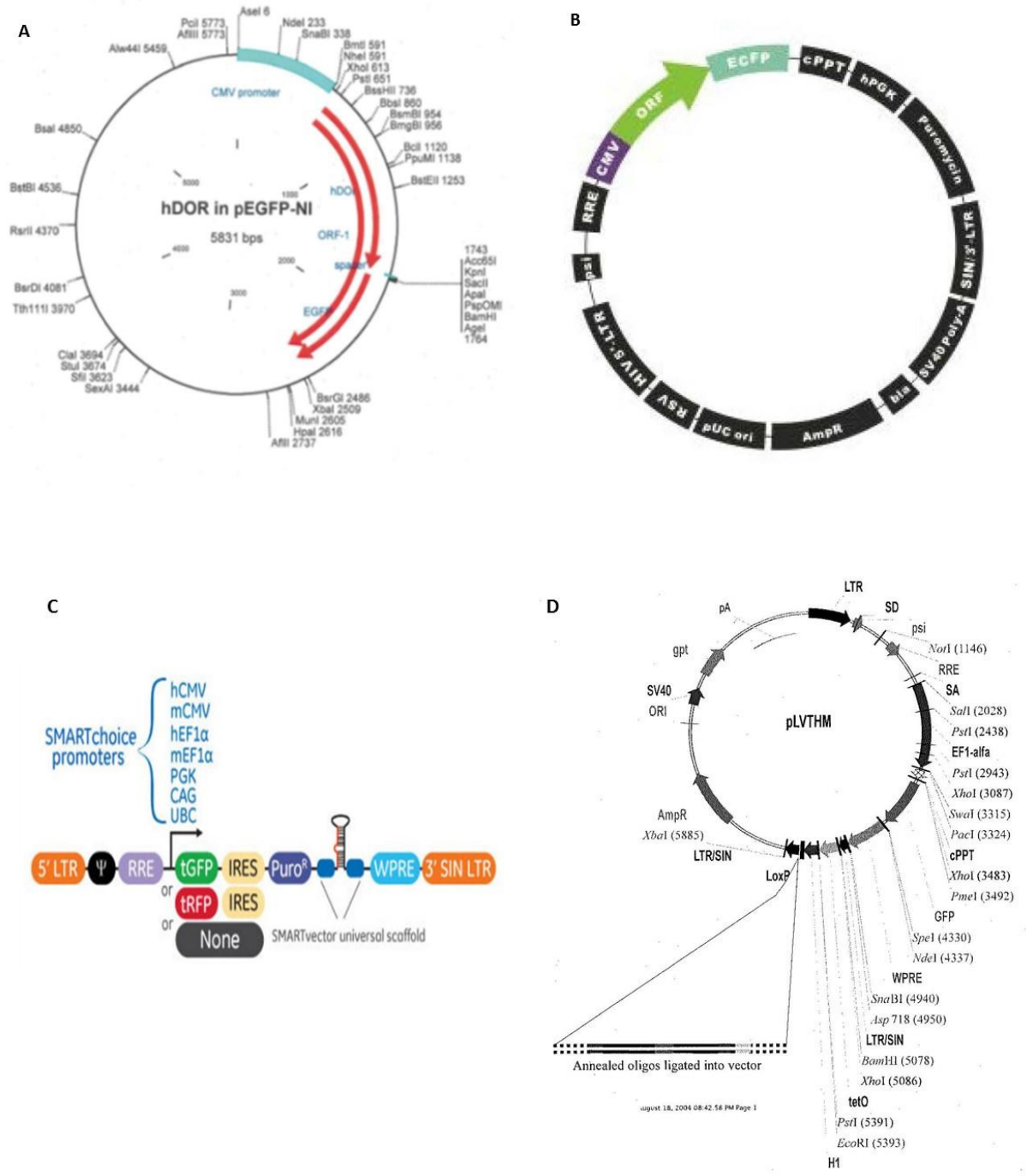


Figure 11 - DNA constructs: (A) The plasmid used for generating lentiviral particles that overexpressed DOPr fused with enhanced Green Fluorescent Protein (GFP) in N/TERT-1 keratinocytes (The infected cells expressed GFP) (B) The plasmid used to generate lentiviral particles that knockdown βarr1 in N/TERT-1 keratinocytes. Cells infected by the virus expressed turbo Red Fluorescent protein (tRFP) (C) The plasmid used to generate lentiviral particles that overexpressed β-arrestin1 fused with enhanced Cyan Fluorescent Protein (ECFP) D) The plasmid used for generating lentiviral particles that knocked down DOPr in N/TERT-1 keratinocytes.

Chromatin Immunoprecipitation

This assay was performed according to the protocol for the SimpleChIP® Plus Enzymatic Chromatin IP Kit (Agarose Beads) from Cell Signaling Technology. The presence of the target gene promoter sequences in both the input DNA and the recovered DNA immunocomplexes was detected by qPCR. The primer pairs for specific promoter regions are shown (refer to DNA constructs in material and methods). The data obtained were normalized to corresponding DNA input control. 4ul of indicated antibody were used for each immunoprecipitation (IP). The details of the antibodies used are Anti-beta Arrestin 1 antibody (ab31868), Anti-GFP antibody - ChIP Grade ab290, Histone H4K16ac (Acetyl Lys16) antibody (GT1271) and Anti- CREB Antibody (PA1-850)

Nuclear extraction

Cells were grown to 70% confluence in 10 cm dishes and were treated without or with 100 nM Metenkephalin for 5 min. They were then washed with PBS and incubated with 2 ml TrypLE. The cells were then resuspended in 5 ml media and centrifuged at 2000 rpm for 3 min and thereafter were similarly resuspended and washed with twice with 5ml PBS. The nuclear fractions were then extracted using the Thermoscientific NE-PER Nuclear and Cytoplasmic Extraction kit. Nuclear extracts (NE extracts) were harvested in nuclear extract buffer (NE-buffer) containing 20 mM NaF and 1 mM phenylmethyl sulphonyl fluoride.

Immunoprecipitation

The NE extracts, which were obtained as described above, were quantified using the Bradford method (Bradford, 1976) with a colorimetric assay Protein Assay (Bio-Rad) according to the manufacturer's instructions using. Thereafter 100 µg of NE extracts were used for each immunoprecipitation reaction. The protein in lysis buffer containing 50 mM Tris-HCl, 150 mM NaCl, 0.5% NP40 with protease inhibitors (Calbiochem) was incubated at 4 °C with indicated antibody

buffer overnight. Thereafter the immune-complexes were precipitated using a 3 h incubation with Protein A Dynabeads (Life Technologies) at 4°C. Immunoprecipitates were washed four times in lysis buffer and subjected to standard western analysis. Antibodies used in this study were anti CFP (Biorbyt, orb256068), Anti-Beta Arrestin 1 antibody (Abcam, ab31868), Anti-Delta opioid receptor antibody (Abcam, ab 176324)

Immunocytochemistry

N/TERT-1 keratinocytes were seeded at 2,000 per well on coverslips in a 12 well plate. Upon reaching 70 percent confluence these cells were then then treated with 100nM Metenkephalin for 5 min or with control KSFM media. These cells were then fixed in cold 1:1 methanol and acetone fixative for 5 minutes. The cells were then washed with 1X PBS and unspecific binding of antibody was achieved by using CAS-Block™ Histochemical Reagent (catalogue number: 00-8120) from Thermoscientific. Thereafter, the cells were treated with primary antibodies for Chicken anti-GFP (Abcam, ab13970) (to target GFP tagged DOPr) and Rabbit anti β-arrestin 1 overnight at 1:5000 dilution in blocking buffer. The secondary antibodies used were goat anti chicken which was conjugated to a fluorophore sensitive to a light of wavelength 488nM (Abcam, ab150169) and donkey anti Rabbit which was conjugated to a fluorophore sensitive to a light of wavelength 594nM (Abcam, ab176324) at 1:2000 dilution in 1X PBS.

Quantitative Real Time Polymerase Chain Reaction (qPCR)

For the quantification of target genes, 100 ng of cDNA template per reaction was amplified using SYBR green master mix and specific Quantitect primers from QIAGEN for BMAL1, PER2, CRY1, CRY2, DEC2, DBP, and TEF. Target gene expression was normalized based on the values of the expression of RPL13A (Forward primer 5'- CTC AAG GTC GTG CGT CTG AA- 3' and reverse primer 5'-TGG CTG TCA CTG CCT GGT ACT-3'). Quantification was performed using the comparative $2^{-\Delta\Delta CT}$ Method.

Protein Extraction

Cells were seeded in 6-well plates. After treatments under experimental conditions were completed cells were lysed in Radioimmunoprecipitation Assay (RIPA) buffer (Sigma R0278) supplemented with 1 mM 1,4-dithiothreitol (DTT), 1 mM Phenylmethylsulfonyl fluoride (PMSF), 10 mM Sodium orthovanadate (Na_3VO_4) and 1X protease inhibitor (Roche Reference No. 11836170001). Cell lysates were quantified using the Bradford assay.

SDS – Polyacrylamide Gel Electrophoresis (PAGE)

50 µg of protein per cell extract were separated on vertical, discontinuous SDS-polyacrylamide gels (Laemmli, 1970). Samples were prepared by addition of 6x SDS sample buffer and heating at 95 °C for 5 min. The composition of the stacking gel was 5% acrylamide in 1.5 mM Tris-HCL (pH 6.8) stacking gel buffer and 12% concentrated in 1.5 mM Tris-HCL (pH 8.8) resolving gel. Electrophoresis was performed in SDS running buffer (25 mM Tris, 192 mM Glycine, 0.1% SDS, pH 8.3) at 30 mA per gel using Mini-PROTEAN® electrophoresis chambers (Biorad). Precison Plus Protein™ Dual Colour Standards was used for size reference.

Ligand Binding Assay

Metenkephalin conjugated to the fluorophore TAMRA (Metenk-TAMRA), was used to detect the expression of DOPr on N/TERT-1 keratinocytes. Cells were seeded in triplicates in 6-well plates and grown to 70% confluence in K-SFM supplemented with BPE, EGF and 0.4 mM Ca^{2+} media. On the day of the experiments BPE- and EGF-free K-SFM was used. Cells were either treated with 1 µM dexamethasone for 1 h or left untreated. Thereafter both groups were trypsinized, washed and resuspended in 500 µl of 1X PBS (Ca^{2+}, Mg^{2+}-free). The cells were then incubated on ice with 100 nM Metenk-TAMRA or left untreated for the unstained control for 20 min, followed by sorting in the BD FACS Canto™ II. The observed cell populations were gated to isolate TAMRA-positive cells. Analysis was carried out by gating the live cell population in unstained/untreated cells. Further gating based

on this selected population helped quantify Metenk-TAMRA positive cells in the dexamethasone treated and untreated cells. This analysis was carried out using the Flowjo software. Obtained population numbers were recorded and then plotted using GraphPad Prism5 (GraphPad Software Inc., San Diego, CA, USA).

Statistical Analysis

Results for 3 experiments are shown as means ± Standard error mean (SEM). The statistical significance of differences between the control group and treatment groups was determined by a Two-Way ANOVA and Bonferroni's post hoc comparison. A 5% level of probability was considered significant.

Cosinor Analysis

To analyse rhythmicity in expression, a single-component cosinor model $\mathrm{RQ(t)} = \mathrm{M} + \mathrm{Acos}\left(\frac{2\,\pi}{\mathrm{P}}\mathrm{t} + \phi\right)$ was fitted to the expression profiles obtained from the qPCR data. M represents the midline statistic of rhythm (mesor), A the amplitude and P the period of oscillation, fixed at 24 h (Cornelissen, 2014). The angle φ is the acrophase, the time at which the maximum RQ occurs in each cycle. For each gene, regression of the model against experimental data yields estimates of M, A and ϕ and associated statistics. The 5 h time point was omitted from the analysis as its inclusion did not yield a proper fit of the cosinor model to the PER2 control data. The reason may be the strong induction of PER2 expression caused by dexamethasone (Cheon et al., 2013).

Results

N/TERT-1 keratinocytes exhibit a robust circadian rhythm upon synchronization with 1µM Dexamethasone

N/TERT-1 keratinocytes were chosen as the cellular model of our investigation since they are immortalized keratinocytes. Their applicability for studies on circadian rhythms was assessed by examining the circadian rhythm of the core clock genes *Per2* and *Bmal1*. It was previously reported that the expression of *Bmal1* peaked at 5-10h and *Per2* peaked at 25h post synchronization in PHKs (Janich et al., 2013). In keeping with this finding, N/TERT-1 keratinocytes in our study exhibit a similar and successive pattern in the expression of *Bmal1* and *Per2* (Fig 12 A and B). The peak expression of *Per2* mRNA was observed at 15-20 h post synchronization. *Bmal1* was the first core clock gene to peak 10 h post synchronization. The time between two successive peaks in *Bmal1* expression was 25 h, which approximately corresponds to a circadian rhythm. These results also indicated that dexamethasone, a glucocorticoid, is a potent synchronizer of N/TERT-1 keratinocytes.

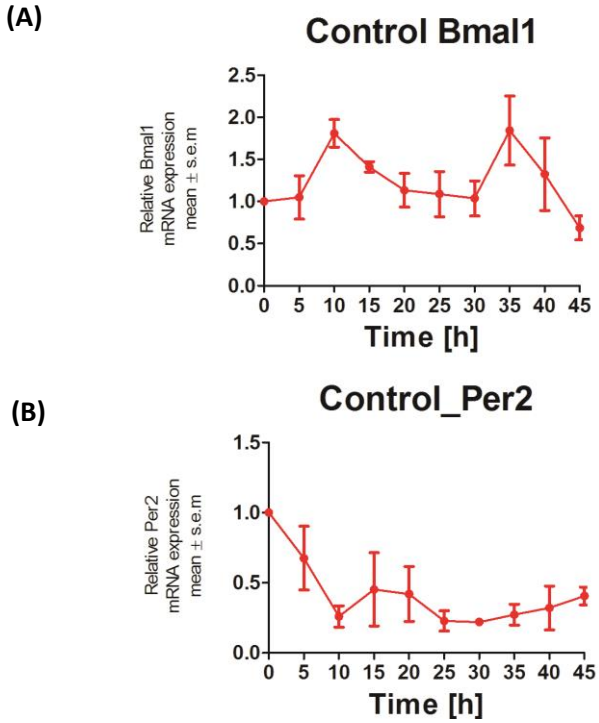


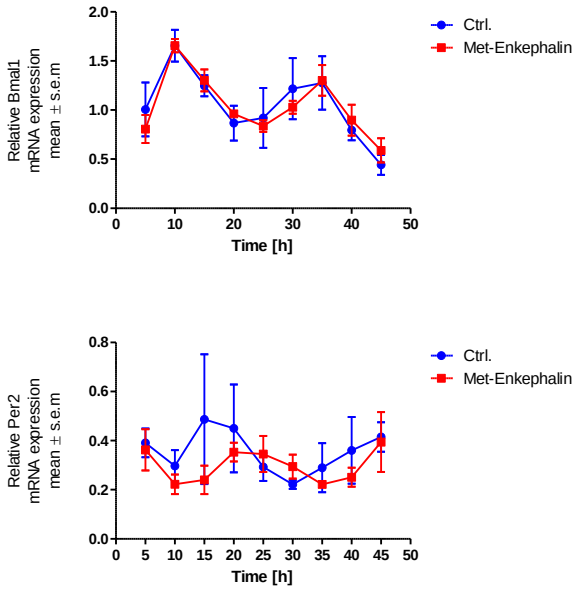
Figure 12- N/TERT keratinocytes exhibit robust circadian rhythms: Upon synchronization with 1uM Dexamethasone: N/TERT-1 keratinocytes were synchronized and gene expression was measured at indicated times. The cells exhibited peak expression in (A) Bmal1 at 10 h and (B) *Per2* at 15-20 h

Metenkephalin (Metenk) treatment induces a phase shift in *Per2* expression

Given the low abuse liability (Negus, Gatch, Mello, Zhang, & Rice, 1998) and lack of physical dependence associated with the DOPr (Brandt, Furness, Mello, Rice, & Negus, 2001) we next sought to assess the effect of the DOPr agonist Metenk on the various parameters that define circadian rhythms. To this end we subjected the obtained gene expression profiles upon Metenk treatment (Figure 13A) to routinely used cosinor analysis.

Figures 13B shows the mathematical fits to the *PER2* and *BMAL1* expression profiles from the control and the Metenk-treated cells. All expression profiles cycle rhythmically. While the mesors and amplitudes are not significantly affected by the treatment, the mean acrophases in the *PER2* expression profiles show a statistically significant 5.6 h delay following Metenk treatment *vs*. control (Figure 13C). For *BMAL1*, an acrophase shift of 1.7 h is obtained, but this difference is not statistically significant. Figures 13 B (b and d) provide a graphical representation of the elliptical 95% confidence regions for the amplitude-acrophase pairs estimated from the cosinor analysis showing the significant shift in peak *PER2* expression caused by Metenk.

(A)



(B)

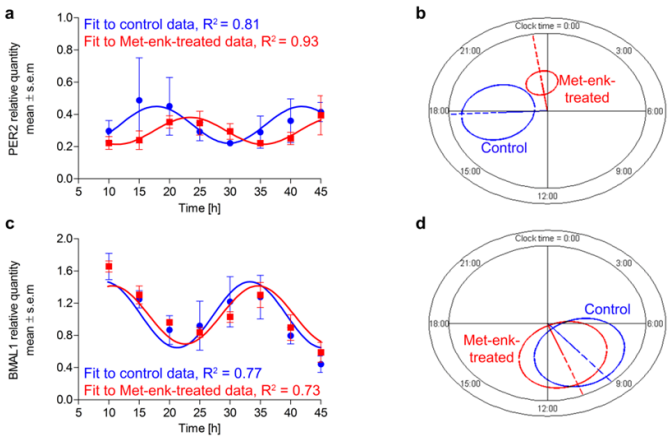


Figure 13 - Metenkephalin (Metenk) treatment induces a phase shift in *Per2* expression: (A) N/TERT-1 keratinocytes were synchronized and gene expression profiles were obtained at indicated times. The upper panel and lower panel show expression profiles of *Bmal1* and *Per2* in control cells and cells treated with DOPr agonist Metenkephalin (Metenk) respectively. It is apparent that the control group of cells exhibited peak expression in *Bmal1* at 10 h, *Per2* at 15-20h whereas the Metenk treated group of cells exhibited peak expression in *Bmal1* at 5–10 h, *Per2* 25 h. n=3 in all panels, results are expressed as mean ± SEM. (B) In all cases the period is fixed at 24 h. The R^2 coefficients indicate goodness-of-fit or percentage of rhythm in the data. (a) Regression of the cosinor model against the *PER2* mRNA expression profiles (pooled data from n = 3 experiments) shows a 5.6 h phase shift in the Metenk-treated (red) cells compared to control (blue). (b) The 95% confidence regions (ellipses) obtained from the *PER2* regressions are distinct; acrophases ϕ (clock times indicated by the dashed lines) are significantly different. (c) Regression against the *BMAL1* control and Met-enk-treatment data yields no difference in rhythmicity. (d) The *BMAL1* 95% confidence regions overlap; A and ϕ are not significantly different

Table 2

Data	M [RQ] (mean ± std. error)	A [RQ] (mean and 95% CI)	ϕ [h] (mean and 95% CI)	p-value*
PER2 control	0.34 ± 0.017	0.11 (0.030 to 0.20)	17.8 (15.0 to 21.0)	0.016
PER2 Met-enk- treated	0.30 ± 0.0077	0.080 (0.050 to 0.12)	23.4 (21.6 to 25.1)	0.0013
BMAL1 control	1.1 ± 0.08	0.41 (0.070 to 0.75)	9.29 (5.34 to 13.3)	0.025
BMAL1 Met-enk-treated	1.1 ± 0.08	0.36 (0.030 to 0.70)	10.6 (5.63 to 15.1)	0.038

Table 2: Results of the cosinor analysis carried out on gene expression profiles of *Bmal1* and *Per2* in control and Metenk treated cells were tabulated. It is apparent that Metenk treatment induced a significant difference (p=0.0013) in the acrophase of *Per2* expression

Metenkephalin treatment induces a change in clock-controlled gene (CCGs) expressions.

It has been shown that Dec2 (BHLHE41/Sharp1), DBP (D site of albumin promoter (albumin D-box) binding protein) and Tef (Thyrotroph Embryonic Factor) belong to the PAR bZIP (Proline and Acidic amino acid-Rich basic leucine ZIPper) family of protein and that they contain E-box sequences in their promoter regions (Wuarin J & Schibler U 2009; Montagner et al., 2016). These sequences are targets for binding of the *Bmal1* and Clock heterodimer which then induce the rhythmic expression of their target genes. *Per2* is known to bind to this heterodimer and thus inhibit the expression of the target genes *Dec2, DBP* and *Tef*. Hence we hypothesized that if the phase shift in *Per2* expression was not a stochastic effect it would affect the expression of the above mentioned genes. We found that the phase shift in *Per2* gene expression in N/TERT-1 keratinocytes does indeed induce significant changes in DBP and Tef expression at the 30^{th} hour and 40^{th} hour post synchronization. However no significant changes were observed in Dec2 expression (Refer to Figure 14)

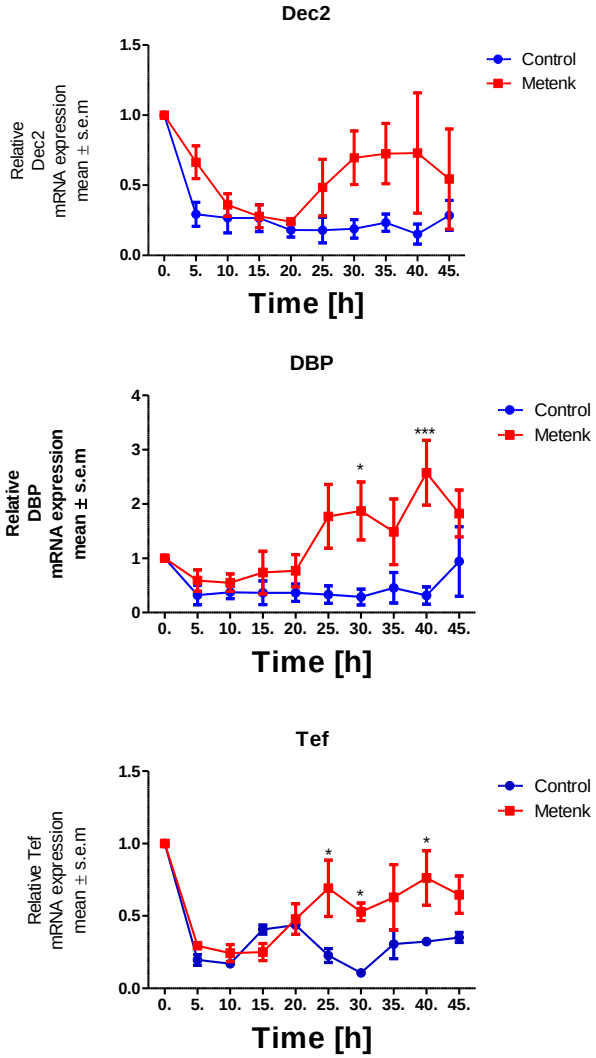


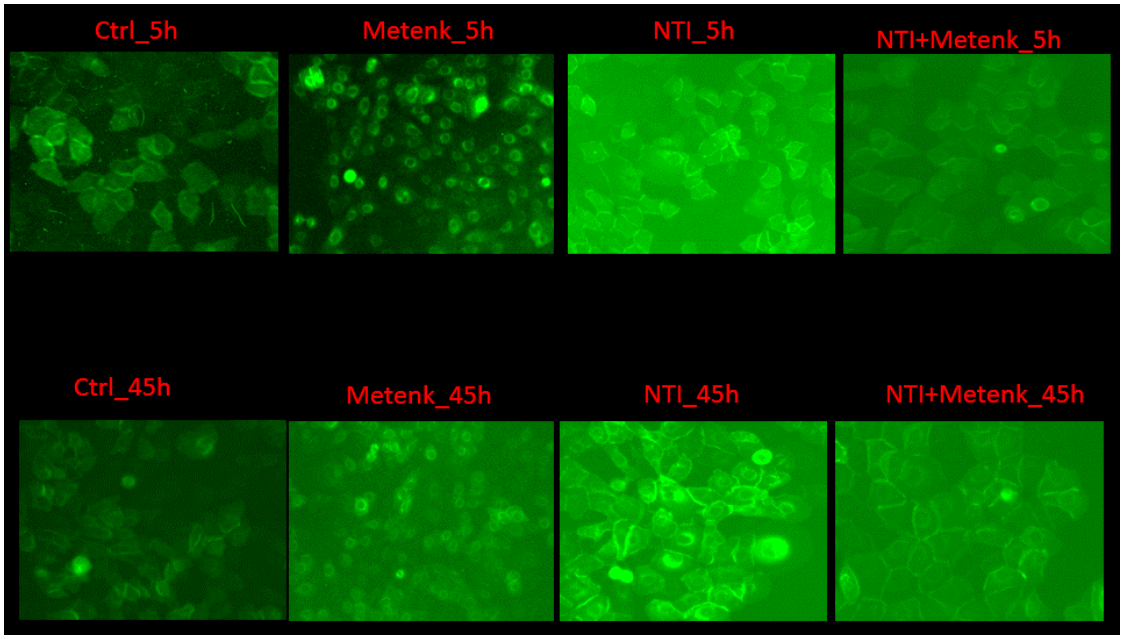
Figure 14 - Metenkephalin treatment induces a change in clock controlled gene expression in N/TERT-1 keratinocytes: There wasn't a significant change in *Dec2* expression as a result of Metenk treatment. However 25h post synchronization the Metenk treated group showed significant changes in *DBP* and *Tef* expression. The data here are the means ± SEM of three independent experiments (n=3). Two way ANOVA reveals *p < 0.05, *** p<0.001

Metenkephalin treatment internalizes the DOPr

In an attempt to decipher the molecular mechanisms involved in inducing a phase shift in *Per2* expression we decided to bias the system by over-expressing the DOPr. Since the DOPr was tagged with GFP we were able to visualize the change in pattern of expression of DOPr upon Metenk treatment. We observed that from being localized at the periphery of the cell the DOPr was internalized and localized to the perinuclear area in the cytoplasm. This condition could be observed from the 5^{th} hr of the experiment until the 45^{th} hr of the experiment (refer to Fig 15 A)

When core circadian clock gene expressions *BMAL1* and *Per2* were studied it was found that Metenk treatment induced a similar pase shift in *Per2* expression in DOPr overexpressing cells (refer to Fig 15 B) as was seen in the WT cells suggesting that DOPr biasing doesn't signicantly affect the phase shifting effects that Metenk has in WT cells. This could perhaps be because of the lack of signalling molecules.

(A)



(B)

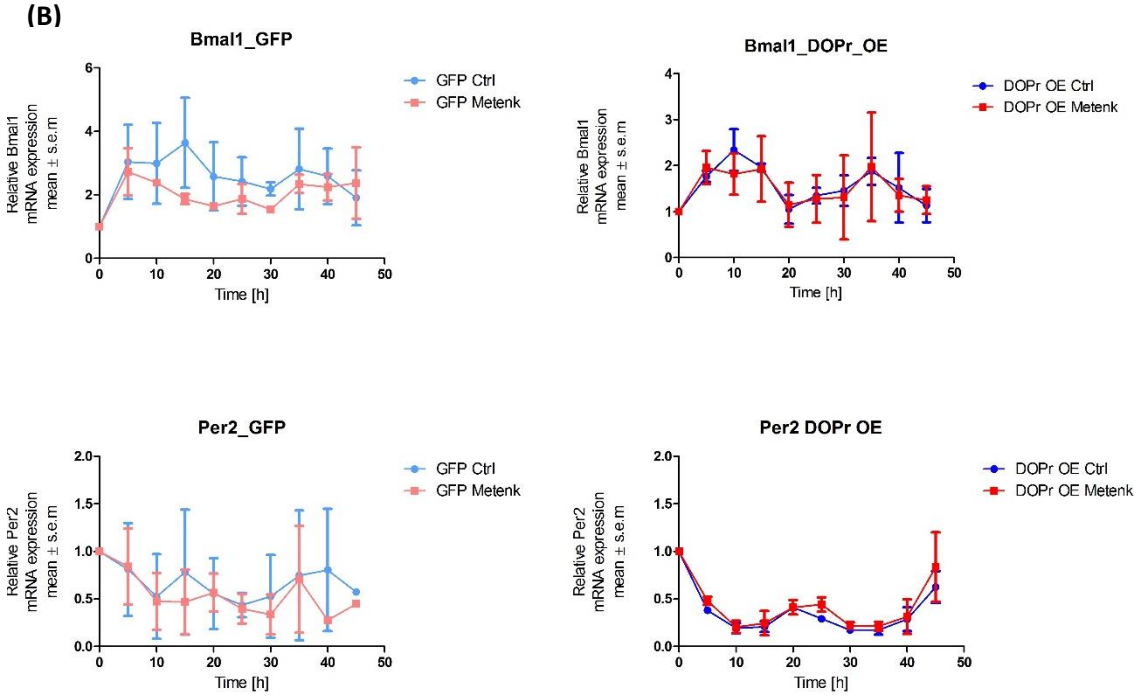


Figure 15 - (A) DOPr-GFP overexpressing (DOPr OE) N/TERT-1 keratinocytes exhibit DOPr localized to the cell membrane in control (untreated) cells. This localization remains unaffected in DOPr antagonist i.e. NTI and combined NTI and agonist (Metenk) treated cells . However upon Metenk treatment the receptor is internalized and appears to undergo relocalization to the perinuclear region in the cytoplasm (B) The analysis of the expression of the core circadian clock genes BMAL1 and Per2 revealed that Metenk treatment induced a similar phase shift in *Per2* mRNA expression as was observed in wildtype N/TERT-1 keratinocytes.

Metenkephalin treatment induced activation of DOPr results in nuclear co-localization of DOPr and βarr1.

It was previously reported that internalization of the DOPr occurs via the beta arrestin pathway (βarr) pathway (Kang et al., 2005). It has also been reported that βarr1 becomes localised to the nucleus (Kang et al., 2005) thus to test the hypothesis that βarr1 undergoes nuclear localization we treated the cells with 100nM Metenk for 5min and we were surprised to find that along with βarr1 even the DOPr undergoes nuclear localization (refer figure 16A and 16 B). These results were further confirmed when the nuclear fractions of cells overexpressing CFP tagged βarr1 and GFP tagged DOPr were subjected to a CFP pulldown using an antiCFP antibody. The pulldown lysate was then subjected to a western blot analysis and tested for the presence of DOPr, CREB, βarr1, CFP and the input control (refer figure 16C).

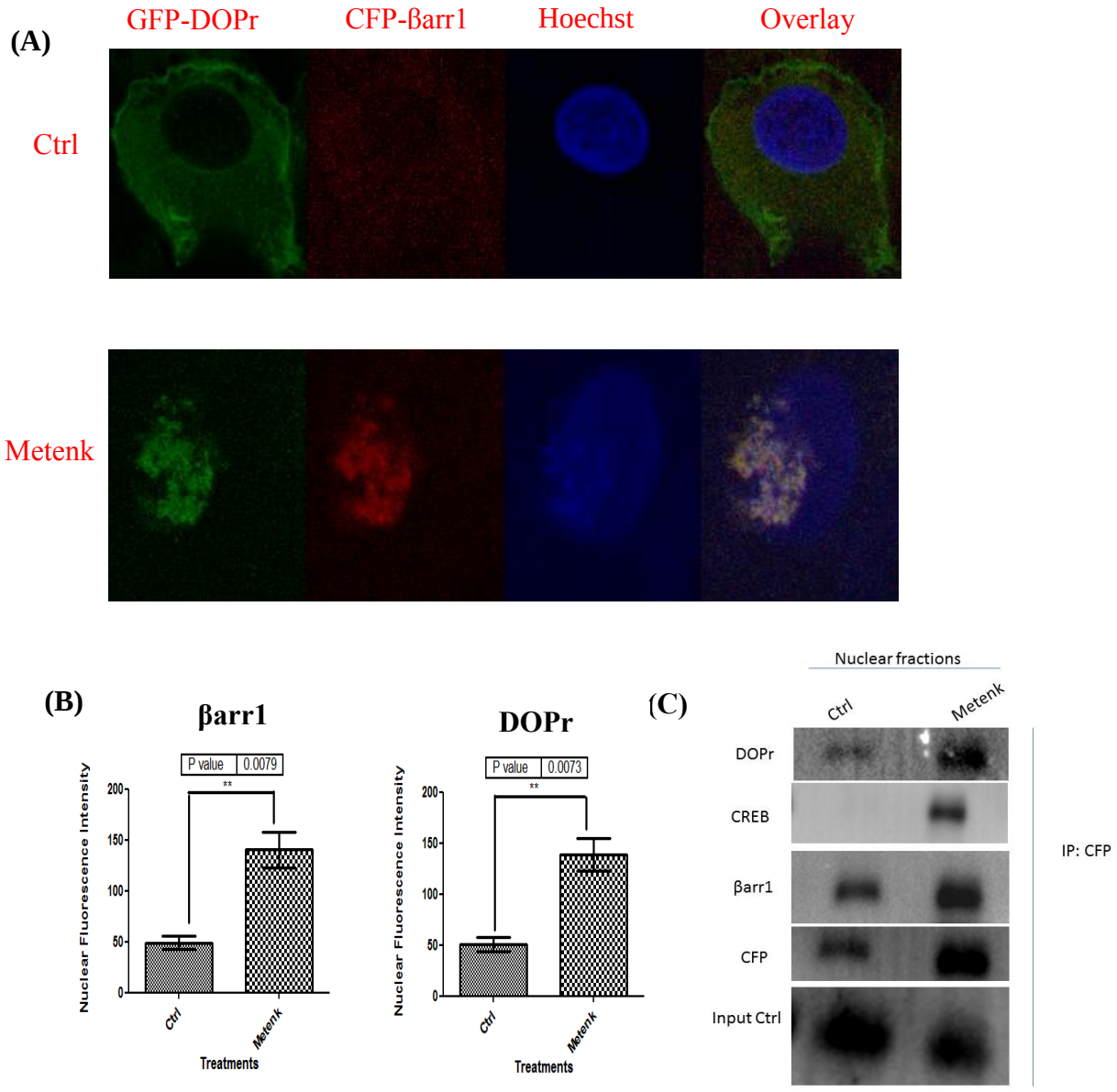


Figure 16 - Activation of DOPr leads to nuclear co-localization of DOPr and βarr1: (A) Confocal visualization of N\TERT-1 keratinocytes overexpressing GFP-DOPr and CFP-βarr1 incubated without (Ctrl) or with 100nM Metenkephalin (Metenk) for 5 min before fixation. It can be seen that upon Metenk treatment both DOPr and βarr1 co-localize in the nucleus. The images shown are representative of three independent experiments n=3. (B) Values of fluorescence signals from acquired images were quantified using Image J software and are expressed as the mean ± SD **p<0.01 (C) N/TERT-1 keratinocytes overexpressing GFP-DOPr and CFP-βarr1 were incubated without (Ctrl) or with 100nM Metenkephalin (Metenk) for 5 minutes. The nuclear extracts were then used for a pulldown using an anti CFP antibody (Biorbyt, orb256068). The pulldown lysates were then tested for DOPr, CREB, βarr1 and CFP using their appropriate antibodies. Anti-Beta Arrestin 1 antibody (Abcam, ab31868), Anti-Delta opioid receptor antibody (Abcam, ab 176324) Anti CREB Antibody (PA1-850) The blots shown are representative of three independent experiments (n=3). The input controls (Input Ctrl) are loading controls to ensure that comparable amounts of protein were loaded. These samples were collected before the Ctrl and Metenk treated nuclear sample fractions were treated with the anti-CFP antibody.

Metenkephalin treatment enhances and induces a phase shift in rhythmical βarr1 binding on the *Per2* promoter

In addition to undergoing nuclear localization βarr1 was reported to bind and acetylate histone protein H4 within target genes' promoters (Kang et al., 2005). Thus to test our hypothesis that βarr1 binds and acetylates histone protein H4 in the promoter of *Per2* we carried out chromatin immunoprecipitation (ChIP) experiments wherein we pulled down βarr1 from chromatin lysates and subsequently carried out a real time quantitative PCR to compare the amounts of βarr1 bound to the *Per2* promoter in control and Metenk treated N/TERT-1 keratinocytes over a 45 hour period. We found that βarr1 binding in control cells peaked at 15th -20th hr and in Metenk treated cells it peaked at 20th -25th hr (refer to figure 17)

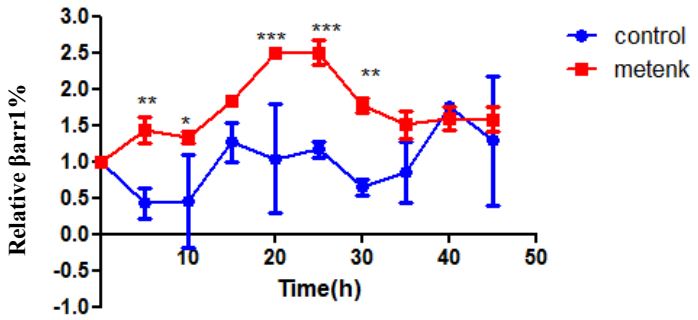


Figure 17 - Metenkephalin treatment enhances and induces a phase shift in rhythmical βarr1 binding on the *Per2* promoter: Chromatin Immunoprecipitation (ChIP) experiments were carried out with anti-βarr1 antibodies and *Per2* promoter sequences in the input DNA and that recovered from antibody-bound chromatin segments were analysed by qPCR. The data were normalized to the corresponding input control and subsequently to the 0h control. All data were then normalized to the corresponding 0hr control. The data shown are the means ± SEM of three independent experiments (n=3). Two way ANOVA reveals *p < 0.05.

Metenkephalin enhances βarr1 binding to acetylated H4 in the *Per2* promoter

We hypothesised that since βarr1 binding to the *Per2* promoter peaks at 25h post synchronization then in order to facilitate the induction in *Per2* expression it must bind to CREB and acetylate histone 4 in the *Per2* promoter. To test this hypothesis we carried out re-ChIP experiments with cells synchronized for 25h with or without Metenk treatment. We then pulled down βarr1 and then subsequently pulled down CREB and acetylated histone 4 (H4K16ac) using anti β-arrestin1, anti CREB and anti H4K16ac antibodies. Upon doing so we found that there was a concomitant increase in binding of CREB to the *Per2* promoter as well as there was increase in H4 acetylation.

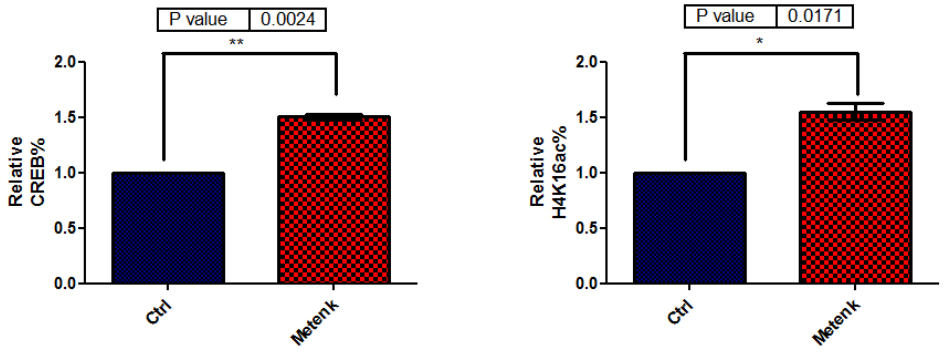


Figure 18 - Metenkephalin enhances βarr1 binding to acetylated H4 in the *Per2* promoter: Re-ChIP experiments were carried out on N/TERT-1 keratinocytes which were synchronized for 25h with or without Metenk treatment. In these experiments, antibodies were first used to target and pulldown βarr1 the resulting eluates were then used for pulling down CREB and acetylated histone 4 (H4K16ac) and the presence of the *Per2* promoter sequences in the input DNA and that recovered from antibody-bound chromatin segments were analysed by qPCR. The data were normalized to the corresponding input control. All data were then normalized to the corresponding 25h control. The means ± SEM of three independent experiments (n=3) are presented. The data was then subjected to a paired two tailed t- test and the P values obtained for CREB and H4K16ac were 0.0024 and 0.0171 respectively.

DOPr expression maintains rhythmicity in *Per2* expression.

We initially knocked down the DOPr to test whether the phase shift seen in *Per2* expression as a result of Metenk treatment, was DOPr mediated. To our surprize we found that DOPr knockdown cells (DOPr KD) didn't exhibit rhythmicity in *Per2* expression at both the mRNA and protein level (Figure 19 A, B, C and E). This finding, together with the findings that (i) both DOPr and βarr1 localize to the nucleus (Figure 16 A, B, C) (ii) βarr1 and DOPr bind to the *Per2* promoter (Figure 17 and Figure 19D), led us to the conclusion that DOPr maintains rhythmicity in *Per2* expression.

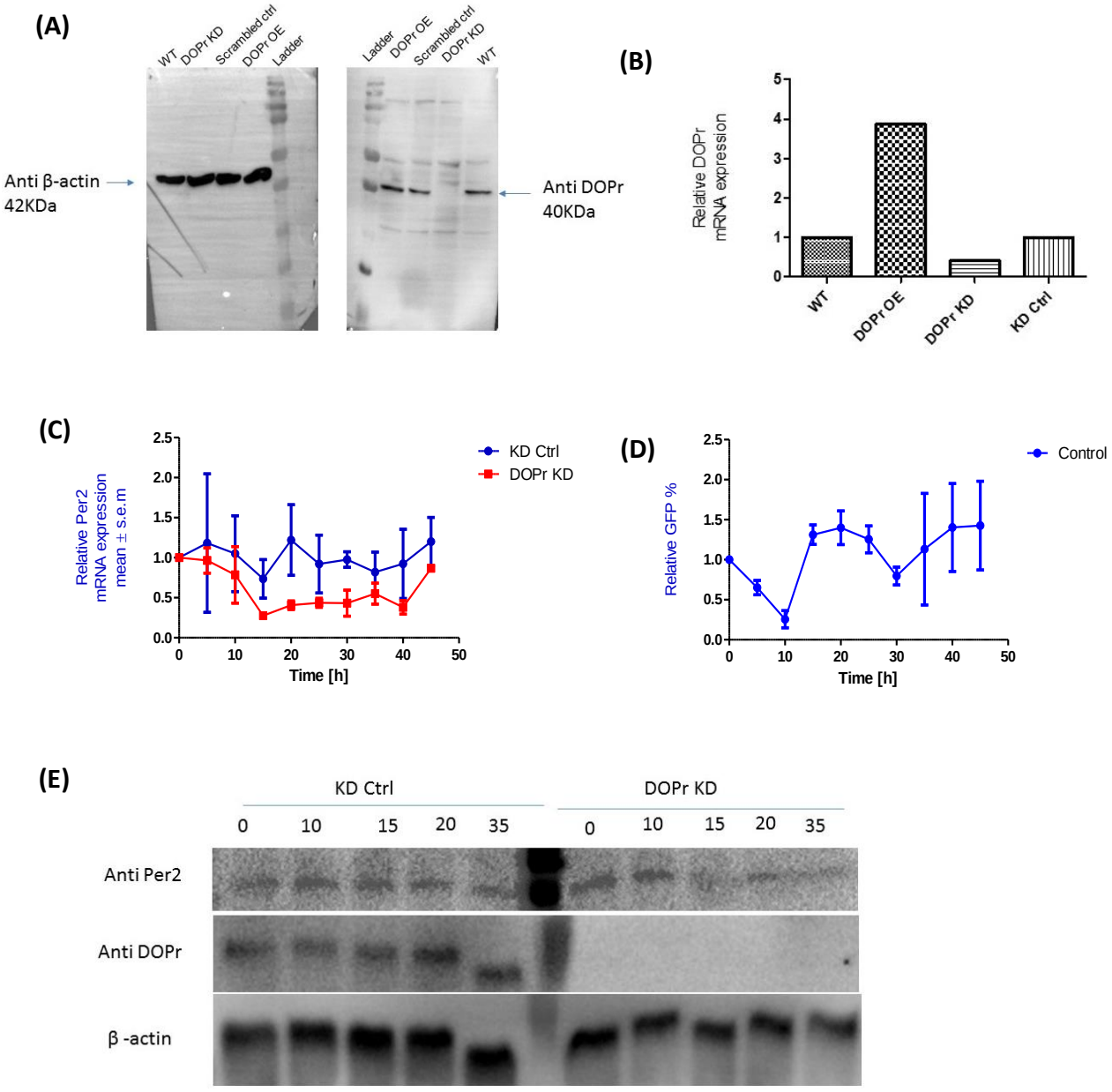


Figure 19 - DOPr expression is essential for maintaining rhythmicity in *Per2* expression: (A) Constitutive knockdown of the DOPr (KD DOPr) was possible using the described lentiviral construct. This was validated by western blot using an anti-Delta Opioid Receptor antibody (ab176324), a band was seen at the 40kDa mark when used at a 1/1000 dilution with anti β-actin antibody (A5441, Sigma) as the loading control and (B) quantitative realtime polymerase chain reaction (qPCR), n=1. To test for rhythmicity in *Per2* expression, qPCRs (C) and western blot analysis (E) were carried out using non targeting control (KD Ctrl) and DOPr KD cells where L, represent the ladder. It was observed that the DOPr KD cells did not exhibit rhythmicity in *Per2* expression when compared to the KD Ctrl cells which exhibited a peak in *Per2* mRNA expression at 20h and protein expression at 10h post synchronization, n=2. (D) Chromatin immunoprecipitation experiments (n=2) targeting the *Per2* promoter in GFP tagged DOPr overexpressing N/TERT-1 keratinocytes showed that DOPr binds rhythmically to the *Per2* promoter.

Metenkephalin recapitulates effects on clock gene expression seen in Primary Human Keratinocytes

In an attempt to validate our findings in another cell line, we tested the effects of Metenk on primary human keratinocytes by using a similar protocol as described in figure 10. We found that Metenk induced a significant increase in *Bmal1* and *Per2* expression at the 20th and 25th hour post synchronization. We also observed a similar phase shift in Per2 expression in PHK as that seen in N/TERT-1 keratinocytes as a result of Metenk treatment (refer to Figure 20B). We also observed a similar trend in increase in *Dec2, DBP* and *Tef* expression as we had seen in N/TERT-1 keratinocytes (Figure 21).

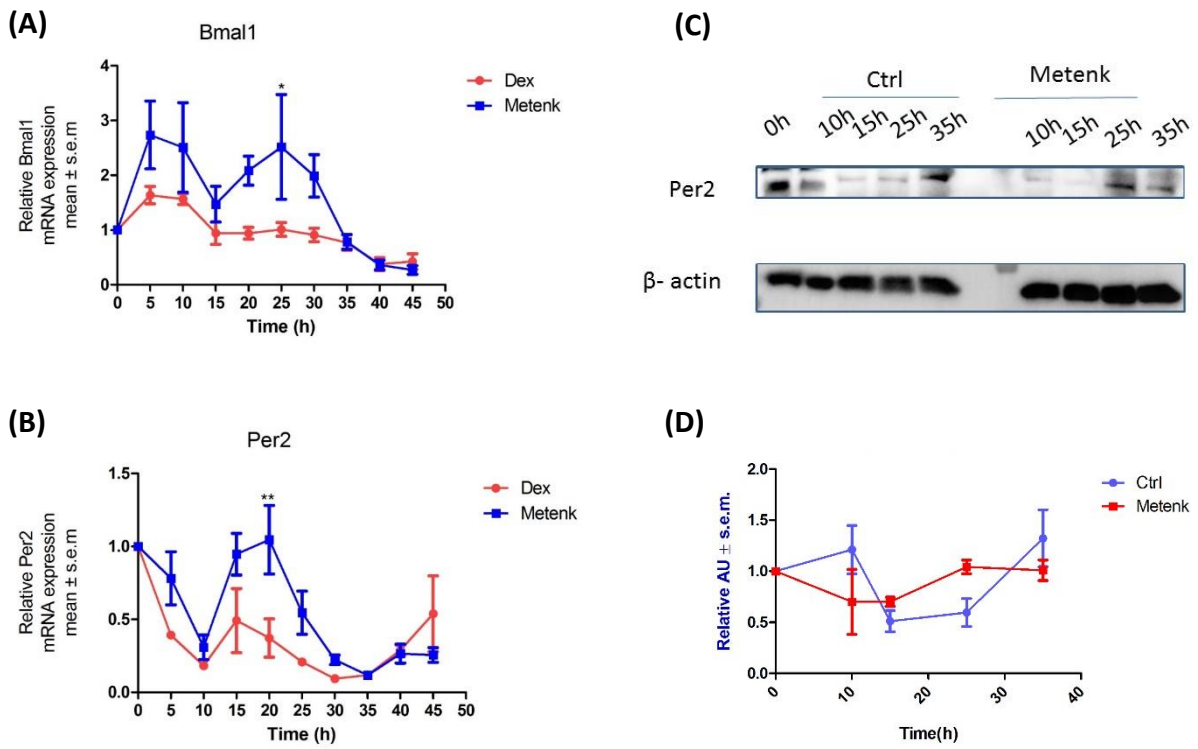


Figure 20 – Metenkephalin (Metenk) treatment induces a change in Bmal1 and Per2 expression in Primary Human Keratinocytes (PHK): There was a significant change in *Bmal1* (A) and *Per2* (B) gene expression as a result of Metenk treatment as compared to the control group (Dex) of cells at 25h and 20h post synchronization respectively. Protein lysates at indicated time points were probed for Per2 and loading control β-actin expression at indicated time points. The target protein signals (C) were quantified using Image J software and the data was normalized to β-actin followed by a further normalization to the 0h control (D). The data here are the means ± SEM of three independent experiments (n=3). Two way ANOVA reveals *p < 0.05, *** p<0.001

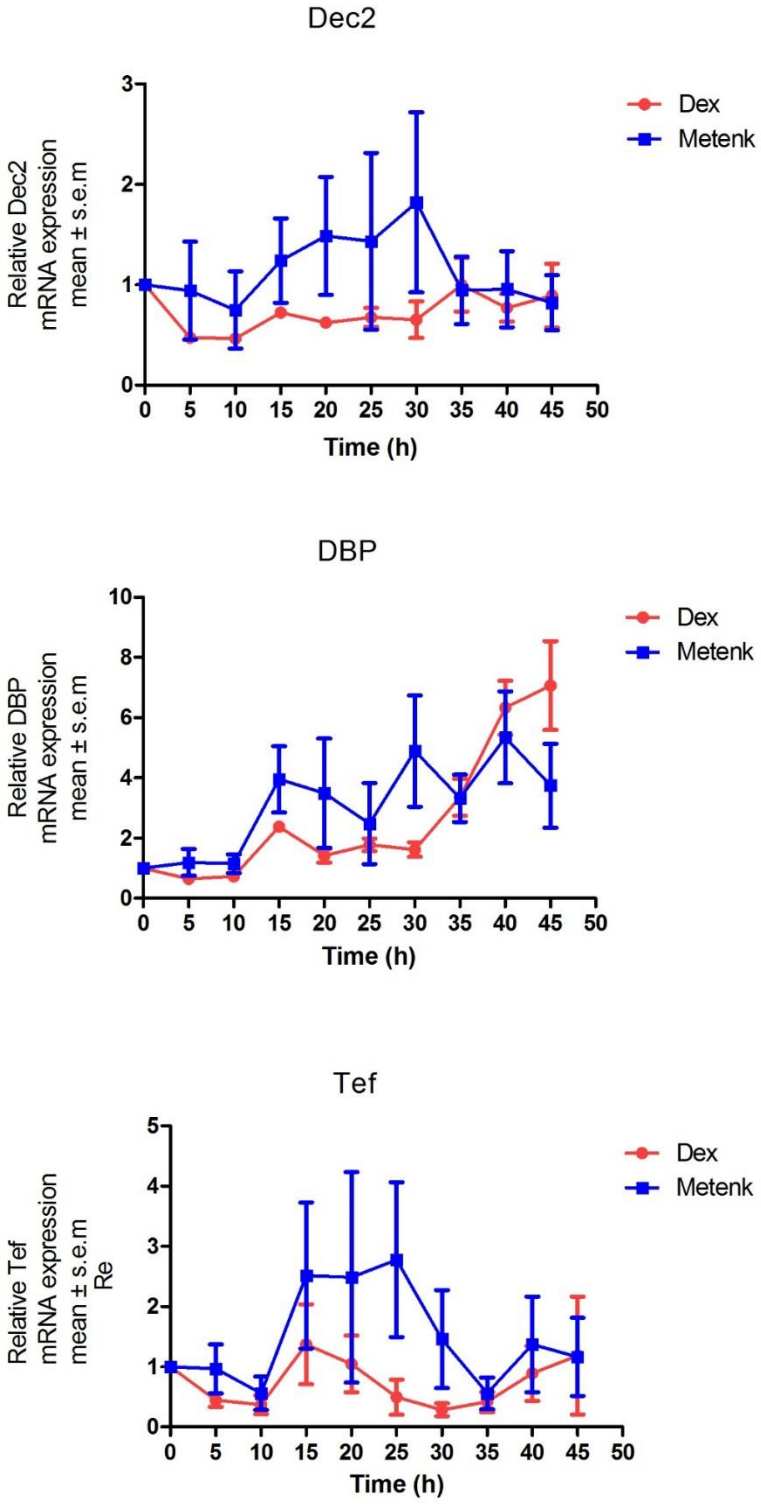


Figure 21 - Metenkephalin treatment induces a change in clock controlled gene expression: There wasn't a significant change in *Dec2, DBP and Tef* expression as a result of Metenk treatment. The data here are the means ± SEM of three independent experiments (n=3) which was then subjected to a two way ANOVA.

Metenkephalin induces changes in *p53*, *CdK1* and *Cyclin D1* in Primary Human Keratinocytes

Previous studies based on buccal mucosa have reported a correlation between phase shifts in *Per2* expression and cell cycle gene expressions such as *p53, Cdk1* and *Cyclin D1* (Tan XM et al., 2015). Likewise cell cycle genes have been reported to be under the control of circadian clock controlled genes (Bjarnason et al., 2001). *Per2* is an important clock gene that regulates many cell cycle genes (Fu L et al., 2002; Hua H et al., 2006). Fu et al., reported that the expression of Cyclin D1, Cyclin A and Cyclin E were increased in Per2 mutant mice. Likewise Hua H et al., reported that Per2 over-expression increased P53 expression and reduced c-Myc expression in Lewis lung cancer cells (LLC) and breast cancer cells (EMT6). In addition, clinical studies have shown that Per2 expression is reduced in cancer patients, and it plays a tumour suppressor role in breast cancer, skin tumours, hepatocellular carcinoma, colorectal cancer and head and neck squamous cell carcinoma (Hsu CM et al., 2012; Lengyel Z et al., 2013; Kuo SJ et al 2009; Lin YM et al., 2008; Sotk M et al., 2013). Hence we asked if the observed phase shift in *Per2* gene expression as a result of Metenk treatment in our study had an effect on the expression of cell cycle genes *p53, Cdk1* and *Cyclin D1*. Indeed we found that Metenk induced a significant changes in gene expressions of *p53* at 10h, and *Cdk1* at 10h and 15h post-synchronization (Figure22). More specifically there was a decrease in *p53* expression and an increase in *Cdk1* expression at the indicated time points as a result of Metenk treatment. However we didn't see any significant changes in *Cyclin D1* expression.

Studies from our laboratory have shown activation of the DOPr by Metenk to drastically inhibit proliferation and differentiation of keratinocytes (Neumann C et al., 2015). This suggests that activation of DOPr in keratinocytes may lead to acquisition of a state of cell cycle arrest (also known as a quiescent) (Coller HA et al., 2006). Furthermore p53 has been

found to induce quiescence and suppress senescence (Zoya N et al., 2010). Hence taking into consideration that DOPr activation reduced p53 expression, we asked if DOPr activation lead to an induction of senescence. Hence we tested for changes in Lamin B1 levels in Metenk treated cells, since elevated levels of Lamin B1 have been shown to trigger senescence (Barascu et al., 2012; Dreesen O et al., 2013) and indeed we found Metenk treated cells to accumulate Lamin B1 (Figure 22 C).

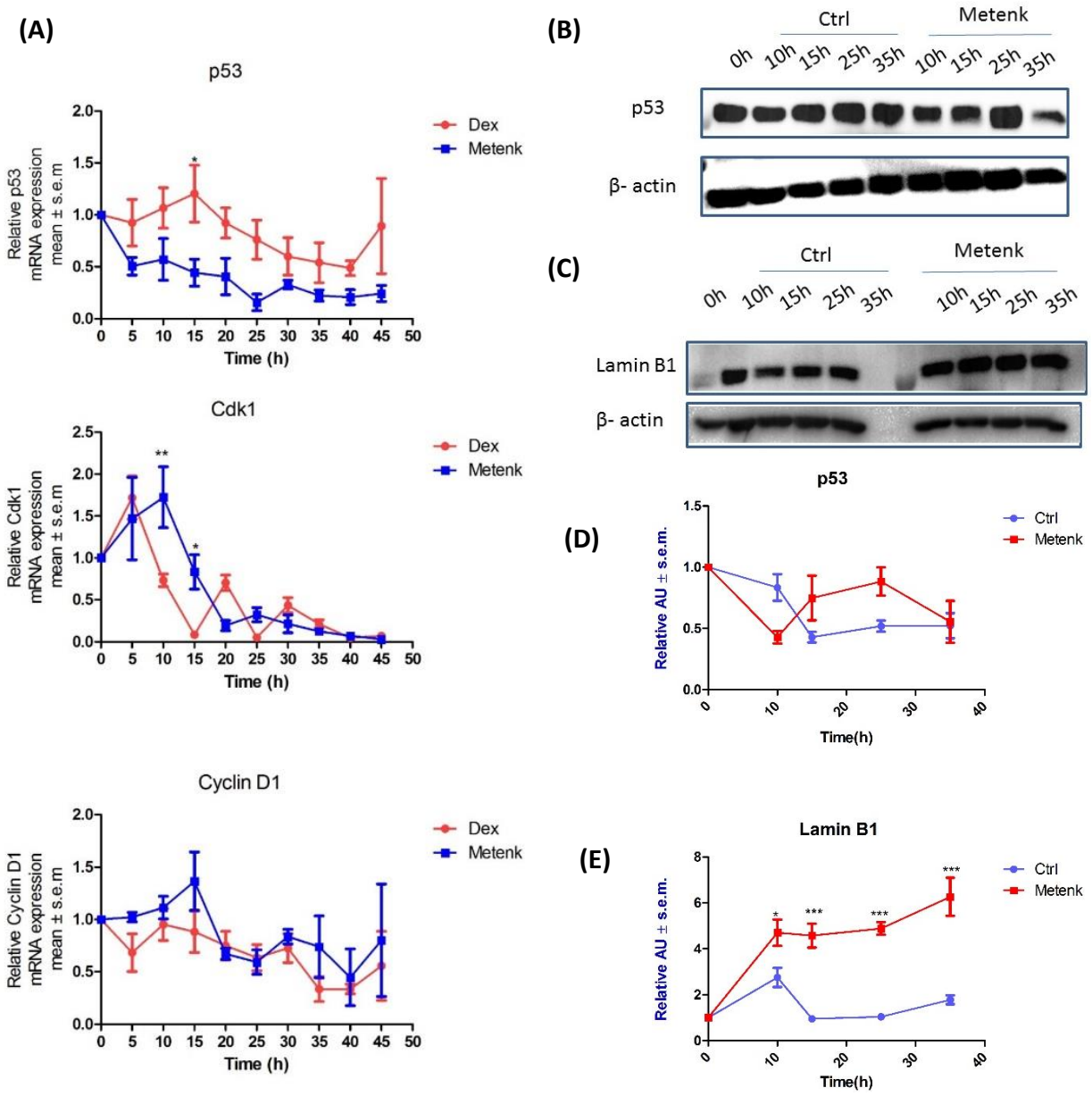


Figure 22 - Metenkephalin treatment induces a change in cell cycle gene expression: There wasn't a significant change in *Cyclin D1* expression as a result of Metenk treatment versus the control in Primary Human Keratinocytes (PHKs). However the Metenk treated group showed significant changes in p53 mRNA expression at the 10h and CdK1 mRNA expression at 10h and 15h post synchronization. Protein lysates were then subjected to a western blot analysis and probed for p53 , Lamin B1 and loading control β-actin using p53 antibody (DO-1): sc126 Santa Cruz Biotechnology; Lamin B1 Antibody (B-10): sc-374015 Santa Cruz Biotechnology and anti β-actin antibody (A5441, Sigma). The target protein signals were quantified using Image J software and the data was normalized to β-actin followed by a further normalization to the 0h control. The data here are the means ± SEM of three independent experiments (n=3 which was then subjected to a two way ANOVA. Two way ANOVA reveals *p < 0.05, *** p<0.001

DOPr expression is not affected by dexamethasone treatment

A ligand binding assay was conducted to detect the variation in receptor expression caused by dexamethasone-mediated circadian synchronisation using Metenk conjugated to fluorophore TAMRA (Metenk-TAMRA). The binding of Metenk-TAMRA indicates that approximately 3.8% of the gated live N/TERT-1 population expresses DOPr. This expression does not undergo significant variation upon treatment with dexamethasone (Figure 23).

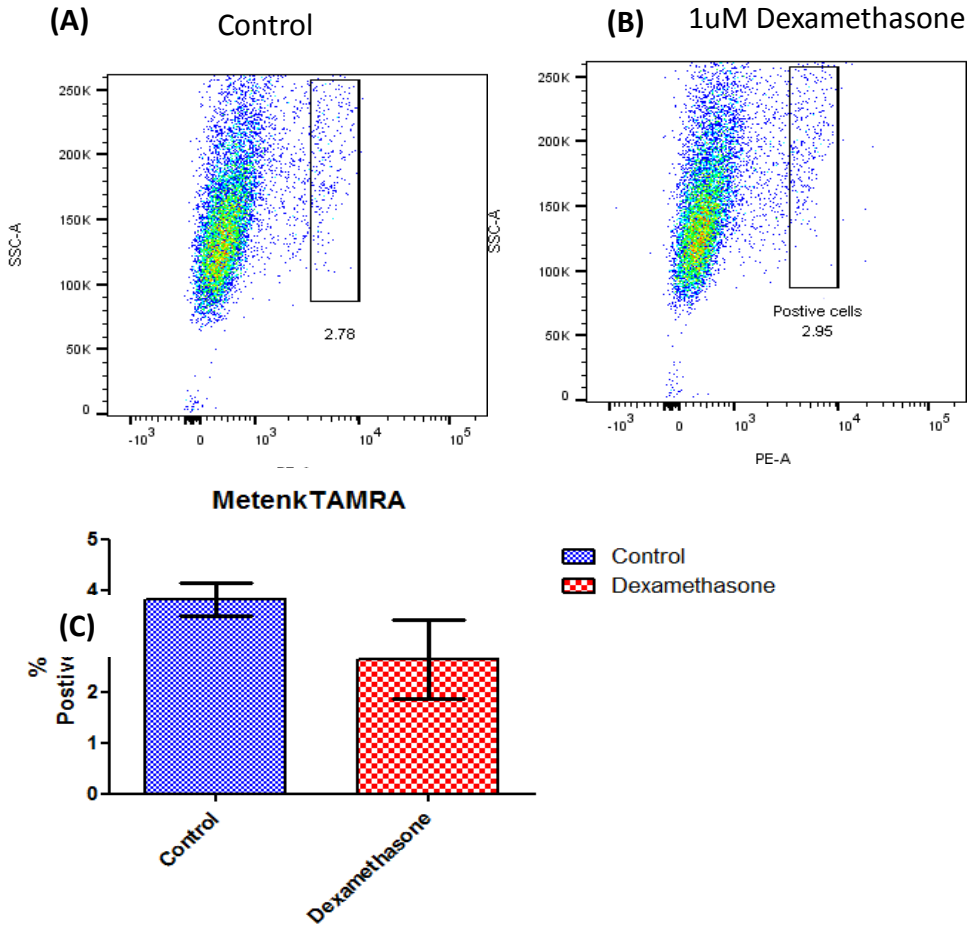


Figure 23. Dexamethasone treatment does not affect DOPr expression in N/TERT-1 keratinocytes. Ligand Metenk-TAMRA binding assay on (A) control N/TERT-1 and (B) dexamethasone treated cells identifies DOPr positive population. (C) Graphical representation of DOPr positive population percentage. n=4, results are expressed as mean ± SEM

Discussion

The Delta Opioid receptor (DOPr) was previously studied in the CNS system wherein its role in maintaining systemic homeostasis (Drolet et al., 2001) and modulating circadian activity was elucidated (Pacesova et al., 2015). Upon the discovery of the DOPr in the periphery such as in the skin and given its similar role in maintaining skin homeostasis (Bigliardi-Qi, Sumanovski, Buchner, Rufli, & Bigliardi, 2004; Bigliardi et al., 1998; Rachinger-Adam, Conzen, & Azad, 2011; A. T. Slominski et al., 2013; C. Stein, 1993, 2003; C. Stein, Hassan, Lehrberger, Giefing, & Yassouridis, 1993) we asked if DOPr activation could have an effect on circadian rhythmicity in skin. This we believe was a relevant question to ask because of the importance of circadian rhythms in regulating skin physiology (Plikus et al., 2015). In the first section of the discussion we will discuss the pathway by which DOPr activation influences circadian rhythms in keratinocytes and then we will discuss the implications of our findings.

As mentioned previously on the molecular level, circadian rhythms are governed by a feedback loop that is auto regulated; the positive arm consists of the *Bmal1* and Clock proteins which dimerize and interacts with the E'box elements of its target genes such *Cry* and *Per* which form the other arm. These proteins heterodimerize and then are phosphorylated in the cytoplasm and subsequently translocated to the nuclear compartment where they break the *Bmal1*-Clock heterodimer and thus repress their own transcription. These cycle of events corresponds to one circadian cycle i.e. they occur over a period of 24 hours (Lowrey & Takahashi, 2004).

Taking these facts into consideration our immediate question then was, does activation of the DOPr affect core clock gene expressions?

N/TERT-1 keratinocytes are a good model for circadian studies in skin:

N/TERT-1 keratinocytes are a cell line that were developed by immortalizing human keratinocytes (Dickson et al., 2000; Rheinwald et al., 2002). The establishment of this cell line as a model to study circadian rhythms in keratinocytes seemed to have several advantages, such as they are cost effective, easy to use, provide an unlimited supply of material and bypass ethical concerns associated with the use of animal and human tissue. As is with most cell lines, this cell line also provides us with a pure population of cells, which is valuable since it would help with generation of consistent samples and reproducible results (Kaur G & Dufour MJ 2012). Hence we asked whether the N/TERT-1 keratinocytes have a circadian rhythm and indeed we found N/TERT-1 keratinocytes to exhibit successive peaks in their core circadian clock gene mRNA expressions. These results were similar to those that were obtained from experiments carried out by us and reported by others on PHKs (Janich et al, 2013).

The method of synchronization used in this study relied upon the use of 1μM Dexamethasone which is a glucocorticoid. This method of synchronization finds its origin in the studies carried out by Prof Ueli Schibler and his group wherein they showed dexamethasone to induce rhythmic circadian gene expression (Balsalobre A et al., 2000). They had based their studies on several facts that were reported by other groups. Firstly, cells in the peripheral organs were reported to express the molecular components of the circadian clock (Dunlap JC 1999). Secondly, in some cases this peripheral clock was found to be entrained by light and signals secreted into the humor of the body by the suprachiasmatic nucleus (SCN) (Brown SA & Schibler U 1999; Emery P et al., 1998; Stanewsky R et al., 1998; Ceriani MF et., 1999). Thirdly, the latter observation was supported by a study which showed that serum could induce circadian gene expressions in rat-1 fibroblasts (Whitmore D, Foulkes NS, Sassone-Corsi P 2000). Fourthly, the plausible candidate in serum that could induce

circadian rhythms in gene expression appeared to be glucocorticoid hormones because their secretion appears to occur in rhythmic cycles and because the glucocorticoid receptor (GR) is expressed in most peripheral cell types. Finally this study by Balsalobre A et al., had concluded that dexamethasone induced synchronization has the advantage of synchronizing peripheral cells without affecting the circadian rhythm of the central circadian pacemaker in the SCN because the SCN lacks GRs.

Lastly, this method of synchronization as compared to synchronization mediated by serum also circumvents the pleiotropic effects that serum has on keratinocyte physiology such as an induction in terminal differentiation in keratinocytes and suppression of colony formation (Bertolero F, Kaighn ME, Camalier RF and Saffiotti U 1986)

Pathway by which DOPr induces a phase shift in *Per2* gene expression.

Given the low abuse liability (Negus, Gatch, Mello, Zhang, & Rice, 1998), lack of physical dependence (Brandt, Furness, Mello, Rice, & Negus, 2001), anti-stress effects (Drolet et al., 2001) and circadian activity modulating effects (Pacesova et al., 2015) exhibited by DOPr activation we asked how does activation of the DOPr by the endogenous ligand Metenkephalin affect the expression of circadian clock genes Bmal1 and Per2?

We found that activation of DOPr in both N/TERT-1 keratinocytes and PHKs resulted in a phase shift in Per2 expression which was validated by using routinely used cosinor analysis. Per2 is an important clock gene and previous research has indicated that Per2 serves a notable function in the occurrence and progression of cancer (Miyazaki et al., 2010; Yang X et al., 2009) and this warranted a further investigation into the relationship between DOPr activation and the phase shift in gene expression of Per2.

We then looked to the overexpression system for clues that would help us identify the molecular mechanisms responsible for this induction in phase shift in Per2 expression. We found that the

activation of the receptor using Metenk results in the internalization of the receptor from the 5th hr of synchronization until the end of the experiment or the 45th hr. Several studies have demonstrated that the βarr pathway leads to the internalization of activated GPCRs and in the case of DOPr binding of the βarr leads to the recruitment of components which are essential to clathrin mediated endocytosis of activated and phosphorylated receptor and eventual degradation and/or recycling of the receptor back to the surface (Lefkowitz & Shenoy, 2005).

A study prior to ours has shown activation of DOPr to trigger the nuclear localisation of βarr1. The same study also showed that upon nuclear localization βarr1 mediates the acetylation of the histones H4 in the promoters of its target genes (Beaulieu & Caron, 2005; Kang et al., 2005). We too, observed that activation of the DOPr by Metenkephalin resulted in the nuclear localization of not just βarr1 but also the DOPr. This re-localization also enhanced the binding of βarr1 to acetylated histone 4 in the promoter of the *Per2* gene and subsequently induced the expression of *Per2*. Recent studies have shown GPCRs such as adrenergic receptors, chemokine receptor CXCR4, and opioid receptors to undergo nuclear translocation and to also subsequently regulate gene expression. While these studies have conclusively shown that these receptors anchor themselves in the nuclear membrane and use canonical pathways to transduce signals that influence gene expression (Tadevosyan, Vaniotis, Allen, Hebert, & Nattel, 2012; Vaniotis, Allen, & Hebert, 2011; Vaniotis, Del Duca, et al., 2011; Wu & O'Connell, 2015a, 2015b) . Our present study shows that the DOPr binds to the *Per2* promoter and possibly induces its expression by acetylation of H4 in the *Per2*'s promoter. The need for the presence of DOPr to sustain circadian rhythms is supported by the fact that *Per2* expression has no apparent rhythm in the DOPr knockdown cells. Hence piecing these two observations together it appears that the expression of DOPr is necessary

for maintaining circadian rhythms and possibly homeostasis in skin. Since molecular signalling mechanisms are conserved in most cell types it seems practical to speculate that a similar mechanism exists in the CNS and hence the DOPr is essential for systemic homeostasis.

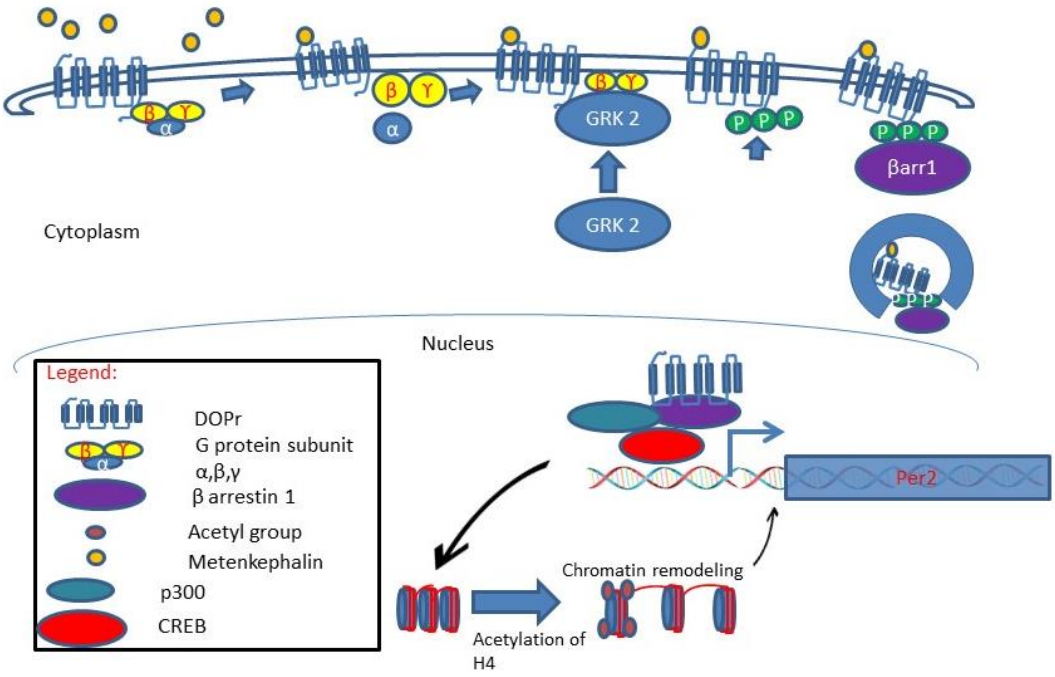


Figure 24: Pathway by which DOPr induces a phase shift in *Per2* gene expression: Stimulation of the delta opioid receptor (DOPr) by 100nM Metenkephalin (Metenk) leads to the hydrolysis of the G protein. G protein receptor kinase 2(GRK2) is recruited by the βϒ component of protein. GRK2 then phosphorylates the activated DOPr. This results in the subsequent recruitment of βarr1 and the necessary endocytic machinery which leads to the internalization of the DOPr. Upon internalization the DOPr and βarr1 co-localize to the nucleus where they bind to CREB in the promoter region of *Per2*. Thereafter histone acetylating factor p300 is recruited and goes on to acetylate histone H4 in the promoter of *Per2* thus inducing *Per2'*s expression.

Perspectives

Given that one of the main aims of research is to find its applicability in the lives of people it does seem rational to discuss steps that would be necessary to find the applicability of the above presented findings in clinical practise. To this end the following sections will deal with

experiments that would be necessary to conclusively to prove that the pathway described in figure 19 is the mechanism by which the activated Delta Opioid Receptor (DOPr) induces a phase shift in *Per2* expression and thus influences circadian rhythms. The following section will deal with the possible applicability of these findings in DOPr mediated therapies

Immediate experiments

Our above described data suggests that activation of the DOPr by Metenkephalin (Metenk) leads to a phase shift in the core clock gene, *Per2*'s expression. Using an overexpression system we have gone on to show that activation of the receptor results in the nuclear co-localization of the βarr1 and DOPr complex. By employing chromatin immunoprecipitation (ChIP) experiments we have been able to show that βarr1 and DOPr bind to the *Per2* promoter and also that Metenk induces a phase shift in βarr1's binding to the *Per2* promoter. Re-ChIP experiments have conclusively shown that treatment with Metenk enhances the binding of βarr1 to the CREB and acetylated histone 4 (H4) proteins in the promoter of *Per2*.

These results suggest but do not conclusively indicate that phase shift in βarr1 binding to the *Per2* promoter results in the phase shift of *Per2*'s gene expression. To make these conclusions it would be necessary to carry out experiments using βarr1 knockdown cells (βarr1 KD). It seems rational to devise experiments similar to those described in figure 10. If Metenk treatment continues to induce a phase shift in *Per2* 's expression it would mean that either βarr1 doesn't play a role in the induction of *Per2*'s phase shift in gene expression or that another protein for example βarr2 assumes the role of βarr1 in the induction of Per2 expression.

Secondly, the proposed pathway based on literature suggests that βarr1 facilitates the acetylation of H4 in the promoter of *Per2* and consequently induces the expression of *Per2*. We have shown here that Metenk treatment not only induces a phase shift in the peak of βarr1 binding to the Per2 promoter but also the amount of βarr1 bound to the *Per2* promoter. To prove that βarr1 induces the acetylation of H4 in the *Per2* promoter we would have to compare the acetylation levels of H4 in the *Per2* promoter in wildtype (WT), βarr1 KD and βarr1 over-expressing cells (βarr1 OE) with and without Metenk treatment. If we observe highest acetylation levels in βarr1 OE followed by WT and lastly βarr1 KD cells this would suggest that βarr1 does indeed mediate H4 acetylation in the *Per2* promoter. Finally if we observe no difference in acetylation levels of H4 in βarr1 KD versus βarr1 Metenk treated cells this would further support the idea that βarr1 mediates H4 acetylation in the *Per2* promoter. Literature suggests that p300 which is an acetylating enzyme is responsible for βarr1 mediated acetylation of H4 (Kang et al., 2005), if this could be proven in our model system this would further support the pathway that has been suggested in figure 19.

DOPr and circadian rhythms: Therapy for the future

We believe that our observations in our present study could have an impact on wound healing and cancer which are clinically relevant conditions. These conditions also contribute significantly to the burden of illness that is borne by our society. They are modulated by both the DOPr and circadian rhythms. Hence studies involving both these parameters might prove to be insightful in resolving these conditions.

Two sides of the same coin: Wound healing and cancer

Wound healing and cancer appear to be two sides of the same coin. The coin or commonality being the processes of migration, adhesion and differentiation of cells

(Sundaram G et al., 2018). In cases of cancer it has been observed that the expression of various core circadian genes such as *Per2* which is known to control the various mathematical parameters that attribute circadian rhythmicity to skin physiology, are downregulated (Lengyel et al., 2013). Cancer like wound healing is characterized by migration, adhesion and differentiation. In the context of cancer a downregulation of factors promoting adhesion between cells and an upregulation of factors promoting migration of cells leads to a lethal phenotype referred to as metastasis. Whereas in case of wound healing a fine balance between adhesion and migration of cells serves as a homeostatic mechanism which restores the integrity of the organ (Rognoni & Watt, 2018). In the recent past *Per2* has been shown to regulate migration, adhesion of fibroblasts (Hoyle et al., 2017) and differentiation of keratinocytes (Janich et al., 2013).

Morbidities are most often remedied in the clinic by targeting GPCRs (Insel AP et al., 2007). The expression of the Delta Opioid Receptor (DOPr) which is a GPCR has been reported in various cells in the periphery. Subsequently it was shown that just as in the CNS where it is crucial for maintenance of systemic homeostasis, it also plays a role in organ homeostasis by regulating differentiation and migration of epithelial cells. Briefly studies in breast cancer have shown that activation of the receptor leads to higher migration of cells whereas blocking of this receptor reverses the effect (Y. C. Wei et al., 2016). Likewise, studies from our lab showed an enhanced migration and impaired differentiation of keratinocytes as a result of DOPr activation.

Finally given the extensive overlap between the roles of the DOPr and circadian component, *Per2* in maintaining tissue homeostasis it does seem likely that incorporating the two into therapies would restore proper organ function. To this end from our observations it would

seem plausible to devise therapies for cancer wherein the cells are synchronized using dexamethasone since cancerous cells lack rhythmicity in clock gene expression. This treatment in combination with DOPr antagonists such as Naltrindole (NTI) to inhibit migration of cells might succeed in providing rhythmicity in skin physiology as well as acquisition of anti-metastatic behaviour of the targeted cells. In context to wound healing topical application of Dexamethasone is regularly used to combat inflammation and promote wound healing (Gauthier A et al., 2018). Hence this treatment in conjunction with the DOPr agonist Metenkephalin might enhance migration of keratinocytes and fibroblasts and thus facilitate wound healing. Taking into account the phase shifting effects of Metenk this treatment could be carried out in humans at 12:00 pm so that *Per2* peaks at the time that it should which is at about 12pm (24 hours later) and hence this would retain rhythmic skin physiology as well as promote wound healing by enhancing migration of keratinocytes.

Receptor heteromers and targeting gene expression

In our study we were able to conclusively show that the DOPr can translocate to the nucleus upon activation and co-localizes with βarr1. Literature shows that the DOPr forms heteromers with other receptors such as the MOPr and chemokine receptor CXCR4 (Pello et al., 2008). Furthermore, its association with other molecules such as the βarrs and other endocytic machinery has also been described (Beaulieu & Caron, 2005; Kang et al., 2005). Hence it could be that along with DOPr and βarr1 other GPCRs and associated molecules could in-fact bind to chromatin thus forming a transcriptional regulatory complex. This complex could then regulate gene expressions which could have an impact on downstream cell physiology and finally on systemic physiology.

The first step in developing therapies that target receptors which form vital components of transcriptional regulator complexes in the nucleus would be to identify genes expressions that are influenced as a result of the receptor binding to the DNA. This can be easily done by carrying out Chromatin Immunopreciptation sequencing (ChIP seq) experiments, wherein the DNA bound target protein is pulled down using its corresponding antibody and the site of binding of the protein is identified by elaborate sequencing techniques. In the case of our future studies we intend to carry out a ChIP seq experiments by pulling down DOPr and subsequently βarr1 thus identifying genes regulated by the DOPr-βarr1 complex. Thereafter we intend to carryout co- immunopreciptation experiments wherein proteins are pulled down using their corresponding antibodies, these lysates will then be subjected to a mass spectrometric (mass spec) analysis to identify the protein components of this complex. Subsequent experiments will confirm the binding of candidate proteins from the mass spec to the genes of interest from the ChIP seq. These studies might provide us with the unique opportunity to silence and activate gene expression by targeting the DOPr.

DOPr activation may regulate metabolism, detoxification and hydration in the epidermis

Initially we had sought to validate the phase shift in *Per2* expression and hence we decided to study the expression of clock-controlled genes (CCGs), such as *DBP*, *Dec2* and *Tef* before and after Metenk treatment on N/TERT-1 keratinocytes and PHKs. We indeed found them to have undergone changes in expression. Although upon comparing the effects of Metenk treatment in N/TERT-1 keratinocytes and PHKs on the expression of *DBP*, *Dec2* and *Tef,* it is apparent that the two cell lines have different responses to DOPr activation, which may be on account of varying availability of downstream molecules. For example it has been shown that cellular pathways that were significantly upregulated in cell lines compared to tumor

cells and normal cells of the same tissue type included ATP synthesis, cell communication, cell cycle, oxidative phosphorylation, purine, pyrimidine and pyruvate metabolism, and proteasome (Chen et al., 2006; Gross et al., 2006; Shahabi et al., 2006; Ertel A et al., 2006). *DBP*, *Dec2* and *Tef* are constituents of a proline and amino acid- rich basic leucine zipper (PARbZip) family of proteins. These PARbZip proteins are a sub family of circadian transcription factors belonging to the bZip family. They are transcriptionally controlled by the circadian molecular oscillators and are suspected to accomplish output functions of the clock. The PARbZip proteins control expression of genes encoding for enzymes involved in metabolism, but also expression of transcription factors which control the expression of these enzymes (Gachon F et al., 2004). Furthermore DBP was found to induce the expression of the gene Aquaporin 3 (*Aqp3*) in the mouse epidermis. *Aqp3* has been shown to regulate skin hydration via transportation of water and glycerol between and into cells (Matsuzaki et al., 1999; Zeuthen & Klaerke, 1999; Sougrat et al., 2002; Verdier-Sèvrian & Bontè, 2007; Voss et al., 2011). Hence we surmise that the changes in gene expression of as *DBP*, *Dec2* and *Tef* as a result of Metenk treatment (and hence DOPr activation) as observed by us might relate to the detoxifying, metabolizing and epidermal skin hydrating functions which potentially could be enhanced by DOPr activation. This could also be a subject of future studies.

Induction of Senescence: Anti-ageing functions mediated by DOPr

A previous study had attempted to decipher the crosstalk between molecular components of the circadian clock and cell cycle gene expression. The same study observed the circadian gene expression profile of the core circadian clock gene, *Per2*, in relation to the gene expression profiles of the cell cycle genes *p53, Cyclin D1* and *CDK1* in cancer, precancerous

and normal tissues (Tan XM et al., 2015). This study was based firstly on previous reports that had shown cell cycle genes to exhibit circadian rhythmicity in their expression as a result of being controlled by circadian clock genes (Bjarnason GA et al., 2001; Bjarnason GA, Jordan RC and Sothern RB 1999). Secondly, alterations of the circadian rhythm were found to accelerate cancer development (Hartwell LH & Kastan MB 1994). Upon reconciling the facts from these two latter mentioned reports this study reported that changes in rhythmic parameters of *Per 2* expression, such as the acrophase, mesor and amplitude, resulted in similar changes in the circadian expression profiles of *p53, Cyclin D1* and *CDK1* (Tan XM et al., 2015).

Hence upon confirming our observation that Metenk treatment induces phase shifts in *Per2* expression, we asked if this might have an effect on cell cycle associated gene expression profiles. To our surprize, we found that there were indeed changes in the expression of *p53, Cyclin D1* and *CDK1*. The most dramatic effect observed was the suppression of *p53* expression as a result of Metenk treatment, which was consistent with the above described study. We then asked what this suppression in p53 expression might mean in terms of keratinocyte physiology. *p53* is an important cell cycle regulator and is known to influence apoptosis, reversible cell cycle arrest, and cellular senescence (Vogelstein B et al., 2000; Brown CJ et al., 2009; Levine AJ et al., 2006; Levine AJ et al & Oren M 2009; Vousden KH & Prives C 2009). More recently, p53 was shown to suppress senescence by inhibiting p21's ability to induce senescence (Zoya N et al., 2010). Likewise studies from our laboratory had shown that activation of DOPr not only enhanced migration of keratinocytes but also reduced the expression of differentiation markers and supressed the proliferation of keratinocytes. Hence we surmised that perhaps DOPr activation leads the cells to acquire a senescent phenotype. To test for senescence, we probed for changes in Lamin B1 levels in

Metenk treated cells, since elevated levels of Lamin B1 have been shown to trigger senescence (Barascu et al., 2012; Dreesen O et al., 2013) and we found that Metenk treated cells accumulate Lamin B1, thus suggesting that DOPr activation could induce senescence.

Cellular senescence is considered to be a complex and irreversible stress response whereby cells with the potential to proliferate irreversibly lose this ability (Campisi, 2013). Aging tissue has been shown to be characterised by a higher number of senescent cells i.e. as tissue ages there is a gradual decline in cell proliferation and physiological tissue repair (Campisi, 2013). Experiments have shown senescent cells to express the tumor suppressor $p16^{INK4a}$ (Ohtani et al., 2004). $p16^{INK4a}$ prevents cell cycle progression from the G1 to S phases by inhibiting two cycle-dependent kinases, CDK4 and CDK6 (Sherr and Roberts, 1999). The expression of $p16^{INK4a}$ is also found to be enhanced with age (Krishnamurthy et al., 2004; Ressler et al., 2006).

Cellular senescence in earlier stages of life has been shown to be beneficial on account of its anti-tumor activity by arresting cell growth in the presence of stress induced DNA damage and oxidative stress (Ben-Porath and Weinberg, 2005; Dasari et al., 2006; McHugh D & Gil J.2018) . Thus senescence can be thought of as a defence mechanism that averts carcinogenesis. (Campisi, 2005; Dimri, 2005). Studies based on UVB and senescence in keratinocytes exemplify this point. UVB is known to induce DNA damage this could lead to the subsequent apoptosis of the DNA damaged cells or these DNA damaged cells may proliferate and facilitate the genesis of cancer. Ligand activated IGF-1R in keratinocytes has been shown to promote the survival of UVB irradiated cells from UVB-induced apoptosis but these UVB irradiated and IGF-1R activated cells are incapable of further replication. Lastly in

the absence of IGF-1R activation, keratinocytes are more sensitive to UVB-induced apoptosis, but the keratinocytes that do survive retain the capacity to proliferate (Kuhn et al., 1999). This suggests that IGF-1R activation prevents the apoptosis of UVB irradiated cells and also prevents their proliferation. This IGF-1R mediated block in cellular replication and apoptosis in UVB irradiated keratinocytes serves as an anti-carcinogenesis mechanism since this induction in senescence prevents the proliferation of UVB induced DNA damaged cells and also simultaneously retains cells numbers to maintain epidermal barrier function (Kuhn et al., 1999).

Amongst its protective functions senescence has also been known to drive tissue regeneration, which may initially seem counter-intuitive. To this end it has been shown that the *Per2* regulates the expression of the transcription factor NONO which impacts senescence of myofibroblasts. This is an essential step in the process of wound healing (Kowalska et al., 2013; Maier & Kramer, 2013). Wound healing is characterised by several phases involving haemostasis, inflammation, proliferation and remodelling (Singer AJ & Clark RA 1999). At the end of the proliferation phase cyclin1 (CCN1) adhesive protein signalling is followed by the activation of both p53-p21 and $p^{16Ink4a}$-pRb senescence pathways which induces replicative senescence in myofibroblasts (Jun JI & Lau LF 2010). Skin wound healing is thought to be aided by senescence of myofibroblasts in two complimentary ways. First cell over-proliferation is effectively checked and second the senescence-associated secretory phenotype (SASP). SASP involves the secretion of multiple immune-modulating cytokines and matrix metalloproteinases (Campisi, J. 2013). These secreted proteins help to develop and mature scar tissue by remodelling granulation. This activity also serves to dampen excessive fibrosis (Jun JI & Lau LF 2010). The molecular mechanisms driving this wave of wound healing have been attributed to a transcription

factor known as NONO (Jun JI & Lau LF 2010). NONO is a binding partner for Per proteins and its rhythmic gene induction has been found to depend on the rhythmic activity of Per2 (Kowalska et al., 2013). Furthermore NONO rhythmically drives the gene transcription of cell cycle inhibitor $p16^{Ink4a}$ which regulates the timing of senescence in myofibroblasts (Maier and Kramer, 2013). NONO and Per1/2 double mutant have disrupted rhythmic activation of $p16^{Ink4a}$ which leads over proliferation of myofibroblasts (Kowalska et al., 2013).

Thus, it can be concluded that an induction of senescence in young skin may facilitate UV protection and wound healing via the circadian clock. While it has been reported that an induction of senescence in keratinocytes prevents UV induced carcinogenesis, it remains to be seen if a similar induction of senescence in keratinocytes as a result of Metenk treatment might help with wound healing.

In context to the result generated by our studies it can be surmised that treatment of keratinocytes with DOPr agonist Metenk prior to UV treatment could induce senescence which could then offer higher UV protection. Lastly, it would also be interesting to see whether this induction in senescence is reversible. To this end I propose the following experiment wherein synchronized keratinocytes can incubated with or without Metenk for a few hours and thereafter irradiated with UVB. The culture may then be washed and allowed grow with or without Metenk for a few hours to a few days. This protocol may then be used to assess for cell proliferation, differentiation, senescence, cell-cycle arrest and DNA damage using appropriate assays.

Similarly in context to wound healing it could be of interest to see whether activation of DOPr could influence wound healing via the induction of senescence in keratinocytes. Evidence of a role for senescence in wound healing does exist and has been described

above. Senescent cells are known to secrete a variety of proteins collectively known as the senescence-associated secretory phenotype (SASP). SASP has also been shown to facilitate embryonic development, wound healing, and even tumor growth via the induction of cell plasticity and stemness. To this end low doses of SASP have been shown to induce the expression of stem cell markers and regenerative capacity of keratinocytes in vivo. Whereas higher doses of SASP reverse this described effect. To confirm that our studies may be of relevance and therapeutically beneficial in a wound healing scenario it would be of interest to carry out assays to test for SASP induced tissue remodelling effects.

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