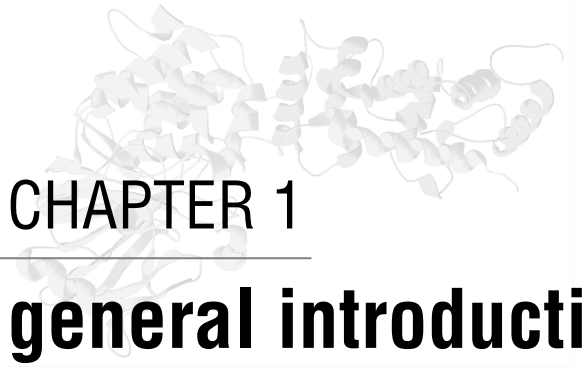

CHAPTER 1

general introduction

CALCINEURIN, IMMUNOSUPPRESSION, AND SKIN CANCER

Calcineurin (Cn) is the *nom de guerre* of protein phosphatase 3 (PP3, formerly PP2B), a heterodimeric member of a small family of serine/threonine phosphatases, in which it distinguishes itself by virtue of its calcium dependence. Cn can be concisely described as the controller of the adaptive immune response. In T helper cells, calcineurin responds to antigen presentation by inducing cytokine secretion; IL-2 is one of the cytokines under control of Cn and acts in both an autocrine and a paracrine fashion to induce clonal expansion and to recruit other types of leukocytes, respectively. Calcineurin inhibitors (CnI), an important class of immunosuppressive drugs, prevent the production of these cytokines and thus block the immune response at an early stage (see figure 1).

Although calcineurin traditionally receives most attention for its role in the immune system, its importance has been established in a multitude of cell and tissue types, for example brain, muscle, and pancreatic tissue. In fact, calcineurin owes its name to its high cerebral abundance. In each organ, calcineurin performs specific functions following activation by a rise in intracellular calcium (see chapter 2). Several muscle and neuronal proteins and enzymes are directly dephosphorylated by Cn (*1*, *2*), but Cn can also couple gene transcription to Ca^{2+} signals by dephosphorylation of several transcription factors, the most celebrated of which is NFAT, the Nuclear Factor of Activated T-cells. Once activated by Cn, NFAT translocates to the nucleus and combines with co-transcription factors (AP-1 and tissue-specific transcription factors (*3*, *4*)) to arrange transcription of a wide portfolio of genes. This results in a broad diversity in cellular responses triggered by Ca^{2+} depending on cell type or cellular differentiation stage. Persistent Cn activity is required to keep NFAT in the nucleus, as nuclear kinases (e.g. GSK-3β) continously try to export NFAT by phosphorylation (see inset of figure 1) (*5-7*). Both the Cn and NFAT families consist of several isoforms that display highly variable tissue distribution and temporal activation kinetics (*8*, *9*).

Currently, one of the most important applications of calcineurin inhibitors lies in the field of transplant medicine. Ever since their spectacular potency in preventing rejection was acknowledged, CnI (e.g. cyclosporin, tacrolimus) have been a mainstay of immunosuppressive regimens after organ transplantation. The immune system of allograft recipients needs to be carefully modulated to prevent rejection of the graft, while maintaining adequate defense against pathogens. In practice, CnI are combined with other drugs, including mycophenolate mofetil and corticosteroids, to achieve optimal efficiency and minimal side-effects of immunosuppressive therapy. Treatment protocols are adjusted to the type of graft and the selection and dosing of drugs can be revised depending on the stage of therapy. However, the severe toxicity of CnI towards multiple organ systems, resulting in kidney damage, hypertension, glycemic dysregulation, and increased cancer risk, particularly in skin (*10-13*), has recently resulted in controversy with

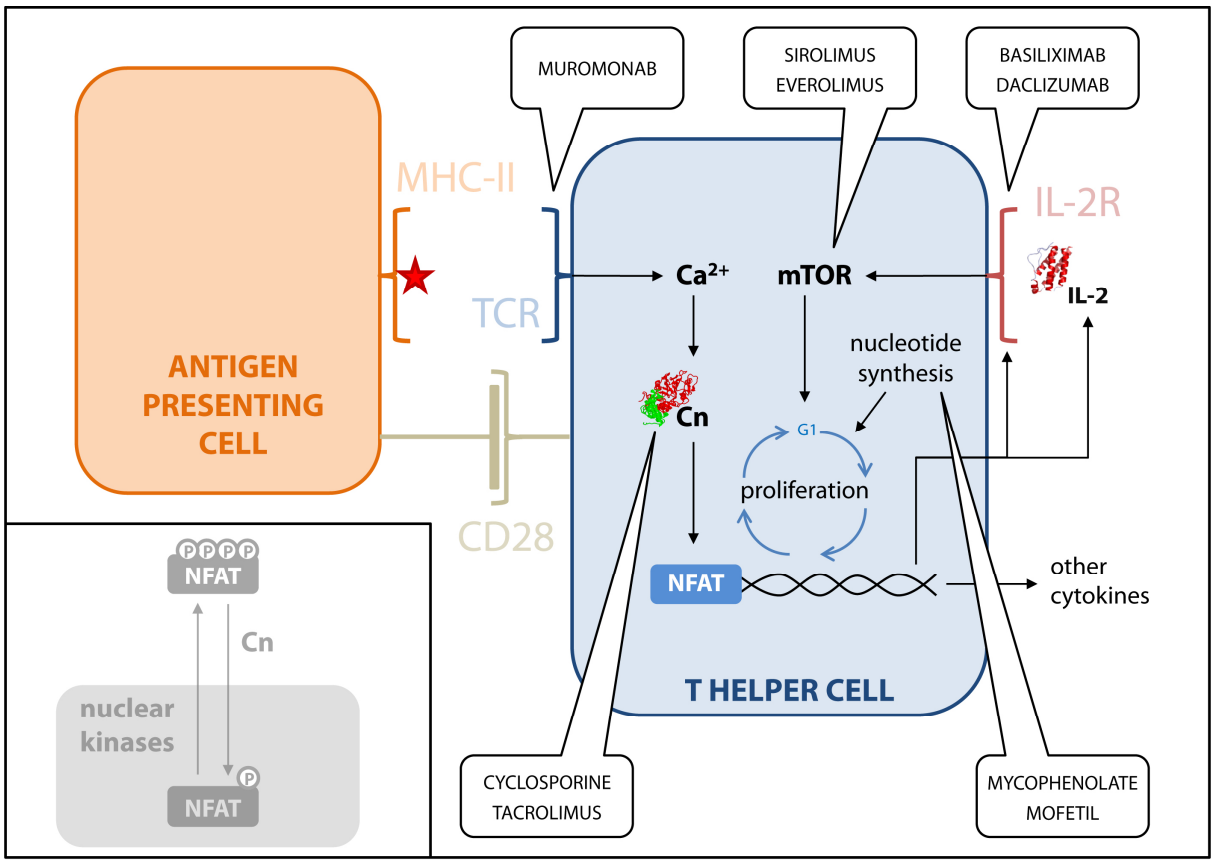

FIGURE 1 Blocking the immune response. Immunosuppressive drugs can act at different stages in the cascade from antigen presentation to T cell recruitment. In short, recognition of antigen by the T cell receptor results in a rise in intracellular calcium and activation of calcineurin, which dephosphorylates NFAT. If a confirmation signal (via CD28) is also present, NFAT, once in the nucleus, will arrange transcription of a large number of genes, including those encoding interleukin-2 and the interleukin-2 receptor. Binding of IL-2 to its receptor results in cellular proliferation. Cyclosporine and tacrolimus inhibit calcineurin, whereas sirolimus and everolimus inhibit the mammalian target of rapamycin (mTOR) downstream of the IL-2 receptor. Antibodies such as muromonab and basiliximab interfere with either antigen recognition by the T cell receptor or IL-2 binding to the IL-2 receptor. Mycophenolate mofetil disrupts nucleotide synthesis by inhibiting inosine monophosphate dehydrogenase, which will also prevent T cell proliferation. See (20) for further details. ***Inset***: Shuttling of NFAT between the cytosol and the nucleus occurs based on its phosphorylation state. Dephosphorylation of NFAT by Cn exposes a nuclear localisation signal; nuclear kinases such as GSK-3β, on the other hand, rephosphorylate NFAT, which results in its export from the nucleus.

regard to the fate of CnI. On one hand, there is a trend to minimize and ultimately withdraw CnI exposure (*14*); a new class of immunosuppressants, inhibitors of the enzyme mTOR (mammalian Target Of Rapamycin, also depicted in figure 1), are already under evaluation as possible replacements of CnI, although their benefits are still uncertain (*15-17*). On the other hand, individual monitoring may further compensate for the high interindividual biological variance in pharmacokinetics of the CnI and thus ensure an optimal balance between effectivity and side-effects. In addition, second

generation CnI are being developed that feature at least equal potency, yet better tolerability (*18*, *19*). Meanwhile, much effort is put into elucidating the pathophysiology of the adverse effects of the CnI. In this dissertation, we will mainly focus on the link between CnI and skin cancer.

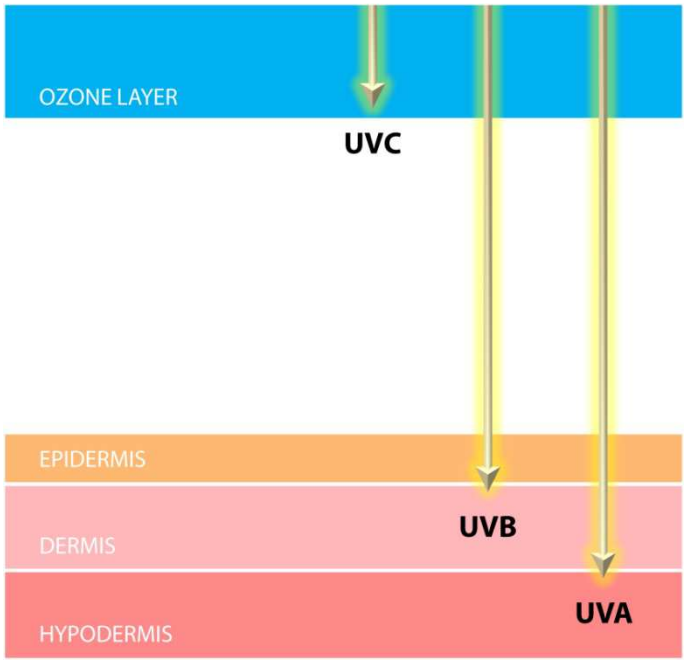

FIGURE 2 Penetrance of UV radiation of different wavelenghts. UVC and a large part of the UVB spectrum are screened out by the ozone layer. UVA, on the other hand, penetrates deeply into the skin.

Skin cancer: a major side-effect of calcineurin inhibitors

Skin cancer is nowadays by far the most commonly diagnosed type of cancer. Non-melanoma skin cancers (NMSC) are the most prevalent and include basal cell carcinomas and squamous cell carcinomas. Although these growths can be cosmetically defiguring, they do not tend to metastasize and the mortality associated with them is very low. Malignant melanoma, on the other hand, represents a far more serious class: despite being much rarer, melanoma is responsible for 75% of all skin cancer deaths, according to the American Cancer Society. As indicated above, the increased incidence of these types of skin cancer, principally NMSC, is a major side-effect of long-term CnI maintenance treatment of transplant patients. These patients are nowadays advised to avoid excessive sun exposure, as the primary risk factor for non-melanoma skin cancers and the primary environmental risk factor for malignant melanoma is cellular exposure to ultraviolet (UV) radiation (*21*, *22*). Spending considerable time in the sun or in tanning facilities, and a history of sunburn all contribute to a higher risk. Although sunlight may be indispensable, if not for vitamin D production, then at least for general wellbeing, it is not exempted from Paracelsus' first law of toxicology. The alarming increase of skin cancer incidence in young people (*23*, *24*) illustrates unawareness and underestimation of the risks of excessive sunbathing by the general public and has recently led to the use of

tanning equipment during youth and adolescence being controlled or prohibited by law in many countries.

UV radiation, cellular damage, and skin cancer development

UV radation emitted by the sun can be classified into three wavebands: UVA, subdivided in UVA1 (340-400 nm) and UVA2 (320-340 nm), UVB (290-320 nm) and UVC (100-290 nm). While UVC radiation is completely absorbed by the ozone layer and atmosphere, UVB is only partly blocked and UVA reaches our skin unhampered (see figure 2). UVB radiation is filtered by the stratum corneum; penetration into the skin is limited to the epidermis. UVB readily and directly damages DNA, causing cyclobutane pyrimidine dimers (CPD) and 6-4 photoproducts (6-4PP). This DNA damage can lead to cell death and underlies the induction of sunburn. It also stimulates production of the UV-absorbing pigment melanin (*25*) and promotes thickening of the epidermis (*26*). Both of these processes are defense mechanisms of the skin by formation of natural barriers against further UV radiation. If DNA damage is not restored by repair mechanisms, UVB signature mutations (C->T and CC->TT tandem transitions) occur that persist through subsequent cell divisions and are often described as the fingerprint of UVB damage (*27, 28*). If mutations occur in tumor suppressor genes such as p53, activation of the DNA damage response pathways is impaired, which represents an important early step in tumorigenesis (*29*).

The health risks of UVA have long been insufficiently recognized, although many scientists believe it is not so much UVB as it is UVA and the accompanying oxidative stress that is the most important risk factor for malignant melanoma (*23, 30, 31*). Due to its higher penetrance, exposure to UVA occurs even on a cloudy day or behind glass. Contrary to UVB, which sets off production of new melanin and thus generates a delayed, yet relatively long-lasting tan, UVA is responsible for short-term tanning due to activation of melanin already present in the skin. Although – due to its higher wavelength – the potential of UVA to cause direct damage to nucleic acids, proteins and other cell components is limited, much harm can still be done through photosensitization by UVA chromophores such as porphyrins and flavins. These molecules undergo photoexcitation upon absorption of UVA radiation. The excited singlet state of the photosensitizer can undergo crossing-over to a longer-lived triplet state that may react either with triplet molecular oxygen to yield singlet oxygen, or with a substrate to generate free radicals that may ultimately react with molecular oxygen to form superoxide (see figure 3). Not all UVA chromophores give rise to photosensitization; some can safely dissipate almost all absorbed UV radiation as heat through internal conversion (*32*). Subtle differences in molecular structure can make the difference between photoprotection and photosensitization. For instance, two types of melanin can be distinguished: eumelanin and pheomelanin. While the black eumelanin qualifies as a “radical sink” – a natural sunscreen –, the reddish pheomelanin, on the other hand, is a photosensitizer: it also

absorbs UV, but converts it to long-lived reactive molecules (*33*). For the same reason, the use of organic compounds as sunscreens is a contentious issue; many of these claimed "sunlight filtering substances" seem to lose their screening ability after long incubation. In fact, some can penetrate the stratum corneum and undergo poorly understood photochemical breakdown reactions that may actually convert them to photosensitizers. This entails that sunscreens could even increase UV-damage through generation of ROS (*34, 35*). Many well-known drugs also qualify as exogenous photosensitizers: users of certain antibiotics such as tetracyclines and fluoroquinolones, contraceptives, neuroleptic drugs (phenothiazines) and HMG-CoA reductase inhibitors (statins) are advised to limit sun exposure, as these compounds are stored in skin and, through photosensitization, can cause photoirritant, photogenotoxic, and photoallergic skin responses (*36*). The power of photosensitizers is medically exploited in the form of photodynamic (PD) therapy. PUVA treatment, for example, combines psoralens with UVA and is particularly effective in clearing severe psoriasis, although its safety is under debate (*37*). Selected types of basal cell carcinoma can be treated using a photosensitizer precursor and localized illumination to inflict fatal oxidative damage to tumor cells (*38, 39*). For instance, administration of 5-aminolevulinic acid results in intracellular production of the photosensitizer protoporphyrin IX, which selectively accumulates in tumor cells due to their intrinsically lower ferrochelatase activity (*40, 41*). Fluorescence of this compound can be used to demarcate the malignant area for surgical intervention (*42*). Incorporation of advanced drug delivery technology and drug targeting strategies may enable the use of PD for treatment of deep tissue tumors (*43*) or even internal pathogens (*44*).

Reactive Oxygen Species

"Reactive oxygen species" (ROS) is a collective term for small, reactive, oxygen-derived species that, despite several valuable roles in cellular messaging and dealing with pathogens, are best known for the fact that they can inflict damage to biomolecules. As shown in figure 3, a number of reactive oxygen species with quite divergent properties can be distinguished, although they can often be interconverted. Singlet oxygen ($^1O_2$) is short-lived and highly reactive; it preferentially attacks double bonds and sulfur-containing groups (*45*). Superoxide ($O_2{\bullet}^-$) by itself is not very reactive, and, by virtue of its preference for one-electron redox reactions, primarily affects enzymes that contain redox-active metal centers. However, it can liberate iron from FeS clusters, which results in Fenton chemistry that may ultimately damage DNA anyway (*46, 47*). In addition, it can combine with NO• radicals to form deleterious peroxynitrite ions. Hydrogen peroxide is also poorly reactive, even at millimolar concentrations, and highly selective in its choice of biomolecules to attack, which are predominantly proteins with reactive –SH groups. H_2O_2 can, however, be converted to hydroxyl radicals (HO•), which will react indiscriminately with practically any cell component at a diffusion-controlled rate.

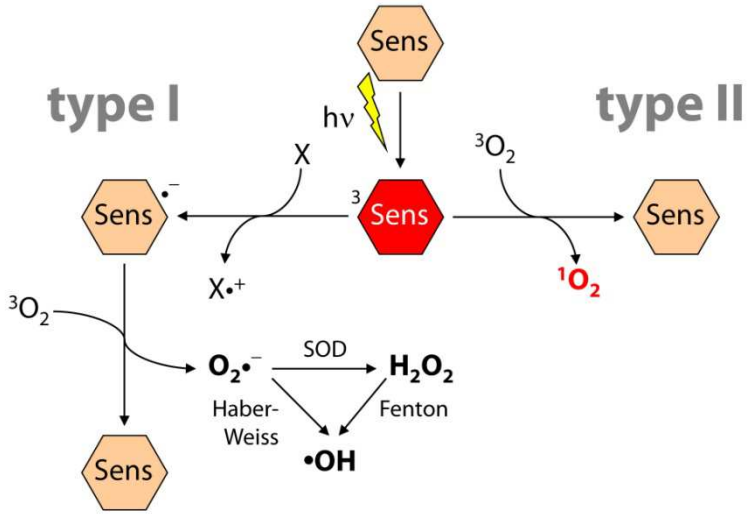

FIGURE 3 From photosensitizer to reactive oxygen species. Upon absorption of radiation (hν), the excited photosensitizer (denoted by a ^{3}triplet sign) can either abstract an electron from a substrate (X) [type I] or transfer its excitation energy to molecular (triplet) oxygen [type II]. The type II mechanism yields highly reactive singlet oxygen. In the type I mechanism, the photosensitizer radical anion can donate an electron to molecular oxygen, producing superoxide radical anion while restoring itself to its original ground state. Superoxide ($O_2^{\bullet-}$) can undergo dismutation to hydrogen peroxide (H_2O_2) or reduce ferric ions to ferrous ions; the latter can, together with hydrogen peroxide, enter the Fenton reaction to generate hydroxyl radicals (Haber-Weiss chemistry).

ROS generated by UVA exposure can cause cataracts and premature aging of the skin by damaging eye lens and connective tissue proteins, respectively (*48*, *49*). In addition, both UVA and UVB induce matrix metalloproteinases, which further degrade connective tissue fibers (*50*, *51*). ROS damage to DNA produces mainly 8-hydroxydeoxyguanosine and ssDNA strand breaks and can ultimately result in T->G transversions (*52*). UVA triggers several signaling cascades, resulting in quick activation of NFκB and AP-1 (via JNK) due to ROS production and iron release (*53-55*). Singlet oxygen produced by UVA induces p38 MAPK in HaCaTs and fibroblasts (*56-58*). Moreover, UV radiation activates the Cn/NFAT pathway (*59*, *60*). The activation of these transcription factors, in turn, upregulates the expression of cytokines, acute phase proteins, growth factors, adhesion molecules, and cyclooxygenase-2 (COX-2) (*61*). High levels of COX-2 are often considered a tell sign that the prelude to the cancer development fugue has been intoned (*62*).

The cellular antioxidant system constitutes the first line of defense against ROS. This system consists of enzymes that catalytically remove ROS (e.g. superoxide dismutase, catalase, and peroxidases), a battery of sacrificial agents (GSH, vitamins C and E, and bilirubin), and some physical quenchers (e.g. carotenoids). Unfortunately, some

ROS have such a short lifetime that they may already have reacted with a biomolecule before encountering an antioxidant molecule. Also, the amount of ROS generated can be so high that it overwhelms the capacity of the antioxidant system, resulting in oxidative stress. Fortunately, cells contain machinery to repair oxidative damage to proteins, for instance using enzymes like thioredoxin (to reduce disulfide bridges) and methionine sulfoxide reductase (to reduce oxidized methionines), and DNA. In addition, via PKC signaling, oxidative stress induces migration of the transcription factor NF-E2-related factor 2 (Nrf-2) to the nucleus, where it binds to genes that contain an Antioxidant Response Element (ARE), promoting the production of detoxification enzymes (*63*) (e.g. heme oxygenase-1 (HO-1) (*64*)). Some cell types are particularly susceptible to oxidative stress. Reduced DNA repair capacity, depletion of antioxidant resources (cysteine / GSH) by pheomelanin synthesis, as well as the photosensitizing properties of pheomelanin make melanocytes highly vulnerable to ROS-mediated damage, which may drive melanomagenesis (*31*, *65*, *66*).

Other risk factors for skin cancer

Although cellular damage following exposure to UV radiation is often the main cause for non-melanoma skin cancer development, several other risk factors exist, such as a fair skin type. In case of melanoma, genetic disposition is often involved, which can be traced back to faulty tumor suppressor genes that should normally confer protection against melanoma (e.g. CDKN2A and CDK4) (*67*). Furthermore, a weakened immune system and exposure to certain toxic compounds such as arsenic can contribute to skin malignancy; these two factors will receive particular attention in this dissertation. While compromised immunity can have a pathological cause, such as AIDS or leukemia, the immune system can also be suppressed by medication, which pertains, for instance, to patients that have received a transplant organ, as mentioned before.

Interestingly, both UVA and UVB also have immunomodulatory properties themselves. Conversion of *trans*-urocanic acid to *cis*-urocanic acid, interference with antigen presentation by dendritic cells, and stimulation of IL-10 and TNF-α release by keratinocytes are among the possible mechanisms by which UVB radiation results in immunosuppression and immunotolerance against skin tumors (*68*, *69*). Consequently, the effects of UV resemble an attack on two fronts, causing DNA damage on one hand, and impairing tumor immunosurveillance on the other. UVA can both stimulate and suppress the immune system, depending on intensity and duration of exposure (*70-72*). The pathways of UVA induced immunosuppression are not yet fully understood, although depletion of infiltrating T cells following UVA-induced apoptosis and loss of B cell function seem plausible mechanisms (*73*, *74*). Several skin conditions, such as psoriasis, vitiligo, subacute lupus erythematodes, atopic dermatitis, and contact hypersensitivity benefit from UVB or UVA phototherapy (*75-78*), resulting in a relieve of

inflammatory symptoms. Topical CnI (tacrolimus, pimecrolimus) have also been proven effective for treatment of many of these conditions (*79, 80*). The conspicuous similarities between CnI and UV radiation also become apparent in the nature of the side-effects of CnI: cancer risk is overall increased, but most notably in skin (*11, 81*).

A role for calcineurin in skin cancer and UVA-induced immunosuppression?

We have already pointed out that, apart from the immune system, Cn signaling has been found of relevance in a large number of organ systems. Just during the last decade, the importance of Cn signaling in skin cells has started to become widely acknowledged, with various roles in cellular growth and development being ascribed to Cn and NFAT. Ironically, most of these functions have in common that dysregulation may result in malignant transformation and tumor formation, sustenance, and progression (*82*). This raises the question to what extent tumorigenicity is due to interference with signaling pathways in skin cells or the result of disturbed immunosurveillance (*83, 84*). Calcineurin is thought to instigate calcium-dependent apoptosis by dephosphorylation of the pro-apoptotic protein Bad (*85*) and upregulation of FasL expression (*86, 87*). Knockdown of calcineurin has been found to reduce nucleotide excision repair of DNA lesions in keratinocytes, possibly via downregulation of xeroderma pigmentosum group A and G proteins (*88-90*). Furthermore, Cn/NFAT signaling seems to be critical for p53-dependent senescence (*91*). NFAT1 regulates cell cycle control via downregulation of cyclin and CDK4 gene expression and upregulation of p21 expression, leading to cell cycle arrest; in essence, it operates the switch between proliferation and differentiation (*92-95*). On the other hand, several reports mention NFAT members appearing to function as inducers of cell cycle progression, cellular proliferation and transformation (*96-98*); in addition, NFAT has been found to effect VEGF-induced angiogenesis via COX-2 upregulation (*99-101*) and to promote cellular metastasis (tissue invasion and migration) by integrin-signaling (*102*). This complex convergence of pro- and anti-oncogenic roles in different members of the NFAT family, which could depend on cell type, presence of co-transcription factors, and the nature of the stimulus, will undoubtedly be a major subject for studies aimed to clarify the relation between Cn signaling and cancer.

AIMS AND OUTLINE OF THIS DISSERTATION

This dissertation aims to address two important issues. The first issue, and the motivation behind these studies, is the question why transplant recipients treated with calcineurin inhibitors display such a high incidence of skin cancer. Although the risk of malignancy development in these patients is clearly higher for most organs (*11*), the risk of skin cancer shows an explosive and unparalleled growth, particularly in countries such

as Australia, where typical UV exposure is generally high (*103*, *104*). The experimental work described in the upcoming chapters was designed to provide mechanistic evidence for our hypothesis that an unforeseen interplay between CnI and UV radiation may be involved in and even lie at the heart of this increased skin cancer incidence. Chapter 2 represents an introductory review of the multitude of mechanisms that underly the regulation of Cn functioning and activity. Normally, endogenous regulatory molecules ensure the adaptation of Cn functioning to different cell types and conditions. However, Cn activity can also be directly affected by exogenous factors such as metal ions, and reactive oxygen species. In chapter 3, we study the effects of UVA1 radiation on calcineurin activity and cytokine production in skin and immune cells. In chapter 4, we subsequently show that the inhibitory effects of UVA and CnI on Cn are additive and cumulative and that UVA inflicts physical damage to Cn *via* production of singlet oxygen and superoxide. This suggests that UVA radiation locally enhances Cn inhibition, and may thus further compromise many of this phosphatase's tumorsuppressive functions. Exposure to several metals, such as nickel and arsenic, also generates intracellular ROS. As chronic exposure to either UVA or arsenic species can cause malignant transformation of human keratinocytes and resistance to apoptosis (*105-107*), we compared the effects of arsenite and UVA radiation on Cn signaling (chapter 5), to further investigate the possibility that Cn inhibition by ROS could be a common starting point on the road towards malignancy formation.

The increasing knowledge on the molecular mechanisms underlying tumor development enables the construction of rudimentary prognostic models for several forms of cancer, incorporating biological markers and genetic features. From a molecular diagnostician's point of view, the prospect of building such a model to establish a patient's predisposition to – in this case – skin malignancy would be highly welcome. The value of Cn activity as a marker for drug monitoring in transplant patients treated with CnI has been thoroughly investigated (see chapter 6). Also, the expression of several NFAT-regulated genes has been correlated with skin cancer incidence (*108*). Ongoing efforts in elucidating the role of Cn/NFAT signaling in tumor formation will have to tell what other prognostic and diagnostic information lies enclosed in this pathway, which is the second focal point of this dissertation. For now, chapter 6 is a critical inventory and evaluation of different players in the Cn signaling cascade as biomarkers, not only in skin and for skin cancer, but also in the many other organ systems, diseases, and conditions in which Cn is implicated.

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