Cover Page

Universiteit Leiden

The handle http://hdl.handle.net/1887/18930 holds various files of this Leiden University dissertation.

Author: Voskamp, Pieter
Title: Local effects of immunosuppressants in the skin and impact on UV carcinogenesis
Date: 2012-05-09

Chapter 6

Summary and general discussion

The overall aim of the study was to provide experimental data which could contribute toward minimizing the (skin) carcinogenic risk from immunosuppressive drugs. The immediate objective of the experiments described in this thesis was to determine modulating effects of immunosuppressants on the responses of skin cells to UV irradiation, and UV induction of skin carcinomas and precursor lesions. Hence, we have determined the effects of the immunosuppressants at several stages of tumor development. Comparison of the different immunosuppressants on their effects on tumor development should contribute to lowering skin cancer risk from immunosuppressive regimens. Unexpectedly, none of the immunosuppressants fed in long-term tolerable dosages (assessed by blood cell counts and/or condition of the animals) enhanced UV carcinogenesis (chapters 2 and 4). CsA even delayed UV carcinogenesis (chapter 4). Evidently, these mouse experiments did not simulate the enhanced risk of skin carcinogenesis in human organ transplant recipients. Our experiments suggest that immunosuppressants do not necessarily accelerate UV carcinogenesis. Feeding CsA in bolus dosages (by gavage) instead of evenly in the diet reproduced the speeding up of UV carcinogenesis as found by Kelly et al.[1] (chapter 5). Thus, our experiments demonstrated the potential importance of the dose schedule and ensuing profiles in blood levels of the immunosuppressive drug. However, even in bolus dosages of CsA the impact on experimental UV carcinogenesis did not appear to match the dramatic increase in risk of skin carcinoma in human organ transplant recipients (60- to 100-fold increase[2]). This suggests that the mouse model we used was not quite adequate, either because mice react differently (e.g. lower rate of DNA repair) or the model is incomplete, e.g. most prominently by the absence of an actual graft (absence of continuous antigenic stimuli and possible tolerance).

Interestingly, the close correspondence between the induction of mut-p53 cell clusters and skin carcinomas reported earlier by our group[3, 4] was not observed when feeding immunosuppressants like CsA and Rapa (chapters 3 and 4). These results indicate that the large majority of mut-p53 cell clusters are probably not potential precursors of skin carcinomas, in contrast with earlier conclusions[3, 5]. Consequently, the frequency of mut-p53 cell clusters in the skin is not a reliable predictor of skin carcinoma risk, again invalidating earlier inferences[3, 4].

Thus, our study has yielded four unanticipated major findings:

a) Immunosuppressants *per se* do not necessarily enhance UV carcinogenesis and in this respect dose scheduling can be of major importance.

b) The mouse model with administration of immunosuppressants appears inadequate in simulating the increase in risk of squamous cell carcinomas in human organ transplant recipients.

c) Mut-p53 cell clusters in majority are not potential precursors of squamous cell carcinomas.

d) Early local carcinogenic effects from various immunosuppressants as expressed by *p53* mutation frequencies in the UV-exposed mouse skin (or rather the lack of such effects) are not predictive of the overall effects on UV carcinogenesis. This latter finding indicates that systemic and not local effects are of major importance for the effects of immunosuppressants on UV carcinogenesis.

In the following paragraphs the effects of immunosuppressive drugs on UV-response in normal skin, *p53* mutations, mutant-p53 overexpressing cells and skin tumors are discussed in more detail. Consequences of these effects for the model of UV-induced tumor development are discussed, followed by concluding remarks and future directions.

Effects of immunosuppressants on epidermal regeneration and responses to UV in human skin equivalents

Some immunosuppressants have direct adverse effects on skin cells, which could contribute to carcinoma risk. CsA affects DNA repair[6, 7] and has been shown to impair apoptotic responses induced by UV in normal human keratinocytes[6] and mice[8]. Aza enhances DNA damage by photosensitization[9]. In chapter 3 and 4 we systematically compared the impacts of the immunosuppressants on epidermis and responses to UV exposure in human skin equivalents (HSEs). Epidermal regeneration (formation of an epidermis by seeded keratinocytes) in HSEs was impaired by Rapa. HSEs treated with Rapa show a thin epidermis with only a few cell layers, as well as a decreased proliferation index and reduced expression of the hyperproliferation associated markers K16 and K17. Decreased proliferation may be relevant to wound-healing problems associated with Rapa treatment in transplantation patients[10]. None of the other immunosuppressants showed any effect on epidermal regeneration.

The apoptotic response after UV exposure in HSEs was increased after Rapa treatment. In our mouse experiments (chapter 3) we did, however, not see any significant increases in apoptosis in Rapa-treated mice which could be attributable to a wide variation in responses. In HSEs CsA pre-treatment caused a decrease in the apoptotic response after UV irradiation, confirming earlier results in cell cultures[6].

We found HIF1α accumulation to be inhibited by Rapa in keratinocytes after UV exposure of HSEs, while the apoptotic response was increased by Rapa. Previously it has been shown that HIF1α is upregulated after hypoxia[11] and UV irradiation[12, 13] and that Rapa can prevent hypoxia-induced HIF1α accumulation[14]. Here we have shown that Rapa is also able to inhibit UV-induced HIF1α accumulation (chapter 3). HIF1α is known to inhibit or induce apoptosis depending on the cell type, and inhibition of HIF1α accumulation may therefore be involved in the pro-apoptotic effect of Rapa in HSEs[15, 16].

Immunosuppressants and UV-induced p53 mutations

In chronically UV-irradiated skin, clusters of cells expressing mutated p53 (mut-p53 cell clusters) develop. These cell clusters harbor similar mutations in the *p53* gene as the skin tumors that arise later[17]. Also the development of these cell clusters correlated with skin tumor development in several studies[3, 4], therefore these cell clusters are generally regarded as precursor lesions for squamous cell carcinomas that arise after continued UV exposure[3, 17-19]. Immunosuppressants do not affect the mutational frequency of *p53* in chronically UV irradiated non-tumor skin in mice (chapter 4). Deep sequencing of two mutational hotspots in the *p53* gene (codons 270 and 275) showed that many cells harbor a mutated allele of *p53*. There was no clear effect of the immunosuppressive treatments on the frequencies of mutated *p53* alleles. On average, 5% of the alleles sequenced were mutated in a stretch of 40 bp, corresponding to 10% of the cells when assuming heterozygously mutated cells (total mutations in *p53* were therefore estimated to amount 20%). These results are in agreement with a recent deep sequencing study of human skin from mid-life individuals that revealed that persistent *p53* mutations had accumulated in 14% of the epidermal cells[20]. In contrast to the mutational frequencies, immunosuppressants did affect formation of mut-p53 cell clusters. CsA and Aza increased the formation of mut-p53 cell clusters in mice, whereas Rapa inhibited mut-p53 cell cluster formation (chapters 3 and 4). The lower number of UV-induced mut-p53 cell clusters formed in the skin of Rapa-treated mice might be caused by early enhanced apoptosis or decreased proliferation, but Ki-67-positive cells were not decreased in Rapa-treated mice. A previous study by our group showed no effect of Aza on mut-p53 cell cluster formation in mice[21], but Aza was administered by i.p. injections in those experiments, and the measurement was limited to one time point with relatively large error margins in the counts. The discrepancy between *p53* mutational frequencies and mut-p53 cell cluster formation is discussed below.

Immunosuppressants and UV-induced tumor development in mice

Effects of immunosuppressants on UV-induced skin cancer were determined in daily UV-irradiated mice fed with drug-containing chow (chapters 2 and 4). Surprisingly, none of the immunosuppressants increased UV-induced tumor development. Remarkably, tumor induction was delayed by CsA treatment. Proliferation of epidermal keratinocytes (e.g. through TGF-β) was not affected by CsA, making it unlikely that reduced keratinocyte proliferation caused the tumor delay. A pioneering study on the effect of CsA on UV carcinogenesis showed a shorter tumor latency time compared to controls[1]. With a different experimental setup, Wulff et al. showed that CsA treatment (by repeated i.p. injections) reduced the rate of new tumor development in mice[22], in agreement with our results. We found that Rapa had no effect on the development of small tumors, but impaired the development of large tumors (chapter 2). This impairment can be attributed to the known anti-tumor, or more specifically, antiangiogenic effects of Rapa[23, 24]. At dosages that maintained allogeneic heart grafts in mice, Rapa was described to inhibit outgrowth of tumor implants whereas CsA enhanced angiogenesis and strongly stimulated tumor outgrowth[25]. Rapa could even block the proangiogenic effect from CsA. In chapter 2 the inhibition of tumor outgrowth by Rapa appeared to be reflected by a significant reduction of Vegf-a positive tumor cells. Interestingly, we did not find any clear effect on the vasculature of tumors that grew in the Rapa-fed groups, but these tumors may be presumed to have been selected for their vascularization and resistance to Rapa whereas those that lagged behind were most sensitive to the antiangiogenic effect of Rapa. Other UV carcinogenesis studies with different treatment schemes have shown either lower tumor yields[22] or higher tumor yields[26] in Rapa-treated mice, indicating that treatment schemes can have important effects on the outcomes of the tumorigenesis experiments.

P53 mutational spectra in SCCs

P53 mutations appear to be involved in the early stages of tumor development[3]. Our hypothesis that Rapa might selectively induce apoptosis in the *p53*-mutated cells, and thus slow down the rate at which tumors are initiated, was not supported by our data because we found no effect on the onset of the smallest perceptible tumors (<1mm) (chapter 2). To check further for local effects of the immunosuppressants on the epidermal cells that might have affected UV carcinogenesis (e.g., disturbed metabolism by Rapa), we investigated the *p53* mutation spectrum of the tumors, which is normally dominated by the UV signature mutations (i.e. C>T transition on a dipyrimidine site)[27]. We found a dramatic shift in the types of mutations in tumors from the Rapa-fed group. Since Rapa-insensitivity can be acquired via increased cellular oxidative stress[28] it is possible that only tumors with high

levels of endogenous oxidative stress could grow into large tumors, hence causing the altered mutational spectrum. The antioxidant effect of MMF[29] may have prevented formation of non-UV-type mutations in tumors of mice on a combination diet with Rapa and MMF, although tumor development was not different in mice on combined MMF and Rapa diet than on Rapa diet. We further determined in chapter 3 that this shift in the type of mutations occurs only in tumors >2mm and not in mut-p53 cell clusters or skin tumors <2mm in mice treated with Rapa. It can therefore be concluded that the altered mutational spectrum is a late event in tumor development. Either it occurred after the initial development of small tumors, or a specific selection of small tumors with altered mutational spectra grew into large tumors, corresponding to the lower yield of large tumors in Rapa-fed mice.

Discrepancies between effects of immunosuppressants at different stages of tumor development in mice

There was no clear effect of the immunosuppressive treatments on the frequencies of mutated *p53* alleles, in contrast with effects on mut-p53 cell cluster formation. Deep sequencing of the mutational hotspot in the *p53* gene (codons 270 and 275) showed that many cells (~20%) harbor a mutated allele of *p53* before the occurrence of tumors. The percentage of skin area containing mut-p53 cell clusters was much lower, approximately 6% in control mice. It can therefore be concluded that a minority of the cells harboring a mutated *p53* show overexpression of mut-p53 in cell clusters. This puts the effects of Rapa, CsA and Aza in a different perspective. A mutation in *p53* is apparently not sufficient to cause overexpression of the p53 protein in mut-p53 cell clusters[30], in agreement with what we found in mut-p53 mice (chapter 3). Tumors may arise from a larger pool of *p53*-mutated cells that do not overexpress the protein. Development of mut-p53 cell clusters and SCCs was not strictly correlated in our experiments. When mice were treated with CsA, numbers of mut-p53 cell clusters were clearly increased compared to controls, but CsA delayed tumor onset. Rapa on the other hand reduced mut-p53 cell clusters without any effect on the onset of small tumors. Numbers of mut-p53 cell clusters had in previous studies always shown a good correlation with the rate of tumor development with chronic UV irradiation[3, 4, 31]. Since most SCCs harbor mutations in the *p53* gene, *p53* mutations were considered early events in SCC formation and therefore mut-p53 cell clusters were thought to be precursors of SCCs (Chapter 4 figure 5A)[18, 32]. However, a retrospective study did not establish differences in numbers of mut-p53 cell clusters between skin from patients with solitary versus multiple skin carcinomas[33]. As we found that immunosuppressants can have opposing effects on mut-p53 cell clusters and SCC formation, this suggests that mut-p53 cell cluster formation cannot be used as a simple indicator of tumor risk. As most mut-p53 cell clusters are

not genuine precursors of SCC, another oncogenic early event is likely to drive early UV carcinogenesis. The necessity of another oncogenic event might also explain the absence of an increased SCC risk among Li-Fraumeni patients, i.e. people heterozygous in germline mutations in *p53*. These results open up an alternative perspective on the developmental stages of skin carcinomas, in which mut-p53 cell clusters do not contribute to skin carcinoma risk (Chapter 4 figure 5B).

Dose schedule of CsA determines effect on experimental UV carcinogenesis

In order to systematically compare different immunosuppressive drugs on their skin cancer risk, several mouse studies have previously been performed. Mouse experiments aimed at mimicking the skin cancer promoting effect of CsA on transplantation patients have resulted in different outcomes. A pioneering study showed enhanced UV-induced skin carcinogenesis with CsA when the drug was administered through force feeding and UV exposure was performed twice weekly[1]. A more recent study showed that CsA administration resulted in larger UV-induced skin tumors compared to control when CsA was administered via i.p. injections daily and UV exposures thrice weekly[26]. Another study from this group showed that CsA decreased UV-induced tumor development when CsA treatment is started and UV treatment was discontinued when the first tumors had occurred[22]. In chapter 4 we showed that CsA inhibited UV-induced tumor formation when CsA was administered in the food and with daily UV exposures. In all these studies the hairless SKH1 mice were used. The main differences between these studies were the experimental procedures regarding UV irradiation and CsA administration, which suggests that the exact experimental protocol determines the effect of CsA on UV-induced skin cancer development. These differential outcomes led us to the hypothesis that when mice are force-fed CsA and repeatedly UV irradiated at times when they have high CsA blood levels that this would increase UV carcinogenesis. In chapter 5 we set out to assess the effects of CsA on UV carcinogenesis in mice using different experimental procedures. No effect of CsA on UV carcinogenesis was apparent in mice treated with dietary CsA after a period of UV exposures. An increase in tumor development occurred in UV-exposed mice that were CsA treated by gavage. This confirmed the results of the earlier experiments by Kelly et al.[1]. However, there was no difference in tumor development in mice UV irradiated during CsA peak blood levels or trough blood levels, indicating that CsA blood level during UV exposures bore no relevance to the increased UV carcinogenesis. Evidently, a more evenly administered CsA lowers (the risk of) UV carcinogenesis.

Concluding remarks and future perspectives

The studies described in this thesis confirm the potential that Rapa, or Rapa analogs like everolimus, may have for decreasing skin cancer in organ transplant recipients. Inhibition of tumor growth by Rapa should be further exploited in immunosuppressive treatment of organ transplant recipients, and by generating proper analogs this beneficial effect might be retained while eliminating adverse side effects.

We have shown that most mut-p53 cell clusters are not genuine precursors of SCCs. It remains likely that SCCs develop from mut-p53 cell clusters, since mutation of the *p53* gene is an early event in tumor development[34]. The effects of immunosuppressants on the development of mut-p53 cell clusters without correspondingly altering tumor onset make it possible to use these drugs in identifying a novel oncogenic event that drives early UV carcinogenesis.

The studies described here have shown that the mouse models of UV carcinogenesis with immunosuppressants may be basically unsuitable to emulate the increased risk of cutaneous SCC in organ transplant recipients. But it is also possible that the models lack an essential component like the graft. Considering the strong immunogenic and tolerogenic effects from a graft and likely interference thereof with skin tumor immunity, it would appear probable that inclusion of a graft in the model will have a great impact.

Table 1: Effects of immunosuppressants on cellular processes in non-immunological cells, based on majority view of literature.

	CsA	Tac	Aza	MMF	Rapa
Proliferation	↓ [35-37]	↓ [37, 38]		↓ [39-42]	↓ [43, 44]
Photogenotoxicity			↑ [9]		
DNA repair	↓ [6, 7, 45-47]	↓ [6, 47]			
Apoptosis	↓ [6, 8]	↓ [6]			↑/↓ [48-52]
Angiogenesis	↑ [24, 25]	↓ [53, 54]		↓ [39, 55, 56]	↓ [24, 25, 53]

Legend: ↓ = inhibitory effect, ↑ = positive effect

References

1. Kelly GE, Meikle W, Sheil AG. Effects of immunosuppressive therapy on the induction of skin tumors by ultraviolet irradiation in hairless mice. Transplantation 1987;44:429-34.
2. Hofbauer GFL, Bavinck JNB, Euvrard S. Organ transplantation and skin cancer: basic problems and new perspectives. Experimental Dermatology 2010;19:473-82.
3. Rebel H, Kram N, Westerman A, Banus S, van Kranen HJ, de Gruijl FR. Relationship between UV-induced mutant p53 patches and skin tumours, analysed by mutation spectra and by induction kinetics in various DNA-repair-deficient mice. Carcinogenesis 2005;26:2123-30.
4. Rebel H, Mosnier LO, Berg RJW, Vries AW-d, van Steeg H, van Kranen HJ, de Gruijl FR. Early p53-positive Foci as Indicators of Tumor Risk in Ultraviolet-exposed Hairless Mice: Kinetics of Induction, Effects of DNA Repair Deficiency, and p53 Heterozygosity. Cancer Res 2001;61:977-83.
5. Athar M, Walsh SB, Kopelovich L, Elmets CA. Pathogenesis of nonmelanoma skin cancers in organ transplant recipients. Archives of Biochemistry and Biophysics 2011;508:159-63.
6. Yarosh DB, Pena AV, Nay SL, Canning MT, Brown DA. Calcineurin inhibitors decrease DNA repair and apoptosis in human keratinocytes following ultraviolet B irradiation. The Journal of Investigative Dermatology 2005;125:1020-5.
7. Kelly GE, Meikle W, Sheil AG. Scheduled and unscheduled DNA synthesis in epidermal cells of hairless mice treated with immunosuppressive drugs and UVB-UVA irradiation. Br J Dermatol 1987;117:429-40.
8. Sugie N, Fujii N, Danno K. Cyclosporin-A suppresses p53-dependent repair DNA synthesis and apoptosis following ultraviolet-B irradiation. Photodermatol Photoimmunol Photomed 2002;18:163-8.
9. O'Donovan P, Perrett CM, Zhang X, Montaner B, Xu Y-Z, Harwood CA, McGregor JM, Walker SL, Hanaoka F, Karran P. Azathioprine and UVA Light Generate Mutagenic Oxidative DNA Damage. Science 2005;309:1871-4.
10. Dean PG, Lund WJ, Larson TS, Prieto M, Nyberg SL, Ishitani MB, Kremers WK, Stegall MD. Wound-healing complications after kidney transplantation: a prospective, randomized comparison of sirolimus and tacrolimus. Transplantation 2004;77:1555-61.
11. Wang GL, Jiang BH, Rue EA, Semenza GL. Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension. Proceedings of the National Academy of Sciences of the United States of America 1995;92:5510-4.
12. Rezvani HR, Dedieu S, North S, Belloc F, Rossignol R, Letellier T, de Verneuil H, Taieb A, Mazurier F. Hypoxia-inducible Factor-1{alpha}, a Key Factor in the Keratinocyte Response to UVB Exposure. J. Biol. Chem. 2007;282:16413-22.
13. Wunderlich L, Paragh G, Wikonkal NM, Banhegyi G, Karpati S, Mandl J. UVB induces a biphasic response of HIF-1alpha in cultured human keratinocytes. Experimental Dermatology 2008;17:335-42.
14. Hudson CC, Liu M, Chiang GG, Otterness DM, Loomis DC, Kaper F, Giaccia AJ, Abraham RT. Regulation of Hypoxia-Inducible Factor 1α Expression and Function by the Mammalian Target of Rapamycin. Molecular and Cellular Biology 2002;22:7004-14.

15. Piret JP, Mottet D, Raes M, Michiels C. Is HIF-1alpha a pro- or an anti-apoptotic protein? Biochem Pharmacol 2002;64:889-92.
16. Greijer AE, van der Wall E. The role of hypoxia inducible factor 1 (HIF-1) in hypoxia induced apoptosis. Journal of Clinical Pathology 2004;57:1009-14.
17. Kramata P, Lu Y-P, Lou Y-R, Singh RN, Kwon SM, Conney AH. Patches of Mutant p53-Immunoreactive Epidermal Cells Induced by Chronic UVB Irradiation Harbor the Same p53 Mutations as Squamous Cell Carcinomas in the Skin of Hairless SKH-1 Mice. Cancer Res 2005;65:3577-85.
18. Jonason AS, Kunala S, Price GJ, Restifo RJ, Spinelli HM, Persing JA, Leffell DJ, Tarone RE, Brash DE. Frequent clones of p53-mutated keratinocytes in normal human skin. Proc Natl Acad Sci U S A 1996;93:14025-9.
19. Ren ZP, Ponten F, Nister M, Ponten J. Two distinct p53 immunohistochemical patterns in human squamous-cell skin cancer, precursors and normal epidermis. Int J Cancer 1996;69:174-9.
20. Stahl PL, Stranneheim H, Asplund A, Berglund L, Ponten F, Lundeberg J. Sun-Induced Nonsynonymous p53 Mutations Are Extensively Accumulated and Tolerated in Normal Appearing Human Skin. J Invest Dermatol 2011;131:504-8.
21. de Graaf YG, Rebel H, Elghalbzouri A, Cramers P, Nellen RG, Willemze R, Bouwes Bavinck JN, de Gruijl FR. More epidermal p53 patches adjacent to skin carcinomas in renal transplant recipients than in immunocompetent patients: the role of azathioprine. Exp Dermatol 2008;17:349-55.
22. Wulff BC, Kusewitt DF, VanBuskirk AM, Thomas-Ahner JM, Duncan FJ, Oberyszyn TM. Sirolimus Reduces the Incidence and Progression of UVB-Induced Skin Cancer in SKH Mice even with Co-administration of Cyclosporine A. J Invest Dermatol 2008;128:2467-73.
23. Tressler RJ, Garvin LJ, Slate DL. Anti-tumor activity of mycophenolate mofetil against human and mouse tumors in vivo. Int J Cancer 1994;57:568-73.
24. Guba M, von Breitenbuch P, Steinbauer M, Koehl G, Flegel S, Hornung M, Bruns CJ, Zuelke C, Farkas S, Anthuber M, Jauch K-W, Geissler EK. Rapamycin inhibits primary and metastatic tumor growth by antiangiogenesis: involvement of vascular endothelial growth factor. Nat Med 2002;8:128-35.
25. Koehl GE, Andrassy J, Guba M, Richter S, Kroemer A, Scherer MN, Steinbauer M, Graeb C, Schlitt HJ, Jauch K-W, Geissler EK. Rapamycin protects allografts from rejection while simultaneously attacking tumors in immunosuppressed mice. Transplantation 2004;77:1319-26.
26. Duncan FJ, Wulff BC, Tober KL, Ferketich AK, Martin J, Thomas-Ahner JM, Allen SD, Kusewitt DF, Oberyszyn TM, VanBuskirk AM. Clinically Relevant Immunosuppressants Influence UVB-Induced Tumor Size Through Effects on Inflammation and Angiogenesis. American Journal of Transplantation 2007;7:2693-703.
27. de Gruijl FR. Skin cancer and solar UV radiation. Eur J Cancer 1999;35:2003-9.
28. Neklesa TK, Davis RW. Superoxide anions regulate TORC1 and its ability to bind Fpr1:rapamycin complex. Proceedings of the National Academy of Sciences of the United States of America 2008;105:15166-71.

29. Krotz F, Keller M, Derflinger S, Schmid H, Gloe T, Bassermann F, Duyster J, Cohen CD, Schuhmann C, Klauss V, Pohl U, Stempfle H-U, et al. Mycophenolate Acid Inhibits Endothelial NAD(P)H Oxidase Activity and Superoxide Formation by a Rac1-Dependent Mechanism. Hypertension 2007;49:201-8.
30. King P, Craft AW, Malcolm AJ. p53 expression in three separate tumours from a patient with Li-Fraumeni's syndrome. J Clin Pathol 1993;46:676-7.
31. Kripke ML, Fisher MS. Immunologic aspects of tumor induction by ultraviolet radiation. Natl Cancer Inst Monogr 1978:179-83.
32. Berg RJ, van Kranen HJ, Rebel HG, de Vries A, van Vloten WA, Van Kreijl CF, van der Leun JC, de Gruijl FR. Early p53 alterations in mouse skin carcinogenesis by UVB radiation: immunohistochemical detection of mutant p53 protein in clusters of preneoplastic epidermal cells. Proceedings of the National Academy of Sciences 1996;93:274 -8.
33. le Pelletier F, Soufir N, de La Salmoniere P, Janin A, Basset-Seguin N. p53 Patches are not increased in patients with multiple nonmelanoma skin cancers. J Invest Dermatol 2001;117:1324-5.
34. Durinck S, Ho C, Wang NJ, Liao W, Jakkula LR, Collisson EA, Pons J, Chan S-W, Lam ET, Chu C, Park K, Hong S-w, et al. Temporal Dissection of Tumorigenesis in Primary Cancers. Cancer Discovery 2011;1:137 -43.
35. Kaplan A, Matsue H, Shibaki A, Kawashima T, Kobayashi H, Ohkawara A. The effects of cyclosporin A and FK506 on proliferation and IL-8 production of cultured human keratinocytes. J Dermatol Sci 1995;10:130-8.
36. Fisher GJ, Duell EA, Nickoloff BJ, Annesley TM, Kowalke JK, Ellis CN, Voorhees JJ. Levels of Cyclosporin in Epidermis of Treated Psoriasis Patients Differentially Inhibit Growth of Keratinocytes Cultured in Serum Free Versus Serum Containing Media. J Investig Dermatol 1988;91:142-6.
37. Karashima T, Hachisuka H, Sasai Y. FK506 and cyclosporin A inhibit growth factor-stimulated human keratinocyte proliferation by blocking cells in the G0/G1 phases of the cell cycle. J Dermatol Sci 1996;12:246-54.
38. Cao XW, Fu ZR, Ding GS. Effects of tacrolimus on proliferation, apoptosis, and fluorouracil sensitivity of liver cancer cell line of SMMC-7721. Hepatobiliary Pancreat Dis Int 2005;4:269-73.
39. Domhan S, Muschal S, Schwager C, Morath C, Wirkner U, Ansorge W, Maercker C, Zeier M, Huber PE, Abdollahi A. Molecular mechanisms of the antiangiogenic and antitumor effects of mycophenolic acid. Mol Cancer Ther 2008;7:1656-68.
40. Kaczmarek R, Zaczynska E, Misiuk-Hojlo M. Antiproliferative properties of mycophenolic acid on human retinal pigment epithelial cells in vitro. Klin Oczna 2010;112:201-4.
41. Amer R, Rabinowich L, Maftsir G, Puxeddu I, Levi-Schaffer F, Solomon A. Mycophenolic acid suppresses human pterygium and normal tenon fibroblast proliferation in vitro. Br J Ophthalmol 2010;94:1373-7.
42. Chang HW, Wu VC, Wu KD, Huang HY, Hsieh BS, Chen YM. In rat renal fibroblasts, mycophenolic acid inhibits proliferation and production of the chemokine CCL2, stimulated by tumour necrosis factor-alpha. Br J Pharmacol 2010;160:1611-20.

43. Horn TD, Boucher CL, Vogelsang GB, Hess AD. The Effects of Rapamycin on Keratinocytes. Journal of Investigative Dermatology 1992;98:650-.
44. Javier AF, Bata-Csorgo Z, Ellis CN, Kang S, Voorhees JJ, Cooper KD. Rapamycin (sirolimus) inhibits proliferating cell nuclear antigen expression and blocks cell cycle in the G1 phase in human keratinocyte stem cells. J Clin Invest 1997;99:2094-9.
45. Weinstein T, Korzets A, Chagnac A, Ori Y, Herman M, Zevin D, Malachi T, Gafter U. Effect of immunosuppressive therapy on DNA repair and cancer incidence in renal transplant recipients. Transplant Proc 2000;32:694-5.
46. Herman M, Weinstein T, Korzets A, Chagnac A, Ori Y, Zevin D, Malachi T, Gafter U. Effect of cyclosporin A on DNA repair and cancer incidence in kidney transplant recipients. Journal of Laboratory and Clinical Medicine 2001;137:14-20.
47. Herman M, Ori Y, Chagnac A, Weinstein T, Korzets A, Zevin D, Malachi T, Gafter U. DNA repair in mononuclear cells: role of serine/threonine phosphatases. J Lab Clin Med 2002;140:255-62.
48. Brown VI, Fang J, Alcorn K, Barr R, Kim JM, Wasserman R, Grupp SA. Rapamycin is active against B-precursor leukemia in vitro and in vivo, an effect that is modulated by IL-7-mediated signaling. Proc Natl Acad Sci U S A 2003;100:15113-8.
49. Schachner T, Oberhuber A, Zou Y, Tzankov A, Ott H, Laufer G, Bonatti J. Rapamycin treatment is associated with an increased apoptosis rate in experimental vein grafts. Eur J Cardiothorac Surg 2005;27:302-6.
50. Stromberg T, Dimberg A, Hammarberg A, Carlson K, Osterborg A, Nilsson K, Jernberg-Wiklund H. Rapamycin sensitizes multiple myeloma cells to apoptosis induced by dexamethasone. Blood 2004;103:3138-47.
51. Ravikumar B, Berger Z, Vacher C, O'Kane CJ, Rubinsztein DC. Rapamycin pre-treatment protects against apoptosis. Hum Mol Genet 2006;15:1209-16.
52. Pan T, Rawal P, Wu Y, Xie W, Jankovic J, Le W. Rapamycin Protects against Rotenone-Induced Apoptosis through Autophagy Induction. Neuroscience 2009;164:541-51.
53. Kidszun A, Schneider D, Erb D, Hertl G, Schmidt V, Eckhard M, Preissner KT, Breier G, Bretzel RG, Linn T. Isolated pancreatic islets in three-dimensional matrices are responsive to stimulators and inhibitors of angiogenesis. Cell Transplant 2006;15:489-97.
54. Siamakpour-Reihani S, Caster J, Bandhu Nepal D, Courtwright A, Hilliard E, Usary J, Ketelsen D, Darr D, Shen XJ, Patterson C, Klauber-Demore N. The role of calcineurin/NFAT in SFRP2 induced angiogenesis--a rationale for breast cancer treatment with the calcineurin inhibitor tacrolimus. PLoS ONE 2011;6:e20412.
55. Koehl GE, Wagner F, Stoeltzing O, Lang SA, Steinbauer M, Schlitt HJ, Geissler EK. Mycophenolate mofetil inhibits tumor growth and angiogenesis in vitro but has variable antitumor effects in vivo, possibly related to bioavailability. Transplantation 2007;83:607-14.
56. Wu X, Zhong H, Song J, Damoiseaux R, Yang Z, Lin S. Mycophenolic acid is a potent inhibitor of angiogenesis. Arterioscler Thromb Vasc Biol 2006;26:2414-6.