

# Isotretinoin and FoxO1

## A scientific hypothesis

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**Key words:** acne, apoptosis, FoxO, isotretinoin, transcriptional regulation, stem cell

**Abbreviations:** AMP, antimicrobial peptide; Akt, Akt kinase (protein kinase B); Apo CIII, apolipoprotein C-III; AR, androgen receptor; Atg1, atrogin 1 (F-box protein 32); ATRA, all-trans-retinoic acid; CDK, cyclin-dependent kinase; C/EBP $\alpha$ , CCAAT/enhancer binding protein- $\alpha$ ; CBP, CREB (cAMP response element-binding); CNS, central nerve system; CPK, creatine phosphokinase; CRABP2, cellular retinoic acid binding protein-2; Cpe, carboxypeptidase E; DBD, DNA binding domain; DHT, dihydrotestosterone; ETC, electron transport chain; FA, fatty acid; FABP5, fatty acid binding protein-5; FGF, fibroblast growth factor; FGFR2, fibroblast growth factor receptor-2; FoxO, forkhead box O; GSK3 $\beta$ , glycogen synthase kinase 3 $\beta$ ; Hmox1, heme oxygenase 1; IGF-1, insulin-like growth factor-1; IGF1R, IGF-1 receptor; I $\kappa$ B, inhibitor of NF $\kappa$ B; I $\kappa$ K, I $\kappa$ B kinase; IL, interleukin; IRAK, IL-1 receptor associated kinases; Isotretinoin, 13-cis-retinoic acid; Lef1, lymphoid enhancer binding factor-1; LPL, lipoprotein lipase; LRP5/6, low density receptor-related proteins 5/6; LXR, liver X receptor;  $\alpha$ -MSH,  $\alpha$ -melanocyte stimulating hormone; MnSOD, manganese superoxide dismutase; MMP, matrix metalloproteinase; MTP, microsomal triglyceride transfer protein; MuRF1, muscle-specific RING finger protein 1; NGAL, neutrophil gelatinase-associated lipocalin; NF $\kappa$ B, nuclear factor  $\kappa$ B; PDK1, 3-phosphoinositide-dependent kinase 1; PDK, pyruvate dehydrogenase kinase-4; PEPCK, phosphoenolpyruvate carboxykinase; PI3K, phosphoinositol 3-kinase; PML, promyelocytic leukemia; PMN, polymorphonuclear neutrophils; POMC, proopiomelanocortin; PPAR $\gamma$ , peroxisome proliferator-activated receptor- $\gamma$ ; PPRE, PPAR response elements; RAR, retinoic acid receptor; ROS, reactive oxygen species; RXR, retinoid X receptor; SREBP-1c, sterol-regulatory-element-binding protein 1c; TAD, transcription activation domain; Tcf, T cell factor; TIMP, tissue inhibitor of metalloproteinase; TG, triglyceride; TLR, toll-like receptor; TNF $\alpha$ , tumor necrosis factor- $\alpha$ ; VDR, vitamin D receptor; VLDL, very-low-density lipoprotein; Wnt, wingless protein

Oral isotretinoin (13-cis retinoic acid) is the most effective drug in the treatment of acne and restores all major pathogenetic factors of acne vulgaris. Isotretinoin is regarded as a prodrug which after isomerisation to all-trans-retinoic acid (ATRA) induces apoptosis in cells cultured from human sebaceous glands, meibomian glands, neuroblastoma cells, hypothalamic cells, hippocampus cells, Dalton's lymphoma ascites cells, B16F-10 melanoma cells, and neuronal crest cells and others. By means of translational research this paper provides substantial indirect evidence for isotretinoin's mode of action by upregulation of forkhead box class O (FoxO) transcription factors. FoxOs play a pivotal role in the regulation of androgen receptor transactivation, insulin/insulin like growth factor-1 (IGF-1)-signaling, peroxisome proliferator-activated receptor- $\gamma$  (PPAR $\gamma$ )- and liver X receptor- $\alpha$  (LXR $\alpha$ )-mediated lipogenesis,  $\beta$ -catenin signaling, cell proliferation, apoptosis, reactive oxygen homeostasis, innate and acquired immunity, stem cell homeostasis, as well as anti-cancer effects. An accumulating body of evidence suggests that the therapeutic, adverse, teratogenic and chemopreventive effects of isotretinoin are all mediated by upregulation of FoxO-mediated gene transcription. These FoxO-driven transcriptional changes of

the second response of retinoic acid receptor (RAR)-mediated signaling counterbalance gene expression of acne due to increased growth factor signaling with downregulated nuclear FoxO proteins. The proposed isotretinoin $\rightarrow$ ATRA $\rightarrow$ RAR $\rightarrow$ FoxO interaction offers intriguing new insights into the mode of isotretinoin action and explains most therapeutic, adverse and teratogenic effects of isotretinoin in the treatment of acne by a common mode of FoxO-mediated transcriptional regulation.

### Introduction

With the observation of Peck et al. in 1979 that isotretinoin (13-cis-retinoic acid) produced marked clearing in patients with nodulocystic acne, a new era in acne treatment began.<sup>1</sup> Since its approval by the US FDA in 1982, isotretinoin has been considered a breakthrough treatment against severe nodulocystic acne.<sup>2,3</sup> Isotretinoin is the most potent known inhibitor of sebum production. Multiple modes of action of isotretinoin, including suppression of sebaceous gland activity, normalization of the pattern of keratinization within the sebaceous gland follicle, inhibition of inflammation, reduction of growth of *Propionibacterium acnes* and normalization of the expression of tissue matrix metalloproteinases make isotretinoin the single most effective drug in the treatment of acne. Isotretinoin not only affects the sebaceous

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follicle but exerts adverse effects on various tissues in the body.<sup>4</sup> Isotretinoin undergoes significant and selective all-trans-isomerization to all-trans-retinoic acid (ATRA) in cultured sebocytes.<sup>5</sup> Isotretinoin has been considered as a prodrug mediating its activity through isomerization to ATRA.<sup>5-7</sup> Despite its multiple actions on proliferation, metabolism, reactive oxygen homeostasis, inflammation, matrix remodeling and sebum suppression, the underlying mode of action and especially its unique sebostatic activity has not been unraveled despite more than 30 years of clinical use. The effectiveness of isotretinoin on all major pathogenetic aspects of acne implies that there is however a fundamental mechanism of action at the regulatory level of gene transcription which cannot be explained by primary transcriptional responses of ATRA to retinoic acid receptor (RAR).<sup>2</sup>

Binding of ATRA initiates changes in interactions of RARs/retinoid X receptors (RXRs) with corepressor and coactivator proteins, activating transcription of primary target genes. Importantly, ATRA/RAR-signaling induces secondary responses in gene expression encoding transcription factors and signaling proteins that further augment a whole cascade of gene expression.<sup>8</sup> These transcription factors of the *secondary response*, especially FoxO proteins, then transcriptionally activate their target genes to generate the whole spectrum of retinoid-mediated transcriptional regulation. It has been speculated that the addition of ATRA leads to major intra- and interchromosomal transcription “interactomes” so that active ATRA-coregulated genes and their regulatory factors cooperate to generate specialized nuclear areas for coordinated transcriptional control.<sup>8</sup> These secondary responses and the full orchestration of transcription factors and coregulators of the second response to ATRA are less well characterized but appear to be crucial for isotretinoin’s mode of action. It has recently been recognized that ATRA increased the expression of transcription factor FoxO3a in neuroblastoma cells.<sup>9</sup> FoxO3a has also been identified as a key regulator for ATRA-induced granulocytic differentiation and apoptosis in acute promyelocytic leukemia.<sup>10</sup> Treating acute promyelocytic leukemia cells with ATRA, FoxO3a phosphorylation was reduced and FoxO3a translocated into the nucleus. Intriguingly, FoxO3a is a strong inducer of the transcription factor FoxO1.<sup>11</sup> FoxO1 expression is stimulated by activated FoxO3a at the promoter of FoxO1 in a positive feed back loop.<sup>11</sup> The transcription of FoxO genes is stimulated by FoxO3 and repressed by growth factors like insulin and insulin-like growth factor-1 (IGF-1) which are increased in puberty and acne-associated syndromes with insulin resistance.<sup>11,12</sup> Thus, there is evidence from translational research for a relationship between retinoid signaling and FoxO-mediated gene regulation. This relationship and the fact, that a multitude of cellular events in acne pathophysiology, isotretinoin action and isotretinoin-induced adverse effects can be related to FoxO regulation resulted in the formulation of a recent hypothesis for the role of FoxO1 in acne pathogenesis and isotretinoin’s mode of action.<sup>13</sup>

Indirect evidence will be provided in this paper which strongly suggests that acne may be explained by a growth factor-induced nuclear deficiency of FoxO1, whereas isotretinoin increases nuclear FoxO1 levels and thus reverses acne-related imbalances of FoxO homeostasis.<sup>13</sup> In fact, all adverse and teratogenic effects

of isotretinoin can be explained by FoxO-mediated proapoptotic signaling. To understand the pluripotent and multifunctional roles of FoxO transcription factors, a brief introduction in the extending network of FoxO transcription factors is helpful.

## FoxO-Transcription Factors

*Forkhead box O* (FoxO) transcription factors FoxO1, FoxO3a, FoxO4 and FoxO6 are important regulatory proteins that modulate the expression of genes involved in cell cycle control, DNA damage repair, apoptosis, oxidative stress, cell differentiation, glucose metabolism, inflammation, immune functions and regulation of stem cell homeostasis.<sup>14-19</sup> FoxO1 represents the predominant FoxO isoform. FoxO1 and FoxO3a are proteins with a length of about 650 amino acids. FoxOs contain a conserved DNA binding domain and either activate or inhibit the transcription of target genes containing a consensus DNA binding sequence TTG TTT AC.<sup>14,19</sup> Furthermore, FoxO proteins can interact with several other transcription factors like androgen receptor (AR) or  $\beta$ -catenin, thereby modifying gene regulation. Central to the regulation of FoxO transcription factors is a shuttling system, which confines FoxO factors to either the nucleus or the cytosol (Fig. 1).<sup>14,15</sup> Among other involved and less important kinases, shuttling of FoxOs requires protein phosphorylation of nuclear FoxOs by phosphoinositol-3-kinase (PI3K)-mediated activation of the serine/threonine kinase Akt (also known as protein kinase B, PKB).<sup>11-14</sup> Activated (phosphorylated) Akt translocates into the nucleus for FoxO phosphorylation. Phosphorylated FoxOs leave the nucleus, thereby changing gene regulation (Fig. 1). Dysregulation of FoxO1 and its nuclear export by insulin, IGF-1, fibroblast growth factors (FGFs) or other growth factors modifying the activation of PI3K/Akt affect the transcriptional activity of key target genes and nuclear receptors involved in acne pathogenesis. Increased growth factor signaling is an endocrinological hallmark of puberty as well as insulinotropic western nutrition with increased consumption of milk and other insulinotropic dairy products and carbohydrates with high glycemic index.<sup>20-23</sup>

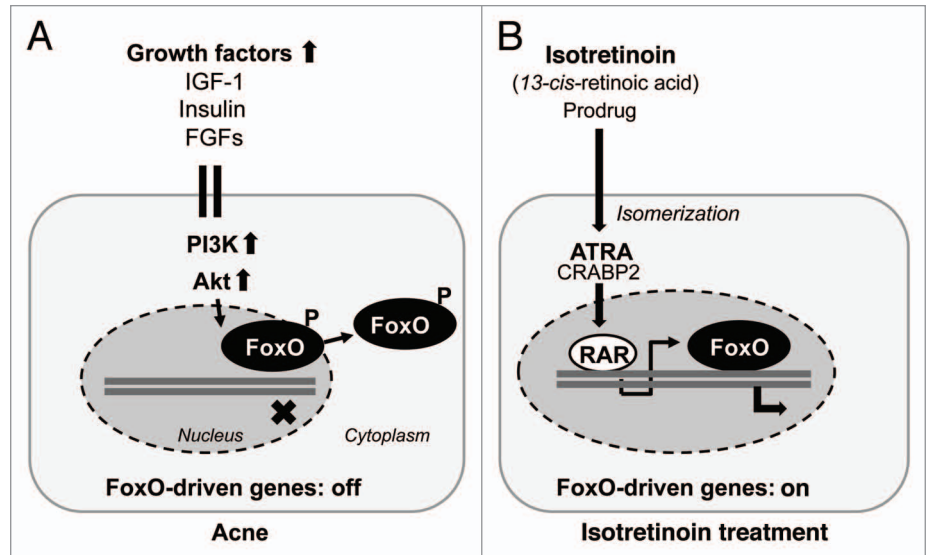
## Isotretinoin, FoxO1 and Suppression of Androgen Receptor Transactivation

Androgen receptor (AR)-mediated signal transduction plays an essential role for the stimulation of the size of sebocytes and sebum production as well as keratinocyte proliferation in the *ductus seboglandularis* and the *acroinfundibulum*. ARs are expressed in basal and differentiating sebocytes and pilosebaceous duct keratinocytes.<sup>24-25</sup> Androgens induce the expression of *sterol regulatory element binding protein* (SREBP), the most important transcription factor of lipogenesis.<sup>26</sup> Androgen-insensitive subjects who lack functional ARs do not produce sebum and do not develop acne.<sup>27</sup> Increased AR protein levels have been determined in skin of acne patients.<sup>28</sup>

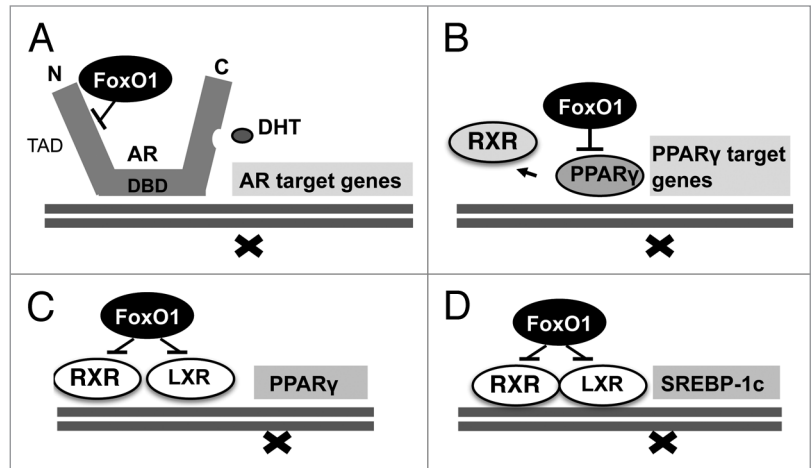
AR is a modular protein organized into functional domains, consisting of an N-terminal transcription activation domain (TAD), a DNA-binding domain and a C-terminal ligand-binding domain for androgens.<sup>29</sup> Ligand-activated ARs induce

the transcription of androgen-responsive target genes. The TAD of AR mediates the majority of AR transcriptional activity and provides the most active coregulator interaction surface.<sup>30</sup> The AR integrates a multitude of regulatory signals and its final transcriptional activity is integrated by the action of more than 150 known coregulators which are either coactivators or corepressors.<sup>31</sup> FoxO1 is an important metabolically regulated AR corepressor and binds to the TAD, where it disrupts p160 coactivator binding and suppresses N-terminal/C-terminal-interaction, which is most important for AR transcriptional activity (Fig. 2A).<sup>32</sup> The AR repressive function of FoxO1 is attenuated by increased growth factor signaling with activation of the PI3K/Akt cascade.<sup>33,34</sup> On the other hand, the expression of several growth factors like IGF-1 and regulatory proteins of cell cycle control and lipogenesis are dependent on androgen signal transduction,<sup>35</sup> pointing to the hierarchical control of AR-mediated gene expression for downstream AR-dependent growth factor signaling. Nuclear FoxO1 extrusion by increased growth factor signaling and upregulation of AR transcriptional activity will thus augment the expression of a substantial set of AR-responsive target genes involved in acne pathogenesis. FoxO1 regulation of AR activity at the genomic level is the connecting piece explaining the functional interaction of insulin/IGF-1 and androgens in the pathogenesis of acne.

Moreover, like an amplification loop, AR receptor signaling increased IGF-1-expression and IGF-1/IGF-1 receptor (IGF1R)-signaling in the ventral prostate gland.<sup>35</sup> Oral isotretinoin treatment has recently been shown to decrease serum IGF-1 levels,<sup>36</sup> which may decrease AR-mediated gene expression. Furthermore, decreased AR protein levels have been observed in skin of male acne patients after oral isotretinoin treatment.<sup>37</sup> These data imply that isotretinoin treatment may downregulate the transcriptional activity of AR by increasing the nuclear concentration of the AR cosuppressor FoxO1. Furthermore, the isotretinoin-induced decrease of IGF-1 serum levels may impair IGF-1/PI3K/Akt-mediated nuclear export of FoxO1. Moreover, IGF-1 is regarded as an androgen-dependent stimulator of 5 $\alpha$ -reductase activity.<sup>38</sup> In fact, experimental evidence has been provided for decreased androgen 5 $\alpha$ -reduction in skin and liver of men with severe acne after oral isotretinoin treatment.<sup>39</sup> The isotretinoin-induced decrease of IGF-1 may reduce the conversion of less potent testosterone to the more potent dihydrotestosterone



**Figure 1.** (A) Nuclear exclusion of FoxO proteins into the cytoplasm by growth factor signaling due to Akt kinase-mediated phosphorylation of nuclear FoxO proteins. (B) Isotretinoin-mediated upregulation of FoxO expression as a secondary response of proapoptotic RAR-signaling. FoxO-regulated genes are switched on. IGF-1, insulin-like growth factor-1; FGFs, fibroblast growth factors; PI3K, phosphoinositid-3 kinase; Akt, Akt kinase (protein kinase B); FoxO, forkhead box class O transcription factor; ATRA, all-trans-retinoic acid; CRABP2, cellular retinoic acid binding protein-2; RAR, retinoic acid receptor.



**Figure 2.** (A) FoxO1-mediated suppression of androgen-receptor (AR) by FoxO1-binding to the AR transcription activation domain (TAD) thereby inhibiting N-/C-terminal interaction of AR resulting in reduced AR transactivation. (B) Direct FoxO1-mediated suppression of PPAR $\gamma$ -regulated target genes. (C) FoxO1-mediated suppression of the PPAR $\gamma$  promoter reducing PPAR $\gamma$  expression. (D) FoxO1-mediated suppression of the SREBP-1c promoter reducing SREBP-1c expression, the key transcription factor of most lipogenic enzymes. DBD, DNA binding domain; DHT, dihydrotestosterone; RXR, retinoid X receptor; PPAR $\gamma$ , peroxisome proliferator-activated receptor- $\gamma$ .

(DHT), thereby decreasing the activity status of the AR ligand binding domain, which binds DHT 10 times stronger than testosterone. Free bioactive IGF-1 is controlled by IGF binding proteins (IGFBPs). In human dermal papilla cells, ATRA induced a significant increase of IGFBP-3,<sup>40</sup> which reduced the bioavailability of free IGF-1 for IGF-1/IGF1R-signaling with potential

impact on nuclear FoxO1 import. Thus, at least four mechanisms of isotretinoin treatment may explain reduced AR transcriptional activity affecting both the FoxO1 regulated N-terminal TAD and the androgen-regulated C-terminal AR ligand binding domain (Fig. 2A).

### Androgen Receptor CAG Repeat Polymorphism and Acne Relapse after Isotretinoin

The high prevalence of acne (>80%) in adolescents of industrialized countries with western life style as well as the increasing persistence of acne into adulthood clearly points to the predominance of environmental and nutritional factors in acne.<sup>41,42</sup> However, there is also clear evidence for a genetic disposition for acne from various twin studies. Sebum excretion exhibited higher correlations in monozygotic vs. dizygotic twins.<sup>43</sup> The proportion of branched fatty acids in the fraction of sebaceous wax esters highly correlated in monozygotic compared with dizygotic twins.<sup>44</sup> Apolipoprotein A1 serum levels were significantly lower in acne twins and a family history of acne was also significantly associated with an increased risk of developing acne.<sup>45</sup> A clinical study evaluating the role of heredity confirmed the importance of heredity as a prognostic factor for the development of acne and showed that a family history of acne is associated with earlier occurrence of the disease, increased number of retentional lesions and therapeutic difficulties.<sup>46</sup> Especially, the risk for a relapse after oral isotretinoin treatment was significantly higher in the population of patients with a positive family history of acne.<sup>46</sup>

AR polymorphism with shortened CAG repeats (<20) encoding the polyglutamine tract of the N-terminal TAD domain of the AR has been associated with increased genetic disposition for acne and other androgen-driven diseases like hirsutism and androgenetic alopecia.<sup>47-49</sup> On the other hand, AR polymorphism with extended CAG repeats results in androgen insensitivity as observed in Kennedy syndrome.<sup>27,50</sup> The N-terminal TAD domain of the AR is the interacting site for AR coregulators,<sup>29,31</sup> which modify N-terminal-C-terminal interaction of the AR protein most important in the regulation of AR transcriptional activity.<sup>29</sup> Intriguingly, FoxO1 binds to the N-terminal domain of AR and inhibits N-terminal/C-terminal interaction of the AR.<sup>32</sup> It is conceivable that a shorter polyglutamine tract of the AR (CAG repeats <20) decreases the ability and affinity for FoxO1 binding, thus increasing coactivator binding and raising the basal state of AR transcriptional activity. Impaired FoxO1 binding to ARs with TAD with shortened polyglutamine tracts could thus explain the increased susceptibility for acne of individuals with AR polymorphisms with shortened CAG repeats (<20) in comparison with individuals with normal (>20) or extended CAG repeats (>30). In acne patients with shortened CAG repeats, isotretinoin-induced upregulation of nuclear FoxO1 would thus have less inhibitory effects on AR transcriptional activity. Impaired FoxO1-AR-TAD interactions may explain the necessity for higher isotretinoin doses to reach therapeutical effects. Taken together, diminished FoxO1 interaction with ARs with shortened polyglutamine tracts (CAG repeats <20) may explain increased relapse rates of isotretinoin treatment in patients with a

high genetic disposition for acne due to AR polymorphism with reduced CAG repeat numbers.

### The Potential Role of FoxO1 for Isotretinoin's Sebum Suppressive Effect

Isotretinoin is the strongest known sebum suppressive drug for the treatment of acne. The sebaceous gland is actively involved in lipid metabolism. Isotretinoin is the most effective retinoid in reducing sebaceous gland size (up to 90%), by decreasing proliferation, disturbing the differentiation of basal sebocytes and suppressing sebum production *in vivo*.<sup>51</sup> During isotretinoin treatment a marked decrease of wax esters, a limited decrease of squalene and a relative increase of cholesterol concentration has been detected in skin surface lipids.<sup>52</sup> Oral isotretinoin was also shown to decrease glyceride fraction, whereas the relative composition of free sterols and total ceramides were increased in comedonal lipids.<sup>53</sup> Isotretinoin exerts pronounced, direct inhibitory effects on proliferation, lipid synthesis and differentiation of human sebocytes *in vitro*.<sup>54</sup> Inhibition of sebocyte proliferation and lipid synthesis were found to be independent mechanisms of isotretinoin activity.<sup>55</sup> Eight weeks of isotretinoin treatment downregulated numerous genes encoding lipid-metabolizing enzymes involved in the synthesis of cholesterol, steroids and fatty acids and increased the expression of genes encoding extracellular matrix proteins like collagen and fibroectin.<sup>56,57</sup> To understand the sebum suppressive effect of isotretinoin, the inhibitory effects of isotretinoin (1) on sebocyte lipid synthesis, (2) the inhibition of sebocyte proliferation, (3) isotretinoin's effect on sebocyte apoptosis and (4) isotretinoin's effects on sebocyte stem cell homeostasis have to be elucidated. Evidence from translational research points to the involvement of the FoxO transcription factors FoxO1 and FoxO3a in all four aspects of isotretinoin action.

### Isotretinoin, FoxO1 and Inhibition of Lipid Metabolism

The sebaceous gland belongs to the type of glands and organs with most active lipid biosynthesis. FoxO transcription factors play a critical role in metabolism and especially in lipid metabolism.<sup>19,58</sup> FoxOs have been implicated in regulating cellular proliferation, stress resistance, apoptosis and longevity. Through the insulin receptor substrate/PI3K/Akt signal cascade, FoxO1 integrates insulin action with the systemic nutrient and energy homeostasis. FoxOs are expressed ubiquitously in mammalian tissues, especially adipose, brain, heart, liver, lung, ovary, pancreas, prostate, skin, skeletal muscle, spleen, thymus and testis.<sup>19</sup> Recently, FoxO1 protein has been detected in human sebaceous glands by immune histochemistry (Liakou A, Zouboulis CC, personal communication).

**FoxO1 and PPAR $\gamma$ .** In human sebocytes, testosterone alone is not able to induce the full program of sebaceous lipogenesis.<sup>59,60</sup> Peroxisome proliferator-activated receptors (PPARs) and their ligands have been identified as important coregulators for sebaceous lipogenesis.<sup>61</sup> Specific agonists of each PPAR isoform ( $\alpha$ ,  $\delta$  and  $\gamma$ ) stimulate sebocyte differentiation. Fatty acids of *n-3*- and

*n*-6 origin and their eicosanoid derivatives play an important role as natural PPAR ligands that modulate PPAR function. PPAR $\gamma$  and its natural ligand prostaglandin J<sub>2</sub> are most important in the regulation of lipid metabolism, sebaceous gland development and function. PPAR $\gamma$  plays a significant role in mediating insulin sensitivity, glucose and lipid homeostasis, and is expressed on sebocytes increasing human sebum production.<sup>62,63</sup> PPAR $\gamma$  is transrepressed by FoxO1 like AR (Fig. 2B). FoxO1 directly binds and represses the PPAR $\gamma$ 2 promoter as well as PPAR $\gamma$  function.<sup>64,65</sup> It has been shown in adipocytes that growth factor signaling with reduced nuclear FoxO1 concentrations augments PPAR $\gamma$  activity required for terminal differentiation and prevents FoxO1-PPAR $\gamma$  interaction which rescues transrepression of genes involved in lipogenesis.<sup>66</sup> In fact, serum IGF-1 levels correlate with facial sebum excretion.<sup>67</sup> PPAR $\gamma$  heterodimerizes with RXR and binds to PPAR response elements (PPRE) in promoters of target genes. One mechanism by which FoxO1 antagonizes PPAR $\gamma$  activity is through disruption of DNA binding as FoxO1 inhibits the DNA binding activity of the PPAR $\gamma$ /RXR $\alpha$  heterodimeric complexes which have recently been detected in sebocytes (Fig. 2B). Thus, growth factor signaling inhibits the transrepressive effect of FoxO1 on AR and PPAR $\gamma$ /RXR $\alpha$  heterodimers, thus amplifying the complete program of sebaceous lipogenesis.

**FoxO1, LXR and SREBP1.** Liver X receptors (LXRs) like PPARs play a critical role in lipid metabolism. Expression of LXR $\alpha$  and LXR $\beta$  has been detected in SZ95 sebocytes and LXR ligands enhance the expression of LXR $\alpha$  stimulating lipid synthesis.<sup>68</sup> LXRs directly control the expression of sterol response element binding protein-1 (SREBP-1).<sup>69</sup> A LXRE motif is present in the PPAR $\gamma$  promoter, on which LXR $\alpha$ /RXR $\alpha$  heterodimer is bound and activated by a LXR ligand.<sup>70</sup> (Fig. 2C) In SZ95 sebocytes activation of LXR $\alpha$  induced lipid synthesis that was accompanied with the induction of SREBP-1 and PPARs.<sup>68,71</sup> In SEB-1 sebocytes, IGF-1 induced SREBP-1 expression and increased lipogenesis via activation of the PI3K/Akt signaling pathway.<sup>72</sup> FoxO1 plays an important role in the regulation of the SREBP-1c promoter activity. In skeletal muscle, SREBP-1c expression is regulated by LXR $\alpha$ /RXR $\alpha$  heterodimer and RXR $\gamma$  or RXR $\alpha$ , together with LXR $\alpha$  have been shown to activate the SREBP-1c promoter,<sup>73</sup> whereas the expression of FoxO1 negatively correlated with SREBP-1c expression (Fig. 2D). Thus, evidence from translational research corroborates the fundamental impact of nuclear FoxO1 on the regulation and SREBP-1c expression, the key transcription factor of multiple lipogenic target genes expressed in adipocyte, hepatocyte, skeletal muscle and sebocyte. Taken together, research data from various cell types with prominent lipid synthesis exhibit the suppressive regulatory effect of nuclear FoxO1 in direct transcriptional regulation of AR and PPAR $\gamma$  as well as coregulation of PPAR $\gamma$ /RXR $\alpha$  and LXR/RXR $\alpha$  heterodimers (Fig. 2).

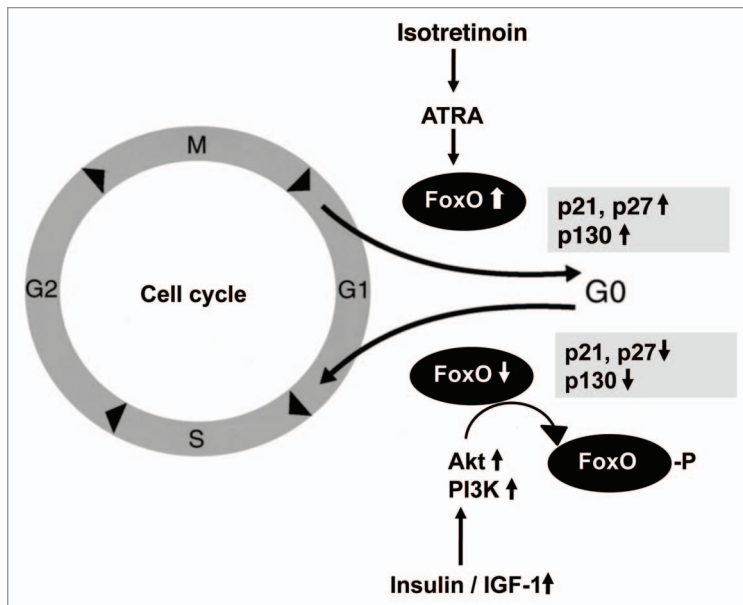
### Isotretinoin, FoxO1 and the Regulation of Cell Proliferation and Apoptosis

Isotretinoin has been found to be superior to other non-aromatic retinoids, such as tretinoin and alitretinoin, in reducing sebocyte

proliferation and suppressing sebum production.<sup>74</sup> This superior effect of isotretinoin has been attributed to the delayed initiation of retinoid inactivation under incubation of sebocytes with isotretinoin, a fact that leads to high intracellular ATRA concentrations. In contrast, incubation with ATRA leads to rapid enhancement of cellular retinoic acid binding protein-2 (CRABP-2) expression, which reduces the free intracellular concentration of ATRA through promotion of its metabolism by cytochrome P450 enzymes, and by induction of CYP1A1 expression, a major xenobiotic metabolizing enzyme, in cultured sebocytes.<sup>5</sup> The antiproliferative activity of retinoids on human sebocytes and rat preputial sebocyte-like cells in vitro was found to be mediated by RAR.<sup>5,75</sup>

Isotretinoin exerts a dose- and time-dependent antiproliferative effect on SEB-1 sebocytes and immortalized SZ95 sebocytes.<sup>5,55,76,77</sup> A portion of this decrease was attributed to cell cycle arrest at the G<sub>1</sub>/S phase of the cell cycle, as evidenced by decreased DNA synthesis, increased p21 protein and decreased cyclin D<sub>1</sub> protein.<sup>76</sup> Isotretinoin-induced apoptosis was not apparent within the first 24-hour treatment period.<sup>78</sup> Marginal induction of apoptosis in SEB-1 sebocytes by isotretinoin was detected after 48 and 72 hours of treatment which already points to delayed secondary responses of transcriptional regulation. The ability of isotretinoin to induce sebocyte apoptosis was not recapitulated by alitretinoin (9-*cis*-retinoic acid) or ATRA. The induction of cell cycle arrest and apoptosis by isotretinoin was specific to sebocytes, as the compound failed to induce apoptosis in HaCaT keratinocytes or normal human epidermal keratinocytes.<sup>76</sup> Furthermore, the RAR pan-antagonist AGN 193109 did not inhibit the apoptosis induced by isotretinoin which suggested an RAR-independent apoptotic mechanism. These observations have been interpreted in a way that, in sebocytes, isotretinoin causes inhibition of cell proliferation after intracellular metabolism to ATRA by an RAR-mediated pathway and cell cycle arrest and apoptosis by an RAR-independent mechanism, which contributes to its sebo-suppressive effect. Induction of cell cycle arrest and apoptosis by isotretinoin is likely to contribute to the overall effect on suppression of sebum, but isotretinoin also inhibits sebaceous lipid synthesis by an RAR- and RXR-mediated pathway.<sup>2,76</sup>

**FoxOs and cell cycle arrest.** There is compelling evidence that retinoids alter the expression of FoxO transcription factors.<sup>8-10</sup> It could be shown in neuroblastoma cells that ATRA induced increased expression of FoxO3a.<sup>9</sup> ATRA treatment of acute promyelocytic leukemia cells increased nuclear levels of FoxO3a which was associated with granulocytic differentiation and apoptosis.<sup>10</sup> FoxO3a is the strongest activator of the FoxO1 promoter, thus increasing the transcription of FoxO1.<sup>11</sup> Upregulation of FoxO3a correlated with the expression of FoxO target genes p27, p130 and manganese superoxide dismutase (MnSOD).<sup>9</sup> FoxO expression induces a cell cycle exit into quiescence. Increased expression of p130 protein is often associated with cell cycle exit and an entry into quiescence or senescence (Fig. 3).<sup>79,80</sup> Intriguingly, the pattern of ATRA-activated FoxO target genes of cell cycle arrest just resembles the observed changes of cell cycle proteins in isotretinoin-treated SEB-1 sebocytes like upregulation of p21 and downregulation of cyclin D<sub>1</sub>



**Figure 3.** FoxO-induced G<sub>1</sub>/S arrest of the cell cycle. Isotretinoin-mediated upregulation of cell cycle inhibitors p21 and p27 by FoxO binding to their promoters. Growth factor-mediated nuclear export of FoxO proteins with consecutive downregulation of p21, p27 and p130. ATRA, all-trans-retinoic acid; Akt, Akt kinase; PI3K, phosphoinositol-3 kinase; IGF-1, insulin-like growth factor-1.

(Table 1).<sup>76</sup> Recent studies on isotretinoin-induced changes in gene expression and apoptosis focused primarily on the regulatory role of RAR and RXR.<sup>78</sup> However, it appears that not the primary ATRA-RAR/RXR interactions are responsible for the proapoptotic effect of isotretinoin but secondary responses due to upregulation of FoxO-transcription factors. Upregulated nuclear FoxO transcription factors are pivotal inducers of apoptosis in various cell systems.<sup>8,10,11,14,15</sup> Increased CRABP-2 expression has been detected in suprabasal sebocytes of sebaceous follicles of isotretinoin-treated acne patients.<sup>81</sup> CRABP-2 was strongly expressed in sebocytes compared to epidermis of isotretinoin-treated patients, pointing to a preferential transport of ATRA to RARs in sebocytes. Proapoptotic activities of ATRA are mediated predominantly by RAR and CRABP-2, its cognate intracellular lipid binding protein which delivers ATRA to RAR, whereas fatty acid binding protein 5 (FABP-5) shuttles the hormone to PPAR $\beta/\delta$  which exert pro-proliferative responses like those observed in keratinocytes.<sup>82</sup>

The ability of ATRA to mediate proapoptotic signaling is thus cell specific and is associated with a high CRABP-2/FABP-5 ratio which results in partitioning of ATRA to RAR signaling.<sup>82</sup> ATRA-induced G<sub>1</sub>/G<sub>0</sub> growth arrest of HL-60 cells is known to require the activation of the RAR $\alpha$  and RXR.<sup>83</sup> Interestingly, FoxO3 has been identified as a key regulator for ATRA-induced apoptosis in acute promyelocytic leukemia.<sup>10</sup> These data show that beside the sebocyte various other cell types are susceptible for isotretinoin/ATRA-induced apoptosis.<sup>84</sup>

There is substantiated evidence that several transcription factors including FoxOs act downstream of ATRA.<sup>8</sup> The high correlation of gene-regulatory effects between known apoptotic

mechanisms of FoxO-transcription factors and isotretinoin-induced apoptosis in SEB-1 sebocytes corroborates the suggestion that isotretinoin mediates its antiproliferative and apoptotic effects by upregulation of FoxO transcription factors, especially FoxO1 and FoxO3a (Table 1).<sup>13</sup>

**NGAL and IGFBP-3.** Isotretinoin treatment of acne patients significantly upregulated the expression of *neutrophil gelatinase-associated lipocalin* (NGAL), which has been identified as an inducer of isotretinoin-mediated sebocyte apoptosis.<sup>85</sup> However, other NGAL-independent mediators of apoptosis could not be excluded. Both isotretinoin and ATRA increased the expression of NGAL in SEB-1 sebocytes ten-fold and seven-fold, respectively.<sup>85</sup> This similar range of NGAL expression allows the conclusion that NGAL-mediated apoptosis is not a specific mechanism of isotretinoin-induced sebocyte apoptosis. Remarkably, a 3.43-fold increased expression of *IGF binding protein-3* (IGFBP-3) during isotretinoin treatment was exclusively observed in sebocytes but not in whole skin.<sup>85</sup> The expression of IGFBP-3 has been shown to be retinoid responsive. For instance, IGFBP-3 is upregulated by ATRA in human dermal papilla cells.<sup>40</sup> IGFBP-3 is a peculiar IGF-1 binding protein, which translocates into the nucleus and interferes with RAR/RXR leading to changes of receptor transactivation.<sup>86,87</sup> Nuclear IGFBP-3

is a potent inducer of apoptosis.<sup>86</sup> Intriguingly, IGFBP-3 is a known FoxO target gene.<sup>15</sup> In prostate cancer cells IGFBP-3 enhanced RXR response element and inhibited RARE signaling. Thus, RXR $\alpha$ -IGFBP-3 interaction leads to modulation of the transcriptional activity of RXR $\alpha$  which is essential for mediating the effects of IGFBP-3 on apoptosis.<sup>86</sup> There might be a common unifying mechanisms of NGAL- and IGFBP-3-mediated sebocyte apoptosis. The promoter region of the *LCN2* gene contains consensus sequences for binding both RAR- and RXR.<sup>85</sup> FoxO-mediated upregulation of IGFBP-3 may interact with RXR on the *LCN2* promoter thus activating the expression of NGAL. This proposed FoxO/IGFBP-3-mediated gene regulatory mechanism of apoptosis would perfectly fit into FoxOs' biological role as inducers of apoptosis, metabolic rest (transcription factor of starvation) and activator of innate immunity associated with increased expression of antimicrobial peptides like defensin- $\beta$ 1.<sup>88</sup> Both antimicrobial peptides and NGAL function as effectors of innate immunity against microbial pathogens.<sup>85,88</sup> It is thus not surprising that the expression of defensin- $\beta$ 1 is upregulated by FoxO as well as isotretinoin treatment.<sup>85,88</sup> Isotretinoin-induced FoxO-activation of the IGFBP-3 promoter might be the underlying cause of isotretinoin-induced sebocyte apoptosis by nuclear IGFBP-3 overexpression. IGFBP-3/RXR $\alpha$ -mediated apoptosis as well as FoxO1-mediated downregulation of the AR transcriptional activity, PPAR $\gamma$  function and SREBP-1c promoter activity all together could thus contribute to the sebum-suppressive and apoptotic effect of isotretinoin treatment.

Isotretinoin-induced nuclear overexpression of FoxO1 and IGFBP-3 might also mediate the anti-comedogenic effects of

**Table 1.** Overlapping gene regulatory functions of FoxO proteins and isotretinoin

Genes & cell functions	FoxO proteins	Ref.	Isotretinoin	Ref.
Cyclin D <sub>1</sub> ↓	FoxO1, FoxO3a, FoxO4	11, 14, 15, 18, 110	Isotretinoin (sebocyte)	76
p21 ↑	FoxO1, FoxO3a, FoxO4	11, 14, 15, 18, 110	Isotretinoin (sebocyte)	76
Apo C-III ↑	FoxO1	191	Isotretinoin (hepatocyte)	190
IGFBP-3 ↑	FoxOs	15	Isotretinoin (sebocyte) ATRA (dermal papilla cells)	40, 85
Defensin β <sub>1</sub> ↑	FoxO	88	Isotretinoin (sebocyte)	85
DNA synthesis ↓	FoxO1, FoxO3a, FoxO4	11, 14, 15, 18, 80, 110	Isotretinoin	76
G <sub>1</sub> /S arrest ↑	FoxOs	11, 14, 15, 18, 80, 103, 110	Isotretinoin (sebocyte)	76
Apoptosis ↑	FoxO1, FoxO3a, FoxO4	11, 14, 15, 18, 80, 110	Isotretinoin (sebocyte)	76
Caspase 3 ↑	FoxOs	267	Isotretinoin (sebocyte) Dalton's lymphoma ascites cells B16F-10 melanoma cells	76, 281, 282
ROS ↓	FoxOs, FoxO3a	171, 172	Isotretinoin (leukocytes)	169
Lipogenesis ↓	FoxO1	19	Isotretinoin (sebocytes) Isotretinoin (keratinocytes)	56, 76
VLDL ↑	FoxO1	19, 187, 188	Isotretinoin (plasma)	184
Insulin resistance ↑	FoxO1	19, 180	Isotretinoin	176, 177
Androgen receptor ↓	FoxO1	32–34	Isotretinoin (skin)	37
MMP-2 ↓ MMP-9 ↓	FoxO1a, FoxO3a	140, 144, 145	Isotretinoin (sebum & keratinocyte)	139

isotretinoin as upregulated IGFBP-3 suppresses proliferation of transient amplifying keratinocytes.<sup>89</sup> Comedo formation results from increased proliferation and retention of infundibular keratinocytes.<sup>90</sup> The antiproliferative activity of nuclear IGFBP-3 has also been confirmed in myeloid leukemia cells, while IGFBP-3 enhances signaling through RXR/RXR homodimers, it blunts signaling by activated RAR/RXR heterodimers.<sup>91</sup> In human breast cancer, ATRA mediated IGFBP-3-promoted apoptosis by enhancing the activity of RXRα.<sup>92</sup> Thus, FoxO-mediated antiproliferative and apoptosis-inducing effects may explain the chemopreventive activity of isotretinoin in certain types of cancers.

### Does Isotretinoin Induce FoxO-Mediated Sebocyte and Sebocyte Stem Cell Arrest?

Fascinating research of the last years has elucidated various signals controlling sebocyte differentiation in vivo and major signaling pathways regulating differentiation of the sebaceous gland, recently reviewed in this journal.<sup>93</sup> Activation of c-myc and hedgehog signaling cascades and repression of β-catenin signaling are important for the differentiation and maturation process experienced by sebocytes. They are essential inductive events responsible for the morphogenesis of the sebaceous gland during embryonal and neonatal development.<sup>93</sup> There is good evidence that activation of *c-myc* in mouse skin results in enhanced sebaceous gland morphogenesis,<sup>94,95</sup> and induction of sebocyte cell fate even within the interfollicular epidermis.<sup>96</sup> The effect of c-myc is somewhat surprising because c-myc is reported to act downstream of β-catenin and is a direct target gene of canonical Wnt signaling.<sup>97,98</sup> In skin, c-myc and β-catenin exert opposing effects on sebocyte differentiation. Analysis of transgenic mice with simultaneous activation of c-myc and β-catenin revealed mutual antagonism: c-myc blocked β-catenin-mediated

formation of ectopic hair follicles and β-catenin reduced c-myc-stimulated sebocyte differentiation.<sup>99</sup> Pulse-chase experiments in mouse skin suggested the existence of slow-cycling cells in the gland and a small cluster of cells at the base of the sebaceous glands expressed the transcriptional repressor Blimp1.<sup>100,101</sup> Blimp1-expressing cells were suggested to be progenitors that give rise to all cells within the sebaceous gland. However, the functional signaling relationship between Blimp1 and c-myc is currently contradictory. On one hand, Blimp1 is not selectively expressed in sebaceous gland progenitor cells, but is also expressed by terminally differentiating cells in the interfollicular epidermis, sebaceous gland and hair follicle.<sup>99,102</sup> A recent study implies that Blimp-1 is expressed late in embryonic development and is restricted to the evolving sebaceous gland and Blimp-1 labels only the most mature cellular constituents.<sup>102</sup> More confusing is the fact that despite Blimp1's known negative regulation of the c-myc promoter,<sup>101</sup> no correlation between Blimp1 and c-myc levels has been found in individual human sebaceous cells.<sup>99</sup> This contradiction suggested that additional factors regulate levels of c-myc protein in sebocytes.<sup>99</sup> Do FoxO transcription factors represent the missing link to understand these controversies in c-myc regulation?

**FoxOs and c-myc.** FoxO transcription factors have been identified as important regulators of stem cell homeostasis.<sup>103</sup> FoxOs play an increasing physiological role in the maintenance and integrity of stem cell compartments in a broad spectrum of tissues.<sup>103</sup> For instance, FoxOs cooperate to affect quiescence of hematopoietic stem cells by regulation of mediators of the G<sub>0</sub>/G<sub>1</sub> and G<sub>1</sub>/S arrest including Rb/p130, cyclin G<sub>2</sub>, p27, p57, p21 and cyclin D<sub>2</sub>.<sup>103</sup> FoxO-mediated stem cell regulation of stem cell quiescence resembles isotretinoin-mediated effects on sebocyte cell cycle arrest. Thus, the question arises whether isotretinoin's sebaceous suppressive effects are related to FoxO-induced quiescence

of sebocyte stem cells? Recent evidence points to a substantial molecular cross talk between FoxO and c-myc dependent signal transduction.<sup>104,105</sup> In colon cancer cells, induction of the transcriptional repressor protein *Mxi1-SR $\alpha$*  of the Mad/Mxd family of proteins by FoxO3a repressed myc-dependent gene expression.<sup>104</sup> FoxO3a activation induced a switch in promoter occupancy from myc to Mxi1 on the E-box containing promoter regions of two studied myc target genes. siRNA-mediated transient silencing of Mxi1 or all Mad/Mxd proteins reduced exit from S phase in response to FoxO3a activation and stable silencing of Mxi1 or Mad1 reduced the growth inhibitory effect of FoxO3a. Thus, the induction of Mad/Mxd proteins contributes to the inhibition of proliferation in response to FoxO3a activation. Direct regulation of Mxi1 by FoxO3a appears to be an additional mechanism through which the PI3K/Akt/FoxO pathway can modulate c-myc function.<sup>104</sup>

There is another important connection between FoxO and c-myc regulation of the p27 cyclin dependent kinase inhibitor. It has been shown in murine WEHI 231 immature B lymphoma cells that inhibition of PI3K/Akt signaling decreased the levels of NF $\kappa$ B and c-myc, which has been shown to repress p27 promoter activity.<sup>105</sup> p27 is coordinately regulated via two arms of a signaling pathway that are inversely controlled upon inhibition of PI3K: induction of the activator FoxO3a and downregulation of the repressor c-myc.<sup>105</sup> FoxO1a, FoxO3a and FoxO4 transactivate the p27 promoter.<sup>105</sup> FoxO3a induced p27 transcription and apoptosis of Ba/F3 cells.<sup>106</sup> The p27 cyclin-dependent kinase (CDK) inhibitor plays an essential role in transition through the G<sub>1</sub> phase, in particular the restriction point, via binding to and inhibiting such complexes as cyclin E-CDK2 and cyclin-A CDK2.<sup>107</sup> There is strong evidence that FoxOs induce G<sub>1</sub> arrest through expression of p27, p21 and p130 and increase the duration of the G<sub>2</sub> phase of the cell cycle by inducing cyclin G<sub>2</sub> (Fig. 3).<sup>80,108-112</sup>

Assuming that this regulatory mechanism operates in sebocytes and sebocyte stem cells as well, the reciprocal control of FoxO3a and c-myc via the PI3K pathway could modify sebaceous gland proliferation via p27 regulation. High levels of growth factors, insulin and IGF-1 in puberty, hyperinsulinemic western diet (hyperglycemic carbohydrates and insulinotropic milk) or acne-associated syndromes with insulin resistance would translocate FoxOs from the nucleus by increased PI3K/Akt signaling, whereas isotretinoin treatment with proposed upregulation of FoxOs counterbalances the effect of increased growth factor signaling in acne and downregulates increased sebocyte proliferation and induces sebocyte apoptosis, the main regulatory features of FoxO transcription factors (Fig. 3).

**Sox9, FoxO and  $\beta$ -catenin.** The earliest known signal necessary for sebaceous gland development is the transcription factor Sox9.<sup>113</sup> The Sox family of transcription factors has emerged as modulators of canonical Wnt/ $\beta$ -catenin signaling in development and diverse disease contexts, recently reviewed elsewhere.<sup>114</sup> Sox physically interact with  $\beta$ -catenin and modulate the transcription of Wnt-target genes.<sup>114</sup> On the other hand, Wnt signaling also regulates Sox expression resulting in feedback regulatory loops that fine tune cellular responses to  $\beta$ -catenin/Tcf activity.<sup>114</sup>

Sox9 in mouse intestinal epithelium requires Wnt signaling, but Sox9 then locally attenuates Wnt-target gene expression.<sup>115,116</sup> These observations clearly demonstrate, that  $\beta$ -catenin maintains a molecular cross-talk with other transcription factors, especially in early steps of stem cell regulation.

FoxO transcription factors not only interact with c-myc signaling but also interact with  $\beta$ -catenin signaling and may be a modulating element between c-myc-driven sebocyte proliferation and Wnt/ $\beta$ -catenin-regulated sebaceous gland morphogenesis. Blocking canonical Wnt signalling during skin development by expression of a dominant negative mutant transcription factor Lef1 ( $\Delta$ NLef1) results in transdifferentiation of hair follicle keratinocytes into mature sebocytes.<sup>117,118</sup> A high proportion of human sebaceous adenomas and sebaceousomas exhibit double nucleotide mutations within the  $\beta$ -catenin binding domain of the *lef1* gene. These mutations within the NH2 terminus of Lef1 prevent  $\beta$ -catenin binding and inhibit expression of  $\beta$ -catenin target genes.<sup>119</sup> Transgenic mice expressing N-terminally deleted  $\Delta$ NLef1 in the skin develop spontaneous sebaceous tumours.<sup>118</sup> Suppression in Wnt/ $\beta$ -catenin signaling activity by overexpression of Smad7 with accelerated cytoplasmic  $\beta$ -catenin degradation resulted in increased sebaceous gland morphogenesis and increased sebocyte differentiation.<sup>120</sup> Sebaceous gland hyperplasia observed in aged UV-exposed skin exhibits upregulation of Smad7 expression,<sup>121</sup> which is associated with reduced  $\beta$ -catenin levels.<sup>120</sup>

Thus, there is good evidence that suppression of Wnt/ $\beta$ -catenin signaling promotes sebocyte differentiation. c-myc is reported to be a direct target gene of canonical Wnt/ $\beta$ -catenin signaling and to act downstream of  $\beta$ -catenin.<sup>97,98</sup> Therefore, it should be expected that downregulation of  $\beta$ -catenin would suppress c-myc as well. However, it is surprising that activation of c-myc in mouse skin enhanced sebaceous gland morphogenesis,<sup>94,95</sup> and induced a sebocyte cell fate even within the inter-follicular epidermis.<sup>96</sup> c-myc and  $\beta$ -catenin exert thus opposing effects on sebocyte differentiation. Analysis of transgenic mice with simultaneous activation of c-myc and  $\beta$ -catenin revealed this mutual antagonism: c-myc blocked  $\beta$ -catenin-mediated formation of ectopic hair follicles and  $\beta$ -catenin reduced c-myc-stimulated sebocyte differentiation.<sup>99</sup>

### Does Isotretinoin Inhibit AR-Mediated Suppression of Wnt/ $\beta$ -Catenin Signaling?

Wingless proteins (Wnts) are secreted lipid-modified proteins that bind to a receptor complex comprising frizzled and the low-density lipoprotein receptor-related proteins 5 or 6 (LRP5 or LRP6).<sup>122</sup> Activation of this receptor complex by Wnts leads to inactivation of glycogen synthase kinase 3 $\beta$  (GSK-3 $\beta$ ), which prevents the proteosomal degradation of the transcriptional coactivator  $\beta$ -catenin and, thereby, promotes its accumulation in the cytoplasm.  $\beta$ -Catenin translocates into the nucleus where it associates with the T-cell factor (Tcf)/lymphoid-enhancer binding factor (Lef) family of transcription factors and regulates the expression of Wnt target genes.<sup>122</sup>

There is recent evidence for a cross-regulation of signaling pathways of nuclear hormone receptors with the canonical Wnt



pathway.<sup>123</sup> The best characterized interaction between nuclear hormone receptors and the canonical Wnt pathway stems from the discovery that RAR binds directly to  $\beta$ -catenin in breast cancer cells.<sup>124</sup> ATRA decreased the activity of the  $\beta$ -catenin-Lef/Tcf signaling pathway.  $\beta$ -catenin interacted directly with the RAR in a retinoid-dependent manner, but not with RXR and RAR competed with Tcf for  $\beta$ -catenin binding.<sup>124</sup> Similar interactions have been discovered for vitamin D receptor (VDR), PPAR $\gamma$ , RXR, LXR $\alpha$  and  $\beta$ , estrogen receptor (ER) and AR.<sup>123</sup>

AR and  $\beta$ -catenin interact by direct binding and complexing, AR/ $\beta$ -catenin interactions are ligand sensitive, whereby complexing occurs in the presence of dihydrotestosterone (DHT).<sup>125</sup> Intriguingly, AR has an inhibitory effect on Tcf/Lef-mediated transcription and can compete with Tcf/Lef molecules for  $\beta$ -catenin binding.<sup>126-128</sup> Repression of the  $\beta$ -catenin/Tcf signaling is mediated by ligand-occupied AR that is in competition with Tcf for nuclear  $\beta$ -catenin.<sup>128</sup> As outlined above, inhibition of Wnt/ $\beta$ -catenin/Tcf/Lef-signaling is a requirement for sebocyte differentiation. The reciprocal relationship between AR and  $\beta$ -catenin on Tcf/Lef-mediated transcription allows the conclusion that a decrease in liganded AR would increase  $\beta$ -catenin-mediated Tcf/Lef-signaling, thus inhibiting sebocyte differentiation. Remarkably, a significant reduction in AR protein expression in skin of acne patients has been observed during oral isotretinoin treatment.<sup>37</sup> However, the time course of reduced AR expression in skin after a usual 3 to 4 month lasting isotretinoin treatment is not known. Moreover, the role of AR and FoxOs in sebocyte stem cell homeostasis has not been studied but may contribute to a prolonged downregulation of Wnt signaling in sebaceous stem cells. It is conceivable that an impairment of sebaceous stem cells would contribute to insufficient epidermal regeneration after epithelial injury. The clinical observation of impaired wound healing after systemic isotretinoin treatment might find here a plausible explanation. Thus, further studies with cultured sebocytes, human sebaceous glands and stem cells should address the possible FoxO1/AR and AR/ $\beta$ -catenin interaction in the presence or absence of isotretinoin.

### Parallels between Adipocyte and Sebocyte Differentiation

There are striking similarities in the regulation of Wnt signaling between sebocyte differentiation and adipogenesis. As already outlined, reduced Wnt signaling is required for sebocyte differentiation, whereas increased Wnt signaling inhibits sebocyte differentiation.<sup>93</sup> When Wnt signaling is off, adipogenesis is initiated, when it is on, adipogenesis is repressed.<sup>129</sup> Thus, Wnt signaling like FoxO1 functions as a lipogenic switch. Wnt signaling maintains preadipocytes in an undifferentiated state through inhibition of the adipogenic transcription factors CCAAT/enhancer binding protein  $\alpha$  (C/EBP $\alpha$ ) and PPAR $\gamma$ .<sup>129</sup> High expression of C/EBP $\alpha$ , C/EBP $\beta$  and PPAR $\gamma$  has been detected in immortalized SZ95 sebocytes which is important for sebocyte differentiation and sebaceous lipogenesis.<sup>130</sup> Intriguingly, the master transcription factors C/EBP $\alpha$  and PPAR $\gamma$  are under direct or

indirect control by members of the FOX family.<sup>131-133</sup> Expression of FoxO1, FoxO3a and FoxO4 is increased during adipogenesis coincident with expression of PPAR $\gamma$  and C/EBP $\alpha$ , but FoxO1 activation is delayed until the end of clonal expansion.<sup>133</sup> Remarkably, expression of constitutively active FoxO1 mutants prevent the differentiation of 3T3-L1 preadipocytes in adipocytes.<sup>131,132</sup> Oral isotretinoin treatment is expected to force high expression of FoxO3a and FoxO1, which may inhibit sebocyte C/EBP $\alpha$  and PPAR $\gamma$  activity. Thus, evidence from translational research clearly demonstrates that FoxOs are involved in the regulation of AR, c-myc, C/EBP $\alpha$ , PPAR $\gamma$ ; LXR $\alpha$  and SREBP-1c, all important regulatory transcription factors involved in differentiation of actively lipid synthesizing cells like sebocytes.

There is another regulatory metabolic relationship between Wnt signaling and ATRA. Wnt suppresses CYP26, an enzyme that is responsible for degrading ATRA into inactive metabolites.<sup>134</sup> Low Wnt signaling would result in less CYP26 suppression with low levels of ATRA, whereas high Wnt signaling would have a stronger inhibitory effect on CYP26 resulting in high ATRA levels. In isotretinoin-treated sebocytes, high intracellular ATRA levels due to isotretinoin isomerization resemble a constellation of high Wnt signaling, thus suppressing sebocyte differentiation.

### FoxO Proteins Interact with $\beta$ -Catenin

Recent evidence corroborated the important role of Wnt signaling for sebocyte differentiation and sebaceous gland morphogenesis.<sup>93,99</sup> In 2005, Essers et al. reported an evolutionarily conserved interaction between  $\beta$ -catenin and FoxO proteins.<sup>135</sup> In mammalian cells,  $\beta$ -catenin interacts with FoxO1 and FoxO3a. This interaction requires armadillo repeats 1 to 8 of  $\beta$ -catenin and the C-terminal half of FoxO proteins.<sup>135</sup> Binding of  $\beta$ -catenin to FoxO enhances the transcriptional activity of FoxO.<sup>135</sup> Interestingly, high Wnt signaling with elevated levels of  $\beta$ -catenin are known to inhibit sebaceous gland morphogenesis and sebocyte differentiation. It is conceivable that high nuclear levels of  $\beta$ -catenin bind to FoxO3a and FoxO1 and augment their transcriptional proapoptotic effects.<sup>14,15,19</sup> It is well demonstrated that FoxOs and Tcf factors compete for the limited nuclear pool of  $\beta$ -catenin.<sup>136,137</sup> These observations confirm the pivotal role of the evolutionarily conserved FoxO/ $\beta$ -catenin interaction and provide new insights into the complex signaling network of AR, FoxO, Wnt, Sox,  $\beta$ -catenin and c-myc in the development and homeostasis of the sebaceous gland.

Retinoids modify this regulatory network at multiple sites: ATRA induces upregulation FoxO3a.<sup>9,10</sup> Moreover, the Wnt pathway can modulate RAR signaling and vice versa.<sup>123</sup> ATRA decreases c-myc-dependent target genes.<sup>124</sup> Isotretinoin reduces IGF-1 serum levels.<sup>36</sup> The activated PI3K/Akt pathway promotes FoxO shuttling from the nucleus to the cytoplasm, inhibits GSK3 $\beta$  which prevents proteasomal degradation of  $\beta$ -catenin.<sup>125</sup> These data imply that a forced intracellular upregulation of ATRA by isotretinoin administration interferes with the activity of multiple important transcription factors involved in gene regulation orchestrated by FoxO transcription factors.

## Isotretinoin and FoxO-Mediated Anti-Inflammatory Effects

Isotretinoin treatment in acne exerts various anti-inflammatory effects including modulation of metalloproteinase function, downregulation of reactive oxygen formation, inhibition of pro-inflammatory NF $\kappa$ B-mediated cytokine signaling and modulation of acquired and innate immunity. It will be shown that upregulated FoxO transcription factors are again most likely candidates which mediate all these anti-inflammatory effects.

**FoxOs and metalloproteinases.** Isotretinoin is known to inhibit scarring in acne and affects dermal tissue remodeling. NF $\kappa$ B and activator protein-1 are activated in acne lesions with consequent elevated expression of inflammatory cytokines and matrix degrading metalloproteinases (MMPs). These elevated gene products have been shown to be molecular mediators of inflammation and collagen degradation in acne lesions *in vivo*.<sup>138</sup> Sebum contains proMMP-9, which was decreased following per os or topical treatment with isotretinoin in parallel to the clinical improvement of acne. Sebum also contains MMP-1, MMP-13, tissue inhibitor of metalloproteinase-1 (TIMP-1) and TIMP-2, but only MMP-13 was decreased following treatment with isotretinoin. The origin of MMPs and TIMPs in sebum is attributed to keratinocytes and sebocytes, since HaCaT keratinocytes in culture secrete proMMP-2, proMMP-9, MMP-1, MMP-13, TIMP-1 and TIMP-2. SZ95 sebocytes in culture secreted proMMP-2 and proMMP-9. Isotretinoin inhibited the arachidonic acid-induced secretion and mRNA expression of proMMP-2 and -9 in both cell types and of MMP-13 in HaCaT keratinocytes.<sup>139</sup>

Thus, there is evidence for the influence of isotretinoin on the regulation of certain MMPs, however there is little information on its regulatory role at the level of gene transcription. The question arises whether FoxOs may regulate the promoter activity of certain MMPs? Interestingly, Tanaka et al. recently investigated the effect of UV-induced changes in FoxO1a expression and the roles of FoxO1a in the regulation of collagen synthesis and MMP expression in human dermal fibroblasts.<sup>140</sup> It should be emphasized that primarily the dermal compartment with its fibroblasts and not the keratinocytes and sebocytes is the primary target of tissue destruction and remodeling in acne. Interestingly, in UVA- or UVB-irradiated fibroblasts the expression of FoxO1a mRNA decreased significantly. The expression of type I collagen also decreased. On the other hand, MMP-1 and MMP-2 mRNA levels increased. FoxO1a small interfering RNA transfection induced the downregulation of FoxO1a expression, it also induced a decrease in type I collagen expression, and it increased MMP-1 and MMP-2 expression. In contrast, the addition of FoxO1a-peptide induced an increase in type I collagen expression and decreased in MMP-1 and MMP-2 expression.<sup>140</sup> Therefore it was concluded that FoxO1a plays a substantial role in skin photoaging, and control of FoxO1a may be a novel approach to prevent the collagen deficiency observed in photoaged skin. This is exactly the rationale of topical ATRA-treatment for aged, UV-damaged skin: to increase collagen synthesis and to reduce the activity of matrix degrading MMPs.<sup>141,142</sup>

There is even more evidence for the regulatory role of FoxOs in MMP expression. In endothelial cells certain vascular endothelial growth factor (VEGF)-responsive genes require FoxO1 activity for optimal expression like MMP-10.<sup>143</sup> Furthermore, resveratrol, a PI3K inhibitor, can enhance the apoptosis-inducing potential of TRAIL by activating FoxO3a and its target genes associated with an inhibition of MMP-2 and MMP-9 expression.<sup>144</sup> Astrocyte-elevated gene-1 (AEG-1) has been reported to be upregulated in several malignant cells and plays a critical role in Ha-ras-mediated oncogenesis through the PI3K/Akt signaling pathway. Interestingly, AEG-1 knockdown induced cell apoptosis through upregulation of FoxO3a activity. This alteration of FoxO3a activity was dependent on reduction of Akt activity in LNCaP and PC-3 cells. AEG-1 knockdown was associated with increased levels of FoxO3a and attenuated the expression of MMP-9.<sup>145</sup> In vascular smooth muscle cells, the C-terminal transactivation domain of FoxO4 is required for FoxO4-activated MMP-9 transcription. FoxO4 activates transcription of the MMP-9 gene in response to tumor necrosis factor- $\alpha$  (TNF $\alpha$ ) signaling.<sup>146</sup> FoxO4 activates the MMP-9 promoter by binding to the transcription factor Sp1, whereas FoxO1 failed to activate the MMP-9 promoter.<sup>146</sup> These data show that distinct FoxO isoforms are able to regulate or coregulate MMP promoters thus linking MMP activity to FoxO signaling. Together, there is an overlap in the inhibitory activity of FoxO1 and FoxO3a and isotretinoin, respectively, regulating the expression of MMP-1, MMP-2 and MMP-3. In conclusion, isotretinoin's suppressive effect on MMP expression can be well explained by isotretinoin-induced upregulation of FoxO1 and FoxO3a modifying MMP promoter activity.

**Isotretinoin, FoxOs, TLRs and NF $\kappa$ B signaling.** The growth factor-stimulated PI3K/Akt pathway activates NF $\kappa$ B signaling that inhibits apoptosis and triggers inflammatory responses and mediates just the opposite of FoxO-mediated gene transcription.<sup>147</sup> Acne in puberty and acne-associated syndromes are associated with increased insulin/IGF-1 signaling.<sup>12,20,21</sup> Excessive insulin/IGF-1 signaling activates the Akt/IKK/NF $\kappa$ B pathway.<sup>147</sup> The canonical pathway of NF $\kappa$ B activation transduces signals from Toll-like receptors (TLRs) and several cytokine receptors like interleukin-1 receptor (IL-1R) mainly to the IKK $\beta$  kinase.<sup>148,149</sup>

Activation of PI3K/Akt signaling by IGF-1 has been shown to increase SREBP-1 expression and sebaceous lipogenesis.<sup>72</sup> Sebaceous triglycerides are a preferred nutrient source of *P. acnes*, a critical milieu factor for *P. acnes* follicular hypercolonization and biofilm formation which trigger TLR-signaling of surrounding cells of the follicular environment. Indeed, TLR expression was found to be increased in the epidermis of acne lesions (TLR2, TLR4) and macrophages (TLR2) in which *P. acnes* induced cytokine production through a TLR2-dependent pathway.<sup>150,151</sup> Distinct strains of *P. acnes* induced selective human  $\beta$ -defensin-2 and IL-8 expression in human keratinocytes through TLRs.<sup>152</sup> *P. acnes*, by acting on TLR2, activates NF $\kappa$ B and stimulates the secretion of IL-6 and IL-8 by follicular keratinocytes and IL-8 and IL-12 by macrophages, giving rise to inflammation. Thus, TLRs play an important role in the induction of innate immunity and inflammatory cytokine responses in acne.<sup>153</sup> Both, the insulin/IGF-1-mediated upregulation of Akt-mediated NF $\kappa$ B-signaling

and *P. acnes*-TLR-mediated NFκB-signaling contribute to the upregulation of inflammatory cytokines in acne. Intriguingly, TLR2 contains a PI3K binding motif and activation of PI3K is particularly important for TLR2 signaling.<sup>154</sup> In response to bacterial ligands, Src family kinases initiate TLR2-associated signaling, followed by recruitment of PI3K and phospholipase Cγ necessary for the downstream activation of proinflammatory gene transcription.<sup>155</sup> PI3K activation is not only associated with TLR signaling but as well as with IL-1/IL-1R signaling, which both converge in increased activation of NFκB.<sup>147</sup> Furthermore, a direct interaction between PI3K and TLRs or their adaptor proteins, such as MyD88, has been reported.<sup>154,156</sup> Thus, growth factor-signaling via PI3K/Akt/NFκB as well as TLR2/PI3K/Akt/NFκB signal transduction are integrated at the level of Akt activation most likely resulting in a nuclear deficiency of FoxOs. Isotretinoin treatment with upregulation of FoxOs will counterbalance the nuclear FoxO deficiency of growth factor-activated PI3K/Akt and will thereby attenuate PI3K/Akt-mediated proinflammatory NFκB signaling.

In a vicious cycle, *P. acnes* might stimulate TLR2 on sebocytes which further increase PI3K/Akt-mediated sebaceous lipogenesis. TLR2 and TLR4 are constitutively expressed on SZ95 sebocytes.<sup>157</sup> Interestingly, *P. acnes* exposure to hamster sebaceous glands has been shown to augment lipogenesis in vivo and in vitro.<sup>158</sup> This observation implicates that TLR2-mediated PI3K/Akt activation might not only be involved in the stimulation of inflammatory responses to *P. acnes* but also to *P. acnes*-triggered TLR2/PI3K/Akt-stimulated sebaceous lipogenesis. Downregulation of PI3K/Akt-mediated sebaceous lipogenesis by isotretinoin-induced upregulation of nuclear FoxOs would just impair lipogenesis and reduce the lipophilic follicular milieu for *P. acnes* overgrowth and *P. acnes*-mediated proinflammatory TLR2/PI3K/Akt/NFκB signal transduction.

### Isotretinoin, FoxOs and Acquired Immunity

It is well known that isotretinoin exerts anti-inflammatory activity.<sup>2,3</sup> Recent studies have highlighted a fundamental role for FoxO transcription factors in immune system homeostasis.<sup>159</sup> In vitro overexpression studies suggested that FoxO1 and FoxO3a are important for growth factor withdrawal-induced lymphocyte cell death. Moreover, FoxO factors importantly regulate cell cycle progression of lymphocytes. FoxOs are of pivotal importance for the control of lymphocyte homeostasis including critical functions in the termination and resolution of an immune response.

There is a functional link between upregulated TLR2-signaling in acne with increased interleukin-1α (IL-1α) production and T-cell mediated acquired immunity because selected IL-1 receptor associated kinases (IRAK-1, 2, M and 4) are bifunctional and can be recruited either to the TLR complex and thus mediate TLR-signaling or can associate with adapter proteins involved in T- and B-cell receptor-mediated signaling pathways linking TLR/IRAK signaling to adaptive immune responses.<sup>160</sup> ATRA has been shown to downregulate TLR2 expression and function.<sup>161</sup> TLR2/PI3K-signaling appears to be the connecting element between upregulated innate and adaptive immune

responses in acne. Increased CD4<sup>+</sup> T cell infiltration and IL-1 activity has been detected in acne-prone skin areas prior to follicular hyperkeratinization and comedo formation.<sup>162</sup>

Intriguingly, FoxO family members play critical roles in the suppression of T cell activation and T cell homing.<sup>16-18</sup> FoxO1 deficiency in vivo resulted in spontaneous T cell activation and effector differentiation.<sup>17,18</sup> Functional studies validated interleukin 7 receptor-α (IL-7Rα) as a FoxO1 target gene essential for FoxO1 maintenance of naïve T cells. These findings reveal crucial functions of FoxO1-dependent transcription in control of T cell homeostasis and tolerance. FoxO1 links homing and survival of naïve T cells by regulating L-selectin, CCR7 and IL-7Rα.<sup>163</sup>

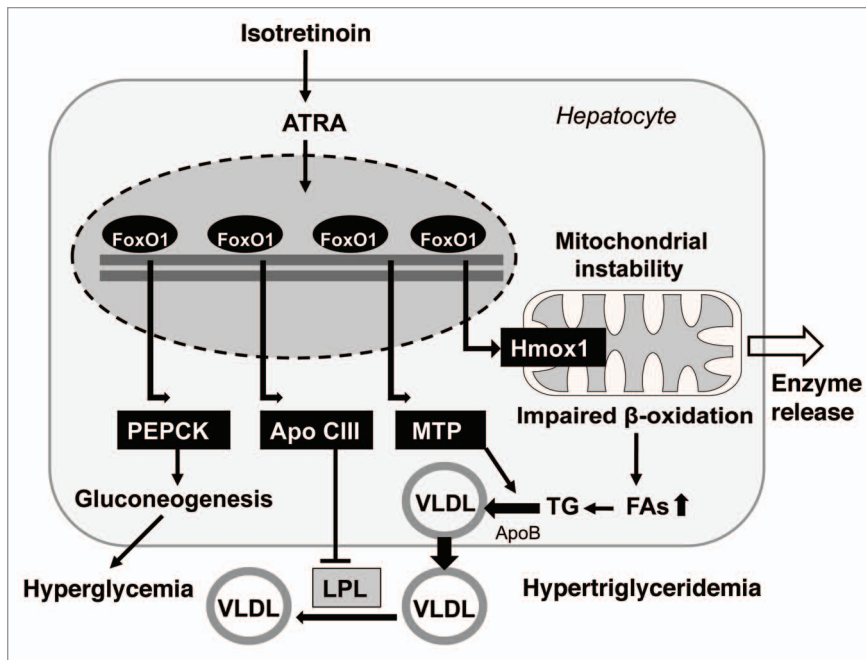
### Isotretinoin, FoxOs and Innate Immunity

There is new evidence that metabolism and growth factor status determine the activity of genes involved in innate immunity which may play a role in *P. acnes* hypercolonization. A recent study underlined the pivotal role of FoxOs in the regulation of innate immunity.<sup>88</sup> In *Drosophila* flies FoxO transcription factor control the expression of several antimicrobial peptides (AMPs) in various tissues including skin.<sup>88</sup> AMP induction is lost in *foxo null* mutants but enhanced when FoxO is overexpressed. In *Drosophila*, AMP activation can be achieved independently of immunoregulatory pathogen-dependent pathways by FoxO, indicating the existence of cross-regulation of metabolism and innate immunity at the promoter level of FoxO-activated AMP genes.<sup>88</sup> In contrast, insulin and IGF-1 dependent signaling with Akt-mediated translocation of FoxO from the nucleus into the cytosol reduces the expression of AMPs. It is thus conceivable that insulinotropic western diet (milk, dairy and hyperglycemic carbohydrates) affects the balance and activity of AMPs. We have to ask whether insulinotropic western diet impairs innate immunity of the pilosebaceous follicle in such a way that *P. acnes* hypercolonization is promoted. Both isotretinoin-induced upregulation of FoxO1 with reduced sebaceous lipogenesis and isotretinoin-induced stimulation of the AMP response are synergistic mechanisms which could explain isotretinoin's suppressive effects on sebaceous lipogenesis, *P. acnes* growth and bacterial follicular colonization.

Taken together, isotretinoin-mediated upregulation of FoxO1 may exert anti-inflammatory effects by downregulation of T-cell responses and upregulation of innate immunity.<sup>16-18</sup> In contrast, growth factor (insulin, IGF-1, FGF)-induced nuclear deficiency of FoxOs would activate T-cell proliferation and decreases expression of AMPs. A decrease of AMPs would increase the number of pathogens (*P. acnes*) stimulating TLR-induced proinflammatory genes. These pro-inflammatory changes of acquired and innate immunity in acne may be counterbalanced by isotretinoin-mediated upregulation of FoxO1.

### FoxO1 and Isotretinoin-Mediated Suppression of Oxidative Stress

Isotretinoin treatment in acne and rosacea has beneficial effects due to its ability to suppress the formation of reactive oxygen



**Figure 4.** Isotretinoin/FoxO1-mediated upregulation of hepatic gene expression at the promoter level: upregulation of phosphoenolpyruvate carboxykinase (PEPCK) results in gluconeogenesis; upregulation of apolipoprotein C-III (Apo CIII) inhibits the activity of lipoprotein lipase (LPL); upregulation of microsomal triglyceride transfer protein (MTP) leads to increased production and secretion of very low density lipoproteins (VLDL). Increased expression of heme oxygenase 1 (Hmox1) results in cytochrome degradation and mitochondrial damage with impaired  $\beta$ -oxidation of fatty acids (FAs) leading to increased formation of hepatic triglycerides (TG).

species (ROS). The ability of neutrophils to produce ROS was significantly increased in patients with inflammatory acne.<sup>164</sup> The involvement of ROS generated by neutrophils appears to play an important role in the disruption of the integrity of the follicular epithelium promoting inflammatory processes of acne. Patients with inflammatory acne showed a significantly increased level of hydrogen peroxide produced by neutrophils compared to patients with comedonal acne and healthy controls.<sup>165</sup> In acne patients, lower levels of superoxide dismutase (SOD) and catalase have been measured in polymorphonuclear neutrophils (PMN) in comparison to controls, which may be responsible for the increased levels of superoxide anion radicals in the epidermis.<sup>166-168</sup> The effect of isotretinoin on the generation of ROS by stimulated human neutrophils showed that isotretinoin exerted an antioxidant activity against the superoxide anion.<sup>169</sup>

One of the most important functions of FoxOs is the protection of cells from oxidative damage by increasing transcription of multiple genes regulating scavenging of ROS.<sup>14,18</sup> Activated FoxO proteins promote stress resistance by binding to the promoters of the genes encoding manganese superoxide dismutase (MnSOD) and catalase, two scavenger enzymes that play essential roles in oxidative detoxification in mammals.<sup>11,135,170-172</sup> FoxO-mediated oxidative-stress resistance is influenced by multiple other pathways like  $\beta$ -catenin which binds directly to FoxO proteins and enhances their transcriptional activity in mammalian cells.<sup>135</sup>

Moreover, FoxO1 controls the promoter activity of the key enzyme of cytochrome synthesis, heme oxygenase.<sup>173</sup> Upregulated FoxO1 downregulates the synthesis of heme, the prosthetic group of hemoglobin and various cytochromes of the mitochondrial respiratory chain involved in ROS formation.<sup>173</sup> Thus, isotretinoin-induced upregulation of FoxO1 explains the suppression of mitochondrial ROS generation and increased ROS catabolism thereby normalizing increased ROS generation in acne.

### FoxO-Upregulation Explains all Adverse Effects of Isotretinoin Therapy

All patients treated with isotretinoin suffer from multiple side effects. This already shows that isotretinoin affects other organ systems. The side-effect profiles qualitatively resemble toxic effects of vitamin A or hypervitaminosis A syndrome.<sup>174</sup>

### FoxO1 and Isotretinoin-Induced Hepatotoxicity

In approximately 15–20% of patients treated with isotretinoin mild-to-moderate transitory elevations of mitochondrial liver enzymes (aspartate aminotransferase and alanine aminotransferase) have been observed.<sup>175</sup> Circulating levels of alkaline phosphatase, lactic dehydrogenase and bilirubin may also become elevated during retinoid therapy.<sup>2,175</sup> Again, we have to ask whether upregulated hepatic FoxO1 is the common cause of liver toxicity resulting in mitochondrial dysfunction with increased release of mitochondrial enzymes and increase in bilirubin?

The critical role of FoxO1 in hepatic glucose and lipid metabolism is well established and reviewed extensively elsewhere in reference 19. Under ordinary conditions, feeding stimulates insulin secretion from pancreatic  $\beta$ -cells, and FoxO1 in the liver is inhibited by insulin signal via IRS/PI3K/Akt cascade (Fig. 1). In fasting state, insulin signal is weak and FoxO1 is activated by translocation into the nuclei to trigger gluconeogenesis for glucose supply. Under insulin resistance conditions, however, hyperactive FoxO1 promotes gluconeogenesis in such an uncontrolled way that it leads to hyperglycemia. It is well known that isotretinoin impairs insulin resistance.<sup>176,177</sup> This fact can be well explained by FoxO1-mediated upregulation of *phosphoenolpyruvate carboxykinase* (PEPCK), the key enzyme of gluconeogenesis.<sup>19</sup> Thus, hyperactive FoxO1 explains impaired insulin sensitivity and an increased disposition for hyperglycemia observed under isotretinoin treatment (Fig. 4).

Furthermore, recent observations indicate that activated FoxO1 impairs fatty acid oxidation. A hepatic increase in fatty acids may promote dyslipidemia which may arise at least in part

from mitochondrial dysfunction (Fig. 4).<sup>178-180</sup> By directly binding the promoter, FoxO1 induces *heme oxygenase-1* (Hmox1) that reduces the heme content required for expression, stability and function of electron transport chain (ETC) components.<sup>178,181</sup> Heme is the functional prosthetic group of all cytochromes in the liver which drive the mitochondrial ETC. FoxO1-mediated induction of Hmox1 disrupts the ETC and impairs mitochondrial metabolism including fatty acid  $\beta$ -oxidation. Hyperactivated FoxO during severe insulin resistance contributes to the accumulation of hepatic lipids.<sup>178-180</sup> Adenoviral delivery of constitutively nuclear FoxO1 to mouse liver promotes hepatic triglyceride accumulation that can progress to steatosis as seen in hypervitaminosis A syndrome.<sup>180</sup> The lipid accumulation is associated with decreased fatty acid oxidation. In rats, administration of isotretinoin (100 mg/kg diet) increased the total hepatic lipid and triglyceride content as well as serum triglyceride concentrations.<sup>182</sup>

FoxO1 mediated increase in heme oxygenase-1 with resultant mitochondrial dysfunction is of fundamental biological importance and explains the isotretinoin-mediated increase of mitochondrial liver enzymes. ATRA, alitretinoin and isotretinoin are able to induce membrane permeability transition observed as swelling and decrease in membrane potential in isolated rat liver cells (Fig. 4).<sup>183</sup> Isotretinoin appeared to be the most effective and stimulated the release of cytochrome *c* from mitochondria, suggesting a potential target of retinoids in the induction of cell apoptosis.<sup>183</sup> Isotretinoin's effect on mitochondrial permeability via FoxO1-mediated inhibition of the ETC not only explains the increased release of mitochondrial liver enzymes but also increased bilirubin levels following increased heme catabolism.

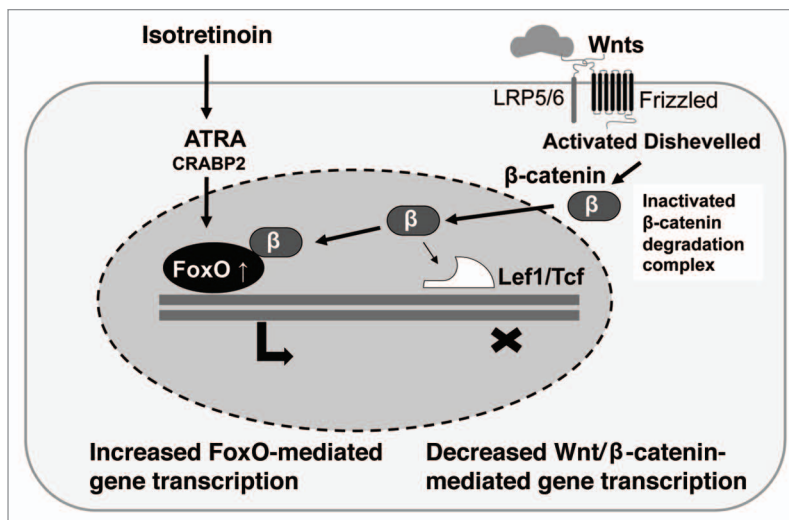
Remarkably, FoxO1-upregulated heme oxygenase-1 and disturbance of mitochondrial function and integrity may be an important trigger for the intrinsic pathway of apoptosis. In this regard, isotretinoin mimics growth factor withdrawal with upregulation of FoxO1/heme oxygenase-induced intrinsic pathway of apoptosis mediated through mitochondrial instability (Fig. 4).

Together, the effects of isotretinoin on hepatic glucose, lipid and heme metabolism as well as mitochondria-dependent apoptosis may be well explained by isotretinoin-induced overexpression of hepatic FoxO1.

### Isotretinoin-Induced Hypertriglyceridemia

Isotretinoin at pharmacological doses elevates plasma triglycerides and induces overt hypertriglyceridemia.<sup>184-186</sup> There are at least three mechanisms involved in the generation of isotretinoin-induced hypertriglyceridemia:

(1) Hepatic triglycerides and free fatty acids are elevated by isotretinoin treatment as a result of FoxO1-mediated upregulation of heme oxygenase-1 with impaired activity of the ETC and *diminished fatty acid oxidation*.<sup>179,180</sup> Increased free fatty acids in the liver are incorporated into triglycerides.



**Figure 5.** Isotretinoin-mediated overexpression of FoxO proteins and divergence of  $\beta$ -catenin signaling from Lef1/Tcf-induced transcription by increased binding of  $\beta$ -catenin to nuclear FoxO proteins. ATRA, all-trans-retinoic acid; CRABP2, cellular retinoic acid binding protein-2; Wnts, Wingless proteins; LRP5/6, low density receptor-related proteins 5/6; Frizzled, Wnt receptor Frizzled;  $\beta$ ,  $\beta$ -catenin; Lef1, lymphoid enhancer-binding factor-1; Tcf, T cell factor.

(2) Hepatic *very low density lipoprotein* (VLDL) production is facilitated by *microsomal triglyceride transfer protein* (MTP) in a rate-limiting step that is regulated by insulin. In hepatocytes, FoxO1 binds and stimulates MTP promoter activity (Fig. 4). Mice that expressed a constitutively active FoxO1 transgene revealed enhanced MTP expression, augmented VLDL production and elevated plasma triglyceride levels.<sup>187</sup> VLDL production is suppressed in response to increased insulin release after meals by insulin-mediated PI3K/Akt activation and reduction of nuclear levels of FoxO1.<sup>188</sup> Thus, isotretinoin-induced upregulation of nuclear FoxO1 would nicely explain increased hepatic VLDL synthesis resulting in retinoid-induced hypertriglyceridemia.

(3) A third mechanism provides indirect evidence for isotretinoin's ability to raise nuclear FoxO1 concentrations. Isotretinoin increases the expression of *apolipoprotein C-III*, a known antagonist of plasma triglyceride catabolism. Apo C-III functions as an inhibitor of lipoprotein lipase and hepatic lipase.<sup>189</sup> In fact, isotretinoin treatment resulted in elevated plasma levels of apo C-III.<sup>190</sup> Recent studies confirmed that FoxO1 stimulated hepatic apo C-III expression and correlated with the ability of FoxO1 to bind to the apo C-III promoter.<sup>191</sup> These observations clearly explain the basic mechanism of isotretinoin-mediated hypertriglyceridemia at the level of upregulated FoxO1-mediated gene transcription and are an excellent proof of the proposed role isotretinoin-induced FoxO transcription in hepatic lipid and lipoprotein metabolism.

### Isotretinoin and FoxO1-Mediated Bone Toxicity

Isotretinoin in high doses and given over prolonged periods (>1 mg/kg body weight, >1 year) disturbs the physiological homeostasis of bone metabolism including demineralization, thinning of the bones and premature closure of the epiphyses as

well as hyperostosis, periostosis (disseminated idiopathic skeletal hyperostosis, DISH syndrome).<sup>192-196</sup> It has been clearly demonstrated that treatment of rats with isotretinoin decreased bone mass.<sup>197</sup> Bone mineral density, bone mineral content, bone diameter and cortical thickness of the femur were reduced in rats treated daily with 10 or 15 mg/kg ATRA or 30 mg/kg isotretinoin.<sup>197</sup> In acne patients receiving high dose isotretinoin (1 mg/kg of body weight) bone density at the Ward triangle significantly decreased by a mean of 4.4% after 6 months of isotretinoin use and some patients showed decreased density of more than 9% at the Ward triangle.<sup>194</sup> However, patients receiving a single course of isotretinoin treatment for 4–6 months until a cumulative dose of 120 mg/kg did not exhibit clinically significant effects on bone metabolism.<sup>195</sup> Most hyperostoses are asymptomatic and clinically insignificant.<sup>2,196,198</sup> High-dose isotretinoin for a period of over 2 years have been shown to appear to induce skeletal hyperostoses and anterior spinal ligament calcification. Bone abnormalities in children, particularly premature closure of the epiphyses, are associated with high isotretinoin doses (>1 mg/kg/day), vitamin A supplementation and long-term treatment.

Again the question: Is there a link between high levels of isotretinoin, FoxOs and bone metabolism? During the last decade, it has been extensively documented that Wnt/ $\beta$ -catenin signaling is a critical determinant of bone mass.<sup>122</sup> The paramount importance of the Wnt/ $\beta$ -catenin/Tcf signaling for bone mass has been explained by the essential role of  $\beta$ -catenin in determining the commitment of multipotential mesenchymal progenitors to the osteoblastic lineage.<sup>199,200</sup> In addition to promoting osteoblastogenesis, Wnt/ $\beta$ -catenin signaling inhibits adipogenesis, an alternative fate of the multipotential mesenchymal progenitors, by blocking the expression of PPAR $\gamma$  and C/EBP $\alpha$  as already outline above.<sup>201</sup> Similar to the Wnt/ $\beta$ -catenin pathway, oxidative stress influences fundamental cellular processes including stem cell fate and has been linked to aging and the development of age-related diseases like osteoporosis.  $\beta$ -catenin has recently been implicated as a pivotal molecule in defense against oxidative stress by serving as a cofactor of FoxO transcription factors.<sup>122</sup> In addition, it has been shown that oxidative stress is a pivotal pathogenetic factor of age-related bone loss and strength in mice, leading to a decrease in osteoblast number and bone formation. These particular cellular changes evidently result from diversion of the limited pool of  $\beta$ -catenin from Tcf- to FoxO-mediated transcription in osteoblastic cells (Fig. 5).<sup>135,137,202</sup> Fascinatingly, attenuation of Wnt-mediated transcription has been linked not only to premature osteoporosis, but also to hyperlipidemia, insulin resistance and diabetes—observed changes of isotretinoin treatment. It is thus conceivable that bone toxicity of isotretinoin may be mediated by increased nuclear FoxO levels which divert  $\beta$ -catenin from to Tcf-binding to FoxO-binding, thereby attenuating Wnt/ $\beta$ -catenin signaling in the bone (Fig. 5).

Interestingly, ATRA treatment of mouse epiphyseal chondrocytes in culture increased Wnt/ $\beta$ -catenin signaling.<sup>203</sup> Cross-regulation of Wnt signaling and retinoid signaling affect chondrocyte function and phenotype and could be quite important in the process of chondrogenesis and proper progression of enchondral ossification during skeletal growth.<sup>203</sup> Thus,

isotretinoin/FoxO-mediated attenuation of epiphyseal chondrocyte Wnt signaling may be a conceivable mechanism explaining premature closure of the epiphyses by isotretinoin treatment.

### Isotretinoin and FoxO-Mediated Adverse Effects on Muscle

Arthralgias and myalgias may occur in up to 2–5% of individuals receiving oral isotretinoin in doses higher than 0.5 mg/kg/day and is more common in adolescents and young adults. In some cases severe muscle pain and temporary disability of movement with early-morning arthralgias were seen. Occasionally, concomitant malaise and fever and increases in creatine phosphokinase (CPK), a specific marker of muscle destruction, may be observed.<sup>2,204,205</sup> CPK, has been found to be elevated, occasionally by up to 100 times the normal value with or without muscular symptoms and signs in a variable percentage of patients receiving isotretinoin treatment and particularly in those undergoing vigorous physical exercise.<sup>206</sup>

Again, the question has to be raised whether isotretinoin affects FoxO transcription in muscle cells? The skeletal muscle is one of the major peripheral tissues that is responsible for insulin-mediated fuel metabolism and energy expenditure. Skeletal muscle accounts for >30% of resting metabolic rate and 80% of whole-body glucose uptake. Expression of FoxO1 is increased in skeletal muscle by energy deprivation such as fasting, suggesting that FoxO1 may mediate the response of skeletal muscle to changes in energy metabolism.<sup>207-209</sup> The maintenance of muscle mass is achieved by a dynamic balance of atrophy and hypertrophy.<sup>210,211</sup> Activation of FoxO1 or FoxO3a in the skeletal muscle, in fasting or diabetic conditions, can increase protein breakdown through ubiquitin-proteasome and autophagy-lysosome pathways, the two major mechanisms causing muscle atrophy (Fig. 6).<sup>212-216</sup> Overexpression of a constitutively active FoxO1 in C2C12 muscle cells promotes expression of *atrogin 1* and *muscle-specific RING finger protein 1*, the two ubiquitin ligases involved in skeletal muscle atrophy.<sup>217</sup> Expression of a dominant-negative FoxO1 construct in myotubes or in rodent muscle decreases atrogin-1 expression and muscle atrophy.<sup>217</sup> Transgenic FoxO1 in skeletal muscle increases expression of cathepsin L, an atrophy-related lysosomal protease, which is associated with reduced skeletal muscle mass and body weight.<sup>218</sup> Moreover, the genes encoding structural proteins of type I muscles (slow twitch, red muscle) are downregulated concomitant with a decreased size of both type I and type II fibers. The coordinate regulation of cathepsin L and type I muscle genes may account at least in part for the loss of muscle mass and glycemic control owing to hyperactivated FoxO.<sup>218</sup>

Furthermore, FoxO1 suppresses SREBP-1c, the key transcription factor of lipogenesis, in skeletal muscle by disrupting the RXR $\alpha$ /LXR heterodimer on the SREBP-1c promoter (Fig. 2C).<sup>219</sup> Mice overexpressing FoxO1 lose their glycemic control and display a lower capacity for physical exercise due to severe muscle loss.<sup>218</sup> Due to clinical experience, isotretinoin is administered with caution to athletes who have to rely on their muscle mass and muscle strength. Skeletal muscle metabolism switches

from oxidation of carbohydrates to fatty acids as the major energy source during fasting when the plasma glucose concentration is low. FoxO1 controls this switch by upregulating 3 enzymes: *pyruvate dehydrogenase kinase-4* (PDK4) that shuts down glucose oxidation by targeting pyruvate dehydrogenase (PDH), lipoprotein lipase that hydrolyzes plasma triglycerides into fatty acids and fatty acid translocase CD36 that facilitates fatty acid uptake into skeletal muscle.<sup>208,220</sup> PDK4 phosphorylates PDH and blocks PDH activity in catalyzing the conversion of pyruvate into acetyl-CoA. This can divert the physiological switch of FoxO1 activity, that is, on in fasting and off in feeding state, which is required for the nutrient/energy homeostasis in the skeletal muscle through carbohydrate/lipid switch. Severe starvation may trigger FoxO1-mediated autophagy and atrophy that break down protein for energy supply, the mechanism that underlies the loss of muscle mass and glycemic control under insulin resistance. It is well known that FoxO1 is a key transcription factor of starvation. Thus, FoxO1 plays a key role in the carbohydrate/lipid metabolic switch in skeletal muscle during fasting/feed cycle. Hyperactivated FoxO1 induces autophagy-related protein degradation through atrogen 1 and muscle-specific RING finger protein 1, which causes atrophy and muscle loss which disturbs metabolic homeostasis (Fig. 6).<sup>12</sup>

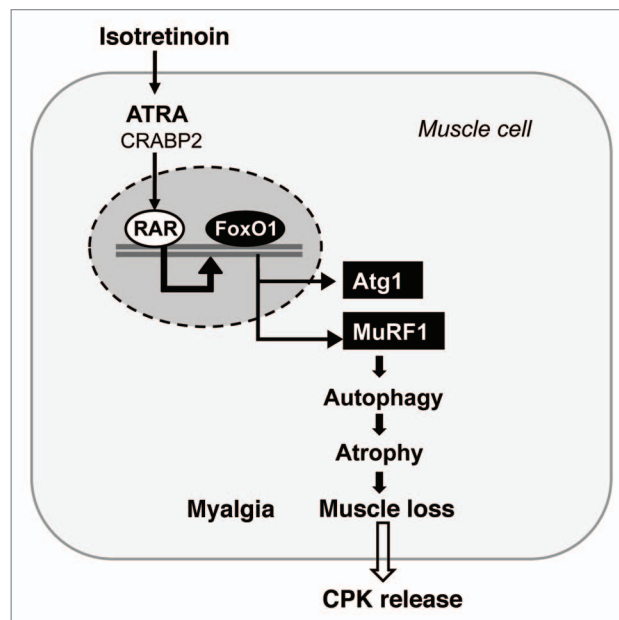
In summary, isotretinoin-induced upregulation of FoxO may imprint an artificial “fasting state” on muscle metabolism switching to catabolic events in muscle cell homeostasis which may very well explain isotretinoin-associated myalgias and increases in CPK due to muscle cell degradation.

### Isotretinoin, FoxO1 and Mucocutaneous Side Effects

Mucocutaneous adverse effects of oral isotretinoin treatment are dose-dependent and predominantly reflect a decreased production of sebum, reduced stratum corneum thickness, and altered skin barrier function.<sup>2,221</sup> Skin xerosis, especially on exposed skin and cheilitis are the earliest and the most frequent side effects that affect almost all treated patients. *Staphylococcus aureus* colonization correlates with the isotretinoin-induced reduction in sebum production and may lead to overt cutaneous infections. Xerophthalmia due to decreased meibomian gland secretion can lead to blepharoconjunctivitis. Dry genitals and anal mucosa may be a side effect and dry nasal mucosa may lead to epistaxis.<sup>2,221</sup>

Pioneering studies have focused on the antikeratinizing effect for retinoid activity which comprised dose-dependent alterations in transepidermal water loss and epidermal and stratum corneum loosening associated with loss of epidermal cohesion and abnormal barrier function.<sup>222-224</sup> Recently however, using large DNA microarrays it has been shown, that ATRA suppresses genes responsible for biosynthesis of epidermal lipids, long-chain fatty acids, cholesterol and sphingolipids in primary human epidermal keratinocytes.<sup>225</sup> Unexpectedly, ATRA regulated many genes associated with the cell cycle and programmed cell death.<sup>225</sup>

The role of epidermal lipids for skin barrier function and antimicrobial defense has been well established.<sup>226,227</sup> The major part of epidermal barrier function is provided by ordered epidermal lipid synthesis and conversion of polar lipids into nonpolar



**Figure 6.** Isotretinoin’s effect on muscle homeostasis is mediated by FoxO1-driven upregulation of atrogen-1 (Atg1) and muscle-specific RING finger protein-1 (MuRF1) which both induce autophagy-related protein degradation with muscle loss and release of creatine phosphokinase (CPK).

ceramides and acylceramides which constitute the intercorneocyte lipid lamellae important in stratum corneum barrier function and control of transepidermal water loss.<sup>226</sup> Free fatty acids, cholesterol and ceramides in appropriate molar ratios are important for barrier function.<sup>226</sup>

There is recent evidence that RAR, PPARs and LXR are also involved in the regulation of epidermal lipid synthesis and barrier function.<sup>226</sup> Whereas activation of PPAR $\alpha$  and LXR improve barrier function, RAR activation worsens it.<sup>226</sup> Both cholesterol and fatty acid synthesis are regulated by SREBPs.<sup>228</sup> Two SREBP genes (SREBP-1 and SREBP-2) encode three proteins: SREBP-1a, SREBP-1c and SREBP-2.<sup>229,230</sup> SREBPs bind to the promoters of multiple SREBP-responsive genes stimulating various enzymes of cholesterol and fatty acid synthesis.<sup>229,230</sup> SREBP-2 is a more important regulator of cholesterol synthesis. SREBP-1a is as effective as SREBP-2 as a regulator of HMG-CoA synthase and HMG-CoA reductase in cholesterol synthesis, but it has a greater effect on fatty acid synthesis than does SREBP-2. SREBP-1c primarily regulates fatty acid synthesis. Nothing is yet known about the role of FoxOs in the regulation of epidermal lipid homeostasis. In muscle cells however, FoxO1 regulates triglyceride content via the RXR $\alpha$ /LXR $\alpha$ /SREBP-1c pathway and has been shown to suppress RXR $\alpha$ /LXR $\alpha$ -mediated SREBP-1c promoter activity (Fig. 2C).<sup>219</sup> In FoxO1 transgenic mice, gene expression of SREBP-1c is downregulated in skeletal muscle. During nutritional changes caused by fasting and feeding, gene expression of RXR $\alpha$  and SREBP-1c in mouse skeletal muscle switches off and on, respectively, whereas expression of FoxO1 shows reverse correlation with SREBP-1c expression.<sup>219</sup> Supposed that isotretinoin-mediated upregulation of FoxO1

would downregulate SREBP1c-mediated epidermal fatty acid- and cholesterol synthesis in epidermal keratinocytes, a functional disturbance of epidermal barrier function may result. Moreover, alterations in fatty acid synthesis, potentially regulated by FoxOs and SREBPs, could indirectly affect ceramide production as the first biosynthetic step in ceramide synthesis catalyzed by serine palmitoyl transferase requires the presence of sufficient amounts of fatty acids.<sup>231-233</sup>

In analogy to sebaceous and epidermal lipid biosynthesis during isotretinoin treatment, a FoxO1-mediated downregulation of lipid synthesis of meibomian glands would explain the isotretinoin-induced blepharoconjunctivitis clinically appearing as “dry eyes.” Indeed, histopathological studies of the eyelids of female New Zealand rabbits after long-term isotretinoin (2 mg/kg) treatment showed “degenerative changes” in the meibomian gland acini, leading to cell necrosis and a decrease in the basaloid cells lining the acini walls without inflammatory changes.<sup>234</sup> Systemic administration of isotretinoin caused a reduction of acinar tissue in the hamster meibomian gland. Histologic examination revealed a decrease in the numbers of mature lipid-laden acinar cells and a reduction of up to 75% in mean volume of meibomian acinar tissue from animals fed a high dose of isotretinoin.<sup>235</sup> Systemic treatment of adult male New Zealand albino rabbits with isotretinoin resulted in a reduction in the size of the meibomian gland and a decrease in acinar tissue.<sup>236</sup>

The isotretinoin-induced reduction of acinar tissue and lipid content of meibomian glands in the presented animal models points again to an isotretinoin-induced apoptosis mechanism. This is comparable to isotretinoin's effect on sebocytes and is most likely driven by FoxO-upregulation. Thus, isotretinoin-mediated stimulation of FoxO-mediated gene expression could explain the defects in the quality and composition of the conjunctival lipid film and the resultant blepharoconjunctivitis observed in 20–45% of the patients treated systemically with isotretinoin.<sup>234</sup>

### FoxOs and Isotretinoin-Induced Hair Loss

Long-term use of isotretinoin in higher doses is associated with increased hair loss in susceptible individuals.<sup>221</sup> Isotretinoin has been demonstrated to affect hair growth.<sup>237</sup> In equine hair follicles in vitro isotretinoin modified sheath-shaft interaction.<sup>238</sup> It has recently been shown that ATRA induces premature hair follicle regression and induced a catagen-like stage in human hair follicles.<sup>239</sup> Hair shaft elongation declined significantly already after 2 days in the ATRA-treated group, and approximately 80% of the ATRA-treated hair follicles had prematurely entered catagen-like stage at day 6, compared with 30% in the control group. This corresponded to an upregulation of apoptotic and a downregulation of Ki67-positive cells in ATRA-treated hair follicles,<sup>239</sup> thus pointing again to the induction of apoptosis, the hallmark of FoxO signaling.

Previous studies have shown that the Wnt signaling pathway plays an important role in the growth and development of hair follicles.<sup>99,113,117,118</sup> Wnts are deeply involved in the proliferation and differentiation of skin epithelial cells. The differentiation of

cultured primary skin epithelial cells toward hair shaft and inner root sheath of the hair follicle via  $\beta$ -catenin stabilization caused by Wnt10b has been reported. In organ cultures of whisker hair follicles in serum-free conditions no hair shaft growth was observed in the absence of Wnt10b, whereas its addition to the culture promoted elongation of the hair shaft, intensive incorporation of BrdU in matrix cells flanking the dermal papilla, and  $\beta$ -catenin stabilization in dermal papilla and inner root sheath cells. These results suggest a promoting effect of Wnt10b on hair shaft growth that is involved with stimulation of the dermal papilla via Wnt10b/ $\beta$ -catenin signaling, proliferation of matrix cells next to the dermal papilla and differentiation of inner root sheath cells by Wnt10b.<sup>240</sup> In contrast, expression of  $\Delta$ Nlefl1 transgene in mouse epidermis, which lacks the  $\beta$ -catenin binding site, resulted in differentiation of hair follicles into squamous epidermal cysts and formation of skin tumours.<sup>118</sup>

Can we explain isotretinoin's adverse effects on hair growth by FoxO-driven apoptosis and induction of catagen? A reasonable explanation would be that isotretinoin-induced FoxO impairs  $\beta$ -catenin signaling which is most important for hair growth. In fact, it has been shown that upregulated nuclear FoxOs binds nuclear  $\beta$ -catenin and divert  $\beta$ -catenin signalling from Tcf/Lef1 interaction (Fig. 5).<sup>135</sup> Thus, upregulated FoxOs by isotretinoin would explain impaired  $\beta$ -catenin signaling leading to apoptosis and reduced growth and differentiation of hair follicles, explaining isotretinoin-induced FoxO-mediated hair loss.

### Isotretinoin, FoxOs and CNS Side Effects

Clinically observed CNS side effects of isotretinoin are rare. However, isotretinoin, has been associated with various psychiatric side effects such as depression, suicidality and psychotic symptoms. A great number of reports on its CNS effects have been published since its introduction into the market. According to the FDA all patients treated with isotretinoin should be observed closely for symptoms of depression or suicidal thoughts, such as sad mood, irritability, acting on dangerous impulses, anger, loss of pleasure or interest in social or sports activities, sleeping too much or too little, changes in weight or appetite, school or work performance going down, trouble in concentrating, mood disturbances, psychosis or aggression. A causal relationship has yet not been established and the link between isotretinoin use and psychiatric events remains controversial.<sup>241</sup> However, six weeks of isotretinoin administration (1 mg/kg) increased depression-related behavior in mice.<sup>242</sup>

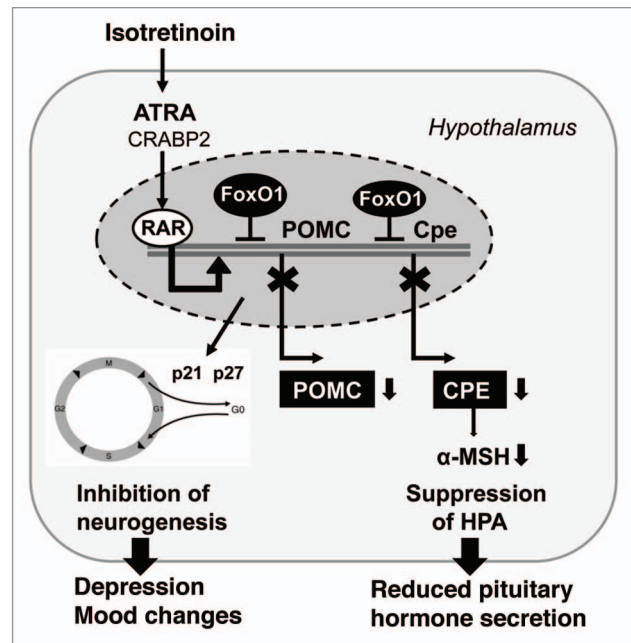
Does isotretinoin modify FoxO regulation in the brain, a metabolically active organ strongly dependent on glucose metabolism? RARs are widely distributed in the brain. The regions of the brain that predominantly exhibit RAR signaling include the limbic system, in particular the hippocampus and the medial prefrontal cortex, the cingulate cortex and subregions of the thalamus and hypothalamus.<sup>243,244</sup> Recent studies have demonstrated that the hippocampus is one of the brain regions where new neurons are constantly born, a phenomenon called *neurogenesis*. One of the theories for the pathogenesis of depression suggests a decreased hippocampal and prefrontal cortex neurogenesis.<sup>245,246</sup>



Moreover, antidepressant treatment seems to operate by an increase in neurogenesis, which is chronologically seen during the same period as the clinical improvement. Another irregularity in the hippocampus associated with depression is the reduction of the hippocampal volume. Intriguingly, isotretinoin treatment of mice results in both decreased hippocampal neurogenesis and a reduction in the hippocampal volume.<sup>247,248</sup> Treatment of GT1-7 hypothalamic cells with 10  $\mu$ M isotretinoin for 48 h decreased cell growth to  $45.6 \pm 13\%$  of control. Griffin et al. hypothesized that the ability of isotretinoin to decrease hypothalamic cell number may contribute to the increased depression-related behaviors observed in mice.<sup>249</sup> Therefore, the isotretinoin-mediated effect on neurogenesis could provide a plausible biological mechanism mediating depressogenic effects.

Intriguingly, FoxO1 is strongly expressed in the striatum and neuronal subsets of the hippocampus, i.e., the dentate gyrus and the ventral/posterior part of the cornu ammonis regions.<sup>250</sup> In wildtype mice, hypothalamic FoxO1 expression is reduced by the anorexigenic hormones insulin and leptin.<sup>251</sup> Upregulation of hippocampal FoxO1 levels may inhibit hippocampal neurogenesis and may thus be responsible for the adverse psychiatric drug effects in some disposed individuals. Remarkably, FoxO1 suppresses the transcription of proopiomelanocortin (POMC) by antagonizing the activity of signal transducer and activator of transcription-3 (STAT3).<sup>251,252</sup> FoxO1 modulates the melanocortin system by regulating the expression of *Agrp* and *Pomc* genes.<sup>252</sup> One of the POMC peptide cleavage products is  $\alpha$ -melanocyte stimulating hormone ( $\alpha$ -MSH). FoxO1 suppresses the expression of both *Pomc* and *Cpe*, which is one of the peptidases (carboxypeptidase E) that processes POMC to  $\alpha$ -MSH (Fig. 7). Remarkably, the proopiomelanocortin system plays an important role as a neuromediator system in controlling the sebaceous gland. It is well known that  $\alpha$ -MSH can stimulate sebocyte differentiation and sebaceous lipogenesis.<sup>253,254</sup> Thus, isotretinoin-mediated upregulation of hippocampal and hypothalamic FoxO1 could inhibit POMC/ $\alpha$ -MSH-signaling to the sebaceous gland.

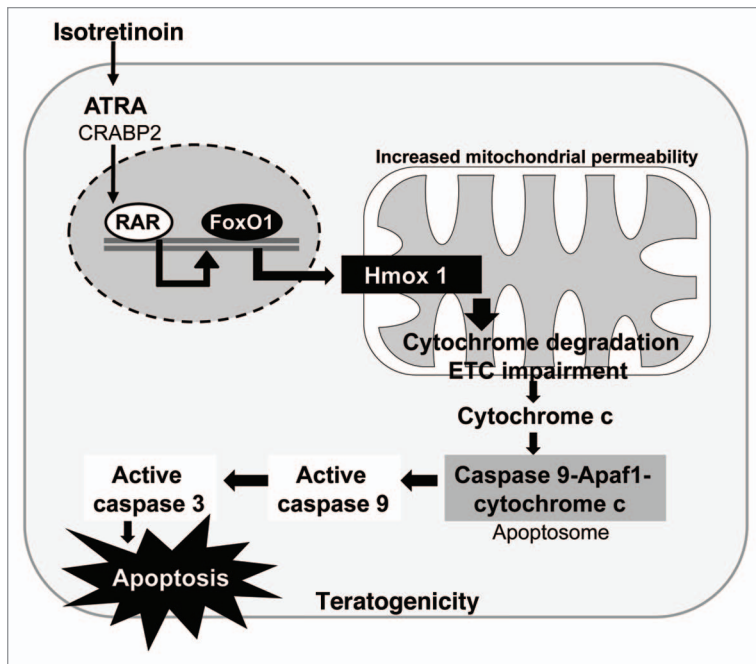
Antidepressant treatment seems to lead to an increase in neurogenesis, which is chronologically seen during the same period as the clinical improvement.<sup>241</sup> Severe acne and acneiform eruptions have been observed with high doses of tricyclic antidepressants and lithium therapy. In brain of mice, lithium significantly decreased FoxO3a levels.<sup>255</sup> As already mentioned, FoxO3a activates the promoter of FoxO1 and is an important inducer of FoxO1 gene expression.<sup>11</sup> The acneogenic effect of lithium therapy may be related to a lithium-induced nuclear deficiency of FoxO1 by suppression of the FoxO1 promoter. In mice, elevated serotonergic activity increased Akt-mediated phosphorylation of FoxO1 and FoxO3a in various brain regions resulting in nuclear deficiency of FoxO1 and FoxO3a.<sup>256</sup> FoxOs in brain of rodents are intensely involved in the regulation of behavioral manifestation.<sup>256</sup> Upregulated serotonin levels by antidepressants reduce nuclear concentrations of FoxO1 and FoxO3 in neuronal cells. FoxO1-deficient mice displayed reduced anxiety, whereas FoxO3a-deficient mice presented with a significant anti-depressant-like behavior.<sup>256</sup> Thus, elevated nuclear content of FoxO1 and FoxO3a by isotretinoin treatment in the human



**Figure 7.** Isotretinoin's effect on the CNS is mediated by FoxO1 up-regulation. In the hypothalamus FoxO1 inhibits neurogenesis associated with the risk of mood changes. FoxO1 suppresses the expression of proopiomelanocortin (POMC) and carboxypeptidase E (Cpe). This results in reduced formation of  $\alpha$ -melanocyte stimulating hormone ( $\alpha$ -MSH) and general suppression of the hypothalamic-pituitary-axis (HPA) with decreased pituitary hormone secretion.

hippocampus and hypothalamic areas of the brain may explain depression and mood changes observed with isotretinoin therapy in some susceptible individuals.<sup>241</sup>

As FoxO1 has been shown to be intimately involved in the regulation of brain metabolisms and brain ROS homeostasis, as well as to suppress the transcription of POMC gene, it is conceivable that isotretinoin exerts effects on the hypothalamic-pituitary-adrenal axis (HPA).<sup>251,252</sup> Moreover, isotretinoin-induced apoptotic effects on hypothalamic cells may have downstream regulatory effects on the pituitary. Thus, a decrease in  $\alpha$ -MSH and adrenocorticotrophic hormone (ACTH) and other pituitary hormones may be expected during isotretinoin treatment. Intriguingly, Karadag et al. recently demonstrated that 3 months of isotretinoin treatment in 47 acne patients reduced free triiodothyronine (T3), thyroid-stimulating hormone (TSH), thyroid-stimulating hormone receptor antibody levels, luteinising hormone, prolactin and total testosterone, morning cortisol and ACTH.<sup>257</sup> In accordance with isotretinoin, central hypothyroidism is a well known adverse effect of the synthetic RXR-selective retinoid bexarotene, approved for the treatment of cutaneous T-cell lymphoma (CTCL).<sup>258-260</sup> Bexarotene was found to cause severe central hypothyroidism with high frequency, associated with marked reductions in serum concentrations of thyroid-stimulating hormone (TSH) and thyroxine.<sup>259</sup> Bexarotene-induced apoptosis of CTCL cells has been hypothesized to be the major mode of action in CTCL.<sup>261</sup> Thus, isotretinoin- and bexarotene-mediated effects on secretory cells of the HPA-axis may share a common proapoptotic mechanism of action. It is thus tempting



**Figure 8.** Isotretinoin-induced and FoxO-mediated apoptosis. FoxO1-induced overexpression of heme oxygenase-1 disrupt the electron transport chain (ETC) with increased release of cytochrome c inducing the intrinsic mitochondrial pathway of apoptosis. Formation of the apoptosome activates caspase 9 which finally activates the executive caspase 3. The pathway explains isotretinoin's teratogenic effects when neuronal crest and CNS cells are affected during embryonic development.

to speculate that isotretinoin suppresses the HPA-axis. In this regard, recent insights into FoxOs role as controlling system of food intake and the regulation of the circadian clock are further arguments for FoxOs important contribution in CNS homeostasis, regulation of the circadian rhythm and pituitary hormone secretion (Fig. 7).<sup>252,262</sup>

### Isotretinoin, FoxO-Upregulation and Teratogenicity

Isotretinoin is well known to exert teratogenic effects in laboratory animals and humans.<sup>263</sup> A characteristic pattern of malformations involving craniofacial, cardiac, thymic and central nervous system structures is seen in humans.<sup>264-266</sup> Some of the most characteristic abnormalities include microtia, anotia, micrognathia, conotruncal heart defects and aortic arch abnormalities, thymic ectopia or aplasia, cerebellar vermis agenesis and various neuronal migration anomalies.<sup>263</sup>

Can we construct a relationship between isotretinoin-mediated upregulation of FoxOs and retinoid-induced teratogenicity during early developmental steps of embryogenesis? FoxOs are expressed especially in adipose, brain, heart, liver, lung, ovary, pancreas, prostate, skeletal muscle, spleen, thymus and testis,<sup>58,91,267</sup> thus in those organs affected by isotretinoin teratogenicity. FoxO3a has been associated with the regulation of neuronal survival, vascular integrity, immune function and cellular metabolism and plays an important role in the brain.<sup>268</sup> During cardiac development, FoxO proteins appear to be necessary to

modulate cardiomyocyte proliferation. Both FoxO1 and FoxO3a are expressed during embryonic development in the developing myocardium. The expression of these FoxO proteins is believed to negatively regulate cardiomyocyte growth, since overexpression of FoxO1 blocks cardiomyocyte proliferation.<sup>269</sup> There is accumulating evidence that FoxOs are pivotal regulatory transcription factors of progenitor cell development, thymic T-cell differentiation, T-cell homeostasis, angiogenesis, cardiovascular function and neuronal development and function.<sup>269</sup> Malformations other than in the CNS are thought to be due to isotretinoin's interference with migration and/or proliferation of the cranial neural crest cells, leading to deficient mesenchyme in the branchial arches. In animal studies, isotretinoin administration was associated with decreased proliferation rates and increased programmed cell death of neural crest cells (Fig. 7).<sup>270,271</sup> Both neural crest and CNS cells expressed high levels of CRABPs during isotretinoin treatment, thus promoting CRABP-mediated proapoptotic signaling.<sup>272,273</sup> Isotretinoin-induced FoxO-mediated apoptosis of neuronal crest cells is thus a conceivable mechanism for isotretinoin's teratogenic effect (Fig. 8).

Recently, it has been demonstrated that excess ATRA repressed blastula Wnt signaling and impaired dorsal development in *Xenopus* embryo.<sup>274</sup> ATRA promoted nuclear accumulation of  $\beta$ -catenin, although, surprisingly, Wnt signaling was repressed. The unexpected reduction in Wnt signaling was explained by the ability of liganded RAR to bind to  $\beta$ -catenin thereby inhibiting  $\beta$ -catenin binding with Tcf4. However, there is another reasonable explanation considering ATRA-mediated upregulation of FoxOs which may attract available nuclear  $\beta$ -catenin, thus impairing  $\beta$ -catenin/Tcf signaling (Fig. 5).<sup>135</sup> Wnt signaling is of crucial importance for osteoblastogenesis and will affect craniofacial morphogenesis.<sup>122,199,200</sup> Isotretinoin-induced diversion of  $\beta$ -catenin from Tcf- to FoxO-mediated transcription may explain the craniofacial and ear abnormalities of retinoid embryopathy. Taken together, an accumulating body of evidence allows the conclusion that overstimulated FoxO-mediated transcriptional regulation with consecutively impaired Wnt/ $\beta$ -catenin signaling may be a major signaling disturbance leading to isotretinoin-induced teratogenicity.

### FoxOs and the Chemopreventive Effect of Isotretinoin

Isotretinoin is used as an adjunct in the treatment of pediatric patients with neuroblastoma, acute promyelocytic leukemia and chemoprevention of high-risk patients with non-melanoma skin cancer.<sup>275-279</sup> Isomerization of the "prodrug" isotretinoin to ATRA is proposed to be of importance for isotretinoin's superior anti-tumor activity in neuroblastoma in comparison to treatments with ATRA.<sup>280</sup> There is recent evidence that isotretinoin induces gene expression and apoptosis in cancer cell which clearly resemble the transcriptional activity of FoxO proteins. For example,

isotretinoin mediates apoptosis in Dalton's lymphoma ascites cells by regulating gene expression with upregulation of caspase-3 and downregulation of bcl-2 expression.<sup>281</sup> Similarly, in B16F-10 melanoma cells isotretinoin induces apoptosis and upregulates caspase-3, the tumor suppressor p53 and downregulates bcl-2.<sup>282</sup> Gene and protein expression profiling during differentiation of neuroblastoma cells triggered by isotretinoin exhibited a downregulation of N-myc, cyclin D<sub>3</sub> and Wnt10B.<sup>283</sup>

Is the chemopreventive effect of retinoids in certain tumors related to their ability to induce increased expression of FoxO proteins which are known to lead to apoptosis and block cell cycle progression?<sup>11,14,15,80</sup> For example, FoxO3a and FoxO4 can promote cell cycle arrest in mouse myoblastic cell lines through modulation of growth arrest and DNA-damage-response protein 45 (GADD45).<sup>284,285</sup> Other work suggests that FoxO proteins utilize the p53 upstream regulator p19(Arf) through myc to block cell cycle induction and lymphoma progression.<sup>286</sup> In cell cultures, overexpression of FoxO1 and FoxO3a in prostrate tumor cell lines also leads to apoptosis, suggesting that FoxO1 and FoxO3a are necessary for limiting prostate cell tumor growth.<sup>287</sup> In addition, it has been shown that inhibition of FoxO3a activity can result in enhanced prostate tumor cell growth while agents that increase FoxO3a activity in both androgen sensitive and androgen insensitive prostate cancer cell lines prevent prostate cancer cell progression.<sup>288</sup> Moreover, it has been shown that astrocyte-elevated gene-1 (AEG-1) can be upregulated in clinical prostate cancer.<sup>145</sup> This possibly leads to activation of Akt that suppresses FoxO3a and inhibits apoptosis in prostate tumor cells.<sup>289</sup> FoxO proteins can function as redundant repressors of tumor growth. For example, somatic deletion in mice of *Foxo1*, *Foxo3a* and *Foxo4* results in the growth of thymic lymphomas and hemangiomas.<sup>290</sup> In addition, the loss of FoxO3a activity may participate in oncogenic transformation in B-chronic lymphocytic leukemia and in the progression of chronic myelogenous leukemia cell line.<sup>291,292</sup> Furthermore, studies suggest that some proteins, such as the Kaposi's sarcoma-associated herpes virus latent protein LANA2, may specifically block the transcriptional activity of FoxO3a to lead to tumor growth.<sup>293</sup> In cell models of endometrial cancer, pre-sensitization of cells to block Akt activation and foster transcription activity of FoxO1 enhances the effect of chemotherapy to limit tumor growth.<sup>294</sup> It has recently been recognized that ATRA increased the expression of transcription factor FoxO3a in neuroblastoma cells.<sup>9</sup> FoxO3a has also been identified as a key regulator for ATRA-induced granulocytic differentiation and apoptosis in acute promyelocytic leukemia.<sup>10</sup>

Isotretinoin-mediated FoxO signaling just reverses the proposed *growth factor/PI3K/Akt pathway of acne* which leads to a nuclear deficiency of FoxO proteins.<sup>13</sup> As most acneigenic stimuli of acne-associated syndromes with increased growth factor-, insulin-, IGF-1- and FGF-signaling converge in the activation of PI3K/Akt, they are more likely to exert nuclear FoxO deficiencies associated with a higher incidence of cancer.<sup>295</sup> Indeed, an epidemiological association between long-lasting acne and prostate carcinoma has been established.<sup>296</sup> Chronically upregulated PI3K/Akt signaling might further explain the increased incidence of cancer in patients with other acne-associated diseases

like acromegaly,<sup>297</sup> polycystic ovary syndrome,<sup>298</sup> syndromes with insulin resistance with consecutive hyperinsulinemia,<sup>12</sup> and Apert syndrome with increased FGF-signaling.<sup>299-303</sup> In this regard, persistent acne in adulthood should be recognized as a serious clinical indicator of dysbalanced growth factor signaling with reduced levels of nuclear FoxOs, an unfavorable condition which increases mitogenic stimulation and cell survival but reduces apoptosis, well-recognized processes in cancer promotion.<sup>267</sup>

Together, substantial evidence exists for the anti-cancer activity of FoxO transcription factors. Isotretinoin appears to confer its chemopreventive activity by upregulation of FoxO-controlled target genes inducing apoptosis and cell death.<sup>267</sup>

## Conclusion and Future Perspectives

In the beginning of the retinoid research era, isotretinoin's mechanism of action was explained only by modulations of CREBP expression and ATRA/RAR interactions. Today, we begin to understand that retinoids exert most important effects on gene regulatory level by inducing secondary responses due to upregulation of further transcription factors including the FoxO family of transcription factors inducing consecutive molecular cross-talk with other signaling systems like Wnt/ $\beta$ -catenin signaling.<sup>8</sup> We have learnt that retinoids provide an essential, early signal that initiates a cascade of events leading to changes in proliferation, differentiation and predominantly apoptosis affecting most CRABP-2 expressing somatic cells as well as the stem cell compartments.<sup>8</sup> We have to appreciate that isotretinoin does not "exclusively" targets apoptosis of the sebaceous glands as pro-apoptotic drug effects have been observed in several unrelated cell systems and explain all adverse effects of isotretinoin and other retinoids (Table 2). The astonishing functional overlap of changes in FoxO-mediated gene transcription and isotretinoin-mediated gene transcription (Table 1) strongly suggests that isotretinoin and its isomerization product ATRA induces upregulation of FoxO-signaling and exerts apoptotic effects in multiple cell types like the muscle, the bone and the brain.

In fact, all isotretinoin-mediated effects on sebocyte apoptosis, sebaceous lipogenesis, anti-inflammatory activity, downregulation of ROS can be explained by upregulation of nuclear levels of FoxO transcription factors. All isotretinoin-induced adverse effect on hepatic glucose and lipid metabolism, retinoid-induced dyslipoproteinemia, loss of bone density, myotoxic effects, mucocutaneous side effects, adverse psychiatric effects, chemopreventive effects and isotretinoin's teratogenicity appear to result from a common mechanism, i.e., FoxO-mediated changes of gene expression. In contrast, increased insulin/IGF-1 signaling of puberty and western diet due to high glycemic load and consumption of insulinotropic milk and milk products downregulates nuclear FoxO levels and thus promotes the development of acne.<sup>13,20,21,304</sup> Similar effects are mediated by insulin resistance with consecutive hyperinsulinemia observed in most acne-associated syndromes like polycystic ovary syndrome, HAIRAN-syndrome, congenital adrenal hyperplasia and others recently reviewed elsewhere in reference 12. Increased FGF-signaling due to a gain-of-function mutation of FGFR2-downstream signaling

**Table 2.** Comparison of retinoid-induced apoptosis in various cell types

Cell type/tissue (Retinoid)	Characteristics of apoptotic signalling and histological changes	Clinical manifestations and clinical signs	Ref.
Sebocytes (Isotretinoin)	G <sub>1</sub> /S arrest, p21 ↑, cyclin D <sub>1</sub> ↓ involution of sebaceous glands, sebocyte apoptosis, sebum suppression	Reduction of seborrhoea, antiinflammatory action, improvement of acne	76
Meibomian gland cells (Isotretinoin)	Reduction of mature lipid-laden acinar cells and meibomian gland volume by 75%	Dry eyes, blepharoconjunctivitis	234–236
Hair follicle keratinocytes (ATRA)	Premature HF regression and change to catagen stage, increase of apoptotic cells, Ki67 ↓	Retinoid induced effluvium	237, 239
Hepatocytes (Isotretinoin, ATRA)	Mitochondrial damage, cytochrome c release, induction of apoptosis, increased free fatty acids and liver triglycerides, increase in VLDL- and apo C-III secretion, gluconeogenesis	Liver toxicity, steatosis, increase in liver transaminases and bilirubin levels, hypertriglyceridemia, insulin resistance	70, 179, 180, 182
Osteoblasts, osteocytes (Isotretinoin)	Reduction of osteoblastogenesis	Loss of bone density	194, 197
Skeletal muscle cells (Isotretinoin)	Reduction in muscle mass	Myalgia, muscle loss, CPK↑	206
Keratinocytes (ATRA)	Suppression of lipid synthesis, downregulation of genes involved in keratinocyte proliferation, upregulation of genes involved in apoptosis	Dry skin, impaired skin barrier function, transepidermal water loss↑	225, 226
Hippocampal cells Hypothalamic cells (Isotretinoin)	Decrease of hippocampal neurogenesis, reduction in hippocampal volume, decrease of hypothalamic cell growth, impaired pituitary hormone secretion	Increase of depressive behaviour, mood change, decrease in pituitary hormones (TSH, T3, T4, LH, prolactin, ACTH)	242, 247–249, 257
Hypothalamus/pituitary (Bexarotene)	Central hypothyroidism	Decrease in TSH, T3, T4	259
Neuronal crest and CNS cells (Isotretinoin)	Decreased cell proliferation, increased apoptosis, disturbance of morphogenesis	Teratogenicity, retinoid embryopathy	270, 271
Neuroblastoma cells (Isotretinoin)	Induction of apoptosis N-myc <sub>1</sub> , cyclin D <sub>3</sub> ↓, Wnt10B <sub>1</sub>	Chemopreventive activity, anti-tumor activity	283
Dalton's lymphoma ascites cells (Isotretinoin)	Apoptosis, caspase-3 ↑, bcl-2 ↓	Chemopreventive activity	281
B16F-10 melanoma cells (Isotretinoin)	Apoptosis, caspase-3 ↑, p53 ↑, bcl-2 ↓	Chemopreventive activity	282
Acute promyelocytic leukemia cells (ATRA)	Apoptosis	Anti-leukemia activity	10
Cutaneous lymphoma T cells (Bexarotene)	Induction of apoptosis	Chemopreventive activity, anti-lymphoma activity	261

in Apert syndrome and acneiform nevus may decrease nuclear levels of FoxO as well.<sup>302,305,306</sup> It is thus not surprising that acne in Apert syndrome and acneiform nevus respond very well to isotretinoin treatment,<sup>306-309</sup> which counteracts increased FGF/FGFR2-signaling with concomitant depletion of nuclear FoxO levels.<sup>305</sup> Thus, isotretinoin corrects through upregulation of FoxOs the relative FoxO deficiency of acne and other acne-associated conditions with increased growth factor signaling, which

all converge in PI3K/Akt-mediated nuclear extrusion of FoxO proteins.<sup>295</sup>

After more than 30 years of clinical use of isotretinoin, we are at the beginning to understand isotretinoin's fundamental mode of action at the level of transcriptional regulation of FoxO transcription factors. Using the powerful tool of translational research we are able to understand the complex pathophysiology of acne and retinoid biology.

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