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Infrared A Radiation Influences the Skin Fibroblast Transcriptome: Mechanisms and Consequences

Christian Calles[1], Maren Schneider[1], Filippina Macaluso[1], Tereza Benesova[1], Jean Krutmann[1,2] and Peter Schroeder[1,2]

Infrared A (IRA) radiation (760–1440 nm) is a major component of solar radiation and, similar to UVR, causes photoaging of human skin by increasing the expression of matrix metalloproteinase-1 in human skin fibroblasts. In this study, we assessed the IRA-induced transcriptome in primary human skin fibroblasts. Microarray analysis revealed 599 IRA-regulated transcripts. The IRA-induced transcriptome differed from changes known to be induced by UV. IRA-responsive genes include the categories extracellular matrix, calcium homeostasis, stress signaling, and apoptosis. Selected results were confirmed by real-time PCR experiments analyzing 13 genes representing these four categories. By means of chemical inhibitors of known signaling pathways, we showed that ERK1/2, the p38-, JNK-, PI3K/AKT-, STAT3-, and IL-6 as well as the calcium-mediated signaling pathways, are functionally involved in the IRA gene response and that a major part of it is triggered by mitochondrial and, to a lesser extent, non-mitochondrial production of reactive oxygen species. Our results identify IRA as an environmental factor with relevance for skin homeostasis and photoaging.

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INTRODUCTION

Human skin is the primary target organ for sunlight. The solar radiation reaching the earth's surface and thus human skin is not only composed of UVR (UVB: $\lambda = 280$–320 nm, UVA: $\lambda = 320$–400 nm), but also it includes infrared (IR) radiation ($\lambda = 760$ nm—1 mm). Although the biological and medical consequences of UV exposure of human skin have been studied extensively, little is currently known about IR radiation-induced effects in human skin (Kochevar *et al.*, 2008). This is somewhat surprising if one imagines that IR, and especially the near-IR range, i.e., infrared A (IRA) radiation (760–1440 nm) accounts for approximately one-third of the solar energy load of human skin. Also, in comparison to UVR, IRA penetrates deeper into human skin, with about half of the energy reaching the dermal compartment and thus viable skin cells, i.e., skin fibroblasts (Schieke *et al.*, 2003; Kochevar *et al.*, 2008). It is exactly this cell type that is of critical importance for the structural integrity and elasticity of the skin (Krutmann and Gilchrest 2006). Accordingly, a number of recent studies have shown that IRA radiation exerts biological effects on human skin fibroblasts and that the net result of these effects is premature skin aging. Specifically, IRA radiation exposure from natural sunlight or artificial irradiation devices was found to induce skin wrinkling, by upregulating the expression of matrix metalloproteinase (MMP-1) but not of its tissue-specific inhibitor tissue inhibitor of MMP-1, both *in vitro* in cultured primary human skin fibroblasts and *in vivo* in the same cell type in mouse or human skin.

The underlying signaling mechanisms are still poorly understood (Schieke *et al.*, 2003; Kim *et al.*, 2005; Schroeder *et al.*, 2008a). IRA radiation appears to initiate a retrograde signaling response that involves the absorbance of IRA radiation at complexes of the mitochondrial respiratory chain (Karu *et al.*, 2001; Karu, 2008) and the subsequent intramitochondrial formation of reactive oxygen species (ROS) (Schroeder *et al.*, 2007, 2008b; Krutmann and Schroeder, 2009). The signal is then transmitted from the mitochondria into the cytoplasm where it causes phosphorylation of mitogen-activated protein kinases (MAPKs) including extracellular signal–regulated kinase 1/2 (ERK1/2) and p38 (Schieke *et al.*, 2002).

To better understand the biological impact of IRA radiation on human skin, we assessed here the IRA radiation-induced transcriptome in primary human skin fibroblasts.

[1]*Institut fuer Umweltmedizinische Forschung (IUF), Duesseldorf, Germany*

[2]*These authors contributed equally to this work.*

Correspondence: Peter Schroeder, Institut fuer Umweltmedizinische Forschung (IUF), Cell Biology/Molecular Aging Research, Auf'm Hennekamp 50, Duesseldorf 40225, Germany. E-mail: peet@gmx.de

Abbreviations: BAX, BCL2-associated X protein; BAD, BCL2-associated agonist of cell death; ECM, extracellular matrix; ER, endoplasmatic reticulum; ERK, extracellular signal–regulated kinase; FASTK, Fas-activated serine/threonine kinase; FN, fibronectin; IL6ST, IL-6 signal transducer; IR, infrared; ITPR, IP_3 receptor; MAPK, mitogen-activated protein kinase; MMP, matrix metalloproteinase; PI3K, phosphoinositide-3-kinase; PIP5K, phosphatidylinositol-4-phosphate 5-kinase; ROS, reactive oxygen species; STAT3, signal transducer and activator of transcription 3; TNFRSF6B, tumor necrosis factor receptor superfamily, member 6b, decoy; VCAM, vascular cell adhesion molecule

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RESULTS AND DISCUSSION

Infrared A radiation regulates a broad range of genes in human dermal fibroblasts

To identify genes differentially regulated after IRA irradiation, we analyzed a set of nine independent sample pairs (IRA irradiated vs sham irradiated), using human primary human dermal fibroblasts from three different donors (termed F1 to F3) by microarray analysis. Cells between passage numbers five to ten were exposed *in vitro* to a dose of 860 J cm^{-2} IRA, which is of physiological relevance and was previously found to be optimal for activating MAPKs and inducing MMP-1 mRNA expression (Schieke *et al.*, 2003).

Unsupervised hierarchical clustering based on all 22,283 transcripts investigated (Figure 1) revealed a heterogeneous distribution of sham- and IRA-irradiated samples, with a closer relation between samples from the same donor than within a treatment group. This indicated that strong inter-individual differences between donors biased the effects of IRA and confirmed our previous notion that different individuals markedly differ in their susceptibility to IRA radiation-induced gene expression (Schroeder *et al.*, 2008b).

To circumvent this bias, we applied a selection algorithm by choosing transcripts that were identically regulated in at least three independent experiments. This supervised filtering process delivered 250 upregulated and 349 downregulated transcripts 24 hours after IRA irradiation (see Supplementary Material online). The magnitude of the changes in gene expression induced by IRA was in the same range as previously shown in cultured fibroblasts on exposure to other types of irradiation (Boerma *et al.*, 2006). For an overview of the identified IRA-regulated genes, a selection of these transcripts, which are related to aging processes, control of apoptosis, stress signaling, or possible involvement in calcium-dependent retrograde mitochondrial signaling, is depicted in Table 1.

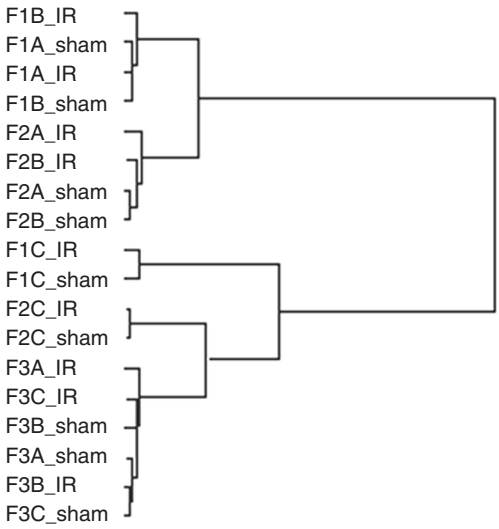

Figure 1. Hierarchical clustering of all 18 samples according to the full probe set list. In the dendogram (average linkage, centered correlation), F1, F2, and F3 are indicating cells from different donors; A, B, C represent different irradiation experiments; IRA are the infrared-A irradiated samples; sham, the respective controls).

Next, the transcriptome data were clustered on the basis of their functional involvement using gene ontology-derived classes. This analysis revealed that the IRA response includes the following four categories: (i) metabolism of the extracellular matrix (ECM), (ii) calcium ion homeostasis, (iii) stress signaling, and (iv) regulation of apoptosis (Table 2, Supplementary Material).

Metabolism of ECM

Our data corroborate and extend the previous observation that IRA regulates the transcriptional expression of genes that are important for the homeostasis of the ECM in skin. In detail, 21 genes relevant for ECM metabolism were differentially expressed on IRA irradiation in skin fibroblasts (Table 2). These genes included MMP-1, which was previously reported to be upregulated at the mRNA and protein level in IRA irradiated cells, respectively (Schieke *et al.*, 2002; Schroeder *et al.*, 2007, 2008b). Although MMP-1 is upregulated, the respective inhibitor tissue inhibitor of MMP-1 does not show a significant change in its expression level after IRA irradiation in our microarray experiments, thus confirming previous studies (Schieke *et al.*, 2002; Schroeder *et al.*, 2008b). The resulting imbalance is thought to be a major mechanism driving the formation of course wrinkles in photoaging of skin (Krutmann and Gilchrest 2006). The functional consequence of upregulated MMP-1 without corresponding changes in tissue inhibitor of MMP-1 expression level is a disturbance of the collagen equilibrium in the dermis, which in the long run causes wrinkle formation and photoaging of the skin (Schroeder *et al.*, 2009).

In independent experiments, we selected two IRA-regulated genes from Table 2, i.e., fibronectin (FN1) and vascular cell adhesion molecule 1 (VCAM-1), and studied their transcriptional expression by real-time PCR analysis. This approach showed downregulation of mRNA expression of both genes after IRA treatment (Figure 2) and thus confirmed the corresponding microarray results (Table 2).

Fibronectin 1 is a structural protein of the ECM involved in cell adhesion, migration, and wound healing, and its transcriptional expression is decreased in aged human dermal fibroblasts (Molinari *et al.*, 2008) and chronically UV-exposed HaCat cells (Lee *et al.*, 2005). The adhesion molecule VCAM-1 is also involved in cell adhesion, migration, and wound healing and has been found to be differentially expressed in oxidatively stressed old tissues (Zou *et al.*, 2006). Integrins have previously been implied to have a role in photoaging (Puschel *et al.*, 1995), and here two integrins, i.e., ITGA10 and ITGB5) (Table 2), were down-regulated after IRA. The same effect was found for Cadherin CDH10, a protein mediating calcium-dependent cell–cell adhesion, which is downregulated during aging processes (Zou *et al.*, 2006) in IRA irradiated human skin fibroblasts. In aggregate, the microarray data of this study further support the concept that IRA radiation is of pathogenetic relevance for photoaging of human skin (Kim *et al.*, 2006) by altering

Table 1. Differentially expressed genes after IRA irradiation

Ref_ID	Unigene no.	x-Times up/down[1]	Gene[2]	Symbol	Gene ontology—biological function	Described functions in the literature
201242_s_at	Hs.78629	−3	ATPase, Na^+/K^+ transporting, beta 1 polypeptide	ATP1B1	Potassium ion transport/ sodium ion transport	Age-modulated in rat brain (Chakraborty *et al.*, 2003). It increases production of mitochondrial ROS and regulates intracellular Ca^{2+} concentration (Xie and Cai, 2003).
209364_at	Hs.76366	+3	BCL2-antagonist of cell death	BAD	Induction of apoptosis	ROS modulated cell survival through PI3K/AKT-pathway. BAD is directly regulating proapoptotic Bad (Clerkin *et al.*, 2008).
211833_s_at	Hs.159428	−3	BCL2-associated X protein	BAX	Negative regulation of survival gene products/ induction of apoptosis	BAX and BAK regulate ITPR1 and calcium efflux from ER (Oakes *et al.*, 2005). Downregulation on protein level and activation of translocation after IRA irradiation (Frank *et al.*, 2004).
214114_x_at	Hs.75087	+3	FAST kinase	FASTK	Induction of apoptosis by extracellular signals	FAST inhibits UV-induced apoptosis (Li *et al.*, 2004).
214701_s_at	Hs.418138	−4	Fibronectin 1	FN1	Cell motility/signal transduction	Protein of the extracellular matrix, involved in cell adhesion, migration, and wound healing, downregulated through glycation endproducts (Lee *et al.*, 2005). Fibronectin is part of a network of longevitiy and age-related disease proteins (Wolfson *et al.*, 2009).
204863_s_at	Hs.71968	−3	IL-6 signal transducer (gp130, oncostatin M receptor)	IL6ST	Cell surface receptor linked signal transduction/immune response	IL-6 induces VEGF expression through IL6ST and ERK (Omori *et al.*, 2004). Soluble IL-6 receptor induces Ca^{2+} flux and selectively modulates chemokine expression in human dermal fibroblasts (Sporri *et al.*, 1999). IL-6 mediates UVA induction of MMP-1 (Wenk *et al.*, 2004).
202662_s_at	Hs.512235	−3	IP_3 receptor, type 2	ITPR2	Calcium channels	Involved in phosphoinositol dependent calcium channeling from ER to the cytosol. Cytochrome *c*, which is released from mitochondria after IRA irradiation (Frank *et al.*, 2004) binds to IP_3 receptors, amplifying calcium release (Boehning *et al.*, 2003).
201187_s_at	Hs.77515	−3	IP_3 receptor, type 3	ITPR3	Calcium channels	Involved in phosphoinositol-dependent calcium channeling from ER to the cytosol. Cytochrome *c*, which is released from mitochondria after IRA irradiation (Frank *et al.*, 2004) binds to IP_3 receptors, amplifying calcium release (Boehning *et al.*, 2003).
202743_at	Hs.372548	−3	Phosphoinositide-3-kinase, regulatory subunit, polypeptide 3 (p55, gamma)	PIK3R3		PI3K is regulated in MnSOD overexpressing *Caenorhabditis elegans* and *Drosophila daf-2* mutants (Curtis *et al.*, 2007). PI3K, acts antiapoptotic through AKT survival pathway, an important intracellular regulator of epidermal homeostasis and repair.
217477_at	Hs.297604	−4	Phosphatidyl-inositol-4-phosphate 5-kinase, type I, beta	PIP5K1B		PIP5K1B is involved in phosphoinositol signaling. Aging decreases PIP_2 level but has no effect on activities of phosphoinositide kinases (Zambrzycka, 2004).
208992_s_at	Hs.421342	+4	Signal transducer and activator of transcription 3	STAT3	Acute-phase response	STAT3 is regulated in MnSOD overexpressing *C. elegans* and *Drosophila daf-2* mutants (Curtis *et al.*, 2007). PKC epsilon overexpression mediates UV-induced cutaneous damage and squamous cell carcinoma by activation of STAT3 (Aziz *et al.*, 2007).
213829_x_at	Hs.348183	+4	Tumor necrosis factor receptor superfamily, member 6b, decoy	TNFRSF6B	Antiapoptosis/ oncogenesis	It acts antiapoptotic and is highly expressed in cancer cells. TNFRSF6B induces transcriptional upregulation of adhesion molecules such as VCAM-1 and the upregulation of IL-8 (Yang *et al.*, 2005).
203868_s_at	Hs.109225	−3	Vascular cell adhesion molecule 1	VCAM1	Cel-cell adhesion	VCAM-1 has a role in wound healing and angiogenesis. It is upregulated in oxidative stressed old rats (Puschel *et al.*, 1995).

[1] + or − combined with numbers indicates how often the genes were up- or downregulated in nine independent microarray experiments.
[2] The genes given in this table were selected for further real-time PCR experiments on the basis of their relation to aging processes, control of apoptosis, stress signaling, or possible involvement in calcium-dependent retrograde mitochondrial signaling.

Table 2. Subordinate classes of IRA-regulated genes according to their processes based on gene ontology clustering

Gene	Symbol	Regulation by IRA	Ref_ID	Unigene no.	Gene ontology—biological function
Extracellular matrix					
Cadherin 10, type 2 (T2-cadherin)	CDH10	Down	220115_s_at	Hs.92489	Homophilic cell adhesion
Carcinoembryonic antigen-related cell adhesion molecule 1 (biliary glycoprotein)	CEACAM1	Down	211883_x_at	Hs.512682	Immune response
Carcinoembryonic antigen-related cell adhesion molecule 4	CEACAM4	Up	207205_at	Hs.12	
Cholinergic receptor, nicotinic, β-polypeptide 3	CHRNB3	Up	207859_s_at	Hs.96094	Synaptic transmission
Chromosome 1 open reading frame 38	C1orf38	Up	210785_s_at	Hs.10649	Cell adhesion
Collagen, type I, α-1	COL1A1	Up	202312_s_at	Hs.172928	Epidermal differentiation
Collagen, type VIII, α-1	COL8A1	Down	214587_at	Hs.114599	Somatic muscle development
Fibronectin 1	FN1	Down	214701_s_at	Hs.418138	Cell motility/signal transduction
Glutamate receptor, ionotropic, *N*-methyl-D-aspartate 2C	GRIN2C	Down	217573_at	Hs.436980	Ion transport
Integrin, α10	ITGA10	Up	206766_at	Hs.158237	Integrin-mediated signaling pathway/cell–matrix adhesion
Integrin, β5	ITGB5	Up	214021_x_at	Hs.149846	Integrin-mediated signaling pathway/cell–matrix adhesion
Matrix metalloproteinase 1 (interstitial collagenase)	MMP1	Up	204475_at	Hs.83169	Collagen catabolism
Neuroligin-1	NLGN1	Down	205893_at	Hs.71132	Calcium-dependent cell–cell adhesion
Neuroligin-4	NLGN4	Up	207703_at	Hs.21107	Cell adhesion
Protocadherin LKC	PC-LKC	Down	220186_s_at	Hs.4205	Homophilic cell adhesion
Sialic acid-binding Ig-like lectin 7	SIGLEC7	Up	216537_s_at	Hs.274470	Cell adhesion
Sialoadhesin	SN	Down	44673_at	Hs.31869	Heterophilic cell adhesion/ cell–matrix adhesion
Spondin 1, (f-spondin) extracellular matrix protein	SPON1	Up	209436_at	Hs.5378	
Vascular cell adhesion molecule 1	VCAM1	Down	203868_s_at	Hs.109225	Cell–cell adhesion
Vascular endothelial growth factor B	VEGFB	Up	203683_s_at	Hs.78781	Positive regulation of cell proliferation
Vesicle-associated membrane protein 2 (synaptobrevin 2)	VAMP2	Up	201557_at	Hs.25348	Nonselective vesicle transport
Calcium ion signaling					
ATPase, Ca++ transporting, plasma membrane 4	ATP2B4	Down	205410_s_at	Hs.343522	Cation transport/calcium ion transport
ATPase, Na+/K+ transporting, beta 1 polypeptide	ATP1B1	Down	201242_s_at	Hs.78629	Potassium ion transport/sodium ion transport
Cadherin 10, type 2 (T2-cadherin)	CDH10	Down	220115_s_at	Hs.92489	Homophilic cell adhesion
Calcium-binding tyrosine-(Y)-phosphorylation regulated (fibrousheathin 2)	CABYR	Down	219928_s_at	Hs.511983	
Calponin 1, basic, smooth muscle	CNN1	Down	203951_at	Hs.21223	Smooth muscle contraction
Casein beta	CSN2	Down	207951_at	Hs.2242	Calcium ion transport
Chemokine (C-C motif) ligand 2	CCL2	Up	216598_s_at	Hs.303649	Calcium ion homeostasis/JAK-STAT cascade
Chloride channel 3	CLCN3	Up	201733_at	Hs.372528	Chloride transport
IP_3 receptor, type 2	ITPR2	Down	202662_s_at	Hs.512235	Calcium channels
IP_3 receptor, type 3	ITPR3	Down	201187_s_at	Hs.77515	Calcium channels

Table 2 continued on the following page

Table 2. Continued

Gene	Symbol	Regulation by IRA	Ref_ID	Unigene no.	Gene ontology—biological function
Neuroligin 1	NLGN1	Down	205893_at	Hs.71132	Calcium-dependent cell–cell adhesion
Phosphatidylinositol-4-phosphate 5-kinase, type I, beta	PIP5K1B	Down	217477_at	Hs.297604	
Phosphatidylinositol-4-phosphate 5-kinase, type I, gamma	PIP5K1C	Up	212518_at	Hs.282177	
Phosphoinositide-3-kinase, regulatory subunit, polypeptide 3 (p55, gamma)	PIK3R3	Down	202743_at	Hs.372548	
Protein tyrosine phosphatase, receptor type, D	PTPRD	Down	214043_at	Hs.323079	Transmembrane receptor protein tyrosine phosphatase signaling pathway
Sodium channel, voltage-gated, type X, alpha	SCN10A	Down	208578_at	Hs.250443	Sodium ion transport
Tachykinin receptor 1	TACR1	Down	208048_at	Hs.1080	G-protein signaling, coupled to IP_3 second messenger
Transient receptor potential cation channel, subfamily V, member 2	TRPV2	Down	219282_s_at	Hs.279746	Cation transport
Stress signaling					
Carcinoembryonic antigen-related cell adhesion molecule 1 (biliary glycoprotein)	CEACAM1	Down	211883_x_at	Hs.512682	Immune response
Caspase recruitment domain family, member 10	CARD10	Up	210026_s_at	Hs.57973	Activation of NF-κB-inducing kinase/apoptosis
Chemokine (C-C motif) ligand 2	CCL2	Up	216598_s_at	Hs.303649	Calcium ion homeostasis/JAK-STAT cascade
Chemokine (C-X3-C motif) ligand 1	CX3CL1	Down	823_at	Hs.80420	Immune response/cell–cell signaling/ cell adhesion
Chemokine (C-X-C motif) ligand 6 (granulocyte chemotactic protein 2)	CXCL6	Up	206336_at	Hs.164021	Inflammatory response/chemotaxis
Chemokine (C-X-C motif) ligand 9	CXCL9	Up	203915_at	Hs.77367	Cell–cell signaling/chemotaxis
Gamma-glutamyltransferase 1	GGT1	Down	207131_x_at	Hs.352119	Glutathione biosynthesis
IL2-inducible T-cell kinase	ITK	Down	211339_s_at	Hs.211576	Cellular defense response/intracellular signaling cascade
Inhibitor of kappa light polypeptide gene enhancer in B cells, kinase gamma	IKBKG	Up	36004_at	Hs.43505	Regulation of transcription/induction of apoptosis
Interferon, alpha 10	IFNA10	Down	208261_x_at	Hs.282275	Defense response
IL-1 receptor-like 1 ligand	IL1RL1LG	Up	203679_at	Hs.446686	Cell–cell signaling
IL-12B	IL12B	Up	207901_at	Hs.674	Regulation of cytokine biosynthesis/ JAK-STAT cascade
IL-16 (lymphocyte chemoattractant factor)	IL16	Down	209827_s_at	Hs.170359	Intracellular signaling cascade
IL-6 signal transducer (gp130, oncostatin M receptor)	IL6ST	Down	204863_s_at	Hs.71968	Cell surface receptor linked signal transduction/immune response
Lymphocyte antigen 6 complex, locus H	LY6H	Down	206773_at	Hs.159590	Cellular defense response
Mannan-binding lectin serine protease 1	MASP1	Up	206449_s_at	Hs.89983	Complement activation
Mitogen-activated protein kinase kinase kinase 8	MAP3K8	Down	205027_s_at	Hs.432453	Cell growth and/or maintenance
Mitogen-activated protein kinase kinase kinase kinase 5	MAP4K5	Down	211081_s_at	Hs.246970	Protein kinase cascade/activation of JUNK
Signal transducer and activator of transcription 3	STAT3	Up	208992_s_at	Hs.421342	Acute-phase response
Thioredoxin domain–containing 4 (endoplasmic reticulum)	TXNDC4	Up	208957_at	Hs.154023	Electron transport/regulation of redox homeostasis
Toll-like receptor 4	TLR4	Down	221060_s_at	Hs.174312	Activation of NF-κB-inducing kinase/ regulation of IL-6 biosynthesis

Table 2 continued on the following page

Table 2. Continued

Gene	Symbol	Regulation by IRA	Ref_ID	Unigene no.	Gene ontology—biological function
Apoptosis signaling					
B-cell CLL/lymphoma 2	BCL2	Down	203685_at	Hs.79241	Antiapoptosis/regulation of cell cycle
BCL2-binding component 3	BBC3	Up	211692_s_at	Hs.87246	
BCL2-antagonist of cell death	BAD	Up	209364_at	Hs.76366	Induction of apoptosis
BCL2-associated X protein	BAX	Down	208478_s_at	Hs.159428	Negative regulation of survival gene products
BCL6 co-repressor	BCOR	Down	219433_at	Hs.186424	
Caspase recruitment domain family, member 10	CARD10	Up	210026_s_at	Hs.57973	Activation of NF-κB-inducing kinase/ apoptosis
Caspase 1, apoptosis-related cysteine protease (IL1, beta, convertase)	CASP1	Down	211368_s_at	Hs.2490	Signal transduction/apoptosis
Caspase 7, apoptosis-related cysteine protease	CASP7	Down	207181_s_at	Hs.9216	Apoptotic program
Fas (TNFRSF6)-associated factor 1	FAF1	Down	218080_x_at	Hs.12899	Apoptosis
FAST kinase	FASTK	Up	214114_x_at	Hs.75087	Induction of apoptosis by extracellular signals
Tumor necrosis factor receptor superfamily, member 6b, decoy	TNFRSF6B	Up	213829_x_at	Hs.348183	Antiapoptosis/oncogenesis

the structure and function of the dermal ECM (Schroeder *et al.*, 2007, 2008b; Buechner *et al.*, 2008).

Calcium ion homeostasis

IRA radiation has been discussed to modulate cellular calcium homeostasis and subsequent signaling processes in sperm (Lubart *et al.*, 1997). Also, in human skin fibroblasts, IRA radiation generated increased levels of mitochondrial ROS, which are well known to be capable of triggering calcium fluxes and initiating calcium-derived intracellular signaling (Ichas *et al.*, 1997; Rosenstock *et al.*, 2004; Biswas *et al.*, 2005). In this study, functional clustering analysis detected 18 IRA responsive genes related to calcium homeostasis (Table 2). From these 18 genes, five genes (ATP1B1, ITPR2, ITPR3, PIK3R3, PIP5K1B) were selected and the results from the microarray analysis (Table 2) were confirmed by real-time PCR (Figure 2).

These five genes are all functionally linked to calcium-induced signaling and aging. The Na^+/K^+-ATPase, ATP1B1, which is downregulated by IRA, activates calcium signaling in different microdomains such as the endoplasmatic reticulum (ER) and the mitochondria (Tian and Xie, 2008), increases the production of mitochondrial ROS, and regulates intracellular calcium fluxes (Xie and Cai, 2003). In association with aging, a decrease in Na^+/K^+-ATPase activity due to oxidative stress was reported (Chakraborty *et al.*, 2003) and Na^+/K^+-ATPase was found to be less expressed in aged cells (Dencher *et al.*, 2007).

Similarly, another important player involved in phosphoinositol signaling, phosphatidylinositol-4-phosphate 5-kinase (PIP5K1B), which produces the IP_3 precursor phosphatidylinositol 4,5-bisphosphate (Oude Weernink *et al.*, 2004), is downregulated after IRA irradiation (Table 1, Figure 2g). It was shown that aged tissues harbor decreased amounts of phosphatidylinositol 4,5-bisphosphate (Zambrzycka, 2004) and that this is the result of a prolonged downregulation of the PIP5K.

IP_3 receptor types 2 and 3 (ITPR2 and ITPR3), which are both downregulated after IRA (Figures 2d and e), are responsible for the phosphoinositol (IP_3)-dependent calcium channeling from the ER to the cytosol. Genes encoding components of the major Ca^{2+} transport pathways, including the IP_3 receptor channels, are thought to be linked in their expression to the Ca^{2+} load of the ER store (Kuo *et al.*, 1997). Both receptors are modulated by binding of cytochrome *c* (Boehning *et al.*, 2003), which is known to be released at sub-apoptogenic amounts from mitochondria after IRA irradiation (Frank *et al.*, 2004).

Phosphoinositide-3-kinase (PIK3R3) was downregulated by IRA exposure as well (Table 1, Figure 2f). PIK3R3 is important for epidermal homeostasis and repair (Pankow *et al.*, 2006). Furthermore, phosphoinositide-3-kinase (PI3K) is up- rather than downregulated in MnSOD overexpressing *Caenorhabditis elegans* and *Drosophila daf-2* mutants, which have an increased lifespan (Curtis *et al.*, 2007).

In addition to these genes, the microarray data in Table 2 provide additional information to support the assumption that calcium fluxes and related signaling processes are part of the IRA response. Accordingly, IRA alters the expression of four genes responsible for calcium binding, transport, and channeling (chemokine (C–C motif) ligand-2 (CCL2), Ca^{2+}-transporting ATPase (ATP2B4), casein beta (CSN2), and calponin-1 (CNN1), of six genes related to phosphate metabolism and phosphate-dependent signaling (phosphatidylinositol-4-phosphate 5-kinase, type I, beta (PIP5K1B), tachykinin receptor-1, phosphatidyl serine receptor, protein

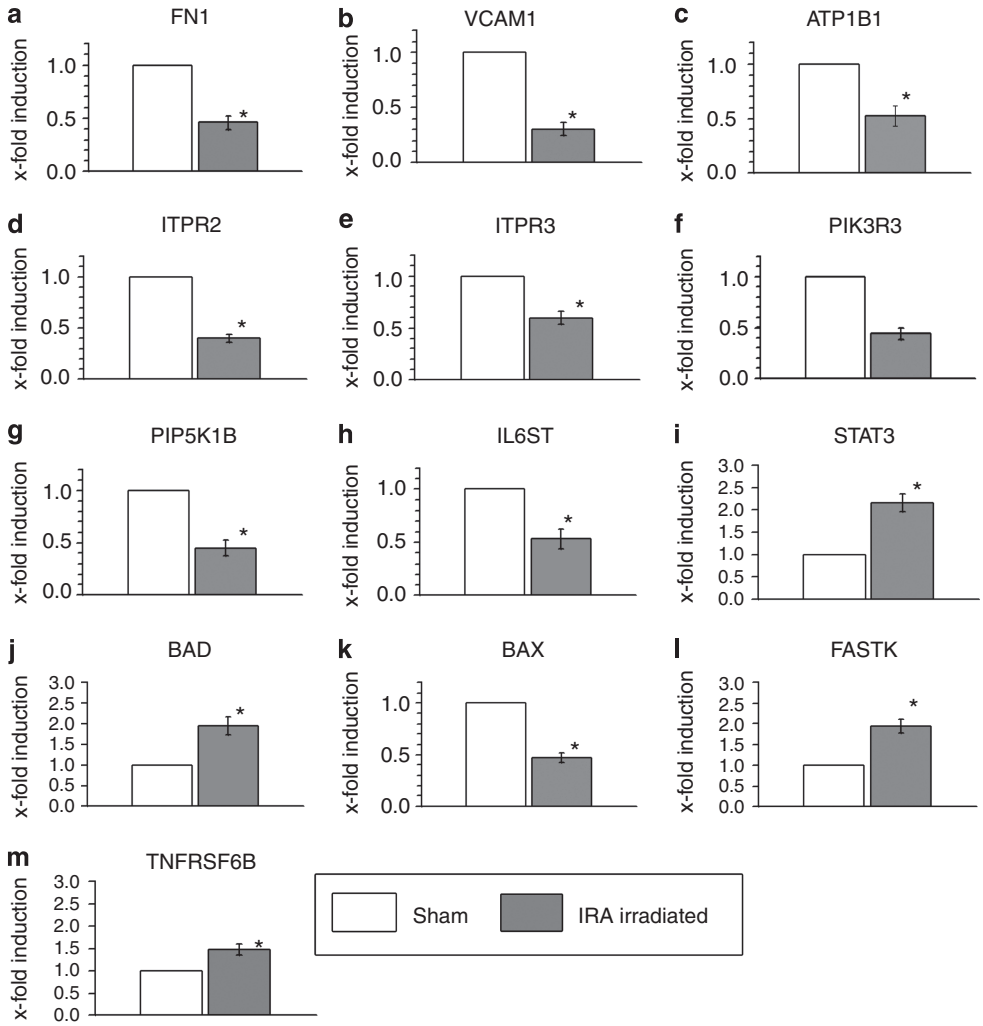

Figure 2. Infrared A-induced gene expression of selected genes measured by real-time PCR. For each gene, the relative mRNA levels were normalized to the 18S rRNA transcript level revered as a housekeeping gene. Differential expression (gray bars) with regard to their respective sham controls (white bars), which are set to 1, are given for the ECM genes FN1 (**a**) and VCAM-1 (**b**); for the calcium signaling genes ATP1B1 (**c**), ITPR2 (**d**), ITPR3 (**e**), PIK3R3 (**f**), and PIP5K1B (**g**); for stress signaling-related IL6ST (**h**) and STAT3 (**i**); and for apoptosis-regulating BAD (**j**), BAX (**k**), FASTK (**l**), and TNFRSF6B (**m**). Fibroblasts were irradiated with 860 J cm^{-2} IRA and harvested 24 hours after irradiation. Data are means ± SEM of at least nine independent experiments. *Significantly different from respective sham ($P<0.05$).

tyrosine phosphatase receptor, and PI3K (PIK3R3) and of five genes involved in other active transport systems that feed back into calcium homeostasis (Lamb *et al.*, 1999); e.g., chloride channel 3.

Taken together, our data are in line with previous reports, suggesting a link between IRA radiation and intracellular calcium homeostasis (Lubart *et al.*, 1997). They suggest that calcium signaling is an integral part of IRA radiation-induced signal transduction. This would be in agreement with the current concept that IRA radiation elicits a retrograde stress response (Schroeder *et al.*, 2007; Karu, 2008).

Stress signaling

In accordance with previous findings on the activation of MAPKs by IRA (Schieke *et al.*, 2002; Shibata *et al.*, 2009), we here identify many IRA-sensitive genes, which are closely related or even part of stress signaling pathways.

In particular, microarray data analysis identified 21 stress-related genes to be regulated by IRA radiation, of which two were further analyzed by real-time PCR analysis. As shown in Figure 2, both the reduced expression of IL-6 signal transducer (IL6ST) and the increased expression of the transcription factor signal transducer and activator of transcription-3 (STAT3) were confirmed by this approach.

Reduced expression of IL6ST (Figure 2h), an important regulator of neoangiogenesis (Omori *et al.*, 2004), points toward an involvement of IL-6 signaling in the IRA response. This is further supported by the capacity of the soluble IL-6 receptor to induce calcium ion fluxes (Sporri *et al.*, 1999) and the effect of IRA on STAT3, a known downstream target of the IL-6 signaling pathway. STAT3 activation is involved in the development of skin cancer (Pedranzini *et al.*, 2004; Aziz *et al.*, 2007) and a target of the IRA inducible MAPKs ERK1/2 (Chung *et al.*, 1997) and the retrograde mitochondrial

mammalian target of rapamycin pathway (Yokogami *et al.*, 2000; Schieke and Finkel, 2006).

Among the IRA responsive genes, we also find ILs (IL12B, IL16) and chemokine ligands (CCL2, CX4CL1, CXCL6, CXCL9), which may interfere with other signaling pathways. A well-known example is the IRA downregulated chemokine ligand-2 (CCL2), which affects both the STAT cascade and calcium homeostasis (Biswas and Sodhi, 2002; Sakamoto *et al.*, 2007; Kok *et al.*, 2009).

These findings are in line with the recently described involvement of ILs and chemokine ligands in anti-inflammatory effects of IRA radiation therapy for rheumatoid arthritis (Shibata *et al.*, 2009). In this study, it was suggested that IRA may act through NF-κB signaling. Accordingly, the present microarray analysis revealed a number of NF-κB-related genes to be IRA regulated (e.g., CARD10, TLR4, IKBKG).

A third group of stress signaling related genes, shown here to be IRA sensitive, contribute to cellular antioxidative defense. These include the thioredoxin domain containing 4, which is present in the ER and upregulated by IRA, and the gamma-glutamyltransferase-1, which is present in the cytoplasm and downregulated by IRA.

Apoptosis and related signaling

In human epithelial cells, IRA irradiation has been shown to exert antiapoptotic effects, if it precedes irradiation with apoptogenic doses of UVB irradiation, but to promote early apoptotic events, if applied alone (Menezes *et al.*, 1998; Frank *et al.*, 2004, 2006; Jantschitsch *et al.*, 2009b). Our microarray analysis supports these findings, as 11 genes related to apoptosis were found to be regulated after IRA irradiation. Once more, independent experiments analyzing four genes from this group confirmed our microarray results.

Among these genes, BAD, the BCL2-antagonist of cell death, was upregulated, whereas expression of the BCL2-associated X protein (BAX) was decreased. Both genes act as proapoptotic activators by binding to BCL-2, thereby reverting the death repressor activity. In the context of the studies mentioned above, IRA-induced downregulation of BAX might be interpreted as an antiapoptotic and BAD upregulation by IRA a proapoptotic effect. Interestingly, ROS-induced modulation of cell survival through the PI3K/AKT pathway directly regulates BAD (Clerkin *et al.*, 2008) and thus there might be a link between IRA-induced modulation of apoptosis and IRA-induced ROS generation (Schroeder *et al.*, 2007). In addition, BAX is known to be closely connected to calcium signaling pathways, i.e., to the regulation of the phosphoinositol receptor and to calcium efflux from the ER (Oakes *et al.*, 2005). Thus, a common denominator of all three activities appears to be their inducibility by IRA radiation.

Real-time PCR analysis also confirmed IRA-induced upregulation of Fas-activated serine/threonine kinase (FASTK) and of tumor necrosis factor receptor superfamily 6, decoy (TNFRSF6B) expression. FASTK is a survival protein inhibiting UV-induced apoptosis (Li *et al.*, 2004), and its upregulation by IRA irradiation is likely to be involved in the promotion of the previously mentioned antiapoptotic effect. TNFRSF6B acts as a decoy receptor, which competes with death receptors for ligand binding and thus exerts antiapoptotic effects as well. It is highly expressed in cancer cells, where it induces upregulation of IL-8 (Yang *et al.*, 2005) and of adhesion molecules such as VCAM-1.

In aggregate, the IRA-induced regulation of these four genes reflects the previously reported, seemingly contradictory, impact of IRA on apoptotic events (i.e., early apoptotic events are triggered, but later stages of apoptosis are blocked by IRA) (Menezes *et al.*, 1998; Frank *et al.*, 2004, 2006; Jantschitsch *et al.*, 2009b).

Our microarray data also confirm that the antiapoptotic effect by IRA is not due to the prevention of initial apoptotic events, but instead to an interruption of later events in apoptosis. Accordingly, caspases CASP-1 and CASP-7, as well as the proapoptotic gene FAF-1 (fas-associated factor-1), are downregulated by IRA irradiation.

Further analysis of IRA radiation induced signaling pathways

The microarray and real-time PCR data described above indicate that IRA radiation induced gene regulatory effects extend not only beyond MMP-1 gene expression—as initially hypothesized—but also beyond the MAPK signaling pathway. In fact, four major signaling pathways appear to be regulated by IRA radiation: (i) the MAPK pathway, (ii) the intracellular calcium signaling pathway, (iii) the IP_3 signaling pathway, and (iv) the IL-6 signaling pathway. To better understand the regulatory pathways responsible for the IRA radiation induced transcriptome, we next performed real-time PCR analysis of 12 IRA-regulated genes in the presence or absence of inhibitors with known specificity for the signaling pathways of interest.

We first reassessed the relevance of MAPK signaling for IRA radiation-induced gene expression by treating cells with specific inhibitors for the three major components of this signaling pathway, i.e., ERK1/2, p38, and JNK. MAPK signaling through ERK1/2 has already been shown to mediate IRA-induced MMP-1 expression (Schieke *et al.*, 2002). We here confirm the pivotal role of ERK1/2 in the IRA signaling response by showing that IRA induced expression of all but two genes depends on a functionally active ERK1/2 pathway. Accordingly, addition of the specific ERK1/2 kinase MEK1/2 inhibitor PD98059 to cultured primary human dermal fibroblasts (Figures 3a, d, g, j) abrogated the IRA-induced gene expression in 10 of 12 genes including ATP1B1, BAX, BAD, FASTK, FN1, ITPR3, IL6ST, PIK3R3, PIK5K1B, STAT3, and TNFRSF6B. Exceptions to be noted were FN1 and VCAM-1.

In addition to ERK1/2, the MAPK p38 was phosphorylated in response to IRA irradiation (Schieke *et al.*, 2002), but its role in IRA-induced gene expression was not known. In this study, use of the p38 inhibitor SB203580 (Figures 3a, d, g, j) altered the expression of nearly all (11/12) IRA-modulated genes (ATP1B1, BAX, BAD, FASTK, ITPR3, IL6ST, PIK3R3, PIK5K1B, STAT3, TNFRSF6B, and VCAM-1) with the exception of FN1.

Inhibition of the third MAPK JNK through SP600125 (Figures 3a, d, g, j) caused a change in the IRA regulation of

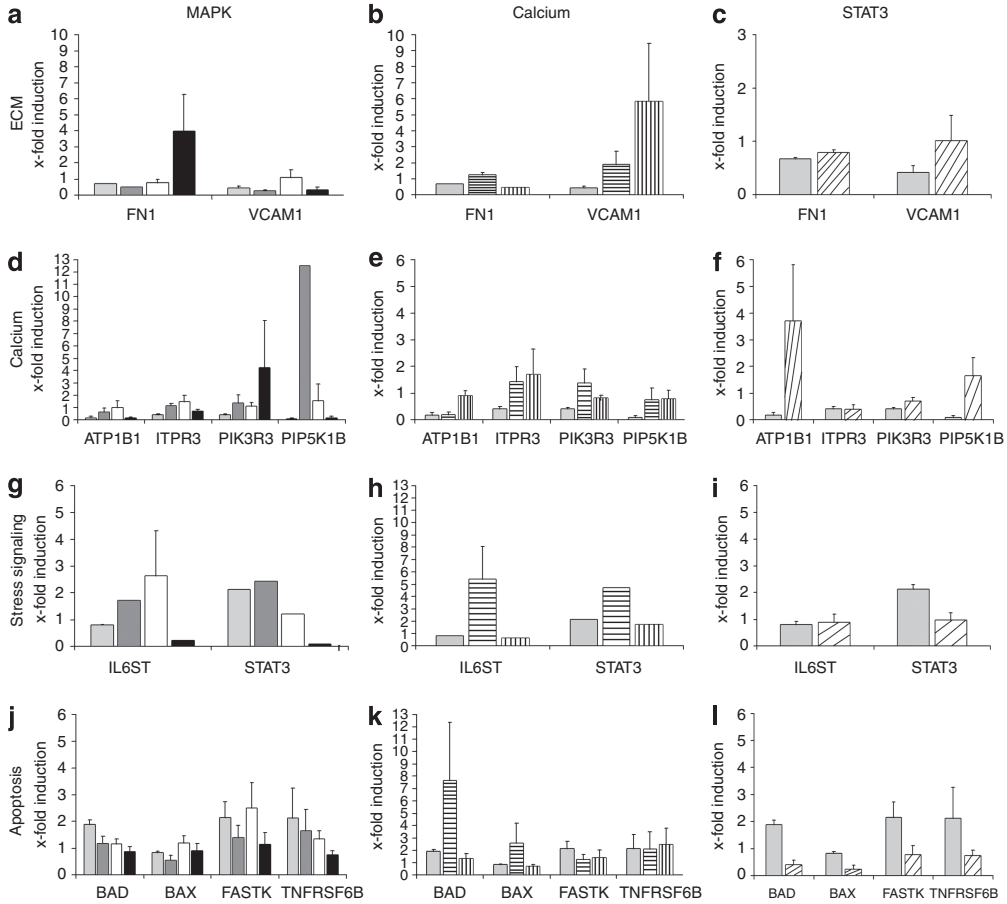

Figure 3. Kinase inhibitors interfere with IRA-induced gene expression differentially. Measurement of mRNA of indicated genes 24 hours after IRA irradiation (860 J cm^{-2}) in lysates from cells treated or not treated (light gray bars) with different inhibitors. Expression of ECM genes (**a**–**c**), genes of the calcium signaling (**d**–**f**), stress signaling (**g**–**i**) and apoptosis genes (**j**–**l**) was analyzed after IRA irradiation combined with inhibitor treatment. Cells were treated with 20 μM ERK1/2 inhibitor PD98059 (dark gray bars, **a**, **d**, **g**, **j**), 10 μM p38 inhibitor SB203580, (white bars **a**, **d**, **g**, **j**), 4 μM JNK inhibitor II SP600125 (black bars, **a**, **d**, **g**, **j**), 20 μM PI3K inhibitor LY294002 (fasciated bars, **b**, **e**, **h**, **k**), 1 μM cyclosporine A (lengthwise striped bars, **b**, **e**, **h**, **k**), or 25 μM STAT3 inhibitor peptide (diagonal striped bars, **c**, **f**, **i**, **l**) 1 hour before and 24 hours after irradiation. Data are means or means ± SEM.

eight genes (8/12): BAD, FASTK, FN1, IL6ST, ITPR3, PIK3R3, STAT3, and TNFRSF6B, whereas the expression of four IRA-sensitive genes (ATP1B1, BAX, PIK5K1B, and VCAM-1) was unaffected**.**

We next assessed the relevance of calcium- and IP_3-signaling for the IRA-induced transcriptome by using two different approaches: calcium-induced signals were targeted by applying cyclosporine A (i.e., by blocking the calcineurin/NFAT pathway) or LY284002 (i.e., by inhibiting IP_3K). Treatment with cyclosporine A (Figures 3b, e, h, k) had a wide influence on the IRA-induced gene expression for the transcripts we investigated in our experiments. Interestingly, only the IRA-induced downregulation of PIK3R3 was unchanged.

Similarly, blocking PI3K with LY294002 (Figures 3b, e, h, k) affected the IRA-induced regulation of most genes analyzed (10/12) (BAX, BAD, FASTK, FN1, ITPR3, IL6ST, PIK3R3, PIK5K1B, STAT3, and VCAM-1) with the exception of TNFRSF6B and ATP1B1.

Targeting of the IL-6 signal cascade using a STAT3 inhibitor peptide (Figures 3c, f, i, l) modulated the IRA-induced changes in gene expression for many (ATP1B1, BAX, BAD, FASTK, FN1, PIK5K1B, STAT3, TNFRSF6B, and VCAM-1) but not all studied genes (9/12), as IL6ST, ITPR3, and PIK3R3 regulation were unaffected.

As summarized in Table 3, IRA exerts cellular effects by substantially influencing intracellular signaling pathways. Besides MAPKs ERK1/2, we also show that the p38-, JNK, PI3K/AKT-, the STAT3-signaling and calcium-mediated signaling pathways, are induced in the IRA response, which is to our knowledge previously unreported.

Functional relevance of ROS in IRA-mediated gene regulation

We and others have previously shown that IRA irradiation causes the formation of ROS in human skin fibroblasts *in vitro* as well as *in vivo* and that this triggers a retrograde mitochondrial signaling cascade, which in turn causes the increased MMP-1 expression (Schroeder *et al.*, 2007, 2008b).

Table 3. Involvement of signaling pathways in IRA-induced gene regulation

	Inhibition of IRA effect by					
	MAPK			Calcium		STAT3
Gene regulated	ERK inhb. PD98059	p38 inhb. SB203580	JNK inhb. SP600125	PI3K inhb. LY284002	Cyclosporine A	STAT3 inhb. peptide
ECM						
FN1	–[1]	–	+	+	+	+
VCAM1	–	+	–	+	+	+
Calcium						
ATP1B1	+	+	–	–	+	+
ITPR3	+	+	+	+	+	–
PIK3P3	+	+	+	+	–	–
PIP5K1B	+	+	–	+	+	+
Stress						
IL6ST	+	+	+	+	+	–
STAT3	+	+	+	+	+	+
Apoptosis						
Bad	+	+	+	+	+	+
BAX	+	+	–	+	+	+
FASTK	+	+	+	+	+	+
TNFRSF6B	+	+	+	–	+	+

[1]Inhibitor treatment that abrogates the IRA-dependent gene expression is marked with "+", IRA-dependent gene expression that was unaffected from the inhibitor treatment is marked in gray with "–".

We therefore next assessed the relevance of ROS to the IRA response by pretreating fibroblasts with two antioxidants. The antioxidant *N*-acetyl-cysteine is a cysteine donor that boosts the intracellular glutathione level and thereby increases the antioxidative defense in all compartments of a cell (Schroeder *et al.*, 2007), whereas MitoQ, a mitochondria-targeted quinone, specifically scavenges ROS originating within the mitochondria (Tauskela, 2007).

As shown in Figure 4, preincubation with *N*-acetylcysteine prevented the IRA-induced expression changes in 12 of 12 genes. Interestingly, the addition of MitoQ affected IRA-induced gene expression in a more specific manner, because regulation of only 8 of 12 IRA-sensitive genes was affected. The remaining four genes (FN1, ATP1B1, IL6ST, and BAX) may thus be regulated by ROS, which are not produced intra but in a different cellular compartment. It is therefore tempting to speculate that in addition to complexes of the mitochondrial respiratory chain (Karu *et al.*, 2001), human fibroblasts possess additional IRA chromophores that may be involved in IRA-induced ROS production. Mechanistically, our data indicate that the mitochondria represent the major cellular compartment involved in IRA responses, but IRA-induced signaling and resulting gene expression changes are not exclusively limited to the mitochondria.

This study unambiguously shows that IRA radiation-induced gene regulatory effects in human fibroblasts clearly extend beyond the regulation of MMP-1 expression and involve up to 599 genes. Figure 5 summarizes the basic scheme of the cellular response to IRA in human dermal fibroblasts. IRA leads to the production of ROS in the cell originating from the mitochondria as well as from other yet unidentified intracellular origins. This leads to IRA-induced signaling events involving MAPKs, Calcium, and the IL6/STAT3 pathway, which in turn modulate the expression of genes relevant to the ECM homeostasis, calcium signaling, stress signaling, and apoptosis. It is therefore likely that in addition to causing premature skin aging, IRA irradiation exerts a variety of other, currently unknown, biological effects on human skin, which may be of relevance not only for photoaging, but also for photocarcinogenesis. This assumption is supported by the recent observation that IRA radiation in combination with UV can influence the formation of skin tumors in mice (Jantschitsch *et al.*, 2009a).

Another important observation is that the IRA-induced gene regulatory response shows similarities but also marked differences with UVB or UVA radiation-induced gene regulation in fibroblasts. In terms of biological end points, there are some similarities, e.g., IRA and UV both upregulate

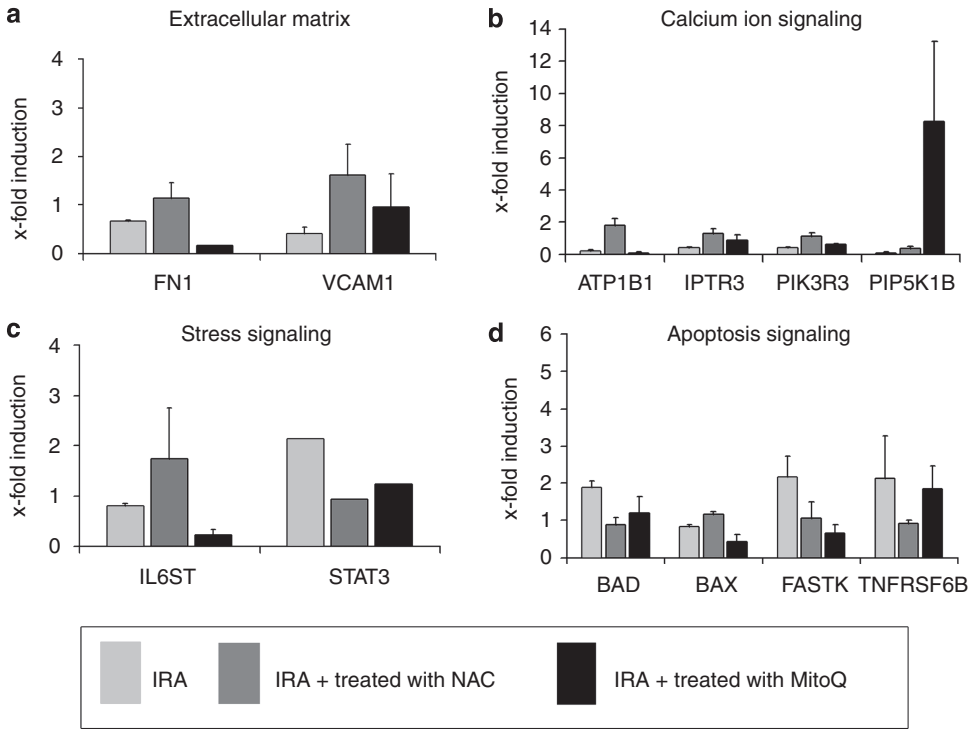

Figure 4. Antioxidants change IRA-induced gene expression. Measurement of mRNA of indicated genes 24 hours after IRA irradiation (860 J cm^{-2}) in lysates from cells treated with 20 mM *N*-acetylcysteine (dark gray bars) or 100 nM MitoQ (black bars) or not treated with antioxidants (light gray bars) 24 hours before and after irradiation for genes of the extracellular matrix (**a**), of calcium signaling (**b**), stress signaling (**c**), and apoptosis signaling genes (**d**). Data are means or means ± SEM.

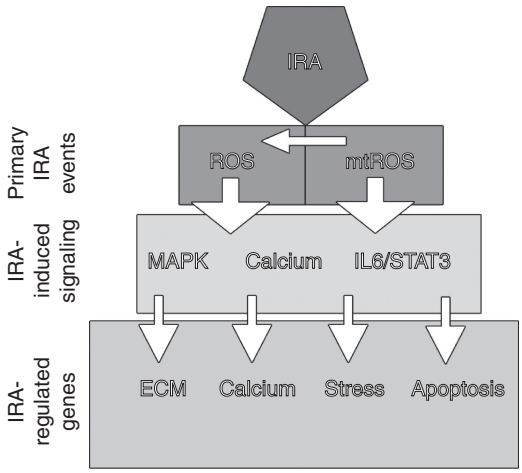

Figure 5. Principles of IRA-induced gene regulation.

MMP-1 (Schieke *et al.*, 2003; Schroeder *et al.*, 2008b) and then downregulate Collα1 (Buechner *et al.*, 2008). In addition, we identified six genes regulated by IRA, which are regulated by UV in an opposite manner: We could observe a downregulation of IL6ST, FN1, and Bax after IRA irradiation, whereas UVB was reported to increase expression of these genes (Enk *et al.*, 2006; Chen *et al.*, 2008). IRA causes upregulation of BAD, STAT3, and TNFRSF6B, whereas UVB downregulates the expression of these genes (Maeda *et al.*, 2001; Sano *et al.*, 2005; Enk *et al.*, 2006). Expression of VCAM-1 was unaffected by UVA and UVB radiation (Heckmann *et al.*, 1994), but IRA led to a significant downregulation of VCAM-1.

In addition, another six genes, which are regulated by IRA (ATP1B1, FASTK, ITPR2, ITPR3, PIK3R3, and PIP5K1B), have not been described to be differentially expressed in fibroblasts after UV exposure. It has to be pointed out that most of these studies examining the gene response of the skin or isolated skin cells to UV focused on UVB, and its effect on keratinocytes. Systematic work on the transcriptome-wide changes in gene expression of human dermal fibroblasts to UVA and UVB are not available.

Taken together, these results strongly indicate that the IRA response is specific in nature and can be clearly distinguished from UVB or UVA responses in human skin fibroblasts.

From a clinical point of view, these observations indicate that the biological effects and/or underlying signaling pathways induced by UVB/UVA and IRA differ, and that protection against IRA radiation requires specific strategies that are not present in conventional sunscreens (Schroeder *et al.*, 2008b; Krutmann and Schroeder, 2009).

MATERIALS AND METHODS

Chemicals

The compounds PD 98059, SB 203580, LY 294002, STAT3 inhibitor peptide, and SP600125 were obtained from Calbiochem Bioscience (La Jolla, CA). Cyclosporine A and *N*-acetylcysteine were obtained from Sigma-Aldrich (Taufkirchen, Germany). MitoQ was a kind gift from Michael P. Murphy (Cambridge, United Kingdom).

Cell culture

Human dermal fibroblasts were isolated from the foreskin of three different donors. Cells cultivated in Eagle's minimum essential medium with Earle's salts (MEM, PAA Laboratories, Pasching, Austria) were supplemented with 10% fetal bovine serum (Gibco, Karlsruhe, Germany), 1% antibiotics/antimycotics (penicillin, streptomycin, amphotericin B), and 1% glutamine (Gibco) and then cultivated on 100 mm plastic culture dishes (Greiner, Solingen, Germany) at 37 °C in humidified air with 5% CO_2. Cells were used between passages five and ten, grown to 100% confluency before treatment. At least 24 hours before IRA irradiation, media were changed to serum-free MEM.

Irradiation

We used a water-filtered Hydrosun 500 H 500 IRA lamp (Hydrosun Medizintechnik GmbH, Müllheim, Germany) with an emission in the range of 760–1400 nm, leading to an irradiance of 360 mW cm^{-2} at a distance of 20 cm measured through the Hydrosun HBM1 irradiance measuring device (Hydrosun). IRA irradiation lasted 40 minutes leading to a total dose of 860 J cm^{-2}. The culture dishes were placed on a cooled plate connected to a thermostated bath (Thermo Haake GmbH, Karlsruhe, Germany) to maintain temperatures below 37 °C during IRA irradiation. For irradiation, medium was replaced by phosphate-buffered saline (37 °C, Gibco). Control cells were held on a 37 °C thermostated plate under similar conditions without irradiation. Following treatment, cells were cultivated for 24 hours with serum-free MEM culture medium at 37 °C.

Gene expression analysis

RNA was extracted using the NucleoSpin RNA II kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. RNA quality and purity were assessed using an Agilent Bioanalyzer 2100 system (Agilent Technologies, Palo Alto, CA). The RNA was then used to synthesize first- and second-strand cDNA by using the Affymetrix One-Cycle cDNA Synthesis Kit (Affymetrix UK, High Wycombe, UK) with a subsequent cleanup with the GeneChip Sample Cleanup Kit (Affymetrix). The *in vitro* transcription into biotinylated cRNA was performed with the IVT Labeling Kit (Affymetrix). After purification, fragmentation and a further quality and purity control with the Agilent Bioanalyzer 2100 system using 9 μg of the labeled cRNA were hybridized on the Affymetrix HG-U133A Microarray according to the manufacturer's protocols.

Data Analysis

The raw and normalized data (normalization, IRA-regulated gene identification and clustering procedures are described in the Supplementary Material from all experiments) have been deposited in NCBI's Gene Expression Omnibus (GEO Series accession number GSE17046, http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE17046)

Real-time PCR measurements of relative mRNA levels

The real-time PCR experiments were performed as described in the Supplementary Material.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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