<table>
<thead>
<tr>
<th>Title</th>
<th>Category</th>
<th>DOI</th>
<th>Authors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Divide or unite—a novel molecular switch in endometrial carcinoma</td>
<td>Clinical Implications</td>
<td>10.1007/s00109-006-0118-5</td>
<td>Martin Götte</td>
</tr>
<tr>
<td>CaMKII, an emerging molecular driver for calcium homeostasis,</td>
<td>Review</td>
<td>10.1007/s00109-006-0125-6</td>
<td>Chad E. Grueter, Roger J. Colbran and Mark E. Anderson</td>
</tr>
<tr>
<td>Proliferation and cell–cell fusion of endometrial carcinoma are</td>
<td>Original Article</td>
<td>10.1007/s00109-006-0104-y</td>
<td>Reiner Strick, Sven Ackermann, Manuela Langbein, Justine Swiatek,</td>
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<tr>
<td>induced by the human endogenous retroviral Syncytin-1 and regulated</td>
<td></td>
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<td>Steffen W. Schubert, Said Hashemolhosseini, Thomas Koscheck,</td>
</tr>
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<td>by TGF-β</td>
<td></td>
<td></td>
<td>Peter A. Fasching, Ralf L. Schild, Matthias W. Beckmann and Pamela L.</td>
</tr>
<tr>
<td>Modification of gene expression of the small airway epithelium in</td>
<td>Original Article</td>
<td>10.1007/s00109-006-0103-z</td>
<td>Ben-Gary Harvey, Adriana Heguy, Philip L. Leopold, Brendan J. Carolan,</td>
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<td>response to cigarette smoking</td>
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<td>Barbara Ferris and Ronald G. Crystal</td>
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<td>CD8⁺ T cells armed with retrovirally transduced IFN-γ</td>
<td>Original Article</td>
<td>10.1007/s00109-006-0107-8</td>
<td>Christian Becker, Stefan Lienenklaus, Jadwiga Jablonska, Heike Bauer and Siegfried Weiss</td>
</tr>
<tr>
<td>Enhanced brain targeting efficiency of intranasally administered plasmid DNA: an alternative route for brain gene therapy</td>
<td>Original Article</td>
<td>10.1007/s00109-006-0114-9</td>
<td>In-Kwon Han, Mi Young Kim, Hyang-Min Byun, Tae Sun Hwang, Jung Mogg Kim, Kwang Woo Hwang, Tae Gwan Park, Woong-Won Jung, Taehoon Chun, Gil-Jae Jeong and Yu-Kyoung Oh</td>
</tr>
<tr>
<td>The roles of endogenous reactive oxygen species and nitric oxide in triptolide-induced apoptotic cell death in macrophages</td>
<td>Original Article</td>
<td>10.1007/s00109-006-0113-x</td>
<td>Xiaofeng Bao, Jun Cui, Yuanyuan Wu, Xiaodong Han, Cheng Gao, Zichun Hua and Pingping Shen</td>
</tr>
</tbody>
</table>
According to current estimates, about 7–8% of the human genome are thought to be derived from retroviral sequences, which have been incorporated into the germ line during evolution [1]. While most of these sequences are not functional due to inactivating mutations or deletions in protein-coding or regulatory regions, some retroviral elements have retained their biological activity. In fact, selected elements have become an integral part of the repertoire of the human body’s physiological functions. Among these retroviral elements is an envelope gene of the HERV-W retrovirus, which encodes the syncytin-1 protein as shown by Mi et al. [2]. Syncytin-1 is initially synthesized as an inactive precursor protein and subsequently processed by subtilisin-like proteases into its mature form composed of an extracellular cell surface domain and a transmembrane domain [2–5]. Syncytin-1 contributes to placental morphogenesis by mediating cell–cell fusion of cytotrophoblasts into syncytiotrophoblasts [2, 5].

The fusogenic activity of syncytin-1 is thought to be mediated through interactions of its extracellular SU domain with the receptor proteins ASCT1 and ASCT2, which were initially described as neutral amino acid transporter systems [3, 5]. The syncytin-2 protein is encoded by the envelope gene of the HERV-FRD endogenous retroviral element. It is expressed in the placenta, shows a hydrophobicity profile similar to syncytin-1, and appears to be fusogenic as well [3].

Syncytiotrophoblasts constitute the invasive front of the blastocyst, and this function has been frequently compared to invasive behavior of tumor cells. In this issue, Strick et al. [6] show that the expression of syncytin-1 mRNA and protein are significantly upregulated in endometrial carcinoma and its pre-stages compared to controls. Endometrial carcinoma is the most common malignancy of the female genital tract [7]. The vast majority of endometrial carcinomas expresses the estrogen receptor and progesterone receptor and is thought to develop from endometrial hyperplasia induced by steroid hormone stimulation [7]. In an elegant set of experiments, Strick et al. [6] demonstrate that syncytin-1 expression is regulated via an estrogen response element in this steroid driven tumor entity, resulting in increased proliferation of primary endometrial carcinoma cells and cell lines. It is of note that the activation of cAMP signaling by forskolin or SP-cAMP also resulted in syncytin-1 upregulation; however, it lead to increased cell–cell fusions instead of proliferation. Chang et al. [8] recently demonstrated forskolin-dependent activation of the transcription factor GCMa, a regulator of placental syncytin-1 expression, and analogous mechanisms may be involved in endometrial cancer. Strick et al. [6] furthermore showed that, after stimulation with steroids or cAMP activation, syncytin-1 is involved in anchorage-independent colony growth and colony fusion. The switch between cell proliferation and cell–cell fusion was shown to be subject to a complex regulatory mechanism involving both steroid hormone- and TGF-β1/TGF-β3-dependent effects. The authors’ findings raise the possibility that syncytins play an important role in the progression of hormone-dependent cancers such as endometrial carcinoma.

In healthy individuals, syncytin-1 expression is usually restricted to the placenta, and it has been suggested that CpG hypomethylation of 5′-LTRs of both syncytin genes is one of the underlying regulatory mechanisms [9]. CpG hypomethylation also appears to be a means of activating syncytin-1 expression in ovarian carcinoma [10]. Most recently, a role for
syncytin-1 in breast cancer was revealed by Bjerrøgaard et al. [11]. Using reverse transcriptase polymerase chain reaction (RT-PCR) and immunohistochemistry, they could demonstrate expression of syncytin-1 in breast cancer cell lines and in 38% of 165 investigated nodal negative ductal breast cancer cases. It is noteworthy that a specific involvement of syncytin-1 in mediating cancer cell and cancer cell–endothelial cell fusion was shown employing antisense RNA and an inhibitory peptide. While the results of this study and the work by Strick et al. [6] imply a direct role for syncytin-1 in mediating cell–cell fusion of carcinoma cells, the pathophysiological consequences are still a matter of debate. Fusions between cancer and untransformed host cells result in aneuploid hybrids, which contain a mixture of the parental chromosomes. Clearly, the fusion event can be expected to lead to profound changes in tumor cell properties and their biological behavior. Studies dating back to the 1960s and 1970s showed that tumor–host cell fusions can result in a suppression of tumorigenicity through the activation of tumor suppressor genes provided by the noncancerous cell (reviewed in [12]). Moreover, it was suggested that sorting and repair of mutant genomic DNA may contribute to a loss of tumorigenicity in the hybrid cells [12]. However, in some instances, tumor cell hybrids are even more aggressive than their parental cells. If fusion of cell nuclei occurs, the genomic instability may promote aberrant sorting and recombination of chromosomes and, consequently, malignant behavior. These events may contribute to the activation of oncogenes such as ras, myc, β-catenin, and ERBB2, or to the inactivation of tumor suppressors such as DNA mismatch repair genes and PTEN, as observed in endometrial carcinoma [7]. Moreover, fusion of cancer stem cells with normal host cells may result in a reprogramming of the normal cell and increased growth rates, as well as resistance to drugs and apoptosis. Since syncytin is upregulated in endometrial carcinoma, the results by Strick et al. suggest that syncytin-1-mediated cell fusions may promote, rather than suppress, tumor growth. Moreover, they demonstrate that syncytin-1 expression and function are highly dependent on hormonal regulation: steroid hormones such as estradiol (E2) induce the expression of syncytin-1 and result in increased cell proliferation and anchorage-independent growth. On the other hand, activation of cAMP signaling promotes both endometrial carcinoma cell fusion and anchorage-independent growth; however, cell fusion can be blocked by TGF-β1/3, which is subject to steroid hormone regulation itself (Fig. 1). Thus, in the presence of steroid hormones, TGF-β provides a switch between syncytin-1-mediated cell fusion and cell proliferation.

Estrogen is a key etiological factor for endometrial carcinogenesis [7]. This entails several problems encountered in a clinical setting: postmenopausal hormone therapy with unopposed estrogens significantly increases endometrial carcinoma risk [13]. Therefore, estrogen needs to be combined with progestin treatment. Selective estrogen receptor modulators such as tamoxifen and raloxifene, which have been used successfully in the treatment of estrogen receptor-positive breast cancer, have also been implicated in the pathogenesis of endometrial carcinoma [7]. Besides surgery and radiation therapy, cytotoxic drugs such as carboplatin/paclitaxel or adriamycin/cisplatin are used to treat endometrial carcinoma patients [13]. In the palliative setting, novel endocrine strategies involving, for example, the antiestrogen fulvestrant, are currently evaluated in addition to the well-established progestagen therapy. Aromatase and steroid sulfatase may convert steroid precursors and metabolites into active estrogens, which can promote endometrial carcinoma. However, aromatase inhibitors have not yet been proven successful in the treatment of endometrial carcinoma. The regulation by TGF-β and the involvement of syncytin-1 add a new dimension to the understanding of endometrial carcinoma progression. These findings show potential new directions for therapeutic approaches, which circumvent the need for potentially risky endocrinological interventions. Several reports have described that a disruption of TGF-β signaling promotes endometrial carcinoma progression, which is in line with the regulatory mechanism uncovered by Strick et al. [6]. Parekh et al. [14] observed that, in contrast to primary endometrial epi-

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**Fig. 1** Schematic representation of the molecular switch steering the fate of endometrial carcinoma cells as reported by Strick et al. [6]
there is a need to evaluate the contribution of cell fusion to the malignant phenotype of endometrial carcinoma cells first to rule out that expression of syncytin is a mere physiological response of the host to the tumor. Such a response could, for example, aim at neutralizing malignant cells via fusion with normal cells expressing tumor-suppressor genes [12]. Besides laboratory approaches, clinical studies correlating syncytin-1 expression in endometrial carcinoma with histopathological and clinicopathological parameters—most notably with patient survival—should help to assess the relevance of this potential novel molecular target.

Martin Götte

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15. Sakaguchi J, Kyo S, Kanaya T, Maida Y, Hashimoto M, Nakamura M, Yamada K, Inoue M (2005) Aberrant expression and intracellular distribution of the encoded receptor protein were recently also noted by Sakaguchi et al. [15] in the endometroid subtype of endometrial carcinoma upon investigation of 27 patient tissues by real-time PCR and mutational analysis. Furthermore, a disruption of TGF-β signaling in endometrial carcinoma, affecting TGFR-II and SMAD4 expression and intracellular distribution of Smads, was observed by Piestrzeniewicz-Ulanska et al. [16]. However, they found an increased expression of TGFR-II protein in infiltrating tumors when compared with non-infiltrating tumors.

The identification of syncytin-1 as a target of TGF-β action in endometrial carcinoma does not only provide novel insight into the molecular mechanism of tumor progression; in addition, these findings may also have important clinical implications. A peptide capable of inhibiting syncytin-mediated cell–cell fusions was recently shown to reduce breast carcinoma cell fusion with human umbilical vein endothelial cells by 50% ($p<0.001$) [11]. While this inhibitor has only been tested in an experimental setting and its toxicity profile has not been evaluated yet, the use of this peptide or of related reagents may be of beneficial effect in the treatment of endometrial and, possibly, other carcinomas. However, the identification of syncytin-1 as a target of TGF-β action in endometrial carcinoma does not only provide novel insight into the molecular mechanism of tumor progression; in addition, these findings may also have important clinical implications. A peptide capable of inhibiting syncytin-mediated cell–cell fusions was recently shown to reduce breast carcinoma cell fusion with human umbilical vein endothelial cells by 50% ($p<0.001$) [11]. While this inhibitor has only been tested in an experimental setting and its toxicity profile has not been evaluated yet, the use of this peptide or of related reagents may be of beneficial effect in the treatment of endometrial and, possibly, other carcinomas. However, this peptide or of related reagents may be of beneficial effect in the treatment of endometrial and, possibly, other carcinomas. However,
CaMKII, an emerging molecular driver for calcium homeostasis, arrhythmias, and cardiac dysfunction

Chad E. Grueter • Roger J. Colbran • Mark E. Anderson

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Abstract Maintenance of cytoplasmic calcium homeostasis is critical for all cells. An exciting field has emerged in elucidating the multiple roles that Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII) plays in regulating Ca\(^{2+}\) cycling in normal cardiac myocytes and in pathophysiological states. Moreover, CaMKII was recently identified as a potential drug target in cardiac disease. This work has given us a closer view of the complexity and therapeutic possibilities of CaMKII regulation of Ca\(^{2+}\) signaling in cardiac myocytes.

Keywords Ca handling • Heart disease • Calmodulin-dependent protein kinase II • Excitation–contraction coupling

Introduction

Calcium (Ca\(^{2+}\)) is a charge carrier and universal mediator of diverse cellular processes. In cardiac myocytes these processes include excitation–contraction coupling, gene transcription, and apoptosis. Thus, cytoplasmic calcium [Ca\(^{2+}\)]\(_i\) operates core functions ranging from contraction that is required for all vertebrate life to programmed cell death. Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII) is one of many specialized proteins poised to respond to Ca\(^{2+}\) signaling in myocytes. Growing evidence has linked CaMKII signaling events to normal and pathological con-
conditions in the heart. In this review, we will focus on the recent advances in understanding CaMKII as a molecular determinant for maintaining [Ca$^{2+}$]$_i$ homeostasis in normal and diseased cardiac myocytes.

CaMKII is a multifunctional kinase that can phosphorylate multiple target proteins sharing consensus motifs containing serine or threonine. CaMKII is able to integrate changes in Ca$^{2+}$ cycling at multiple cell membrane-delimited protein targets. This includes regulation of Ca$^{2+}$ entry into the cell through L-type Ca$^{2+}$ channels (LTCC), Ca release from the intracellular sarcoplasmic reticulum (SR) stores through ryanodine receptors (RyR) and by SR [Ca$^{2+}$], uptake through the sarcoplasmic/endoplasmic reticulum Ca$^{2+}$-ATPase (SERCA).

CaMKII is a downstream effector of multiple signaling pathways activated under physiological and pathophysiological conditions (Fig. 1). CaMKII expression and activity are upregulated in structural heart disease [1] (Fig. 2). Multiple model systems were utilized to study the role of CaMKII in cardiac disease, including transgenic mouse models overexpressing CaMKIIα-B [2], CaMKIIαC [3, 4], and CaMKIV [5] and transgenic mouse models with universal [6] and targeted [7, 8] chronic CaMK inhibition. The prominent role of CaMKII in heart disease makes it an attractive candidate for targeted therapy.

**CaMKII structure determines function**

CaMKII is a dodecameric holoenzyme. Four genes encode CaMKII subunits (α, β, γ, and δ), but only γ and δ were detected in the heart [9, 10]. The catalytic and regulatory domains of all four gene products are about 90% identical. Upon activation the catalytic domain transfers the γ phosphate from ATP to a serine/threonine within the substrate. The regulatory domain binds Ca$^{2+}$/CaM and contains multiple phosphorylation sites, including threonine 287. The variable region of CaMKII links the association domain to the catalytic and regulatory domains and is responsible for a majority of the differences between splice variants (Fig. 3). The association domain binds other CaMKII association domains [11], allowing for assembly of the dodecameric holoenzyme. Variable splicing allows for distinct localization of CaMKII to specific compartments, thus enhancing specificity [12, 13]. CaMKIIδα was demonstrated to be the predominant isoform in mammalian heart [14]. According to studies by Hagemann et al. [10], δB (also called δ3) predominates in the adult heart while δC (δ2) is abundant in the embryonic heart. The δB isoform has a nuclear localization sequence in the hypervariable domain, located at the N-terminal region of the association domain that directs δB to the nucleus. CaMKIIδC lacks this sequence and so is predominantly resident in cytoplasm. Heteromultimeric complexes form between the different CaMKII isoforms and splice variants. The localization of the holoenzyme complex is determined “democratically” according to whether the majority of monomers express or lack a nuclear localization sequence [12].

In the absence of Ca$^{2+}$, parts of the regulatory domain bind to the catalytic domain, occluding the binding of nucleotides and protein substrates. Ca$^{2+}$/CaM binds to the regulatory domain, presumably causing a change in protein conformation [15]. Ca$^{2+}$/CaM activation of CaMKII occurs to different degrees depending upon the frequency, amplitude, and duration of [Ca$^{2+}$], transients [16]. Threonine 287 within the autoregulatory domain of CaMKII is a critical phosphorylation site. Transautophosphorylation of threonine 287 requires activation of two adjacent monomers (Fig. 3). Phosphorylation of CaMKIIα at threonine 286 enhances the binding affinity for calmodulin by 1,000-fold from nanomolar to picomolar [17]. Phosphorylation at threonine 287 also confers 20–80% Ca$^{2+}$/CaM-independent activity, depending on the experimental conditions [17, 18]. The ability of CaMKII to sustain its activity through autophosphorylation, even in the absence of elevated Ca$^{2+}$/CaM, confers remarkable flexibility for extending CaMKII-dependent regulation over time. Thus, CaMKII is well configured to integrate and regulate [Ca$^{2+}$], and to “connect” these transients to [Ca$^{2+}$]-dependent transcriptional tasks that are important for production and maintenance of the contractile apparatus in cardiac myocytes.

**CaMKII and Ca$^{2+}$ homeostasis**

[Ca$^{2+}$], can simultaneously regulate multiple diverse physiological processes requiring the precise regulation of local and global [Ca$^{2+}$]$_i$ concentrations. This is achieved by grading Ca$^{2+}$ influx through the plasma membrane, modulating Ca$^{2+}$ release and reuptake into the SR, Ca$^{2+}$ efflux
through Ca$^{2+}$ pumps and exchangers, and Ca$^{2+}$ buffering by a multitude of Ca$^{2+}$ binding proteins [19, 20]. Well established functions of Ca$^{2+}$ in cardiac myocytes include enzyme activation [18], regulation of gene transcription [21], and activation of signaling molecules such as CaM [22–24]. CaMKII is a key CaM-dependent regulator of cardiac Ca$^{2+}$ cycling [18, 25]. Alterations in Ca$^{2+}$ transients in cardiac myocytes lead to disease states, including arrhythmias and apoptosis [26–29].

CaMKII localization

In addition to the direct regulation of enzymatic activity by Ca$^{2+}$/CaM activation and autophosphorylation, CaMKII appears to be dynamically targeted to its substrate in diverse subcellular compartments. Recent studies have demonstrated direct interactions between CaMKII and the RyR [30], the inositol 1,4,5-triphosphate receptor (IP$_3$R) [31], as well as the $\beta_{2a}$ and $\alpha_{1c}$ subunits of the LTCC complex [32, 33]. Indirect evidence also suggests that a localized pool of CaMKII exists at the longitudinal SR that regulates Ca$^{2+}$ uptake into the SR [7, 8]. Unlike protein kinase A (PKA), which is targeted to specific microdomains by scaffolding proteins known as A-kinase anchoring proteins [34], studies involving CaMKII localization suggest that CaMKII is targeted by direct interaction with the signaling complex. The mechanisms for CaMKII interactions also vary depending on the binding partner. For example, CaMKII interacts with a sequence within the LTCC $\beta_{2a}$ subunit resembling the CaMKII regulatory domain upon activation of the kinase [32]. Thus CaMKII is poised to respond to changes in local Ca$^{2+}$ events.

CaMKII regulation of LTCC

Calcium channels are uniquely regulated by [Ca$^{2+}$], whereas sodium or potassium channels are not known to have biological feedback mechanisms to regulate the concentration of permeant ions in the cytoplasmic or extracellular domains, the predominant cardiac Ca$^{2+}$ channel (CaV1.2) is highly responsive to [Ca$^{2+}$], signals for feedback control. Our laboratory [25, 36–40] and others [22, 23, 33] have demonstrated an important role for Ca$^{2+}$, CaM, and CaMKII as feedback mechanisms for LTCC regulation of cardiac contraction and arrhythmias in model systems [36–38, 41]. Ca$^{2+}$-dependent facilitation of $I_{Ca}$ (enhanced peak $I_{Ca}$ and decreased inactivation) was first described in 1982 by Marban and Tsien. CaMKII was first implicated in $I_{Ca}$ facilitation in smooth muscle [42] and later three labs found CaMK inhibitors prevented $I_{Ca}$ facilitation in cardiac myocytes [43–45]. Dzhura et al.
cardiac myocytes enhances RyR Ca²⁺ release while blocking CaMK inhibitor peptide AC3I in acutely isolated rabbit cardiac myocytes. Endogenous CaMKII is associated with purified RyR, then later identified as the β and α subunits, respectively, PKA, PKC, PKG, and casein kinase II all phosphorylate both the α₁ and β subunits but not the α₂δ or γ subunits (this work was done before the function of the auxiliary subunits was known) [52]. We recently showed that CaMKII regulation of single LTCCs requires the β subunit when transiently expressed in heterologous cells and that mutating β₂₅ threonine 498 to alanine ablates CaMKII-dependent regulation of LTCC in heterologous cells and in primary adult cardiomyocytes [35]. Other groups have demonstrated an α₁c-dependent mechanism for CaMKII regulation of I_{Ca} in oocytes and voltage-dependent facilitation [33, 53]. Together these reports suggest multiple modes for CaMKII actions at the LTCC complex.

RyR

The second phase of the Ca²⁺ cycle, Ca²⁺ release from internal stores, occurs via Ca²⁺ activation of the RyR. The RyR is a signaling complex with four RyR subunits forming the pore of the channel and a multitude of binding proteins. Included in this constellation of associated proteins are CaM and CaMKII [54]. Depending on the experimental conditions, CaMKII was shown to both increase and decrease Ca²⁺ release via RyR [25, 55, 56]. Endogenous CaMKII is associated with purified RyR, then reconstituted in lipid bilayers, decreasing the channel open probability [30]. Consistent with this effect, the use of CaMK inhibitor peptide AC3I in acutely isolated rabbit cardiac myocytes enhances RyR Ca²⁺ release while blocking CaMK-dependent I_{Ca} facilitation, thus enhancing the gain of excitation–contraction coupling. Addition of a constitutively active CaMKII (over a period of minutes) yielded the opposite result suggesting that CaMK can act as a functional link between LTCC and RyR during excitation–contraction coupling [25]. In contrast, acute (24 h) over-expression of CaMKII in cultured cardiac myocytes by viral transduction enhances the phosphorylation of RyR at both Ser2815 (CaMKII site) and 2809 (also a PKA site) and also increases in Ca²⁺ sparks [57]. Chronic overexpression of CaMKII-c results in altered Ca²⁺ handling with severe cardiac hypertrophy. Enhanced Ca²⁺ sparks from the RyR and reduced SR content were both observed. This could be a direct result of CaMKII; however, the chronic over-expression of CaMKII also leads to changes in the protein expression level of key Ca²⁺ cycling proteins such as a decrease in sarcoplasmic/endoplasmic reticulum Ca²⁺ ATPase (SERCA2) and phospholamban (PLB) expression coupled with an increase in the Na/Ca²⁺ exchanger [3, 4]. Thus, although it is clear that CaMKII is an important regulator of SR Ca²⁺ release, the details of how this process works mechanistically remain to be fully elucidated.

SERCA/PLB

Ca²⁺ reuptake into the SR occurs via SERCA on the longitudinal SR. SERCA was proposed to be directly regulated by CaMKII [58]. Another evidence suggests that CaMKII can regulate the frequency dependence of relaxation in the absence of PLB [59]. However, most studies have focused on SERCA regulation by PLB. SERCA is negatively regulated by PLB [60]. Upon phosphorylation of PLB by PKA [61] or by CaMKII, the inhibition of SERCA is removed, allowing for regulated uptake of cytosolic Ca²⁺ back into the SR [60, 62, 63]. Threonine 17 (CaMKII site) phosphorylation can occur independently of serine 16 (PKA site) phosphorylation in vitro and threonine 17 phosphorylation is directly enhanced by increasing stimulation frequency [64]. However, physiologica increases in threonine 17 phosphorylation probably follow catecholamine-stimulated increases in chronotropy and inotropy [65]. Thus, threonine 17 phosphorylation potentially correlates with an adaptive response to sudden changes in heart rate. Chronic CaMKII inhibition reduces the variability of Ca²⁺-induced Ca²⁺ release in cardiac myocytes by regulation of both SR Ca²⁺ release from stores and Ca²⁺ reuptake by SERCA [35]. As a result of CaMKII inhibition, PLB threonine 17 phosphorylation is significantly reduced [35]. Transgenic mice with targeted inhibition of CaMKII at the longitudinal SR results in a decrease in PLB threonine 17 phosphorylation. These mice exhibit dilated heart failure when stressed by gestation and parturition [7], but this result should be interpreted cautiously because the targeting approach requires over-expression of protein in the SR membrane and no model has yet been developed to control for potential side effects of this strategy. Further studies in these mice also suggest that CaMKII activity at the longitudinal SR contributes to cardiac contractility and Ca²⁺ handling. The rate of contraction and relaxation in whole heart and in isolated myocytes is decreased [8].
IP3R

The IP3R is a Ca2⁺ channel activated by IP3 and Ca2⁺. It is localized on intracellular membranes, the nuclear membrane [66], and SR in neonatal cardiomyocytes [67] and forms a tetrameric complex similar to the RyR. The expression of the receptor complex in cardiomyocytes is about 50-fold lower than RyR. IP3R2 is the predominant expression of the receptor complex in cardiomyocytes. The IP3R and the IP3R is a CaMKII substrate [31]. IP3R is activated by IP3 and Ca2⁺. It is implicated in activation of a fetal gene program via an interpreter and a modulator of local Ca2⁺ concentrations [81]. During the cardiac Ca2⁺ cycle local [Ca2⁺], range from ~0.1 μM at diastole to more than 100 μM in the microdomain of the LTCC and RyR during systole [76]. It was demonstrated that CaMKII has a tonic level of activity under basal conditions and activated (threonine 287 autophosphorylated) CaMKII localizes to the sarcolemma with a T-tubule staining pattern [45]. The major questions remaining include: Under what physiological or pathological conditions is CaMKII activated and what is its function within specific pathways (Fig. 1)? Many studies have begun to address these questions. These include experiments showing that increased frequency and action potential duration both enhance CaMKII activity [16, 77, 78].

CaMKII regulation of MEF2 activity

Ca2⁺ signaling requires precise mechanisms to discriminate between changes in local Ca2⁺ concentrations. CaMK was implicated in activation of a fetal gene program via myocyte enhancer factor 2 (MEF2) signaling [5, 72]. MEF2 regulates structural genes and genes involved in growth, stress response, and apoptosis. Mice overexpressing CaMKIV have a 100-fold increase in MEF2 activity when interbred with transgenic mice expressing a MEF2 sensor [5]. CaMKII regulates gene expression; one example of CaMKII regulation of MEF2 transcription activity involves CaMKII interaction with class II HDACs. HDAC4 and 5 are transcriptional repressors that are targeted by multiple serine/threonine kinases including CaMKII at two conserved serine residues. Phosphorylation of HDACs at these serines creates a binding site for 14-3-3 protein that occludes a nuclear retention signal to favor movement of HDAC to the cytoplasm. Because HDAC is a MEF2 repressor, prevention of HDAC binding with or without export to the cytoplasm results in enhanced MEF2 activity [73, 74]. Specifically, CaMKII binding to and signaling via HDAC4 results in hypertrophy [75]. Thus CaMKII acts as an interpreter and a modulator of local Ca2⁺ concentrations that regulate long-term changes in cardiac myocyte gene expression.

Pathways mediating CaMKII activation

During the cardiac Ca2⁺, cycle local [Ca2⁺], range from ~0.1 μM at diastole to more than 100 μM in the microdomain of the LTCC and RyR during systole [76]. It was demonstrated that CaMKII has a tonic level of activity under basal conditions and activated (threonine 287 autophosphorylated) CaMKII localizes to the sarcolemma with a T-tubule staining pattern [45]. The major questions remaining include: Under what physiological or pathological conditions is CaMKII activated and what is its function within specific pathways (Fig. 1)? Many studies have begun to address these questions. These include experiments showing that increased frequency and action potential duration both enhance CaMKII activity [16, 77, 78].

The β-adrenergic system is the most prominent regulator of cardiac function and “β-blockers” are the major therapy for treating patients with structural heart disease and myocardial dysfunction [79, 80]. The traditional pathway mediating β-adrenergic signaling involves activation of Gs-protein–coupled receptor. Activation of Gs leads to activation of adenyl cyclase, enhancing cAMP production and ultimately activation of PKA. PKA targets many of the same proteins in the Ca2⁺ cycle such as CaMKII leading to enhanced Ca2⁺ mobilization. Recent studies revealed other means of β-adrenergic signaling via CaMKII [6, 28, 81]. Our recent findings support the hypothesis that CaMKII is a key downstream effector of the β-adrenergic receptor signaling cascade. It is intriguing that CaMKII inhibition does not appear to affect the “fight or flight” responses to βAR activation. However, transgenic overexpression of AC3I, a CaMKII inhibitory peptide, mitigates the deleterious impact of myocardial infarction on left ventricular function. Chronic inhibition of CaMKII in AC3I mice and acute inhibition using KN93 also protects from cardiomyopathic responses to chronic βAR stimulation. These studies show that CaMKII activity directly contributes to loss of Ca2⁺ homeostasis in two cardiac disease models associated with βAR activation. Zhu et al. studied the effects of β₁ adrenergic activation regulating apoptosis and found that activation of CaMKII, independent of PKA, is responsible for increased programmed cell death during excessive isoproterenol treatment in vitro. They found that CaMKII activation was required for this response. Subsequent findings support CaMKII activation upon β₁AR stimulation as an integral part of the enhanced cardiac contractility [81]. Our group found that CaMKII inhibition protected against apoptosis in vivo during myocardial infarction or excessive β₁AR stimulation with isoproterenol.
These studies suggested that the proapoptotic actions of CaMKII were related to its regulation of SR Ca\(^{2+}\) content because the benefits of CaMKII inhibition for reducing apoptosis were lost when chronic CaMKII inhibitor mice were bred into a PLB null background with SR Ca\(^{2+}\) overload.

The α-adrenergic signaling cascade activates CaMKII by a mechanism requiring PKC activation. The functional outcome of α-adrenergic stimulation of CaMKII is enhanced I\(_{\text{Ca}}\) from LTCC and a change in activated CaMKII localization from the plasmalemma to T-tubules where LTCC are more densely expressed [82]. Activation of the α-adrenergic system also turns on genes involved in cardiac hypertrophy, which is prevented by CaMKII inhibition [13]. This suggests that CaMKII activation by the α-adrenergic system could potentially regulate local [Ca\(^{2+}\)]\(_i\) signaling and global gene expression.

### CaMKII as a signal in structural heart disease: fulfilling Koch’s postulates

Robert Koch established a systematic guideline in the 1890s to identify a microorganism as the source of a disease [83]. Simply stated, the microorganism must be found in all cases of the disease. It must be isolated from the host and grown in pure culture. It must reproduce the original disease when introduced into a susceptible host and must be found in the experimental host so infected [84]. Koch’s postulates can be adapted and partially applied to structural heart disease [84]. Molecules fulfilling Koch’s postulates would first need to have altered expression or be misregulated in disease. In the case of CaMKII, knocking out the protein or inhibiting its activity would be protective against the disease. Third, reintroducing an excess or constitutively active mutant would lead to the disease in a previously normal tissue. Finally, over activity of CaMKII would then be verified in the host tissue. Much work was published implicating CaMKII as an integral part of many forms of cardiomyopathy in human and animal models.

CaMKII expression and activity are increased in patients [1, 41] and animal models [38, 85] of heart disease. Transgenic mice overexpressing CaMKII\(_c\) develop dilated cardiomyopathy and sudden death [4]. According to Koch’s postulates, the cause of the disease must be isolated from the experimental host. To isolate CaMK as a significant focal point in cardiomyopathy, a transgenic mouse was engineered with a CaMK inhibitory peptide. These mice exhibit a significant cardioprotective effect after myocardial infarction and chronic βAR stimulation [6]. Transgenic mice overexpressing CaMKIV in a heart have severe cardiomyopathy; in addition, constitutively active CaMKII “restores” I\(_{\text{Ca}}\) facilitation [86]. While Koch’s postulates for infectious disease do not provide a completely parallel analysis for the study of structural heart disease, it is becoming increasingly clear that CaMKII is a critical cardiomyopathic signal in structural heart disease in patients and in numerous animal models of cardiomyopathy [87].

### CaMKII in structural heart disease

Structural heart disease is characterized by (1) electrical instability and arrhythmias, (2) myocardial dysfunction, and (3) myocardial hypertrophy and chamber dilation [88]. One possibility is that targeted inhibition of CaMKII could improve the fundamental changes in structural heart disease [89].

Action potentials are the result of a highly coordinated series of ionic currents through selective ion channel complexes in response to an electrical stimulus [20]. The cardiac action potential is unique in that it has a prolonged depolarized state termed the plateau phase where Ca\(^{2+}\)-ion influx is balanced by potassium ion efflux. Electrical changes are seen in heart disease due to remodeling of Ca signaling proteins and other ion transport proteins. In heart disease, CaMKII protein and activity levels are increased along with changes in ion channel expression. The changes in proteins involved in electrical propagation and Ca\(^{2+}\) cycling may result in an increase in the probability of developing arrhythmias [27, 90]. In addition, structural heart disease creates a substrate that favors arrhythmias. The formation of a physical barrier for electrical conduction along with remodeling of key Ca\(^{2+}\)-regulatory proteins and increase neurohormonal stimulation combine to provide favorable circumstances for arrhythmias to occur. These include action potential prolongation (electrical remodeling) and prolongation of the [Ca\(^{2+}\)] transient ("[Ca\(^{2+}\)]\(_i\)" remodeling).

Due to the high electrical resistance of the cell membrane at the plateau phase of the cardiac action potential, slight changes in I\(_{\text{Ca}}\) or I\(_k\) lead to dramatic changes in the action potential, including early afterdepolarizations [91]. Chronic CaMKII overexpression leads to electrical remodeling and increased susceptibility to sudden death [3] and CaMKII is proarrhythmic in various models of action potential prolongation in structurally normal hearts and isolated cardiomyocytes [77, 78, 92–94]. CaMKII actions at LTCCs is particularly important under voltage and SR Ca\(^{2+}\) release conditions present at the action potential plateau [95]. Enhanced RyR Ca\(^{2+}\) leak or general increases in [Ca\(^{2+}\)]\(_i\) due to altered CaMKII activity can lead to increased Na/Ca\(^{2+}\) exchanger activity, thus enhancing the probability of producing delayed afterdepolarizations. CaMKII inhibition reduces the Na/Ca\(^{2+}\) exchanger current.
(also called the transient inward current) by reducing SR Ca\(^{2+}\) release under conditions of cellular Ca\(^{2+}\) overload [78]. Because CaMKII can enhance I\(_{\text{Ca}}\), it was thought to potentially play a role in mediating early afterdepolarizations. Indeed, in studies where early afterdepolarizations were induced, CaMKII inhibition significantly decreased the occurrence of early afterdepolarizations [78, 86]. Taken together, these diverse mechanisms for CaMKII signaling in heart disease conspire to build proarrhythmic inward current under conditions of adverse electrical and Ca\(^{2+}\) remodeling that are a fundamental characteristic of structural heart disease.

Other triggers for arrhythmias are neurohormonal activation of signaling pathways such as the β-adrenergic pathway, which leads to increased heart rate and frequency. Activation of this pathway also recruits CaMKII and enhances Ca\(^{2+}\) cycling [6, 28]. These findings suggest to us that the efficacy of βAR antagonist drugs (β-blockers) in preventing sudden cardiac death may be in part related to their inhibition of CaMKII activity.

### Strategies for targeting CaMKII

Protein kinases are second only to G-protein-coupled receptors as therapeutic drug targets. Most kinase inhibitors, however, target the ATP-binding domain, limiting the identification of selective inhibitors for specific kinases [96]. It is important to note that the structural information that is steadily becoming available for many kinases provides useful clues for development of more specific inhibitors. Among the recently crystallized structures is a structure of the regulatory and catalytic domains of CaMKII [15]. This new information suggests that specific inhibitory agents could be developed by allosterically altering the ATP-binding pocket. As pointed out by Cohen [96], critical studies that remain are a detailed analysis of inhibitors and their effects on catalytic and regulatory properties.

CaMKII is emerging as an attractive candidate for drug therapy in structural heart disease and arrhythmias. However, there are many caveats to targeting this multifunctional kinase. Among the many known roles for CaMKII is gene regulation and molecular memory [97, 98]. New studies of CaMKII target proteins are providing evidence for the mechanism of CaMKII action. An attractive model may be to target CaMKII substrates that are critical components of Ca\(^{2+}\) cycling, thus taking a more specific approach to regulating CaMKII in Ca\(^{2+}\) signaling.

CaMKII inhibitors such as KN62 and KN93, whose mode of action is to bind to the Ca\(^{2+}\)/CaM domain and inhibit the CaMKII activation, have provided useful information on the function of CaMKII in cardiac myocytes. However, these data should be interpreted with care because of the effects of these drugs on multiple ion channels [77]. More selective CaMKII inhibitors are needed.

Endogenous inhibitors of CaMKII were identified: The kinase itself contains an autoregulatory domain that reversibly binds to the catalytic domain and inhibits the kinase (as discussed previously). A brain-specific protein CaMKIIN\(\beta\) was identified by yeast-2-hybrid and found to selectively inhibit CaMKII [99]. Another endogenous inhibitor of CaMKII is the NR2B subunit of the NMDA receptor (a glutamatergic ligand-gated ion channel). It contains a motif mimicking the autoregulatory domain of CaMKII that directly interacts with the kinase [100–104] and this interaction inhibits CaMKII activity in vitro. Zhang et al. [6] showed marked protection of cardiac function in a structural and neurohormonal model by chronic inhibition of CaMK throughout the cell. Some studies were done looking at the effect of inhibition of localized pools of CaMKII; however, more work is needed [7, 8].

The use of viral-mediated transduction of specific gene products or mutants has become a valuable tool for identifying the role of CaMKII target proteins in vivo [35, 57, 105, 106]. Work has been done demonstrating the possibility of injecting virus directly into live animals and studying the effect on whole heart physiology [107]. Viral approaches provide an alternative approach to production of transgenic mice and are more easily used to study effects in other animal models such as rabbits, whose cardiac physiology is more closely related to humans.

### Closing remarks

In cardiac disease it was well documented that there is an underlying mishandling of Ca\(^{2+}\). Whether alterations in Ca\(^{2+}\) cycling is a cause or effect of the disease is still under investigation. It is important to note that key Ca\(^{2+}\) signaling molecules such as the multifunctional CaMKII are emerging as focal points for studying cardiac disease. CaMKII was identified as a determinant for the severity of the outcome of a structural and neurohormonal model of cardiomyopathy with inhibition of CaMKII having a significant functional benefit. Still, further studies are needed to identify the mechanisms for CaMKII regulation of the proteins regulating Ca\(^{2+}\) cycling and the contribution of these points to cardiac disease. Increased effort to develop novel clinically relevant strategies for CaMKII inhibition are clearly warranted.

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Pigment epithelium-derived factor as a multifunctional antitumor factor

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Abstract The design of new therapeutic strategies for cancer treatment is based on the combination of drugs directed against different tumor compartments, including the tumor cells themselves and components of the stroma, such as the tumor vasculature. Indeed, several antiangiogenic compounds have entered clinical trials for use alone or in combination with conventional cytotoxic drugs. Pigment epithelium-derived factor (PEDF) is a multifunctional natural peptide with complex neurotrophic, neuroprotective, antiangiogenic, and proapoptotic biological activities, any of which could potentially be exploited for therapeutic purposes. This review summarizes recent studies that reveal the antitumor potential of PEDF based on its antiangiogenic properties and its newly discovered direct antitumor effects, which involve the induction of differentiation or apoptosis in tumor cells. We also discuss possible therapeutic applications of PEDF, based on these mechanistic insights and on the identification of functional domains that retain specific biological activities.

Keywords Pigment epithelium-derived factor · Tumor angiogenesis · Antiangiogenic factors · New cancer therapeutics

Abbreviations
PEDF pigment epithelium-derived factor
TSP-1 thrombospondin-1
VEGF vascular endothelial growth factor
bFGF basic-fibroblast growth factor
NFATc2 nuclear factor of activated T cells
c-FLIP cellular-FLICE-like inhibitory protein
VEGF-R1 vascular endothelial growth factor receptor-1
NF-κB  nuclear factor-κB  
JNK  c-Jun NH2-terminal kinase  
MMP-9  matrix metalloproteinase-9  
Ad-PEDF  adenovirus-PEDF  

**Introduction**

Pigment epithelium-derived factor (PEDF), or early population doubling level cDNA-1, is a 50-kDa secreted glycoprotein that was first identified as a neurotrophic factor purified from the conditioned medium of cultured retinal pigment epithelial cells [1]. It was later described as a marker that distinguishes young cultured fibroblasts from senescent ones [2]. Ten years later, PEDF emerged as one of the most potent natural inhibitors of both physiological senescent ones [2]. Ten years later, PEDF emerged as one of the most potent natural inhibitors of both physiological and pathological ocular vascularization [3].

Numerous studies have shown that tumor vascularization is essential for cancer progression and have demonstrated the efficacy of antiangiogenic compounds at halting tumor growth in animal models. Despite extensive efforts to replenish the arsenal of antiangiogenic drugs, the results of clinical trials of their use as single therapy point to the urgent need for more effective agents. Thus, the development of endogenous inhibitors of tumor angiogenesis into therapeutic agents remains both promising and challenging. In the past 5 years, a number of studies have evaluated the potential use of PEDF’s antiangiogenic properties in the treatment of cancer and the role that this factor may play in human disease. In this review we bring into focus the complex, multifunctional nature of PEDF, especially its disparate effects on distinct cell types, and highlight how these effects translate in potent activity against newly arising and established tumors.

**Indirect antitumor action: PEDF as an inhibitor of tumor angiogenesis**

PEDF exerts specific biological effects on endothelial cells. It inhibits endothelial cell proliferation and migration and induces apoptosis in activated endothelium, a surprising finding given its survival function in neuronal cells. A remarkable characteristic of PEDF is its potent antiangiogenic activity compared with other endogenous inhibitors of neovascularization such as thrombospondin-1 (TSP-1), angiostatin, and endostatin [3]; this property makes PEDF an excellent candidate for drug development.

One of the important mechanisms underlying the antivascular activity of endogenous inhibitors of angiogenesis is their selective capability to induce endothelial cell apoptosis in actively remodeling vessels [4, 5]. At the root of PEDF’s selective destruction of remodeling vasculature is the activation of the Fas/Fas-L death pathway [6]. Endothelial cells stimulated by vascular endothelial growth factor (VEGF) or basic-fibroblast growth factor (bFGF) expose Fas (the receptor for the death ligand, FasL) on their surfaces. PEDF, meanwhile, induces the expression of FasL. Thus, in this scenario, only endothelial cells of remodeling vessels are vulnerable to the induction of apoptosis by PEDF [6]. Underlying this mechanism is a complex cross talk between the signaling pathways generated by inhibitors and inducers of angiogenesis. On one hand, proangiogenic inducers trigger dephosphorylation of the transcription factor nuclear factor of activated T cells (NFATc2), causing its translocation to the nucleus where it up-regulates the expression of cellular-FLICE-like inhibitory protein (c-FLIP), an endogenous dominant negative variant of caspase-8. On the other hand, PEDF triggers c-Jun NH2-terminal kinase (JNK)-mediated rephosphorylation of the terminus of vascular endothelial growth factor-receptor 1 (VEGF-R1) [8]. In addition, PEDF interferes with the phosphorylation of VEGF-R1, and consequently, may modulate angiogenic signaling via vascular endothelial growth factor-receptor 2 (VEGF-R2). These data underscore the complexity of cross talk between inducers and inhibitors of angiogenesis.

A recent study by Cai et al. unveils another mechanism underlying the antiangiogenic action of PEDF, involving the activation of γ-secretase-dependent cleavage of the C terminus of vascular endothelial growth factor-receptor 1 (VEGF-R1) [8]. In addition, PEDF interferes with the phosphorylation of VEGF-R1, and consequently, may modulate angiogenic signaling via vascular endothelial growth factor-receptor 2 (VEGF-R2). These data underscore the complexity of cross talk between inducers and inhibitors of angiogenesis.

The antiangiogenic activity of PEDF has been studied mostly in the context of ocular vascularization, where a skewed balance between angiogenesis inducers and inhibitors leads to multiple pathologies. However, PEDF is also found in many other organs, such as liver, testes, ovaries, placenta, brain, pancreas, and kidney. There is evidence that, at least in some organs, the antiangiogenic action of PEDF contributes to the maintenance of an appropriate angiogenic balance throughout the course of development. Animals knocked-down for PEDF display deficiencies in the development of prostate, pancreas [9], and kidney [10], possibly caused by an abnormal vascularization.

PEDF’s ability to restrict neovascularization in normal tissues implies that its loss during malignant tumor progression would create an environment permissive for aberrant tumor vascularization. The first evidence for such an antiangiogenic action of PEDF in the context of tumor growth was discovered in neuroblastomas by Crawford et al. [11], who found that PEDF produced by the Schwannian stromal cells present in neuroblastomas counterbalances the
proangiogenic effect of high levels of VEGF secreted by neuroblastoma cells.

PEDF depletion during prostate development results in severe hyperplasia [9]. This prompted treatment of prostate xenografts with purified recombinant PEDF, which effectively suppressed tumor growth [9]. The expression of PEDF in the prostate is furthermore negatively regulated by androgens and hypoxic conditions. Therefore, an increase in PEDF levels could be important for the regression of the stromal vasculature upon androgen ablation therapy, which causes tumor involution [9].

The effect of PEDF overexpression has been evaluated in diverse tumor cell lines. In experiments with pancreatic adenocarcinoma cells [12] and studies with melanoma cell lines [13, 14], overexpression of PEDF caused a reduction of tumor microvessel density that translated into a significant antitumor effect. Consistent with the mechanistic traits described for PEDF, we found that after human melanoma cells overexpressing PEDF were grafted into mice, the remnant vessels were densely covered by pericytes, suggesting that only immature (remodeling) vessels are vulnerable to PEDF’s antiangiogenic action [13]. Importantly, PEDF is able to normalize the angiogenic balance of melanoma cells via down-regulation of stimulatory VEGF and a simultaneous induction of angiopoietin-2, a factor that counters maturation of the new vessels [13].

Finally, there is evidence supporting a role for endogenous PEDF in the control of tumor progression. PEDF levels are inversely correlated with the metastatic potential and tumor grade of prostate adenocarcinoma [15], pancreatic adenocarcinoma [16], glioblastoma [17], hepatocellular carcinoma [18], and Wilm’s tumor [10]. PEDF has also been suggested to prevent the invasion of osteosarcoma into cartilage by counterbalancing chemotaxis driven by VEGF [19, 20].

The effects of PEDF on primary tumor growth and metastasis development could be explained as resulting solely from the inhibition of tumor neovascularization we have described above; however, the results reviewed below suggest that the nature of PEDF’s action is much richer and more complex.

Direct antitumor action. PEDF as a multifunctional factor impinging on tumor cell differentiation, migration, and survival

Effect of PEDF on tumor cell differentiation

PEDF causes differentiation of Y79 retinoblastoma cells, which manifests itself by the emergence of neurite-like extensions, the expression of neuronal differentiation markers, and growth arrest [21]. The neurotrophic function of PEDF was reported by Houenou et al. in chick spinal cord cultures, where PEDF also induces neurite formation [22]. However, this effect appears to be cell-type-dependent, as treatment with PEDF at similar concentrations enhances the survival of cerebellar granule cells in culture without any sign of differentiation [23]. Survival and differentiation are processes that usually occur in concert, so these effects of PEDF may utilize common mechanisms. PEDF has prosurvival activity under a variety of conditions that lead to neuronal damage, including excitotoxicity [22–25], oxidative stress [26], ischemia [27, 28], and light-induced degeneration of rod photoreceptors [29, 30]. A recent study by Ramirez-Castillejo et al., demonstrated that vascular-derived PEDF drives neuronal stem cell renewal in the supraventricular zone of the mouse brain, a finding which highlights the connection between neuronal and vascular cells and identifies PEDF as a bridging factor between these two niches [31].

In tumors of neuronal origin, PEDF promotes differentiation, which may account for the reduced malignant phenotype. The neurodifferentiating effect in Y79 retinoblastoma cell lines first suggested that PEDF may act directly on tumor cells. As mentioned above, PEDF secreted by Schwannian stromal cells of neuroblastomas not only creates an antiangiogenic microenvironment, but also induces the differentiation of neuroblastoma cells towards a less malignant phenotype [11]. PEDF is also produced by the more differentiated S-type neuroblastomas, suggesting an inverse correlation between PEDF levels and the progression of neuroblastomas towards a more malignant and less differentiated phenotype. This finding provides an explanation of the reduced malignancy associated with the enrichment in the Schwann-cell component. Another noteworthy finding of this study is that PEDF acts as a survival factor for Schwann and ganglion cells, generating an autocrine loop which is beneficial for tumor prognosis. This study was thus the first to report both antiangiogenic and neurotrophic antitumor effects of PEDF in the same tumor model. Recently, a similar dual antitumor effect has been described by Filleur et al., who showed that PEDF induces neuroendocrine differentiation in prostate cancer cell lines and tumors, suggesting that the prodifferentiation activity of PEDF could be beneficial in multiple tumor types [32].

Recent reviews by Tombran-Tink cover in detail the molecular mechanisms mediating the neurotrophic and neuroprotective activities of PEDF [33, 34]. Briefly, PEDF activates nuclear factor-κB (NF-κB) by inducing the phosphorylation and degradation of its inhibitor IκB, leading to nuclear translocation of the p65 subunit, and consequently increases the phosphorylation and DNA-binding activity of c-AMP-responsive element binding protein [35]. In immature cerebellar granule cells, PEDF also induces the
expression of antiapoptotic molecules such as bcl-2, bcl-x, the inhibitor of apoptosis protein, c-FLIP, and superoxide dismutase; all of these factors are potential transcriptional targets of NF-κB [35]. Moreover, in mature neurons, PEDF increases the expression of several neurotrophic factors such as nerve growth factor, brain-derived neurotrophic factor, and glial cell-derived neurotrophic factor [35].

It is not yet clear how PEDF causes opposing effects in neuronal and endothelial cells (survival vs apoptosis) [33]. The specific activation by PEDF of diverse signaling cascades or its opposing regulation of key modulators may be required for achieving the appropriate response in each cell type. c-FLIP has been described as one of the switches used by PEDF to control cell type specific fates. PEDF inhibits transcription of c-FLIP in activated endothelial cells [7], whereas it activates c-FLIP transcription through NF-κB in neuronal cells [35]. Thus, c-FLIP acts as a switch that can direct the signal of PEDF towards Fas-L-induced apoptosis in endothelium or towards survival and differentiation in neuronal cells. A second possible mechanism could involve differential regulation of the levels of NF-κB p50/p50 homodimers or p65/p50 heterodimers, each of which might activate different target genes. A third mechanism could involve cell-type-specific responses achieved by endothelial and neuronal cells expressing different PEDF receptors, coupled to different signaling pathways. There is evidence for a putative 80-kDa receptor in neural cells [36–38] and for a 60-kDa receptor in endothelial cells [39].

This notion of two distinct receptors is corroborated by the fact that PEDF induces distinct signaling events in endothelial and neuronal cells: a rapid, short-lived activation of JNK in endothelial cells [7] and a late and sustained induction of extracellular regulated kinases 1,2 in neuronal cells [40]. A final possibility could be the extracellular phosphorylation of PEDF by protein kinase A or casein kinase 2, which differentially regulate the antiangiogenic or neurotrophic activity of PEDF [41, 42].

Effect of PEDF on tumor cell apoptosis

The analysis of PEDF’s antitumor effect has revealed a third biological activity of this multifunctional factor. Doll et al. have shown that PEDF triggers the apoptosis of cultured prostate tumor cells, and this effect was augmented when cells were exposed to hypoxic conditions [9]. In the last 2 years, several studies have confirmed a direct apoptotic effect of PEDF in cell lines of glioma [43], osteosarcoma [44], and melanoma [13, 14]. PEDF can thus act as an antitumor agent via two routes: it directly sensitizes tumor cells to stress-induced apoptosis and it promotes apoptosis indirectly by disrupting angiogenesis, and thus causing hypoxic stress. This dual antitumor and antiangiogenic effect of PEDF doubles the efficacy of PEDF as a possible therapeutic agent, in comparison with other antiangiogenic factors that only affect the tumor vasculature.

The molecular mechanisms implicated in the induction of apoptosis by PEDF in tumor cells are still poorly understood. Abe et al. reported that stable transfection of melanoma cells with PEDF increases the apoptotic rate; this effect could be mediated by Fas/Fas-L cascade, as it was blocked by neutralizing antibodies against Fas-L [14]. This result was confirmed by Takenaka et al. in the MG63 osteosarcoma cell line [44]. We detected apoptosis in melanoma tumor xenografts, which could be attributed not only to the inhibition of angiogenesis, but also to a direct induction of apoptosis by PEDF in tumor cells because PEDF treatment of cultured melanoma cells enhanced apoptosis under stress due to ant-survival conditions such as serum withdrawal or substrate detachment [13]. Furthermore, in xenografts of UCD-Mel-N melanoma cells, we have found constitutive expression of the receptor Fas both in control and in PEDF-transduced cells; however, only PEDF-overexpressing cells were positive for Fas-L expression (Thomas, Fernandez-Garcia, Volpert, and Jimenez unpublished results). These results suggest a model in which tumor cells that express Fas receptor are susceptible to PEDF-induced apoptosis mediated via the expression of Fas-L, a mechanism very similar to that observed in endothelial cells.

Altogether, these data provide evidence supporting the therapeutic potential of PEDF as an agent that renders tumor cells more susceptible to apoptosis, which is the therapeutic output expected to be achieved by standard radio- and chemotherapy. Sensitization of tumor cells to apoptosis by a wide range of stress conditions, such as low serum, hypoxia, impaired matrix attachment, and reactive oxygen species, makes PEDF an attractive factor that may synergize with chemotherapeutic drugs, thereby providing a wider therapeutic window for diverse tumor types.

Effect of PEDF on tumor cell migration, invasion, and metastasis

PEDF exerts antimigratory activity on a number of cell lines, including not only endothelial cells but also melanoma cells, glioma cells, and others. Our group has reported a reduction in the in vitro migratory potential of melanoma cells that overexpress PEDF [13]. Similar results were reported in glioma [17, 43], where PEDF overexpression downregulates matrix metalloproteinase-9 (MMP-9), which may account for the reduced invasiveness observed. A negative regulatory loop has also been reported, in which activation of MMP-2 and MMP-9 by hypoxia or VEGF results in a proteolytic inactivation of PEDF that abolishes its prosurvival and antiangiogenic activities [45].
One possible mechanism of inhibition of migration by PEDF could be mediated through binding to collagens I and III. PEDF secreted by colon cancer cells was found to bind collagens I and III, and its expression levels inversely correlate with the metastatic capacity of these cells, thus suggesting that PEDF inhibits migration by competing with integrins for the binding to extracellular-matrix components [46]. Similar results were found for Maspin, like PEDF, a member of the serine protease inhibitor (SERPIN) superfamily of proteins [47], which inhibits tumor cell migration and invasion and whose expression inversely correlates with breast cancer progression [48]. Maspin’s anti-invasive properties may also be mediated by its binding to collagens I and III through a collagen-binding motif that is highly conserved in SERPINs [49]. The region responsible for collagen binding in PEDF locates to a negatively charged surface, which is distinct from the heparin binding site, the neurotrophic and antiangiogenic active site, and the exposed SERPIN loop [50].

The modulation by PEDF of the production of chemotactants by tumor cells also contributes to its ability to suppress migration. PEDF down-regulates the production of the proangiogenic factors VEGF and bFGF in glioma cells [43], and VEGF in osteosarcoma [44] and melanoma cells [13]. At the same time it up-regulates antiangiogenic TSP-1 in glioma cells [43] and angiopoietin-2 in melanoma [13]. Therefore, the metastatic potential of tumor cells could be diminished by PEDF not only via the disruption of the vascular network that provides them with an escape route from the primary tumor towards metastatic foci, but also by direct inhibition of the migratory and invasive capacities of the tumor cells themselves. Our group has reported that PEDF overexpression in UCD-Mel-N cells dramatically reduces metastasis formation in the mouse tail-vein injection model [13]. PEDF also exerts an antimetastatic effect in a mouse model that uses a colon adenocarcinoma cell line [51]. This effect is likely mediated by the modulation of the survival and migratory capacities of tumor cells. We have found that PEDF compromises the survival of melanoma cells when detached from substrate, an effect that could severely impair the viability of tumor cells in transit towards the target organ during the process of metastasis [13].

Therapeutic implications

To best utilize PEDF as an antitumor agent, it is important to devise strategies that exploit its multifunctional nature and cell-type-specific activities. Currently known biological responses underlying PEDF’s antitumor potential are summarized in Fig. 1.

Recent studies have only just begun to define the structural domains responsible for the diverse biological activities ascribed to PEDF. These structure–function studies have identified small PEDF peptides that display specific and distinct activities, and which have potential as rational therapeutic tools, used alone or as part of combination treatments. Structural studies have mapped the regions responsible for the neurotrophic/neuroprotective and antiangiogenic functions, respectively. The region encompassing residues 58–101, termed the 44-mer, replicates PEDF’s neurotrophic function in several neuronal cell types [36] and inhibits vascular permeability induced by
VEGF [52]. 44-mer also causes neuroendocrine transdifferentiation of prostate cancer cells [32]. The region responsible for antiangiogenic function has been mapped to the sequence containing residues 24–57 (termed the 34-mer) [32]. Antiangiogenic 34-mer and prodifferentiative 44-mer are exposed as separate epitopes on the surface of the full-length molecule [53]. 44-mer, previously characterized as neurotrophic and neuroprotective, is also capable of inducing angiogenesis, an activity that is likely concomitant with the ability to block vascular leakage, a function that has also been attributed to several other proangiogenic factors [54]. It thus appears likely that the dual antitumor effect of full-length PEDF in the prostate would be the result of the combined antiangiogenic activity of the 34-mer and the neurotrophic activity of the 44-mer, likely activating their respective receptors.

For other previously characterized multifunctional inhibitors such as TSP-1, identification of functional domains has been a major breakthrough towards therapeutic applications [55–57]. TSP-1 peptide mimetics have recently entered phase II clinical trials in sarcoma, renal carcinoma, and lymphoma, and remain promising as potential tumor-angiogenesis-targeting molecules [58]. The antiangiogenic properties of the 34-mer make it an attractive new candidate for the list of cancer therapeutics. The identification of a smaller peptide, TGA, that retains the antiangiogenic properties of 34-mer [32] is another step forward in optimizing therapeutic applications of PEDF in cancer. Detailed studies of PEDF’s structure and function, as well as the search for and characterization of specific receptors, will open avenues for the design of therapeutic strategies based on an understanding of the mechanisms underlying its biological roles.

Gene therapy strategies aimed at transducing PEDF into the tumor mass are also being investigated. Such approaches have already been developed for the treatment of ocular pathologies, where the first clinical trials have begun [59–63]. Several recent reports point to the efficacy of PEDF gene therapy strategies in mouse models of solid tumors. Wang et al. reported that systemic administration of an adenovirus encoding PEDF (ad-PEDF) resulted in marked remission of established hepatocellular carcinoma [64]. The same result was achieved by intratumoral administration of a plasmid encoding PEDF in preestablished hepatocellular carcinomas [18]. Also, in a mouse model of lung carcinoma and in a pulmonary metastasis model of colon carcinoma, intratumoral and intrapleural administration of ad-PEDF, respectively, significantly reduced tumor and metastatic burden [51]. Lentiviral vectors have been tested in a mouse model of pancreatic adenocarcinoma [12], and adenov-associated-virus PEDF effectively blocks neuroblastoma growth in syngeneic mouse models, causing both inhibition of vascularization and induction of tumor cell differentiation [65]. Finally, in a study of syngeneic murine mesothelioma, intratumoral administration of ad-PEDF in combination with adeno-s-flt (a soluble form of the human VEGF-R1) reduced tumor size and microvessel density more profoundly than either agent when administered alone [66].

In summary, PEDF has emerged as a promising new antitumor agent that acts in a cell-type-specific manner against a wide range of tumors. PEDF combines direct antitumor effects, ranging from direct induction of apoptosis to the cessation of growth as a result of cell differentiation, and an antiangiogenic function, which is again both direct, by inducing apoptosis of activated vascular endothelium, and indirect, by suppressing the angiogenic potential of tumor cells. Deciphering the mechanisms underlying the multifunctional nature of PEDF presents a challenge, but nonetheless, this multifunctional nature remains the most attractive feature of this endogenous factor.

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Proliferation and cell–cell fusion of endometrial carcinoma are induced by the human endogenous retroviral Syncytin-1 and regulated by TGF-β

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Abstract Endometrial carcinomas (EnCa) predominantly represent a steroid hormone-driven tumor initiated from prestages. The human endogenous retrovirus HERV-W envelope gene Syncytin-1 was significantly increased at the mRNA and protein levels in EnCa and prestages compared to controls. Steroid hormone treatment of primary EnCa cells and cell lines induced Syncytin-1 due to a new HERV-W estrogen response element and resulted in increased proliferation. Activation of the cAMP-pathway also resulted in Syncytin-1 upregulation, but in contrast to proliferation, classic cell–cell fusions similar to placental syncytiotrophoblasts occurred. Cell–cell fusions were also histologically identified in endometrioid EnCa tumors in vivo. Clonogenic soft agar experiments showed that Syncytin-1 is also involved in anchorage-independent colony growth as well as in colony fusions depending on steroid hormones or cAMP-activation. The posttranscrip-

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Abstract Endometrial carcinomas (EnCa) predominantly represent a steroid hormone-driven tumor initiated from prestages. The human endogenous retrovirus HERV-W envelope gene Syncytin-1 was significantly increased at the mRNA and protein levels in EnCa and prestages compared to controls. Steroid hormone treatment of primary EnCa cells and cell lines induced Syncytin-1 due to a new HERV-W estrogen response element and resulted in increased proliferation. Activation of the cAMP-pathway also resulted in Syncytin-1 upregulation, but in contrast to proliferation, classic cell–cell fusions similar to placental syncytiotrophoblasts occurred. Cell–cell fusions were also histologically identified in endometrioid EnCa tumors in vivo. Clonogenic soft agar experiments showed that Syncytin-1 is also involved in anchorage-independent colony growth as well as in colony fusions depending on steroid hormones or cAMP-activation. The posttranscrip-
TGF-β3 were identified as main regulative factors, due to the finding that steroid hormone inducible TGF-β1 and TGF-β3 inhibited cell–cell fusion, whereas antibody-mediated TGF-β neutralization induced cell–cell fusions. These results showed that induced TGF-β could override Syncytin-1-mediated cell–cell fusions. Interactions between Syncytin-1 and TGF-β may contribute to the etiology of EnCa progression and also help to clarify the regulation of cell–cell fusions occurring in development and in other syncytial cell tumors.

**Keywords**  
Tumorigenesis · HERV-Endometrial carcinoma · Cell fusion · TGF-beta

**Introduction**

EnCa is the most common invasive gynecologic cancer in Western industrialized countries, mainly occurring in postmenopausal women. In the USA, the EnCa incidence has been rising over the last 10 years from 41% in 1994 to currently 50%, making EnCa the eighth most common cause of cancer deaths [1, 2]. Over 85% of all EnCa cases are histologically classified as endometrioid (type I), mainly express the estrogen receptor (ER) and progesterone receptor (PR) and most likely develop from endometrial hyperplasia driven by hormonal changes, like estrogen stimulation [3]. Two percent of complex hyperplasias, but 52% of atypical hyperplasias can progress into a carcinoma [4]. The rare nonendometrioid EnCa (type II) is more aggressive, often lacks ER and PR expression, and may develop from transformed endometrial surface epithelium [1]. Although unclear in the staging of EnCa, benign endometrial polyps can internally progress into hyperplasias (11–24%) but also malignancies (1–3%) [5, 6].

According to the International Federation of Gynecology and Obstetrics, EnCa is staged according to tumor location (Ia is endometrial to IVb with distant metastasis) and graded according to the growth pattern (G1-3). A causal relationship between hormone replacement therapies and EnCa has been previously established [7, 8]. An increase of relative EnCa risk with selective estrogen receptor modulators, like Tamoxifen used for breast cancer prevention and for adjuvant trials was found to be >twofold [9].

Genetic markers noted in EnCa, which show correlations in tumors with and without complex atypical hyperplasia are, e.g., microsatellite instability, DNA nondiploidy, mutations in PTEN, p53, β-catenin 1, K-ras, and amplifications in c-myc and INT2 [1]. PTEN mutations occurred equally in both early and advanced EnCa; however, c-myc amplification was more frequent in advanced tumors, supporting an earlier and later role for PTEN and c-myc, respectively. Loss of heterozygosity of BRCA1, p53, and TCRD genes in EnCa tumors was associated with tumor grade, younger age, and family cancer history, respectively [10].

Cell–cell fusions leading to multinucleated or syncytial cells represent a central and essential event during embryogenesis and morphogenesis. In humans, besides sperm cell–oocyte fusion, other cell–cell fusion events include mononucleated hematopoietic or myoblast precursors fusing to syncytial osteoclasts or myofibrils during bone and muscle differentiation, respectively [11, 12]. During the first 11 days of pregnancy, while the blastocyst implants into maternal endometrium, placental cytrophoblasts fuse to multinucleated syncytiotrophoblasts forming the maternal–fetal cellular interface [13]. Syncytiotrophoblasts also represent the invasive front of the blastocyst.
which have similarities to invasive tumors. The so-called giant syncytial cells have also been identified in cells infected with enveloped viruses and in certain tumors, like EnCa [11, 14]. However, with the exception of enveloped viruses, the molecular mechanisms of cell fusion and the function of these multinucleated cells in tumors are not understood.

Human endogenous retrovirus (HERV) families represent remnants of past retroviral infections of germline cells of human ancestors and embody 7.7% of the human genome [15]. HERVs have been classified according to their gag, pol, and envelope gene homology with exogenous retroviruses, like, e.g., HIV and HTLV [15, 16]. So far, over 200 HERV families are described in the human genome, with sequences on different chromosomes, where most elements produce no functional proteins due to deletions and mutations [15, 17]. A functional HERV-W envelope gene, also called Syncytin-1, locates along with inactive gag and pol genes and intact flanking LTRs at 7q21-22 (Fig. 1). It is interesting to note that Syncytin-1 has evolved as being essential for human placental morphogenesis, where Syncytin-1 mediates cell-cell fusions of cytotrophoblasts into syncytiotrophoblasts [17, 18]. Syncytin-1 is regulated in cytotrophoblasts by the transcription factor "glial cells missing homolog" (GCMa) and by CpG-methylation [18–20], and is expressed as an inactive protein precursor, processed through cellular subtilisin-like proteases into a predicted extracellular surface domain (SU) and a transmembrane domain (TM) (Fig. 1) [21, 22].

Results from exogenous retroviral envelope proteins showed that the TM and SU remained associated after cleavage, where the SU recognized the host cell receptor and the TM promoted virus-cell fusion [23]. Proposed membrane receptors for Syncytin-1 are the Na⁺-dependent neutral amino acid transporters ASCT1 and ASCT2 [24]. ASCT2 is described as a general retrovirus-receptor for simian immunosuppressive type D retroviruses [25].

Transforming growth factor-beta (TGF-β) proteins regulate proliferation, differentiation, and can induce transformation of various cell types. TGF-β has a dual effect in carcinogenesis as a tumor suppressor or activator depending on the cell type [26]. High levels of TGF-β can be a marker for poor prognosis in cancer of, e.g., breast, colon, brain, and prostate [27]. The TGF-β signaling pathway appears disabled in both endometrial hyperplasias and EnCa showing a loss of growth inhibition, e.g., the TGF-β antagonist Smad7 was found significantly upregulated in EnCa [28, 29]. In addition, steroid hormones were found to regulate TGF-β3 gene expression in ER positive cells [30]. Specifically for endometrial stromal cells, 17β-estradiol (E2) induced TGF-β1 and β3 transcription, but not TGF-β2 [31].

This report analyzed the gene expression of Syncytin-1 in endometrioid EnCa, control endometrium and other benign endometrial tissues. The role of Syncytin-1 and TGF-β in steroid hormone stimulated cell proliferation and in cAMP-induced cell–cell fusions unraveled pivotal control points in EnCa.

Materials and methods

Patient tissues Handling of patients and tissues was approved by the Ethics Committee at the University of Erlangen-Nuremberg. All patients gave a written informed consent. Tissues were collected between 2003 and 2004, classified by the Institute of Pathology (Erlangen) and flash
frozen in liquid nitrogen. EnCa tumors of endometrioid histology were collected from 24 patients (age 51–88; mean 66.3) and grouped according to stages (Ib–IVb) and growth patterns (G1-3): stage Ib/G1 ($n=5$), Ib/G2 ($n=3$), Ib/G3 ($n=2$), Ic/G1 ($n=2$), Ic/G3 ($n=1$), IIb/G1 ($n=1$), IIb/G2 ($n=7$), IIIa/G2 ($n=1$), IIIc/G2 ($n=1$), IVb/G2 ($n=1$). In addition, a colorectal carcinoma from an EnCa Ic diagnosed in 1997 (#771), a vaginal tumor from an EnCa Ic diagnosed in 2002.
TaqMan-Assays (Applied Biosystems). Coamplification of TGF-β and the two isoforms PR A/B (exon 4–5), Ki-67 (exon 11–12), ASCT1 (exon 7–8), ASCT2 (exon 1–2), TGF-β1 (exon 1–2), and TGF-β3 (exon 1–2) gene expression were analyzed using TaqMan-Assays (Applied Biosystems). Coamplification of 18S-rRNA and one patient cDNA were used as internal controls for a standard curve in semiquantitation analysis. Gene expression for GCMA was analyzed according to Hashemolhosseini and Wegner [19]. For quantitative real-time PCR (qPCR) of Syncytin-1, a QuantiTect Multiplex Assay (Qiagen) was designed: primer 1 (CCCTATGAC CATCTACAC) and 2 (GCACTCCTGCTCTATAAACA) with FAM-labeled probe (ATCTAAGCCCACGAAC) including a 18S-rRNA control. As an external standard, cloned Syncytin-1 DNA with known copy numbers was used to generate a standard curve with the CT value against the log of amount of standard. The CT of Syncytin-1 was calculated according to the standard curve.

Northern and Southern analysis Thirty μg DNAase I-treated RNA was electrophoresed on 1% formaldehyde agarose gels and for Southern 10 μg DNA was analyzed on TBE-agarose gels. Gels were electroblotted onto GeneScreen Plus (NEN) and hybridized. The cloned Syncytin-1 cDNA 748 bp [32P]dCTP labeled fragment was used as a probe (Fig. 1).

Immunoblot analysis Proteins were isolated from tissues using a microdismembranator and resuspended 10-fold of wet weight in: 0.125 M Tris–HCl pH 6.8, 2% sodium dodecyl sulfate (SDS), 5% glycerol, 2 mM EDTA, 0.1% TritonX100, 1 mM Na3VO4, 1.5 mM NaF, and PI-cocktail I: 1 mM PMSF, 10 μg/ml of each Pepstatin, Leupeptin, and Chymostatin. An amount of 44 μg of each cell lysate were resolved on a 7.5–12.5% acrylamide gradient SDS-gel according to Strick et al. [32], then transferred to a polyvinylidene fluoride membrane using a CAPS-transfer buffer (10 mM CAPS, 10% methanol, 0.01% SDS, pH 10.4) at 0.5 A at 4°C. Immunoblot analyses were done with a Syncytin-1 polyclonal antibody (1:500) (gift of Dr. Chen, Taiwan); β-actin monoclonal antibody (mab) (1:1,000) along with secondary peroxidase labeled antibodies and a blocking reagent (all Sigma–Aldrich). Antibodies were stripped off immunoblots with 2% SDS at 50°C for 20 min and then reprobed.

DNA binding analysis Purified human ERα (Invitrogen) was incubated with either [32P]labeled Syncytin-1-ERE (5′CTAGCTGGGAAGTGACCCATGCTTACCGTACCCTTTAAA CACG) or control ERE from the Xenopus laevis A2 vitellogenin gene at position −306 to −342 (5′CTGTCCCA AAGTCAGGTCACAGTGACCGCCACTTTAAA CACG) and 80 mM NaCl, 10 mM Tris–HCl pH 7.5, 0.5 mM DTT, 0.2 mM EDTA, 5 μM ZnCl2, 5% glycerol and 30 ng/μl poly dIdc for 15 min at 21°C. DNA protein complexes were fractionated on a 6% nondenaturing acrylamide gel at 150 V and RT, then the gel was dried and autoradiographed.
**Cell culture** Primary EnCa tumor cultures and the cell lines RL95-2, Kle and HEC-1-A [human endometrial carcinoma (HEC)] derived from poorly and moderately differentiated EnCa and the BeWo cell line derived from a choriocarcinoma (ATCC) were grown in phenol red free RPMI with 5% charcoal treated serum (CTS) (HyClone). For steroid hormone treatment, cells were seeded at 200,000 cells and 48 h later incubated with 10⁻⁵⁻⁵₀ nM E₂, 4-OH-E₂, 2-OH-E₂, estrone, estriol, or 500 nM progesterone (Sigma-Aldrich) for an additional 72 h in RPMI+2% CTS and then growth curves determined (Celldyne, Abbott). For isolation and culturing of primary cytotrophoblasts, placentas were minced, incubated with 250 mg trypsin and 120 mg DNAase I per 150 mg of tissue for 45 min at 37°C and cytotrophoblasts isolated using a continuous Percoll gradient centrifuged at 18,000×g for 50 min.

**Cell–cell fusion analysis** Cells were seeded at 230,000 and after 72 h, cell–cell fusions were induced with 40 μM Forskolin or the SP isomer of cAMP (SP-cAMP) (Sigma-Aldrich) or induced with 1 μg/ml mabs against TGF-β₁ or -β₃ (R&D) in the presence of steroid hormones and then incubated for an additional 72 h. Cells were analyzed for DNA ploidy by, (1) microscopy using a May–Gruenwald and Giemsa stain (Sigma-Aldrich), or using the plasma membrane stain Wheat germ agglutinin with Alexa 594 and the nuclear Hoechst 33342 stain (Molecular Probes), or by (2) fluorescence activated cell sorting (FACS). For inhibition of cell–cell fusions, 5 ng/ml purified human recombinant TGF-β₁ or TGF-β₃ proteins (R&D) or 20 nM E₂ were added 1 day before addition of Forskolin or SP-cAMP. Cell supernatants from steroid hormone, Forskolin, SP-cAMP, RP-cAMP, TGF-β₁, or β₃ or anti-TGF-β₁ or

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**Table 1** Gene expression profile of endometrial tissues, placenta, and cell lines

<table>
<thead>
<tr>
<th>Genes/a</th>
<th>Postmenopausal endometrium (n=24)</th>
<th>Endometrial polyps (n=16)</th>
<th>Hyperproliferative endometrium (n=11)</th>
<th>Endometrial hyperplasia (n=7)</th>
<th>EnCa (n=24)</th>
<th>Placenta (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASCT1</td>
<td>1.65±0.58</td>
<td>0.72±0.09</td>
<td>1.58±0.14</td>
<td>1.81±0.29</td>
<td>2.65±1.25</td>
<td>26.26±6.65</td>
</tr>
<tr>
<td>ASCT2</td>
<td>0.73±0.29</td>
<td>0.72±0.09</td>
<td>0.5±0.11</td>
<td>1.07±0.34</td>
<td>2.84±1.24</td>
<td>2.4±0.38</td>
</tr>
<tr>
<td>GCMa</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Ki-67</td>
<td>1.18±0.49</td>
<td>0.46±0.15</td>
<td>2.95±1.08</td>
<td>2.56±1.09</td>
<td>6.61±1.5</td>
<td>1.1±0.21</td>
</tr>
<tr>
<td>TGF-β₁</td>
<td>0.29±0.06</td>
<td>nd</td>
<td>nd</td>
<td>0.52±0.12</td>
<td>0.54±0.11</td>
<td>1.0±0.10</td>
</tr>
<tr>
<td>TGF-β₃</td>
<td>2.41±0.52</td>
<td>nd</td>
<td>nd</td>
<td>2.05±0.36</td>
<td>3.77±0.95</td>
<td>6.63±0.68</td>
</tr>
</tbody>
</table>

± Represents standard error of the mean (SEM), nd not done

a 2⁻ΔΔct results of real-time PCR experiments

b p values were calculated with postmenopausal endometrium

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**Table 2** Gene expression profile of cell lines treated with E₂ and SP-cAMP

<table>
<thead>
<tr>
<th>Genes/proteins</th>
<th>BeWo (n=12)</th>
<th>BeWo+E₂ (n=12)</th>
<th>BeWo+SP-cAMP (n=12)</th>
<th>RL95-2 (n=12)</th>
<th>RL95-2+E₂ (n=12)</th>
<th>RL95-2+SP-cAMP (n=12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASCT1a</td>
<td>1.05±0.05</td>
<td>2.82±0.12</td>
<td>1.82±0.06</td>
<td>1.15±0.07</td>
<td>1.91±0.75</td>
<td>1.3±0.24</td>
</tr>
<tr>
<td>ASCT2a</td>
<td>1.01±0.03</td>
<td>2.64±0.02</td>
<td>1.78±0.03</td>
<td>1.1±0.11</td>
<td>1.5±0.1</td>
<td>1.4±0.03</td>
</tr>
<tr>
<td>GCMa</td>
<td>1.0±0.0</td>
<td>1.0±0.0</td>
<td>3.47±1.29</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>β-hCGb (mU/ml)</td>
<td>158.0±32.9</td>
<td>130.3±6.7</td>
<td>2,113.0±264.1</td>
<td>1.67±0.21</td>
<td>1.22±0.17</td>
<td>1.2±0.17</td>
</tr>
<tr>
<td>TGF-β₁b (pg/ml)</td>
<td>74.4±7.3</td>
<td>312.1±33.7</td>
<td>nd</td>
<td>167.5±16.6</td>
<td>922.9±50.8</td>
<td>144.9±13.1</td>
</tr>
</tbody>
</table>

± Represents standard error of the mean (SEM), nd not done

a 2⁻ΔΔct results of real-time PCR experiments

b ELISA results of cell supernatants

c p values were calculated with untreated cells

d Incubation with 2OH-E₂ (n=2) resulted in TGF-β₁ levels of: 284.0±5.21 (pg/ml)

e Incubation with 4OH-E₂ (n=2) resulted in TGF-β₁ levels of: 1,364.2±33.5 (pg/ml)
siRNA transient transfections and soft agar assays For the posttranscriptional gene silencing of Syncytin-1 two different siRNAs were designed: (1) 5′TGCCCTGTAAGCG TATTAA, Syncytin-1-siRNA-1 in the SU-domain and the (2) 5′TCGGAAATCTGACTGAGAAA, Syncytin-1-siRNA-2 in the TM-domain (Qiagen). For optimal loss of function effects, the Syncytin-1-siRNA 1 or 2 were titrated and analyzed for Syncytin-1 gene expression and cell toxicity. RL95-2 or BeWo cells were seeded at 200,000 cells in RPMI + 5% CTS and on day 3, 60 nM Syncytin-1-siRNA 1 or 2 were transfected at a 1:9 ratio with lipofectin for 5.5 h. Mock transfections using neg-siRNA-Alexa 488 (Qiagen) demonstrated >80% transfection efficiency. Twenty-four hours posttransfection, cells were trypsinized and set up for treatment with steroid hormones, SP-cAMP, or Forskolin using growth curves or soft agar assays. Non-transfected, mock and Syncytin-1-siRNA transfected cells were plated in triplicate with soft agar in RPMI +5% CTS (bottom 0.33% agar, top 0.30% agar with cells) at 30,000 cells/well. After 7–12 days, cell colonies were scored and measured in six fields per well using a Leitz Axiovert microscope and photographed.

Statistical analyses A two-tailed t test and in addition an ANOVA test between the groups were performed using SPSS13.0 (SPSS), with p<0.05 as statistically significant. For each mean value, a standard error of the mean (SEM) was calculated using SPSS. The box-plot graphs were performed using SPSS13.0.

Results

Syncytin-1 overexpression in EnCa and prestages

Syncytin-1 mRNA was amplified, cloned, and sequenced from three EnCa, one control pre- and one postmenopausal endometrium. Results confirmed sequence identity with no premature stop codons or deletions and insertions to the HERV-W envelope gene (7q21-q22) from placenta (Fig. 1a). Using RT-PCR and comparative qPCR, Syncytin-1 expression was high in placenttal tissues (138.35±16.37 Syncytin-1 copies/ng total RNA; p<0.001), but lower in premenopausal endometrium (36.07±8.84; p=0.004), and even more reduced in myometrium (6.74±2.58) thus demonstrating tissue specificity (Fig. 1c). Syncytin-1 expression was then compared between a collective of control, benign and malignant endometrial tissues, including placenta using qPCR, RT-PCR, Northern, and Western analysis. Results showed that Syncytin-1 was upregulated in all benign and malignant tissues with the highest expression in EnCa above controls including placenta (Fig. 1c,d). Syncytin-1 mRNA copies per nanogram of total RNA were determined for control postmenopausal endometrium with a mean (M) of 15.93±2.25; endometrial polyps: M=72.41±6.26; hyperproliferative endometrium: M=53.14±3.57; endometrial hyperplasia: M=124.5±16.75 and for EnCa the values ranged from 109.1–824.5 with an M=363.58±47.14. All quantitatively determined Syncytin-1 mRNA values from benign and malignant tissues were statistically significant as compared with postmenopausal controls (p<0.001). Northern and long-range RT-PCR analysis of control, benign, and malignant tissues also detected three Syncytin-1 transcripts similar to placenta [17]: a full-length 7.0, 3.0, and 1.0 kb, the last corresponding to the cytoplasmic domain (Fig. 2d).

Examining Syncytin-1 protein levels using an antibody against the SU domain, Western analysis demonstrated an upregulation of all Syncytin-1 protein species in benign and malignant tissues as well as in EnCa metastatic tumors, in contrast to patient matched control (Fig. 1e). Specific Syncytin-1 proteins corresponding to a full-length glycocluster preprotein of 66 kD (gp66-env), a protein of 50 kD (gp50SU) harboring the SU domain, and a third protein of 92 kD (gp92SU-TM) consisting presumably of linked SU and TM domains were detected (Fig. 1b,e). Although the fully processed SU and TM units are probably linked over disulfide bonds, the gp92SU-TM was still detected even after a 5-min, 100°C treatment in the presence of 1–10% β-mercaptoethanol, but was not resistant against 100 mM DTT and 30 min at 100°C (data not shown). Although Syncytin-1 was aberrantly overexpressed at the RNA and protein level, Syncytin-1 was not amplified genomically in all tissues tested, supporting transcriptional regulation (Fig. 1f).

In addition, real-time PCR gene expression analyses was performed to address if the putative Syncytin-1 receptors ASCT1, ASCT2, the Syncytin-1 GCMa transcription factor, as well as the proliferation cell marker Ki-67 were also
overexpressed in the collective tissues. Only Ki-67 showed a significant induction in EnCa tissues (p=0.002) indicating increased tumor cell proliferation, whereas all other transcripts were not upregulated significantly (Tables 1 and 2).

Steroid hormone regulation of Syncytin-1

A strong association exists between steroid hormones and EnCa and its clinical prestages [3], therefore a possible link between Syncytin-1 overexpression and steroid hormone regulation was investigated. First, examining the steroid hormone receptor status using real-time PCR, a significant higher ratio of ERα (p=0.010), but not ERβ or PR A/B was detected in EnCa compared to patient matched controls (Fig. 2a). Endometrial polyps (p=0.017) and hyperplasia (p=0.002) showed a significant increase of ERα, but in contrast to EnCa, a significant increase of PR A/B was found for both benign tissues (p<0.001) as compared to postmenopausal endometrium. Growth curves of RL95-2 and BeWo cells following either 10 nM E2, 2-OH-E2, 4-OH-E2, or 500 nM progesterone treatments determined a two- to threefold stimulation of cellular proliferation above control cells on the third day demonstrating steroid hormone responsiveness (Fig. 2b, Supplementary Fig. S1). Addition of either 10 nM E2, 2-OH-E2, 4-OH-E2, or 500 nM progesterone to ER or PR A/B positive EnCa primary cells, RL95-2, and BeWo, showed an ~20-fold induction of Syncytin-1 RNA on the third day of growth correlating with the peak of cellular proliferation (Fig. 2c, Supplementary Fig. S2a). In addition to a steroid hormone upregulation of Syncytin-1 detected by RT-PCR, the incubation of RL95-2 and BeWo cells with steroid hormones also demonstrated an upregulation of all three Syncytin-1 transcripts (Fig. 2c,d). In contrast, the addition of 17α-estradiol, the inactive form of E2, as well as the weak estrogens estrone and estril to the same cells as above, had no stimulatory effect on Syncytin-1 gene expression (Supplementary Fig. S2a). No induction of cellular proliferation and Syncytin-1 expression were detected in the presence of steroid hormones with the ER-negative cell line Kle or with cultured uterine fibroblastoid cells from an EnCa tumor (Fig. 2c, Supplementary Fig. S1).

Further gene sequence analysis of HERV-W revealed an estrogen response element (ERE) with 85% homology in the U3 region of the 5′ and 3′LTR (Fig. 2d). DNA binding assays showed that purified ERα specifically bound to the Syncytin-1 ERE, however, when compared to the control ERE from the Xenopus laevis vitellogenin A2 gene promoter region, an ~20-fold lower DNA binding affinity was observed (Fig. 2e). All of the above results support a specific regulation of Syncytin-1 via steroid hormones and receptors.

cAMP-induced cell–cell fusions of EnCa cells

The activation of cell–cell fusion involving placental cytotrophoblasts and BeWo cells has been shown previously to be stimulated by the activation of the cAMP-pathway [22]. Therefore, studies were initiated to analyse EnCa cell–cell fusion regulation. In contrast to steroid hormone studies, treatment of EnCa primary cells and cell lines with the adenylyl cyclase activator Forskolin or with SP-cAMP for 48 to 72 h did not induce cell proliferation, but stimulated the classic cell–cell fusion phenotype compared to BeWo cells or spontaneously fused syncytiotrophoblasts (Figs. 2 and 3b, Supplementary Fig. S1). Forskolin or SP-cAMP treated EnCa cells also demonstrated Syncytin-1 overexpression of up to 20-fold correlating with cell–cell fusions (Fig. 2c,d). In contrast, the inactive RP-isomer of cAMP (RP-cAMP) had no effect on cell–cell fusion or Syncytin-1 expression. When 2′,5′-dideoxy-adenosine (ddA), an inhibitor of the adenylyl cyclase, was added to E2 or progesterone-treated RL95-2 or BeWo cells, both stimulated cell proliferation and Syncytin-1 overexpression was not inhibited (Supplementary Figs. S1 and S2). This finding proved that Syncytin-1 can be upregulated by two distinct cellular pathways, involving steroid hormones or cAMP, leading either to cell proliferation or cell–cell fusion, respectively.

Quantitative FACS analysis of SP-cAMP-treated EnCa cells showed an increase in cell–cell fusions up to 22.5-fold, with a range of DNA content from 4n–32n (Fig. 3c). In addition, microscopic analysis of EnCa and BeWo cells also demonstrated cell–cell fusions >32n after cAMP-activation similar to syncytiotrophoblasts (Fig. 3a,b). Fluorescent microscopy of SP-cAMP treated RL95-2 with specific plasma membrane and nuclear stains confirmed single multinucleated cells (Fig. 4a). A secreted protein marker for cytotrophoblast cell–cell fusions in vivo and in vitro is the human chorionic gonadotropin hormone (β-hCG) where high levels indicate fusion [13]. After Forskolin or SP-cAMP treatment of primary cytotrophoblasts and BeWo cells, β-hCG levels peaked at 72 h, whereas in primary EnCa cells as well as Kle and RL95-2 no induction of β-hCG occurred, except for a threefold stimulation of HEC-1-A (Table 2, Supplementary Table 2). In addition, treatment of BeWo or RL95-2 cells with SP-cAMP or Forskolin did not result in induced gene expression of the putative Syncytin-1 receptors ASCT1 and ASCT2, however, GCMa was stimulated with SP-cAMP or Forskolin in BeWo, but not in RL95-2 cells indicating a GCMa-specific regulation of Syncytin-1 in choriocarcinoma and placenta (Table 2).

Importantly, histologically examining a series of eosin–haematoxylin stained endometrioid EnCa biopsies revealed cell–cell fusions or syncytial (trophoblast-like)
cells (Fig. 4b). These findings implicate that cell–cell fusions are part of endometrioid tumors and as shown above, could be due to aberrant cAMP-activation.

Inhibition of cell proliferation and cell–cell fusion after siRNA silenced Syncytin-1 expression

The correlation of both steroid hormone-induced proliferation and cell–cell fusion with increased Syncytin-1 gene expression led to RNA interference-studies investigating, if Syncytin-1 is essential for both pathways. The posttranscriptional gene silencing of Syncytin-1 was performed with two independent siRNAs (Syncytin-1-siRNA 1 and 2), where each demonstrated silencing specificity. Steroid hormone-treatment of transfected RL95-2 cells with Syncytin-1-siRNA 1 or 2 resulted in an ∼90% reduction of Syncytin-1 gene expression and inhibition of induced cell proliferation after 72 h postset-up (Fig. 5b,d, and data not shown). In addition, Forskolin or SP-cAMP treatment of transfected RL95-2 and BeWo cells with Syncytin-1-siRNA 1 or 2 caused a >95% inhibition of Syncytin-1 gene and protein expression and cell–cell fusions (Figs. 3c and 5a,f).

After 7 days post Syncytin-1-siRNA 1 or 2 transfection, Syncytin-1 RNA and protein amounts returned to normal and cell proliferation and cell–cell fusions could be induced with E2 and SP-cAMP, respectively, demonstrating a true siRNA rescue (Supplementary Fig. S2b).

To analyze the role of Syncytin-1 in anchorage-independent colony growth, clonogenic soft agar experiments were performed with Syncytin-1-siRNA 1 or 2, mock-siRNA transfected or nontransfected RL95-2 cells in the presence or absence of steroid hormones, Forskolin or SP-cAMP (Fig. 5c). Nontransfected or mock-siRNA transfected RL95-2 cells treated with either E2, 4-OH-E2 or progesterone and seeded in soft agar, demonstrated a 37.8–41.6% increase of colony number and an increase of colony size above controls after 7 days (n = 3). In contrast, when RL95-2 cells were transfected with Syncytin-1-siRNA 1 or 2, the steroid hormone induction of colonies was blocked resulting in colony number and size similar to transfected, untreated cells. Treatment of mock-siRNA transfected RL95-2 cells with SP-cAMP or Forskolin resulted in an expansion of the average colony size with a fusion-like appearance. This colony fusion effect of SP-cAMP or

Fig. 4 Cell–cell fusions in EnCa. a RL95-2 cells incubated with 40 μM SP-cAMP and then the plasma membrane of live cells was stained with wheat germ agglutinin (Alexa 594) (red) and the nuclear DNA with Hoechst 33342 (blue). Fused cells with over 10 nuclei (top) and with six nuclei (bottom) are shown. Bars 50 μm. b Two different endometrioid EnCa biopsies (stage Ic) embedded in paraffin were hematoxylin–eosin stained and then analyzed for cell–cell fusions or syncytia, also called giant trophoblastic cells (arrows). Note the presence of infiltrating granulocytes and glandular structures. Bars 50 μm.
Forskolin was repressed after Syncytin-1-siRNA 1 or 2 transfection of RL95-2 cells (Fig. 5c). These experiments showed that Syncytin-1 is also involved in anchorage-independent colony growth and fusion depending on steroid hormones or cAMP-activation.

Regulation of cell proliferation and cell–cell-fusion by TGF-β1 and TGF-β3

A complex regulation of cell proliferation and cell–cell fusion in primary EnCa cells and cell lines, as well as in primary syncytiotrophoblasts and BeWo cells was further unraveled between steroid hormones, TGF-β1 and TGF-β3 and the activated cAMP-pathway (Figs. 5d–f and 6, Supplementary Fig. S3). TGF-β1 and TGF-β3 gene expression analyses of endometrial hyperplasia, EnCa and placenta showed an increase of both gene products in comparison to matched control endometrium, supporting a role of TGF-β in these tissues (Table 1). Treatment of RL95-2 and BeWo cells with either 4-OH-E2, 2-OH-E2 or E2 also induced TGF-β1 protein levels significantly and correlated with Syncytin-1 upregulation during cell proliferation (Table 2, Fig. 2, Supplementary Figs. S1 and S2a). In contrast, TGF-β1 protein levels were not induced with Forskolin or SP-cAMP (Table 2). A monoclonal antibody-directed neutralization of TGF-β1 or TGF-β3 in E2-treated RL95-2 or BeWo cells resulted in cell–cell fusions similar to cAMP-activation, thus reversing the E2-induced cell proliferation (Fig. 5f, Supplementary Fig. S3). These results suggested that TGF-β has an inhibitory role in cell–cell fusion even in the presence of high Syncytin-1 levels. Indeed, addition of purified TGF-β1 or TGF-β3 proteins alone or before SP-cAMP or experiments with E2 addition before Forskolin or SP-cAMP treated primary EnCa, RL95-2 or BeWo cells inhibited the cell–cell fusion activity by >80% as determined by FACS and microscopy as well as decreasing β-hCG levels for BeWo (Figs. 3c and 5f, Table 2, Supplementary Fig. S3), however, Syncytin-1 levels remained high (Fig. 5d).

For comparison, the TGF-β regulatory action on cell–cell fusion was examined with cytotrophoblasts. Addition of TGF-β1 or TGF-β3 proteins blocked spontaneous fusion of cytotrophoblasts at 72 h, as analyzed by microscopy and decreasing β-hCG levels, with Syncytin-1 levels unchanged (Fig. 5e,f, Supplementary Table 2). Endogenous TGF-β levels were also analyzed. At day 3 of culturing, the cell–cell fusion peak of syncytiotrophoblasts showed low TGF-β1 gene expression (2−ΔΔct:0.02±0.01 by real-time PCR), whereas on day 8, when cell–cell fusion activity decreased, TGF-β1 levels had risen to over 10-fold (2−ΔΔct:0.22±0.09). This rise of endogenous TGF-β1 protein levels between days 3–8 could also be confirmed using enzyme-linked immunosorbent assay (ELISA).

In summary, TGF-β1 and TGF-β3 represent dominant negative regulators of cell–cell fusion, especially during cell proliferation due to steroid hormone induction of Syncytin-1 in EnCa, whereas Syncytin-1 in the absence of TGF-β contributes to cell–cell fusions.

Discussion

Models similar to the multistep evolution of colorectal carcinoma by Fearon and Vogelstein [33] have been proposed for EnCa [34, 35]. This report determined that Syncytin-1 increased in transcript copy number and protein levels from benign endometrial tissues to EnCa stages and metastasis, and was not due to genomic amplification (Fig. 1). We therefore propose that Syncytin-1 in addition
to other genes, like K-ras, c-myc, and PTEN is important in the multistep genetic evolution to EnCa.

A strong connection exists between endometrioid EnCa and its pregestages and steroid hormones or unopposed estrogen therapy; however, steroid hormone receptor expression demonstrated differences. In all tissues, the expression of ERβ was equally low, but ERα expression was the highest in EnCa (Fig. 2a). It is interesting to note that PR-expression was the lowest in EnCa possibly due to silencing of PR-B by hypermethylation [36]. In addition, the ratios of PR A/B and ERα demonstrated differences between the stages, a 2:1 ratio in hyperplasia, compared to 0.06:1 ratio in EnCa, thus, supporting progestin for treatment of hyperplasias. It has previously been demonstrated that EnCa tumors have 58% higher than normal E2 hormone levels compared to matched controls [37]. The ERE-based upregulation of Syncytin-1 by steroid hormones along with induced cell proliferation shed light on the connection between steroid hormones and EnCa growth. This clinical connection was further supported by experiments using primary EnCa cell lines with and without ER expression, high Ki-67 levels and especially with Syncytin-1-siRNA (Figs. 2 and 5, Tables 1 and 2, Supplementary Fig. S1). Taken together, these findings establish Syncytin-1 as an essential gene for steroid hormone-induced cell proliferation in EnCa staging.

Other examples of steroid hormone-induced envelope genes of endogenous retroviruses are HERV-K, which was also inducible up to 10-fold in breast cancer cells when treated with steroid hormones and the endogenous Jaagsiekte sheep retrovirus (enJSRV), which is highly related to the exogenous lung oncogenic JSRV retrovirus [38, 39]. The enJSRV was expressed in the endometrium epithelia of the ovine uterus and detected in trophoectoderm syncyta. Like Syncytin-1, the enJSRV plays a role in early placentogenesis and could be induced with progesterone, whereas JSRV was not inducible [38]. Recently, Syncytin-1 was found upregulated in ovarian carcinoma, which also could involve steroid hormone induction [40]. The correlation of Syncytin-1 with EnCa in this report and other published associations of HERV genes with human diseases, like the upregulation of HERV-K envelope transcripts in most breast tumor tissues support a connection of HERVs with tumors and tumor progression [39].

In addition to the essential role of cell–cell fusions in development, syncyta are also more commonly found in tumors, e.g., in renal cell carcinoma and in mamma adenocarcinoma [41, 42]. Multinucleated, syncytial or so-called trophoblast-like cells are described in EnCa tumors of serous papillary histology, but are also found in endometrioid EnCa (Fig. 4b). EnCa cases with trophoblastic cells were described as very aggressive with an unusually rapid and progressive clinical course [43]. This report supports that cAMP-activated Syncytin-1 is responsible for cell–cell fusions in EnCa. Cell–cell fusions of EnCa cells induced by the cAMP-signaling pathway were quantitated by FACS and verified with specific membrane stains demonstrating multinuclei surrounded by one cellular membrane (Figs. 3 and 4), in contrast to the phenomenon of endomitosis, as in megakaryocytes demonstrating a single polyploidated nucleus with >2n ploidy. EnCa as well as BeWo cell–cell fusions were mediated by Syncytin-1 because an inhibition of cell–cell fusion was achieved with Syncytin-1-siRNA 1 or 2 (Fig. 5). Other publications have showed that Syncytin-1 transfected BeWo cells increased cell–cell fusions, which could be inhibited with Syncytin-1 antibodies [22].

This report also identified TGF-β1 and TGF-β3 as key regulators of cell proliferation and cell–cell fusions in EnCa, choriocarcinoma, and placenta (Fig. 6). Treatment of EnCa, BeWo cells, or primary cytotrophoblasts with purified TGF-β1 or TGF-β3 proteins even in the presence of Forskolin or SP-cAMP inhibited cell–cell fusion and β-hCG expression (Figs. 3c and 5e,f; Tables 1 and 2). TGF-β, but not SP-cAMP treatment of EnCa and BeWo cells induced endogenous TGF-β1 expression resulting in cell proliferation and inhibiting cell–cell fusion. For example, noninduced RL95-2 endogenous TGF-β1 levels were >twofold when compared to BeWo cells and which significantly increased upon E2 treatment (Tables 1 and 2). Although, TGF-β can inhibit growth in some cell types, it is possible that higher endogenous EnCa TGF-β1 levels like in RL95-2 prevent an inhibition of cell proliferation even after addition of exogenous TGF-β1 proteins. These findings are supported by others using various HEC cell
lines [44]. In addition, primary cytotrophoblasts showed an increase of endogenous TGF-β1 or TGF-β3 with a decrease of cell–cell fusion activity after 8 days of culturing. Other examples of the TGF-β-inhibitory activity of cell–cell fusions have been found with osteoclasts and myoblasts [45–47]. In contrast, an antibody mediated neutralization of TGF-β1 or TGF-β3 in steroid hormone treated EnCa cells or cell lines led to cell–cell fusions due to the steroid hormonal induction of Syncytin-1 (Figs. 5f and 6, Supplementary Fig. S3). Thus, high Syncytin-1 levels, but low TGF-β resulted in cell–cell fusions, whereas high Syncytin-1 and high TGF-β resulted in cell proliferation. One proposal for the above regulation of cell proliferation and cell–cell fusion by TGF-β could be explained by the capability of TGF-β to increase the cholesterol efflux from cellular membranes and therefore modifying the membrane location and function of Syncytin-1 [48]. For example, cholesterol depletion of host cells resulted in resistance against syncytia formation by HIV-1 and HTLV1 [49].

In summary, this study showed an overexpression of Syncytin-1 in EnCa and pregestates linked with steroid hormone-induced cell proliferation and cAMP-stimulated cell–cell fusions. As a key regulatory gene in mediating both cell phenotypes in EnCa and placental cells, the steroid hormone inducible TGF-β1 and TGF-β3 were identified. Especially, the posttranscriptional knock-down of Syncytin-1 gene expression and concomitant functional silencing of induced cell proliferation and cell–cell fusion with two different siRNAs directed against the SU and TM unit proved the essential role of Syncytin-1 in these cellular processes. Although the fate or function of multinucleated cells in tumors is presently unknown, recently, it was shown that bone-marrow-derived cells fused with, e.g., hepatocytes and contributed to the development and maintenance of these cells [50]. Spontaneous cell–cell fusions between mouse tumor and normal cells in vivo, led to the hypothesis that these cells play a role in tumor progression and emergence of metastatic cells [51]. In addition, cell–cell fusions between human fibroblasts were found to occur due to a monkey retrovirus and oncogene expression, and resulted in transformed cells [52]. It is therefore plausible that cell–cell fusions between tumor cells and normal cells could change the phenotype and potency of normal cells to a cancer phenotype, which could be called “trans-carcinogenesis”. Syncytin-1 mediated cell–cell fusions between EnCa cells could also be involved in silencing the immune response, which was proposed for syncytiotrophoblasts protecting the fetus [17]. Escaping the immune response was shown for the envelope proteins of exogenous retroviruses, HERVs [15] and mouse tumor cells expressing MMLV envelope [53]. The immunosuppressive activity of the HERV-W envelope Syncytin-1 could involve modulation of the cytokine synthesis, inhibition of lymphocyte proliferation and natural killer cell action as shown with the related MMLV-envelope protein ([53] and references therein). Therefore, in addition to cell proliferation and cell–cell fusion, a role for Syncytin-1 in promoting tumor growth could also include controlling the immune system.

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Modification of gene expression of the small airway epithelium in response to cigarette smoking

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Abstract The earliest morphologic evidence of changes in the airways associated with chronic cigarette smoking is in the small airways. To help understand how smoking modifies small airway structure and function, we developed a strategy using fiberoptic bronchoscopy and brushing to sample the human small airway (10th–12th order) bronchial epithelium to assess gene expression (Affymetrix HG-U133A and HG-133 Plus 2.0 array) in phenotypically normal smokers (n=16, 25±7 pack-years) compared to matched nonsmokers (n=17). Compared to samples from large (second to third order) bronchi, the small airway samples had a higher proportion of ciliated cells, but less basal, undifferentiated, and secretory cells, and contained Clara cells. Even though the smokers were phenotypically normal, microarray analysis of gene expression of the small airway epithelium of the smokers compared to the nonsmokers demonstrated up- and downregulation of genes in multiple categories relevant to the pathogenesis of chronic obstructive lung disease (COPD), including genes coding for cytokines/innate immunity, apoptosis, mucin, response to oxidants and xenobiotics, and general cellular processes. In the context that COPD starts in the small airways, these gene expression changes in the small airway epithelium in phenotypically normal smokers are candidates for the development of therapeutic strategies to prevent the onset of COPD.
**Keywords** COPD · Smoking · Microarray

**Introduction**

Chronic obstructive pulmonary disease (COPD) associated with chronic cigarette smoking is characterized physiologically by limitation of expiratory airflow that, unlike asthma, is not reversed by pharmacologic intervention with bronchodilators [1–3]. The primary site of the airflow limitation is the small airways, defined as bronchi <2 mm in diameter [4–6]. While many affected individuals with COPD also have loss of elastic recoil and increased compliance secondary to destruction of central lobular alveoli, the initial site of the pathology in COPD is in the small airways [1–7]. Consistent with this concept, morphologic abnormalities are found in the small airways of cigarette smokers who are asymptomatic and have normal lung function [8–12]. Disease of the small airways is always a feature of COPD, independent of stage [13, 14], and the extent of small airway disease correlates with the extent of emphysema [13, 14].

Extensive data generated by many investigators supports the concept that the abnormalities of the small airways in COPD result from a combination of the toxic elements in cigarette smoke, a localized inflammatory host response, and biologic changes in the cells comprising the small airways, initially in the epithelium [1–4, 6, 7, 10, 14–17]. To help define the responses of the human airway epithelium to the stress of cigarette smoke, we along with others have employed microarray technology to assess the expression of the transcriptome of the large airways, using fiberoptic bronchoscopy and brushing to obtain pure populations of the epithelium of the second to third order bronchi [18–21]. While this approach has yielded valuable data regarding the responses of large airway epithelium to the stress of smoking, the large airways are not the initial site of airway injury in smokers [4–6]. This fact, and the knowledge that there are differences in the relative proportion of epithelial cell types in the small airways compared to the large airways (more ciliated cells, fewer goblet and basal cells) and the inclusion of a different cell type (Clara cells in the small, but not large, airways), leads to the question: What are the gene expression responses of the small airway epithelium to the stress of cigarette smoking?

To evaluate this question, we have developed methods utilizing fiberoptic bronchoscopy and brushing to sample the epithelium of small (10th to 12th order) airways of humans in high purity and in sufficient quantities to carry out microarray analysis. Assessment of the epithelial cell types recovered by small airway sampling demonstrated a cell composition consistent with small airways, including the presence of Clara cells, a cell type not present in the large airways. With this technology, we compared the expression of genes in the small airway epithelium of normal nonsmokers to that of phenotypically normal smokers with an average 25±7 pack-years of smoking, a smoking history that places these individuals at the cusp of risk for the development of smoking-induced lung disease [22–24]. Analysis of the microarray data demonstrated a large number of small airway epithelial genes that were significantly up- or downregulated in response to smoking. To place this in the context of the current concepts of pathogenesis of COPD, we have identified classes of genes previously implicated in the pathogenesis of COPD that our analysis demonstrated were significantly up- or downregulated in the small airway epithelium of smokers. While by no means complete, this subset of smoking-modulated genes provides a working list of potential targets for therapeutic intervention to prevent the development of COPD, and to assess the efficacy of therapies related to COPD.

**Materials and methods**

**Study population**

Normal nonsmokers and normal current cigarette smokers were recruited by posting ads in local newspapers. There were two groups: group A with n=11; 6 healthy smokers and 5 healthy nonsmokers, and group B with n=22; 10 healthy smokers and 12 healthy nonsmokers All 33 individuals were evaluated in the Weill Cornell NIH General Clinical Research Center under Institutional Review Board approved clinical protocols. All individuals were HIV-negative and determined to be phenotypically normal based on standard history, physical exam, complete blood count, coagulation studies, liver function tests, urine studies, chest X-ray, EKG, and pulmonary function tests (Supplemental Table 1). To verify smoking status, a complete smoking history was obtained and urine samples were evaluated for nicotine and cotinine, and venous blood was evaluated for carboxyhemoglobin. All chest X-rays and pulmonary function tests (spirometry, lung volumes, and diffusion capacity) were normal. The 16 normal smokers had a 25±7 pack-years smoking history, actively smoking 1.0±0.3 pack/day. There were no differences in age (p>0.2), sex (p>0.6), or race (p>0.7) among the smokers and nonsmokers.

**Sampling the airway epithelium**

Fiberoptic bronchoscopy was used to collect airway epithelial cells. After mild sedation was achieved with
Demerol and Versed, and routine anesthesia of the vocal cords and bronchial airways with topical lidocaine, the fiberoptic bronchoscope (Pentax, EB-1530T3) was positioned distal to the opening of the desired lobar bronchus. To obtain small airway epithelial cells, a 1.2-mm-diameter brush was advanced approximately 7 to 10 cm distally from the third order bronchial branching under fluoroscopic guidance (Supplemental Fig. 1). The distal end of the brush was wedged at about the 10th to 12th generation branching of the right lower lobe, and small airway epithelial cells were obtained by gently gliding the brush back and forth on the epithelium five to ten times in ten different locations in the same general area. The cells were detached from the brush by flicking into 5 ml of ice-cold bronchial epithelial basal cell medium (BEBM, Clonetics, Walkersville, MD, USA). An aliquot of 0.5 ml was used for differential cell count and to develop slides for immunohistochemistry studies (typically 2×10^4 cells per slide). The remainder (4.5 ml) was processed immediately for RNA extraction. To compare cell types obtained from sampling the small airways to the cell types obtained from brushing the large airways, samples of the large airway epithelium were obtained in the same individuals using 2.0-mm disposable brushes to sample the epithelium of second and third order bronchi in the right lower lobe as previously described [18–20]. All individuals tolerated the fiberoptic bronchoscopy well; radiologic and fluoroscopic evaluations after fiberoptic bronchoscopy with sampling of large and small airways showed no evidence of pneumothorax.

Morphology of airway epithelial cells

The total number of cells recovered by bronchial brushing was determined by counting on a hemocytometer. To quantify the percentage of epithelial and inflammatory cells and the proportions of ciliated, basal, secretory, and undifferentiated epithelial cells, aliquots of 2×10^4 cells were prepared by centrifugation (Cytospin 11, Shandon Instruments, Pittsburgh, PA, USA) and stained with Diff-Quik (Dade Behring, Newark, NJ, USA). Aliquots were also assessed by immunohistochemistry with antibodies directed against surfactant protein A (SPA, Lab Vision, Fremont, CA, USA) and Clara cell protein 10 (CC10, BioVendor, Candler, NC, USA). Cytospin preparations were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) at pH 7.4 for 20 min at 23°C. Incubation with anti-SPA and anti-CC10 was carried out overnight at 4°C; subsequently, cytospins were washed with PBS, followed by incubation with a secondary peroxidase-coupled antibody for 30 min at 23°C. The final step included incubation with a 3,3′-diaminobenzidine chromogenic substrate detection system (Dako, Carpinteria, CA, USA), which rendered positive cells into brown. All cytospins were counterstained with hematoxylin. Species and subtype-matched antibodies were used as negative controls.

To assess the cell populations by transmission electron microscopy, the brushed airway epithelial cells were suspended in BEBM medium, pelleted, fixed, stained, cut, and viewed on a JSM 100 CX-II electron microscope (JEOL, Peabody, MA, USA) operated at 80 kV as previously described [25]. Images were recorded on Kodak 4489 Electron Image Film (Electron Microscopy Sciences) and then digitized on an Epson Expression 3200 Pro Scanner at 800 dpi (Epson America, Long Beach, CA, USA).

RNA and microarray processing

The HG-U133A and the HG-U133 Plus 2.0 arrays (Affymetrix, Santa Clara, CA, USA), including probes representing ~22,000 and ~39,000 full-length human genes, respectively, were used to evaluate gene expression. Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA), yielding 2 to 4 μg from 10^6 cells. Quality control included an A260/A230 ratio of 1.7 to 2.3. First and second strand cDNA were synthesized from 6 μg (HG-U133A chip) or 3 μg (HG-U133 2.0 Plus) of RNA, in vitro transcribed, and fragmented using the recommended Affymetrix reagents and kits. The quality of the RNA labeling was verified by hybridization to a test chip, and only test chips with a 3′ to 5′ ratio of <3 were deemed satisfactory. Samples passing the quality control criteria were then hybridized to the HG-U133A or the HG-U133 Plus 2.0 array, processed by the fluids station to receive the appropriate reagents/washes, and then transferred to the scanner for duplicate scanning. The captured image data for HG-U133A arrays was processed using the Affymetrix Microarray Suite version 5 (MAS5) algorithm. Image data from the HG-U133 Plus 2.0 arrays was processed using MAS5 and also by the robust multi-array average (RMA) algorithm [26], using GeneSpring version 7.2 software (Agilent Technologies). MAS5 takes into account the perfect match and the mismatch values, while the RMA method utilizes only the perfect match values. MAS5-analyzed data were normalized using GeneSpring as follows: (1) per array, by dividing the raw data by the 50th percentile of all measurements; and (2) per gene, by dividing the raw data by the median of the expression level for the gene in all samples. RMA preprocessed data was normalized to the median measurement for the gene across all the arrays in the data set because the per array normalization step is included in this method.

Microarray data analysis

To determine the normal gene expression profile (the normal transcriptome) of the small airway epithelium in
healthy nonsmokers, RNA from the small airway epithelium of healthy nonsmokers was assessed for gene expression with the HG-U133 Plus 2.0 microarray. Expressed was defined as having an Affymetrix detection call of “Present” in >50% of the samples. The probe sets were grouped into functional categories, using the database from the Affymetrix NetAffx Analysis Center (http://www.affymetrix.com/analysis/index.affx) by the Gene Ontology (GO) Biological Processes classification.

Initial assessment of differentially expressed genes in small airway epithelium of smokers compared to nonsmokers was carried out in 11 healthy individuals (5 nonsmokers and 6 smokers, for convenience referred to as part A of the study; see below). To identify the categories of small airway epithelial genes up- and downregulated by smoking in these individuals, and to provide an overview of the relative fold changes of these genes by gene category relevant to the pathogenesis of COPD, microarray analysis was carried out using the Affymetrix HG-U133A microarray. Genes were considered significant if \( p < 0.05 \) and the fold change (up- or down-regulation) was >2-fold between the two groups. The fold change was calculated by dividing the geometric mean expression value in all smoker samples by the geometric mean value in nonsmoker samples. The genes were categorized according to the GO annotations, in categories relevant to COPD pathogenesis, and additional general categories, such as signal transduction and transcription. Based on our assessment of patterns of gene expression in small airways of healthy smokers vs healthy nonsmokers, and from the data in the literature regarding molecular pathways in airway epithelium previously implicated in the pathogenesis of COPD, we generated a list of categories of genes expressed in the small airway epithelium relevant to the pathogenesis of COPD, including cytokines/innate immunity, apoptosis, profibrotic, mucin, response to oxidants, antiproteases, and general cellular processes. From the preliminary data comparing genes up- and downregulated in the small airway epithelium of smokers to nonsmokers in the first 11 individuals studied, a total of 152 genes with known function were identified and placed into various categories. From this catalog of genes, we chose examples representing the genes with (1) the highest fold differences in each group; and (2) literature suggesting that the pathway that includes the gene may be involved in the pathogenesis of COPD.

We independently assessed the small airway epithelial gene expression in an entirely new group of healthy individuals (\( n = 22 \), 10 healthy smokers and 12 healthy nonsmokers, referred to as group B; see below) who shared similar phenotypic characteristics as the initial 11 individuals studied in group A. Assessment of the small airway epithelium gene expression of these new 10 healthy smokers vs 12 healthy nonsmokers (group B) was carried out with the newest generation Affymetrix chip, the HG-U133 Plus 2.0. Genes were considered significant if \( p < 0.05 \) and the fold change (up- or downregulation) was >1.5-fold between the two groups in both the MAS5 and the RMA-generated datasets. To limit the number of false positives, we applied the Benjamini and Hochberg false discovery rate multiple test correction to both the MAS5 and the RMA-generated datasets [27]. Fold change was calculated by dividing the geometric mean expression value in all smoker samples by the geometric mean expression value in nonsmoker samples. Similar to the assessment of gene expression in group A, the genes differentially expressed in group B were classified according to categories relevant to COPD pathogenesis as described above. All data was deposited at the Gene Expression Omnibus site (http://www.ncbi.nlm.nih.gov/geo/), a high-throughput gene expression/molecular abundance data repository curated by the National Center for Bioinformatics site [28]. The accession number for the HG-133A data set is GSE3320, and for the HG-U133 Plus 2.0 dataset is GSE4498.

**Cluster analysis**

Unsupervised classification of samples was carried out by hierarchical cluster analysis, by gene and by individual sample, using the standard correlation, with the GeneSpring software (Agilent Technologies), using the expression levels of the expressed genes (called present in at least one array by the MAS5 algorithm) and by genes (up- and downregulated) modulated by smoking obtained by assessment of gene expression in group B. The goal of the cluster using the significant genes was to obtain a graphical representation of general variability within this population.

**TaqMan RT-PCR**

TaqMan real-time reverse transcriptase (RT) PCR was carried out for eight nonsmokers and eight smokers from group B, using the same RNA samples that had been used for the microarray analysis. First strand cDNA was synthesized from 2 \( \mu \)g of RNA in a 100-\( \mu \)l reaction volume, using the TaqMan Reverse Transcriptase Reaction Kit (Applied Biosystems), with random hexamers as primers. The cDNA was diluted 1:100 or 1:50, and each dilution was run in triplicate wells. Five microliters were used for each TaqMan PCR reaction in 25 \( \mu \)l of final reaction volume, using premade kits from Applied Biosystems. Relative expression levels were calculated using the \( \Delta \Delta C_t \) method (Applied Biosystems), using ribosomal RNA as the internal control (Human Ribosomal RNA Kit, Applied Biosystems), and the average value for nonsmokers, as the calibrator. The rRNA probe was labeled with VIC and the probes for the genes of interest with FAM. The PCR reactions were run in an Applied
Individuals in group B was also used for trix HG-U133 Plus 2.0. Small airway epithelium RNA from was assessed with the newest microarray chip, the Affymetrix HG-U133A microarray. Gene expression in airway samples from individuals in nonsmokers with the Affymetrix HG-U133A microarray demonstrated that airway epithelial cells from small airways, to Clara cells in samples obtained from small airways, to determine the presence of Clara cells in human small airways [35]. Because Clara cells are found only in airways <3 mm in diameter [35–37], the observation of Clara cells in the small airway samples confirms that the samples were, in fact, from the small airways.

As further evidence that the small airway epithelium was being sampled, independent of smoking status, gene expression of airway epithelium from small airways (evaluated in the individuals in group A) revealed the expression of surfactant apoprotein A2, surfactant apoprotein B, and surfactant apoprotein C genes (Fig. 2). Consistent with prior studies of surfactant gene expression in the small airway epithelium [34–36], the surfactant apoprotein genes were not expressed in the large airway epithelial samples.

Genes expressed in the small airway epithelium of normal nonsmokers

To determine the normal small airway epithelium transcriptome, RNA from small airway epithelial cells from the 12 healthy nonsmokers from group B was assessed with the HG-U133 Plus 2.0 microarray. In this analysis, 27,244 of the total 54,675 probe sets were “Present” or expressed according to the MAS5 algorithm in >50% of the samples. These genes were functionally grouped into 14 different

Results

Study population

The study individuals were divided into two groups (A and B). Airway epithelial samples from individuals in group A (n=11; 6 healthy smokers and 5 healthy nonsmokers) were used to establish the morphologic differences between large and small airway epithelium, to determine the presence of Clara cells in samples obtained from small airways, to demonstrate that airway epithelial cells from small airways but not the large airways expressed surfactant apoproteins-related genes, and to carry out preliminary assessment of the differences in gene expression among smokers compared to nonsmokers with the Affymetrix HG-U133A microarray chip. Gene expression in airway samples from individuals in group B (n=22; 10 healthy smokers and 12 nonsmokers) was assessed with the newest microarray chip, the Affymetrix HG-U133 Plus 2.0. Small airway epithelium RNA from individuals in group B was also used for TaqMan RT-PCR confirmation of a selected group of differentially expressed genes among smokers vs nonsmokers.

Sampling of the small airway epithelium

From a total of 5 to 10×10^6 epithelial cells, more than 95% of cells recovered from small and large airways from smokers and nonsmokers were epithelial (Table 1). The percentage of inflammatory cells in the large and small airways of smokers did not differ from that of the nonsmokers (p>0.4, both comparisons). Independent of smoking history, albeit low (≤5%, both large and small airways), the percentage of inflammatory cells in small airways evaluated in group A was higher than in large airways (p<0.005). Less than 1% of cells recovered from large and small airways were squamous cells in both smokers and nonsmokers (p>0.7). Assessment with Diff-Quik stain of the airway epithelial cells identified the four main epithelial cell types present in the human airways [ciliated, basal, undifferentiated, and secretory [29–33] in both the large and small airways (Table 1 and Fig. 1a)]. Evaluation by immunohistochemistry with SPA antibody [34] demonstrated the presence of Clara cells only in airway epithelial cells obtained from the small airways, not from large airways (Fig. 1b,c).

Assessment of the large and small airway epithelial cell populations by transmission electron microscopy demonstrated cells typical of Clara cells only in the small airway epithelial cell populations (Fig. 1d). These cells had 1 to 2 μm dense granules in the apical cytoplasm and contained microvilli, but not cilia, typical of ultrastructural descriptions of Clara cells in human small airways [35]. Because Clara cells are found only in airways <3 mm in diameter [35–37], the observation of Clara cells in the small airway samples confirms that the samples were, in fact, from the small airways.

Consistent with prior morphologic studies describing the composition of airway epithelial cells in the human lung, the small airways had a higher proportion of ciliated cells than large airways (nonsmokers p<0.001, smokers p<0.001). In contrast, the large airways had higher proportion of basal (nonsmokers p<0.001, smokers p<0.001), undifferentiated (nonsmokers p<0.001, smokers p<0.001), and secretory (nonsmokers p<0.04, smokers p<0.04) cells than small airways (Table 1).

Nonmicroarray-related statistical analyses

Comparison of the percentage cell types and demographic parameters in the nonsmokers and smokers was performed by two-tailed Student’s t test. A two-way ANOVA with smoking status (smokers vs nonsmokers) and method (microarray vs TaqMan) as independent factors was carried out using StatView version 5.0 (SAS Institute) to demonstrate that smoking was significant but the methodology was not, therefore confirming the agreement between the two methodologies.
categories. Forty percent, representing 10,935 probe set IDs, were classified as unknown function and were not used to generate the data on the distribution of types of genes expressed. The remaining genes were classified in the general biological processes categories. The largest categories were transcription, transport, metabolism, signal transduction, followed by cell cycle, apoptosis, and cell adhesion; other categories included differentiation, immune response, proteolysis, electron transport, cell growth, and cell signaling related genes (Fig. 3).

Differentially expressed genes in the small airway epithelium of phenotypically normal smokers compared to normal nonsmokers

Relevant to the pathogenesis of COPD, assessment of gene expression in the small airway epithelium of smokers compared to nonsmokers showed a significant up- and downregulation of several genes in various functional categories (Table 2, Figs. 4 and 5). Initial assessment of gene expression in a small number of individuals (group A, n=11, 6 smokers vs 5 nonsmokers) demonstrated 152 genes differentially expressed, 103 genes upregulated, and 49 genes downregulated in several functional categories in the small airway epithelium of healthy smokers compared to nonsmokers (Supplemental Table 2). Of these 152 genes, 133 genes were of known function and were grouped into biologically relevant categories. Based on the assessment of the small airway gene expression and a review of the molecular pathways shown in the literature to be related to the pathogenesis of COPD, we chose the most relevant six of these categories, to generate a representative “small airway epithelial smoking-induced phenotype.” These categories included cytokine/innate immunity, apoptosis, profibrotic, response to oxidants and xenobiotics, antiproteases, and general cellular processes (Supplemental Table 3).

After the initial assessment of differential gene expression in the first group of healthy individuals studied, we sought to verify these changes by studying a larger group of healthy individuals (group B, n=22, 10 smokers vs 12 nonsmokers). It is interesting to note that consistent with the initial assessment using the HG-U133A chip (group A), genes in similar categories were differentially expressed in small airway epithelium of healthy smokers compared to nonsmokers assessed with the HG-U133 Plus 2.0 chip (group B; Table 2). The group B assessment, which was subject to a more rigorous analysis (see “Materials and methods”), demonstrated a more restricted number of genes up- or downregulated [118 genes, 48 upregulated and 70 downregulated (Supplemental Table 4)] compared to the initial gene list of 152 observed in the initial analysis of group A (Supplemental Table 2). The 118 genes differentially

---

Table 1 Comparison of the cell types removed by brushing the airway epithelium

<table>
<thead>
<tr>
<th></th>
<th>Group A&lt;sup&gt;a&lt;/sup&gt;</th>
<th></th>
<th>Group B&lt;sup&gt;b&lt;/sup&gt;</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Small airways</td>
<td>Large airways</td>
<td>Small airways</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Nonsmokers</td>
<td>Smokers</td>
<td>Nonsmokers</td>
<td>Smokers</td>
</tr>
<tr>
<td>Total number of cells recovered (x10&lt;sup&gt;6&lt;/sup&gt;)</td>
<td>10±6</td>
<td>7±4</td>
<td>7±2</td>
<td>9±3</td>
</tr>
<tr>
<td>Percentage of total cells</td>
<td>96±4</td>
<td>96±4</td>
<td>98±5</td>
<td>98±1</td>
</tr>
<tr>
<td>Epithelial</td>
<td></td>
<td></td>
<td>99±1</td>
<td>97±1</td>
</tr>
<tr>
<td>Inflammatory</td>
<td>4±3</td>
<td>4±4</td>
<td>1±1</td>
<td>1±1</td>
</tr>
<tr>
<td>Squamous</td>
<td>0</td>
<td>0</td>
<td>1±1</td>
<td>1±1</td>
</tr>
<tr>
<td>Percentage of epithelial cells</td>
<td>80±5</td>
<td>75±6</td>
<td>50±2</td>
<td>43±3</td>
</tr>
<tr>
<td>Ciliated</td>
<td></td>
<td></td>
<td>78±7</td>
<td>75±10</td>
</tr>
<tr>
<td>Secretory&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4±1</td>
<td>4±3</td>
<td>9±4</td>
<td>10±2</td>
</tr>
<tr>
<td>Basal</td>
<td>5±3</td>
<td>8±2</td>
<td>20±3</td>
<td>27±4</td>
</tr>
<tr>
<td>Undifferentiated</td>
<td>8±2</td>
<td>9±3</td>
<td>21±4</td>
<td>20±2</td>
</tr>
<tr>
<td>Clara&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2±1</td>
<td>4±1</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

ND: Not determined

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<sup>a</sup>Large airway epithelial cells were collected from second to third generation bronchi and small airway epithelial cells were collected from airways at the 10th to 12th generation under fluoroscopic guidance by advancing the brush 7 to 10 cm beyond the third generation bronchi. Cytospin preparations were stained with Diff-Quick to determine the percentage of epithelial vs inflammatory cells, and identification of ciliated, secretory, basal, and undifferentiated epithelial cells. The data is presented as mean±SD for each cell type in small and large airways for nonsmokers (n=5) and smokers (n=6).

<sup>b</sup>Small airway epithelial cells were collected from airways at the 10th to 12th generation under fluoroscopic guidance as described in table note a. The data is presented as mean±SD for each cell type in small airways for nonsmokers (n=12) and smokers (n=10).

<sup>c</sup>Secretory cells (percentage of total epithelial cells) include Clara cells and non-Clara cells.

<sup>d</sup>Immunostaining of large and small airway epithelial cells with SPA antibody with hematoxylin counterstaining was used to quantify Clara cells within the secretory subset (data as percentage of total epithelial cells); percentage of Clara cells were not determined for group B.
expressed in smokers vs nonsmokers in group B included genes in the categories cytokine/innate immunity, apoptosis, response to oxidants and xenobiotics, proteases/antiproteases, and general cellular processes (Supplemental Table 5). A comparison of the results of the HG-U133 2.0 Plus analysis with those from the HG-U133A microarray showed
Functional categories of genes expressed in the small airway epithelium of normal nonsmokers. The pie chart shows the different functional categories of the small airway epithelium transcriptome in 12 healthy nonsmokers; small airway epithelium gene expression was assessed with the Affymetrix HG-U133 Plus 2.0 microarray chip. The distribution data represents the gene expression in nonsmokers with unknown function and were not used for the final analysis

Expression of genes potentially relevant to the pathogenesis of COPD

Independent assessment of gene expression by RMA and MAS5 demonstrated that the small airway epithelium of smokers vs nonsmokers downregulated several immune-related genes. These genes included the interleukin-4 (IL4) receptor gene ($p<0.002$), the chemokine (C-X-C motif) ligand 1 ($p<0.02$), also known as fractalkine, and the spondin 2 ($p<0.04$); these genes are involved in many inflammatory functions in human airways [38–41] (Table 2 and Fig. 5a).
We also observed differentially expressed apoptosis-related genes: consistent with prior studies demonstrating upregulation of pirin, a proapoptotic gene, in the large airways of smokers \[20, 21, 42–45\], we observed upregulation of pirin in the small airway epithelium of healthy smokers compared to nonsmokers \(p < 0.03\; \text{Table 2 and Fig. 5b}\). Similarly, the proapoptosis-related genes HIV-Tat interactive protein 2, 30 kDa gene also known as TIP30,

### Table 2: Examples of genes differentially expressed in the small airway epithelium in nonsmokers and smokers in functional categories that are relevant for the pathogenesis of COPD

<table>
<thead>
<tr>
<th>Category</th>
<th>Gene</th>
<th>Gene symbol</th>
<th>References in literature that suggested pathways relevant for COPD pathogenesis&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Group A</th>
<th>Group B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytokine/ innate immunity</td>
<td>Chemokine (C-X3-C motif) ligand 1</td>
<td>CX3CL1</td>
<td>[7, 16, 38–41]</td>
<td>−2.97</td>
<td>&lt;0.040</td>
</tr>
<tr>
<td>Apoptosis</td>
<td>Pirin</td>
<td>PIR</td>
<td>[42–48, 56–61]</td>
<td>2.78</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Growth arrest and DNA damage-inducible, beta</td>
<td>GADD45B</td>
<td>[42–48, 56–61]</td>
<td>−2.25</td>
<td>&lt;0.043</td>
</tr>
<tr>
<td>Response to oxidants and xenobiotics</td>
<td>Cytochrome P450, family 1, subfamily B, polypeptide 1</td>
<td>CYP1B1</td>
<td>[7, 16, 21, 49, 62]</td>
<td>17.69</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Aldo-keto reductase family 1, member B10</td>
<td>AKR1B10</td>
<td>[7, 16, 21, 49, 62]</td>
<td>11.73</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Aldehyde dehydrogenase 3 family, member A1</td>
<td>ALDH3A1</td>
<td>[7, 16, 21, 49, 62]</td>
<td>6.63</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Alcohol dehydrogenase 7</td>
<td>ADH7</td>
<td>[7, 16, 21, 49, 62]</td>
<td>6.24</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Glutathione peroxidase 2</td>
<td>GPX2</td>
<td>[7, 16, 21, 49, 62]</td>
<td>5.16</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>NAD(P)H dehydrogenase, quinone 1</td>
<td>NQO1</td>
<td>[7, 16, 21, 49, 62]</td>
<td>4.41</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Aldo-keto reductase family 1, member C3</td>
<td>AKR1C3</td>
<td>[7, 16, 21, 49, 62]</td>
<td>3.09</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>General cellular processes</td>
<td>ubiquitin carboxyl-terminal esterase L1</td>
<td>UCHL1</td>
<td>[63, 64]</td>
<td>11.75</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Group A includes 11 healthy individuals (5 healthy nonsmokers and 6 healthy smokers) in whom small airway epithelial gene expression was assessed with the Affymetrix HG-U133A gene chip. Group B includes 22 healthy individuals (12 nonsmokers and 10 smokers) in whom small airway epithelial gene expression was assessed with the Affymetrix HG-U133 Plus 2.0 gene chip; for group B, expression values were independently generated using RMA and MAS5. Genes were considered expressed when they had Affymetrix present “P” calls in >50% of any given group of samples (nonsmokers or smokers) in both group A and group B study individuals.

<sup>a</sup>These references directly implicate the specific genes in some cases, or implicate pathways in which these genes are involved.

<sup>b</sup>Smokers (S) vs nonsmokers (NS) fold change was calculated by dividing the average expression value in the smokers by the average expression value in the nonsmokers.

<sup>c</sup>p values were calculated using the Welch \(t\) test (assuming unequal variances) using the Affymetrix HG-U133A gene chip; expression values were generated using MAS5.

<sup>d</sup>p values were calculated using the Welch \(t\) test (assuming unequal variances) using the Affymetrix HG-U133A Plus 2.0 gene chip; expression values were generated using RMA with Benjamini–Hochberg correction.

*Same as table note e except expression values were generated using MAS5.*

We also observed differentially expressed apoptosis-related genes: consistent with prior studies demonstrating upregulation of pirin, a proapoptotic gene, in the large airways of smokers [20, 21, 42–45], we observed upregulation of pirin in the small airway epithelium of healthy smokers compared to nonsmokers \(p < 0.03\; \text{Table 2 and Fig. 5b}\). Similarly, the proapoptosis-related genes HIV-Tat interactive protein 2, 30 kDa gene also known as TIP30,
and the homeodomain interacting protein kinase genes [46, 47] were upregulated in smokers compared to nonsmokers ($p<0.03$). In contrast, the growth arrest and DNA damage-inducible, $\beta$-related gene, another proapoptotic gene [48], was downregulated in small airway epithelium of healthy smokers ($p<0.03$).

In agreement with prior gene expression studies in large airways of phenotypically normal smokers [18, 21], several oxidative stress and xenobiotic-related genes were differentially expressed in the small airway epithelium of smokers compared to nonsmokers (Table 2, Fig. 4, Fig. 5c). For example, the aldo-keto reductase family 1, member C1, and member C2 gene, the aldehyde dehydrogenase 3 family, member A1 gene, and the glutathione peroxidase 2 gene were upregulated in small airway epithelium of smokers compared to nonsmokers ($p<0.002$). Similarly, in the category of xenobiotics metabolism, the cytochrome P450, family 1, subfamily B, polypeptide 1 gene was upregulated in the small airway epithelium of healthy smokers compared to nonsmokers ($p<0.04$; for the entire list of genes in this category see Supplemental Tables 2 and 4).

Several genes involved in general cellular processes were differentially expressed in small airway epithelium of healthy smokers; for example, the ATPase H$^+$ transporting, lysosomal V0 subunit a isoform 4, a gene involved in acidification of intracellular organelles for various intracellular processes such as protein sorting, receptor mediated endocytosis, and synaptic vesicle proton gradient generation, was upregulated in healthy smokers ($p<0.03$). In contrast, the coiled-coil alpha-helical rod protein 1 (CCHCR1) gene, which is involved in metabolism and cell differentiation, the forkhead box A2 (FOXA2) gene, important in cell differentiation, and the frizzled homolog 8 (Drosophila) FZD8 gene, involved in signal transduction, were downregulated ($p<0.03$; Fig. 5d and Table 2).

**TaqMan RT-PCR**

Independent analysis of differentially expressed genes in small airway epithelium of smokers vs nonsmokers in group B by real-time quantitative TaqMan RT-PCR confirmed the findings demonstrated by microarray assessment in a selected group of nine genes (Fig. 6). A two-way ANOVA with smoking status (smokers vs nonsmokers) and method (microarray vs TaqMan) as independent factors confirmed that expression levels of these nine genes were significantly affected by smoking status ($p<0.05$, all cases), and that method was not a significant factor ($p>0.2$, all cases).

![Gene expression analysis](image-url)
While COPD associated with chronic cigarette smoking eventually involves all levels of the airways, the earliest smoking-induced changes are in the small airway epithelium [4–6, 13, 14]. To begin to understand the responses of the small airway epithelium to the stress of cigarette smoking, we developed a strategy with fiberoptic bronchoscopy and airway brushings to obtain highly pure epithelial cells from human small airways, and analyzed the epithelial cells gene expression with microarray technology.

The small airway epithelial samples from healthy smokers and nonsmokers differed in composition from that of large airway samples, with the small airways having more ciliated cells, and less undifferentiated, basal, and secretory cells than the large airways. Consistent with the known composition of the secretory subset of airway epithelium in the small airways, epithelial cells of the small, but not large airways, demonstrated the presence of Clara cells and expression of surfactant apoprotein genes irrespective of smoking status.

Initial assessment of small airway epithelium gene expression in a small group (five nonsmokers and six smokers, group A) of healthy individuals with the HG-133A microchip array demonstrated 152 differentially expressed genes.

**Fig. 5** Examples of genes up- and downregulated in the small airways of smokers compared to nonsmokers. The data is based on 10 healthy smokers compared to 12 healthy nonsmokers assessed with the Affymetrix HG-U133 Plus 2.0 microarray chip, with Benjamini-Hochberg correction for multiple comparisons. The abscissa shows the specific genes; the ordinate shows the normalized gene expression levels. Each symbol represents an individual. Open symbols represent nonsmokers (NS); closed symbols represent smokers (S). p values are shown below each gene symbol. **a** Expression of cytokine/innate immune-related genes. The genes shown are the IL-4 receptor (IL4R) gene, the spondin 2 (SPON2) gene, the sushi domain containing four (SUSD4) genes, and the chemokine (C-X3-C motif) ligand 1 gene, also known as fractalkine (CX3CL1). **b** Expression of apoptosis-related genes. The genes shown are the HIV-Tat interactive protein 2 (HTATIP2) gene; the pirin (PIR) gene, the growth arrest and DNA damage-inducible, β-related (GADD45B) gene; and homeodomain interacting protein kinase 2 (HIPK2) gene. **c** Examples of expression of oxidative stress and xenobiotic-related genes. Shown are the cytochrome P450, family 1, subfamily B, polypeptide 1 (CYP1B1) gene; the aldo-keto reductase family 1, member C1 (AKR1C1) gene; the aldo-keto reductase family 1, member C2 (AKR1C2) gene; and the glutathione peroxidase 2 (GPX2) gene. **d** Examples of expression of general processes and differentiation genes. Shown are the ATPase H+ transporting, lysosomal V0 subunit a isoform 4 (ATP6V0A4) gene; the coiled-coil alpha-helical rod protein 1 (CCHCR1) gene, which is involved in metabolism and cell differentiation; the forkhead box A2 (FOXA2) gene, important in cell differentiation; and the frizzled homolog 8 (Drosophila) (FZD8) gene, involved in signal transduction.
expressed genes (103 upregulated, 49 downregulated) in response to cigarette smoking; 133 of which are of known function and belong to several functional categories. After these initial assessments, we carried out an independent study in an entirely new and larger group of healthy smokers vs nonsmokers (12 nonsmokers vs 10 smokers, group B). This analysis, assessed with the HG-U133 Plus 2.0 microarray chip, with MAS5 and RMA (independently), and with Benjamini–Hochberg correction for false discovery rate, demonstrated 118 differentially expressed genes in healthy smokers vs nonsmokers.

Based on review of the literature on the different molecular pathways implicated in the pathogenesis of COPD involving the airway epithelium, and on the assessment of the differentially expressed genes in the small airways of smokers compared to nonsmokers, we developed a working list of genes divided into categories relevant to the pathogenesis of COPD. The categories of genes generated on the assessment of group A and group B included cytokine/innate immunity, apoptosis, response to oxidants and xenobiotics, proteolysis/antiproteases, and general cellular processes. These genes represent a snapshot of the early molecular changes in the small airway epithelium of healthy individuals who actively smoke, and are therefore at risk to develop COPD. These genes may represent COPD susceptibility genes and protective genes, and the risk for COPD may depend on an individual’s specific pattern of combined expression for susceptibility.

![Graph showing normalized expression levels of various genes](image_url)

**Fig. 6** Confirmation of microarray results with TaqMan real-time RT-PCR. Expression levels of six genes upregulated by smoking and three genes downregulated by smoking on initial assessment by microarray analysis (RMA-based) with the Affymetrix HG-U133 Plus 2.0 chip were confirmed with TaqMan real-time RT-PCR. To allow direct comparisons of values obtained using the two independent methods, TaqMan expression levels were normalized by dividing individual values by the median expression level of all nonsmokers and smokers for that method, as was done for microarray analysis. Relative expression levels (ordinate) are shown for six genes upregulated by smoking, as follows: four genes involved in the response to oxidative stress or xenobiotics, the NAD(P)H dehydrogenase, quinone 1 (NQO1) gene; the aldehyde dehydrogenase 3 family, member A1 (ALDH3A1) gene; the aldo-keto reductase family, member C3 (AKR1C3) gene; the alcohol dehydrogenase 7 (ADH7) gene; two genes involved in apoptosis, the pirin (PIR); and the homeodomain interacting protein kinase 2 (HIPK2) genes; and for three genes downregulated by smoking: the cyclin-dependent kinase inhibitor 1C (CDKN1C) gene, also known as p57 or Kip2, a cell cycle arrest protein; the transcription factor forkhead box A2 (FOXA2) gene, involved in transcription of the surfactant genes; and the chemokine (C-X3-C motif) ligand 1 (CX3CL1). A two-way ANOVA with smoking status (smokers vs nonsmokers) and method (microarray vs TaqMan) as independent factors confirmed that expression levels of these nine genes were significantly affected by smoking status ($p<0.05$, all cases), and that method was not a significant factor ($p>0.2$, all cases).
and protective genes. Furthermore, these genes may represent only a subset of the genes underlying the pathogenesis of COPD.

Small airways, smoking, and COPD

The small airways (<2 mm) represent the main site of airway obstruction in individuals with COPD [4–6, 13, 14]. Asymptomatic smokers display evidence of small airway inflammation; for example, Niewoehnner et al. [8] studied the lungs of 19 young smokers and 20 nonsmokers, and demonstrated that the small airways of smokers had definitive pathologic abnormalities with denuded epithelium and increased number of mural inflammatory cells. These data are consistent with the concept that the small airways represent the earliest site of smoking-induced structural changes before the development of COPD [4–6, 8–14]. It is interesting to note that the extent of small airway disease correlates with the extent of alveolar destruction [13, 14].

Small airway epithelial smoking-induced phenotype

The airway epithelium plays an important role in controlling many airway functions and is capable of up- and downregulating genes in several categories as well as producing and secreting mediators important in several aspects of airway function [16–18, 21, 33, 49–54]. These genes and mediators, among others, include cytokines, chemokines, apoptosis-related, profibrotic-related, oxidative stress-related, proteolysis/antiproteases-related, mucin-related, and genes related to general processes [16–18, 21, 33, 49–54]. In this context, persistent activation of the small airway epithelium with the insult of cigarette smoke leads to an alteration of the “resting” state of the small airway epithelium with up- and downregulation of genes in different categories as observed in our study population.

The cytokine, innate, and immunomodulatory responses of the small airway epithelium to the insult of cigarette smoke play an important role, over time, in the eventual development of the small airway inflammatory component characteristic of individuals with established COPD. The small airways from healthy smokers demonstrate inflammation, as do asymptomatic smokers, and individuals at different COPD stages [Global Initiative for Chronic Obstructive Lung Disease (GOLD) stages 1–4] [1, 2, 8–12]. It is interesting to note that although some degree of overlap in small airway inflammation is observed among asymptomatic smokers and individuals with COPD GOLD stages 0–3 [12], increased numbers of CD8⁺ T lymphocytes are observed only in smokers who develop COPD [11]. This suggests that the cytokine/innate immune response seems to be nonspecific at earlier stages of smoking, but over time, for those individuals who develop COPD, the immune response undergoes a more specific change, which results in increased accumulation of CD8⁺ T lymphocytes.

We found downregulation of the IL4 receptor, a mediator of several proinflammatory functions in human airways [38], and downregulation of chemokine (C-X3-C motif) ligand 1 (CX3CL1), which is involved in cell adhesion and recruitment of monocytes and T lymphocytes cells [39, 40]. It is interesting to note that microarray analysis of lung tissue from individuals with COPD demonstrated upregulation of the CX3CL1 gene in individuals with later stage COPD compared to individuals with early COPD [55]. Although lower mRNA levels do not necessarily reflect lower protein levels, it can be speculated that the downregulation of CX3CL1 in our study suggests that phenotypically healthy smokers attempt to maintain a balance in the inflammatory response in the small airways by suppressing signals that could potentially injure the epithelium.

The role of apoptosis in the pathogenesis of COPD is well recognized, and increased apoptosis of airway epithelial cells from individuals with COPD was documented even after cessation of smoking [56–61]. In our study, assessment of the small airway epithelium of healthy smokers showed smoking-related modulation of several proapoptotic genes [42–48], suggesting ongoing attempts of an “apoptosis balance” in the small airway epithelium of phenotypically healthy smokers.

It is well recognized that the oxidative stress of cigarette smoking plays an important role in the pathogenesis of COPD [49, 62]. We observed upregulation of several oxidative stress-related and xenobiotic genes in the small airway epithelium. This suggests that similar to studies of increased expression of oxidative stress-related genes in the large airway epithelium of healthy smokers [18, 21], the small airway epithelium responds to the insult of cigarette smoking by upregulating several oxidative stress-related and xenobiotic genes.

Several genes relevant to general cellular processes were upregulated, consistent with the increased energy expenditure observed in healthy smokers and in individuals with COPD [63, 64].

Implications for the understanding and treatment of COPD

Assessment of the molecular changes of the small airway epithelium in healthy smokers is relevant to establishing patterns of gene expression for comparison with the gene expression in small airways of individuals at various stages of COPD. The differential gene expression observed in the small airway epithelium of healthy smokers represents the initial deviations of gene expression observed in the main site of potential disease in individuals at risk for COPD.
This study may help in the identification of novel genes not related to already known mechanisms of COPD pathogenesis. Assessment of the expression of these genes in the small airway epithelium of individuals with COPD should be useful in identifying mechanisms relevant to the pathogenesis of COPD and potential new therapeutic targets for intervention.

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References


Upstream transcription factor 1 gene polymorphisms are associated with high antilipolytic insulin sensitivity and show gene–gene interactions

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Abstract Upstream transcription factor 1 (USF1) regulates the expression of many genes involved in lipid and glucose metabolism, among them genes regulating lipolysis. USF1 specifically regulates the expression of the hormone-sensitive lipase gene (HSL) in adipocytes and the hepatic lipase gene (LIPC) in the liver, which was found to be involved in liver fat accumulation. The usf1s1 C>T and usf1s2 G>A single-nucleotide polymorphisms (SNPs) in USF1 are associated with increased in vitro catecholamine-induced lipolysis in adipocytes. We investigated first whether SNPs in USF1 affect the lipolysis-suppressing action of insulin in vivo, and second, whether they interact with the −60C>G SNP in HSL on lipolysis and the −514C>T SNP in LIPC on liver fat. The usf1s1 C>T and usf1s2 G>A SNPs, together with the SNPs in HSL and LIPC, were determined in 407 Caucasians. Lipolysis was estimated as a change in free fatty acid (FFA) levels from baseline to 2 h of a 75-g oral glucose tolerance test (OGTT). Fifty-four subjects had data from a euglycemic hyperinsulinemic clamp with calculation of antilipolytic insulin sensitivity. Subjects carrying the minor alleles (T of usf1s1 and A of usf1s2) had lower 2 h FFA (p = 0.01) and a larger decrease in FFA concentrations during the OGTT (p = 0.02). Antilipolytic insulin sensitivity was higher in these individuals (p = 0.03). No interaction of the usf1s1 C>T and usf1s2 G>A SNPs with the −60C>G SNP in HSL on antilipolytic insulin sensitivity was detected. Liver fat, measured by 1H magnetic resonance spectroscopy, was elevated only in subjects who were both homozygous for the major alleles of usf1s1 and usf1s2 and carriers of the T allele of the −514C>T SNP in LIPC (p = 0.01). In
conclusion, subjects carrying the T allele of SNP usf1s1 and the A allele of SNP usf1s2 have a higher antilipolytic insulin sensitivity. Moreover, both SNPs may interact with the −514C>T SNP in LIPC to determine liver fat.

Keywords USF1 · Diabetes · Obesity · Glycerol

Introduction

Upstream transcription factor 1 (USF1) and related upstream transcription factor 2 (USF2) are ubiquitously expressed transcription factors that were found to be important for the regulation of expression of a variety of genes involved in lipid and glucose metabolism [1].

In adipocytes, USFs regulate gene expression of the rate-limiting enzyme of lipolysis, the hormone-sensitive lipase (HSL) [2], and mediate the insulin-responsive expression of acetyl-CoA carboxylase [3]. These data indicate an important role for USF1 in coordinating lipid metabolism in response to glucose and insulin levels. However, hitherto only few data about the effect of USFs in humans exist. Two recent studies showed a strong association of familial combined hyperlipidemia with the single-nucleotide polymorphisms (SNPs) usf1s1 (rs3737787) and usf1s2 (rs2073658) within the gene coding for USF1 (USF1) [4, 5]. Among them, the SNP usf1s2 was found to be functionally relevant [6]. In another study, three other SNPs, but at least two of them in almost complete linkage disequilibrium with the SNPs usf1s1 and usf1s2, were tested. Significant haplotype associations with peak glucose after an oral glucose tolerance test (OGTT) and interactions with HSL −60C>G on triglycerides were reported [7]. Hoffstedt et al. [8] found that SNPs usf1s1 and usf1s2 are associated with increased in vitro catecholamine-induced lipolysis in subcutaneous adipocytes from obese women. More recently, SNPs in USF1 were found to be associated with cardiovascular disease in women [9].

Among other genes [1, 10–14] in hepatocytes, USFs control the expression of hepatic lipase [15]. Hepatic lipase is a liver-specific enzyme that controls hepatic lipoprotein metabolism [16–18]. The −514C>T SNP of the hepatic lipase gene (LIPC) is associated with decreased plasma hepatic lipase activity and was found to affect total- and high-density lipoprotein cholesterol levels [19–21]. The −250G>A substitution in the promoter of LIPC, which is in almost complete linkage disequilibrium with the −514C>T variant, was associated with increased insulin resistance [22], reduced glucose tolerance [23], and conversion to type 2 diabetes in the Finnish Diabetes Prevention Study [24, 25]. We have recently found that the −514C>T SNP of LIPC is associated with high liver fat and low insulin sensitivity [26].

In the present study, we addressed the following questions: first, whether the SNPs usf1s1/usf1s2 are associated with insulin-mediated suppression of lipolysis as assessed by the decrease in free fatty acid (FFA) concentrations during an OGTT in a large cohort of nondiabetic subjects. To directly assess the lipolysis-suppressing action of insulin, we additionally determined the associations of the SNPs with whole-body antilipolytic insulin sensitivity in subjects who underwent a euglycemic hyperinsulinemic clamp. Second, because USFs regulate the expression of HSL in adipocytes, we determined whether the SNPs usf1s1/usf1s2 interact with the −60C>G SNP in HSL on lipolysis. Third, because USFs control the expression of LIPC in the liver and because the −514C>T SNP is associated with the accumulation of fat in the liver, we tested whether the SNPs usf1s1/usf1s2 interact with the −514C>T SNP in LIPC on liver fat and insulin sensitivity.

Material and methods

Subjects

We analyzed data from a total of 407 nondiabetic Caucasians from the southern part of Germany, who participated in an ongoing study to investigate the pathophysiology of type 2 diabetes. All subjects underwent a 75-g OGTT. At a random time point, 54 consecutive participants who agreed to undergo more invasive procedures underwent additionally a euglycemic hyperinsulinemic clamp with direct measurements of antilipolytic insulin sensitivity [27, 28]. The participants did not take any medication known to affect glucose tolerance or insulin sensitivity. They were considered healthy according to a physical examination and routine laboratory tests. All studies were done after a 10-h overnight fast, and the subjects were asked to refrain from smoking for the same period. Informed written consent was obtained from all participants, and the local medical ethics committee had approved the protocol.

Body composition and body fat distribution

Body composition was measured by bioelectrical impedance as percentage body fat. BMI was calculated as weight divided by the square of height (kilogram per square meter). Waist circumference was measured in the supine position as an index of body fat distribution.

OGTT

All subjects underwent an OGTT. At 8 a.m., they ingested a solution containing 75 g of dextrose. Venous blood samples were obtained at 0, 30, 60, 90, and 120 min for de-
termination of plasma glucose and insulin concentrations. Insulin sensitivity was calculated from glucose and insulin values during the OGTT as proposed by Matsuda and DeFronzo [29]. Serum FFAs, triglycerides, and total LDL- and HDL-cholesterol were measured at 0 min and FFA levels also at 2 h of the OGTT. Sensitivity to insulin as an antilipolytic hormone was calculated during the OGTT as the suppression of serum FFAs between 0 and 2 h as previously reported [30, 31].

Three-step and standard euglycemic hyperinsulinemic clamp

The clamp procedures were previously reported [27, 28]. In brief, using data on plasma glycerol appearance from a three-step and a standard euglycemic hyperinsulinemic clamp, an insulin sensitivity of systemic lipolysis index (EC50; in picomoles per liter) was assessed as the serum insulin concentration that effectively suppressed plasma glycerol appearance rate (in micromoles per-kilogram per-minute) by 50% of the maximal suppression.

1H magnetic resonance spectroscopy

Liver fat was determined by 1H magnetic resonance spectroscopy (1H-MRS) as previously described [26]. Percentage of liver fat was calculated as the ratio of lipid resonances (methylene + methyl signals) and water signal serving as internal reference.

Genotyping

Genomic DNA was isolated from whole blood using a commercial DNA isolation kit (NucleoSpin; Macherey-Nagel, Düren, Germany). Genotyping of the SNPs usf1s1 C>T and usf1s2 G>A were in complete linkage disequilibrium (D’=1.0). All subjects homozygous for the major alleles in usf1s1 (C/C) were also homozygous for the major (G/G) alleles for SNP usf1s2, and this held true for the homozygous subjects for the minor alleles. Therefore, the data for only one of the polymorphisms, usf1s2 G>A, are presented. The allele frequency for the major G allele of SNP usf1s2 was 0.704. The genotypes were in Hardy-Weinberg equilibrium (p=0.58).

Analytical procedures

Blood glucose was determined using a bedside glucose analyzer (glucose-oxidase method; YSI, Yellow Springs Instruments, Yellow Springs, CO, USA). Plasma insulin was determined by microparticle enzyme immunoassay (Abbott Laboratories, Tokyo, Japan), and serum FFA concentrations were determined with an enzymatic method (WAKO Chemicals, Neuss, Germany). Lipoprotein concentrations were measured with a standard colorimetric method using the Roche/Hitachi analyzer (Roche Diagnostics, Mannheim, Germany).

Statistical analyses

Data were given as mean±standard error (SE). Statistical comparison of genotype groups was performed using logarithmically transformed data (for nonnormally distributed parameters). Hardy-Weinberg equilibrium was tested by the χ² test. Differences in anthropometrics and metabolic characteristics between genotypes were tested using multivariate regression models. In these models, the trait was the dependent variable, whereas, e.g., age, gender, and genotype were the independent variables. Interaction between the genotypes was tested by inclusion of the genotypes as well as their interaction term in the models. When this term was significant, genotype groups were generated for further testing of differences between the variants. Due to the relatively small number of subjects who were homozygous for the rare alleles, besides the additive model, a dominant model was used. A p value ≤0.05 was considered to be statistically significant. The statistical software package JMP (SAS Institute, Cary, NC, USA) was used. Pairwise linkage disequilibrium (D’) was determined using the THESIAS program [32].

Results

Relationships of SNPs usf1s1 and usf1s2 with anthropometrics and metabolic characteristics

The SNPs usf1s1 C>T and usf1s2 G>A were in complete linkage disequilibrium (D’=1.0). All subjects homozygous for the major alleles in usf1s1 (C/C) were also homozygous for the major (G/G) alleles for SNP usf1s2, and this held true for the homozygous subjects for the minor alleles. Therefore, the data for only one of the polymorphisms, usf1s2 G>A, are presented. The allele frequency for the major G allele of SNP usf1s2 was 0.704. The genotypes were in Hardy-Weinberg equilibrium (p=0.58).

The anthropometrics and metabolic characteristics of the 407 subjects are summarized in the Table 1. There were no differences between the genotypes with respect to gender, age, and percentage of body fat. Also, no difference was noted regarding fasting plasma glucose, fasting insulin levels, fasting serum FFA levels, lipoprotein levels, and insulin sensitivity as estimated during the OGTT after adjustment for gender, age, and percentage of body fat.

There was a small effect regarding 2 h glycemia and insulinemia. In the dominant model, subjects carrying the A
allele of SNP usf1s2 had lower 2 h plasma glucose and insulin levels compared to subjects who were homozygous for the G allele of SNP usf1s2. In contrast, there was a larger effect on 2 h FFA concentrations. Subjects with the A allele of SNP usf1s2 had lower 2 h serum FFA concentrations compared to subjects who were homozygous for the G allele of SNP usf1s2 both in the additive and the dominant models after additional adjustment for 2 h insulin levels. Furthermore, serum FFA concentrations decreased during the OGTT in subjects with the A allele of SNP usf1s2 more than in individuals who were homozygous for the G allele of SNP usf1s2 (additive model \( p = 0.045 \), and dominant model \( p = 0.02 \)) after adjustment for gender, age, percentage of body fat, and 2 h insulin and fasting FFA levels. Data from subjects carrying the A allele are also shown in a dominant model (X/A). (\( p \)) using ANOVA.

Relationship of the SNP \(-60C>G\) in HSL with antilipolytic insulin sensitivity and interactions with the SNP usf1s2

Because the \(-60C>G\) SNP in HSL was found to interact with SNPs in USF on determination of triglyceride levels after fat load [7], we investigated whether this SNP is associated with antilipolytic insulin sensitivity and whether there is an interaction of the SNP in HSL with the SNP usf1s2 on antilipolytic insulin sensitivity. No associations

\begin{table}
\centering
\begin{tabular}{|c|c|c|c|c|c|}
\hline
Genotype & G/G & G/A & A/A & \( p^a \) & X/A & \( p \) \\
\hline
Gender (F/M) & 112/86 & 87/91 & 19/12 & 0.22 & 106/103 & 0.24 \\
BMI (kg/m\(^2\)) & 27.1±0.4 & 27.1±0.4 & 28.5±1.5 & 0.98 & 27.3±0.4 & 0.89 \\
Waist circumference (cm) & 92.2±1.1 & 92.7±1.2 & 94.5±3.8 & 0.98 & 93.0±1.2 & 0.86 \\
Age & 37.4±0.9 & 37.2±0.9 & 42.2±2.0 & 0.09 & 37.9±0.8 & 0.50 \\
Body fat (%) & 28.3±0.7 & 27.6±0.7 & 28.8±2.0 & 0.46 & 27.8±0.7 & 0.93 \\
Fasting insulin (\( \mu \)U/ml) & 8.9±0.4 & 8.2±0.4 & 10.1±1.3 & 0.16 & 8.5±0.4 & 0.26 \\
2 h insulin (\( \mu \)U/ml) & 63.1±4.2 & 53.1±3.9 & 60.1±10 & 0.14 & 54.1±3.7 & 0.047 \\
Fasting glucose (mg/dl) & 90.4±0.5 & 89.6±0.7 & 91.8±1.8 & 0.40 & 90.0±0.7 & 0.25 \\
2 h glucose (mg/dl) & 110±2 & 106±2 & 105±5 & 0.11 & 106±2 & 0.046 \\
Fasting FFA (\( \mu \)M) & 548±17 & 509±15 & 575±35 & 0.39 & 518±14 & 0.48 \\
2 h FFA (\( \mu \)M) & 88±3 & 67±3 & 69±8 & 0.02 & 66±2 & 0.01 \\
Total cholesterol (mg/dl) & 189±3 & 191±3 & 192±5 & 0.78 & 192±3 & 0.55 \\
HDL-cholesterol (mg/dl) & 56±1 & 56±1 & 54±2 & 0.19 & 60±1 & 0.52 \\
LDL-cholesterol (mg/dl) & 118±2 & 119±2 & 123±5 & 0.80 & 119±2 & 0.55 \\
Triglycerides (mg/dl) & 116±7 & 115±6 & 115±14 & 0.98 & 115±5 & 0.84 \\
IS\(_{\text{OGTT}}\) (AU) & 18.8±0.8 & 19.8±0.9 & 19.8±2.8 & 0.19 & 19.8±0.8 & 0.15 \\
\hline
\end{tabular}
\caption{Characteristics of the subjects who underwent an OGTT divided according to the genotype of the usf1s2 (rs2073658) G>A polymorphism in the USF1 gene}
\end{table}

\( Data \) represent means±SE. IS\(_{\text{OGTT}}\) (AU): insulin sensitivity in arbitrary units estimated from the oral glucose tolerance test [29]. \( p \) values were obtained using (\( \chi^2 \) test)\(^b\) or multivariate linear regression models. Body fat was adjusted for age and gender, and except for 2 h FFA levels, all metabolic variables were adjusted for age, gender, and body fat. Two-hour FFA levels were additionally adjusted for their determinant 2 h insulin levels. Data from subjects carrying the A allele are also shown in a dominant model (X/A). \( (p)^a \) using ANOVA.

EC\(_{50}\) was lower in subjects carrying the A allele of SNP usf1s2 compared to individuals who were homozygous for the G allele of SNP usf1s2 \( (p=0.03 \) after adjustment for gender, age, and percentage of body fat, Fig. 1). These data indicate that less insulin was needed to suppress lipolysis in carriers of the A allele of SNP usf1s2.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig1}
\caption{Associations between the genotypes of the usf1s2 (rs2073658) G>A polymorphism and EC\(_{50}\) (insulin concentration resulting in half-maximal suppression of glycerol appearance) in the dominant model after adjustment for gender, age, and percentage of body fat}
\end{figure}

Fifty-four subjects had data on antilipolytic insulin sensitivity measured during the euglycemic hyperinsulinemic clamp. Forty-eight of them also had measurements of FFAs at baseline and at 2-h of the OGTT. Due to the small number of subjects homozygous for the rare A allele (\( n=3 \)), only the dominant model (G/G vs X/A) was used. There were no differences between the groups with respect to gender (G/G: women 9, men 14; X/A: women 16, men 15, \( p=0.33 \)), age (29.1±1.7 vs 29.2±1.3 years, \( p=0.96 \)), BMI (23.1±0.5 vs 23.2±0.7 kg/m\(^2\), \( p=0.95 \)), and percentage body fat (19.7±1.5 vs 22.4±1.7%, \( p=0.73 \)). However, the
with antilipolytic insulin sensitivity and no interactions with the SNP usf1s2 on antilipolytic insulin sensitivity, both measured in the clamp and estimated from the OGTT, were detected (all \( p > 0.23 \), data not shown).

Relationships of the SNP usf1s2 and the \(-514C>T\) SNP in \(LIPC\) with liver fat and insulin sensitivity

Data on liver fat were available in 114 individuals. In respect to the characteristics shown in the Table 1, in this smaller group, except for lower fasting FFAs \((p=0.04)\) in carriers of the A allele of SNP usf1s2, no significant differences were seen between the genotypes. The different results in respect to fasting FFA levels compared to the larger group could be a result of a type 1 error due to the small sample size and the relative high variability in the measurements of serum FFAs. In contrast, the measurement of liver fat by \(^1\)H-MRS is highly reproducible. The usf1s2 G>A SNP was not associated with liver fat which was adjusted for age, gender, and percentage body fat \((G/G, n=59, 7.73 \pm 1.02\% \text{ and } X/A, n=55, 6.25 \pm 1.05\%, p=0.35)\). In contrast, as previously reported \([26]\), the T allele of the \(-514C>T\) SNP in \(LIPC\) was associated with higher liver fat \((C/C, n=74, 5.67 \pm 0.76\% \text{ and } X/T, n=40, 9.48 \pm 1.47\%, p=0.01)\). However, this association differed on the background of the SNP usf1s2 \((p \text{ for interaction } = 0.01)\). Liver fat was elevated only in subjects who were both homozygous for the major alleles of SNP usf1s2 and carriers of the T allele of the SNP in the \(LIPC\) gene (Fig. 2).

Furthermore, we investigated whether the SNP usf1s2 also displays interaction with the \(-514C>T\) SNP in \(LIPC\) on insulin sensitivity both estimated from the OGTT and determined during the euglycemic hyperinsulinemic clamp. Similar to the results for liver fat, although this was statistically not significant, insulin sensitivity was lower particularly in subjects who were both homozygous for the major allele of the SNP usf1s2 and carriers of the T allele of the SNP in \(LIPC\) \((p \text{ for interaction } = 0.06 \text{ for the OGTT and } p=0.08 \text{ for the clamp, data not shown).}

Discussions

In the present study, we determined the impact of genetic variations in \(USF1\), specifically the rs3737787 (usf1s1) C>T and rs2073658 (usf1s2) G>A SNPs, on antilipolytic insulin sensitivity. We found that carriers of the T allele of SNP usf1s1 and A allele of SNP usf1s2 were more sensitive to the antilipolytic effect of insulin than the homozygous carriers of the C allele of SNP usf1s1 and G allele of SNP usf1s2. To our knowledge, this is the first study showing an impact of genetic variations in \(USF1\) on lipolysis in vivo. As mentioned before, Hoffstedt et al. \([8]\) found that the T allele of SNP usf1s1 and A allele of SNP usf1s2 were associated with increased in vitro catecholamine-induced lipolysis in subcutaneous adipocytes of obese women. The recent studies of Pajukanta et al. \([4, 5]\) and Coon et al. \([5]\), which showed a tight statistical association of the same SNPs with familial combined hyperlipidemia (FCHL, protective effect of the T allele of SNP usf1s1 and the A allele of SNP usf1s2) provide additional indirect support for an important role of the SNPs for lipolysis. This is supported by the data showing that catecholamine-induced lipolysis is impaired in FCHL \([33]\). The aforementioned studies \([4, 5]\) also reported an association of the SNPs with high serum triglycerides and LDL-cholesterol levels and small LDL-particle size. Three other SNPs within \(USF1\), with at least two of them in tight linkage disequilibrium with usf1s1 and usf1s2, were not associated with serum basal and postprandial lipid levels in healthy young men with a family history of premature coronary heart disease \([7]\). Consistent with this study and the findings of Hoffstedt et al. \([8]\), we found no association of the usf1s1/usf1s2 SNPs with the fasting plasma lipid profile.

The mechanism for the increased antilipolytic insulin sensitivity in carriers of the T allele of SNP usf1s1 and A allele of SNP usf1s2 is not known. An explanation may be the reduced activity of the rate-limiting enzyme of lipolysis, the HSL. The \(HSL\) promoter contains an E-box binding
USFs, and HSL mRNA levels are decreased in adipose tissue of USF1 and USF2-deficient mice [2]. Putt et al. [7] reported an interaction of SNPs in USF with the −60C>G SNP in HSL on triglyceride levels after fat but not after glucose load. Hoffstedt et al. [8] found the effects of the T allele of SNP usf1s1 and A allele of SNP usf1s2 to be limited in the maximum lipolytic action of catecholamines, which suggests that the effect is exerted in a postreceptor level where HSL is also active. No effects of the SNPs on basal lipolysis were found. Consistent with this finding, we also did not find an effect of the SNPs usf1s1 and usf1s2 on fasting FFA concentrations. In addition, no interaction of the SNP usf1s1 with the −60C>G SNP in HSL on triglyceride levels after glucose load or on antilipolytic insulin sensitivity was detected. Because in the study by Putt et al. [7] an interaction between both SNPs on triglycerides was found after fat—but not after glucose load, fatty acids seem to determine the interaction of both genes.

In the present study, the lower 2 h FFA concentrations were accompanied with a marginally better glucose tolerance. It is plausible to suppose that the decreased release of FFA in carriers of the T allele of SNP usf1s1 allows a more efficient suppression of glucose production by the liver under insulin-stimulatory conditions. This would implicate a better hepatic insulin sensitivity in these subjects. This is supported by the data showing that USF proteins are involved in the regulation of the expression of genes involved in lipid metabolism in the liver [1, 10–14]. Therefore, apart from the effect on lipolysis, the polymorphisms may have a more pronounced effect on the liver than on muscle, the main tissues influencing whole-body insulin sensitivity. However, because we did not measure hepatic insulin sensitivity, we can only speculate on this relationship. Nevertheless, if this was the case, then this could be an explanation for the lack of effects of the polymorphisms on whole-body insulin sensitivity, despite of an effect on glucose tolerance and 2 h insulin levels, both of which were shown to be strongly determined by hepatic insulin sensitivity [34].

Also, if these subtle relationships with insulin sensitivity exist, then they may not be powerful enough to affect glucose tolerance. This is supported by more recent data showing that genetic variants in USF1, among them both SNPs that are presented in the current analysis, were not associated with type 2 diabetes in French Caucasians [35]. In contrast, in the Chinese population, the usf1s1 C allele was associated with a higher risk for type 2 diabetes in a family-based, but not in a population-based, study [36]. Thus, unlike the well-documented effects of USF1 on triglycerides in syndromes such as FCHL [4, 5], the effects of USF1 on type 2 diabetes are small. An explanation may be that USF1 regulates many genes that are involved in lipid metabolism such as apolipoproteins, fatty acid synthase, acetyl-CoA carboxylase, and LIPC [37]. In contrast, there are only few genes, such as glucagon receptor, that are regulated by USF1 and are directly involved in glucose metabolism [37]. USF1 may only have an impact on insulin resistance and type 2 diabetes indirectly by modulating genes that are involved in lipid metabolism and affect insulin resistance by regulation of lipolysis or ectopic fat accumulation in the liver as shown for LIPC [26].

A limitation of our study is the lack of a real replication of the findings on antilipolytic insulin sensitivity in another population. Furthermore, the results of the clamp studies and of the liver fat measurements are based on a relatively small number of subjects. This is due to the fact that this precise phenotyping, particularly for the measurement of antilipolytic insulin sensitivity, is rather complex and elaborative. For the findings on liver fat content, although we adjusted for multiple comparisons, multiple hypothesis testing still may be an issue.

In summary, our findings suggest that subjects carrying the T and A alleles in the SNPs usf1s1 and usf1s2 of USF1 are more sensitive to suppression of lipolysis by insulin than those carrying the common alleles C and G. The data also indicate an interaction with the −514C>T SNP in LIPC on liver fat. These findings need to be replicated in larger epidemiologic studies.

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CD8+ T cells armed with retrovirally transduced IFN-γ

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Abstract Interferon-gamma (IFN-γ) is considered a key cytokine involved in the preventive and defensive responses of T cells against infectious pathogens and tumors. Therefore, the transgenic expression of IFN-γ in specific T cells appears to be an obvious therapeutic possibility. To directly examine whether IFN-γ production can be increased in T cells, we introduced an IFN-γ encoding cDNA into IFN-γ−/− and IFN-γ+/+ CD8+ effector populations by retroviral transduction. Here, we show that CD8 T cells can be equipped with IFN-γ that increases their capacity to secrete the cytokine. Despite constitutive retroviral IFN-γ mRNA transcription, translation and secretion of IFN-γ protein was tightly regulated and only observed in activated T cells. Neither proliferation nor cytolytic activity of CTL was affected by IFN-γ transduction. Importantly, CD8+ T cells retrovirally transduced with IFN-γ exhibit augmented tumor suppressive capacity upon adoptive transfer into IFN-γ−/− mice. Thus, T cells can be readily armed with IFN-γ without risking immunopathology by dysregulated production of this highly potent proinflammatory cytokine.

Keywords IFN-γ · Retroviral transduction · CD8+ T cell

Introduction

Antigen-specific CD8+ T cells are protective against infectious pathogens and tumors by two mechanisms: lysis of infected cells or tumor cells and secretion of cytokines. Among the cytokines produced by activated CD8+ T cells, interferon-gamma (IFN-γ) seems to be of particular importance. Only IFN-γ producing CD8 T cells are able to clear certain viral infections [1–3], and viral clearance is observed before significant cytotoxicity can be demonstrated [1, 2]. Similarly, in murine experimental tumor systems, protective immunity depends on a population of tumor-specific, IFN-γ producing CD8 T cells [4–6]; in tumor patients, CD8+ T cells with tumor rejection potential reside
in the fraction of IFN-γ producing CD8+ T lymphocytes [7, 8]. In addition, IFN-γ deficient mice and IFN-γ receptor deficient mice show impaired T-cell dependent immunity against certain infections [3] and tumors [9, 10]. In the absence of IFN-γ, T cells are not able to clear viral infection [11] and fail to enter the tumor tissue [9]. These and other data imply that IFN-γ secretion is a critical effector mechanism of CD8+ T cells in vivo. Hence, transgenic expression of IFN-γ in specific T cells appears to be an obvious therapeutic possibility.

Here, we show that CD8+ T cells can be readily equipped with IFN-γ by retroviral infection, increasing their IFN-γ secreting capacity and augmenting their therapeutic function.

Materials and methods

Mice

Inbred BALB/c mice were obtained from Harlan Olac (Bicester, UK), and BALB/c IFN-γ knockout mice were bred and maintained under standard housing conditions in the animal facility of the HZI.

The Clone-4 (CL4) TcR transgenic mouse line expresses an H-2Kd restricted TcR (Vα10, Vβ8.2) against a transmembrane epitope of hemagglutinin (HA) from influenza virus comprising amino acids 512–520 (IYSTVASSL) of the strain A/PR/8/34 [12]. The transgenic line has been backcrossed over more than 12 generations onto the BALB/c background and was bred in the HZI animal facility. All mice used in the experiments were sex- and age-matched.

T cell activation and phenotype differentiation

Splenocytes from CL4TcR-transgenic mice were activated by influenza virus hemagglutinin peptide 512–520 (0.3 μg/ml) at 3 × 10^6 cells/ml in Iscove’s modified Dulbecco’s medium. Splenocytes from IFN-γ knockout mice were activated with plate-bound anti-TcR mAb (2C11) and anti-CD28 (37.51; 1 and 4 μg/ml respectively, both from Pharmingen) for 48 h. Cells were collected, washed, and transferred into medium containing 10 U/ml rhIL-2 and 5 ng/ml rmIL-15. For analysis of IL-12/IL-18-induced IFN-γ production, sorted populations were incubated at a density of 2 × 10^5 cells/well in a 96-well plate with 10 U/ml rmIL-12 and 10 ng/ml rmIL-18 (both R&D).

Retroviral construct and retroviral transduction

The IFN-γ coding sequence was amplified by reverse transcription polymerase chain reaction (RT-PCR) from the transfectant X63/BCMG NEO IFNγ (kindly provided by Dr. Karasuyama, Tokyo, Japan), using the forward primer GCCGCCACTAGTGCCACCATGAAACGTACA CACTGC and the reverse primer ATAGAGGGCCGCCT CAGCAGGACTCTTTTCCGGC, and cloned into the pBluescript via SpeI/NcoI resulting in the plasmid pBS-IFNγ. The IFN-γ coding fragment was excised from pBS-IFNγ using BamHI and NotI. This fragment was cloned into a XhoI-digested, blunt-ended, and subsequently BglII-digested GFP-RV retroviral vector containing an internal ribosomal entry site (IRES) element for bi-cistronic expression (a kind gift from Dr. Kenneth Murphy, Howard Hughes Medical Institute, MD, USA) resulting in IFN/GFP-RV. Thus, the final vector contains the coding sequence for IFN-γ without untranslated regions of the gene, followed by an IRES and the GFP coding sequence. Integrity and function were confirmed by enzymatic digestion of the vector and analyzing supernatants from plasmid-transfected 3T3 fibroblasts by IFN-γ ELISA.

Phoenix-Eco packaging cells (Nolan/Stanford CA, purchased through ATCC) were transfected according to Dr. Nolan’s protocol. Primary T cells were activated as described above and infected after 48 h using 1 vol of viral supernatant and polybrene (6 μg/ml, Sigma), centrifuged at 1,800 rpm for 45 min at room temperature, and incubated at 37°C for 48 h, before being supplied with fresh media and expanded until day 8 after primary activation.

RT-PCR

Transcription of the retroviral DNA was analyzed using RT-PCR. Retrovirally infected, CD8+/GFP+-sorted CL4 TcR Tc1 cells were either stimulated for 24 h with plate-bound anti-CD3 antibodies or left unstimulated. RNA extraction and reverse transcription was carried out using standard procedures. Primers used for PCR were: GAPDH,
ATCTTCTTGCACTGACCAGG (forward) and ACTCATACCTACACCC (reverse); total IFN-γ, AGTGCATAGATGTGGAAGAAAAGTCTCTTCTTG (forward) and GGGCTGGACCTGTGGGTGG (reverse); retroviral IFN-γ, CGTCATTGAATCACACCTG (forward) and GAACCTAGGGCTAGCTT (reverse, binding to GFP); endogenous IFN-γ, AGTTCTGGGCTTCTCCTCCT (forward) and GTCAACATCTTTTGGCCAGT (reverse).

Intracellular IFN-γ staining

For intracellular staining of IFN-γ, cells were fixed for 20 min at room temperature in phosphate-buffered saline (PBS) 1% paraformaldehyde, followed by incubation in 0.1% NP40 in PBS for 3 min on ice. After washing, cells were incubated with PE-conjugated anti-IFN-γ mAb or the appropriate PE-conjugated isotype control (both Pharmingen) for 30 min on ice. Cells were analyzed by flow cytometry using a FACSCalibur (Becton Dickinson).

Cytotoxicity assay

The cytotoxic activity of CL4TCR CTL was determined using the JAM assay [13]. Briefly, 5,000 [3H]-thymidine-labeled P815 were incubated in 96-well U-bottom plates with or without 1 μg/ml HA peptide 512–520 for 1 h. CL4TCR effector T cells were added at different ratios and the plates were incubated for a further 4 h. Percent cytotoxicity was calculated from the mean of triplicate wells.

HA tumors and tumor cell injections

Full-length HA cDNA from the Mt. Sinai strain of the PR8 influenza virus was subcloned into the pCDNA3 expression vector. The BALB/c colon carcinoma CT26 was transfected with this vector using standard methodology. CT26-HA were selected and grown in complete medium supplemented with G418 (500 μg/ml). The level of HA expression on transfected cells was determined by flow cytometry analysis, using the fluorescein isothiocyanate-conjugated HA-specific monoclonal antibody H36-4-5 [14].

BALB/c IFN-γ knockout mice, 8–10 weeks of age, were inoculated s.c. with 1×10⁵ CT26-HA transfectants with high HA expression and monitored for tumor growth every day. Tumor size was measured in two dimensions and given as the mean diameter. Explants of CT26-HA, obtained after 20 days of tumor progression, demonstrated that expression of HA was maintained in vivo as determined by staining with the antibody H36-4-5 against HA (data not shown).

Results

CD8+ T cells can be retrovirally equipped with IFN-γ

To test whether IFN-γ production can be increased in T cells by ectopic expression of the IFN-γ gene, we retrovirally introduced an IFN-γ encoding cDNA into CD8+ T cells. A bi-cistronic retroviral vector was used to co-express murine IFN-γ together with GFP under the control of the murine stem cell virus long-terminal repeat (MSCV LTR; IFN/GFP-RV; Fig. 1a). GFP-RV (Fig. 1a) served as negative control for these experiments. Using GFP as a reporter gene, transduced CD8+ T cells from transgenic CL4 mice were routinely enriched by cell sorting to purities greater than 95% (Fig. 1b,c).
First, we determined whether IFN-γ can be expressed in CD8+ T cells via retroviral transduction. Therefore, we infected CD8+ T cells from IFN-γ knockout mice after anti-CD3 stimulation. Sorted GFP+ CD8+ T cells infected with IFN/GFP-RV (Fig. 2a), but not CD8+ T cells infected by control vector, secreted IFN-γ after restimulation with anti-CD3. When comparing transduction efficiency under Tc1 or Tc2 culture conditions, IFN-γ secretion was found to occur under both priming conditions, although TcR-activated Tc2 cells secreted less recombinant IFN-γ per cell than Tc1 cells did (Fig. 2a). Interestingly, no IFN-γ production could be observed in resting T cells (Fig. 2a), although the IFN-γ encoding cDNA was under the control of the viral LTR and the IFN/GFP-RV retrovirus did not contain any upstream regulatory elements of the IFN-γ gene [15, 16]. Pertinent to this observation, fibroblasts constitutively secreted the cytokine upon infection with IFN/GFP-RV virus (Fig. 2b).

When IFN-γ competent CD8+ T cells were transduced with IFN/GFP-RV, an increase in TcR-induced IFN-γ secretion was observed compared to the levels produced by GFP-RV infected control CD8+ T cells (Fig. 2c) or GFP− cells from IFN/GFP-RV-infected cultures (not shown). Similar data were obtained when IFN/GFP-RV infected CD8+ T cells were stimulated with stimulator cells and peptide instead of anti-CD3 (Fig. 2d). Together these data demonstrate that IFN-γ can be enhanced in CD8+ T cells by retroviral transduction.

However, despite retroviral LTR control of the cDNA, IFN-γ secretion depends on activation of the T cells.

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**Fig. 2** Retroviral expression of IFN-γ in IFN-γ sufficient and deficient CD8+ T cells. a Retroviral expression of IFN-γ in IFN-γ−/− CD8+ T cells. CD8+ T cells were prepared from BALB/c IFN-γ−/− spleen cells and stimulated with plate-bound anti-CD3 and anti-CD28 mAb for 48 h under either Tc1 or Tc2 conditions as indicated. At 48 h, cells were infected with retroviral GFP-RV or IFN/GFP-RV supernatants containing Tc1 or Tc2 cytokines and appropriate mAb in addition to 10 ng/ml rhIL-2. On day 8, cells were harvested, washed, and stimulated at 1×10⁶ cells/well in a 96-well plate with 1 μg/ml plate bound anti-CD3 mAb. IFN-γ production was measured by ELISA from supernatants taken after 24 h of activation. The dotted line indicates the detection limit of the ELISA. b Constitutive secretion of IFN-γ by transduced fibroblasts. 3T3 fibroblast were infected with GFP-RV or IFN/GFP-RV, respectively. Cells (1×10⁵) were seeded into wells of a 24-well plate and supernatants were taken 24 h later. c Retroviral expression of IFN-γ in CD8+ T cells from CL4 transgenic mice sufficient for IFN-γ. CL4 transgenic primary T cells were activated under Tc1 conditions and infected with GFP-RV or IFN/GFP-RV on day 2. GFP−/CD8+ T cells were purified by cell sorting on day 8 (as in Fig. 1) and restimulated at a density of 1×10⁵ cells/well in a 96-well plate using 1 μg/ml plate bound anti-CD3 mAb. IFN-γ production was measured by ELISA from supernatants taken after 24 h of activation. d Same cells as under c were restimulated with irradiated feeder cells and 1 μg/ml peptide. Data are representative for at least three independent experiments. Note that the scales are different in the three panels.
Constitutive transcription of retroviral IFN-γ in resting T cells transduced with IFN/GFP-RV

It was unexpected that the secretion of retrovirally derived IFN-γ driven by the viral LTR was dependent on activation of the transduced T cells. This regulation could be either on a transcriptional or translational level. Since only the IFN-γ coding sequence was introduced into the vector and the reporter protein GFP (co-encoded by bi-cistronic mRNA) was found to be expressed, it was unlikely that regulation was on the transcriptional level. This was confirmed by RT-PCR on resting transduced T cells. Using primers that distinguished the virally derived IFN-γ PCR on resting transduced T cells. Using primers that was on the transcriptional level. This was confirmed by RT-reporter protein GFP (co-encoded by bi-cistronic mRNA) coding sequence was introduced into the vector and the a transcriptional or translational level. Since only the IFN-γ of the transduced T cells. This regulation could be either on IFN-γ. It was unexpected that the secretion of retrovirally derived cells transduced with IFN/GFP-RV

T cells [17]. Uninfected cells from the same culture (GFP

Constitutive transcription of retroviral IFN-γ was driven by the viral LTR was dependent on activation of the transduced T cells. This regulation could be either on a transcriptional or translational level. Since only the IFN-γ coding sequence was introduced into the vector and the reporter protein GFP (co-encoded by bi-cistronic mRNA) was found to be expressed, it was unlikely that regulation was on the transcriptional level. This was confirmed by RT-PCR on resting transduced T cells. Using primers that distinguished the virally derived IFN-γ specific mRNA from the endogenous transcript, we could show that significant amounts of IFN-γ encoding mRNA were present in resting T cells transduced with IFN/GFP-RV (Fig. 3a). This mRNA could not be detected in T cells transduced with the GFP-RV control vector (Fig. 3b). The amount of virally derived IFN-γ mRNA did not change upon the stimulation of the T cells, although mRNA encoding endogenous IFN-γ became readily detectable under these conditions.

Despite the presence of functional mRNA no IFN-γ protein could be detected by intracellular staining in resting T cells transfected with IFN/GFP-RV (Fig. 3c). In contrast, when such T cells were stimulated with plate-bound anti-CD3, strong IFN-γ production could be detected intracellularly in most of the GFP+ T cells. Thus, the absence of production of recombinant IFN-γ by resting T cells is not simply a block in secretion. Rather, our results suggest a T cell specific regulation of IFN-γ production at the post-transcriptional or post-translational level.

Influence of Tc1 and Tc2 stimulating conditions on retroviral IFN-γ expression

To analyze the influence of cytokines on IFN-γ production by IFN-γ transduced CD8+ T cells, we infected CL4-TcR-transgenic T cells with IFN/GFP-RV under Tc1 or Tc2 inducing conditions. IFN-γ and IL-4-secretion of sorted GFP+CD8+ and GFP+CD8+ T cells were analyzed subsequent to the antigenic expansion of the sorted populations. Transduction of IFN-γ highly augmented TcR-induced IFN-γ secretion in Tc1, and significantly in Tc2 CL4-TcR CD8+ T cells, after 8 and 16 days of expansion, respectively (Fig. 4). Under Tc2 conditions, IFN-γ transduction partially repressed IL-4 production in IFN/GFP-RV transduced cells (Fig. 4). This is likely due to a feedback loop of IFN-γ acting on the IL-4 promoter via IRF-1 and IRF-2 as previously described for human CD4+ T cells [17]. Uninfected cells from the same culture (GFP) were not affected, suggesting that a short exposure to IFN-γ present in the viral supernatant during infection does not alter IL-4 production by CD8+ Tc2 cells. As observed by others [18, 19], Tc2 cells remain capable of secreting IFN-γ upon stimulation with antibodies against the T cell receptor. Together these results demonstrate that IFN-γ transduction enforces IFN-γ production in CD8+ T cells even under Tc2 conditions.

Enhanced IFN-γ production in IFN-γ transduced CD8+ T cells is stable

To examine whether IFN-γ production in IFN/GFP-RV transduced CD8+ T cells is transient or stable, transduced CD8+ T cells were expanded in vitro by restimulation on stimulator cells plus peptide under Tc1 inducing conditions. Already after the first round of restimulation, such T cells produced IFN-γ and T cells transduced with IFN/ GFP-RV exhibited increased production of IFN-γ (Fig. 5). This property was maintained over several rounds of restimulation. No influence on the viability and growth were observed throughout a 3- to 4-week period of cell culture (not shown). Thus, IFN/GFP-RV transduction stably augments TcR-regulated IFN-γ secretion in IFN-γ sufficient Tc1 polarized cells during expansion and differentiation.

Retroviral IFN-γ transduction increases IL-12/IL-18-induced IFN-γ secretion

At least two receptor-mediated pathways can induce IFN-γ secretion in T cells. Besides a TcR-dependent pathway, an alternative antigen-independent pathway for IFN-γ secretion is implemented by the upregulation of both IL-12 and IL-18 receptor expression [20]. Triggering via the two cytokine receptors also results in IFN-γ production. We therefore analyzed IFN-γ production of transgenic CD8+ T cells transduced with GFP-RV or IFN/GFP-RV in the presence of IL-12 and IL-18. As shown in Fig. 6, stimulation with these cytokines resulted in a strong increase in IFN-γ secretion by IFN/GFP-RV infected Tc1 cells as compared to GFP+ cells from the same culture (uninfected internal control) or GFP+ cells from GFP-RV infected T cells (vector control). Thus, retroviral IFN-γ expression enhanced the antigen independent IFN-γ secretion in CD8+ T cells.

Retroviral IFN-γ transduction does not alter specific cytolytic activity

An influence of IFN-γ on the homeostasis of T cells has been shown before [21], although mature CD8+ T cells seem to be insensitive to IFN-γ due to reduced IFN-γR2 chain expression [22]. No significant difference in cytolytic
activity could be discerned between GFP and IFN/GFP-RV transduced CL4 T cells. When the cytolytic activity of T cells sorted for GFP+CD8+ was compared at day 8 or 16 after retroviral infection, IFN/GFP-RV infected cells exhibited only a slightly higher unspecific cytolytic activity than vector controls (Fig. 7a). Similar results were obtained...
with GFP and IFN/GFP-RV transduced CL4 T cells additionally expanded for 10 days in culture (data not shown). Thus, IFN/GFP-RV transduction does not significantly affect the induction of a fully competent cytolytic response.

Tumor protective potential of CTL retrovirally transduced with IFN-γ

Adoptive transfer of IFN/GFP-RV transduced CL4-Tc1 cells into syngeneic mice that have received a challenge with HA expressing CT26 tumor cells, enabled us to evaluate the antitumor potential of such T cells in vivo. We compared two CD8+ T cell populations that only differed in their capacity to produce IFN-γ. Control mice received no T cells at all, or CL4 Tc1 cells that had been infected with the GFP-RV control vector. Adoptive transfers were carried out in mice deficient for IFN-γ production to exclude contribution of IFN-γ by host cells.

Under these circumstances, IFN/GFP-RV transduced CL4 T cells exhibited improved therapeutic potency compared to GFP-RV-infected Tc1 cells (Fig. 7b). Thus,
under limiting conditions, IFN-γ production driven by retrovirus transduction is able to improve the therapeutic function of CD8+ T cells.

Discussion

This study makes several noteworthy observations. First, we show that antigen-specific CD8+ T cells can readily be armed with IFN-γ by retroviral transduction. Interestingly, a tight regulation of IFN-γ production in CD8+ T cell was observed and only activated T cells were able to secrete the cytokine. In contrast, fibroblasts constitutively produced the cytokine upon infection with the same retroviral vector, demonstrating that regulation of IFN-γ production is intrinsic for the T cell.

Downregulation of IFN-γ was not due to the inactivation of transcription when the T cells went into quiescence, as has been observed by others [23, 24]. Virally derived mRNA encoding IFN-γ was unambiguously demonstrated in resting T cells by RT-PCR, and the amount of virally derived specific mRNA was not further enhanced upon T cell stimulation. The controversy might be due to a different retroviral constructs used in our work or to our selection of GFP expressing T cells by cell sorting. Thus, we would have selected for cells that constitutively express the LTR driven genes. In favor of this argument is that IFN-γ secretion is resumed upon T cell stimulation but virally derived mRNA is not increased.

This suggests that although on/off cycling of IFN-γ production by T cells is thought to be transcriptionally regulated [25], post-transcriptional mechanisms contribute to the control of IFN-γ production in CD8+ T cells. In agreement with our finding, it was recently shown that primed T helper cells can regulate cytokine secretion by controlling ribosomal loading of cytokine mRNA [26]. Since only the coding sequence of IFN-γ was introduced into the retroviral vector, appropriate regulatory motifs should be restricted to this region.

Viritually derived IFN-γ mRNA was apparently not translated in resting IFN/GFP-RV CD8+ T cells, although these cells remained GFP+. It is possible that the translation of the complete bi-cistronic mRNA might be downregulated and cells might remain GFP+ due to the longevity of the GFP protein. However, it is more likely that the IRES element sufficiently uncouples the translation of both cistrons to allow independent GFP expression.

Whereas retroviral IFN-γ is downregulated in resting IFN/GFP-RV CD8+ T cells, it contributes to CD8+ T cell IFN-γ production under T-cell stimulating conditions. This is most evident in IFN/GFP-RV transduced CD8+ T cells from IFN-γ deficient mice. In IFN-γ competent CD8+ T cells, IFN/GFP-RV transduction resulted in increased T-cell receptor driven and IL-12/IL-18-induced IFN-γ secretion as compared to GFP-RV transduced T cells. Thus, the antigen-dependent, as well as the antigen-independent, activation pathway induces translation and/or secretion of recombinant IFN-γ in CD8+ T cells.
Retrovirally derived IFN-γ can be secreted by CD8⁺ T cells under different polarizing conditions. In such cases, secretion of retrovirally derived IFN-γ can be modulated in a similar way as endogenous IFN-γ is. Thus, Tc1 cells exhibit stronger secretion of recombinant IFN-γ than Tc2 cells, an observation most obvious at day 16 after stimulation. On the other hand, retroviral IFN-γ expression participates in the polarization of CD8⁺ T cells, as illustrated by a decrease in IL-4 production by transduced Tc2 cells.

IFN-γ plays a key role in the homeostatic control of CD8⁺ T cells [21]. Thus, expression of this cytokine by retroviral transduction might impair T-cell function. However, in our hands, infected Tc1 cells that over-expressed IFN-γ did not exhibit a significantly diminished viability. Growth and cytotoxic function of these T cells were also not altered throughout a 3- to 4-week restimulation period in vitro, in spite of stably increased IFN-γ throughout a 3- to 4-week restimulation period in vitro. Growth and cytotoxic function of these T cells were also not altered throughout a 3- to 4-week restimulation period.

It was shown that IFN-γ can directly inhibit tumor cell growth [27, 28], enhance tumor immunogenicity by increasing tumor antigen presentation [29], and recruit and activate innate antitumor responses [30, 31]. Furthermore, IFN-γ suppresses tumor angiogenesis by inducing angiogenesis-inhibitory chemokines [22] and, most notably, invasion of antitumor T cells into the tumor stroma seems to depend on IFN-γ signaling [9]. Comparison of IFN-γ deficient CD8⁺ T cells and/or Tc1 and Tc2 populations [18, 19] in different experimental tumor systems suggested that the capacity to secrete large amounts of IFN-γ is the most critical antitumor effector mechanism mediated by adaptively transferred CD8⁺ T cells in vivo. In the present study, we show that retrovirally armed CD8⁺ T cells producing increased amounts of IFN-γ delay the outgrowth of an experimental tumor when transferred into IFN-γ deficient mice. This observation suggests that CD8⁺ T cells can be readily equipped with IFN-γ to augment their therapeutic function.

<table>
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<th>Infection</th>
<th>Stimulation</th>
<th>pg IFN-γ/ml</th>
<th>% specific lysis</th>
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<tr>
<td>GFP-RV</td>
<td>IL-12/IL-18</td>
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<tr>
<td>IFN/GFP-RV</td>
<td>IL-12/IL-18</td>
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<td>IFN/GFP-RV</td>
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**Fig. 6** Retroviral expression of IFN-γ enhances IL-12/IL-18-induced IFN-γ production by CD8⁺ T cells. CL4 transgenic primary T cells were activated under Tc1 conditions and infected by GFP-RV or IFN/GFP-RV on day 2. GFP⁺/CD8⁺ T cells from the GFP-RV infected culture, as well as GFP⁺/CD8⁺ T cells and GFP⁺/CD8⁺ T cells from the IFN/GFP-RV infected culture were purified by cell sorting on day 8, and a fraction of the sorted populations was restimulated at a density of 2 × 10⁶ cells/well in a 96-well plate with 10 U/ml IL-12 and 10 ng/ml IL-18. Supernatants were harvested after 24 h, and IFN-γ content of the supernatants was determined by ELISA. Addition of either cytokine alone was not sufficient to induce secretion of IFN-γ (data not shown). The dotted line indicates the detection limit of the ELISA. Data are representative for at least three independent experiments.

**Fig. 7** IFN-γ transduction does not affect specific cytolytic activity but enhances tumor immunity upon adoptive transfer. a CL4TcR-transgenic primary T cells were activated under Tc1 and infected by GFP-RV (squares) or IFN/GFP-RV (circles) on day 2. GFP⁺/CD8⁺ T cells were purified by cell sorting on day 8 as described in Fig. 1 and incubated with 5000 [³H]-thymidine-labeled P815 cells in 96-well, U-bottom plates with (circles) or without (squares) 1 μg/ml HA peptide 512-520 for 1 h. Sorted CL4 effector T cells were added at different ratios and plates incubated for 4 h. Percent cytotoxicity was calculated from the mean of triplicate wells as described. b IFN-γ deficient mice were injected s.c. with 1 × 10⁶ CT26-HA cells. On the same day mice received 3 × 10⁶ GFP-RV infected (filled circles) or IFN/GFP-RV-infected (open circles) CL4 Tc1 cells i.v. or were left untreated (filled squares). The mean tumor diameter after tumor cell injection is shown. P values (Student’s t test) are shown for mice that received GFP-RV infected T cells vs IFN/GFP-RV-infected T cells. *P < 0.05, **P < 0.005.
One other group has previously transduced murine tumor-specific CD8\(^+\) T lymphocytes with the IFN-\(\gamma\) gene [30, 31]. In these studies, a tumor-specific T-cell line that did not produce IFN-\(\gamma\) was transduced with a retrovirus encoding a mono-cistronic IFN-\(\gamma\) cDNA. One T-cell clone, obtained from the IFN-\(\gamma\) transduced cell line, was shown to constitutively secrete IFN-\(\gamma\) in vitro and displayed enhanced cytolytic activity against a tumor in vivo. In contrast, CD8\(^+\) T cells transduced with the IFN-\(\gamma\) gene still depended on physiological signals to secrete the cytokine in our experimental setting. This result corroborates the general observation that IFN-\(\gamma\) secretion and cytotoxicity are regulated independently in CD8\(^+\) T cells [32]. Thus, our study establishes compellingly that CD8\(^+\) T cells can be selectively equipped with interferon \(\gamma\) without the risk of immunopathology by deregulated production of this proinflammatory cytokine.

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Enhanced brain targeting efficiency of intranasally administered plasmid DNA: an alternative route for brain gene therapy

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Abstract Recently, nasal administration has been studied as a noninvasive route for delivery of plasmid DNA encoding therapeutic or antigenic genes. Here, we examined the brain targeting efficiency and transport pathways of intranasally administered plasmid DNA. Quantitative polymerase chain reaction (PCR) measurements of plasmid DNA in blood and brain tissues revealed that intranasally administered pCMVβ (7.2 kb) and pN2/CMVβ (14.1 kb) showed systemic absorption and brain distribution. Following intranasal administration, the β-galactosidase protein encoded by these plasmids was significantly expressed in brain tissues. Kinetic studies showed that intranasally administered plasmid DNA reached the brain with a 2,595-fold higher efficiency than intravenously administered plasmid DNA did, 10 min post-dose. Over 1 h post-dose, the brain targeting efficiencies

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were consistently higher for intranasally administered plasmid DNA than for intravenously administered DNA. To examine how plasmid DNA enters the brain and moves to the various regions, we examined tissues from nine brain regions, at 5 and 10 min after intranasal or intravenous administration of plasmid DNA. Intravenously administered plasmid DNA displayed similar levels of plasmid DNA in the nine different regions, whereas, intranasally administered plasmid DNA exhibited different levels of distribution among the regions, with the highest plasmid DNA levels in the olfactory bulb. Moreover, plasmid DNA was mainly detected in the endothelial cells, but not in glial cells. Our results suggest that intranasally applied plasmid DNA may reach the brain through a direct route, possibly via the olfactory bulb, and that the nasal route might be an alternative method for efficiently delivering plasmid DNA to the brain.

Keywords Nasal route · Brain gene delivery · Gene expression · Biodistribution

Introduction

Intranasal gene therapy has recently drawn attention as a noninvasive method for administering therapeutic genes. For example, intranasal administration of interleukin-12 genes has been studied as a method for treating osteosarcoma lung metastasis in mice [1]. Intranasal dosing of a plasmid DNA encoding transforming growth factor β1 was shown to prevent the development of murine experimental colitis [2]. Moreover, the nasal route has been studied as a new route for administering cystic fibrosis transmembrane conductance regulator genes during treatment of patients with cystic fibrosis [3].

Increasing evidence has suggested that numerous pharmaceutical agents can reach the brain by the nasal route [4]. Intranasally administered antiepileptic drugs have been delivered to the brain [5], and intranasal administration of neurotrophic factors was found to offer some degree of brain targeting with minimal invasiveness [6]. The use of the sniffing neuropeptide has been suggested as a transnasal approach for targeting agents to the human brain [7]. Recently, intranasal administration of nerve growth factor protein was shown to rescue recognition memory deficits in a transgenic mouse model [8]. However, few studies have examined the delivery of genes to the brain via the nasal route. Previous studies have examined the extent of absorption and the distribution of intranasally administered plasmid DNA [9, 10]. However, it remains unclear whether there is limit to the size of plasmid DNA that may be absorbed intranasally. Moreover, the nose-to-brain transport pathways and functional expression levels of intranasally administered plasmid DNA in brain tissues have not been fully elucidated. Here, we report that intranasally administered plasmid DNAs up to 14.1 kb in size could be delivered to the brain, and their encoded genes could be expressed. Moreover, we provide evidence that the intranasally administered plasmid DNA may reach the brain via the olfactory bulb.

Materials and methods

Preparation of plasmid DNA

Three plasmid DNAs of different sizes were used in this study. pCMVβ encoding the β-galactosidase gene (7.2 kb, Clontech, Palo Alto, CA, USA) and pVAXmIL-2 encoding murine interleukin-2 (3.5 kb) were prepared as described previously [9, 11]. To construct pN2/CMVβ encoding the β-galactosidase gene (14.1 kb), a β-galactosidase cDNA fragment spliced to a cytomegalovirus early promoter/enhancer sequence was cut from pCMVβ and inserted into the XhoI site of pN2 vector (kindly provided by Dr. KH Baek, Pochon CHA University, South Korea). For construction of pN2/dmCMVβ (14.1 kb), a deletion mutant of pN2/CMVβ, a smaller size of β-galactosidase cDNA fragment (4.31 kb) was cut from pdmCMVβ using PstI enzyme, blunt-ended, and ligated to the blunt-ended XhoI site of pN2 vector. All the plasmid DNAs used in this study were amplified using Escherichia coli DH5α and purified with a Qiagen plasmid giga kit (Qiagen, CA, USA). The purity of DNA preparations was confirmed on a 1% agarose gel.

Competitive and quantitative polymerase chain reaction (PCR)

Competitive PCR was performed by adding various concentrations of the internal standard (IS) plasmids to the reaction mixtures containing the sample DNAs. IS plasmids for pCMVβ and pVAXmIL-2 were constructed as described previously [9, 11]. Sense and antisense primers were used for co-amplification of 449- and 261-bp fragments from pN2/CMVβ and pN2/dmCMVβ, respectively. The sense primer was 5′-TTGACCTCCATAGAAGACACCGG-3′ and the antisense primer was 5′-CCCAACTTATCGCCTTGAGG-3′. Competitive PCR was performed in a 0.2-ml reaction tube containing 50 μl of PCR mixture composed of 1 μl sample DNA, 1 μl of the competitor plasmid, 4 μl, 2.5 mM dNTPs, 5 μl×10 reaction buffer (50 mM Tris, pH 8.0, 100 mM NaCl, 0.1 mM EDTA, 1 mM dithiothreitol, 50% glycerol, and 1% Triton X-100), 10 pmol of each primer, and 1 U Taq polymerase. The mixture was cycled in a Perkin-Elmer/ model 2400 thermocycler at 94°C for 30 s, 57°C for 30 s, and 72°C for 30 s for 35 cycles. The products of each
reaction were separated on a 2% agarose gel. A comparison of the PCR products was then used to determine the quantity of the target plasmids in the samples. The quantitation was made graphically. The amount of target DNA in the samples was read from the x-axis intercept where the log ratio of target/IS density became zero.

Intranasal or intravenous administration of plasmid DNA

Male ICR mice were supplied from Daehan Experimental Animal Center (Seoul, South Korea). The mice were used at 6 to 8 weeks of age. Animals received food and water ad libitum. Before the intranasal administration of plasmid DNA, mice were anesthetized with a single intraperitoneal dose of ketamine hydrochloride (80 μg/g). Plasmids were introduced into the nostril using a gel-loading tip. Each mouse received a total 100 μg of plasmid DNA in 20 μl of phosphate-buffered saline (PBS) for intranasal administration or in 100 μl of phosphate-buffered saline for tail vein injection.

Preparation of biological samples

At various times after intranasal or intravenous administration of plasmid DNA, approximately 30 μl of blood was collected from the tail vein using a capillary tube with 1.1-mm diameter. Total DNA from serum was prepared according to a method described by Zerbini [12] with slight modification. Serum samples were heated at 90°C for 3 min to inactivate nucleases and centrifuged at 15,000×g for 1 min to precipitate proteins. The supernatant was then used in the competitive PCR. For brain distribution study, mice were sacrificed at 5, 10, and 60 min after administration of plasmid DNA. Total brain or nine regional brain tissues were harvested. To minimize the influence of plasmid DNA in blood circulating through the tissues at the time of sampling, the samples were thoroughly washed several times with saline, blotted dry, and weighed. The samples were then suspended into DNAzol® (Gibco BRL, NY, USA) with a concentration of 50 mg tissue per ml and homogenized using Teflon tissue grinders. The tissue grinders were changed after each tissue homogenization to avoid the cross-contamination of the tissues. The homogenates (500 μl) were then loaded onto Wizard® DNA clean up columns (Promega, WI, USA). After the washing steps, the DNA was eluted with 50 μl of TE buffer.

Reverse transcription PCR analysis

To determine the mRNA expression of plasmid DNA in the brain tissues, the mice were sacrificed 24 h after intranasal or intravenous administration of plasmid DNA (50 μg/mouse), and brain tissues were removed. As a control, the brain tissues of untreated mice were treated in parallel. Total RNA was extracted from the brain tissues using a TRIzol® reagent (Gibco BRL). The cDNA was prepared using a First-Strand cDNA synthesis kit (Boehringer Mannheim, IN, USA). The mRNA levels of pCMVβ and pN2/CMVβ were semiquantitatively determined by using the expression levels of the glyceraldehyde-3-phosphate dehydrogenate (GAPDH) housekeeping gene as an endogenous reference. PCR amplification of a 1,036-bp segment of the β-galactosidase gene was performed using primers as described previously [13]. The primer pair to amplify a 360-bp segment of GAPDH gene was 5′-ATCACCATCTCCGAGGAGC-3′ for the sense and 5′-AGAGGCGCCATCCAGTCTTC-3′ for the antisense directions. mRNA levels of pVAXmIL-2 was quantitatively determined by quantitative reverse transcriptase (RT)-PCR using a synthetic RNA of IL-2 as an internal standard [11]. All PCR products were analyzed by electrophoresis on a 2% agarose gel.

β-Galactosidase enzyme assay

Protein expression resulting from delivery of pCMVβ in the relevant tissues were tested using a β-galactosidase enzyme assay system (Promega, WI, USA) according to the manufacturer’s instructions [13]. Mice were sacrificed 24 and 48 h after nasal administration of plasmid DNA (100 μg/mouse). Brain tissue samples were then suspended in the reporter lysis buffer and homogenized. The homogenates (500 μl) were then centrifuged at 10,000×g for 30 min. The supernatants were used for β-galactosidase assay.

Detection of plasmid DNA in brain cells

At 15 min after intranasal administration of plasmid DNA (pCMVβ), microvessel endothelial cells and microglial cells were isolated for detection of plasmid DNA. Microvessel endothelial cells were prepared as described previously [14]. In brief, isolated brains were minced into small pieces, and the suspension was spun at 10,000×g for 10 min. The pellet was resuspended in 0.02% dispase solution. After centrifugation of the dispase suspension, the pellet was resuspended in 15% dextran solution and spun. The resulting tissue pellet was resuspended and filtered through a mesh. After dissociation of the microvessel suspension using 0.1% collagenase/dispase, the microvessels were harvested and layered onto Percoll gradient. After the Percoll gradient centrifugation, the microvessel endothelial cell pellet was collected. Meanwhile, microglial cells were prepared as described previously [15]. In brief, brain tissues were minced and enzymatically digested by type II collagenase and DNase I (Sigma, MO, USA) in dissociation buffer. The digested brain material was resuspended in 5 ml of Hanks’ buffer and layered onto Percoll gradients. Following centrifugation, microglial cells were collected from the interfaces.
of two consecutive density gradients (1.124 and 1.088 g/ml layer). The microvessel endothelial cells and microglial cells were then suspended into DNAzol®, and the levels of plasmid DNA were measured using quantitative PCR.

Statistics

Statistical analysis of data was performed using the Student’s t test and analysis of variance (ANOVA). A P value of less than 0.05 was considered significant. Student–Newman–Keuls test was used as a post hoc test.

Results

Effect of plasmid sizes on systemic absorption following intranasal administration

The systemic absorption of intranasally administered plasmids in mice was compared using a quantitative polymerase chain reaction (PCR) strategy. Quantitative PCR is based on co-amplification of the sample template together with various amounts of internal standard (IS) molecules bearing the same primer recognition sites, but differing from the sample in size [16]. Previously, the IS plasmid, pdmCMVβ, was used for quantitative PCR measurement of pCMVβ. In this study, a deletion mutant of pN2/CMVβ, pN2/dmCMVβ, was designed to share the same sense and antisense primers as the parent plasmid used for target amplification (Fig. 1a). The expected PCR products were 449- and 261-bp for the target pN2/CMVβ and the IS competitor, respectively. Co-amplification of the pN2/CMVβ target with various amounts of the IS (pN2/dmCMVβ) generated PCR products of different band densities (Fig. 1b). Quantitation of PCR products by densitometry allowed generation of a calibration curve that showed a linear relationship over an extended range of DNA concentrations. A representative internal standard curve is shown in Fig. 1c. The femtogram masses of the plasmid could be detected using this quantitative PCR. The amounts of pCMVβ and pN2/CMVβ in the biological samples were calculated from the internal standard curve based on the ratio between the sample-based PCR product and the corresponding internal standard competitors. The internal standard curve was generated for each run.

All plasmid DNAs were detected in systemic circulation within 15 min of intranasal administration, and peak levels were attained within 2 h (Fig. 2). The plasmids differed in terms of their maximum serum concentrations (C_max) and their areas under the curve. The C_max of pVAXmIL-2 (3.5 kb) showed the highest C_max followed by pCMVβ (7.2 kb) and pN2/CMVβ (14.1 kb). The C_max of pCMVβ was sixfold higher than that of pN2/CMVβ. The areas under the curve, calculated using the WinNonlin™ program (Pharsight, CA, USA), were 23,989±12,007 pg/ml h for pVAXmIL-2, 1,438±807 pg/ml h for pCMVβ, and 251±114 pg/ml h for pN2/CMVβ. These results indicate that the sizes of intranasally administered plasmid DNA might affect the efficiency of systemic absorption.

Brain distribution and expression of intranasally administered plasmid DNA

Intranasally administered pCMVβ and pN2/CMVβ reached the brain, resulting in the protein expression of their encoded gene, β-galactosidase, in the brain tissues. At 15 min post-dose, the brain level of pCMVβ, measured by quantitative
PCR, was 5.2-fold higher than that of pN2/CMVβ (Fig. 3). The plasmid DNA levels in the brain peaked at 4 h, with pCMVβ showing 3.8-fold higher levels than pN2/CMVβ. These findings revealed that intranasally administered pCMVβ and pN2/CMVβ reached the brain and that the smaller plasmid was more effectively distributed to the brain following intranasal administration.

The functional integrity of the plasmid DNA in the brain was tested by measuring the protein expression levels of encoded genes, using β-galactosidase activity assay. Both pCMVβ and pN2/CMVβ showed significant protein expression levels of β-galactosidase in brain tissues after intranasal administration (Fig. 4). In contrast, brain β-galactosidase expression in mice intravenously injected with plasmid DNA was not significantly higher than that in untreated controls. These results revealed that intranasally administered DNA could be expressed in the brain regardless of plasmid size.

Brain targeting efficiency of plasmid DNA following intranasal or intravenous administration

To test the brain targeting efficiency of plasmid DNA following intranasal or intravenous administration, the plasmid DNA levels were measured from brain and serum following administration of plasmid DNA by the two different routes. After intravenous administration (Fig. 5a), the brain levels of plasmid DNA were 72-, 222-, and 37-fold lower than the serum levels of plasmid DNA, 5, 10, and 60 min post-dose, respectively. In contrast, the brain levels of DNA following intranasal administration were 6-, 11-, and 9-fold higher than the serum levels of DNA following intravenous administration 5, 10, and 60 min post-dose, respectively (Fig. 5b). The brain targeting efficiency was determined by calculating the brain-to-serum ratios of plasmid DNA concentrations. Over 60 min post-administration, the brain targeting efficiencies were consistently higher for intranasally administered plasmid DNA than for intravenously administered plasmid DNA (Fig. 5c). The highest difference was observed at 10 min post-dosing,
with intranasal administration showing 2,595-fold higher efficiency. These results indicate that the brain targeting efficiency of plasmid DNA significantly differed depending on the route, with intranasal administration showing significantly higher efficiency.

Brain regional distribution kinetics of plasmid DNA following intranasal or intravenous administration

Given the significant differences in the brain distribution efficiency of plasmid DNA following intravenous and intranasal administration, we hypothesized that intranasally administered plasmid DNA may have a direct nose-to-brain transport pathway. To test this hypothesis, we studied the early brain regional distribution kinetics of plasmid DNA following intranasal or intravenous administration. At 5 min post-dose, the brain levels of intravenously administered plasmid did not differ significantly among the nine regions examined (Fig. 6a). The regions include olfactory bulb, septal area, striatum, hypothalamus, thalamus, midbrain, hippocampus, medulla oblongata, cerebellum. However, the regional levels of intranasally administered plasmid showed significant differences, with the olfactory bulb showing significantly higher levels than the other regions. Regions far from the nose like the medulla oblongata and thalamus showed no detectable levels of plasmid DNA at 5 min. At 10 min post-dose, intranasally administered plasmid DNA was distributed to the olfactory bulb, septal area, and striatum, with significantly higher levels noted as compared to intravenously administered cases (Fig. 6b). At 60 min after intravenous administration, the levels of plasmid DNA were significantly decreased in all regions of brain tissues as compared to 10 min post-dose (Fig. 6c). Moreover, the regional levels of plasmid DNA after intravenous administration were lower than those after intranasal administration. Among the nine regions, the olfactory bulb showed the highest level of plasmid DNA following intranasal administration (Fig. 6c). Unlike the intravenous route, the intranasal administration showed increased level of plasmid DNA in medulla oblongata and cerebellum at 60 min as compared to 10 min.

To study the distribution of plasmid DNA in brain cells, the microvessel endothelial cells and microglial cells were isolated after intranasal administration of pCMVβ at two doses. The plasmid DNA was observed in the microvessel endothelial cells but not in the microglial cells (Fig. 7). In the microvessel endothelial cells, the levels of plasmid DNA increased in proportion to the intranasal dose of plasmid DNA.
Discussion

In this study, we demonstrated that intranasally administered plasmid DNA could reach the brain via the olfactory bulb and that intranasal administration may provide higher brain targeting efficiency than intravenous administration. We observed that intranasally administered plasmid DNAs up to 14.1 kb in size could be systemically absorbed. The absorption mechanism for large molecules like plasmid DNA is unknown, but it is possible that a transport system may facilitate the absorption of plasmid DNA across the nasal mucosa. Our observation that the smaller plasmid DNA, pCMVβ, showed higher systemic absorption may suggest that the smaller plasmid DNAs might pass through the nasal epithelial membranes more easily. The systemic absorption rate of pCMVβ (7.2 kb) after intranasal dosing was relatively slow compared to some other intranasally administered small molecules such as the migraine drug, alniditan. The time to reach $C_{\text{max}}$ ($T_{\text{max}}$), an index of the speed of absorption, was longer for intranasally administered pCMVβ (90 min) than for nasally administered alniditan (11 min) [17].

Although the systemic absorption of plasmid DNA peaked within 2 h, the brain levels of plasmid DNA were highest at 4 h post-dose. This difference between systemic absorption and brain distribution rates may support the notion that intranasally administered plasmid DNA may reach the brain via a direct, nonsystemic route. To test this possibility, we measured the distribution levels of plasmid DNA in nine different brain regions following intranasal or intravenous administration (Fig. 6). Currently, it is hard to describe quantitatively what proportion of plasmid in the brain might be derived from the systemic circulation following intranasal delivery. However, the different regional distribution patterns
at early (5 min) and late (60 min) time points after intravenous and intranasal administrations (Fig. 6) imply that the major portions of plasmid DNA in the brain after intranasal administration might be from the direct absorption from the nasal cavity rather than systemic circulation. Moreover, the highest plasmid DNA levels in the olfactory bulb following intranasal administration and the higher levels of plasmid DNA at all the time point (Fig. 6c) support the notion that the olfactory bulb might play an important role in the absorption of intranasally administered plasmid DNA into the brain.

In this study, following the nasal administration of plasmid DNA encoding β-galactosidase, we measured the mRNA and protein level expression of β-galactosidase using quantitative RT-PCR and colorimetric enzyme function assay, respectively. Both methods support that plasmid DNA was absorbed in the form intact enough to be transcribed and expressed in vivo. However, as we used the tissue homogenates for quantitative RT-PCR and enzyme function assay, there is limited information regarding the cell types and intracellular localization of expressed proteins. Immunohistochemistry or immunofluorescence techniques may be needed to visualize the brain cellular distribution patterns of proteins expressed from intranasally administered plasmid DNA in the future study.

The brain targeting efficiency of plasmid DNA was significantly higher following intranasal administration vs intravenous administration. It has been reported that the distribution levels of plasmids to the brain were about 145-fold lower than that to the liver 20 min after intravenous injection of DNA [18]. We previously observed that the level of plasmid DNA in the brain was only 15-fold lower than that in the liver 15 min after intranasal administration [9]. The higher brain targeting efficiency of plasmids after intranasal administration might be at least partly attributed to the fact that the traditional blood–brain barrier is not present at the interface between the nasal epithelium and the brain [19].

Although the exact mechanisms by which intranasally administered plasmid DNA confers higher brain targeting efficiency remains to be studied, our regional distribution kinetic data suggest that intranasally administered plasmid DNA might reach the brain via the olfactory bulb. In agreement with our observation, other studies have suggested that intranasally administered small molecules might be delivered to the brain via the olfactory epithelium [19]. Moreover, a protein neurotrophic factor such as insulin-like growth factor-I (m.w., 7.65 kDa) was shown to reach the rat brain and spinal cord along olfactory and trigeminal pathways following intranasal administration [20]. The brain delivery route was also observed following intraocular administration of a recombinant adeno-associated viral vector, via the optic nerve [21].

The enhanced brain targeting following intranasal administration of plasmid DNA would be advantageous for delivery of genes having therapeutic activities on brain disorders such as Alzheimer or Parkinson’s diseases. Recently, ex vivo nerve growth factor gene therapy was used to treat Alzheimer disease [22], while in vivo viral gene transfer of dopamine-synthetic enzymes was reported to reverse motor impairments in an animal model of Parkinson’s disease [23]. Given the noninvasive nature of intranasal delivery, the nasal route might be further developed as a new mode of brain gene therapy. However, it should be noted that the higher brain targeting efficiency of nasally administered plasmid DNA could be disadvantageous for administration of agents not intended for brain targeting. Daily nasal inoculation with the insulin gene complexed to cationic liposomes was shown to ameliorate diabetes in mice [24]. Moreover, intranasal immunization with HIV-1 DNA resulted in neutralizing humoral mucosal and systemic immunity [25]. Additional work will be required to determine whether the high brain distribution of intranasally administered DNA for systemic therapy could exert any side effects after repeated dosings.

We observed that nasally administered plasmid DNA was mainly detected in the microvessel endothelial cells, but not in glial cells. Our result indicates that the nasal route might be useful for targeting plasmid DNA to brain endothelial cells. Recently, small interfering RNA (siRNA) was systemically injected to target brain endothelial cells [26]. For in vivo delivery, large dose of siRNA was intravenously administered using hydrodynamic technique in the study. Given the therapeutic potential of siRNA for brain diseases, it might be interesting to test whether nasal route may also provide more effective brain endothelial cell targeting of siRNA as compared to systemic injection.

For clinical application, it might be necessary to select devices that favor various biodistributions of intranasally administered plasmid DNA. The bidirectional nasal delivery device was reported to minimize the lung deposition occurring during conventional nasal inhalation from a nebulizer [27]. Given that droplet size determines the likelihood of deposition within the nasal cavity by inertial impaction [28], the development of nasal devices which can enhance the local distribution of plasmid DNA to the olfactory regions in the nasal cavity may increase the feasibility of intranasal route for brain gene therapy.

In conclusion, we, herein, show that intranasally administered plasmid DNA may reach the brain with enhanced targeting efficiency over systemic administration. Moreover, our data provide evidence that intranasally administered plasmids may reach the brain through a more direct route, most likely via the olfactory bulb. These novel findings will hopefully form the basis for important therapeutic advances in the gene therapy of brain diseases.
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References

The roles of endogenous reactive oxygen species and nitric oxide in triptolide-induced apoptotic cell death in macrophages

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Abstract Triptolide, a major active component extracted from the root of *Tripterygium wilfordii* Hook f, has been shown to possess potent immunosuppressive and anti-inflammatory properties. In the present report, we reported that triptolide increased the generation of reactive oxygen species (ROS) and nitric oxide (NO) and induced apoptosis of RAW 264.7 cells in a dose-dependent manner (5–25 ng/ml). The antioxidant, reduced glutathione (GSH), significantly inhibited triptolide-induced apoptosis and inhibited the degradation of Bcl-2 protein, disruption of mitochondrial membrane potential, release of cytochrome c from mitochondria into the cytosol, activation of caspase-3, and cleavage of poly-(ADP-ribose)-polymerase. The inducible nitric oxide synthase-specific inhibitor 1400w blocked triptolide-induced apoptosis, but did not alter mitochondria disruption and caspase-3 activation. These results, for the first time, implicated that the increased endogenous ROS and NO co-mediated triptolide-induced apoptosis in macrophages. ROS initiated triptolide-induced apoptosis by the mitochondria signal pathway, while the apoptotic cell death mediated by NO was not via mitochondria collapse and caspase-3 activation. In addition, combining mathematical calculation and computer simulation based on our conventional experimental results, we set and validated the apoptotic model and provided more dynamic processes of triptolide-induced apoptotic cascade in macrophages.

Keywords Triptolide · Macrophage · Apoptosis · Reactive oxygen species · Nitric oxide · Simulation

Introduction

Extracts of the root of *Tripterygium wilfordii* Hook f (TWHf) have been used in traditional Chinese medicine...
for the treatment of a variety of autoimmune diseases such as rheumatoid arthritis, nephritis, systemic lupus erythematosus, psoriasis, and dermatomyositis for centuries [1–4]. Triptolide, a diterpene triepoxide, is one of the major components of most functional extracts of TWHf and is known to have immunosuppressive and anti-inflammatory properties [5]. Studies have shown that triptolide inhibits mitogen-stimulated lymphocyte proliferation and possesses anti-proliferative activity against leukemic cells and transformed cell lines [6, 7]. Clinical and experimental studies have also demonstrated that Tripterygium extracts effectively prolong allograft survival [8]. Recently, reports have revealed that triptolide can promote apoptosis in a variety of cell types including lymphocytes, leukemic cells, tumor cells, and monocytes [9–12].

Apoptosis is an autonomously programmed cell death mechanism that is utilized extensively during the development and maintenance of tissue and organ homeostasis. Cell shrinkage, membrane blebbing, chromatin condensation, and formation of a DNA ladder on an agarose gel electrophoresis are characteristics of apoptosis [13]. Numerous studies have shown that the mitochondrial pathway plays an important role in apoptotic cell death and members of the Bcl-2 protein family control the apoptotic signals [14]. During apoptosis, the anti-apoptotic proteins including Bcl-2 and Bcl-XL are inhibited, while the pro-apoptotic proteins, such as Bax and Bad, dominate. These changes lead to the alteration of the mitochondrial membrane potential (ΔΨm) and result in some key effector proteins such as cytochrome c release from the mitochondria into the cytosol, eventually leading to apoptosis [14–17].

Macrophage plays a pivotal role in innate immune system by recognizing and destroying altered host compounds and invading microorganisms. However, when the control mechanisms go awry, the inflammatory response of macrophage can result in persistent swelling, pain, and eventually tissue injury. Studies showed that the immune dysfunction of thermal injury was related to the hyperactivity of macrophages leading to increased release of pro-inflammatory factors [18]. The increased number of macrophages in the joint, in both the lining and sublining areas of rheumatoid arthritis synovium, greatly correlated with the severity of cartilage destruction [19, 20]. Accordingly, the studies aiming to illustrate the effects of triptolide on macrophages become very significant. Kim et al. [21] found that triptolide inhibited transcription of the inducible nitric oxide synthase (iNOS) gene in macrophage cell line RAW 264.7 by suppressing the activity of NF-κB and c-jun NH2-terminal kinases. In our previous works, we showed that triptolide exhibited an immunosuppressive effect on macrophages and could attenuate NF-κB activation and multiple proinflammation cytokines gene expressions [22]. However, the molecular mechanisms underlying triptolide-induced apoptosis of macrophages are poorly understood.

The immunosuppressive property of triptolide is correlated with its activity in inducing apoptosis. It was demonstrated that triptolide inhibited proliferation and induced apoptosis in a caspase-3-dependent and Bcl-2-regulated manner in T cell lines [9]. In dendritic cells, triptolide could induce apoptosis through sequential p38 phosphorylation and caspase-3 activation [12]. In this study, we used the RAW 264.7 macrophage cell line as a model to investigate the molecular mechanisms of triptolide on the induction of apoptosis in macrophages. We confirmed that triptolide could increase the generation of reactive oxygen species (ROS) and nitric oxide (NO), and the elevation of ROS and NO regulated the process involved in triptolide-induced apoptosis. Furthermore, we examined a sequence of events related to the mitochondria disruption, including the changes of the Bcl-2 family of proteins, alteration of ΔΨm, release of cytochrome c from mitochondria into the cytosol, activation of caspase-3, and cleavage of poly-(ADP-ribose)-polymerase (PARP) protein. The data showed that increased intracellular ROS mediated triptolide-induced apoptosis in macrophages through the mitochondria signaling pathway. In contrast, apoptosis mediated by endogenous NO was not via mitochondria destruction and caspase-3 activation. In addition, based on our conventional experimental results, we proposed a hypothetical apoptotic model of triptolide-induced signal pathway. By using computer simulations, we validated the model and further explain the apoptotic cascade induced by triptolide in macrophages.

Materials and methods

Reagents

Crystalline triptolide (PG490, molecular weight 360) was obtained from Alexis (Carlsbad, CA). Triptolide was dissolved in dimethyl sulfoxide (DMSO) and stored at 2 mg/ml at −20°C. Triptolide was freshly diluted with culture medium to the indicated concentrations before use. DMSO concentration in experimental conditions never exceeded 0.01% (V/V). RPMI 1640 medium was supplied by Hyclone (Logan, UT). Reverse transcription (RT) and polymerase chain reaction (PCR) reagents were purchased from Promega (Madison, WI). The iNOS-specific inhibitor N-3-aminomethyl-benzyl-acetamidine (1400w) was purchased from Calbiochem (San Diego, CA). Antibody against cytochrome c was obtained from BD PharMingen (San Diego, CA). Antibodies against Bcl-2, Bax, caspase-3, p53, and PARP were purchased from Cell Signaling Technology (Beverly, MA). All chemicals were purchased from Sigma Chemicals (St. Louis, MO) unless otherwise stated.
Cell culture

RAW 264.7 and U937 macrophage-like cell lines were obtained from Shanghai Institutes for Biological Sciences. The cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO₂ at 37°C. All experiments were performed using RPMI 1640 medium with the same supplementation, except that fetal bovine serum was at 1%.

Annexin V staining

Triptolide-induced cell death was determined by annexin V–fluorescein isothiocyanate kit according to the manufacturer’s instruction. Macrophages incubated with triptolide for 24 h were harvested and washed with cold phosphate-buffered saline (PBS) twice. Briefly, cells were resuspended in binding buffer at a concentration of 1×10⁶ cells/ml and 200 μl cell suspension was subsequently transferred to a 5-ml culture tube, treated with 5 μl annexin V and 10 μl propidium iodide (PI) at 37°C for 15 min in the dark. The cells were then diluted with 200 μl of binding buffer and 10,000 cells in each group were analyzed on a FACS Calibur (Becton Dickinson, USA) equipped with a single laser emitting excitation light at 488 nm.

DNA fragmentation assay

The cells (4×10⁶) were allowed to adhere to the culture plates overnight. The cells were treated with triptolide for 24 h before they were harvested; they were then lysed in 20 μl lysis buffer (100 mM Tris–HCl, pH 8.0; 20 mM EDTA; 0.8% sodium dodecyl sulfate) at 37°C for 6 h. Ten microliters of RNase (2 mg/ml) was then added to the cellular lysate and incubated at 37°C for 4 h, followed by addition of 10 μl proteinase K (20 mg/ml) and incubation at 50°C overnight. Fragmented DNA in the lysate was then analyzed electrophoretically on 1% agarose gels containing 0.1 mg/ml ethidium bromide.

Nitrite assay

After treatment with triptolide, the cells were harvested and total RNA was extracted by using TriPure Isolation Reagent (Roche Molecular Biochemicals, IN). cDNA was prepared from 1 μg RNA with avian myeloblastosis virus reverse transcriptase. PCR reaction was performed in a 50-μl mixture on the Gene Amp 480 System (Perkin-Elmer, Wellesley, MA). Primer sequences were as follows: iNOS, forward primer 5′-CTGACGACTTGGATCAGGAACCTG-3′, reverse primer 5′-GGGAGTGCGCCTGTGGCAGAAC-3′; GAPDH, forward primer 5′-AAGCACCCTTCATTGACC-3′, reverse primer 5′-TCAGATGCCTGCTTACCAC-3′. PCR products were electrophoresed on 1% agarose gels containing 0.1 mg/ml ethidium bromide and visualized under ultraviolet illumination. Band intensity was calculated densitometrically using Gelwork 1D Intermediate, UVP software. Levels of iNOS mRNA were expressed as the ratio of band intensity for iNOS to that for GAPDH.

Intracellular ROS determination

The generation of intracellular ROS was detected by using the fluorescence probe dihydrorhodamine 123 (DHR). Cells were plated in a 96-well plate (1×10⁵ cells/well). After 24 h, cells were treated with different doses of triptolide for 12 h and then incubated in 1 μM DHR at 37°C for 30 min. The fluorescence intensity was measured at an excitation wavelength of 488 nm and an emission of 530 nm by using a fluorescent Mutli-label Counter (Safire, TECAN, Austria). The relative amount of intracellular ROS production was expressed as the fluorescence ratio of the treatment to control.

Mitochondrial membrane potential (ΔΨ) assay

To assess the ΔΨ, the green fluorescent lipophilic cationic dye 3,3′-dihexyloxacarbocyanine iodide (DiOC6) was used. Cells (1×10⁶) were exposed to triptolide for 12 h. After exposure, cells were harvested and washed with cold PBS twice. Cells were then resuspended in 400 μl medium and cultured in 40 nM DiOC6 at 37°C for 30 min. ΔΨ was determined by FACS analysis.

Western blotting

Cells cultured as above were harvested and then suspended in 300 μl lysis buffer (50 mM Tris–HCl, pH 8.0; 150 mM NaCl; 1 mM EDTA, 10% glycerol; 1 mM DTT; 1% NP-40; 1 mM PMSF) at 4°C for 30 min. Cell debris were removed by centrifugation at 12,000×g for 10 min at 4°C, and the supernatants were collected and stored at −70°C until used. Protein concentration of cell lysate was determined by the Bradford method. The proteins (50 μg per sample) were
electrophoresed on 8, 10, 12, or 15% sodium dodecyl sulfate polyacrylamide gels, and then electrophoresed onto a polyvinylidene fluoride membrane (Amersham, UK). After being blocked in PBS containing 0.1% Tween 20 with 5% skimmed milk powder at 37°C for 4 h, the membrane was incubated with primary antibody overnight. The detection step was performed with horseradish peroxidase-conjugated antibody. The target proteins were visualized with an Enhanced Chemiluminescent Method kit (SABC, PRC).

Determination of cytochrome c release from mitochondria

Cells were treated with triptolide for 24 h and harvested from culture. After washing with PBS twice, the cells were suspended in 200 μl lysis buffer (250 mM sucrose, 1 mM EDTA, 50 mM Tris–HCl, 1 mM DTT, 1 mM PMSF, pH 7.4) [23]. After incubation in an ice bath for 10 min, the cells were homogenized with a glass homogenizer for 30 strokes. The homogenate was centrifuged at 1,000×g for 10 min at 4°C, and the supernatants were transferred to a new tube and centrifuged at 12,000×g at 4°C for 30 min. The supernatants were collected and used as the cytosol fraction. The cytochrome c level in the cytosol fraction was determined by Western blotting analysis.

Statistical analysis

Data was expressed as the mean±SE. Statistical analysis was performed by Student’s t test. P<0.05 or P<0.01 was considered statistically significant and was indicated by a single asterisk or two asterisks, respectively.

Mathematical modeling

Previously, mathematical models have been successfully applied to simulate the dynamic behaviors of apoptosis [24–27]. In this report, we developed a theoretical model based on our hypothesis to describe a possible mechanism of apoptosis induced by triptolide. The possible interactions of triptolide, ROS, and other apoptotic factors include the following:

1. Triptolide prompted the production of ROS in macrophages.
2. ROS could initiate the degradation process of Bcl-2 that could bind to Bax to prevent its activation.
3. Active Bax formed tetrameric channels on the mitochondrial outer membrane and released cytochrome c.
4. Cytochrome c then activated caspase-3 by forming apoptosome with the help of ATP, Apaf-1, and caspase-9.

The differential equations for this model are shown below:

\[
\frac{d TP_{out}}{dt} = -k_1 TP_{out}
\]

(1)

\[
\frac{d TP_{in}}{dt} = k_2 TP_{out} - d_1 TP_{in}
\]

(2)

\[
\frac{d ROS}{dt} = p_1 + k_3 \frac{TP_{in}^2}{c_1 + TP_{in}^2} - d_2 ROS
\]

(3)

\[
\frac{d Bcl2}{dt} = p_2 - k_4 \cdot ROS \cdot Bcl2 - d_3 Bcl2
\]

(4)

\[
\frac{d Bax}{dt} = p_3 - k_5 \cdot Bax \cdot Bcl2 - d_4 Bax
\]

(5)

\[
\frac{d cyt}{dt} = k_6 (cyt_{total} - cyt) \frac{Bax^4}{c_2 + Bax^4} - d_s cyt
\]

(6)

\[
\frac{d C3}{dt} = k_7 (C3_{total} - C3) \frac{cyt^2}{c_3 + cyt^2} - d_o C3
\]

(7)

Table 1 The biological meanings of the abbreviations found in the equations

<table>
<thead>
<tr>
<th>Abbreviations</th>
<th>Meaning</th>
</tr>
</thead>
<tbody>
<tr>
<td>TP_{out}</td>
<td>Triptolide outside cell</td>
</tr>
<tr>
<td>TP_{in}</td>
<td>Triptolide inside cell</td>
</tr>
<tr>
<td>ROS</td>
<td>Superoxide anion</td>
</tr>
<tr>
<td>Bcl2</td>
<td>Bcl-2</td>
</tr>
<tr>
<td>Bax</td>
<td>Bax</td>
</tr>
<tr>
<td>cyt</td>
<td>Cytochrome c</td>
</tr>
<tr>
<td>C3</td>
<td>Caspase-3</td>
</tr>
</tbody>
</table>

Table 2 Biological interpretation of the parameters found in the equations

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Biological Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>p_i</td>
<td>Normal production or activation coefficient of ROS or related protein</td>
</tr>
<tr>
<td>d_i</td>
<td>Degradation coefficient of ROS or related protein</td>
</tr>
<tr>
<td>k_i</td>
<td>Reaction coefficient of ROS and related proteins</td>
</tr>
<tr>
<td>c_i</td>
<td>The significant effect constant of related variable</td>
</tr>
<tr>
<td>cyt_{total}</td>
<td>Total number of cytochrome c (in or out mitochondria)</td>
</tr>
<tr>
<td>C3_{total}</td>
<td>Total number of caspase-3 (include pro-caspase-3)</td>
</tr>
</tbody>
</table>

subroutines, and all the graphics were performed using Matlab 6.5.

Results

Triptolide-induced apoptosis in macrophage cell line RAW 264.7

To verify triptolide-induced cell death, RAW 264.7 macrophage cells were exposed to increasing concentrations of triptolide (5, 10, 15, 20, or 25 ng/ml), and apoptosis was assessed 24 h later by annexin V staining. Triptolide induced a significant increase in annexin-V positive, PI-negative cells progressively, at the meantime annexin-V positive, PI-positive cells remained few (Fig. 1a), indicating that triptolide treatment resulted in macrophage apoptosis in a dose-dependent manner while no obvious necrosis occurred. Cells treated with doses of either 0, 5, 10, 15, 20, or 25 ng/ml triptolide had values of 0.55, 1.56, 3.77, 22.36, 55.30, and 65.67% apoptotic cells, respectively.

One hallmark of apoptosis is the degradation of chromosomal DNA at internucleosomal linkages [13]. To further confirm that the cell death induced by triptolide was due to apoptosis, the induction of DNA fragmentation was demonstrated by treating cells with different concentrations of triptolide for 24 h. The typical ladder pattern, internucleosomal fragmentation of DNA, was observed when the cells were treated with 15, 20, or 25 ng/ml triptolide. The amount of fragmented DNA peaked at the highest dose of 25 ng/ml (Fig. 1b). Consistent with the results of annexin V staining, the pattern of increased DNA fragmentation was also dose-dependent. This result suggested that the death of RAW 264.7 cells induced by triptolide at 24 h was due to apoptosis, and 15 ng/ml was the turning point.

Triptolide altered production of NO, ROS, and expression of iNOS mRNA

To investigate the effect of triptolide on NO production, we used Griess reagent to measure the accumulation of nitrite in the cell culture media after exposing the cells to different doses of triptolide for 24 h. As shown in Fig. 2a, triptolide concentrations lower than 15 ng/ml suppressed the NO productions, while higher than 15 ng/ml increased the production, compared to the untreated cells serving as the control. The increases of the NO level were 9.31, 31.63, and 52.44% at doses of 15, 20, and 25 ng/ml, respectively. Since NO production was both upregulated and down-regulated by triptolide, we assessed iNOS mRNA levels to determine whether triptolide might modulate iNOS gene transcription in view of iNOS catalyzes NO production. As illustrated in Fig. 2c, the expression of iNOS mRNA was inhibited at 5 and 10 ng/ml. In contrast, at 15 ng/ml or higher, iNOS mRNA was elevated gradually corresponding to increasing triptolide concentrations. The data showed that triptolide was capable of modulating NO generation by modulating iNOS expression.

Fig. 1 Triptolide-induced apoptosis in macrophage RAW 264.7. Cells were exposed to 0, 5, 10, 15, 20, and 25 ng/ml triptolide for 24 h. a After treatment, cells were harvested and assayed by annexin V–PI staining. The percentage of cells positive for annexin V was determined by flow cytometry. b After treatment, genomic DNA was extracted and subjected to 1.0% agarose gel electrophoresis. DNA fragmentation was analyzed by digitally imaged after staining with ethidium bromide. The results were representative of three independent experiments.
The intracellular ROS was determined as described in the "Materials and methods" section. The relative levels of ROS were expressed as the fluorescence intensity ratio of the triptolide-treated groups to the control (the untreated). As shown in Fig. 2b, at 12 h, the intracellular ROS was increased by 4.03, 11.15, 31.02, 42.99, and 63.61% at 5, 10, 15, 20, and 25 ng/ml triptolide, respectively.

Antioxidant GSH and iNOS-specific inhibitor 1400w inhibited triptolide-induced apoptotic cell death

Because triptolide treatment led to the elevation of ROS and the alterations of NO, it was possible that the changes in the cellular superoxidant state might regulate triptolide-induced apoptosis. To examine this hypothesis, antioxidant GSH and the iNOS-specific inhibitor 1400w were used to perturb the generation of ROS and NO in RAW 264.7 cells. The data in Fig. 2a,b showed that triptolide-induced ROS and NO were suppressed by co-treatment with 30 mM GSH and 50 μM 1400w. After co-treatment, annexin V-positive PI-negative cells were reduced at 24 h (Fig. 3). At a dose of 25 ng/ml triptolide, co-treatment with 30 mM GSH strikingly reduced triptolide-induced apoptotic cells from 65.67 to 21.60%, while co-treatment with 50 μM 1400w reduced triptolide-induced apoptotic cells from 65.67 to 41.03% (Fig. 3), which is a relatively smaller effect. Both agents inhibited triptolide-induced apoptotic cell death significantly, suggesting that both ROS and NO were involved in triptolide-induced apoptosis in RAW 264.7 macrophages.

To further confirm the involvement of ROS and NO in triptolide-induced apoptosis in macrophages, another macrophage-like cell line, U937, was employed to perform additional experiments. As shown in Fig. 6a, at a dose of 25 ng/ml triptolide, co-treatment with 30 mM GSH reduced triptolide-induced apoptotic U937 cells from 31.03 to 10.52%, while co-treatment with 50 μM 1400w only reduced apoptotic U937 cells from 31.03 to 29.09%, not exhibiting statistically significant effect. The results from these two cell lines indicated that both ROS and NO were involved in triptolide-induced apoptosis in macrophages, and ROS play a more important role.

Triptolide-induced disruption of ΔΨm, release of cyttochrome c, degradation of Bcl-2 protein, activation of caspase-3, and cleavage of PARP

Mitochondria play a pivotal role in apoptosis. We investigated the possibility that triptolide-induced apoptosis might be related to mitochondria. As seen in Fig. 4a, the exposure of the cells to triptolide for the indicated time induced a gradual left shift of the DiOC6 fluorescence...
Therefore, we measured the levels of subunits after the release of cytochrome to generate an active heterodimer of 20 and 12 kDa pro-apoptotic Bax dropped.

expression; nevertheless, the ratio of anti-apoptotic Bcl-2 to treated. However, there were no notable changes in the Bax by more than 80%, compared with the cells that were not treated. 25 ng/ml triptolide, the level of Bcl-2 protein was reduced to 25 ng/ml triptolide, the cytochrome c level in the cytosol fraction was slightly elevated. In contrast, a substantial increase of cytosol cytochrome c was detected at 15 ng/ml and the increase peaked at 25 ng/ml.

Evidence suggested that the Bcl-2 protein family regulated the mitochondria cytochrome c release [14–16]. Thus, we tried to reason that cytochrome c release caused by triptolide was the result of Bcl-2 family’s modulation. Figure 4b showed an overview of the expression of Bcl-2 and Bax after triptolide exposure. A significant decrease of Bcl-2 expression was detected, as shown in Fig. 4b. At 25 ng/ml triptolide, the level of Bcl-2 protein was reduced by more than 80%, compared with the cells that were not treated. However, there were no notable changes in the Bax expression; nevertheless, the ratio of anti-apoptotic Bcl-2 to pro-apoptotic Bax dropped.

Caspase-3 is activated by cleaving the 32-kDa precursor to generate an active heterodimer of 20 and 12 kDa subunits after the release of cytochrome c from the mitochondria [28]. Therefore, we measured the levels of the active 20-kDa subunit to examine the effect of triptolide on caspase-3 activation. A gradual increase of the 20-kDa band intensity was detected on Western blot using caspase-3-specific monoclonal antibody (Fig. 4b), suggesting that caspase-3 was activated by the treatment. Next, the cleavage of PARP, a result of caspase-3 activation during apoptosis, was also detected after triptolide exposure. The data in Fig. 4b indicated that the triptolide treatment led to the accumulation of the 89-kDa fragment together with the dissipation of the 116-kDa protein, indicating a dose-dependent cleavage of PARP.

The p53 protein is an important regulator of apoptosis, and RAW 264.7 cells harbor the wild-type p53 gene. We then detected the p53 status in triptolide-induced apoptosis in RAW 264.7 cells. As shown in Fig. 4b, there were no obvious changes in p53 protein expression after triptolide treatment, suggesting that p53 might be not involved in triptolide-induced apoptosis in macrophages.

Since triptolide modulated ROS and NO production (Fig. 2) and antioxidant GSH and the iNOS-specific inhibitor 1400w could obviously attenuate triptolide-induced apoptosis (Fig. 3), we supposed whether triptolide-induced mitochondria-associated apoptosis was mediated by ROS and NO. As displayed in Fig. 5a, after co-incubation with 30 mM GSH, triptolide-induced, left shift of the DiOC6 fluorescence curves was reversed, and the profile was nondistinctive to the untreated control. The release of cytochrome c from the mitochondria into the cytosol and the degradation of Bcl-2 protein were also inhibited after co-incubation with GSH, as compared with triptolide treatment alone (Fig. 5b). However, co-incubation with 50 μM 1400w did not reverse the left shift of fluorescence curves induced by triptolide (Fig. 5a) and did not show discernible inhibition on cytochrome c release and Bcl-2 degradation (Fig. 5b). In U937 cells, triptolide-induced cytochrome c release was also inhibited after co-treatment with 30 mM GSH, while co-treatment with 50 μM 1400w did not reveal an inhibitory effect (Fig. 6b). The results indicated that triptolide-induced destruction of the mitochondria in RAW 264.7 and U937 cells were blocked by GSH, while 1400w could not protect the cells against the mitochondria disruption.

Subsequently, we examined whether GSH and 1400w could block caspase-3 activation and PARP cleavage induced by triptolide. The co-treatment with GSH exhibited clear suppressive effects on both caspase-3 activation and PARP cleavage, but no perceptible inhibitory effect was detected for 1400w (Fig. 5b).
Discussion

Triptolide, a major active component of TWHf, has been widely used as an effective immunosuppressor in the clinical application [1–5]. Our previous work have documented that triptolide exposure suppressed activity of NF-κB and inhibited gene expression of some key proinflammatory cytokines [22]. It has been reported that triptolide causes apoptosis in a range of cell lines [9–12]. In this paper, we further explored the role of triptolide in inducing apoptosis of macrophages and the delineation of the molecular mechanisms for its immunosuppressive activities. We showed that, by annexin V staining and DNA fragmentation, triptolide induced apoptosis in RAW 264.7 cells in a

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**Fig. 4** Triptolide-induced disruption of $\Delta \Psi_m$, release of cytochrome $c$ from mitochondria into cytosol, degradation of Bcl-2 protein, activation of caspase-3, and cleavage of PARP. Cells were treated with triptolide at indicated doses. **a** At 12 h, cells were harvested and incubated in the presence of 40 nM DiOC6 at 37°C for 30 min. $\Delta \Psi_m$ was determined by FACS analysis. **b** At 24 h, triptolide-induced release of cytochrome $c$ into cytosol, changes of Bcl-2 and Bax protein, activation of caspase-3, and cleavage of PARP were analyzed by Western blotting. The results were representative of three independent experiments.
dose-dependent manner, and the results were further confirmed in another macrophage-like cell line U937 (Figs. 1, 3, and 6). This work also demonstrated that triptolide treatment induced intracellular ROS and NO and resulted in the activation of a series of signaling molecules. However, we also documented that the triptolide-induced macrophage apoptosis mediated by ROS is distinct from that by NO. As far as we know, this is the first evidence of roles for ROS and NO in triptolide-induced macrophage apoptosis.

Most apoptotic signaling processes converge on the mitochondria, and the loss of \( \Delta \Psi_m \) is implicated as an earlier event in the induction of apoptosis by mitochondria [14, 17]. In this work, we demonstrated a gradual decrease in \( \Delta \Psi_m \) in responding to increasing triptolide doses (Fig. 4a), implying that the mitochondrion was correlated with triptolide-induced apoptosis. Mitochondrial permeability transition (MPT) could be triggered by the disruption in \( \Delta \Psi_m \), and the opening of the permeability transition pore deflated the proton gradient and led to the swelling of the mitochondria, resulting in the release of cytochrome \( c \) from the mitochondria into the cytosol [14, 16]. Consistent with the collapse of \( \Delta \Psi_m \), a dose-dependent release of cytochrome \( c \) induced by triptolide was demonstrated in our experiments (Fig. 4b), providing further evidence that the breakdown of the mitochondria was involved in triptolide-induced apoptosis in macrophages.

The Bcl-2 family is a number of potent regulators of apoptosis that can influence the permeability of the outer mitochondrial membrane and the release of cytochrome \( c \) [14–16]. In the present work, the changes of Bcl-2 family protein expression were detected (Fig. 4b). Although no apparent changes occurred in total Bax protein level, the ratio of Bcl-2/Bax dropped. During apoptotic stimulation, cytosolic Bax inserts into the mitochondria membrane after oligomerization and Bid-induced conformation change, where it exerts its apoptotic function by opening the mitochondrial transition pore [29–31]. By binding with Bax, the anti-apoptotic Bcl-2 prevents the opening of the mitochondrial transition pore and subsequently blocks apoptosis by stabilization of \( \Delta \Psi_m \) and inhibition of cytochrome \( c \) release [14, 32, 33]. Therefore, the declined Bcl-2/Bax protein ratio in our observations should lead to the dismissal of the Bcl-2/Bax complex and the release of

Fig. 5 Effect of antioxidant GSH and the iNOS-specific inhibitor 1400w on triptolide-induced mitochondria-associated apoptotic cascade. Cells were pretreated with 30 mM GSH or 50 \( \mu \)M 1400w for 1 h, and then cultured with triptolide at indicated doses. a At 12 h, cells were harvested and incubated in the presence of 40 nM DiOC6 at 37°C for 30 min. \( \Delta \Psi \) was determined by FACS analysis. b At 24 h, triptolide-induced release of cytochrome \( c \) into cytosol, changes of Bcl-2 and Bax protein, activation of caspase-3, and cleavage of PARP were analyzed by Western blotting. The results were representative of three independent experiments.
free active Bax, and ultimately initiated the following apoptotic cascades. To certify our imagination that the decreasing Bcl-2/Bax ratio led to the release of free active Bax, we used computer calculation combined with a mathematical model to simulate and further explain this process. As seen in Figs. 7 and 8, increasing active Bax was obtained compared with the comparatively stable level of total Bax after exposure of macrophages to triptolide. By detecting the translocation of Bax from cytosol into the mitochondria, Bax activation after triptolide treatment predicted from mathematical modeling was testified (Fig. 9). These results suggested that it was the ratio of Bcl-2/Bax that modulated mitochondria-associated apoptosis induced by triptolide in macrophages. Our conclusion is consistent with other studies [34, 35].

ROS have been shown to mediate apoptosis in a variety of cell types [17, 36, 37]. In this study, we detected an elevation of ROS after triptolide exposure (Fig. 2b), suggesting the possible roles of endogenous ROS in apoptotic cell death induced by triptolide. We tested this supposition by co-treatment of macrophages with GSH, an effectual antioxidant that blocked intracellular ROS induced by triptolide (Fig. 2b), to rescue triptolide-induced apoptotic cells. Indeed, GSH strongly protected the RAW 264.7 and U937 macrophage-like cells against apoptotic impact of triptolide (Figs. 3 and 6a). Moreover, GSH suppressed triptolide-induced $\Delta \Psi_m$ collapse, cytochrome c release, and caspase-3 activation (Figs. 5 and 6a), indicating that ROS generation was a required part for triptolide-induced

Fig. 6 Triptolide-induced apoptosis in macrophage-like cell U937. Cells were pretreated with 30 mM GSH or 50 $\mu$M 1400w for 1 h, and then cultured with triptolide at 25 ng/ml. a At 24 h, cells were harvested and assayed by annexin V–PI staining. The data were expressed as the apoptotic cells after GSH or 1400w co-incubation compared with triptolide alone. Asterisks denote a response that was significantly different from the positive control (**$P<0.05$, ***$P<0.01$). b At 24 h, the release of cytochrome c was analyzed by Western blotting. The results were representative of three independent experiments.

Fig. 7 Simulation of triptolide-induced generation of ROS, degradation of Bcl-2 protein, liberation of active Bax protein, release of cytochrome c from mitochondria into the cytosol, and activation of caspase-3 by a mathematical model in macrophages.
apoptosis by the mitochondrial pathway in macrophages. These results were consistent with other reports that intracellular ROS mediated apoptosis by mitochondria destruction and cytochrome c release [17, 36]. It was known that Bcl-2 protein was involved in ROS-mediated apoptosis. However, the evidence that ROS generation was the cause or the consequence of Bcl-2 protein down-regulation was uncertain. A recent study showed that ROS reduced endogenous Bcl-2 levels [37], whereas another showed that Bcl-2 over-expression could block the ROS generation [17]. Our data revealed that GSH reversed the reduction of Bcl-2 expression (Fig. 5b), suggesting that the generation of ROS might be an upstream event to the downregulation of Bcl-2 protein in triptolide-induced apoptosis in macrophages.

At low-dose treatments (5, 10 ng/ml), triptolide inhibited the NO production by downregulating iNOS mRNA expression in macrophages (Fig. 2a,c), which is in agreement with the previous works [21, 22]. On the contrary, the NO production and the expression of iNOS

**Triptolide (ng/ml)**

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**Fig. 9** Triptolide-induced translocation of Bax from cytosol into mitochondria. At 6 h, cells were harvested and fractionated into cytosol (cyto) and mitochondria (mito) fractions and analyzed by Western blotting. The result was representative of three independent experiments.
mRNA increased at high-dose treatments (20, 25 ng/ml; Fig. 2a,c). We presumed that the elevation of NO production at high-dose treatments possessed an important role in triptolide-induced apoptosis. Recent literature had implicated the involvement of endogenous NO in the modulation of apoptosis [38]. To test this possibility in our work, we investigated whether iNOS-specific inhibitor 1400w exposure could reduce triptolide-induced apoptosis. Data showed that 1400w obviously rescued triptolide-induced apoptotic cells (Fig. 3), suggesting the importance of the endogenous NO in triptolide-induced apoptosis in macrophages. However, 1400w did not block the reduction of Bcl-2 protein, collapse of ΔΨm, release of cytochrome c, and activation of caspase-3 (Fig. 5), ruling out a possible role for NO in regulating the mitochondria-mediated process.

The p53 protein is an important regulator of apoptosis. Several reports indicated that triptolide-induced apoptosis was p53-independent in HL-60 (promyelocytic leukemia) cell lines and solid tumors [7, 39], whereas some others showed that a functional p53 was required for the apoptotic effect of triptolide in HT1080 (fibrosarcoma) and AGS (gastric cancer) cell lines [40, 41]. In this study, we found that there were no obvious changes in p53 protein expression after triptolide treatment in RAW 264.7 cells harboring the wild-type p53 gene (Fig. 4b), indicating that p53 might be not involved in triptolide-induced apoptosis in macrophages.

Based on our experimental results, we proposed a hypothetical model of pathway on the apoptotic effect of triptolide in macrophages (Fig. 10). In this pathway, triptolide firstly triggered intrinsic ROS and NO, which subsequently initiated apoptosis in different processes. The increased ROS attenuated Bcl-2 protein level, bringing on the liberation of active Bax. The active Bax then oligomerized and translocated into mitochondrial membrane, resulting in the disruption of ΔΨm and release of cytochrome c. The released cytochrome c activated caspase-3 through apoptosome complex and eventually led to apoptotic cell death. On the contrary, the NO-mediated apoptosis was not through the mitochondria disruption and caspase-3 activation. The molecular mechanism of this process requires further investigation.

To consider the reliability of this hypothesis, we developed a mathematical model to simulate the variation of ROS and apoptosis-related molecules during the triptolide-induced apoptosis (Fig. 7). Encouragingly, with the given parameter set, all the simulation results were perfectly consistent with the experimental data, strongly providing the evidence that our hypothetical model was reliable. Furthermore, through computational simulations, the concentration vs time kinetic processes of the changes of ROS, Bax, Bcl-2, cytochrome c, and caspase-3 were given, shown in Fig. 8. These dynamic results provided more information to illustrate the apoptotic action of triptolide in macrophages.

During inflammatory responses, macrophages that accumulate at sites of injury tissue are activated to secrete a variety of proinflammatory and cytotoxic mediators, which can augment tissue injury and result in inflammatory processes [42]. Therefore, anti-inflammatory therapy that aims to depress infiltrating macrophages is considered an effective strategy. Many therapeutic medicines such as nonsteroidal anti-inflammatory drugs have been used in clinics to reduce the inflammation by inhibiting activated macrophages and macrophage activation. Moreover, apoptosis is known to play an important role in inflammation by eliminating activated macrophages when there is no longer a need for them [42, 43]. Accordingly, inducing infiltrating macrophage apoptosis could be another promising therapeutic means to diminish inflammatory symptoms. In the present study, we illuminated for the first time that triptolide induced apoptosis in macrophages by upregulating the intracellular ROS and NO expressions. Our findings
suggested that further exploration of this potential immunosuppressor for the treatment of inflammation was desirable.

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References