Results

1. Expression of two PPARδ isoforms in vitro.

As mentioned above, PPARδ is known to be expressed in the epidermis of psoriasis patients, localizing to the nucleus as well as in the cytoplasm of lesional and non-lesional skin (Westergaard *et al.*, 2003). In order to investigate whether PPARδ overexpression is an attribute of psoriatic keratinocytes the biopsy-derived primary keratinocytes were cultivated *in vitro*. In parallel, primary keratinocytes from two healthy donors were treated accordingly. After approximately two weeks in culture nuclear and cytosolic extracts were prepared. Western blot analysis revealed significant differences in PPARδ expression levels in keratinocytes expanded from lesional, non-lesional psoriatic skin or from control skin (Fig. 3A) suggesting that the overexpression of PPARδ in lesional psoriasis may indeed represent a cell-autonomous phenotype of keratinocytes from psoriatic individuals.

Interestingly, there are two distinct bands visible upon probing the blot of primary keratinocytes protein extracts with an antibody against PPARδ. These bands are localized approximately 2–3 kDa apart at the expected size of PPARδ (55 kDa), with the shorter one being the predominant in the nuclear fraction. HaCaT protein extracts (shown on right) produced only one band at the expected size regardless of the protein fraction considered. Since these results raised some doubts as to the used antibody specificity, a peptide competition experiment was performed. Figure 3B presents the upper band in cytoplasmic extracts and lower band in nuclear fraction (marked with arrows) as being specifically recognized by a N-terminally directed PPARδ antibody, thus ruling out an antibody-derived artifact.

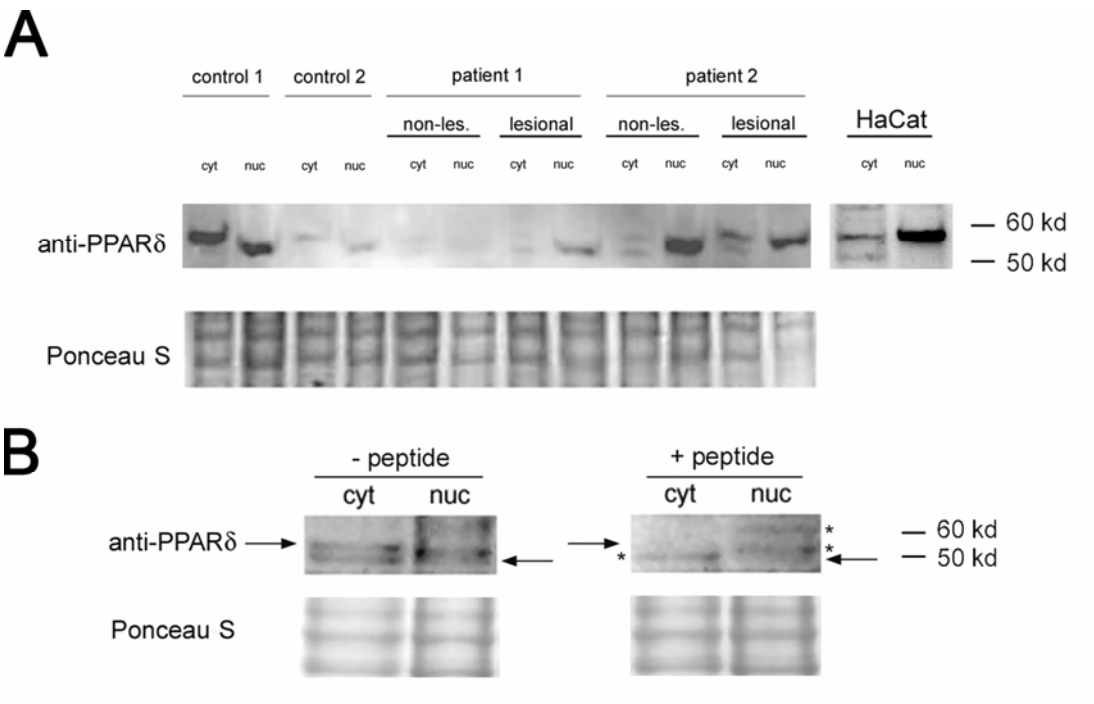

Figure 3. *Expression of PPARδ in primary adult keratinocytes.* **A.** Western blot of nuclear and cytosolic fractions from keratinocytes expanded from non-lesional, or lesional skin, as well as from healthy control skin, as indicated. 20 µg of nuclear and cytosolic fractions were loaded per lane. Ponceau S staining is shown on the bottom as a control for even loading. **B.** Cytosolic and nuclear fractions of primary keratinocytes from healthy control skin were prepared and processed exactly as in (A), except that the PPARδ antibody was preincubated with 10 µg/ml of antigenic peptide (+ peptide) for 30 min. prior to incubation of the blot with antibody. Non-specific bands not competed by peptide are marked by a star (*). Bands specifically recognized by PPARδ antibody are marked with arrows.

In order to study the subcellular distribution of PPARδ *in vitro* the transcription factor was activated with the synthetic ligand L-165041. A Western blot performed after 48 hours of treatment showed again varying levels of PPARδ expressed in keratinocytes from each of the three donors examined. Addition of synthetic ligand specific for PPARδ failed to result in consistent translocation of the transcription factor of the nucleus, as shown in figure 4. These results that PPARδ is not uniformly overexpressed in psoriasis-derived keratinocytes and that neither its expression nor its subcellular distribution is dependent on ligand binding.

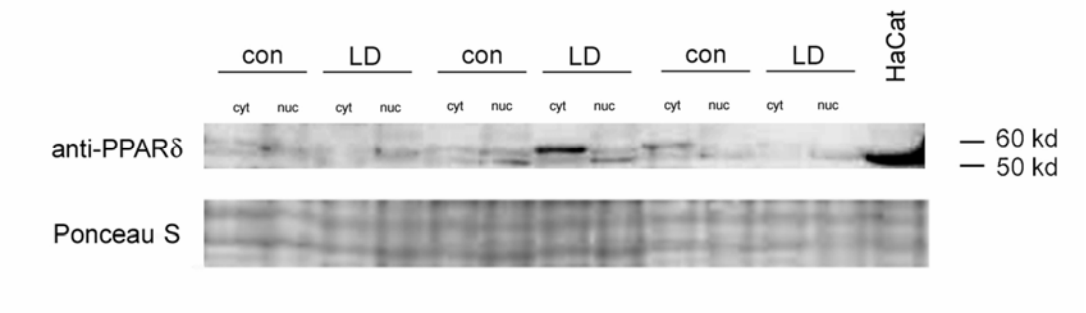

Figure 4. *Subcellular localization of PPARδ* in vitro. Western blot of nuclear and cytosolic protein fractions from keratinocytes isolated from three control individuals stimulated *in vitro* with the PPARδ - specific ligand L-165041 (LD) or vehicle (con) for 48h. Nuclear extract from HaCaT keratinocytes was included as positive control (rightmost lane).

2. Localization of NF kappa B is independent of PPARδ.

The ubiquitously expressed transcription factor NFκB plays a major role in the regulation of immune and inflammatory responses. Its activation has been reported to produce a psoriasis-like phenotype in a mouse model (Chen *et al.*, 2000). It has been also shown in GST-pull down experiments that PPARδ physically interacts with the N-terminal Rel homology domain of NFκB p65 subunit (p65/RelA) (Westergaard *et al.*, 2003). It was therefore of interest to examine whether functional interaction between PPARδ and p65/RelA could induce nuclear translocation coupling transcriptional activity of NFκB with PPARδ activation in the keratinocytes. On the Western blot of primary human keratinocytes re-probed with p65 antibody the protein is observed in the cytosolic fraction (Fig.5), and not in the nucleus as PPARδ. Activation of PPARδ with

the L-165041 compound for 48 h prior to protein extraction did not induce a nuclear accumulation of p65. Based on these findings, PPARδ - p65/RelA interaction is not likely to be the main mechanism contributing to the PPARδ activity keratinocytes.

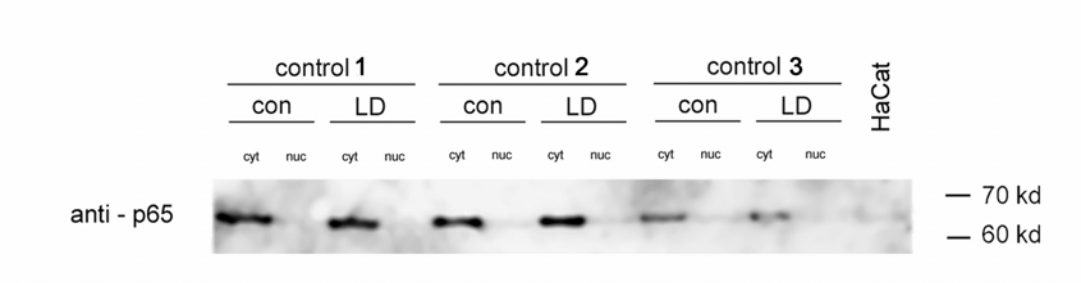

Figure 5. *Localization of NF kappa B in primary keratinocytes*. Western blot of nuclear and cytosolic fractions from keratinocytes isolated from three control individuals stimulated with the PPARδ - specific ligand L-165041 (LD) or vehicle (con) for 48h. Membrane showed on fig. 4 was re-probed using the p65 antibody. Nuclear extract from HaCaT keratinocytes was included as positive control (rightmost lane).

3. PPARδ is regulated through AP1 activation in keratinocytes.

PPARδ functionally acts as a "fatty acid sensor" in the cell. It facilitates intercellular cross talk that coordinates the different cellular events involved in the inflammation and the energy homeostasis and as such is bound to be exposed to tight and multi-level control mechanisms. These include the classic allosteric control of the transcription factor activity (ligand specifity, co-activators and co-repressors binding, heterodimeryzation, see "Introduction") as well as transcriptional regulation. The transcriptional regulation of PPARδ has been shown to proceed through pro-inflammatory signals, such as TNFα and IFNγ, which are able to trigger the stress-associated signalling pathway, activate the transcription factor complex AP1, and finally stimulate the PPARδ gene (Tan *et al.*, 2001). The complex mechanism that involves an inhibitory effect on AP1 activity and DNA binding resulting in an inhibition of the AP1-driven induction of the PPARδ promoter is facilitated by transforming growth factor-β1 (TGF-β1)/Smad3 action. In response to TGF-β1, Smad3 interferes with AP1 subunit c-jun mediated transactivation by inhibiting the binding of the c-jun-p300 complex on the PPARδ promoter (Tan *et al.*, 2004b). The majority of pathways

regulating transcriptional regulation of PPARδ in murine keratinocytes have been related to an AP1 site in the PPARδ promoter. Therefore it was decided to assess the contribution of this cis-acting element to PPARδ regulation in human keratinocytes. The expression of two AP1 subunits active in adult epidermis, c-jun and junB, was blocked in HaCaT keratinocytes by RNA-interference (RNAi). As shown in figure 6, knock-down of junB greatly reduced steady-state PPARδ expression while c-jun knock-down had only a minor effect. These results confirm that AP1 dependent transcription is a major determinant of PPARδ expression levels in keratinocytes.

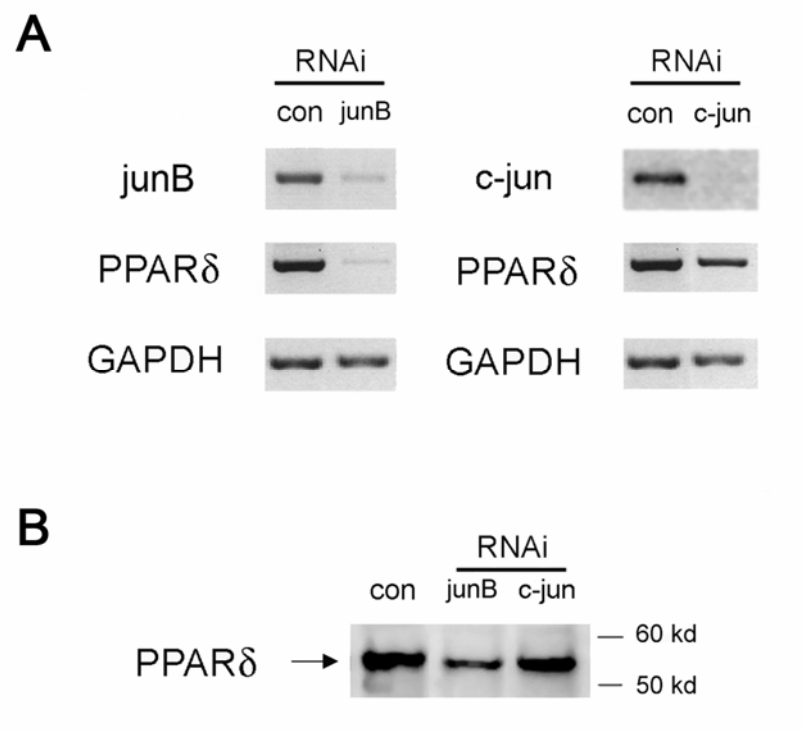

Figure 6. *Transcriptional regulation of PPARδ expression by AP1* subunits *in human keratinocytes.* **A.** Reverse transcription-polymerase chain reaction (RT-PCR) of human HaCaT keratinocytes transfected in the absence (con) or presence of small interfering RNA (siRNA) specific for junB or c-Jun, as indicated. **B.** Western blot of human HaCaT keratinocytes transfected in the absence (con) or presence of siRNA specific for junB or c-Jun, as indicated. RNA (A) or protein (B) was isolated 48h after transfection with siRNA specific for junB or c-Jun.

In human colorectal cancer cell lines, PPARδ is regulated by β-catenin through a T-cell factor (TCF)/lymphocyte enhancer factor (LEF) (TCF/LEF) site (He *et al.*, 1999). Therefore, the lentivirus-mediated knock-down of β-catenin was performed next in order to assess the contribution of this regulatory site to the regulation of PPARδ expression. To this end, HaCaT cells were infected with lentiviral particles harboring shRNA specific for β-catenin. The efficiency of infection exceeded 90% as shown in figure 7A. Subsequently, cells were cultured in standard, as well as calcium-free medium. Calcium is an important regulator of keratinocyte differentiation *in vitro* and *in vivo* and since calcium-induced differentiation proceeds through effector transcription factors including CCAAT enhancer binding protein (C/EBP) which, in turn, inhibits PPARδ basal promoter activity in mouse keratinocytes (Di-Poi *et al.*, 2005) and AP1 (Jang & Steinert, 2002). HaCaT cells cultured in calcium-free medium show very little, if any, activation of NF-κB which correlates with a low differentiation state of the cells (Pleguezuelos & Kapas, 2006) and resembles a lack of NF-κB activation observed in primary keratinocytes (see figure 5). Therefore the investigation of the possible contribution of β-catenin to transcriptional regulation of PPARδ included high- and low-differentiated HaCaTs. The cells were cultured in either RPMI 1640 medium containing 0,4 mM Ca^{2+} or KBM-2 calcium-free medium for 7 days after knock-down of β-catenin. Protein and RNA extracts were prepared and analyzed with Western blot or RT-PCR, respectively. Knock-down of β-catenin had a negligible effect on PPARδ expression at RNA (figure 7B) and protein level (figure 7C), both in calcium-containing and in calcium-free medium showing that PPARδ expression in human keratinocytes is primarily regulated via AP1 activation, specifically by junB, while canonical Wnt signalling has no effect, independent on the differentiation state of the keratinocytes.

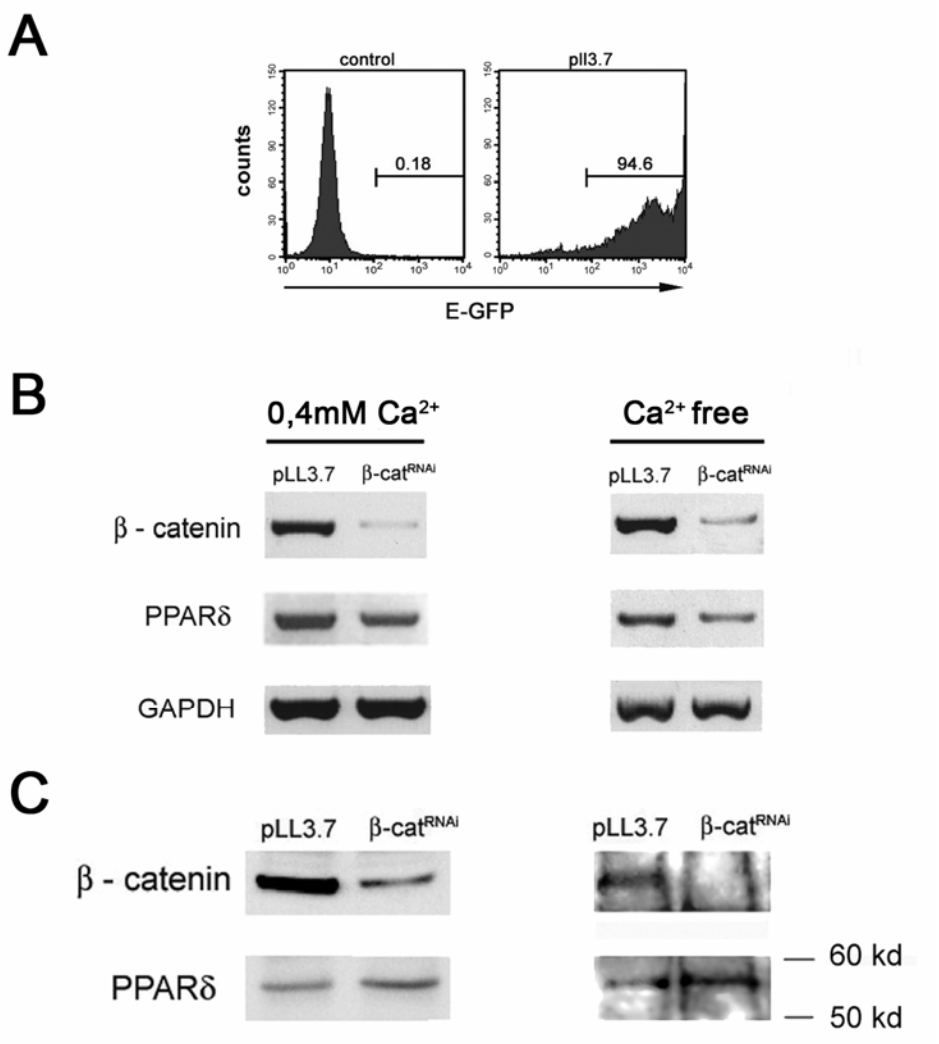

Figure 7. *Transcriptional regulation of PPARδ expression by canonical Wnt pathway in human keratinocytes.* **A.** Fluorescence Activated Cell Sorting (FACS) analysis of human HaCaT keratinocytes 48 h after infection with empty control lentivirus (pLL3.7). Normal, not infected HaCaTs served as control. Enhanced Green Fluorescent Protein (E-GFP) reporter expression indicates infection ratio over 90%. Results are representative for all experimental conditions used: infection with empty control lentivirus (pLL3.7) or with lentivirus harboring a β-catenin specific siRNA sequence (β-cat^{RNAi}), 48h or 7days after infection. **B.** RT-PCR analysis of RNA isolated from HaCaT infected with empty control lentivirus (pLL3.7) or lentivirus harboring a β-catenin-specific siRNA sequence (β-cat^{RNAi}) cultivated for 7 days after infection either in RPMI 1640 medium containing 0,4 mM Ca^{2+} or the calcium-free medium (KBM-2). GAPDH served as control for even loading. **C.** Protein analysis by Western blot for the expression of β-catenin and PPARδ. Whole protein extracts were prepared from human HaCaT keratinocytes treated as indicated above.

4. PPARδ enhances proliferation in keratinocytes.

Hyperproliferation of keratinocytes is one of the most prominent phenomena in psoriasis. Up to date there is still great controversy about the role of PPARδ on cellular proliferation, since some reports suggest that ligand activation of PPARδ potentiates cell growth (Piqueras *et al.*, 2007) while other reports propose PPARδ as a factor responsible for cell growth inhibiton (Burdick *et al.*, 2007). Therefore, the effect of PPARδ on proliferation was examined in human keratinocytes. To this end the fast cycling HaCaT keratinocytes were infected with a lentivirus containing a RNAi sequence recognizing PPARδ or empty control lentivirus. As shown in figure 8, the infection ratio for both vectors, quantified by FACS analysis of the E-GFP reporter 3 days after infection, was appr. 94 % for both vectors. However, thirteen days after infection, the percentage of E-GFP-positive cells had decreased significantly only after infection with $PPAR\delta^{RNAi}$, but not after control virus infection, indicating a proliferative disadvantage in the absence of PPARδ.

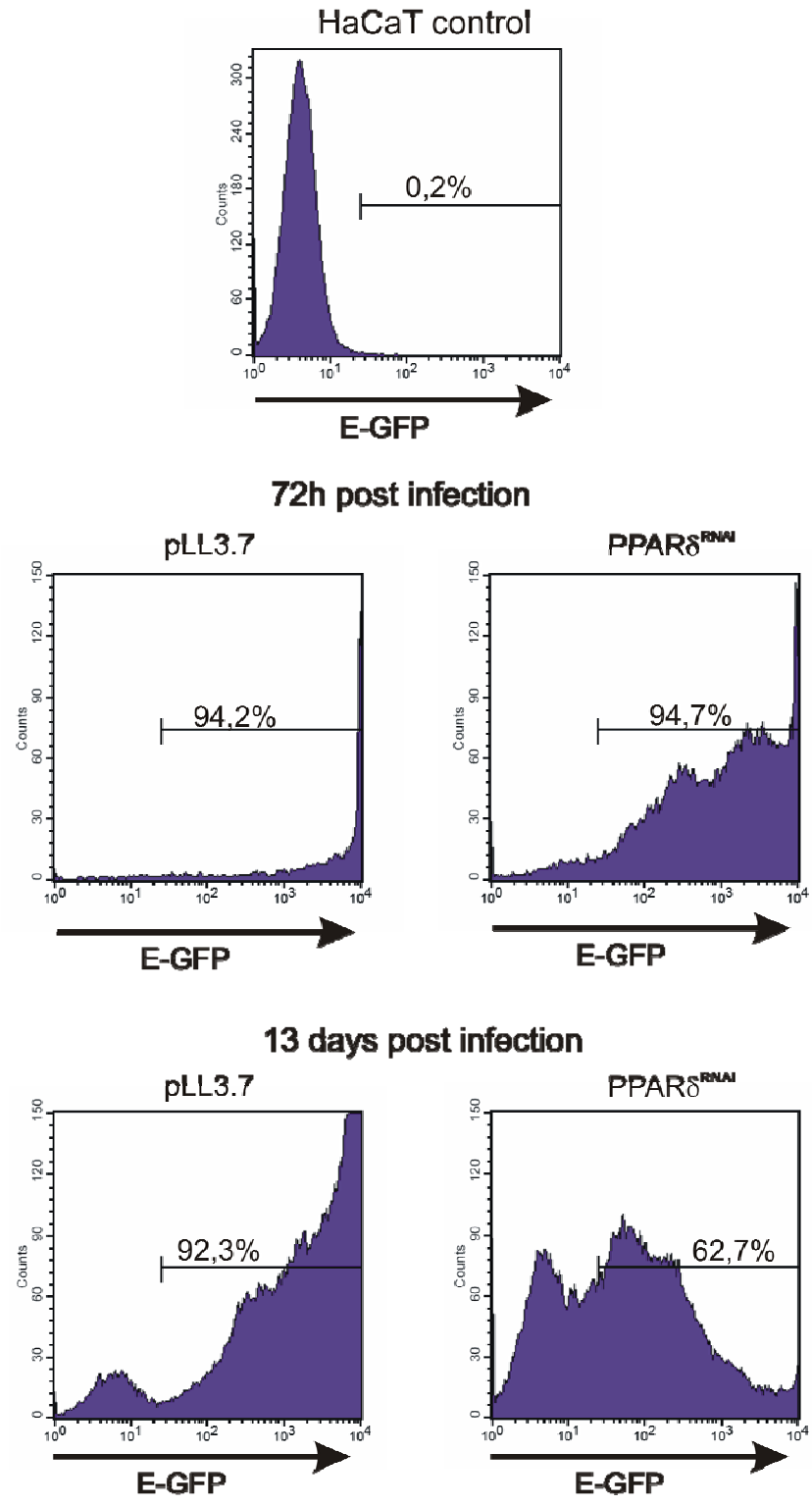

Figure 8. *Effect of PPARδ on HaCaT keratinocytes proliferation.* HaCaT keratinocytes were infected with control virus (pLL3.7) or lentivirus harboring a PPARδ – specific siRNA sequence (PPARδ^{RNAi}). The frequency of infected cells was determined by FACS analysis of the E-GFP reporter gene on the time points indicated.

In a complementary approach, adult primary epidermal keratinocytes, which proliferate much slower than HaCaT cells, were expanded from healthy donors and than exposed to the PPARδ - specific ligands L-165041 or GW-501516 for seven days. As shown in figure 9A and 9B, stimulation with either ligand caused a highly significant increase in cell number of keratinocytes expanded from three independent donors. These data indicate that PPARδ augments, rather than inhibits, keratinocytes proliferation, suggesting that this effect may contribute to keratinocyte hyperproliferation in psoriasis.

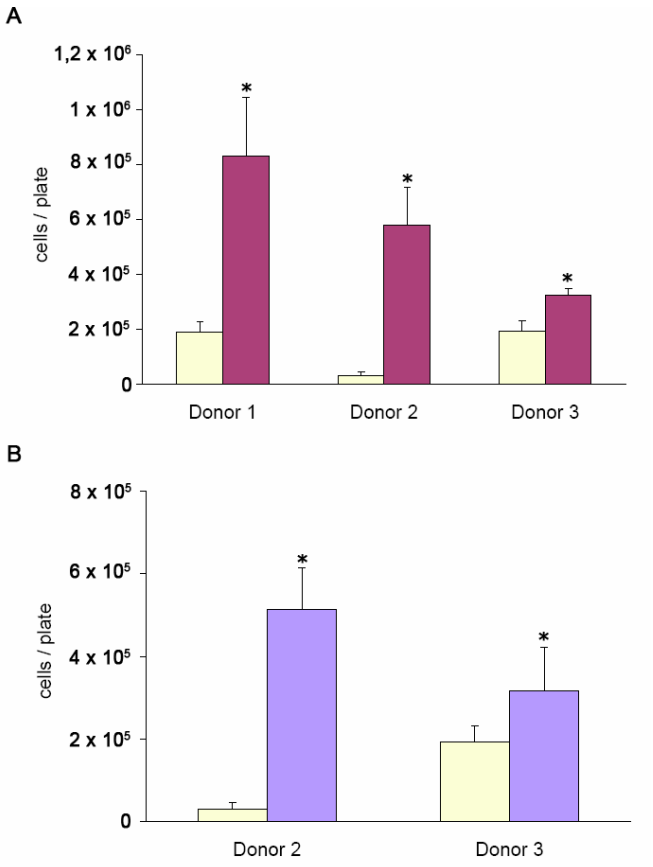

Figure 9. *Effect of PPARδ on adult primary keratinocytes proliferation.* **A.** Proliferation of adult primary keratinocytes *in vitro* grown for seven days in the presence of 1 μM of the PPARδ specific ligand L-165041 (purple columns) or adequate concentration of vehicle (pale yellow). **B.** Proliferation of adult primary keratinocytes *in vitro* grown for seven days in the presence of 5 μM of the PPARδ specific ligand GW-501516 (lavender columns) or adequate concentration of vehicle (pale yellow). Data represent mean ± s.d. of experiments performed in duplicate or triplicate with the cells isolated from three independent donors. * $p < 0.01$.

5. Heparin-binding EGF-like growth factor is a target of PPARδ.

To gain insight into the mechanism connecting PPARδ and increased proliferation of primary keratinocytes the target genes transcribed by PPARδ activation were identified using gene expression profiling. Primary keratinocytes from four independent donors were grown in the presence of L-165041, or vehicle, for 48h when total RNA was isolated. Total gene expression was then analyzed using the PIQUOR SkinPatho array which contains appr. 1100 transcripts relevant to epidermal biology. The magnitude of gene induction by PPARδ has been found to lie in the range of 1.5 – 3 fold in previous microarray studies (Tan *et al.*, 2001; Tanaka *et al.*, 2003; Tachibana *et al.*, 2005). Since this moderate induction is close to the background level, a threshold for a minimum –fold change of greater than three standard deviations of the mean –fold change observed across all genes was applied (fig.10). Although this conservative approach reduced sensitivity, thus preventing detection of further potential target genes, it largely eliminates identification of false-positives. Table 1 summarizes the observed changes. Several of the listed genes typical to fatty acid metabolism have previously been identified as PPARδ targets in other cell types (Tanaka *et al.*, 2003; Tachibana *et al.*, 2005), thereby confirming the present data. One gene, heparin-binding EGF-like growth factor (HB-EGF), was of particular interest because it is also up-regulated 3.6-fold in vivo in lesional psoriasis (table 1) and because it is known to enhance keratinocyte proliferation.

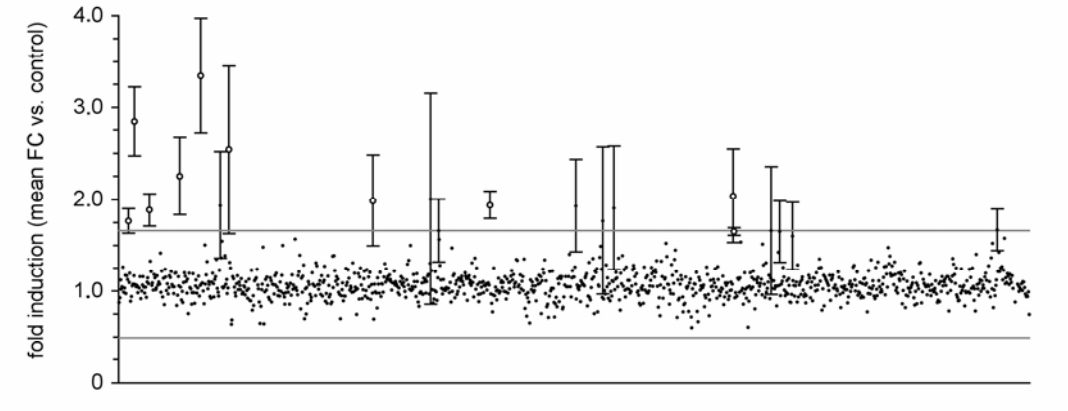

Figure 10. *Potential PPARδ target genes in primary keratinocytes.* Scatter plot of mean fold changes induced by stimulation of primary adult epidermal keratinocytes with the PPARδ- specific ligand L-165041. Shaded horizontal lines indicate the threshold applied for significant changes (three standard – deviations of the mean change across all genes). Error bars indicate standard deviation (s.d.) for all genes exceeding the threshold. Open symbols denote genes listed below in table 1.

Table 1. *Genes induced by PPARδ activation in keratinocytes.* Data shown represent mean ± s.d. of -fold changes (L–165041 vs. 0.1% DMSO) for cells from four independent donors.

Gene ID	NAME	FC	p-value[1]	FC *in vivo*[2]
ABCC3	multispecific organic anion transporter 2	1.8 ± 0.1	< 0.001	**2.4**
ACADVL	acyl-CoA dehydrogenase, very long chain	2.8 ± 0.4	< 0.001	n.s.
AKR1B1	aldose reductase	1.9 ± 0.2	< 0.001	n.s.
ATP12A	K-transporting ATPase (non-gastric)	2.3 ± 0.4	0.001	n.t.
BG1	very long-chaing acyl-CoA synthase	4.6 ± 0.6	0.001	n.t.
CAT	catalase	2.5 ± 0.9	0.008	n.s.
ECHB	3-ketoacyl CoA thiolase	2.0 ± 0.5	0.012	n.t.
HB-EGF	heparing-binding EGF-like growth factor	1.9 ± 0.1	0.009	**3.6**
KRT75	cytokeratin type II (K6HF)	1.9 ± 0.5	< 0.001	n.s.
OACT5	O-acyltransferase domain containing 5	2.0 ± 0.1	0.002	n.s.

[1] As calculated by a two-sided paired student's t-test.

[2] Upregulation in lesional vs. non – lesional psoriatic skin, as determined by microarray – based expression profiling (Romanowska *et al.*, 2007). n.s.- non significant ($p > 0.05$), n.t.- transcript not present or not detected on U95A microrray used for this study.

The observed upregulation of HB-EGF was confirmed by quantitative real-time PCR. Cell death inducing DFF45-like effector A (CIDE-A), a pro-apoptotic gene previously shown to be indirectly down-regulated by PPARδ (Tan *et al.*, 2001) served as a positive control. Expression of PPARδ itself was used as a negative control. As shown in figure 11, both HB-EGF and CIDE-A were moderately, but statistically significant, induced by PPARδ activation in case of four independent donors, whereas expression of PPARδ itself did not change, in confirmation of the microarray data.

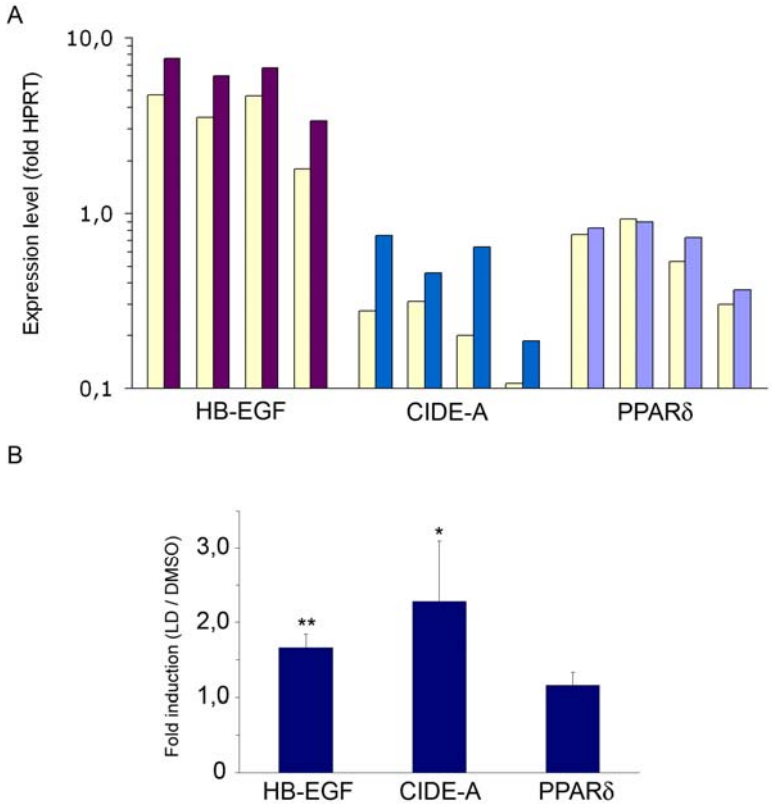

Figure 11. *HB-EGF expression in primary keratinocytes upon PPARδ activation.*
A. Quantitative real-time PCR showing gene expression relative to Hypoxanthine-guanine phosphoribosyltransferase (HPRT) in primary keratinocytes from four independent donors stimulated with L-165041 for 48h. **B.** Mean ± s.d. of the changes for all individuals. * $p < 0.01$; ** $p < 0.001$.

Results obtained on the RNA level were confirmed with Western blot of whole cell extracts prepared from primary keratinocytes treated with synthetic PPARδ ligand L-165041 for 48h. In the complementary approach primary keratinocytes were infected with a lentivirus containing a RNAi sequence recognizing PPARδ or empty control lentivirus. On the protein level, HB-EGF expression was increased upon activation of PPARδ (fig. 12A) and decreased after lentivirus-mediated knock-down of PPARδ in primary keratinocytes (fig. 12B), confirming the regulation of HB-EGF expression by PPARδ.

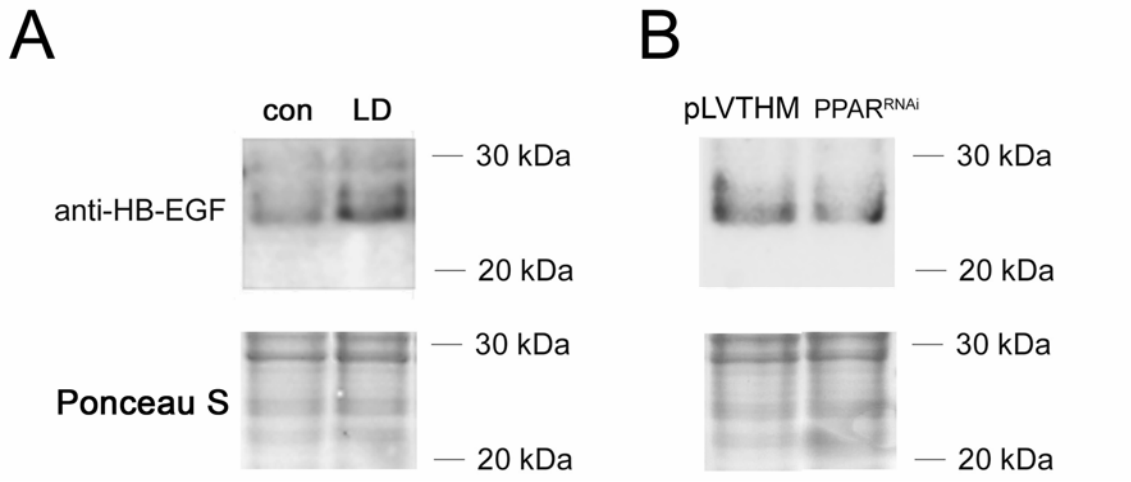

Figure 12. *HB-EGF protein level in primary keratinocytes upon activation or knock-down of PPARδ* . **A.** Whole-cell lysates separated by SDS-PAGE, and blots probed with anti-HB-EGF. **B.** Primary keratinocytes were infected either with empty control virus (pLVTHM) or virus containing a PPARδ RNAi-sequence ($PPAR^{RNAi}$) for 48h prior to cell harvesting. Ponceau S staining is shown as a loading control.

Finally, the question whether HB-EGF is directly or indirectly induced by PPARδ was addressed. To this end, primary keratinocytes were incubated in the absence, or presence, of L-165041, with or without a protein synthesis inhibitor – cycloheximide (CHX). Subsequently, total RNA was extracted and HB-EGF expression was determined by RT-PCR. An established PPARδ target gene acyl-Coenzyme A dehydrogenase, very long chain (ACADVL) was amplified as positive control. CIDE-A, previously shown to be indirectly induced by PPARδ (Tan *et al.*, 2001), was used as a negative control. As shown in figure 13, induction of CIDE-A by PPARδ activation can not take place when protein synthesis is suppressed, whereas this was not the case for ACADVL and HB-EGF. Thus, HB-EGF is a direct target gene of PPARδ.

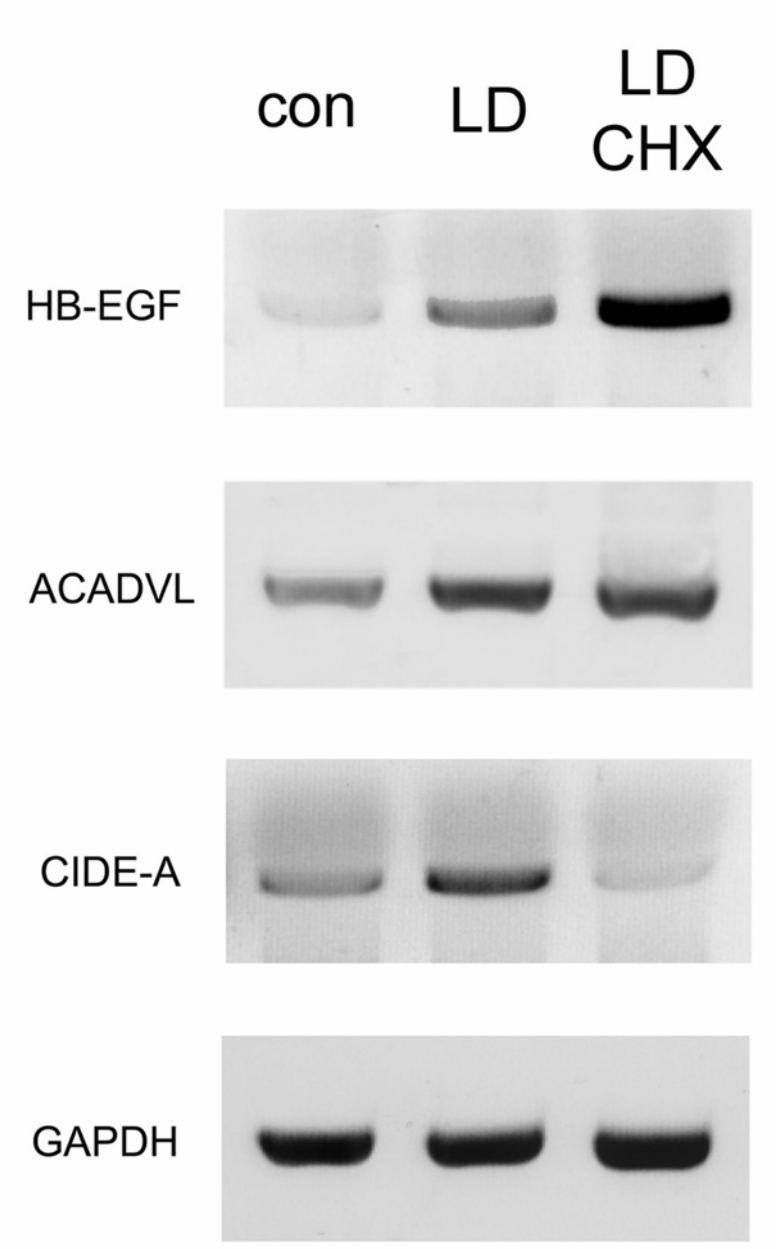

Figure 13. *HB-EGF is a direct target gene of PPARδ.* RT-PCR analysis of primary keratinocytes after 24h stimulation with L-165041, vehicle - 0.5% DMSO (con), or 5μg/ml cycloheximide (CHX). GAPDH served as a control for even loading.

6. *Engineering of lentiviral plasmid for inducible expression of constitutively active PPARδ* in vivo.

Although the *in vitro* data presented above suggested PPARδ involvement in psoriasis pathogenesis, further investigation of its role in a complex interplay between keratinocytes and immune system demands usage of an *in vivo* system. Up to date various transgenic mouse models have been created each replicating some of the pathologic features observed in psoriasis. In most of these models, increased expression of specific genes is directed to the basal ((Sano *et al.*, 2005), (Xia *et al.*, 2003)) or to the suprabasal ((Blessing *et al.*, 1996), (Hobbs *et al.*, 2004)) layer of the epidermis. However, to investigate the function of PPARδ ubiquitous expression of the gene seems to be desirable, in order to target all potentially relevant cell types (lymphocytes, keratinocytes, dendritic cells, endothelial cells, macrophages). In development PPARδ mediates embryo implantation (Lim *et al.*, 1999) and muscle development (Luquet *et al.*, 2003), potentially impeding generation of viable transgenic animals. To address this problem a tetracycline-inducible system was chosen requiring tetracycline derivative - doxycycline (Dox) to switch on PPARδ overexpression *in vivo*. Lentiviral vectors are now recognized as an efficient transgene delivery system (for review see (Park, 2007)). The ability of this vector type to efficiently deliver transgenes to the early embryo makes the lentiviral derived vector injection into the perivitelline space the method of choice for generating transgenic animals.

5LN-33 CAM-PPARδ/pIGETOn lentiviral transfer plasmid was engineered on the pTK272 (Haack *et al.*, 2004) backbone kindly provided by Tal Kafri. Originally present tetracycline responsive transactivator (tTA) was replaced with the reverse transactivator (rtTA^{S}-M2) derived from pUHrT62-1 vector (a kind gift of W. Hillen), while the latter has highly improved properties with respect to specificity, stability and inducibility (Koponen *et al.*, 2003). The transactivation domain (78 amino acid residues, corresponding to 413–490 residues) of VP16 amplified from Herpes simplex virus was fused in frame with the N-terminus of mouse PPARδ yielding constitutively active mouse PPARδ (CAM-PPARδ). PPARδ requires a specific ligand for its activity and acts in a dominant-negative manner when ligand-deplated (Piqueras *et al.*, 2007). The CAM-PPARδ was generated to ensure transcriptional activity of the transgene without

additional treatment of founder animals with synthetic PPARδ ligands. CAM-PPARδ followed by Internal Ribosome Entry Site (IRES) -coupled EGFP was inserted upstream to a cytomegalovirus promoter (CMVp) driving rtTA^{S}-M2 constitutive expression. A detailed cloning strategy is contained in the Methods section of this thesis. Expression of EGFP as a second cistron allows virus titration during virus production and concentration as well as monitoring of infection efficiency. Furthermore, a tetracycline responsive promoter (TRE) is localized upstream of the Bovine Growth Hormone polyA (BGHpA) sequence and a self-inactivating 3' Long Terminal Repeat with deletion in the U3 region (3'ΔLTR) (fig. 14A). TRE consists of a minimal cytomegalovirus promoter fused to seven tetracycline operator sequence repeats (7xTetO) and drives the transgene expression as a result of reverse transcription and duplication of LTRs upon genomic integration of the vector.

In order to produce viral particles co-transfection of the 5LN-33 CAM-PPARδ/pIGETOn transfer plasmid along with VSV-G coding envelope plasmid (pMD2.G), and psPAX2 packaging plasmid containing *gag, pro* and *pol* coding sequences into producer cells (293T/17) is necessary. Transcription of vector full-length RNA is initiated from the CMV promoter at the 5'LTR and subsequently the transfer plasmid RNA is packaged into infectious pseudoviral particles. After infection of the target cell, viral RNA undergoes reverse transcription and the construct integrates into host genome, in the process of integration TRE from the 3'ΔLTR is transferred to the 5'LTR and duplicated. The proviral DNA form of complete 5LN-33 CAM-PPARδ/pIGETOn construct is shown schematically in figure 14B. To switch on transgene expression in the infected cells Dox is administrated. Dox induces a conformational change in the rtTA which subsequently binds to and activates TRE.

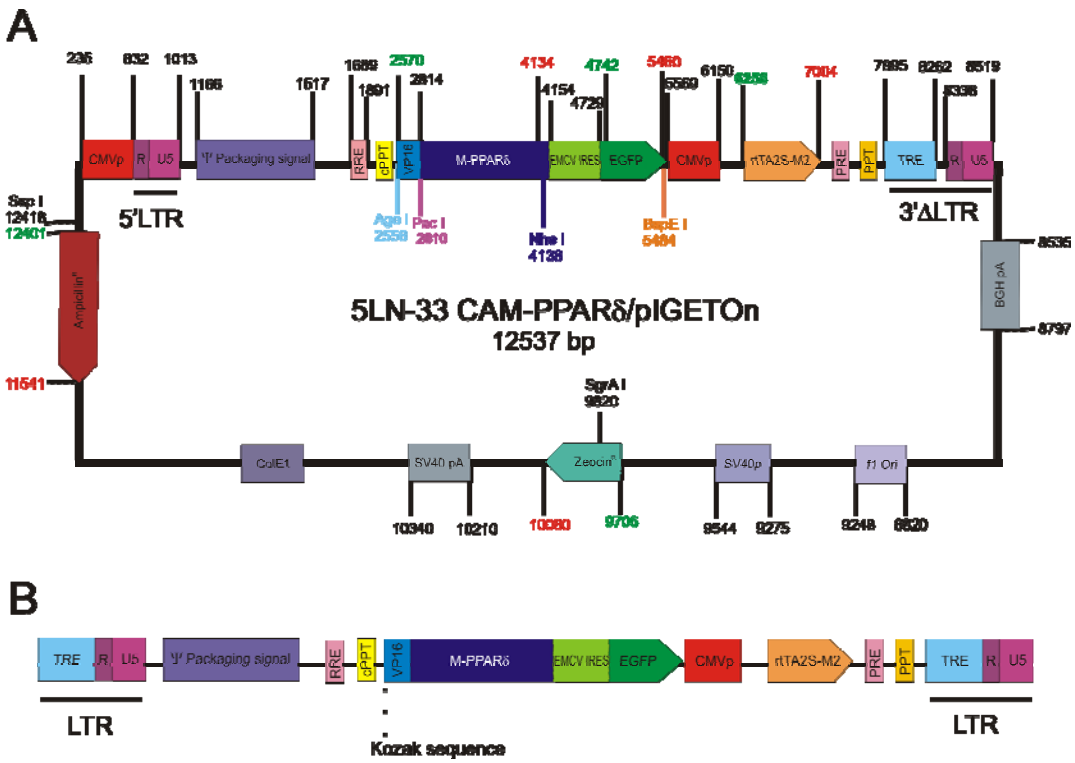

Figure 14. *Schematic of the lentiviral construct facilitating CAM-PPARδ expression under the control of tetracycline-induced promoter.* **A.** 5LN-33 CAM-PPARδ/pIGETOn plasmid map. Unique restriction sites are indicated. **B.** Genetic organization of 5LN-33 CAM-PPARδ/pIGETOn-derived provirus. Long terminal repeats (LTRs) are composed of R and U5 elements, the tetracycline responsive element (TRE) has been relocated to the U3 region promoting expression of the transgene. CMVp - cytomegalovirus promoter; 5'LTR - long terminal repeat; Ψ - packaging signal; RRE - rev response element; cPPT - central poly-purine tract; VP16 - the transactivation domain (78 amino acid residues, corresponding to 413–490 residues) of VP16 from Herpes simplex virus; M-PPARδ - murine peroxisome proliferator activator receptor delta; EMCV IRES - internal ribosomal entry site sequence of encephalo-myocarditis virus; EGFP - Enhanced Green Fluorescent Protein; rtTA2S-M2 - synthetic tetracycline-controlled transactivator; PRE - Woodchuck hepatitis virus posttranscriptional regulatory element; PPT - poly-purine tract; TRE - tetracycline responsive element (promoter); 3'ΔLTR - self-inactivating 3' LTR with deletion in U3 region; BGHpA - bovine growth hormone transcription termination and polyadenylation; f1 ori - f1 origin of replication; SV40p - SV40 promoter; SV40pA - SV40 transcription termination and polyadenylation; ColE1 - ColE1 origin of replication. Vector elements are not drawn to scale.

Mouse primary neonatal keratinocytes were used to verify the inducibility of the 5LN-33 CAM-PPARδ/pIGETOn construct by doxycycline. Cells isolated from two day-old mice were cultivated in KBM-2 medium containing 0,05 mM Ca^{2+} to 60% confluence and than infected with lentivirus for 12 hours. Subsequently, transgene expression was induced with doxycycline (1 μg/ml) for 24 hours. The E-GFP reporter gene expression increased in dox-treated cells upon induction about 10-fold (3,7 to 32,6), as determined with FACS analysis of fluorescence intensity (fig.15). Thus, the 5LN-33 CAM-PPARδ/pIGETOn construct provides transgene induction level suitable for effective regulation of transgene in vitro. Nevertheless, further investigation is necessary in order to verify the construct ability of effective expression of functional, transcriptionaly active PPARδ on the RNA and protein level.

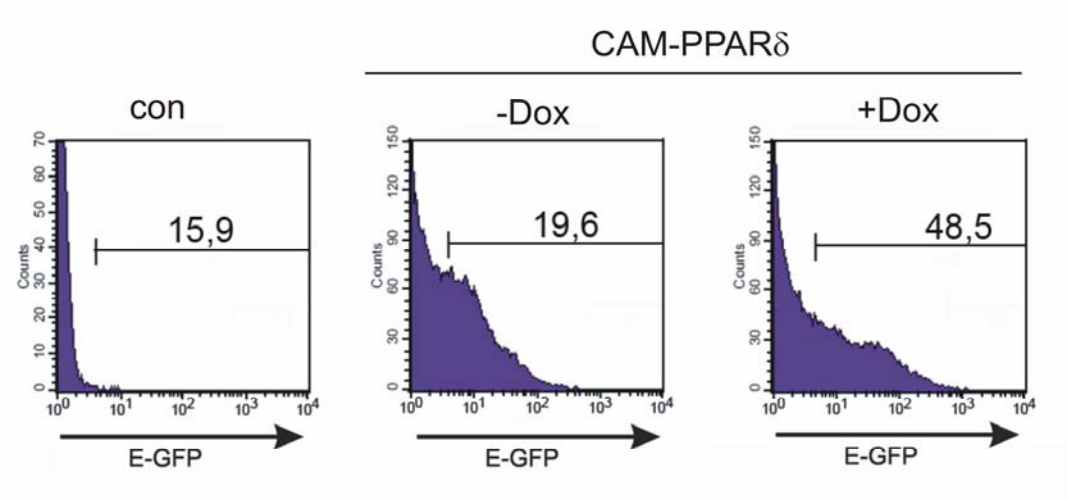

Figure 15. *Verification of 5LN-33 CAM-PPARδ/pIGETOn construct inducibility* in vitro. Primary neonatal mouse keratinocytes were infected with 5LN-33 CAM-PPARδ/pIGETOn – derived lentivirus for 12 hours and subsequently CAM-PPARδ expression was induced with 1μg/ml doxycycline (+Dox), or with PBS (-Dox) for the next 24 hours, not infected cells (con) were used as control. The induction level was determined by FACS analysis of the E-GFP reporter gene expression. The mean fluorescence intensity indicates an about 10-fold induction (3,7 to 32,6 fluorescence units) of E-GFP expression after Dox treatment.