Title: PreImplantation Factor (PIF\*) Regulates Stress-induced Adrenal Steroidogenesis and Anti-inflammatory Cytokines: Potential Application for Bioartificial Adrenal Transplant

Short running title: Effect of PIF on Adrenocortical Cells

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Stefan R. Bornstein designed research, Mariya Balyura and Evgeny Gelfgat performed research and analyzed data, Barbara Ludwig provided analytic tools, Eytan R Barnea provided PIF and conceptual ideas, Mariya Balyura and Evgeny Gelfgat wrote the paper with Eytan R. Barnea and Enrico Ullmann.

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**Abstract**

The main treatment algorithm for adrenal insufficiency is hormonal replacement, however inadequate hormone substitution often leads to severe side effects. Adrenal cell transplantation could be a more effective alternative but would require life-long immune suppressive therapy. PreImplantation Factor (PIF) is an endogenous peptide secreted by viable human embryos that leads to maternal tolerance without immunosuppression. PIF could be effective for allogeneic cell transplantation such as of bovine adrenocortical cells (BAC), which are used for bioartificial adrenal gland development that may more effectively restore complex adrenal functions. We report here that PIF exerts a dual regulatory effect on BAC by targeting mostly hyper-activated cells to specifically reduce adrenocorticotropic hormone (ACTH)-stimulated cortisol secretion. Reverse transcription real time PCR analysis revealed that PIF modulates the expression of two genes in the cortisol synthesis pathway, Steroidogenic Factor 1 (SF1), an activator of steroidogenesis, and the downstream steroidogenic enzyme Cytochrome P450 17A1 (CYP17A1). PIF increased basal expression of SF1 and CYP17A1 regardless of the activation level of the adrenocortical cells. In contrast, following ACTH stimulation, PIF reduced SF1 expression and induced expression of the immune suppressing anti-inflammatory cytokine IL10 only in the hyper-activated cells, suggesting both a protective and immune tolerant function. In conclusion, PIF regulates stress-induced adrenal steroidogenesis and immune tolerance in BAC, supporting a potential clinical application to reduce rejection by the host’s immune response following xenotransplantation.

**Keywords:** adrenocortical cells, immune tolerance, immune regulation

**Introduction**

Adrenal insufficiency describes the inability of the adrenal gland to release sufficient hormones through the limbic hypothalamic pituitary adrenal (LHPA) axis. Congenital Adrenal Hyperplasia (CAH) due to deficiency of 21-hydroxylase is one of the most common genetic adrenal disorders in humans. It is associated with clinical symptoms of virilization, neuroendocrine perturbations, and metabolic disease [1]. The current treatment algorithm with glucocorticoid substitution can only partially reverse these symptoms, is associated with severe side effects, and often fails to prevent adrenal crisis [1]. As an alternative, restoration of normal adrenal function could be achieved by adrenal cell transplantation. Transplanted adrenocortical cells could respond to physiological demands and reconstitute endocrine feed-back including the ultra- and circadian rhythms of hormone secretion. However, this strategy is extremely limited due to the requirement for life-long use of immunosuppressive drugs [2, 3]. These can result in serious side effects such as infection and malignancy [4], which lowers compliance causing rejection of the organ [5]. Intense efforts are ongoing to overcome these deleterious limitations of transplantation, for example by using organ encapsulation of bovine adrenal cells (BAC), as recently reported [6]. Transplantation of BACs has been shown to create a functionally responsive bioartificial adrenal gland to treat adrenal insufficiency in rats [6]. However, further improvements in immune regulation are required to establish adrenocortical cell transplants as a standard therapy in humans.

One promising therapeutic agent that might improve the outcome of adrenocortical cell transplantation without systemic immune suppression is PreImplantation Factor (PIF) [7]. PIF is a peptide secreted by viable human and most other mammalian embryos from the two-cell stage onwards [8]. After implantation, PIF levels in the maternal circulation correlate with a favorable pregnancy outcome [9]. PIF promotes implantation and trophoblast invasion, and regulates systemic immunity consequently leading to tolerance, which would obviate the need for deleterious immune suppression in a transplant setting [10, 11]. PIF has a protective effect negating adverse environments [8, 12] and targets the developing embryo to reduce oxidative stress and protein misfolding, which are critical for survival [13, 14]. PIF targets the innate immune system through antigen presenting cells (APCs) and also regulates the adaptive arm of immunity [15, 16]. Short-term PIF administration following semi/allogeneic bone marrow transplant in mice reduces graft vs. host disease (GVHD) and systemic inflammation [17]. PIF presents a very high safety profile: it received Fast-Track designation by the FDA and successfully completed a university-sponsored Phase I clinical trial for autoimmune disease. Moreover, PIF's ability to eliminate apoptotic cells, reduce oxidative stress, and prevent protein misfolding in damaged cells could also be beneficial for creating bioartificial organs [13, 18].

Considering the significant potential of PIF in transplantation tolerance and maintenance, we assess here its effect on the function of bovine adrenocortical cells (BAC) and thereby its potential as a therapeutic agent to support bioartifical transplants for treating adrenal insufficiency.

**Material and methods**

**Experimental layout**

To study the influence of PIF on the functionality of cultured BAC, we performed three series of experiments. In the first set we analyzed the influence of PIF on cortisol production. Based on the different degree of effect of PIF on cortisol secretion in the cells, isolated from different adrenal glands, subsequent analysis was implemented to define three groups of cells, determined by quantile analysis of stimulation index. Highly Responsive Cells (HRC) were defined by a stimulation index computed by quantile analysis >Q=75; range 16.3 – 20.6, n=12), Normally Responsive Cells, (NRC) computed by quantile analysis Q=30-70 (median ± 20); range 5.9 – 10.5, n=12). BAC with weak response to ACTH stimulation (stimulation index computed by quantile analysis Q<25; stimulation index <5) showed a short functional life span (data not shown), therefore would have no practical value for creating bioartificial adrenal glands and were not included in the subsequent analyses. The relationship between cell proliferation, viability, and apoptosis, with release of cortisol and expression of genes SF1 and CYP17A1 were investigated by correlation analysis, see below.

**Cell preparation and culture**

Adrenocortical cells were isolated from bovine adrenal glands shortly after the slaughtering of 1-3 year old cattle as previously described [6]. Briefly, adrenal glands were transported to the laboratory in cold (+4°C) Euro Collins Solution supplemented with 1% (vol/vol) penicillin-streptomycin solution (Thermo Fisher Scientific). The glands were then liberated from fat and connective tissue and rinsed several times with PBS through the central vein to remove remaining blood. Afterwards, a longitudinal incision was made to cut the adrenals in half, the medulla was removed and the cortex was scraped off the capsule and cut in small pieces. Adrenal cortex was digested for 50 min in Dulbecco’s modified Eagle’s /Ham’s F12 (DMEM/F12) medium (Thermo Fisher Scientific), containing 2 mg/ml collagenase and 0.1 mg/ml DNase (both from Sigma-Aldrich) at 37°C while shaking. Collected cells were washed with culture medium, pelleted by centrifugation (8 min, at 300g) and filtered through a 100-µm cell strainer (Becton Dickinson). Then, primary adrenocortical cells were placed in cell culture flasks (Thermo Fisher Scientific) and cultivated at 37°C in a humidified atmosphere (95% air, 5% CO2) in DMEM/F12 medium with 10% (vol/vol) FBS, 10% (vol/vol) horse serum (both from Thermo Fisher Scientific), 0.1 ng/ml recombinant FGF-2 (PromoCell GmbH) and 1% (vol/vol) penicillin-streptomycin solution for 24 hours.

**Experiments with PIF.**

PIF, MVRIKPGSANKPSDD, was provided by Bio-Synthesis, Inc. (Lewisville, TX). Peptide identity was verified by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry and amino-acid analysis, and the peptide was purified to >95% by HPLC, as documented by mass spectrometry.

PIF was diluted in cell culture medium to a concentration of 0.1 µg/ml. BAC were cultivated with PIF containing medium for 3 days starting the day after cell isolation. During this period, the cells received freshly prepared standard or PIF containing medium every day.

**Steroid release and measurement.**

For cortisol release experiments, the day after isolation the cells from different adrenals were seeded in a 24 well plate, 5x104 cells per well, in six replicates for each condition. The cells were cultured with (experimental group) or without (control group) PIF in the cultivation medium. After 48 hours of cultivation in 24 well plates, three wells of the control group of cells from each adrenal gland were stimulated with medium, containing 3 ng/ml ACTH1-24 (Synacthen, Sigma-tau Arzneimittel GmbH) and the other three with standard medium (basal). The experimental group received PIF containing medium with or without ACTH.

Basal and ACTH stimulated cortisol secretion was measured in cell culture supernatants after 24 hours of cultivation by cortisol ELISA (IBL). The stimulation index was determined by division of ACTH stimulated cortisol by basal cortisol.

**Reverse-transcription and semi quantitative real-time PCR.**

Total RNA from bovine adrenocortical cells was isolated using the RNeasy Micro kit (Qiagen) according to the manufacturer’s protocol. For reverse transcription, up to 1 μg of total RNA was converted to first-strand cDNA using M-MLV reverse transcriptase, reaction buffer, RNase inhibitor, dNTP mix and oligo(dT) 15/random hexamer primer according to the manufacturer’s instructions (Promega).

Semi quantitative real-time PCR was performed using SYBR green (Qiagen) and a Roche Light Cycler 1.5 (Roche). Primers (Tab.1) were designed by Primer-BLAST – NCBI software to span at least one intron to prevent unspecific amplification of DNA remnants. To normalize data, the *RPS9* gene was used as an internal control. Evaluation of different housekeeping genes in our laboratory (*GAPDH*, *β-actin*, *TBP*) revealed that *RPS9* is the most stable gene in our system. Typical genes used as internal controls (*GAPDH* and *β-actin*) increased their expression in cultured cells in response to traumatization compared to freshly isolated cells. These data correspond with previously published results [19]. PIF and ACTH effects were defined by comparison of gene expressions for all groups with the mean value of the same gene in the control group.

**Assessment of Proliferation, Apoptosis and Viability.**

The day after isolation, BAC from different adrenals were seeded in 96 well plates, 1x104 cells per well, in triplicates for each group. Control cells were incubated with standard cell culture medium, whereas the medium for experimental cells contained PIF. The cells were cultivated this way for 3 days before assessment of cell proliferation, apoptosis and viability.

Proliferation was measured using Cell Proliferation ELISA, BrdU (Roche) following the manufacturer’s protocol. Apoptosis was assayed by determination of caspase 3/7 activity using Caspase-Glo 3/7 Assay (Promega) according to the manufacturer’s instructions. Viability of BAC was defined by Cell Proliferation Kit II (XTT) (Roche) following the manufacturer’s instructions.

**Statistical analyses**

Quantitative data is represented as mean ± s.e.. Statistical significance was determined by a two-tailed Student’s t-test, one-way analysis of variance (ANOVA) with the post hoc Bonferroni’s multiple comparison test, Spearman’s rank correlation coefficient or quantile analysis where appropriate. A value of p ≤ 0.05 was considered as significant in all tests. Graph Pad Prism 5.0 (GraphPad Software, Inc., La Jolla, USA) was used for statistical analysis.

**Results**

**PIF influences cortisol production of BAC.**

To examine the effect of PIF on isolated BACs we first measured cortisol secretion in response to stimulation by pituitary adrenocorticotropic hormone (ACTH). We found that BACs isolated from different adrenal glands secreted a particularly wide range of cortisol levels upon ACTH stimulation. In the cells that responded to ACTH by secreting the highest levels of cortisol, as computed by quantile analysis (>Q=75; n=12, see methods), designated as highly responsive cells (HRC), PIF significantly decreased ACTH-stimulated cortisol release by 84% (Fig.1A). In normally responsive cells (NRC) (Q=30-70, median ± 20, n=12), this effect of PIF was not observed (p>0.1). In the absence of ACTH, PIF had no effect (p>0.1) on cortisol secretion in either group (Fig. 1B). Thus, PIF decreases cortisol production specifically in activated BACs i.e., only those highly responsive to ACTH stimulation.

**Characterization of groups with normal and heightened response to ACTH stimulation.**

Basal and ACTH-stimulated cortisol production of NRC and HRC cells in the absence of PIF are presented in Fig. 2A (basal cortisol 47±2 ng/ml for NRC and 63±4 ng/ml for HRC respectively, p<0.01; and ACTH stimulated - 313±98 ng/ml for NRC and 1052±117 ng/ml for HRC, p<0.05). To investigate the underlying mechanism of this differential response we analyzed gene expression levels of two factors involved in the cortisol synthesis pathway of BACs associated with adrenal gland function, steroidogenic factor 1 (SF1), and the steroidogenic enzyme, cytochrome P450c17 (CYP17A1). SF1 plays an essential role in homeostatic proliferation of the adult adrenal gland. It acts as an obligatory activator of most steroidogenic enzymes in the adrenal cortex and participates in both proliferation and differentiation (steroidogenesis) of the adult gland [20]. The CYP17A1 gene is a cytochrome P450 17A1 enzyme involved in the synthesis of steroids from cholesterol. ACTH stimulation activates CYP17 transcription by promoting the binding of SF1 [21]. We found that in HRC, the CYP17A1 and SF1 genes were much more strongly expressed than in NRC under basal conditions (CYP17A1 expression was 0.16±0.01 for NRC and 0.83±0.07 for HRC, p<0.01; and SF1 - 0.29±0.1 for NRC and 1.4±0.23 for HRC respectively, p<0.001; Fig.2B and 2C). Upon ACTH stimulation, cortisol release and expression of SF1 and CYP17A1 were increased in both groups of cells (expression of SF1 increased 9.6 fold in NRC and by 4.9 fold in HRC groups, p<0.001; and expression of CYP17A1 was upregulated by 4482 fold in NRC and by 3016 fold in the HRC group, respectively, p<0.001; Fig.2B and 2C). The significant difference between the NRC and HRC groups with regard to cortisol production and expression of SF1 and CYP17A1 remained unchanged following ACTH stimulation (p<0.05 for SF1 and p<0.01 for CYP17A1; Fig.2B and 2C).

Differing from HRC, the NRC group showed a higher rate of cell proliferation (absorbance 0.33±0.02 OD for NRC and 0.24±0.01 OD for HRC, p<0.05; Fig.3A). Cell viability and apoptosis were also higher in the NRC group as compared to HRC (absorbance 1.46±0.03 OD for NRC and 1.18±0.04 OD for HRC, p<0.05 for cell viability and luminescence 218900±2149 RLU for NRC and 204370±4227 RLU for HRC, p<0.05; Fig.3B and 3C). Considering that PIF affects production of the anti-inflammatory cytokine IL-10 [16], which reduces the immune response to transplantation [22], we analyzed initial gene expression levels of this interleukin in the BAC culture. We found that NRC also had much higher levels of IL-10 than the HRC population (gene expression of 1.38±0.51 for NRC and 0.18±0.08 for HRC, p=0.08; Fig. 3D).

Furthermore, we found a negative correlation between cell proliferation and the level of cortisol production (r=-0.87, n=12, p < 0.001), cell proliferation and the expression of SF1 (r=-0.92, n=12, p < 0.05) and cell proliferation and the expression of CYP17A1 (r=-0.81, n=12, p < 0.05). ACTH stimulation also induced a significant reduction in proliferation of BAC (69 ± 7.9% of control, n=6, p<0.05). Thus, functional BACs (i.e., those highly responsive to ACTH stimulation), are characterized by higher expression of steroidogenic genes, and decreased proliferation.

**PIF affects expression of cortisol synthesis pathway genes.**

Given that the hyper-activatable BAC, which produced higher levels of cortisol and expressed higher levels of cortisol synthesis pathway genes, were more sensitive to PIF, we next investigated the role of those genes as possible targets for PIF action. Under basal conditions, PIF increased expression of CYP17A1 in both the NRC and HRC groups. Interestingly, in the NRC group, PIF induced a far greater increase in the expression of CYP17A1 when compared to the HRC group (13 fold vs. 4.5 fold respectively, Fig.4A). Similarly, basal levels of SF1 following PIF exposure in both groups of cells were significantly elevated. Indeed, an increase in SF1 expression after PIF application in the NRC group reached the initial level of expression found in the HRC group (1.08±0.1 for NRC and 1.37±0.23 for HRC respectively; Fig.4B).

After ACTH stimulation, PIF reduced CYP17A1 expression 1.7 fold in the NRC group, whereas in the HRC group it was downregulated by 5 fold (p<0.05 for NRC and p<0.01 for HRC respectively; Fig. 4C). PIF also significantly downregulated SF1 expression in the HRC group (2.8 fold, p<0.05) without affecting SF1 expression in the NRC group (p>01). Interestingly, in HRC cells, PIF reduced SF1 to almost the same levels as in the NRC group (2.4±1.01 for HRC and 2.82±0.85 for NRC; Fig.4D).

**PIF affects IL-10.**

We also analyzed the effect of PIF on IL-10 levels, which we earlier showed were lower in the NRC than in the HRC group. PIF's effects on the expression of IL-10 in BAC were significant but opposite in the two tested groups: PIF significantly downregulated IL-10 in the NRC group (p<0.05). In contrast, in the HRC group, where initially IL-10 levels were lower, PIF significantly upregulated IL-10 expression (21 fold, p<0.001) (Fig.4E).

**Discussion**

This study demonstrates that PIF, originally an embryo-secreted peptide, made synthetically, exerts potent regulatory effects on cortisol production in primary cultures of BAC. We demonstrate that the action of PIF depends on the initial functional status of the cells, basal or activated, determined by the degree of their response to ACTH stimulation. The regulatory effect of PIF primarily consists of its selective ability to reduce cortisol release in cells that have a heightened response to ACTH stimulation while not affecting cells with a “normal” response to ACTH. These results reveal the selective activity of PIF on BAC, consistent with results in systemic mononuclear cells: the effect of PIF on activated cells was more pronounced compared to unstimulated cells [15, 16]. Therefore, we document that this dual regulatory mechanism also applies to non-immune BAC. We showed that PIF affects the cortisol synthesis pathway of BAC likely by targeting SF1 and CYP17A1 gene expression. This regulatory effect of PIF on SF1 and CYP17A1 in adrenocortical cells was dependent on their initial level of expression: PIF markedly increased SF1 and CYP17A1 in cells where basal expression was low, and, in contrast, had only a mild effect in cells where the basal expression was already relatively high. ACTH significantly upregulated SF1 and CYP17A1 expression while PIF significantly downregulated them in NRC, and even more effectively in HRC. Notably, the observed effects of PIF depended on the rate of response to ACTH stimulation.

The mechanism of PIF's action on BAC is to block stimulation by a specific physiological activator. Consequently, there is a selective suppression of cells associated with a greater response by the cytochrome p450 enzyme complex. Thus, PIF targets hyper functioning cells that could become exhausted and damaged. This reveals a unique protective mechanism of PIF action, which may enable long-term cell survival. PIF’s inhibitory effect begins when activation of cytochrome p450 reaches a certain level. Involvement of PIF in additional local protective pathways may also involve adrenodoxin reductase, a p450 mitochondrial enzyme, which has the highest expression in the adrenal cortex [23]. PIF may act by regulating this enzyme’s oxidoreductase activity thus reducing oxidative stress and protein misfolding [13, 16, 24] .

The protective mechanism of PIF's action is further revealed by the observed reduction in BAC hyper functionality, which was coupled with an increase in IL-10, a key anti-inflammatory cytokine that reduces the immune response in transplantation [22]. IL-10 expression may be connected to local defense mechanisms mitigating stimulated immune cell activity as well as protecting overactive BAC. Moreover, IL-10 also promotes proliferation and cell differentiation [25, 26]. PIF was already shown to promote IL-10 both *in vitro* as well *in vivo,* to increase cell viability and reduce apoptosis [27, 28]. However, in our study, the effect of PIF on BAC proliferation and apoptosis was not observed, possibly due to the short observation period or the low number of immune cells present in culture.

In summary, PIF is a promising agent for bioartificial transplant applications. PIF features address most currently known limitations of allo- and xenotransplant use and stand to improve the outcome of transplantation of a bioartificial adrenal gland. By regulating BAC function, PIF may enable development of a safe and functional bioartificial adrenal organ.

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**Legends of tables and figures**

**Tab.1 List of primers and amplification conditions for RT-PCR**

**Fig.1**. **Effect of PIF on cortisol production by adrenocortical cells.** **A** – Influence of PIF on ACTH stimulated cortisol release. **B** – Effect of PIF on basal cortisol production. All data presented as mean ± s.e.; n≥6 for each time point; \*p≤0.05; \*\*p ≤0.01; \*\*\*p≤0.001.

**Fig.2. Characteristics of cells with different responses to ACTH stimulation. A** – Characterization of normally (NRC) and highly responsive cells (HRC) by basal and ACTH stimulated cortisol production, relative gene expression of SF1 (**B**) and CYP17A1 (**C**). All data presented as mean ± s.e.; n≥6 for each time point; \*p≤0.05; \*\*p ≤0.01; \*\*\*p≤0.001. Reference gene – *RPS9*.

**Fig.3. Characteristics of processes occurring in cells with different responses to ACTH stimulation.** A – Proliferation, viability (B), apoptosis (C) and relative mRNA gene expression of IL-10 (D) of normally and highly responsive cells. All data presented as mean ± s.e.; n≥6 for each time point; \*p≤0.05.

**Fig.4. Effect of PIF on gene expression**. **A** – Effect of PIF on basal gene expression of CYP17A1, and SF1 (**B**). **C** - Effect of PIF of ACTH-stimulated expression of CYP17A1 and SF1 (**D**). Effect of PIF on expression of IL-10. All data presented as mean ± s.e.; n≥6 for each time point; \*p≤0.05; \*\*p ≤0.01; \*\*\*p≤0.001. Reference gene – *RPS9*.