Supplemental Results

Gonadotropin dependent up-regulation of PHB positively correlates with steroidogenesis in granulosa cells

Correlation analysis was used to demonstrate the relationship between expression levels of PHB versus secretion of E2 and P4 (supplemental figure 1). Phase contrast photomicrograph pictures (Supplemental figure 1A) show that GCs are fibroblastic in appearance in vitro in serum free medium (control GCs), and was only slightly altered in presence of either T or FSH alone. However, GCs showed altered cellular appearance in presence of T plus FSH with an epitheloid morphology, characteristics of more highly differentiated GCs. Treatment of T and FSH alone did not show any effect on E2 and P4 secretions (Supplemental figure 1B). However, T plus FSH potently induced E2 and P4 secretion in GCs significantly when compared to T or FSH treated group alone (p<0.05; Newman-keuls’ test). Western blot analyses shown in supplemental figure 1C revealed that PHB protein expression were increased in T, FSH and FSH plus T groups compared to the untreated controls (p<0.05). Interestingly, PHB expression was significantly higher in FSH plus T group compared to GCs treated with either FSH or T alone. Moreover, immunocytochemical colocalization studies (Supplemental figure 1D) have further confirmed that the FSH plus T dependent up-regulated PHB co-localizes with cytochrome c in mitochondria.

Granulosa cells (GCs) survival state in prohibitin (PHB) knock-down GCs treated with gonadotropin

Based on the significant positive correlation and up-regulation of PHB with E2 and P4 secretions in FSH plus T treated GCs, we hypothesized that up-regulation of PHB in FSH plus T treated GCs is necessary for E2 and P4 synthesis and secretions. Therefore, to elucidate the physiological and functional responses of PHB in FSH-induced steroidogenesis, we performed knock-down studies of Phb gene expression in GCs using adenoviral shRNA (AdshRNA). Greater than 90% of infected GCs in primary culture exhibited adenovirus expression of the green
fluorescent protein (eGFP) reporter gene without any adverse effects on cellular functions. Studies to determine the efficiency (dose and time dependent) and specificity of AdshRNA dependent knock-down of Phb gene expression were performed in GCs infected with equal viral titers (achieved by adding the appropriate amount of empty adenoviral vectors, Ad-scrambled), and analyzed by western blot and immunofluorescence microscopy (Chowdhury et al., 2007, 2011, 2013).

Since PHB is an important survival factor. We have shown in previous studies that knockdown of PHB sensitized the cells such that any further treatment with pro-apoptotic factors or cell death inducing factors or unfavorable stress conditions including serum shock are able to tilt the balance towards apoptosis or cell death (Chowdhury et al., 2007, 2011, 2013). Therefore, further studies aimed at delineating the FSH effects after PHB knock-down in GCs were done under our previous experimental conditions. Phase contrast and epifluorescence (eGFP-shPhb or eGFP-scrambled) photomicrograph images and Hoechst 33248 staining post 48h post-treatment suggest that Ad-eGFP-scrambled infection of GC had no adverse effects on GCs survival under these experimental conditions (Supplemental figure 2A and 2B). In contrast, adenoviral dose (MOI) dependent knock-down of PHB had differential effects on survivability of the GCs. Survivability of GCs after knock-down of PHB at 20 MOI was 70-75% at 48h post-FSH treatment. Furthermore, analysis of cytosolic caspase-3 enzymatic activities post-FSH treatment for 48 h showed no significant changes in Ad-eGFP-scrambled infected cells, and at lower dose (5 MOI) of Ad-eGFP-shPhb and in the uninfected parallel groups. However, a significant (P < 0.05) increase in caspase-3 activity was observed in GCs infected with Ad-eGFP-shPhb at 20 MOI compared to Ad-eGFP infected group (Supplemental figure 2C). The morphological differences observed and quantitative measures of the extent of survival in the GCs suggest that higher levels of knock-down of PHB tilted the balance towards cell death in a time dependent manner.
Moreover, the correlation coefficient among percentage apoptosis and Caspase-3 activity were determined by linear regression analysis. Correlation analysis demonstrated that percentage apoptosis and Caspase-3 activity have a significant positive correlation (y = 3.799x - 8.419, R² = 0.89163, P<0.001) (graph not included). Ad-eGFP-shPhb at 20 MOI compared to Ad-eGFP at 20 MOI infected group in presence of FSH plus T dependent up-regulated caspase-3 activity in GCs and have significantly positive correlations with percentage apoptosis.