

Effect of serum starvation and cell density in the expression of VEGF, bFGF and Angiogenin, and also the effect of VEGF up regulate in the expression of bFGF and Angiogenin

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Abstract- We investigated the effects of serum starvation and cell density expression of Vascular endothelial growth factor (VEGF), Basic fibroblast growth factor (bFGF) and Angiogenin by used PC3M cells, we demonstrated that low density increase the expression of VEGF and bFGF but no effects on Angiogenin in the mRNA levels, while no deferent in high density. However the VEGF and bFGF was decrease when the cells was starved 24h but no effects on angiogenin in the mRNA levels, also We transfected the VEGF gene in the sense orientation into PC3M cells, and obtained stable VEGF overexpressing transfectants. The bFGF and angiogenin was detected ,and we obtain the VEGF up regulate increase of bFGF expression level, and negative correlation was found between up regulate VEGF and angiogenin expression level.

Index Terms- PC3M cells, VEGF, bFGF, angiogenin, of serum starvation, cell density

I. INTRODUCTION

Prostate cancer is a form of cancer that develops in the prostate, a gland in the male reproductive system. Most prostate cancers are slow growing; however, there are cases of aggressive prostate cancers. On the other hand, the more aggressive prostate cancers account for more cancer-related mortality than any other cancer except lung cancer(Siegel *et al.*, 2011). Many factors, including genetics and diet, have been implicated in the development of prostate cancer(Djulbegovic *et al.*, 2010).

Vascular endothelial growth factor (VEGF), also known as vascular permeability factor (VPF), is an important mediator of these angiogenic processes in both normal and diseased conditions. As one of the most important cytokines, VEGF is widely expressed by a number of human and animal tumors and has the ability to regulate most of the steps in the angiogenic signal cascade(Sutapa *et al.*, 2009).

Basic fibroblast growth factor (bFGF), amember of a family of heparin binding multifunctional polypeptides, is one of the most potent angiogenic factors. It is highly expressed in a variety of tumor cells and tissues(Bikfaivi *et al.*,1997).

Angiogenin is localizes on chromosome 14q11, Its expression increases in various types of human cancers, including breast, cervical, colon, colorectal, endometrial, gastric,

liver, kidney, prostate(Katona *et al.*, 2005). Four aspects of ANG have been discovered to be necessary for the process of ANG-induced angiogenesis, including ribonuclease activity, basement membrane degradation, signaling transduction, and nuclear translocation(Xiangwei and Zhengping, 2008).

In recent years, the inhibitors of some angiogenic factors were expected to be used in tumor therapy, and themechanism of the interaction between angiogenic factors became important and appealing. The present study may be helpful in finding a potential therapeutic approach to human prostate cancer.

The single most important element that determined whether cancer cells were responsive to angiogenin or not was cell density. Other details such as the cell passage number, confluence status, attachment factors, and stimulation times had some influence, but were not as critical as cell density and starvation.

To investigate the effect of cell density in three gene the VEGF, bFGF and Angiogenin in prostate cancer. To investigate the effect of serum starvation in three gene the VEGF, bFGF and Angiogenin in prostate cancer. To further understand the biological role of VEGF,angiogenin and bFGF, we investigated the effect of VEGF up regulation on the expression of angiogenin, bFGF and PC3M cell growth.

II. MATERIALS AND METHODS

Cell Culture

Cell Lines and Culture Conditions. The human Prostate cancer cell line PC-3M was obtained from the Northeast Normal University Lap. These cells were cultured and maintained in IMDM supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin and kept in a humidified atmosphere of 5% CO₂. They also use the Dimethyl sulfoxide 50µM for kept cells in -80. For induction of serum starvation, cells were grown to 100% confluence in IMDM and 10% fetal bovine serum, and the medium was then changed to serum-free medium. Cells were grown to 100% confluence to avoid any variations in VEGF, bFGF and Angiogenin expression that might occur when cells are grown to a lesser confluence, as demonstrated previously. a lesser confluence, as demonstrated previously. To determine the effects of serum starvation and high and low density, cells grown under these serum-free and high

and low density conditions were harvested after various, time periods, and mRNA was obtained.

Transfection experiments

The day before transfection, PC3-M cells were seeded at a density of 3×10^5 cell/cm². twenty-four hour later, when cells reached 70-80% confluency, the decoy ODNs was transfected into PC-3M cells by LipofectaminTM2000 according to the manufacturer's instructions. PC-3M cells were divided into two groups. VEGF decoy was transfected into first group, Control decoy was not transfected into second group, was used 1.6-2 μ g /50 μ l decoy DNA and 4-5 μ l/50 μ l lipofectamin were diluted into 50 μ l medium without serum. Then the above two were combined and layed at room temperature for 20 mins. The complexes were added onto the cells cultured with serum-free medium. After 15-20 h, PC-3M cells were detected.

Reverse transcriptase-PCR (RT-PCR) Analysis

Expression of VEGF, bFGF and Angiogenin gene was determined by RT-PCR. Total RNA was extracted using TRIzol Reagent (in vitro). For oligonucleotide treated cells, RNA expression was performed 15 or 20 h after termination of oligonucleotide treatments. Two microgram of total RNA were reverse transcribed using 200 units of murine reverse transcriptase under recommended conditions. One-fourth of this cDNA was used as the template in a PCR using the following upstream and downstream primers:

VEGF sense primer 5'-
ACATCTCCAGGAGTACCCTGATGAG-3'
VEGF antisense primer 5'-
GCATTCACATTTGTTGTGCTGT-3'
Angiogenin sense primer 5' **CATCATGAGGAGACGGGG**-
3'
Angiogenin antisense primer 5'-
TCCAAGTGGACAGGTAAGCC-3'
bFGF sense primer 5'-
ATGGCAGCCGGGAGCATCACC-3'
bFGF antisense primer 5'-
CACACACTCCTTTGATAGACACAA-3'
 β -actin sense primer 5'-
TGGTCAGAAGGATTCCTATGT-3'
 β -actin antisense primer 5'-
CAGCCTGGATAGCAACGTACA-3'

PCR for the detection of VEGF, bFGF and Angiogenin gene in field isolates for all reactions was done using Model Gene Amp[®] Chinese PCR system (Tiangen biotech) in the following conditions: one cycle at 94 °C for 5 minutes; 28 cycles at 94 °C for 30 second, 58 °C for 30 second, and 72° C for 30 second; and a final extension at 72° C for 10 minutes. This bFGF, but for Angiogenin gene was used 25 cycles, and for VEGF was used 26 cycle, For each series of samples, water was used β actin as Internal control. Each PCR product was run on an 1.5% gel electrophoresis at 100V for 30 min and the gel was scanned.

III. RESULT

studies were performed to examine effect of cell density and serum starvation in three gene the VEGF, bFGF and Angiogenin in prostate cancer, and the effect of up-regulated VEGF expression in bFGF and Angiogenin expression, two low density (1.5×10^5 cells/well), high density (6×10^5 cells/well), and 3×10^5 was used as control in 6 well dish.

When cells were plated sparsely (1.5×10^5 cells/well), there a significant increase in bFGF expression that occurred when decrease cells branched out to touch other cells via long this processes; this increase in bFGF levels remained as the cells became confluent. cells were plated high densities (6×10^5 cells/well), high densities cells was present lower than lower densities cells for bFGF expression

Despite the dramatic changes between low densities and 24h in bFGF expression, but high and 0h expressed did not change significantly at densities of 6×10^5 - 3×10^5 cells per well.

Levels of the VEGF, bFGF and Angiogenin were compared when cells were grown in serum-replete medium vs. serum-starved conditions at 0 and 24h, only significant decrease in VEGF and bFGF was observed after cells were serum-starved for 24h, while the VEGF levels was increase at the low density.

While Angiogenin expression in cells grown in serum-replete medium and serum-starved showed no significant difference.

Using PCR, we genotyped the cells expression VEGF, bFGF, and angiogenin and found that, A strong correlation was found between up regulate VEGF and increase of bFGF expression level, and negative correlation was found between up regulate VEGF and angiogenin expression level.

IV. DISCUSSIONS

To test the hypothesis that constitutively active oncogenes or oncogene homologs can prevent the switch to a protective maintenance mode in response to starvation. Expression of VEGF, bFGF and Angiogenin mRNA by Serum Starvation, high and low density. We first performed differential stress to determine the effects of serum starvation, high and low density on VEGF, bFGF and Angiogenin expression. Our hypothesis was that the combination of these genetic manipulations with starvation, PC-3M cells were grown to 100% confluence in IMDM supplemented with 10% fetal bovine serum. The medium was then changed to serum-free medium, and the cells were harvested at intervals thereafter. and other cells it put in high and low density To determine the effects of these conditions on VEGF, bFGF and Angiogenin expression.

There is morphological difference was observed among the cells between starved cells and un starved cells, but There is no morphological difference was observed among the cells between high and Low density cells. To measure the proliferation rate of cells in serum starvation is difference, it was fall down when the time is increase, also there is no difference between low and high density in the growth rates. The number of cells 3×10^5 in serum starvation, 6×10^5 in high density and 1.5×10^5 low density in each dish, was counted the cell before put in dish. No significant difference was found in the growth rates of the high

and low density (results not shown), but there is significant difference was found in the growth rates of the starved cells.

This phenotypic change would presumably allow for motility and, eventually, wound contraction. Indeed, changes in motility induced by fibroblast growth factor have been demonstrated in cells at low cell density, while the same growth factor induces mitogenesis at higher cell density (Thomas *et al.*, 1999).

When cells were plated sparsely (1.5×10^5 cells/well), there a significant increase in bFGF expression that occurred when decrease cells branched out to touch other cells via long thin processes; this increase in bFGF levels remained as the cells became confluent. Cells were plated high densities (6×10^5 cells/well), high densities cells was present lower than lower densities cells for bFGF expression. Same to Thomas *et al.*, 1999.

Despite the dramatic changes between low densities and 24h in bFGF expression, but high and 0h expressed did not change significantly at densities of 6×10^5 - 3×10^5 cells per well.

Levels of the VEGF, bFGF and Angiogenin were compared when cells were grown in serum-replete medium vs. serum-starved conditions at 0 and 24h, only significant decrease in VEGF and bFGF was observed after cells were serum-starved for 24h, while the VEGF levels was increase at the low density.

While Angiogenin expression in cells grown in serum-replete medium and serum-starved showed no significant difference. The mitogenic and proliferative activities of angiogenin are relatively low compared with those of bFGF and VEGF. These data also indicate that the endogenous angiogenin secreted by endothelial cells is neither necessary for nor inhibitory to the actions of bFGF and VEGF (Guo *et al.*, 1997). Nuclear translocation of angiogenin in endothelial cells decreases as cell density increases and ceases when cells are confluent (Takanori *et al.*, 2005).

We have examined Levels of the VEGF after 24h starvation, VEGF was decrease compare with control, while the VEGF levels was increase at the low density. Other researchers found that the expression of VEGF increased after the growth of tumor cells was inhibited. The inhibition of tumor cell growth by conventional forms of therapy such as irradiation or low dose chemotherapy led to an increase of VEGF expression (Polytarchou *et al.*, 2004; Fersis *et al.*, 2004). Gene expression studies comparing dense and sparse growing tumor cells have shown that VEGF gene expression increased in confluent slow growing H460 cells compared with sparse fast growing cells (Kuhn *et al.*, 2004). VEGF is a key factor for angiogenesis in development, and lack of one allele of the VEGF gene causes embryonal death (Carmeliet *et al.*, 1996).

Other researchers demonstrated that serum starvation induces increased VEGF expression in protein levels. Serum starvation has been shown variously to activate apoptosis pathways through NF- κ B, to up-regulate IFN regulatory factor in cultured Swiss 3T3 cells, and to up-regulate expression of cyclins D and E in rat fibroblasts (Young *et al.*, 1999).

A statistically significant correlation was found between the number of angiogenic factor. A strong correlation was found between VEGF and bFGF levels.

Angiogenin was independently correlated with VEGF. A negative significant correlation was found between VEGF levels and Angiogenin (Fig 3).

Yasuaki *et al.*, 1999 demonstrate that bFGF induces VEGF mRNA expression (2.8-fold) and, thus, demonstrate a potential mechanistic explanation for the synergistic activity of VEGF and bFGF.

bFGF is not an endothelial cell-specific growth factor such as VEGF; it acts as a growth factor for many different cell types. bFGF levels seem to be important for the degree of tumor in our cells lines, because there is a positive correlation between bFGF and VEGF.

Human Prostate cancer is an angiogenesis dependent tumor. As VEGF, bFGF and Angiogenin is a potentially important angiogenic factor for Prostate cancer progression, we obtained stable VEGF over-expressing transfectants by transfecting the VEGF gene in the sense orientation into PC3M cells. A strong correlation was found between up regulate VEGF and increase of bFGF expression levels in mRNA levels, but there is no correlation between up regulate VEGF and Angiogenin in mRNA level.

In the present study, we have examined the effects of VEGF on the expression of bFGF and angiogenin in PC3M cells to further approach the interaction between VEGF, bFGF and angiogenin. Such results demonstrated that VEGF negatively affected the expression of angiogenin and positively affected the expression of bFGF in PC3M cells.

It has been reported that aFGF, bFGF, and FGF8 are co-localized in human prostate cancer (Dorkin *et al.*, 1999). We have demonstrated that VEGF mRNA was affected in bFGF expression in PC3M cells. According to this result, we presumed that VEGF and bFGF may have a synergistic effect or that they may be in the same signal pathway in PC3M cell growth and progression.

bFGF activates stress-activated protein kinases (SAPK)/c-Jun N-terminal kinase (JNK) and p44/p42 mitogen-activated protein (MAP) to stimulate VEGF release, and bFGF-activated p38 MAPK negatively regulates the VEGF release (Yasuda *et al.*, 2005). Angiogenin is also reported to activate SAPK/JNK (Xu *et al.*, 2001). MAP kinase superfamily mediates intracellular signaling and plays a crucial role in cellular functions such as proliferation, differentiation, and cell death in a variety of cells (Widmann *et al.*, 1999).

It has been reported that the 18-kDa bFGF interacts with a transcription factor in the nucleus and directly regulates rRNA transcription and ribosome biogenesis, a rate-limiting process in cell growth (Sheng *et al.*, 2003; Dailey *et al.*, 2005). bFGF first binds and activates high-affinity cell surface receptors, and after internalization it can translocate into the nucleus and nucleolus. Therefore, a change in bFGF expression level may affect the rRNA transcription and cell proliferation rate (Pederson, 1998; Claus *et al.*, 2003; Xu *et al.*, 2003; Sheng *et al.*, 2004).

In conclusion, angiogenesis has become a new target of tumor treatment. Some angiogenesis factors and their inhibitors are expected to be used in tumor treatment, diagnose and prognosis. Our study indicates that there is a feedback regulation between VEGF and bFGF in human Prostate cancer cells. However, the mechanism of the interaction between VEGF and bFGF and angiogenin still needs further study, and the

interrelationships among other angiogenic factors are also unclear. How do these factors coordinate to induce angiogenesis? Are they simultaneously involved in all steps in the angiogenesis process or individually responsible for different steps? Are they interdependent or are their actions additive or synergistic? These questions will be studied in our future work, and we expect to answer some of these questions through our future investigations to guide the treatment of human Prostate cancer.

Multiple environmental stimuli important to tumor growth and metastasis may regulate the expression of VEGF mRNA. Some effects may be additive, whereas others may be mediated by common pathways. A better understanding of the signaling pathways activated in response to environmental stimuli may be important to therapeutic strategies that target angiogenesis of tumor cells.

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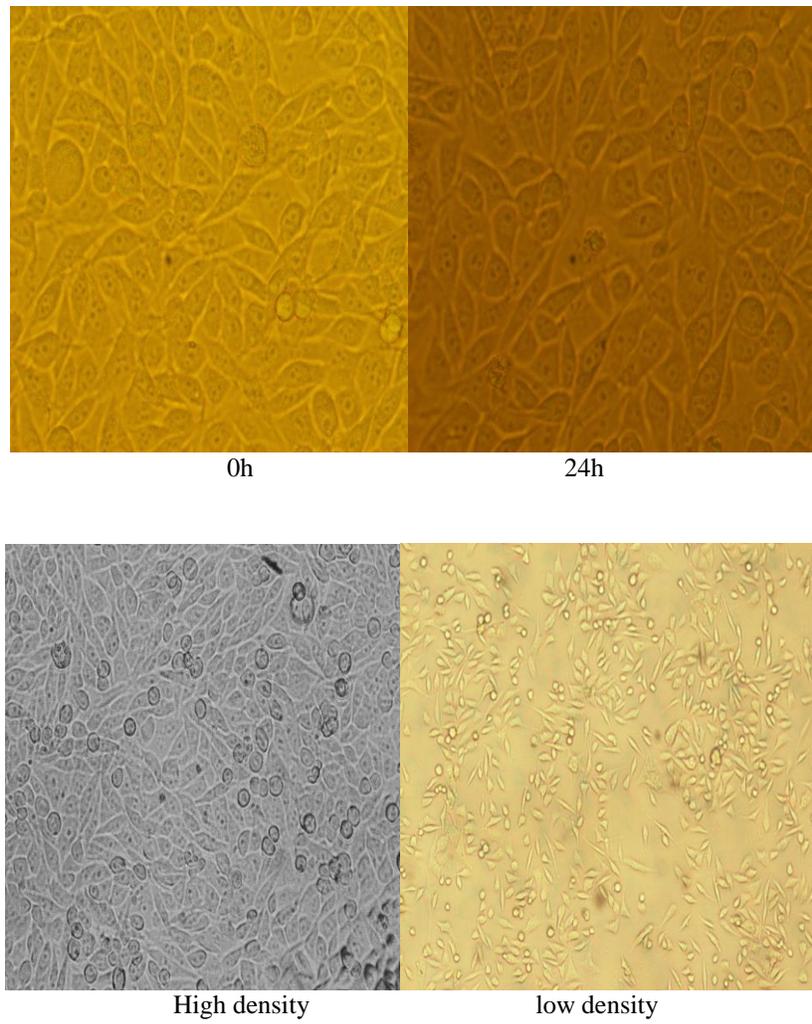


Figure 1- serum starved cells, cell density and control.

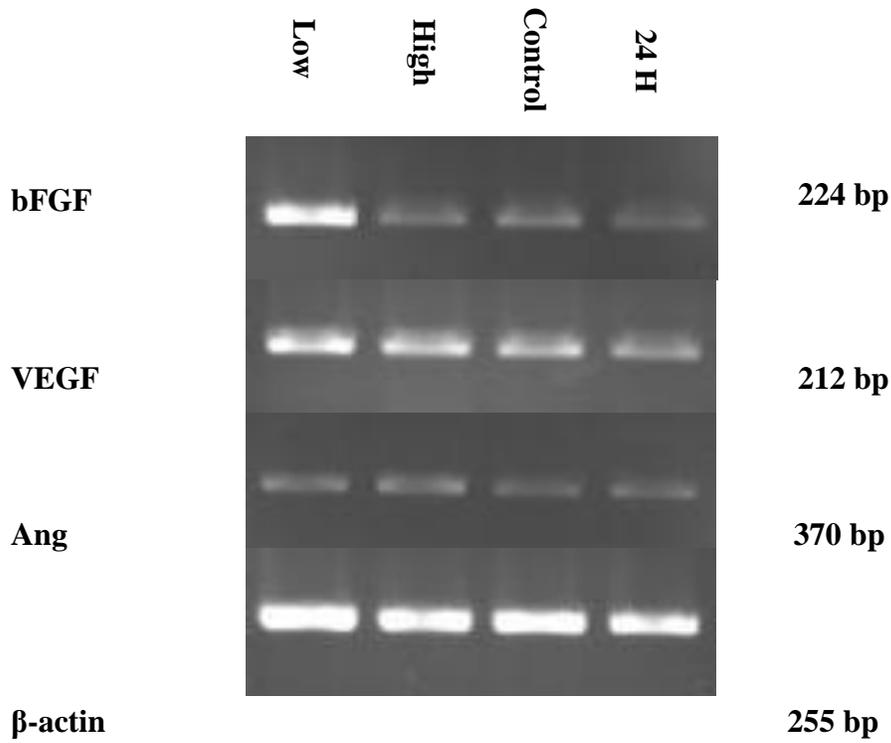


Figure 2 - The mRNA expression of basic fibroblast growth factor (bFGF), Vascular endothelial growth factor (VEGF), Angiogenin, and β -actin used as an internal control. In low and high density, starved cell and control.

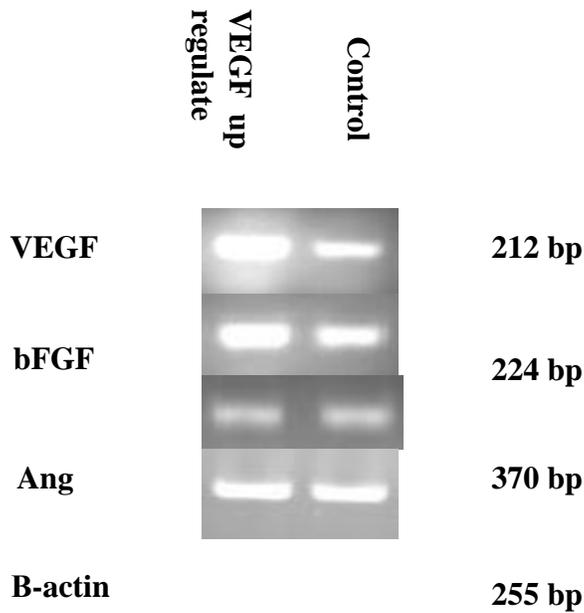


Figure 3 - The mRNA expression of Vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), Angiogenin, and β -actin used as an internal control. In VEGF transfected cells and control.