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13. ABSTRACT (Maximum 200 Words)

Prostate cancer cells express multiple types of FGF receptor and increased expression of FGF receptor-1 (FGFR-1) is present in poorly differentiated human prostate cancers *in vivo*. We have proposed to evaluate biological affects of DN FGFR expression in human primary prostate epithelial cells and prostate cancer cell lines. The findings in this report support that prostate cancer cells are dependent upon FGFR signaling for survival and cells treated with DN FGFR are arrested G2/M phase of cell cycle followed by cell death. FGF signaling modulated *CDC25C* activity in prostate cancer, and in this manner can promote progression through the G2/M checkpoint. *CDC25C* protein is upregulated in comparison to normal prostate tissue and is present almost exclusively in its active dephosphorylated form. Determining other molecules involved in this pathway contributing tumor growth and survival will facilitate the development of cancer therapies to target FGF signaling pathway

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INTRODUCTION:

Alterations in FGF signaling pathway have been implicated in the pathogenesis of variety of malignancies including prostate cancer by *in vitro* and *in vivo* studies(1-4). FGFs produce their mitogenic and angiogenic effects in target cells by signaling through four distinct cell-surface tyrosine kinase receptors, FGFR-1 through FGFR-4. Prostate epithelial cells express FGF receptors and require FGFs for growth in primary culture. Prostate cancer cells express multiple types of FGF receptor and increased expression of FGF receptor-1 (FGFR-1) is present in poorly differentiated human prostate cancers *in vivo*(3,4). We hypothesized that FGF receptor signaling is essential for viability of human prostate cancer cells and disruption of this signaling via expression of a dominant negative FGF receptor-1 protein in human prostate cancer cells might contribute to the death of cancer cells and can be used as adjuvant to current treatment options especially to radiotherapy since it has been also administered locally. Furthermore, analysis of gene expression profile in FGFR DN transfected cells might help our understanding of how FGFR DN works and the differentially expressed genes determined by microarray analysis can be used as targets for prostate cancer therapy.

BODY:

Task 1: We have proposed to evaluate biological affects of DN FGFR expression in human primary prostate epithelial cells and prostate cancer cell lines. We have obtained different recently established prostate cancer cell lines from other investigators and American Type Culture Collection (ATCC). Among these are 22Rv1(5) obtained from ATCC, LAPC4 (6) from Dr. Charles Sawyers of University of California, Los Angeles, C4, C4-2 and C4-2B (7) from Dr. Leland Chung of University of Virginia and MDA PCa 2b (8) from Dr. Nora Navone of University of Texas M.D. Anderson Cancer Center. These cell lines have been frozen in liquid nitrogen for further experiments. Normal prostate biopsy specimens were also cultured for the purpose of obtaining primary prostate epithelial cells. All cell lines have been tittered for optimal multiplicity of infection (MOI) to be used in subsequent experiments. Viral particles was used to establish MOIs in cell lines since this has been suggested as a reliable approach in the literature (9-11). This data is summarized in Table 1.

Table1. Prostate cancer cell lines and appropriate MOIs used in adenovirus infection

Name of the cell line	MOI used (in thousand particles)
22 Rv1	8
LAPC4	4
MDA Pca 2b	6
C4	5
C4-2	5
C4-2B	5

To analyze the affect of DN FGFR in human prostate cell proliferation and/or viability, cells were counted by Coulter counter after 24, 48 and 72 hr of infection with DN FGFR and LacZ. As seen in Figure 1, LAPC4 cells had the most dramatic effect on the inhibition of cell proliferation when infected with DN FGFR adenovirus. Over 90 % of the cells died after 72 hours of infection with DN FGFR; however, there was a 16%

increase on the number of cells infected with LacZ as control. MDA Pca 2b cells also showed decreased in the number of proliferating cells after infection with DN FGFR adenovirus as compared to control at all three time points (Figure 1). After 72 hours of infection cells treated with DN FGFR stopped proliferating as contrast to the cells infected with control adenovirus which continued to grow and more than doubled in number in 72 hours. The 22 Rv1 cell line only had significant reduction on the number of proliferating cells after 72 hours of infection.

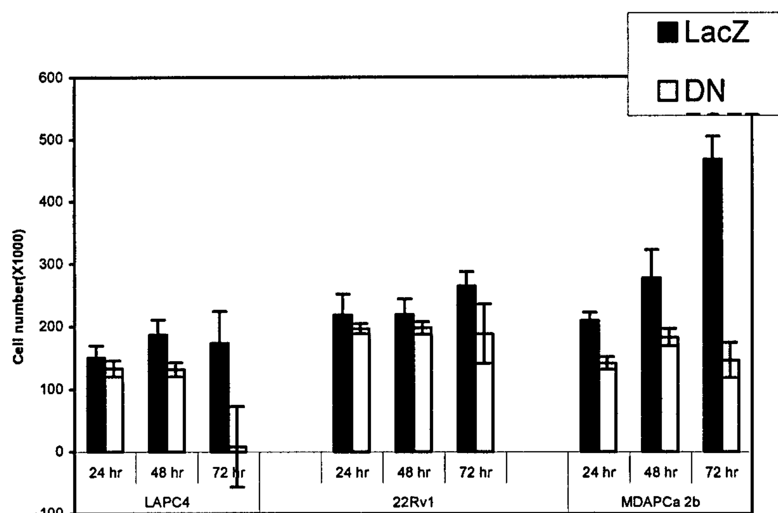


Figure 1. Effect of DN FGFR on prostate cancer cell proliferation and viability. LAPC4 22Rv1 and MDA PCa 2b cells were plated at 5×10^4 cells per 35-mm dish and infected with Ad FGFR or Ad Lac Z as control. The cell number was determined by counting with the use of Coulter counter at 24, 48, and 72 hours after infection. All values are the mean of triplicate determinations.

A series of lineage-related LNCaP cell sublines that reflect the various steps of prostate carcinogenesis and progression has been derived (12). An androgen-independent (AI) cell line, C4-2, reproducibly and consistently follows the metastatic patterns of hormone-refractory prostate cancer by producing lymph node and bone metastases when injected either s.c. or orthotopically in either hormonally intact or castrated hosts (13). This LNCaP model will help improve our understanding of the mechanisms of androgen-dependent to androgen-independent prostate cancer progression. As seen on Figure 2, although DN FGFR adenovirus treatment did decrease the proliferation of all three LNCaP derivatives, The biological effect was not as dramatic as it was in parental LNCap cells. We have previously observed 50- 70% decrease in cell number by 72 hours after DN FGFR treatment (2). One possible explanation of this difference could be the difference in the

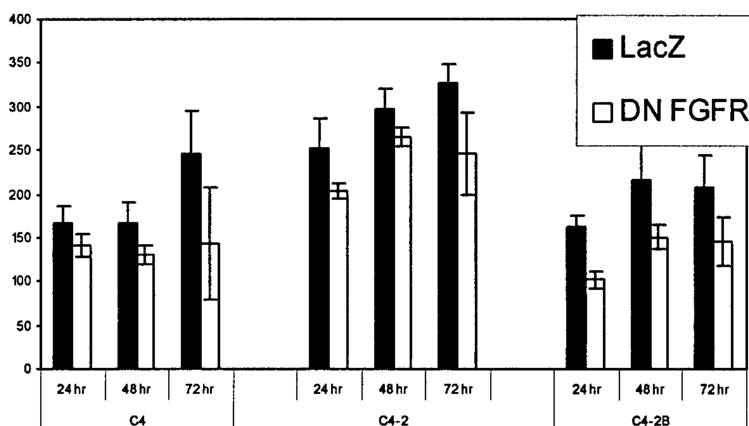
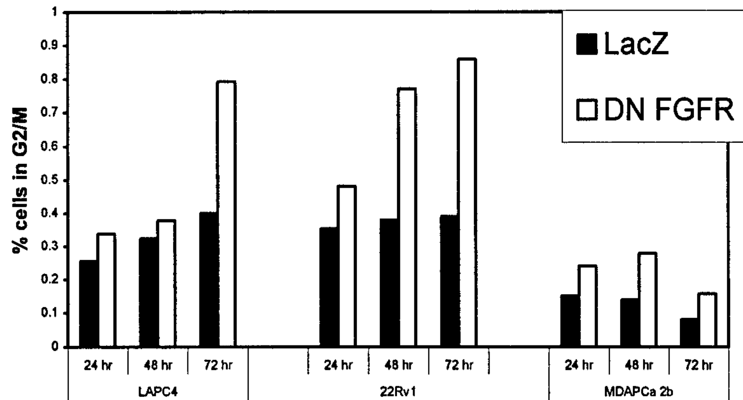


Figure 2. Effect of DN FGFR on prostate cancer cell proliferation and viability on LNCaP prostate cancer sublines C4, C4-2 and C4-2B.

endogenous FGF2 responds to the DN FGFR treatment of these cell lines. This possibility is currently under investigation. It is interesting, however, to note that the original LNCaP cell line was androgen sensitive in contrast to C4-2 sublines. Two of the cell lines, LAPC4 and MDA PCa 2b, mentioned earlier to have the most biological effect after DN FGFR treatment are also androgen sensitive prostate cancer cell lines. It might be interesting to test the effect of DN FGFR on different androgen sensitivity conditions.

For the examination of the effect of the DN FGFR on cell cycle progression, prostate cells were infected with AdDN FGFR and AdLacZ and flow cytometry analysis was performed after different time points (24, 48 and 72 hours). A summary of these results is shown in Figures 3 A and B.
All cell lines tested except C4-2 accumulated in G2/M 72 hours after the infection with DN FGFR. In 48 hours of infection with DN FGFR, C4-2 cells, however showed 20% increase in the number of cells in G2/M phase. The accumulation of cells in G2/M did not correlate the biological effect of DN FGFR in every cell line. For example, treatment of DN FGFR showed more biological effect on MDA PCa 2b cells as compared to 22Rv1 cells after 72 hours of infection (28% reduction in cell number after 72 hours of infection in 22Rv1 cells vs 68% reduction in MDA PCa 2b cells). However, The percentage of the cells in G2/M were 85 and 15 in 22 Rv1 and MDA PCa cells, respectively. This might require further investigation. 22 Rv1 cells might need more time to acquire the biological effect of DN FGFR. We are planning to do more experiments in this

A.



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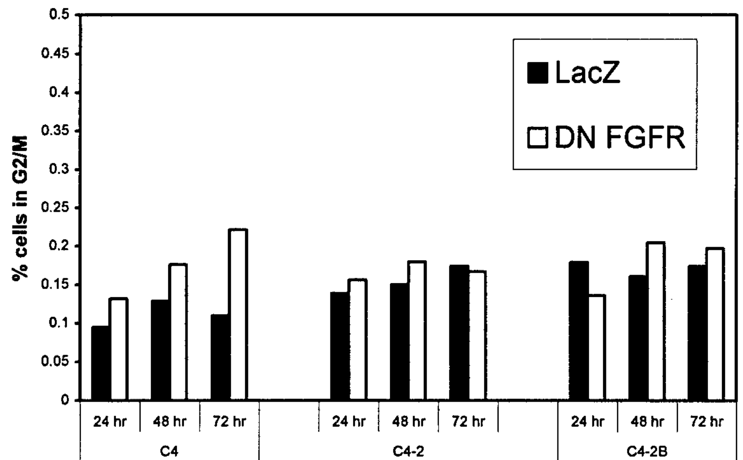

Figure 3. Prostate cancer cells, LAPC4, 22Rv1 and MDA Pca 2b (A) and C4, C4-2 and C-42B (B) were infected with AdDN FGFR and AdLacZ and flow cytometry analysis was performed after 24, 48 and 72 hours. Percent of the cells in G2 is shown in each on the y-axis.

respect in coming year. Cell cycle analysis of DN FGFR treated LAPC4 cells as a representation is shown in Figure 4.

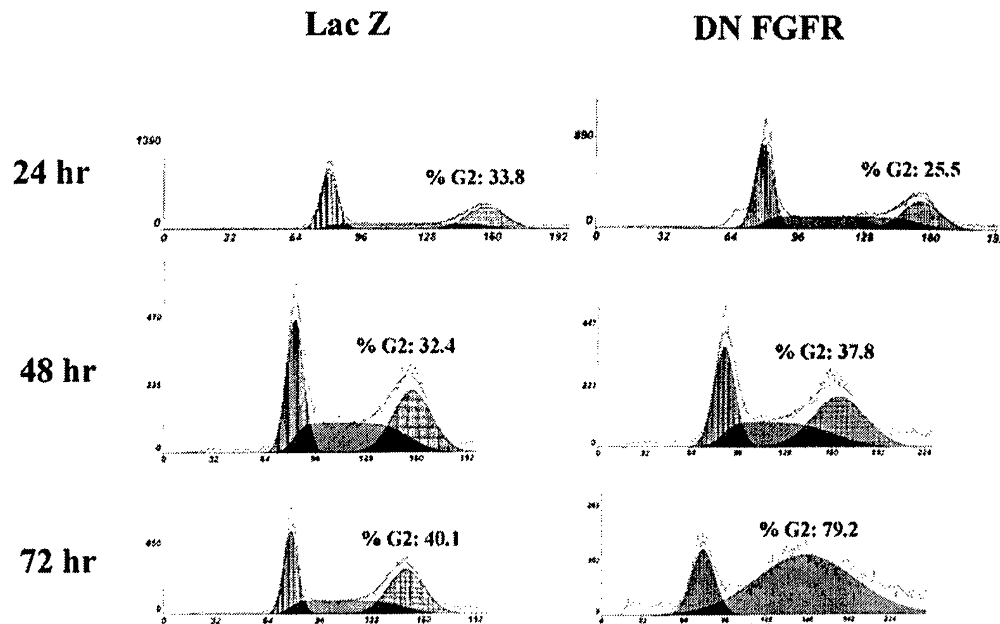

Figure 4. Cell cycle analysis of DN FGFR infected LAPC4 cells. In each case, cell number is represented on the y-axis, with the corresponding fluorescence at 550 nm is shown on the x-axis. Flow cytometry data are seen as a line, and filled area represents the result of cell cycle analysis by the use of Multi Cycle software. The percantage of G2/M as determined with this software is indicated.

Task 2: Since our production of AdDN FGFR, further developments have been made in the area of adenoviral delivery to enhance the transduction ability of the vectors such as Ad5 and Ad35. The entry pathway for Ad5 consists of initial binding to the cell, which is mediated by the association of the Ad5 fiber protein and a 46-kd membrane protein called CAR, followed by internalization. CAR is a member of the immunoglobulin superfamily and also serves as the receptor for coxsackie B virus. Recently, a new adenovirus vector has been developed in which the fiber protein of adenovirus type 35 (Ad 35) has been substituted for the fiber protein of Ad5 that allows the virus to enter cells in a CAR-independent fashion. It has been shown that this new vector could efficiently transfer genes into hematopoietic stem cells and human bone marrow mesenchimal stem cells(14-16). We have done some preliminary experiments using Ad5F35gfp adenovirus on DU145 and PC3 human prostate cancer cells. We were able to infect these cells with as

low as 10-20 MOI. This is a tremendous improvement as compared to our current vector since we were not able to infect PC3 cells with our adenovirus at as high as 10 thousand MOI. Since we need high transduction efficiency of DN FGFR without seeing much toxicity of the adenovirus itself, this approach will expedite our research. Currently the construction of Ad5F35 DN FGFR is in progress with collaboration of our colleagues Drs. Elizabeth Olmsted-Davis and Alan Davis of Vector Development Core Facility at our Institution. We already have some lysates to test the expression of DN FGFR and we are hoping to expend and have the virus in less than 2 months. Since this new vector will enhance the efficiency of delivery of DN FGFR especially in vivo, we have not initiated our in vivo experiments yet. We are planning to initiate these experiments in the upcoming year and have the data by the end of second year.

Task 3: Although we have not done extensive microarray experiments to compare the differentially expressed genes in DN FGFR treated prostate cancer cell lines, in our preliminary experiments one of the differentially expressed genes in AdDN FGFR treated cells was CDC25C. CDC25 phosphatases belong to the tyrosine phosphatase family and play a critical role in regulating cell cycle progression by dephosphorylating cyclin dependent kinases at inhibitory residues. In human cells, cdc25 proteins are encoded by a multigene family, consisting of *CDC25A*, *CDC25B*, and *CDC25C(17,18)*. In late G2, the *CDC25C* dephosphorylates Cdc2 on both threonine 14 and tyrosine 15, leading to the activation of Cdc2/cyclin B complexes(19-21) and progression through the G2/M checkpoint. Phosphorylation of serine 216 of *CDC25C* throughout interphase and upon G2 checkpoint activation has been found to negatively regulate the enzymatic activity of *CDC25C* (22,23) and a positive feedback loop has been proposed between cdc2 and *CDC25C* (17,21,23,24). Activated Chk kinases can inactivate *CDC25C* via phosphorylation at serine 216, blocking the activation of cdc2 and transition into M-phase (25). Another aspect of Cdc25 regulation is alternative splicing that may produce at least five *CDC25B* variants (26). Splice variants are also reported for *CDC25A* and *C* (27,28). The activity and regulation of *CDC25C* in prostate carcinoma has not been previously examined, despite its potentially important role in the G2/M transition in this common malignancy. To determine whether *CDC25C* plays a role in prostate cancer, we have examined the expression of *CDC25C* and its alternatively spliced variant in human prostate cancer. *CDC25C* protein is upregulated in comparison to normal prostate tissue and is present predominantly in its active dephosphorylated form. In addition, expression of a biologically active alternatively spliced *CDC25C* isoform is increased in prostate cancer. In addition, we have found, by expression of dominant negative fibroblast growth factor (FGF) receptors, that FGF signaling modulated *CDC25C* activity in prostate cancer, and in this manner can promote progression through the G2/M checkpoint.
This study on CDC25C has been prepared as manuscript titled "Increased CDC25C phosphatase activity in prostate cancer: the role of fibroblast growth factor receptor signaling" and submitted to Cancer Research and is currently under review as attached. By comparing and clustering differentially expressed genes in DN FGFR treated cells, we are hoping to identify more genes in this pathway that can be used in targeted therapy as we proposed to do in our approved statement of work for 12-36 months.

KEY RESEARCH ACCOMPLISHMENTS:

- Verification of DN FGFR effect on proliferation, survival and cell cycle of additional prostate cancer cell lines to determine FGF receptor signaling is essential for viability of human prostate cancer cells and disruption of this signaling via expression of a dominant negative FGF receptor-1 protein in human prostate cancer cells might contribute to the death of cancer cells.

- The G2/M transition may be a critical checkpoint in prostate cancer.

- Identification of increased CDC25C phosphatase activity in prostate cancer and the role of fibroblast growth factor receptor signaling in its activity.

REPORTABLE OUTCOMES:

A manuscript in review: Ozen M and Ittmann M: Increased CDC25C phosphatase activity in prostate cancer: role of fibroblast growth factor receptor signaling. Cancer Research (submitted).

CONCLUSIONS:

A research in this report supports that FGF receptor signaling is essential for viability of human prostate cancer cells and disruption of this signaling via expression of a dominant negative FGF receptor-1 protein in human prostate cancer cells might contribute to the death of cancer cells. These findings reveal that prostate cancer cells treated with DN FGFR is arrested G2/M phase of cell cycle and eventually die. FGF signaling modulated *CDC25C* activity in prostate cancer, and in this manner can promote progression through the G2/M checkpoint. *CDC25C* protein is upregulated in comparison to normal prostate tissue and is present almost exclusively in its active dephosphorylated form. Expression of a biologically active alternatively spliced *CDC25C* isoform is also increased in prostate cancer. A better understanding of the mechanism by which FGF signaling is regulated and determining other molecules involved in this pathway contributing tumor growth and survival will facilitate the development of cancer therapies to target FGF signaling pathway.

REFERENCES:

1. Polnaszek, N., Kwabi-Addo, B., Peterson, L. E., Ozen, M., Greenberg, N. M., Ortega, S., Basilico, C., and Ittmann, M. Fibroblast growth factor 2 promotes tumor progression in an autochthonous mouse model of prostate cancer. Cancer Research, *63*: 5754-5760, 2003.

2. Ozen, M., Giri, D., Ropiquet, F., Mansukhani, A., and Ittmann, M. Role of fibroblast growth factor receptor signaling in prostate cancer cell survival. Journal of the National Cancer Institute, *93*: 1783-1790, 2001.

3. Takahashi, H. Studies on the expression of fibroblast growth factors and fibroblast growth factor receptors in human prostate cancer. Nippon Hinyokika Gakkai Zasshi 89, 836-845. 1998.
4. Giri, D., Ropiquet, F., and Ittmann, M. Alterations in expression of basic fibroblast growth factor (FGF) 2 and its receptor FGFR-1 in human prostate cancer. Clinical Cancer Research, *5*: 1063-1071, 1999.

5. Sramkoski, R. M., Pretlow, T. G., Giaconia, J. M., Pretlow, T. P., Schwartz, S., Sy, M. S., Marengo, S. R., Rhim, J. S., Zhang, D. S., and Jacobberger, J. W. A new human prostate carcinoma cell line, 22R upsilon 1. In Vitro Cellular & Developmental Biology-Animal, *35*: 403-409, 1999.

6. Craft, N., Shostak, Y., Carey, M., and Sawyers, C. L. A mechanism for hormone-independent prostate cancer through modulation of androgen receptor signaling by the HER-2/neu tyrosine kinase. Nature Medicine, *5*: 280-285, 1999.

7. Thalmann, G. N., Sikes, R. A., Wu, T. T., Degeorges, A., Chang, S. M., Ozen, M., Pathak, S., and Chung, L. W. K. LNCaP progression model of human prostate cancer: Androgen-independence and osseous metastasis. Prostate, *44*: 91-103, 2000.

8. Navone, N. M., Olive, M., Ozen, M., Davis, R., Troncoso, P., Tu, S. M., Johnston, D., Pollack, A., Pathak, S., von Eschenbach, A. C., and Logothetis, C. J. Establishment of two human prostate cancer cell lines derived from a single bone metastasis. Clinical Cancer Research, *3*: 2493-2500, 1997.

9. Green, A. P., Huang, J. J., Scott, M. O., Kierstead, T. D., Beaupre, I., Gao, G. P., and Wilson, J. M. A new scalable method for the purification of recombinant adenovirus vectors. Human Gene Therapy, *13*: 1921-1934, 2002.

10. Yotnda, P., Onishi, H., Heslop, H. E., Chen, D., Chiu, W., Piedra, P. A., Takahashi, S., Barry, M., Davis, A., Templeton, N. S., and Brenner, M. K. Targeting adenovectors to hemopoietic cells. Blood Cells Molecules and Diseases, *28*: 347, 2002.

11. Olmsted-Davis, E. A., Gugala, Z., Gannon, F. H., Yotnda, P., McAlhany, R. E., Lindsey, R. W., and Davis, A. R. Use of a chimeric adenovirus vector enhances BMP2 production and bone formation. Human Gene Therapy, *13*: 1337-1347, 2002.

12. Thalmann, G. N., Sikes, R. A., Wu, T. T., Degeorges, A., Chang, S. M., Ozen, M., Pathak, S., and Chung, L. W. K. LNCaP progression model of human prostate cancer: Androgen-independence and osseous metastasis. Prostate, *44*: 91-103, 2000.

13. Thalmann, G. N., Sikes, R. A., Wu, T. T., Degeorges, A., Chang, S. M., Ozen, M., Pathak, S., and Chung, L. W. K. LNCaP progression model of human prostate cancer: Androgen-independence and osseous metastasis. Prostate, *44*: 91-103, 2000.

14. Yotnda, P., Onishi, H., Heslop, H. E., Shayakhmetov, D., Lieber, A., Brenner, M., and Davis, A. Efficient infection of primitive hematopoietic stem cells by modified adenovirus. Gene Therapy, *8*: 930-937, 2001.

15. Olmsted-Davis, E. A., Gugala, Z., Gannon, F. H., Yotnda, P., McAlhany, R. E., Lindsey, R. W., and Davis, A. R. Use of a chimeric adenovirus vector enhances BMP2 production and bone formation. Human Gene Therapy, *13*: 1337-1347, 2002.

16. Shayakhmetov, D. M., Papayannopoulou, T., Stamatoyannopoulos, G., and Lieber, A. Efficient gene transfer into human CD34(+) cells by a retargeted adenovirus vector. Journal of Virology, *74*: 2567-2583, 2000.

17. Hoffmann, I. The role of Cdc25 phosphatases in cell cycle checkpoints. Protoplasma, *211*: 8-11, 2000.

18. Turowski, P., Franckhauser, C., Morris, M. C., Vaglio, P., Fernandez, A., and Lamb, N. J. C. Functional cdc25C dual-specificity phosphatase is required for S-phase entry in human cells. Molecular Biology of the Cell, *14*: 2984-2998, 2003.

19. Graves, P. R., Lovly, C. M., Uy, G. L., and Piwnica-Worms, H. Localization of human Cdc25C is regulated both by nuclear export and 14-3-3 protein binding. Oncogene, *20*: 1839-1851, 2001.

20. Dunphy, W. G. and Kumagai, A. The Cdc25 Protein Contains An Intrinsic Phosphatase-Activity. Cell, *67*: 189-196, 1991.

21. Strausfeld, U., Labbe, J. C., Fesquet, D., Cavadore, J. C., Picard, A., Sadhu, K., Russell, P., and Doree, M. Dephosphorylation and Activation of A P34Cdc2 Cyclin-B Complex Invitro by Human Cdc25 Protein. Nature, *351*: 242-245, 1991.

22. Graves, P. R., Yu, L. J., Schwarz, J. K., Gales, J., Sausville, E. A., O'Connor, P. M., and Piwnica-Worms, H. The Chk1 protein kinase and the Cdc25C regulatory

pathways are targets of the anticancer agent UCN-01. Journal of Biological Chemistry, *275*: 5600-5605, 2000.

23. Peng, C. Y., Graves, P. R., Thoma, R. S., Wu, Z. Q., Shaw, A. S., and PiwnicaWorms, H. Mitotic and G(2) checkpoint control: Regulation of 14-3-3 protein binding by phosphorylation of Cdc25C on serine-216. Science, *277*: 1501-1505, 1997.

24. Izumi, T. and Maller, J. L. Phosphorylation and Activation of the Xenopus Cdc25 Phosphatase in the Absence of Cdc2 and Cdk2 Kinase-Activity. Molecular Biology of the Cell, *6*: 215-226, 1995.

25. Zeng, Y., Forbes, K. C., Wu, Z. Q., Moreno, S., Piwnica-Worms, H., and Enoch, T. Replication checkpoint requires phosphorylation of the phosphatase Cdc25 by Cds1 or Chk1. Nature, *395*: 507-510, 1998.

26. Baldin, V., Cans, C., SupertiFurga, G., and Ducommun, B. Alternative splicing of the human CDC25B tyrosine phosphatase. Possible implications for growth control? Oncogene, *14*: 2485-2495, 1997.

27. Wegener, S., Hampe, W., Herrmann, D., and Schaller, H. C. Alternative splicing in the regulatory region of the human phosphatases CDC25A and CDC25C. European Journal of Cell Biology, *79*: 810-815, 2000.

28. Bureik, M., Rief, N., Drescher, R., Jungbluth, A., Montenarh, M., and Wagner, P. An additional transcript of the cdc25C gene from A431 cells encodes a functional protein. International Journal of Oncology, *17*: 1251-1258, 2000.

APPENDICES:

A manuscript by Ozen M and Ittmann M: Increased CDC25C phosphatase activity in prostate cancer: role of fibroblast growth factor receptor signaling. Cancer Research (submitted).

Increased CDC25C phosphatase activity in prostate cancer: role of fibroblast growth factor receptor signaling.

Mustafa Ozen and Michael Ittmann

Department of Pathology, Baylor College of Medicine and Michael E. DeBakey Dept. of Veterans Affairs Medical Center, Houston, Texas 77030

Running Title: CDC25C activity is increased in prostate cancer

Key Words: CDC25C, fibroblast growth factor receptor, prostate cancer

Abbreviations: fibroblast growth factor (FGF); fibroblast growth factor receptor (FGFR); polymerase chain reaction (PCR); dominant negative (DN); prostate specific antigen (PSA); β-galactosidase (LacZ), benign prostatic hyperplasia (BPH), fetal bovine serum (FBS)

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ABSTRACT

Alterations in proteins regulating cell cycle progression have been implicated in the pathogenesis in a wide variety of malignant neoplasms, including prostate cancer. CDC25 phosphatases belong to the tyrosine phosphatase family and play a critical role in regulating cell cycle progression by dephosphorylating cyclin dependent kinases at inhibitory residues. CDC25C plays an important role in the G2/M transition by activating Cdc2/Cyclin B1 complexes. To determine whether CDC25C activity is altered in prostate cancer, we have examined the expression of CDC25C and its alternatively spliced variant in human prostate cancer. CDC25C protein is upregulated in prostate cancer in comparison to normal prostate tissue and is present almost exclusively in its active dephosphorylated form. Expression of a biologically active alternatively spliced CDC25C isoform is also increased in prostate cancer and is correlated with the occurrence of biochemical (PSA) recurrence. By expression of dominant negative fibroblast growth factor receptors (FGFR), we have found that loss of fibroblast growth factor signaling decreases CDC25C activity in prostate cancer by multiple mechanisms, implying that the increased CDC25C activity in prostate cancer is linked to increased FGF signaling in this malignancy. A better understanding of the mechanism by which FGF signaling regulates cell cycle progression will facilitate the development of cancer therapies that target the FGF signaling pathway.

INTRODUCTION

Abnormal expression and/or activity of cell cycle regulatory proteins have been identified in a wide variety of malignant neoplasms, including prostate cancer (1,2). Cell cycle progression is controlled by the sequentially activities of cyclin dependent kinases, whose activities are tightly regulated by cyclins, cyclin dependent kinase inhibitors and a variety of other proteins. Several groups have shown increased expression of cyclin B1, which plays a critical role in the G2/M transition, in human prostate cancers (1,2). Recent work by Maddison et al. (3) has demonstrated increased levels of cyclin B1 in poorly differentiated and androgen independent prostate cancers in the TRAMP mouse model of prostate cancer. During G2, the Cdc2/Cyclin B complex is kept inactive by phosphorylation of Cdc2 by Wee1. At the onset of mitosis Cdc2/Cyclin B complex are dephosphorylated by the phosphatase CDC25C leading to increased kinase activity (4-6). Our laboratory has demonstrated previously that disruption of fibroblast growth factor (FGF) signaling in prostate cancer cells leads to decrease in Cdc2 kinase activity and arrest in G2, followed by cell death (7). Together, these findings imply that the G2/M transition may be a critical checkpoint in prostate cancer.

CDC25 phosphatases belong to the tyrosine phosphatase family and play a critical role in regulating cell cycle progression by dephosphorylating cyclin dependent kinases at inhibitory residues. In human cells, CDC25 proteins are encoded by a multigene family, consisting of *CDC25A*, *CDC25B*, and *CDC25C* (8). In late G2, the CDC25C protein dephosphorylates Cdc2 on both threonine 14 and tyrosine 15, leading to the activation of Cdc2/Cyclin B complexes (9-11) and progression through the G2/M checkpoint. Phosphorylation of serine 216 of CDC25C throughout interphase and upon G2 checkpoint activation has been found to negatively regulate the enzymatic activity of CDC25C (12,13) and a positive feedback loop has been proposed

between Cdc2 and CDC25C (11,13-15). Activated Chk kinases can inactivate CDC25C via phosphorylation at serine 216, blocking the activation of Cdc2 and transition into M-phase (16). Another aspect of CDC25 regulation is alternative splicing that may produce at least five CDC25B variants (17), and splice variants are also reported for CDC25A and CDC25C (18,19). The activity and regulation of CDC25C in prostate carcinoma has not been previously examined, despite its potentially important role in the G2/M transition in this common malignancy.

To determine whether CDC25C plays a role in prostate cancer, we have examined the expression of CDC25C and its alternatively spliced variant in human prostate cancer. CDC25C protein is upregulated in comparison to normal prostate tissue and is present predominantly in its active dephosphorylated form. In addition, expression of a biologically active alternatively spliced CDC25C isoform is increased in prostate cancer. Finally, we have found, by expression of dominant negative (DN) fibroblast growth factor receptors (FGFR), that FGF signaling modulates CDC25C activity in prostate cancer, and in this manner can promote progression through the G2/M checkpoint.

MATERIALS AND METHODS

Tissue acquisition and extraction: Normal peripheral zone, hyperplastic transition zone (BPH) and cancer tissues were collected from men undergoing radical prostatectomy for clinically localized prostate cancer by Baylor prostate cancer SPORE Tissue Core and snap frozen. Benign tissues were confirmed to be free of cancer and cancer tissues contained at least 70% carcinoma. RNAs were extracted from 17 normal peripheral zone tissues, 7 BPH tissues and 58 prostate cancers using TRIzoL Reagent (Invitrogen, Carlsbad, CA) as described in the manufacturer's protocol. We analyzed 20 cancers with no evidence of prostate specific antigen (PSA) recurrence after 5 years of follow-up, 19 cancers with delayed PSA recurrence (mean time to recurrence

34.1 months) and 19 cancers with early recurrence i.e. less than one year (mean time to recurrence 4.5 months). PSA recurrence was defined as serum PSA greater than 0.2 ng/ml. Protein extracts were prepared as described previously (20) from 10 cancers and 8 normal peripheral zone tissues.

Reverse transcriptase-PCR and agarose gel elecrophoreisis: RNAs extracted from the prostate tissues were first reverse transcribed as previously described (20) and analyzed for the presence of two different cDNAs for CDC25C using primers flanking the deletions of the CDC25C sequence: forward, 5'-AGA GAG AAG CTT ATG TCT ACG GAA CTC TTC TCA TCC-3'; reverse, 5'-CCC AAA TAT TTC ATT TCA CTG TCC-3' as described previously by Bureik et al. (19). Thirty-five PCR cycles of were performed as follows: denaturation at 94° C for 1 min, annealing at 60° C for 1.5 min, elongation at 72° C for 1 min followed by a final 5 min extension at 72° C. The reaction was performed with the Takara kit (Takara Bio Inc, Japan) following manufacturers protocol. The PCR products were analyzed on a 1.5 % agarose gels and stained with ethidium bromide.

Cell lines and viruses: PC-3, DU145, and LNCaP human prostate cancer cell lines were cultured in RPMI-1640 medium supplemented with 1% antibiotic and antimycotic (Gibco-BRL, Grand Island, NY) and 10% fetal bovine serum (FBS). Dominant negative (DN) FGFR, green fluorescent protein (GFP) and β-galactosidase (LacZ) expressing, replication incompetent adenoviruses have been described previously (7).

Adenovirus infection: Cells were infected with adenovirus in presence of infection media (RPMI-1640 supplemented with 2% FBS), incubated 1 hour at 37° C with shaking, and transferred to an incubator (37° C, 5% $C0_2$) after adding complete medium as described previously (7).

Western blot analysis: Cells were plated at 1×10^6 cells per 10 cm dish and infected with adenoviruses as described above. The cells were collected at each timepoint and lysed in lysis buffer (7) and cleared by centrifugation for 10 min in a microcentrifuge at 4° C. Protein concentration was determined using a BioRad protein assay (BioRad, Hercules, CA). The lysates were then boiled in sample buffer, centrifuged and the 30 ug of supernatant protein subjected to sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) electrophoresis using a 10% gel. The resolved proteins were electrotransferred to nitrocellulose membranes and then blocked with phosphate buffered saline with 0.5% Tween 20 (PBST) containing 5% fat-free milk. Western blot for CDC25C was performed using 500 ng/ml polyclonal anti-CDC25C antibody (C20, Santa Cruz Biotechnology, Santa Cruz, CA) or anti-phosphoCDC25C (ser 216) antibody (901, Cell Signaling Technology, Beverly, MA) at 4^0 C for 16 hrs The membranes then were washed with PBST and treated with appropriate secondary antibody. The antigen-antibody reaction was visualized using an enhanced chemiluminescence (ECL) assay (Amersham, Arlington Hts, IL) and exposure to ECL film (Amersham). Control primary antibody was an anti-β actin monoclonal antibody (A5316, Sigma, St Louis, MO) at a 1:5000 dilution.

RESULTS

CDC25C in clinically localized human prostate cancers.

To evaluate the *in vivo* activity of CDC25C in prostate cancer tissues, we determined the levels of total and serine 216 phosphorylated CDC25C protein in the lysates from normal prostate peripheral zone and prostate cancer tissue samples. Out of 9 evaluable cancer samples, only two had detectable amounts of phosphorylated CDC25C, while total CDC25C levels was detectable in 6 of 9 cases. In contrast, normal prostate peripheral zone samples had detectable phosphorylated CDC25C proteins in 6 of 8 samples whereas only 2 of them showed detectable

total CDC25C protein (Figure 1). Thus, in cancer tissues there is both markedly increased CDC25C protein and much less of its inactivate, phosphorylated form. In addition to the full length CDC25C protein examined here, there is an alternatively spliced of CDC25C transcript that has been detected in a number of tissues and in colon cancers (18,19). This variant protein could not be evaluated because of overlap with the immunoglobulin heavy chain that is always present in large amounts in clinical tissue samples. We therefore analyzed RNAs from clinically localized prostate cancers for the presence of the CDC25C variant mRNA using RT-PCR and electrophoreisis on agarose gels (Figure 2). The alternatively spliced CDC25C transcript is present in both LNCaP and DU145 prostate cancer cell lines. The results of analysis of the tissue samples are summarized in Table 1. The variant CDC25C transcript was detectable in 29 of 58 prostate cancer RNAs. In contrast, only 3 of 17 normal peripheral zone samples and none of the seven BPH samples had this variant. The difference between the cancer and benign samples was statistically significant ($p=0.002$, Fisher exact test). In addition, the presence of the variant was strongly associated with the occurrence of biochemical (PSA) recurrence. Overall, the variant was present in cancer samples from 23 of 38 patients with biochemical recurrence but only 6 of 20 without PSA recurrence. This difference was statistically significant ($p=0.027$, Pearson's chi square). Since PSA recurrence is associated with aggressive disease and decreased patient survival, this observation implies that expression of the variant mRNA is higher in aggressive prostate cancers. No correlation of expression of the CDC25C variant with preoperative PSA or pathological stage was detected.

Dominant negative FGF receptor expression alters CDC25C activity in prostate cancer cell lines.

Given that expression of dominant negative (DN) FGF receptors is associated with G2 arrest and decreased Cdc2 kinase activity in prostate cancer cells, we examined whether CDC25C activity is dependent on FGF signaling. LNCaP and DU145 prostate cancer cells were infected with adenovirus carrying DN FGFR constructs as described previously (7) and cell lysates were prepared 6, 16, 24, and 48 hr after infection. Previous experiments have shown that expression of DN FGFR in these cell lines leads to a marked increase in cells in G2 by 24 hours after infection. Western blot analysis revealed that expression of DN FGFR caused increased phosphorylation of CDC25C at serine 216 by 6 hrs after infection in LNCaP cells (Figure 3A). A similar phenomenon has observed in DU145 cells (data not shown). As seen on Figure 3B, total CDC25C levels did not change after 16 hours of DN FGFR expression, but there was a marked downregulation of a the protein arising from the alternatively spliced variant of CDC25C (marked as variant 1 in Figure 3B). The specificity of this band was confirmed by preincubating the antibody with the peptide used to generate this antibody (Figure 3B). At later times after expression of DN FGFR, there was a marked decrease in full length CDC25C protein. As seen on Figure 4, total CDC25C protein levels were decreased 48 hours after infection with very minimal amounts detected after 72 hours of DN FGFR expression. As observed for LNCaP cells, there was marked downregulation of the alternatively spliced smaller variant of CDC25C by 24 hrs after infection with DN FGFR adenovirus in DU145 cells. We performed semi-quantitative RT-PCR analysis to determine whether there was a similar alteration in CDC25C mRNA levels in DU145 cells infected with DN FGFR adenovirus or control GFP adenovirus. As shown in Figure 5, there is a marked decrease in full length CDC25C transcript visible after 25 cycles of

PCR, which occurs within 24 hours after expression of DN FGFR. The band corresponding to the alternatively spliced transcript is not visibly decreased within 24 hours after infection at 25 cycles.

Dominant negative FGF receptor does not change Wee 1 levels.

During G2, Wee 1 counteracts the effects of CDC25C by keeping the Cdc2/Cyclin B complex inactive by phosphorylation of Cdc2. It is possibly that the observed decrease in CDC25C activity could be negated by loss of Wee 1 in response to DN FGFR expression. We therefore determined the levels of Wee 1 protein in DN FGFR treated cells. As shown in Figure 6, Wee 1 levels did not change even after 72 hours of DN FGFR expression. Taking together, these data indicate that CDC25C, but not Wee 1, is affected by DN FGFR expression in prostate cancer cells.

DISCUSSION

CDC25A and CDC25B have been shown to collaborate with either mutation in the RAS oncogene or loss of RB in transformation and, in this initial report, CDC25B protein was increased in 32% of human breast cancers (21). Subsequently, increased expression of CDC25A has been demonstrated in head and neck (22), non-small cell lung (23), gastric (24) and colon cancers (25) while CDC25B is increased in non-Hodgkin's lymphomas (26), as well as head and neck (22), non-small cell lung (23), gastric (24), colon (25), pancreatic (26) and prostate cancers (27). In contrast, increased expression of CDC25C has only been reported in a fraction of colon (25) and endometrial cancers (28). We have shown that the majority of prostate cancers have both increased total CDC25C protein and less phosphorylated CDC25C when compared to normal prostatic tissue. In addition to the full length CDC25C mRNA, we detected an alternatively spliced CDC25C in both human prostate cancers *in vivo* and in prostate cancer cell

lines. This alternatively spliced transcript has a deletion of exons 3 and 5 of the CDC25C gene and encodes a smaller protein containing the carboxy terminal catalytic domain and 17 unique amino acids. This alternatively spliced variant can complement a *CDC25C* mutant strain of *S. Pombe* (19). It is interesting to note that this variant leads to an increased uncoupling of the onset of mitosis and the completion of DNA synthesis in this mutant strain of *S. Pombe*, implying poor regulation of the activity of this variant protein. Of note, we did not observe any phosphorylation of this variant on serine 216 in prostate cancer cells, despite the fact that the variant protein retains this negative regulatory site. This alternatively spliced RNA was detected in one half of the prostate cancers analyzed but in only 13% of the benign tissues. The presence of this variant was significantly correlated with biochemical (PSA) recurrence following radical prostatectomy, which is associated with aggressive disease and worse patient outcome. Although this variant is only a fraction of total CDC25C mRNA *in vivo*, it may have significant biological effects that lead to more aggressive diseases, perhaps through poor regulation of its activity. Thus, prostate cancer is characterized by multiple alterations in CDC25C that can increase its activity *in vivo*.

Disruption of FGF signaling by expression of dominant negative receptors leads to marked decrease in CDC25C activity. This loss of CDC25C activity has three components. First, there is a marked increase in CDC25C phosphorylation on serine 216 as early as 6 hours after expressing of the DN FGF receptor. The mechanism by which loss of FGF signaling leads to increased CDC25C phosphorylation is unclear. To date, we have been unable to demonstrate changes in phosphorylation of Chk1 or Chk2 kinases in response to disruption of FGF signaling (unpublished observations). Other kinases, such as C-TAK1 may also phosphorylate CDC25C (29), and these have not yet been evaluated. In addition, there is a marked decrease in CDC25C

mRNA within 24 hours, due to either decreased transcription or increased degradation. This results in decreased full length CDC25C protein by 48 hours after disruption of FGF signaling. Finally, there is a marked decrease in the level of an alternatively spliced CDC25C protein. Interestingly, despite the rapid decrease in full length CDC25C mRNA, there is little change in the level of the alternatively spliced transcript, implying that the rapid loss of the variant CDC25C protein is either due translational or post-translational mechanisms. Thus, through multiple mechanisms, there is a marked decrease in active CDC25C in prostate cancer cells in response to disruption of FGF signaling, but determination of the mechanism by which these changes occur will require further investigations.

Alterations in FGF signaling pathway have been implicated in the pathogenesis of variety of carcinomas including prostate and pancreatic carcinoma as well as melanoma by *in vitro* and *in vivo* studies (7,20,30-39). FGFs produce their mitogenic and angiogenic effects in target cells by signaling through four distinct cell-surface tyrosine kinase receptors, FGFR-1 through FGFR-4. Prostate epithelial cells express FGF receptors and require FGFs for growth in primary culture. Prostate cancer cells express multiple types of FGF receptor and increased expression of FGF receptor-1 (FGFR-1) is present in poorly differentiated human prostate cancers *in vivo* (31,34). Autocrine expression of FGFs and expression of FGF receptors is present in the commonly used prostate cancer cell lines i.e. PC-3, DU145 and LNCaP (35-37) and there is increased expression of FGF2 (34), FGF6 (38) and FGF8 (39) in human prostate cancers *in vivo*. Given the strong evidence that FGF receptor activity is significantly increased in prostate cancer, and that loss of FGF receptor activity leads to decreased total CDC25C protein and increased phosphorylation of CDC25C *in vitro*, it is reasonable to conclude that the increase in CDC25C activity in human prostate cancers *in vivo* is due, at least in part, to increased FGF receptor activity in the prostate

cancer cells. Loss of FGF receptor signaling resulted in a rapid decrease in the variant protein *in vitro*, so this protein may be also be increased by FGF signaling in prostate cancers. While some of the decrease in phosphorylated CDC25C in prostate cancer may reflect increased numbers of cells at the G2/M transition, prostate cancers have a relatively low rate of proliferation, so that it is unlikely that a significant fraction of cells are undergoing the G2/M transition at any given time. If FGFs directly increase CDC25C activity this would facilitate progression through this checkpoint in prostate cancer cells.

In summary, we have described, for the first time, the increased activity of CDC25C phosphatase in prostate cancer. We have found, by expression of dominant negative fibroblast growth factor FGF receptor, that FGF signaling modulates CDC25C activity in prostate cancer, and in this manner promote progression through the G2/M checkpoint. Further studies are underway to determine the mechanisms by which FGF signaling can modulate CDC25C activity. A better understanding of the mechanisms by which FGF signaling regulates CDC25C activity and determination of how other molecules involved in the FGF signaling pathway contribute to tumor growth and survival will facilitate the development of cancer therapies that target FGF signaling pathway.

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REFERENCES:

1. Mashal, R. D., Lester, S., Corless, C., Richie, J. P., Chandra, R., Propert, K. J., and Dutta, A. Expression of cell cycle-regulated proteins in prostate cancer. Cancer Research, *56*: 4159-4163, 1996.

2. Kallakury, B.V., Sheehan, C.E., Rhee, S.J., Fisher, H.A., Kaufman, R.P. Jr, Rifkin, M.D., and Ross, J.S. The prognostic significance of proliferation-associated nucleolar protein p120 expression in prostate adenocarcinoma: a comparison with cyclins A and B1, Ki-67, proliferating cell nuclear antigen, and p34cdc2. Cancer, *85*:1569-1576, 1999.

3. Maddison, L. A., Huss, W. J., Barrios, R. M., and Greenberg, N. M. Differential expression of cell cycle regulatory molecules and evidence for a "cyclin switch" during progression of prostate cancer. Prostate, *58*: 335-344, 2004.

4. Booher, R. N., Holman, P. S., and Fattaey, A. Human Myt1 is a cell cycle-regulated kinase that inhibits Cdc2 but not Cdk2 activity. Journal of Biological Chemistry, *272*: 22300-22306, 1997.

5. Draetta, G. and Eckstein, J. Cdc25 protein phosphatases in cell proliferation. Biochimica et Biophysica Acta-Reviews on Cancer, *1332*: M53-M63, 1997.

6. Taylor, W. R. and Stark, G. R. Regulation of the G2/M transition by p53. Oncogene, *20*: 1803-1815, 2001.

7. Ozen, M., Giri, D., Ropiquet, F., Mansukhani, A., and Ittmann, M. Role of fibroblast growth factor receptor signaling in prostate cancer cell survival. Journal of the National Cancer Institute, *93*: 1783-1790, 2001.

8. Turowski, P., Franckhauser, C., Morris, M. C., Vaglio, P., Fernandez, A., and Lamb, N. J. C. Functional cdc25C dual-specificity phosphatase is required for S-phase entry in human cells. Molecular Biology of the Cell, *14*: 2984-2998, 2003.

9. Graves, P. R., Lovly, C. M., Uy, G. L., and Piwnica-Worms, H. Localization of human Cdc25C is regulated both by nuclear export and 14-3-3 protein binding. Oncogene, *20*: 1839-1851, 2001.

10. Dunphy, W. G. and Kumagai, A. The Cdc25 Protein Contains An Intrinsic Phosphatase-Activity. Cell, *67*: 189-196, 1991.

11. Strausfeld, U., Labbe, J. C., Fesquet, D., Cavadore, J. C., Picard, A., Sadhu, K., Russell, P., and Doree, M. Dephosphorylation and Activation of A P34Cdc2 Cyclin-B Complex Invitro by Human Cdc25 Protein. Nature, *351*: 242-245, 1991.

12. Graves, P. R., Yu, L. J., Schwarz, J. K., Gales, J., Sausville, E. A., O'Connor, P. M., and Piwnica-Worms, H. The Chk1 protein kinase and the Cdc25C regulatory pathways are targets of the anticancer agent UCN-01. Journal of Biological Chemistry, *275*: 5600-5605, 2000.

13. Peng, C. Y., Graves, P. R., Thoma, R. S., Wu, Z. Q., Shaw, A. S., and PiwnicaWorms, H. Mitotic and G(2) checkpoint control: Regulation of 14-3-3 protein binding by phosphorylation of Cdc25C on serine-216. Science, *277*: 1501-1505, 1997.

14. Hoffmann, I. The role of Cdc25 phosphatases in cell cycle checkpoints. Protoplasma, *211*: 8-11, 2000.

15. Izumi, T. and Maller, J. L. Phosphorylation and Activation of the Xenopus Cdc25 Phosphatase in the Absence of Cdc2 and Cdk2 Kinase-Activity. Molecular Biology of the Cell, *6*: 215-226, 1995.

16. Zeng, Y., Forbes, K. C., Wu, Z. Q., Moreno, S., Piwnica-Worms, H., and Enoch, T. Replication checkpoint requires phosphorylation of the phosphatase Cdc25 by Cds1 or Chk1. Nature, *395*: 507-510, 1998.

17. Baldin, V., Cans, C., SupertiFurga, G., and Ducommun, B. Alternative splicing of the human CDC25B tyrosine phosphatase. Possible implications for growth control? Oncogene, *14*: 2485-2495, 1997.

18. Wegener, S., Hampe, W., Herrmann, D., and Schaller, H. C. Alternative splicing in the regulatory region of the human phosphatases CDC25A and CDC25C. European Journal of Cell Biology, *79*: 810-815, 2000.

19. Bureik, M., Rief, N., Drescher, R., Jungbluth, A., Montenarh, M., and Wagner, P. An additional transcript of the cdc25C gene from A431 cells encodes a functional protein. International Journal of Oncology, *17*: 1251-1258, 2000.

20. Polnaszek, N., Kwabi-Addo, B., Peterson, L. E., Ozen, M., Greenberg, N. M., Ortega, S., Basilico, C., and Ittmann, M. Fibroblast growth factor 2 promotes tumor progression in an autochthonous mouse model of prostate cancer. Cancer Research, *63*: 5754-5760, 2003.

21. Galaktionov, K., Lee, A. K., Eckstein, J., Draetta, G., Meckler, J., Loda, M., and Beach, D. Cdc25 Phosphatases As Potential Human Oncogenes. Science, *269*: 1575-1577, 1995.

22. Gasparotto, D., Maestro, R., Piccinin, S., Vukosavljevic, T., Barzan, L., Sulfaro, S., and Boiocchi, M. Overexpression of CDC25A and CDC25B in head and neck cancers. Cancer Research, *57*: 2366-2368, 1997.

23. Wu, W. G., Fan, Y. H., Kemp, B. L., Walsh, G., and Mao, L. Overexpression of cdc25A and cdc25B is frequent in primary non-small cell lung cancer but is not associated with overexpression of c-myc. Cancer Research, *58*: 4082-4085, 1998.

24. Kudo, Y., Yasui, W., Ue, T., Yamamoto, S., Yokozaki, H., Nikai, H., and Tahara, E. Overexpression of cyclin-dependent kinase-activating CDC25B phosphatase in human gastric carcinomas. Japanese Journal of Cancer Research, *88*: 947-952, 1997.

25. Hernandez, S., Bessa, X., Bea, S., Hernandez, L., Nadal, A., Mallofre, C., Muntane, J., Castells, A., Fernandez, P. L., Cardesa, A., and Campo, E. Differential expression of cdc25 cell-cycle-activating phosphatases in human colorectal carcinoma. Laboratory Investigation, *81*: 465-473, 2001.

26. Guo, J. C., Kleeff, J., Li, J. S., Ding, J. Y., Hammer, J., Zhao, Y. P., Giese, T., Korc, M., Buchler, M. W., and Friess, H. Expression and functional significance of CDC25B in human pancreatic ductal adenocarcinoma. Oncogene, *23*: 71-81, 2004.

27. Ngan, E. S. W., Hashimoto, Y., Ma, Z. Q., Tsai, M. J., and Tsai, S. Y. Overexpression of Cdc25B, an androgen receptor coactivator, in prostate cancer. Oncogene, *22*: 734-739, 2003.

28. Tsuda, H., Hashiguchi, Y., Inoue, T., and Yamamoto, K. Alteration of G2 cell cycle regulators occurs during carcinogenesis of the endometrium. Oncology, *65*: 159-166, 2003.

29. Peng, C. Y., Graves, P. R., Ogg, S., Thoma, R. S., Byrnes, M. J., Wu, Z. Q., Stephenson, M. T., and Piwnica-Worms, H. C-TAK1 protein kinase phosphorylates human Cdc25C on serine 216 and promotes 14-3-3 protein binding. Cell Growth & Differentiation, *9*: 197-208, 1998.

30. Ozen, M, Medrano, E. E, and Ittmann, M. Inhibition of proliferation and survival of melanoma cells by adenoviral-mediated expression of dominant negative fibroblast growth factor receptor. Melanoma Research 14, 13-21. 2004.

31. Takahashi, H. Studies on the expression of fibroblast growth factors and fibroblast growth factor receptors in human prostate cancer. Nippon Hinyokika Gakkai Zasshi 89, 836-845. 1998.

32. Kleeff, J., Kothari, N. H., Friess, H., Fan, H., and Korc, M. Adenovirus-mediated transfer of a truncated fibroblast growth factor (FGF) type I receptor blocks FGF-2 signaling in multiple pancreatic cancer cell lines. Pancreas, *28*: 25-30, 2004.

33. Wagner, M., Lopez, M. E., Cahn, M., and Korc, M. Suppression of fibroblast growth factor receptor signaling inhibits pancreatic cancer growth in vitro and in vivo. Gastroenterology, *114*: 798-807, 1998.

34. Giri, D., Ropiquet, F., and Ittmann, M. Alterations in expression of basic fibroblast growth factor (FGF) 2 and its receptor FGFR-1 in human prostate cancer. Clinical Cancer Research, *5*: 1063-1071, 1999.

35. Payson, R. A., Chotani, M. A., and Chiu, I. M. Regulation of a promoter of the fibroblast growth factor 1 gene in prostate and breast cancer cells. Journal of Steroid Biochemistry and Molecular Biology, *66*: 93-103, 1998.

36. Chandler, L. A., Sosnowski, B. A., Greenlees, L., Aukerman, S. L., Baird, A., and Pierce, G. F. Prevalent expression of fibroblast growth factor (FGF) receptors and FGF2 in human tumor cell lines. International Journal of Cancer, *81*: 451-458, 1999.

37. Rudra-Ganguly, N., Zheng, J. P., Hoang, A. T., and Roy-Burman, P. Downregulation of human FGF8 activity by antisense constructs in murine fibroblastic and human prostatic carcinoma cell systems. Oncogene, *16*: 1487-1492, 1998.

38. Ropiquet, F., Giri, D., Kwabi-Addo, B., Mansukhani, A., and Ittmann, M. Increased expression of fibroblast growth factor 6 in human prostatic intraepithelial neoplasia and prostate cancer. Cancer Research, *60*: 4245-4250, 2000.

39. Dorkin, T. J., Robinson, M. C., Marsh, C., Bjartell, A., Neal, D. E., and Leung, H. Y. FGF8 over-expression in prostate cancer is associated with decreased patient survival and persists in androgen independent disease. Oncogene, *18*: 2755-2761, 1999.

FIGURE LEGENDS:

Figure 1. CDC25C protein levels in clinically localized human prostate cancers.

Normal peripheral zone, hyperplastic transition zone (BPH) and cancer tissues were collected from men undergoing radical prostatectomy for clinically localized prostate cancer by Baylor prostate cancer SPORE Tissue Core and snap frozen. Protein lysates were prepared as described in Materials and Methods. Western blot for CDC25C was performed using polyclonal anti-CDC25C antibody or a serine 216 phosphorylation site specific antibody. An anti-β-actin monoclonal antibody was used as loading control. The numbers representing samples are obtained from our clinical database. Sample 24 was not included in the analysis due to low β-actin signal, indicating inadequate protein in this lane.

Figure 2. Expression of full length and alternatively spliced CDC25C mRNA in prostate cancer and normal or hyperplastic prostate.

RNAs were extracted from normal peripheral zone tissues, BPH tissues and prostate cancer tissue samples using TRIzoL Reagent as described by the manufacturer's protocol and analyzed by RT-PCR as described in Materials and Methods. Bands corresponding to the full length (512 bp) and variant mRNA (293 bp) are indicated. Three samples each of cancer, normal and BPH groups are shown.

Figure 3. Dominant negative FGF receptor expression alters CDC25C activity in LNCaP prostate cancer cells. LNCaP cells were infected with adenovirus in presence of infection media as described in Materials and Methods. The cells were collected at each timepoint, lysed, and lysates boiled in sample buffer, centrifuged and the 30 ug of supernatant protein subjected to sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) electrophoresis using a 10% gel. A serine 216 phosphorylation site specific antibody (A) and a polyclonal anti-CDC25C antibody detecting total CDC25C levels (B) were used for Western blotting. The alternatively spliced variant of CDC25C is marked as variant 1 (B). The specificity of this band was confirmed by preincubating the antibody with the peptide used to generate this antibody (B).

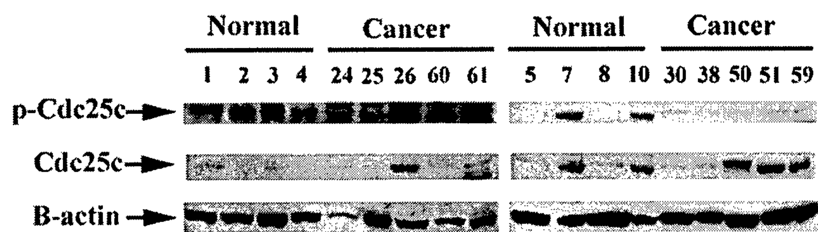
Figure 4. Effect of dominant negative FGF receptor expression on CDC25C levels on DU145 cells. DU145 prostate cancer cells were infected with adenovirus carrying dominant negative FGF receptor as described. Cell lysates were prepared 24, and 48 and 72 hr after infection. Total CDC25C protein was detected using a polyclonal antibody. The smaller spliced variant is marked as variant 1. Western blot for β-actin was used as loading control.

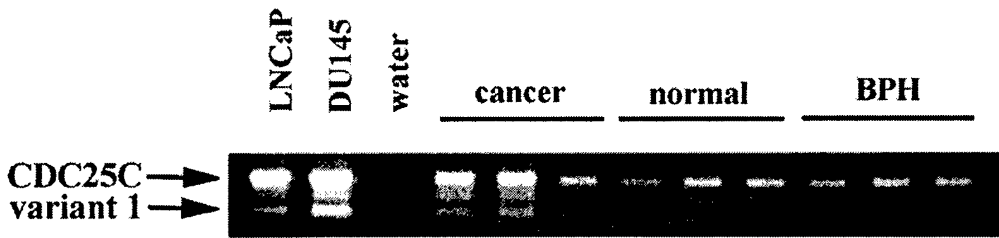
Figure 5. Semi-quantitative RT-PCR analysis of alternatively spliced CDC25C mRNA transcripts after FGFR DN expression. DU145 cells infected with adenovirus expressing DN FGFR or GFP (control). Total RNA was isolated from the cells 24, 48 and 72 hours after infection, reverse transcribed and subjected to 25 and 35 cycles of PCR amplification as described in Materials and Methods. The band corresponding to full length CDC25C was 512 bp and a spliced variant 293 bp in size. Uninfected DU145 cells and water were used as positive and negative controls, respectively.

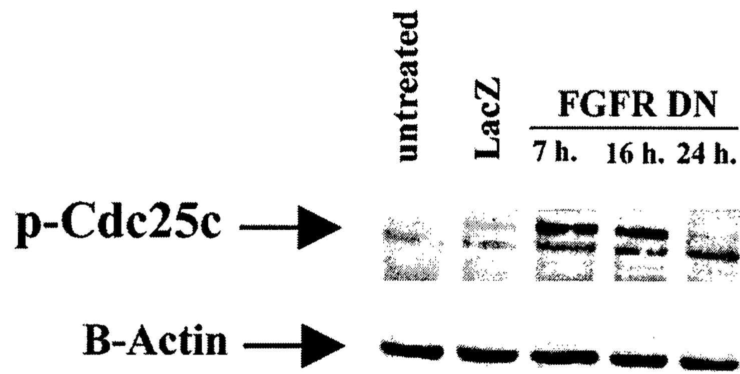
Figure 6. Wee 1 protein expression in DU145 cells treated with adenovirus expressing dominant negative FGF receptor. DU145 prostate cancer cells were infected with adenovirus carrying DN FGFR. Cell lysates were prepared 24, 48 and 72 hours after the infection and subjected to Western blotting with the antibodies detecting Wee 1 and β-actin proteins as described in Materials and Methods.

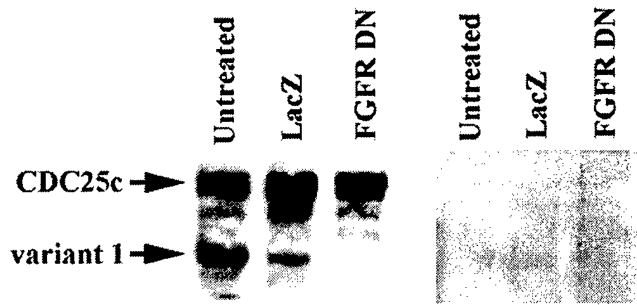
Table 1. Determination of expression of CDC25C alternatively spliced variant in clinical prostate specimens

	Variant present (%)	*Variant absent (%)*	*Total*
Recurrent	23 (60.5)	15 (39.5)	38
Non-recurrent	6 (30)	14 (70)	20
Normal	3 (17.6)	14 (82.4)	17
BPH	0 (0)	7 (100)	7


Normal
Cancer
Normal
Cancer
1 2 3 4 24 25 26 60 61 5 7 8 10 30 38 50 51 59
p-Cdc25c
Cdc25c
B-actin


LNCaP
DU145
water
cancer
normal
BPH
CDC25C
variant 1


untreated
LacZ
FGFR DN
7 h.
16 h.
24 h.
p-Cdc25c
B-Actin



Blocking peptide

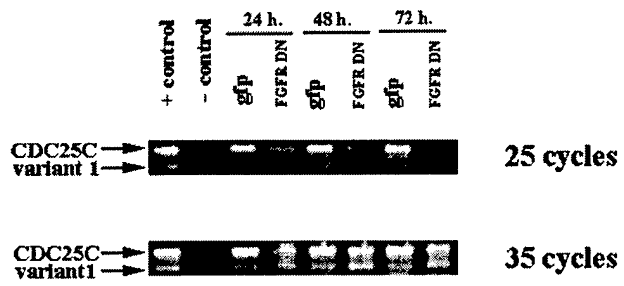
24 h. 48 h. 72 h.

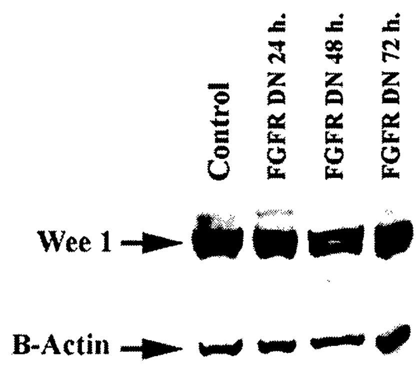
gfp FGFR DN gfp FGFR DN gfp FGFR DN

Cdc25c

variant 1

B-Actin


24 h.
48 h.
72 h.
+ control
- control
gfp
FGFR DN
gfp
FGFR DN
gfp
FGFR DN
CDC25C
variant 1
25 cycles
CDC25C
variant1
35 cycles


Control
FGFR DN 24 h.
FGFR DN 48 h.
FGFR DN 72 h.
Wee 1
B-Actin