

ROLE OF c-erb B GENE FAMILY IN PROSTATE  
CANCER

BY  
Karen Zhi Ching

A thesis  
Submitted to the Faculty of  
Graduate Studies in Partial  
Fulfillment of the Requirements for  
the Degree of Doctor of Philosophy

Department of Physiology  
Faculty of Medicine  
University of Manitoba  
July 1993



National Library  
of Canada

Acquisitions and  
Bibliographic Services Branch

395 Wellington Street  
Ottawa, Ontario  
K1A 0N4

Bibliothèque nationale  
du Canada

Direction des acquisitions et  
des services bibliographiques

395, rue Wellington  
Ottawa (Ontario)  
K1A 0N4

*Your file* *Votre référence*

*Our file* *Notre référence*

The author has granted an irrevocable non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of his/her thesis by any means and in any form or format, making this thesis available to interested persons.

L'auteur a accordé une licence irrévocable et non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de sa thèse de quelque manière et sous quelque forme que ce soit pour mettre des exemplaires de cette thèse à la disposition des personnes intéressées.

The author retains ownership of the copyright in his/her thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without his/her permission.

L'auteur conserve la propriété du droit d'auteur qui protège sa thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

ISBN 0-315-92134-X

Canada

Name Karen Zhi Ching

*Dissertation Abstracts International* is arranged by broad, general subject categories. Please select the one subject which most nearly describes the content of your dissertation. Enter the corresponding four-digit code in the spaces provided.

PHYSIOLOGY

SUBJECT TERM

0	4	3	3	U·M·I
SUBJECT CODE				

**Subject Categories**

**THE HUMANITIES AND SOCIAL SCIENCES**

**COMMUNICATIONS AND THE ARTS**

Architecture ..... 0729  
 Art History ..... 0377  
 Cinema ..... 0900  
 Dance ..... 0378  
 Fine Arts ..... 0357  
 Information Science ..... 0723  
 Journalism ..... 0391  
 Library Science ..... 0399  
 Mass Communications ..... 0708  
 Music ..... 0413  
 Speech Communication ..... 0459  
 Theater ..... 0465

**EDUCATION**

General ..... 0515  
 Administration ..... 0514  
 Adult and Continuing ..... 0516  
 Agricultural ..... 0517  
 Art ..... 0273  
 Bilingual and Multicultural ..... 0282  
 Business ..... 0688  
 Community College ..... 0275  
 Curriculum and Instruction ..... 0727  
 Early Childhood ..... 0518  
 Elementary ..... 0524  
 Finance ..... 0277  
 Guidance and Counseling ..... 0519  
 Health ..... 0680  
 Higher ..... 0745  
 History of ..... 0520  
 Home Economics ..... 0278  
 Industrial ..... 0521  
 Language and Literature ..... 0279  
 Mathematics ..... 0280  
 Music ..... 0522  
 Philosophy of ..... 0998  
 Physical ..... 0523

Psychology ..... 0525  
 Reading ..... 0535  
 Religious ..... 0527  
 Sciences ..... 0714  
 Secondary ..... 0533  
 Social Sciences ..... 0534  
 Sociology of ..... 0340  
 Special ..... 0529  
 Teacher Training ..... 0530  
 Technology ..... 0710  
 Tests and Measurements ..... 0288  
 Vocational ..... 0747

**LANGUAGE, LITERATURE AND LINGUISTICS**

Language  
 General ..... 0679  
 Ancient ..... 0289  
 Linguistics ..... 0290  
 Modern ..... 0291  
 Literature  
 General ..... 0401  
 Classical ..... 0294  
 Comparative ..... 0295  
 Medieval ..... 0297  
 Modern ..... 0298  
 African ..... 0316  
 American ..... 0591  
 Asian ..... 0305  
 Canadian (English) ..... 0352  
 Canadian (French) ..... 0355  
 English ..... 0593  
 Germanic ..... 0311  
 Latin American ..... 0312  
 Middle Eastern ..... 0315  
 Romance ..... 0313  
 Slavic and East European ..... 0314

**PHILOSOPHY, RELIGION AND THEOLOGY**

Philosophy ..... 0422  
 Religion  
 General ..... 0318  
 Biblical Studies ..... 0321  
 Clergy ..... 0319  
 History of ..... 0320  
 Philosophy of ..... 0322  
 Theology ..... 0469

**SOCIAL SCIENCES**

American Studies ..... 0323  
 Anthropology  
 Archaeology ..... 0324  
 Cultural ..... 0326  
 Physical ..... 0327  
 Business Administration  
 General ..... 0310  
 Accounting ..... 0272  
 Banking ..... 0770  
 Management ..... 0454  
 Marketing ..... 0338  
 Canadian Studies ..... 0385  
 Economics  
 General ..... 0501  
 Agricultural ..... 0503  
 Commerce-Business ..... 0505  
 Finance ..... 0508  
 History ..... 0509  
 Labor ..... 0510  
 Theory ..... 0511  
 Folklore ..... 0358  
 Geography ..... 0366  
 Gerontology ..... 0351  
 History  
 General ..... 0578

Ancient ..... 0579  
 Medieval ..... 0581  
 Modern ..... 0582  
 Black ..... 0328  
 African ..... 0331  
 Asia, Australia and Oceania ..... 0332  
 Canadian ..... 0334  
 European ..... 0335  
 Latin American ..... 0336  
 Middle Eastern ..... 0333  
 United States ..... 0337  
 History of Science ..... 0585  
 Law ..... 0398  
 Political Science  
 General ..... 0615  
 International Law and  
 Relations ..... 0616  
 Public Administration ..... 0617  
 Recreation ..... 0814  
 Social Work ..... 0452  
 Sociology  
 General ..... 0626  
 Criminology and Penology ..... 0627  
 Demography ..... 0938  
 Ethnic and Racial Studies ..... 0631  
 Individual and Family  
 Studies ..... 0628  
 Industrial and Labor  
 Relations ..... 0629  
 Public and Social Welfare ..... 0630  
 Social Structure and  
 Development ..... 0700  
 Theory and Methods ..... 0344  
 Transportation ..... 0709  
 Urban and Regional Planning ..... 0999  
 Women's Studies ..... 0453

**THE SCIENCES AND ENGINEERING**

**BIOLOGICAL SCIENCES**

Agriculture  
 General ..... 0473  
 Agronomy ..... 0285  
 Animal Culture and  
 Nutrition ..... 0475  
 Animal Pathology ..... 0476  
 Food Science and  
 Technology ..... 0359  
 Forestry and Wildlife ..... 0478  
 Plant Culture ..... 0479  
 Plant Pathology ..... 0480  
 Plant Physiology ..... 0817  
 Range Management ..... 0777  
 Wood Technology ..... 0746  
 Biology  
 General ..... 0306  
 Anatomy ..... 0287  
 Biostatistics ..... 0308  
 Botany ..... 0309  
 Cell ..... 0379  
 Ecology ..... 0329  
 Entomology ..... 0353  
 Genetics ..... 0369  
 Limnology ..... 0793  
 Microbiology ..... 0410  
 Molecular ..... 0307  
 Neuroscience ..... 0317  
 Oceanography ..... 0416  
 Physiology ..... 0433  
 Radiation ..... 0821  
 Veterinary Science ..... 0778  
 Zoology ..... 0472  
 Biophysics  
 General ..... 0786  
 Medical ..... 0760

**EARTH SCIENCES**

Biogeochemistry ..... 0425  
 Geochemistry ..... 0996

Geodesy ..... 0370  
 Geology ..... 0372  
 Geophysics ..... 0373  
 Hydrology ..... 0388  
 Mineralogy ..... 0411  
 Paleobotany ..... 0345  
 Paleocology ..... 0426  
 Paleontology ..... 0418  
 Paleozoology ..... 0985  
 Palynology ..... 0427  
 Physical Geography ..... 0368  
 Physical Oceanography ..... 0415

**HEALTH AND ENVIRONMENTAL SCIENCES**

Environmental Sciences ..... 0768  
 Health Sciences  
 General ..... 0566  
 Audiology ..... 0300  
 Chemotherapy ..... 0992  
 Dentistry ..... 0567  
 Education ..... 0350  
 Hospital Management ..... 0769  
 Human Development ..... 0758  
 Immunology ..... 0982  
 Medicine and Surgery ..... 0564  
 Mental Health ..... 0347  
 Nursing ..... 0569  
 Nutrition ..... 0570  
 Obstetrics and Gynecology ..... 0380  
 Occupational Health and  
 Therapy ..... 0354  
 Ophthalmology ..... 0381  
 Pathology ..... 0571  
 Pharmacology ..... 0419  
 Pharmacy ..... 0572  
 Physical Therapy ..... 0382  
 Public Health ..... 0573  
 Radiology ..... 0574  
 Recreation ..... 0575

Speech Pathology ..... 0460  
 Toxicology ..... 0383  
 Home Economics ..... 0386

**PHYSICAL SCIENCES**

**Pure Sciences**  
 Chemistry  
 General ..... 0485  
 Agricultural ..... 0749  
 Analytical ..... 0486  
 Biochemistry ..... 0487  
 Inorganic ..... 0488  
 Nuclear ..... 0738  
 Organic ..... 0490  
 Pharmaceutical ..... 0491  
 Physical ..... 0494  
 Polymer ..... 0495  
 Radiation ..... 0754  
 Mathematics ..... 0405  
 Physics  
 General ..... 0605  
 Acoustics ..... 0986  
 Astronomy and  
 Astrophysics ..... 0606  
 Atmospheric Science ..... 0608  
 Atomic ..... 0748  
 Electronics and Electricity ..... 0607  
 Elementary Particles and  
 High Energy ..... 0798  
 Fluid and Plasma ..... 0759  
 Molecular ..... 0609  
 Nuclear ..... 0610  
 Optics ..... 0752  
 Radiation ..... 0756  
 Solid State ..... 0611  
 Statistics ..... 0463  
**Applied Sciences**  
 Applied Mechanics ..... 0346  
 Computer Science ..... 0984

Engineering  
 General ..... 0537  
 Aerospace ..... 0538  
 Agricultural ..... 0539  
 Automotive ..... 0540  
 Biomedical ..... 0541  
 Chemical ..... 0542  
 Civil ..... 0543  
 Electronics and Electrical ..... 0544  
 Heat and Thermodynamics ..... 0348  
 Hydraulic ..... 0545  
 Industrial ..... 0546  
 Marine ..... 0547  
 Materials Science ..... 0794  
 Mechanical ..... 0548  
 Metallurgy ..... 0743  
 Mining ..... 0551  
 Nuclear ..... 0552  
 Packaging ..... 0549  
 Petroleum ..... 0765  
 Sanitary and Municipal  
 System Science ..... 0790  
 Geotechnology ..... 0428  
 Operations Research ..... 0796  
 Plastics Technology ..... 0795  
 Textile Technology ..... 0994

**PSYCHOLOGY**

General ..... 0621  
 Behavioral ..... 0384  
 Clinical ..... 0622  
 Developmental ..... 0620  
 Experimental ..... 0623  
 Industrial ..... 0624  
 Personality ..... 0625  
 Physiological ..... 0989  
 Psychobiology ..... 0349  
 Psychometrics ..... 0632  
 Social ..... 0451



ROLE OF c-erb B GENE FAMILY IN PROSTATE CANCER

BY

KAREN ZHI CHING

A Thesis submitted to the Faculty of Graduate Studies of the University of Manitoba  
in partial fulfillment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

© 1994

Permission has been granted to the LIBRARY OF THE UNIVERSITY OF MANITOBA  
to lend or sell copies of this thesis, to the NATIONAL LIBRARY OF CANADA to  
microfilm this thesis and to lend or sell copies of the film, and LIBRARY  
MICROFILMS to publish an abstract of this thesis.

The author reserves other publication rights, and neither the thesis nor extensive  
extracts from it may be printed or other-wise reproduced without the author's written  
permission.

亲爱的丈夫邢健和可爱的女儿邢奕灵

我远在大洋彼岸的父母，覃民权先生和张成婉女士

献给：

## ACKNOWLEDGMENTS

I would like to thank my supervisor, Dr. Janice Dodd, for her excellent guidance throughout my research and writing of this project, and for her encouragement throughout my entire studies in her Lab. As well, I extend my gratitude to the members of my advisory committee, for their assistance and encouragement. I would like to thank Dr. Robert Shiu, Dr. Patricia McNicol and Dr. Martin Gleave for their effort in reviewing this thesis.

A very special thanks to Ms. Sally Downes for her excellent technical support, critical discussion and friendship since joining the Lab.

Finally, I would like to thank my husband, Jian, and my parents, Cheng-wan Zhang and Min-Quan Qin, for their encouragement and support. Without them this endeavour would not have been possible.

## ABSTRACT

Prostate carcinoma is the second most common cancer among males in developed countries. Development of the prostate and maintenance of adult structure and function, as well as its pathological disturbances, benign prostatic hyperplasia (BPH) and prostate cancer, are strongly influenced by testicular androgens. Unfortunately, present therapeutic approaches for prostate cancer which achieve androgen ablation are unsatisfactory. Such treatment frequently yields an initial beneficial response; however, nearly all patients eventually relapse to a hormone-insensitive state and succumb to progression of the disease. Our understanding of the continuing growth of prostate cancer cells in the absence of androgens is incomplete. Recent efforts have investigated the hypothesis that polypeptide growth factors and their receptors act as mediators of prostate cell growth in vivo and may sustain cancer cell growth in the absence of androgens. The autocrine hypothesis has served as a useful working hypothesis to study this important clinical observation.

This thesis is focused on the contribution of the erbB gene family to the control of prostate cancer cell growth. Two complementary approaches have been used: first, the examination of human prostate tumors for erbB gene expression or perturbation, and second, the experimental manipulation of human prostate cancer cell lines to study erbB-mediated growth control.

Elevated expression of the erbB1 gene, the epidermal growth factor (EGF) receptor, and its ligands, EGF and transforming growth factor alpha (TGF- $\alpha$ ) was demonstrated in prostate tumor samples and human prostate cancer cell lines. In two cell lines, Du145

and LNCaP, exogenous EGF and TGF- $\alpha$  stimulated cell growth, while antibodies to the extracellular domain of the EGF receptor inhibited growth in a dose-dependent manner. A third cell line, PC-3, was refractory to these manipulations; although PC-3 cells express EGF receptor and bind EGF, ligand-dependent autophosphorylation of the receptor did not occur. This result indicates that mechanisms other than androgen- or EGF receptor-mediated growth are present in PC-3 cells.

Overexpression of the erbB2 (NEU) gene also was demonstrated in prostate tumor samples and cell lines, and was associated in many instances with gene amplification. In the cell lines, monoclonal antibodies to the c-erbB2/NEU receptor inhibited proliferation and caused a decrease in the mRNA for the nuclear transcription factor c-fos. In LNCaP cells, a line which has retained responsiveness to androgens, the expression of c-erbB2/NEU mRNA and protein was enhanced in the presence of androgen or estrogen, both apparently acting through the mutated androgen receptor. However, c-erbB2/NEU expression was evident in all three prostate cancer cell lines in the absence of steroid hormones.

These data confirm the involvement of the erbB gene family in regulating prostate cancer cell growth. Targetting the EGF receptor and the c-erbB2/NEU receptor may provide new avenues to inhibit prostate cancer growth in the clinical setting.

# TABLE OF CONTENTS

	Page
ACKNOWLEDGMENTS	I
ABSTRACT	II
TABLE OF CONTENTS	IV
LIST OF ABBREVIATIONS	VII
LIST OF FIGURES & TABLE	X
INTRODUCTION AND REVIEW	1
Prostate gland	2
Morphology	2
Growth regulation	3
Hormone regulation for growth	3
Hormone regulation for function	5
Involvement of growth factors	6
Prostate cancer	8
Stages of prostate cancer	9
Causes	10
-Common factors	10
-Growth factors	11
Treatments	13
-Surgery	14
-Radiation therapy	14
-Chemotherapy	15
-Hormonal therapy	15

<b>The erb B gene family</b>	<b>21</b>
<b>The v-erb B gene</b>	<b>21</b>
<b>Growth factors and their receptor</b>	<b>22</b>
The c-erb B1 (EGF receptor)	23
The c-erb B2	27
The c-erb B3	30
The c-erb B4	31
Ligands for the erb B gene family	31
Receptor mediated signal transduction	35
Function of the erb B gene family	39
<b>The erb B gene and cancer</b>	<b>42</b>
<b>Evidence for EGF receptor (erb B1) and its ligands</b>	<b>42</b>
<b>c-erb B2/NEU and cancer</b>	<b>50</b>
<b>The erb B3 and cancer</b>	<b>53</b>
<b>The erb b4 and cancer</b>	<b>53</b>
<b>erb B gene family and prostate cancer</b>	<b>53</b>
<b>HYPOTHESIS</b>	<b>57</b>
<b>MATERIALS AND METHODS</b>	
<b>Materials</b>	
Prostate tissue	58
Cell lines	58
Antibodies, growth factors	59
Hormones	60
DNA and cDNA probes	60

Primers	61
Conditioned media	62
<b>Methods</b>	
Cell culture	62
Radio-receptor assay	65
Genomic DNA preparation and Southern analysis	66
RNA preparation and analysis	68
Reverse transcription polymerase chain reaction (RT-PCR) analysis	69
Fmol DNA sequencing	72
Protein preparation and analysis	73
<b>RESULTS AND DISCUSSIONS</b>	<b>76</b>
<b>CHAPTER ONE</b>	
<b>Role of c-erb B1 (EGF receptor) in prostate cancer cell growth</b>	<b>77</b>
A. Expression of EGF receptor, EGF and TGF- $\alpha$	79
1. Introduction	79
2. Results	80
3. Discussion	88
B: Autocrine regulated growth by the c-erb B1 pathway	91
1. Introduction	91
2. Results	92
3. Discussion	105
<b>CHAPTER TWO</b>	
<b>Role of c-erb B2/NEU in prostate cancer cell growth</b>	<b>109</b>
A: Analysis of genetic alterations of c-erb B2/NEU in prostate tumor	109
1. Introduction	109
2. Results	109

3. Discussion	119
B: Autocrine regulation growth by the c-erb B2/NEU	122
1. Introduction	122
2. Results	123
3. Discussion	132
C: Regulation of c-erb B2/NEU gene expression by steroid hormones in prostate cancer cells	135
1. Introduction	135
2. Results	137
3. Discussion	146
<b>CONCLUSION</b>	<b>148</b>
<b>REFERENCES</b>	<b>154</b>

## LIST OF ABBREVIATIONS

Ab	antibody
AMV	avian myelocytomatosis virus
bp	base pairs
BPH	benign prostatic hyperplasia
°C	degrees centigrade
CaP	prostatic carcinoma
cpm	counts per minute
CO <sub>2</sub>	carbon dioxide
CsCl	cesium chloride
DHT	5 $\alpha$ -dihydrotestosterone
DMEM	Dulbecco's modified Eagle's medium
DNA/cDNA	deoxyribonucleic acid/ complementary
DNase	deoxy ribonuclease
DTT	dithiothreitol
E2	17 $\beta$ -estradiol
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ER	estrogen receptor

erb	erythroblastoma
FGF	fibroblast growth factor
g/mg/ug/ng	grams/milli-/micro-/nano-
GAP	triphosphatase activating protein
GH	growth hormone
H <sub>2</sub> O	water
HCl	hydrochloric acid
HEPES	4(2-hydroxyethyl)-1-piperazine ethanesulfonic acid
hPRL	human prolactin
IGF	insulin like growth factor
IU	international Units
KCl	potassium chloride
Kd	kilodaltons
Kb	kilobase
L/ml/ul	liters/milli-/micro-
MEM	minimum essential medium
M/mM/uM/nM	molar/milli-/micro-/nano-
MgCl <sub>2</sub>	magnesium chloride
NaCl	sodium chloride
PCR	polymerase chain reaction
PI3	phosphatidylinositol 3'

PKC	protein kinase C
PLC	phospholipase C
RNA/mRNA/tRNA	ribonucleic acid/messenger-/transfer-
TGF- $\alpha$	transforming growth factor alpha
TGF- $\beta$	transforming growth factor bata
Tris	tris(hydroxymethyl) amino methano
v/v	volume/volume
v/w	volume/weight

## LIST OF FIGURES & TABLE

	Page
Fig. 1-1. Northern blot analysis of expression of EGF, TGF- $\alpha$ and EGF receptor from prostatic carcinoma cell lines	83
Fig. 1-2. Representative dot blot analysis of expression of EGF, TGF- $\alpha$ and EGF receptor in BPH and CaP	84
Fig. 1-3. Relative expression of EGF, TGF- $\alpha$ and EGF receptor mRNA in total RNA from prostatic carcinoma cell line	85
Fig. 1-4. Relative expression of EGF, TGF- $\alpha$ and EGF receptor from prostate tissue	86
Fig. 1-5. Genomic Southern analysis of DNA from prostatic carcinoma cell lines	87
Fig. 1-6. Growth response of prostatic carcinoma cell lines (DU145 and LNCaP) to exogenous EGF, TGF- $\alpha$ and Ab EGF receptor antibody	97
Fig. 1-7. Inhibition of growth response of prostatic carcinoma cell lines of varying dose of Ab EGF receptor antibody	98
Fig. 1-8. Growth response of prostatic carcinoma cell lines (DU145	

	and LNCaP) to conditioned media and Ab EGF and Ab TGF- $\alpha$ antibodies	99
<b>Fig. 1-9-A.</b>	Radio-ligand binding assay of Pc-3 and Du145 cells	100
<b>Fig. 1-9-B.</b>	Growth response of prostatic carcinoma cell line, PC-3, to exogenous EGF, TGF- $\alpha$ and Ab EGF receptor antibody	101
<b>Fig. 1-10.</b>	Growth response of prostatic carcinoma cell line, PC-3, to conditioned media and Ab EGF and Ab TGF- $\alpha$	102
<b>Fig. 1-11.</b>	Western blot analysis of autophosphorylated EGF receptor in DU145 cells	103
<b>Fig. 1-12.</b>	Western blot analysis of autophosphorylated EGF receptor in PC-3 cells	103
<b>Fig. 1-13.</b>	Schematic representation of the EGF receptor	104
<b>Fig. 2-1.</b>	Genomic Southern analysis of c-erbB2/NEU gene from prostatic carcinoma cell lines and breast cancer cell lines	113
<b>Fig. 2-2.</b>	Genomic Southern analysis of c-erbB2/NEU gene from prostatic carcinoma cell lines, breast cancer cell line and human placenta tissue.	114
<b>Fig. 2-3.</b>	Genomic Southern analysis of c-erbB2/NEU gene from human prostate tissue.	115
<b>Fig. 2-4.</b>	Analysis of c-erbB2/NEU gene amplification	116

Fig. 2-5.	Western blot analysis of c-erbB2/NEU protein from prostatic carcinoma cell lines, CaP and BPH tissue	118
Fig. 2-6.	Northern blot analysis of expression of c-erbB2/NEU from prostatic carcinoma cell lines and breast cancer cell lines	126
Fig. 2-7.	Western blot analysis of c-erbB2/NEU protein from prostatic carcinoma cell lines and breast cancer cell lines	127
Fig. 2-8.	Inhibition of growth response of prostatic carcinoma cell lines to varying dose of Ab c-NEU Ab-2 antibody	128
Fig. 2-9.	Inhibition of growth response of prostatic carcinoma cell lines to Ab c-NEU Ab-2 antibody	129
Fig. 2-10.	Assay of reversal of growth inhibition, LNCaP cell line, to Ab c-NEU Ab-2 antibody	130
Fig. 2-11.	Northern blot analysis of regulation of c-fos expression by Ab c-NEU antibody in prostatic carcinoma cells	131
Fig. 2-12.	Growth response of LNCaP cell to DHT and E2	140
Fig. 2-13.	Northern blot analysis. Time course of c-erbB2/NEU in DHT and E2 treated LNCaP cells	141
Fig. 2-14.	Western blot analysis. Time course of c-erbB2/NEU protein in DHT and E2 treated LNCaP cells	142

<b>Fig. 2-15.</b>	Northern analysis of effects of Tamoxifen, DHT and E2 on expression of c-erbB2/NEU	143
<b>Fig. 2-16.</b>	Northern blot analysis of expression of ER in prostatic carcinoma cell lines and breast cancer cell line	144
<b>Fig. 2-17.</b>	PT-PCR analysis of expression of ER in prostatic carcinoma cell lines and breast cancer cell line	145
<b>Table 2-1.</b>	Analysis of c-erbB2/NEU gene amplification from prostate tissue of BPH and CaP and prostatic carcinoma cell lines	117

## INTRODUCTION & REVIEW

Cancer cells are defined by two heritable properties: they and their progeny reproduce in defiance of the normal restraints (Cairns, 1975; Nowell, 1976) and, invade and colonize territories normally reserved for other cells. It is the combination of these features that makes cancers peculiarly dangerous. The emerging dominant role for cancer as a cause of death is reflected in the cancer mortality rate, which has increased by 6% between 1950 and 1987. There is the potential for cancer incidence to continually increase by the year 2000 (Henderson *et al.*1991).

Prostate cancer is common in the male population worldwide. In past few decades, the incidence of prostate cancer has increased by greater than 15% (Henderson *et al.*1991), which makes prostate cancer the second most common cancer among males in developed countries. Although the incidence varies among different countries (Silverberg *et al.*1983), in the United States, prostatic cancer is now the most commonly diagnosed malignancy in males of all ages (Silverberg *et al.*1989). Each year, it is estimated that more than 90,000 new cases of prostate cancer present, resulting in approximately thirty thousand deaths in USA (Silverberg *et al.*1990). This mortality rate makes prostate cancer the second most common fatal tumor in males of all ages in America, and the leading cause of cancer death in man over the age 55 (Silverberg *et al.*1989). It is estimated now that there is a 10 percent chance that a 50 year old man will have a clinically detected prostatic cancer in his life time; it is estimated also based on autopsy data that at least 9 out of 10 such cancers remain undetected. The actual prevalence of

the disease is higher than the cancer diagnoses. Autopsy data show that more than 30% of men age 50, have evidence of prostatic carcinoma (Stamey *et al.*1989). In addition, the annual incident rate of clinical prostatic cancer has increased steadily since 1930 to the present time (Henderson *et al.*1991). This indicates that prostate cancer has become one of the most serious clinical problems.

## PROSTATE GLAND

### **Morphology**

The prostate is an exocrine gland which is associated with the urethra immediately below the urinary bladder. Based on histological three dimensional, reconstruction of the adult prostate, four morphologically distinct zones have been identified: the anterior fibromuscular stroma, the peripheral zone, the central zone, and the transition zone (McNeal, 1968). Prostatic adenocarcinoma arises almost exclusively in the peripheral zone. In humans, the prostate is composed of compound tubuloalveolar or tubosaccular glands (Cunha *et al.*1987). Prostatic ducts originating from the urethra radiate peripherally to completely surround the urethra. The ductal networks within the prostate are derived from solid epithelia out growths ( prostatic buds) that emerge from the endodermal urogenital sinus (UGS) immediately below the developing bladder and grow into the surrounding mesenchyme. The epithelium of the acini ranges from pseudostratified columnar to cuboidal. The distal ducts are lined by simple cuboidal or columnar epithelium, while transitional epithelium is seen in the proximal ducts as they

emerge from the urethra. It has been demonstrated that columnar prostatic epithelial cells exhibit all of the cytological features typical of secretory function (Tenniswood *et al.*1990). Individual ducts are lined by a pseudostratified columnar secretory epithelium, whose height varies with the functional state of the gland. Interspersed along the basement membrane are nonsecretory basal epithelial cells which do not reach the ductal lumen (Dermer, 1978; Mao *et al.*1966). The significance of these basal cells in the normal prostate and during prostatic pathogenesis is unknown. It has been suggested that they may be reserve or stem cells capable of differentiating into columnar secretory cells (Mao *et al.*1966) or giving rise to prostatic squamous metaplasia in response to estrogen (Merk *et al.*1982; Kroes *et al.*1972). Others have suggested a role for basal cells in the transporting of material between secretory epithelial cells and the extracellular space and in regulating the function of the columnar epithelia cells (Ichihara *et al.*1985). Observations by other investigators emphasize the role of prostatic basal cells in a variety of pathological conditions and suggest that basal cells may be targets of neoplastic agents (Dermer, 1978; Brawer *et al.*1985).

## **Growth regulation**

### **Hormone regulation for growth**

Normal human prostatic ductal morphogenesis and growth occurs in two separate periods, prenatally and pubertally, while pathological growth in BPH is initiated in the fourth decade (Farnsworth, 1970). During all processes of prostate development, androgen plays an important role for regulating prostatic growth (Bruchovsky *et al.*1968a;

Bruchovsky *et al.*1968b; Wilson *et al.*1981).

In the human fetal stage, absence or surgical removal of testes during the ambisexual period of sex differentiation, inhibits development of male internal sex glands including the prostate (Griffiths *et al.*1991). Similarly chemical castration through administration of estrogens or anti androgens inhibits development of the prostate. In organ culture, testosterone induces the female UGS to form prostatic buds and stimulates their formation in the male UGS. During the postnatal period, castration will greatly inhibit continued prostate growth, while administration of exogenous testosterone to immature males accelerates prostatic growth and maximal prostate size is achieved precociously (Cunha *et al.*1987; Lasnitzki *et al.*1977; Takeda *et al.*1986).

Maintenance of morphology and functional activity of the adult prostate is also influenced by androgens. The most active intracellular form of androgen is dihydrotestosterone (DHT) which is made from testosterone by  $5\alpha$ -reductase when puberty is reached and in adulthood (Ofner *et al.*1970; Wilson *et al.*1981). The effect of DHT is mediated by intracellular androgen receptors. During development this receptor is expressed first in mesenchyme and the also in the epithelium (Shannon *et al.*1983; Takeda *et al.*1985; Wasner *et al.*1983). A deficiency of androgen receptors such as occurs in Tfm (testicular feminization), the absence of androgens such as in castration, or the failure to produce DHT such as occurs in  $5\alpha$ -reductase deficiency will cause the prostate to fail to develop completely, or exhibit a striking reduction in size (Griffin *et al.*1984). Similarly reports from deKlerk, Coffey's group and English's group have demonstrated that most reduction occurs in the number of epithelial cells, although

reduction is also found in stromal cells as a result of castration (DeKlerk *et al.*1978; English *et al.*1985). This effect of androgens on the epithelium will affect prostatic function since the major secretory function of prostate is carried out by epithelial cells.

### **Hormone regulation for function**

One of the important functions of the prostate is to produce and secrete numerous proteins. These proteins have been the subject of study because of their biological properties and their regulation by androgens through effects on the gene expression.

In man, prostatic acid phosphatase (PAP) and prostate-specific antigen (PSA) are glycoproteins and are considered as the predominant androgen-regulated protein of the prostate. PSA and PAP have also been characterized as cell type specific markers for prostate epithelial cells by using immunohistochemical localization. The biological function of these prostate antigens has remained unclear. Both PSA and PAP, particularly PAP, appear to show a gradual diminution of expression as the prostate proceeds from the normal to the malignant state (Ablin, 1972).

The PSA is synthesized exclusively in prostate epithelial cells and secreted into the prostatic tubules to become a component of the seminal fluid (Papsidero *et al.*1981). PSA plays a fibrinolytic role in semen has been suggested (Lilja *et al.*1984) since a physiological substrate (a human seminal protein) has been observed (Lilja, 1985). However, the precise physiological function of PSA has not been determined. Expression of PSA in prostatic tissue has been correlated with fluctuating androgen levels during development in man. Other studies also have shown that the level of PSA protein may be

reduced when prostatic cells either lose their androgen response or increase their anaplasticity (Hasenson *et al.*1989). Therefore, PSA should be a useful marker for studying androgen action in human normal or abnormal prostatic cells. Lin and Li reported that the PAP may play a role as a phosphotyrosine protein (P-Tyr protein) phosphatase in determining the phosphorylation state of phosphotyrosine-containing proteins (Lin *et al.*1986; Li *et al.*1984). This observation suggests that the PAP may contributed to the malignant growth of the prostate.

### **Involvement of Growth factors**

In the past decade, numerous peptide growth factors and receptors have been found in normal or abnormal prostate tissue. Five major growth factor families including fibroblastic growth factor (Mydlo *et al.*1988a; Nakamoto *et al.*1992), epidermal growth factor (Elson *et al.*1984; Fowler *et al.*1988), insulin like growth factor, (Fiorelli *et al.*1991; Knabbe *et al.*1991) and the transforming growth factors have been implicated in the control of prostate growth. In contrast to classical endocrine hormones, growth factors are generally of local origin and have local effects tissues, although some factors, such as TGF- $\beta$  may originate from cellular elements of the circulatory system (Mori *et al.*1990; Wilding, 1992). Several lines of evidence, most come from animal models, suggest that there are correlations between growth factors and androgen in regulating the prostate gland. For example, ligand binding and immunohistochemical analyses indicate that the EGF receptor family is a candidate for regulation by androgen. EGF receptor binding also appears to be modulated by androgen. Castration increased EGF receptor number

in rat ventral prostate and receptor number decreased with subsequent administration of dihydrotestosterone (DHT) (Traish *et al.*1987; St Arnaud *et al.*1988). The level of EGF in the mouse submaxillary gland was increased by androgen (Byyny *et al.*1972). Castration of adult male mice greatly reduced the amount of EGF in ventral prostate. Testosterone administration to castrates increased EGF to the level found in intact animals.

For insulin-like growth factors (IGF), the low levels of IGF-I are sufficient to support proliferation of both normal and tumor derived cultured prostate epithelial and mesenchymal cell proliferation with or without addition of androgen (McKeehan *et al.*1984). Elevated IGF-I gene expression is seen in regenerating normal prostate after androgen treatment of castrated rats and appears to be constitutive in slow growing, androgen-responsive and highly malignant transplantable tumors (McKeehan, 1991). IGF-I gene expression appears to occur predominantly in the mesenchymal cells of both regenerating normal prostate and slow growing tumor.

For FGF family, the expression has been detected in both BPH and prostate cancer specimens (Mydlo *et al.*1988b; Nakamoto *et al.*1992). The basic FGF has been shown to be stimulatory to human prostate cancer cell growth in culture system (Gleave *et al.*1991). It implicate that FGF may play some role in regulation of prostate cancer growth.

For transforming growth factor  $\beta$  (TGF- $\beta$ ), expression is very low in normal rat prostate tissue. However, exogenous TGF- $\beta$  completely abrogates the mitogenic effect of EGF and TGF- $\alpha$  on epithelial cells (Wilding, 1992). These observations are still far

from resolving all the questions, and the mechanisms of growth factor influence in prostate cells need be established.

Little is known of the function and regulation of growth factors in the development of human normal prostate, even though the EGF family (Shaikh *et al.*1990), basic fibroblast growth factor (bFGF), (Story *et al.*1987), TGF- $\beta$  (Mori *et al.*1990), platelet derived growth factor (PDGF) (Sitaras *et al.*1988) and IGF (McKeehan, 1991) have been found expressed in human normal, BPH and cancerous prostate tissue.

## PROSTATE CANCER

The prostate gland is composed of acinar glands which are lined by a two layer epithelium. The peripheral cell layer contains the basal cells; the luminal layer contains the principle cells (Catalona, 1990). More than 95% of prostate cancers arise in the acinar epithelium. Most prostate cancers are multifocal in origin (Catalona, 1990; McNeal, 1968). Normally, proliferation occurs only in the basal layer, generating cells that then move outward toward the surface, differentiating into flattened, keratin rich, non dividing cells, and finally being sloughed off from the surface. Although most carcinomas in humans are not diagnosed until a relatively late stage, it is generally thought that the development of such cancers occurs in stages from normal cells through dysplasia, carcinoma in situ, invasive carcinoma and finally metastatic cancer. In the dysplasia stage, cell division is no longer confined to the basal layer and there is some disorder in the process of differentiation. During the stage of carcinoma in situ, the usual

pattern of cell division and differentiation is much more severely disrupted, and all the layers of the epithelium consist of undifferentiated proliferating cells, which are often highly variable in size and karyotype. However, the abnormal cells are still confined, but toward the epithelial side of the basal lamina. When cells break out of the epithelium by crossing the basal lamina and begin to invade the underlying connective tissue, this represents from in situ stage to invasive carcinoma (Nicolson, 1979). Surgical cures become progressively more difficult as the invasive growth spreads. Invasiveness usually implies an ability to break loose, enter the bloodstream or lymphatic vessels, and form secondary tumors at other sites in the body. For prostate cancer the most common early metastatic tumors appear in the pelvic lymph nodes followed by bone and brain, but the specific targeting factors remain unknown (Saitoh *et al.*1984). The wider a cancer metastasizes, the harder it becomes to eradicate.

### **Stages of prostatic cancer**

The staging of prostatic cancer is of central importance to any discussion of therapy and clinical results. Staging has become more elaborate, with the possibility of increased definition of lesions by imaging and the use of tumor markers. Many methods for staging prostatic cancer have been established; the most elaborate, Hopkins system, is subdivided to make clinically important distinctions as follows: stage A: microscopic, not a clinically palpable tumor; stage B: palpable, macroscopic tumor; stage C: tumor with extracapsular extension, but still clinically localized; and stage D: demonstrated metastatic tumor (Gittes, 1991). Measurement of the prostatic tumor markers, prostate specific

antigen (Allhoff *et al.*1983; Stamey *et al.*1989), prostatic acid phosphatase (Oesterling *et al.*1987), and a total body bone scan (Hricak, 1988; Paolson *et al.*1979) constitute the usual initial stage in evaluation of a patient with proven prostatic cancer. A positive bone scan, is usually correlated with the level of prostate specific antigen, identifies the patient's disease as stage D and makes elaborate local staging unnecessary.

## Causes

### Common factors

As with many human cancers, the causes of prostate cancer are not fully understood. Epidemiological studies have found no consistent correlation of prostatic cancer with venereal disease, sexual habits, smoking, or occupational exposure. In contrast, age, race, diet (Gleave *et al.*1992a), hormone and growth factors have been observed as correlates of prostate cancer.

Statistical studies show the incidence of prostate cancer is dramatically increased with age. Every decade of aging nearly doubles the incidence of such tumors from 10 percent of men in their 50s to 70 percent of men in their 80s. Autopsy studies indicate that 30% of men have latent carcinoma (intraprostatic microscopic foci) by the age of 50 while only about 1% of the American male population over the age of 45 with latent carcinoma present annually with clinically manifest disease (Rich, 1935; Griffiths *et al.*1989).

The incidence of prostatic carcinoma is influenced not only by age but also by race. Prostatic cancer is more common among blacks and much less common among Asians

than among whites, with the incidence of clinically diagnosed prostatic cancer ranging from 0.8 case per 100,000 population in Shanghai, China, to 100.2 per 100,000 among blacks in Alameda county, California (Ross *et al.* 1986a; Miller, 1988). The mortality rate among men with prostatic cancer is also much higher among blacks (Levine *et al.* 1979). Since black men have serum testosterone levels 15% percent higher than white men (Ross *et al.* 1986a), an attractive but conjectural hypothesis is that higher serum testosterone levels predict some if not all of the epidemiologic spectrum of prostatic cancer.

Environmental or dietary factors also play a part in the disease etiology. The study of migrating people indicates that the mortality rate from prostatic carcinoma although low in Japan, increases to about half that of the indigenous American population for those Japanese who move to the USA (Haenzel *et al.* 1968; Wynder *et al.* 1971).

Although the familial clustering of prostate cancer cases is not as strong as in breast cancer, it is estimated that men who have both an affected first degree relative (a brother or father) and an affected second degree relative (an uncle or grandfather) have an significant increase in risk (Steinberg *et al.* 1990). These observations indicate that genetic pre-disposition is one of the factors of in prostate cancer etiology.

### **Growth factors**

Recent studies have established that growth promoting polypeptides related to epidermal growth factor, fibroblast growth factor, insulin-like growth factor, multiple insulin-like growth factor binding proteins and insulin are present in extracts of normal, hyperplastic and carcinomatous prostatic tissues (Fiorelli *et al.* 1991).

Immunohistochemical study has localized EGF in BPH and prostate cancer specimens; EGF staining was seen in only 6% of BPH specimens, whereas 68% of prostate cancer specimens stained with EGF antiserum (Fowler *et al.*1988). EGF receptor has been characterized in tissue specimens of BPH and carcinomatous prostate tissue (Davies *et al.*1988). The role for EGF receptor family and their ligand(s) for cancer growth, including prostate cancer, will discuss further in later sections below in this thesis. PC-3 cells is one of the first source to isolate TGF- $\beta$  1 and 2. In vitro studies have provided evidence that a variety of growth factors affect prostatic epithelial growth, with or without interceding androgenic regulation (Chaproniere *et al.*1986; Schuurmans *et al.*1988; Wilding *et al.*1989b). Although at this time no definite link between growth factors and prostatic growth has been established in vivo, hypotheses regarding the role of growth factor in BPH and prostatic carcinoma have received considerable attention. Among them, it has been postulated that autocrine, paracrine and interacrine interactions may be an important feature of prostatic growth aberration.

Human prostatic cancer is unique in that it is the only cancer to consistently produce osteoblastic metastases in bone. More than 90% of bone lesions due to prostatic cancer are osteoblastic rather than osteolytic. Using histomorphometric analysis of bone metastases, it was concluded that a local trophic factor released by the cancer cells could account for the histological picture (Charhon *et al.*1983). Extracts of normal prostate, BPH and prostate cancer tissue were reported to be mitogenic for fetal rat calvarial derived osteoblasts (Jacobs *et al.*1979). Other investigators have provided additional evidence that osteoblast-specific factors may be present in the prostate. Prostate cancer

tissue, but not normal prostate or BPH, contains a mitogen with specificity for MC3T3-E1 mouse osteoblasts. (Nishi *et al.*1988). A mitogen with specificity for human osteoblasts over fibroblasts was identified in the medium conditioned by PC-3 cells (Perkel *et al.*1990). PC-3 mRNA microinjected into *Xenopus* oocytes was reported to directed the synthesis of substances mitogenic for osteoblastic cells (Simpson *et al.*1985). This factor(s) has not been characterized, but it identifies a linkage between growth factors and the metastases of prostate cancer. One of the most interesting group of factors is bone morphogenetic proteins (BMPs) which have been identified as an important group of bone-inducing factors. BMPs have the capacity to induce new bone formation when implanted ectopically into experimental animals (Urist, 1965). BMPs have also been found in human normal and abnormal prostate tissue. Some members of the BMPs, such as BMP-6, were only found in the prostate tissue with clinically defined metastatic prostate adenocarcinoma, but was not detected in non-metastatic or benign prostate sample or in ocular melanoma tissue (Bentley *et al.*1992). This funding suggests that prostate cancer cell may have the capacity to induce osteoblastic growth. In vitro mitogenic assay shown that a bidirectional stimulation between prostate carcinoma cell line LNCaP and bone or prostate fibroblast conditioned media (Gleave *et al.*1992b). It implicated that a putative bone paracrine growth factors or extracellular matrix factors may contribute to the site specificity of prostate cancer metastasis and a passible mechanism of bidirectional paracrine interactions may exist between prostate and bone cell.

## TREATMENT

## **Surgery**

Radical prostatectomy offers a curative treatment in patients with early disease that is limited to the prostate (stage A and B) (Schroeder, 1989). The main goals of a radical prostatectomy or of any surgical treatment for cancer, is to cure the patient and to control the tumor locally. The success of the radical prostatectomy is obvious. A 15 year tumor-free survival rate following radical prostatectomy is reported as 93 percent at the Mayo clinic (Blute *et al.* 1989), 89 percent at the Mason clinic (Gibbons, 1988), and 86 percent at Johns Hopkins Hospital (Lepor *et al.* 1988). It indicates that radical prostatectomy or potency-sparing radical prostatectomy is sufficient for achieving a complete cure in an early stage of prostate cancer.

## **Radiation therapy**

Like surgery, radiation therapy is recommended for the treatment of localized prostate cancer, and also is used in combination with another therapy, such as surgery or hormone treatment. Superior results to surgery were claimed in terms of preserving potency and avoiding incontinence. Reports of up to 90 percent disease-free patients five years after radiation treatment make this therapy comparable to surgery (Bagshaw *et al.* 1988). Because the prostate gland is situated deep in the pelvis, high energy linear accelerators are required to minimize radiation injury to surrounding normal tissues (Gittes, 1991).

Although surgical and radiation therapy both show success in the control of prostate cancer, the limitations are also obvious. Besides the injury to surrounding normal tissue,

especially in radiation treatment, the biggest disadvantage of both methods is that they are suitable for early stages of prostate cancer only. Unfortunately early diagnosis is limited.

### **Chemotherapy**

Chemotherapy has been widely used in later stages of androgen-independent or metastatic cancer treatment. Unfortunately, cytotoxic chemotherapy has been largely ineffective in the treatment of prostate cancer (Eisenberger *et al.*1987). Most of the commonly used drugs have been evaluated and objective response rates are approximated at 15 percent and are usually short lived. Drugs which have shown some activity include 5-fluorouracil, cyclophosphamide, methotrexate, doxorubicin, and mitomycin. There is no evidence that combination chemotherapy is more effective than single agent therapy (Rasmussen, 1989). The median survival time of patients treated with chemotherapy is approximately 6 months to 1 year with androgen-independent diseases (Eisenberger *et al.*1987)

### **Hormonal therapy**

The central observation of endocrine investigations involving the prostate has been that growth, maintenance and function in the normal gland is dependent on testosterone secreted by the testes. The action of androgens on the prostate is, to some extent, elucidated. Hormones released from the hypothalamus induce the pituitary to release Luteinizing hormone (LH), which causes the Leydig cells of the testes to produce

testosterone. Furthermore, Adrenocorticotrophic hormone (ACTH) acts on the adrenal glands to secrete androgen precursors that are later converted to testosterone. Most plasma testosterone is bound to serum hormone binding globulin (SHBG). Free testosterone diffuses into the cells where it may be reduced by 5-alpha-reductase to dihydrotestosterone (DHT), which joins a specific receptor. This steroid-receptor complex undergoes a complex transformation involving phosphorylations and eventually binds to specific DNA elements, thus altering transcription and protein synthesis. Since Charles Huggins established the correlation between androgens and prostate cancer in the early 1940's (Huggins *et al.* 1940; Huggins *et al.* 1941a; Huggins *et al.* 1941b), the most common treatments used clinically have involved androgen withdrawal, especially in late stages of prostate cancer ( stages C and D).

Since the majority of androgens in circulation of men come from testes, surgical castration has been identified as successfully withdrawing most of the testosterone from circulation, and has been used as a standard endocrine therapy for prostatic cancers. However castration can be psychologically unacceptable to many patients and other forms of reducing testicular androgen production have been sought.

The hormone-releasing action of the testes is controlled by the hypothalamus-pituitary-peripheral gland axis (LHRH-LH-gonadotrophin), and inhibited by negative feedback loops and inhibin. Any therapy that can decrease Luteinizing hormone-releasing hormone (LHRH) release or LH concentration in circulation will eventually inhibit testosterone production in the testes. Both estrogens and androgens are products of gonads, and have been recognized as strong inhibitors for LHRH and LH

through long feedback loops. Using estrogen to treat prostate cancer patients inhibits the release LHRH and LH from the hypothalamus-pituitary to such a point that circulating testosterone is essentially at castrate levels, based on the negative feedback regulation mechanism. The use of estrogen or estrogen analogues also has the advantage of raising the serum concentration of SHBG, thereby decreasing the amount of free testosterone. It is also possible that estrogens have a cytotoxic effect on prostatic cancer cells (Rasmussen, 1989). Concern about the side effects of estrogens increased when it was demonstrated that they could alter platelet adhesiveness and increase the incidence of thromboembolic phenomena in patients treated for prostatic cancer (de Voogt *et al.* 1986). Other drugs used to decrease the amount of testicular androgens are the steroid anti-androgens, cyproterone acetate and megestrol acetate, that act centrally to produce a decrease in LH release, thereby decreasing testosterone production. To reduce the formation of androgen-independent tumorigenic stem cells during androgen withdrawal treatment, the intermittent androgen suppression method has been developed and it has been shown that successful to induce multiple apoptotic regressions of a tumor (Akakura *et al.* 1993).

LHRH analogues in pharmacological doses also decrease the amount of testosterone in circulation, but another working principle is involved. LHRH analogues, a group of peptides including leuprolides, buserelin and goserelin, require administration by parenteral injection, and the action is to occupy the receptors of LHRH in the pituitary. Initially, LHRH analogues produce a significant increase in serum testosterone (flare reaction). However, within a few weeks, serum testosterone concentration is reduced to

castrate levels. This effect is due to depletion of pituitary LH and negative feedback by high circulating androgens. Combination therapy using LHRH analogues and estrogen or anti-androgens will overcome the unwanted effect termed as flare reaction during LHRH analogue treatments (Labrie *et al.*1982; Crawford *et al.*1989); this is needed to reduce the potential rapid worsening of a patient's condition during the initial two weeks of paradoxical stimulation of testosterone release (Kuhn *et al.*1989).

Besides the vast amount of testosterone being released from the testes, there is another source of androgens in men. The adrenal glands are able to produce androgen precursors which will later be converted into testosterone (Walsh, 1975). This process is controlled by ACTH, another hormone released by hypothalamus-pituitary. A small amount of androgens, around 5 percent of total androgens, produced in the adrenal glands have clinical importance and will not be removed by surgical castration or inhibited by administering gonadotrophin or LHRH analogues. Another strategy is to block the pathway of androgen function on target tissues without reducing the concentration of androgen in circulation. Anti-androgens, especially non-steroidal anti-androgens, such as flutamide, anandron and ICI 176,334, are commonly used in this therapy. These non-steroidal anti-androgens function to inhibit the translocation of the androgen receptor complex from the cytoplasm to the nucleus, or by competing with androgens at the receptor steroid binding sites in previously sensitive cells, normal or malignant. Most non-steroidal anti-androgens, except ICI 176,334, have little or no central effects, but significant peripheral effects as they compete with androgens for steroid binding sites. ICI 176,334, in human studies, showed both central effects that inhibit LH and peripheral

effects (MacFarlane *et al.* 1985; Lund *et al.* 1988; Labrie *et al.* 1983a; Labrie *et al.* 1983b). Another class of drugs currently under investigation for the treatment of metastatic prostate cancer are specific inhibitors of  $5\alpha$ -reductase which block most effects of androgens on prostatic cells by preventing the formation of DHT (McConnell, 1990). However, the  $5\alpha$ -reductase inhibitors have not produced clinical results that are equal to castration.

Several disadvantages of withdrawing androgens from prostate cancer patients have been described above, but the most serious failure of hormone therapy is the almost universal relapse of metastatic prostate cancer to an androgen-insensitive state. Even if there was initial positive response to androgen withdrawal therapy, following relapse additional forms of anti-androgen therapy are ineffective regardless of how aggressively given (Scott *et al.* 1980; Schulze *et al.* 1987; Smith *et al.* 1984; Menon *et al.* 1979; Griffiths *et al.* 1989). Because of this nearly universal relapse phenomenon, the annual death rate from prostatic cancer has not decreased at all over the subsequent 40 years since androgen withdrawal became standard therapy. The phenomenon of human prostate cancer in which tumor progression is associated with a loss of androgen-dependency, has led to the suggestion that prostatic cancer within an individual patient is heterogeneously composed of clones of both androgen-dependent and independent cancer cells even before hormone therapy has begun. The role of androgens in the androgen-independent prostate cancer may be indirect, being mediated by other hormones, growth factors and intracellular regulators. Our understanding of the continuing growth of prostate cells and tumors in the absence of androgens is incomplete.

At present we must assume that androgen-independent growth represents an altered ability to respond to non-androgenic growth signals and/or to ignore inhibitory control. The autocrine hypothesis (Todaro *et al.*1978; Sporn *et al.*1985b) has served as a useful working hypothesis to study this phenomenon. The hypothesis states that transformed cells produce their own growth factors which act on the producer cells to maintain a continuous signal for proliferation. According to this hypothesis, a number of growth factors and their receptors have potential roles in prostate cancer. We have investigated the c-erbB gene family of growth factor receptors because: 1) Human EGF has been found in prostatic fluid (Gregory *et al.*1986; Elson *et al.*1984; Shaikh *et al.*1990) and in extracts of prostate tissue (Yang *et al.*1993). EGF receptor has been demonstrated biochemically and immunocytochemically in human BPH and CaP tissue (Seidman *et al.*1985; MacFarlane *et al.*1985). 2) The v-erbB oncogene codes for a product homologous to the cytoplasmic domain of the EGF receptor thus confirming the oncogenic potential of this gene family. 3) TGF- $\alpha$  is produced by many transformed cells and has been identified in several tumor cell lines (Liu *et al.*1990; Smith *et al.*1987; Tateishi *et al.*1991). The amplification of EGF receptor gene has been documented in some tumors, such as squamous cell carcinomas, malignant gliomas, breast, gastric, lung, brain and renal carcinomas (Berger *et al.*1987; Perez *et al.*1984; Tuzi *et al.*1991) confirming the association of this gene family with other human cancers.

## The erb B gene family

The erbB gene family is a small family which includes four distinct proto-oncogene members, named erbB1 (EGFR), erbB2 (NEU, HER2), erbB3 (EGFR3, HER3) and erbB4 (HER4). Since the first member of this gene family, EGF receptor, is highly homologous to the products encoded by avian erythroblastosis virus (v-erbB) transforming gene (Downward *et al.* 1984b), this group of genes has been named as the erbB gene family. However, since the cellular products of these proto-oncogenes are highly structurally related to the EGF receptor and appears to function as cell surface receptors, it is also sometimes referred to as the EGF receptor family.

### The v-erb B gene

The v-erb B gene encodes a product which is an analog of the receptor for EGF. This protein has undergone truncation events that have led to the loss of its extracellular ligand-binding domain and a portion of its extreme carboxyl terminus (Downward *et al.* 1984a), which includes the primary site for autophosphorylation (Downward *et al.* 1984a; Downward *et al.* 1984b; Wells *et al.* 1988). The biochemical consequence of this deletion is to generate an oncoprotein which is a constitutively active protein-tyrosine kinase (Goustin *et al.* 1986). This observation suggests a mode of oncogene activation in which the erbB protein might function to relay a mitogenic signal even in the absence of ligand binding (Cooper, 1990).

## Growth factors and their receptors

Growth factor is a term applied to a polypeptide that either stimulates or inhibits cell proliferation and may influence cell differentiation as well. The effects of a growth factor on cells depends on a variety of factors, such as the nature of the growth factor, the cell type and the physiological conditions of the responding cell and its environment. Thus, individual factors may stimulate some cell types and inhibit others, depending on the conditions (Sporn *et al.* 1988).

Generally, growth factors cause cells in the resting state to enter and proceed through the cell cycle. In general, the mitogenic response can be considered to occur in two parts; first, the quiescent cell must be advanced into the G1 phase of the cell cycle by "competence" factors, then traverse the G1 phase, and become committed to DNA synthesis under the influence of "progression" factors (Pledger *et al.* 1977; Pledger *et al.* 1978). It has been demonstrated that different growth factors selectively effect different points or periods in the cell cycle. For example, EGF, FGF and PDGF act as competence factors to recruit the quiescent cells into the cell cycle, while IGF-1 or insulin work as progression factors to transit cells through the G1 phase (Pardee, 1989).

Cells of most, if not all, major tissue types are targets of growth factors. The target cells for growth factors are characterized by the expression of specific transmembrane receptors that bind the factor and stimulate the cell to respond in one of a number of different ways. Transmembrane growth factor receptors can be divided into an extracellular domain and an intracellular domain. These are connected physically and functionally through the cell membrane by a membrane spanning region (transmembrane

domain) or regions.

### **The erb B1 (EGF receptor)**

EGF receptor is the first member of the erbB gene family to be isolated (Cohen *et al.*1982) and biochemically characterized, and has served as a paradigm for other growth factor receptors. The EGF receptor is detected in a large variety of cells by measurement of EGF binding or by crosslinking of labeled EGF to its receptor or by using monoclonal antibodies (Schreiber *et al.*1981). The EGF receptor was first purified to near homogeneity in 1980 by the use of affinity chromatography from A431 cells (Haigler *et al.*1978; Cohen *et al.*1980; Stoscheck *et al.*1983). The A431 cell line was derived from a human squamous carcinoma of the vulva and has been identified as expressing an increased number, about 50 times more, of EGF receptors (Fabricant *et al.*1977). The purified receptor had an apparent molecular mass of 150,000; subsequent purification methods that eliminated calcium from the buffers indicated the actual mass to be 170,000 (Cohen *et al.*1982). This corresponded well to mass estimations obtained from earlier studies in which <sup>125</sup>I-EGF was chemically crosslinked to the receptor (Das *et al.*1977; Wrann *et al.*1979). Most cell homogenates contain a calcium activated protease that cleaves the native molecule of 170,000 daltons to the lower molecular mass (150,000) species (Cassel *et al.*1982).

Although the full X-ray structural analysis for the EGF receptor is still unclear, biochemical study indicates that the EGF receptor is a single chain glycoprotein composed of three different domains: a ligand binding domain which lies external to the plasma

membrane, a transmembrane domain and a cytoplasmic kinase domain which has both the kinase activity and the autophosphorylation site (Yarden *et al.*1988). Isolation of the EGF receptor resulted in copurification of the growth factor sensitive tyrosine kinase activity, suggesting that the ligand binding site and kinase might be physically coupled. Consistent with this idea was the demonstration that antibodies to the 170,000 dalton EGF receptor were able to precipitate the growth factor-sensitive kinase activity (Cohen *et al.*1982). Treatment of the EGF receptor in membrane vesicles with a radiolabeled ATP affinity reagent demonstrated the presence of an ATP binding site on the 170,000 dalton receptor (Buhrow *et al.*1982; Buhrow *et al.*1983). These studies also demonstrated that if the tyrosine kinase was inactivated by mild heating or N-ethylmaleimide exposure, subsequent incubation with the ATP reagent failed to label the receptor. This result suggested that the EGF receptor contains both the ligand binding domain and the active kinase domain (Greenfield *et al.*1988).

Sequence analysis of tryptic peptides derived from the EGF receptor (Downward *et al.*1984b) led to cDNA cloning of the receptor (Downward *et al.*1984b; Downward *et al.*1984a) and the deduced amino acid sequence of the entire molecule (Ullrich *et al.*1984). These elegant studies have provided a structural basis on which to understand the ligand binding and tyrosine kinase activities of the EGF receptor as domains of a single polypeptide chain.

The mature receptor contains a single polypeptide chain of 1186 amino acid residues which is divided into two domains by one hydrophobic membrane anchor sequence. The extracellular domain is comprised of about half the total receptor sequence which is

around 621 amino acid residues (Ullrich *et al.*1984). This domain must convolute to accommodate high affinity ligand binding. Chemically, this portion of the receptor has been demonstrated as having a cystine-rich (10%) region which is assumed to form disulphide bonds although the exact disulphide content has not been quantified. It is proposed that the region between the two half-cystine-rich clusters is involved in ligand binding (Lax *et al.*1988). Several N-Linked glycosylation sites are present in this domain too, and have been shown to be important for ligand binding (Sliker *et al.*1985; Mayes *et al.*1984; Cummings *et al.*1985).

A single transmembrane region of 23-26 residues has been found to couple the extracellular ligand binding domain to the catalytic tyrosine kinase domain (Yarden *et al.*1988). The components of the transmembrane domain amino acids must play a role in the passive anchor of the receptor to the membrane as well as receptor lateral and rotational diffusion. The several basic residues of the cytoplasmic and of the putative transmembrane stretch are believed to act as a "stop transfer" function for the insertion of the polypeptide chain into the membrane. It is well known that EGF receptors are randomly distributed on the cell surface (Schlessinger *et al.*1978), and they undergo rapid lateral and rotational diffusion. Results of an experiment using an EGF receptor mutated in the transmembrane domain suggests that the EGF receptor transmembrane region does not directly influence the signal transduction pathway (Kashles *et al.*1988), including ligand binding characteristics, dimerization and stimulation of the protein kinase activity on substrates. However, evidence of enhancement of transforming ability by a NEU/erbB2 receptor with a point mutation within the transmembrane domain (Bargmann

*et al.*1986b; Segatto *et al.*1988) and by an EGF receptor with an altered transmembrane region in a created mutation mode suggests an active role for the transmembrane region in signal transduction.

The cytoplasmic domain of the EGF receptor is composed of 542 amino acids, most of them basic residues (Schlessinger, 1988). At least 65 putative kinase active sites have been identified in this region and share extensive sequence homology even between different classes of receptor (Hanks *et al.*1988). The kinase domain from amino acid 250 to 300 in human EGF receptor (30 Kd) is the most conserved cross the family and is homologous to the catalytic domain of the protein-tyrosine kinase encoded by the src gene family (Hunter *et al.*1985). Within this tyrosine kinase region, considered to be the catalytic domain, there is a lysine (residue 721) that has been labeled with an ATP affinity reagent (Russo *et al.*1985). The initial phosphorylation studies indicated that ATP and/or GTP is used as a phosphate donor in EGF receptor type receptor-mediated signal transduction (Carpenter *et al.*1979b). Replacement of a single lysine residue in the ATP (adenosine triphosphate) binding site (Honegger *et al.*1987) completely abolishes receptor kinase activity and destroys the biological function of EGF receptor, although ligand binding properties are not altered (Yarden *et al.*1988; Chen *et al.*1987). This result indicates the ATP binding site is absolutely required for receptor signalling.

Most of the carboxyl terminal region of the EGF receptor contains sequences not found in other tyrosine kinases. Tryptic phosphopeptide analysis suggests that there are several phosphorylation sites (Iwashita *et al.*1984), and most of these sites are within the 30-40 Kd tryptic fragment containing the protein-tyrosine kinase domain (Chinkers *et*

*al.*1984). Near the carboxyl terminus of the receptor are four sites of EGF-dependent tyrosine autophosphorylation (Downward *et al.*1984a; Margolis *et al.*1989). According to mutagenesis studies, including point mutation, deletion and truncation, it is most likely that the autophosphorylation of multiple tyrosine residues is necessary for phosphorylation of certain intracellular substrates and receptor kinase activity (Sorkin *et al.*1991; Honegger *et al.*1987; Velu *et al.*1989; Helin *et al.*1991). However, one reports suggests loss of three major autophosphorylation sites in the EGF receptor does not block the mitogenic action of EGF in a mutant chinese hamster ovary (CHO 11) cell line (Clark *et al.*1988). In summary, there are several features of the EGF receptor that are common to all receptors with tyrosine kinase activity, including an ATP-binding site, sequences determining substrate specificity that control which intracellular proteins can be phosphorylated, and autophosphorylation sites, which may serve in regulating the kinase activity towards endogenous substrates (Tornqvist *et al.*1988; Downward *et al.*1985; Margolis *et al.*1989; Wahl *et al.*1989; Shalaby *et al.*1992).

### **The erb B2**

The human c-erbB2 (HER2 and NEU) gene encodes another member of the erbB gene family (Bargmann *et al.*1988a). It is related but distinct from the human erb B1 gene (Schechter *et al.*1984). The erb B1 gene is located on chromosome 7 and encodes mRNA transcripts of 5.8 kb and 10 kb mRNA for a protein product of 170 kDa molecular weight. In contrast, the c-erbB2 gene is located on chromosome 17, and generates a messenger RNA of 4.8 kb and produces a protein with 185 kDa (Coussens

*et al.*1985; Semba *et al.*1985; Schechter *et al.*1985; Popescu *et al.*1989). This gene was originally identified as a result of transfection studies with DNA from chemically induced rat neuroglioblastomas (Shih *et al.*1981) and called NEU. In separate work using v-erbB and human EGF receptor clones as probes to screen human genomic and complementary DNA (cDNA) libraries, two genes named as HER2 and c-erbB2 were isolated (Coussens *et al.*1985; Semba *et al.*1985). Subsequent sequence analysis and chromosomal mapping studies revealed all three genes (NEU, erbB2 and HER2) to be the same (Semba *et al.*1985; Schechter *et al.*1985). Like the EGF receptor protein, c-erbB2/NEU has an extracellular domain that includes two cysteine-rich repeat clusters, an intracellular domain, and a transmembrane domain (Schechter *et al.*1984). It has been demonstrated conclusively that the c-erbB2/NEU product does not bind EGF but appears to have a distinct ligand (Yarden *et al.*1989). However, activation of the EGF receptor can result in cross-phosphorylation of the c-erb b2 protein in cells that express both receptors (King *et al.*1988; Kokai *et al.*1988; Stern *et al.*1988b). This might indicate that there is the heterodimer of the EGF receptor and the c-erbB2 protein induced by EGF binding (Wada *et al.*1990; Goldman *et al.*1990). The synergistic action of EGF receptor and c-erbB2 protein has been seen in rat cells (Kokai *et al.*1989; Di Fiore *et al.*1990b; Segatto *et al.*1991), but the mechanisms involved in this cooperation are still unclear (Wada *et al.*1990; Goldman *et al.*1990). Overexpression of the c-erbB2 growth factor receptor occurs, usually as a consequence of gene amplification, in several tumors including breast, stomach, pancreatic, bladder and ovarian cancer (Slamon *et al.*1988; Slamon *et al.*1989; Hall *et al.*1990; Lemoine *et al.*1992; Soomro *et al.*1991). Overexpression is

associated with poor prognosis in breast and ovarian cancers and possibly non-small cell lung cancer (Slamon *et al.*1989; Parkes *et al.*1990; Shi *et al.*1992).

The c-erbB2 protein is expressed widely on normal human and animal tissue (Gullick *et al.*1987; Quirke *et al.*1989; Natali *et al.*1990; Press *et al.*1990). To determine whether c-erbB2 can convey a mitogenic signal, a created chimeric protein consisting of the extracellular ligand binding domain of the EGF receptor fused to the cytoplasmic sequence of the human c-erbB2 protein or the equivalent rat protein, NEU, has been tested. The chimeric protein bound EGF, which stimulated autophosphorylation and mitogenesis, and was shown to induce increased transcription of fos and jun (Sistonen *et al.*1989). In a rat experimental carcinogenesis model, a single point mutation in the transmembrane region of the c-erbB2 gene (valine to glutamic acid), led to tumor development (Bargmann *et al.*1988a). The mechanisms of activation of c-erbB2 to a transforming oncogene is currently uncertain, but some evidence suggests that the single transmembrane mutation stabilizes receptors in a dimeric form in which they are catalytically active (Weiner *et al.*1989; Segatto *et al.*1991). Introduction of the same mutation into the human gene will transform cells in culture (Segatto *et al.*1988) and the same mutation in the *Drosophila* EGF receptor greatly increases its tyrosine kinase activity (Wides *et al.*1990). However, this activating point mutation of c-erbB2 has not found in naturally occurring in human tumours (Hall *et al.*1990; Lemoine *et al.*1992; Lemoine *et al.*1990; Tuzi *et al.*1991; Slamon *et al.*1989; Saya *et al.*1990). The oncogenic function of c-erbB2 in human cancer may be contributed by the aberrantly high expression of the receptor protein. Gene amplification appears to be predominant, at least

in breast cancer, but overexpression by increased mRNA transcription alone does occur in a minority of cases (Kraus *et al.*1987; Parkes *et al.*1990). Overexpression of c-erbB2 has been found to correlate with high rate of cell proliferation in invasive and in situ breast cancer, but experimental studies suggest that there may not be a simple relationship between expression of c-erbB2 and human breast cancer cell proliferation in vivo and in vitro (Salomon *et al.*1987b; Barnes *et al.*1991; Dati *et al.*1991). There is also evidence that c-erbB2 may contribute to the invasion and spread of tumors. Clinical studies shown that c-erbB2 amplification and overexpression are correlated with aggressive tumor growth and poor prognosis in breast and ovarian cancer (Slamon *et al.*1989) and experimental studies demonstrated that the rat NEU oncogene can induce the metastatic phenotype in transfected cells (Yu *et al.*1991). The elements involved in the control of c-erbB2 expression are under investigation; it has been report that estrogen may have an inhibitory effect on c-erbB2 transcription in breast cancer cells (Warri *et al.*1991).

### **The c-erb B3**

Human erbB3, also named as EGFR3 and HER3, was identified recently as a protein related to the EGF receptor (Kraus *et al.*1989; Plowman *et al.*1990b). In the tyrosine kinase domain, 60% of the residues are identical between EGF receptor and c-erbB3. The mature protein is extensively glycosylated and has a molecular weight of 160 kDa. This protein is widely expressed in normal epithelia and some mesenchymal tissues. It is not generally found in haematopoietic or lymphoid cells (Prigent *et al.*1992). The c-erbB3 protein is a candidate receptor for an EGF-like growth factor, such as amphiregulin

(Ciardiello *et al.*1991). The 6.2 kb mRNA for c-erbB3 has been detected in a variety of human fetal and adult tissues (Kraus *et al.*1989). Overexpression of c-erbB3 mRNA was found in breast cancer cell lines but not in haematopoietic malignant cell lines (Kraus *et al.*1989).

#### **The c-erb B4**

The fourth member of EGF receptor family, termed erbB4/HER4 just recently was described. The erbB4 gene encodes a 180 kd transmembrane protein with an extracellular domain which is most similar to the erbB3 receptor and a cytoplasmic kinase domain exhibiting 79% and 77% identity with EGF receptor and erbB2 respectively (Plowman *et al.*1993). The erbB4 protein is a possible receptor for a heparin-binding protein. The erbB4 protein has been found that predominantly express in several breast carcinoma cell lines, and in normal tissue including skeletal muscle, heart, pituitary, brain and cerebellum.

#### **Ligands for erb B gene family**

Cell-cell interaction is an essential requirement for the integrated function of a multicellular organism during development and natural life. Secreted molecules and their specific cell surface receptors are key components of this cellular communication network. Ligand-receptor interaction on the cell surface is translated into activation of intracellular pathways, initiating a sequence of events that eventually result in a specific cellular response. (Yarden *et al.*1988). The functioning of the erbB gene family is a series of

ligand-dependent events.

The ligand for the *erbB1* gene is EGF which was first found in mouse submaxillary glands (Cohen, 1962), and later discovered in human urine as urogastrone (Reddy *et al.*1980; Cohen *et al.*1975). EGF was described first as a single polypeptide chain, the mature form with 52 amino acids displaying 3 internal disulphide bonds (Cohen, 1962). The cDNA cloning revealed that EGF is synthesized from a precursor which is as long as 128 kDa with approximately 1200 residues (Gray *et al.*1983; Scott *et al.*1983; Reddy *et al.*1980). The sequence of this precursor includes not only the sequence of EGF but also eight EGF-like units near the carboxyl terminus, and a hydrophobic sequence characteristic of an integral membrane protein. Subsequent studies with transfected cells have demonstrated that prepro-EGF can exist as a glycosylated membrane protein (Mroczkowski *et al.*1989). The means by which EGF is processed from the precursor molecule is not known, and there is substantial interest in other functions of the precursor such as intracrine function, since the prepro-EGF retains EGF-like biological activity (Mroczkowski *et al.*1989). The observation that the EGF precursor is not often processed into mature EGF (Rall *et al.*1985) has led to the suggestion that the EGF precursor may be a receptor for an unknown ligand (Pfeffer *et al.*1985). There is as yet no experimental confirmation of this hypothesis.

According to the results of two dimensional nuclear magnetic resonance analysis EGF has a globular structure (Burgess *et al.*1988). Sequence analysis of EGF revealed the presence of six conserved cysteine residues which cross-bond to create three peptide loops. Several other peptides, also members of EGF family, contain the same general

motif as EGF. These peptides include TGF- $\alpha$ , amphirgulin, heparin-binding EGF and cripto (Massague, 1983; Lin *et al.*1988; Higashiyama *et al.*1991; Hommel *et al.*1991). The secondary structural conservation and the disulphide bond configuration in the core sequences of the EGF and EGF like peptides are the basis for the ability of these factors to interact with the same receptor, usually referred to as the EGF receptor (Derynck, 1992). An interesting feature of these peptides is that they are all synthesised as much larger membrane-bound, glycosylated precursors. These EGF-like sequences are not confined to growth factors but have been observed in a variety of cell-surface and extracellular proteins which have properties in cell adhesion, protein-protein interaction and development (Derynck, 1992).

Besides EGF, this family also contains TGF- $\alpha$  (transforming growth factor alpha)(Marquardt *et al.*1984), amphiregulin (AR)(Shoyab *et al.*1988), and heparin-binding EGF-like growth factor (HB-EGF) (Higashiyama *et al.* 1991). As a family, they all share structural homology, but are encoded by distinct genes (Carpenter *et al.*1990), and different mRNA(Goustin *et al.*1986). Due to the structure homology, TGF- $\alpha$  is able to share the same receptor, although antibodies against different epitopes of the EGF receptor ligand-binding domain indicate the TGF- $\alpha$  and EGF may not bind to the same site on the receptor (Korc *et al.*1991). It has been demonstrated that TGF- $\alpha$  is highly expressed in fetal stages, and it may be the case that EGF is the adult form of the embryonic growth factor TGF- $\alpha$  (Goustin *et al.*1986).

The ligand for c-erbB2/NEU remains unclear, although there are a number of reports proposing molecules with c-erbB2/NEU ligand-like activity (Holmes *et al.*1992; Dobashi

*et al.*1991; Lupu *et al.*1990).

A 30kd glycoprotein, termed as gp30 was secreted by the MDA-MB-231 human breast cancer cell line, and was able to stimulate phosphorylation of both c-erbB2 p185 and the EGF receptor directly and independently (Lupu *et al.*1990). The gp30 protein was shown to specifically inhibit the growth and suppress the soft agar colony formation of SKBR-3 cells. The anti-EGF receptor antibody had no effect on the inhibition of SKBR-3 cell colony formation produced by gp30. The direct binding of gp30 to c-erbB2 growth factor receptor was confirmed by binding competition experiments with anti-c-erbB2 antibody. It thus appears that gp30 is a ligand for c-erbB2, and also for the EGF receptor.

A 45 kd glycosylated protein termed as Heregulin, also has been proposed as the specific ligand for the c-erbB2 receptor. The Heregulin possesses the EGF-like general structure and is synthesised as a larger precursor, but only binds to and activates the c-erbB2 receptor (Shepard *et al.*1991).

Several other proteins also have been reported as the ligand for the c-erbB2 including a 35 kd glycoprotein which is heat-stable but sensitive to reduction. This protein was partially purified from the conditioned media of cell transformed with activated ras oncogene. This factor could induce phosphorylation of the NEU p185 protein and stimulate proliferation in haemopoietic cells transfected with the rat NEU oncogene. The factor is also active on the EGF receptor (Yarden *et al.*1989; Yarden *et al.*1991). A 25kd polypeptide also has been reported to interact with the rat NEU receptor (Dobashi *et al.*1991). It is possible that all the proteins described above are

ligands for the c-erbB2 receptor, as is the case for the multiple ligands of the EGF receptor. However, since all the genes encoding these proteins have not been cloned and the proteins have not been purified to homogeneity, there is no sequence information available for comparison of these possible ligands for the c-erbB2 receptor at the present time.

AR is a secreted heparin-binding growth factor originally isolated from a human breast carcinoma cell line (Shoyab *et al.* 1988; Shoyab *et al.* 1989). The 1.4 kb transcript for AR encodes a 252 amino acid transmembrane precursor that is proteolytically processed to release the 78 amino acids, secreted molecule (Plowman *et al.* 1990a). It has been observed that AR shares significant structural and functional homology with EGF and TGF- $\alpha$  (Shoyab *et al.* 1989), and it has been suggested that it is a putative ligand for c-erbB3. Support for this view comes from evidence that both AR and c-erb B3 are highly expressed in colon cancer (Ciardiello *et al.* 1991).

Since a partially purified heparin-binding protein specifically stimulates the intrinsic tyrosine kinase activity of erbB4 receptor while having no effect on the phosphorylation of EGF receptor, erbB2 and erbB3 (Plowman *et al.* 1993), it has been suggested that the heparin-binding protein is a potential ligand for erbB4 receptor.

### **Receptor-mediated signal transduction**

Currently, two general mechanisms of cellular signal transduction are relatively well understood: coupling of receptors to various effectors by means of G proteins (Gilman, 1987) or coupling through the activation of a tyrosine-specific protein kinase activity that

is intrinsic to the receptor molecule. It has been demonstrated that the EGF receptor-mediated signal transduction is a direct protein-tyrosine kinase rather than a G protein coupling pathway. The means by which kinase activity is induced in all growth factor receptors remains unclear but the interaction of epidermal growth factor with its receptor is perhaps the best characterized.

The transduction of signals from the plasma membrane involves receiving an extracellular mitogenic signal and translocating that signal across the plasma membrane; then a cascade of events are initiated that eventually result in a specific cellular response. The machinery includes the growth factor receptor, its substrates, a number of key enzymes (including kinases and lipases), cytoskeletal proteins, transcriptional factors, DNA-binding proteins, and lastly a complex of enzymes which channel deoxy and ribonucleotide precursors into the growth forks of DNA replication (Reddy *et al.* 1980). Cell surface receptors are key components of this cellular communication network.

The members of the EGF receptor family are cell surface transmembrane molecules with extracellular domain which is composed of ligands binding sites and allow initiation of the interaction of ligand-receptor. In response to the ligand binding, the receptor may undergo many changes. The most widely cited model for receptor response is the "cluster" proposal. In this model, EGF binding induces an intermolecular allosteric change which involves receptor redistribution in the membrane, or an association with other membrane proteins, most likely dimerization (homodimer or heterodimer) (Schlessinger, 1988). After ligand binding, receptors can also be seen to cluster on the cell surface (aggregation) prior to down regulation as the ligand-receptor complex is

internalized into receptosomes (Pastan *et al.*1981). It has been suggested that the aggregation of the receptor prior to internalization is essential for activation of the kinase domain. Although it has been suggested that dimer formation may be a consequence of ligand binding, rather than a prerequisite for kinase activation and hence signal transduction (McDonald *et al.*1989), the experiments done on EGF receptor and c-erb B2/NEU interaction indicate that dimers play a positive role in the activation of the intracellular substrate (Goldman *et al.*1990; Stern *et al.*1988a).

The residues phosphorylated in all members of the erbB gene family are now known to be tyrosine. Four major autophosphorylation sites (Try 1173, Try 1148, Try 1086 and Try 1068 ) of EGF receptor intracellular domain have been proposed as essential for the induction of intracellular events which are necessary for mitogenesis (Chen *et al.*1987; Stern *et al.*1988a). Thus in addition to phosphorylating intracellular substrates, receptor kinase activity is required for autophosphorylation of multiple tyrosine residues. The presence of these tyrosine residues are essential for maximal biological activity of the receptor (Helin *et al.*1991). The tyrosine-phosphorylated carboxyl-terminal fragment of the EGF receptor has been shown to represent an association site for phospholipase C, mediated by the src homology 2 (SH2) domains of phospholipase C (Cantley *et al.*1991).

Recently, substantial progress has been made in identifying tyrosine kinase substrates for membrane receptors. This permits construction of a potential mitogenic signal pathway. It depicts five proteins as tyrosine kinase substrates: PLC (Margolis *et al.*1989), PI-3 kinase (Kaplan *et al.*1987), src and src-like tyrosine kinase (Molloy *et al.*1989;

Ralston *et al.*1985) and raf kinase (Morrison *et al.*1989). Although this intracellular signal pathway is not complete, it has been noted that the complex signal transduction cascade terminates with the nuclear transcription of genes, such as c-fos and c-myc that directly regulate cell cycle progression (Ullrich *et al.*1990; Koskinen *et al.*1990).

Several recent studies demonstrated that members of the steroid hormone receptor superfamily are phosphoproteins and that they could be phosphorylated in steroid-independent manner by a membrane receptor ligand, the neurotransmitter dopamine (Power *et al.*1991b; Power *et al.*1991a). This observation indicates there is a very complex network involved in the signal transduction pathway. Peptide membrane receptors may interact with steroid hormone receptors to control gene expression. It has been shown that the phosphorylation of the steroid hormone receptors, including the progesterone receptor (PR) (Power *et al.*1991b; Denner *et al.*1990), the chicken ovalbumin upstream promoter transcription factor (COUP-TF) (Power *et al.*1991a) and the glucocorticoid receptor (GR) (Hoeck *et al.*1990) can occur in vitro when treated with dopamin or 8-bromo-cyclic adenosine monophosphate (8-Br cAMP), an activator of cAMP-dependent protein kinase A (PKA), in the absence their natural steroidal ligands. Phosphorylation has been shown to modulate the steroid hormone receptor-mediated transcription in vivo (Denner *et al.*1990). However, It is not clear how the phosphorylation modulates the biological activity of steroid receptors. Previous studies have implied that phosphorylation is required to create the DNA binding state of GRs and ERs (Mendel *et al.*1986; Migliaccio *et al.*1989). It remains unclear how the ligand-independent phosphorylation occurred and what site of the receptor is involved in the

activating phosphorylation. Experimental study has suggested that the protein kinase A is involved in phosphorylation of the PR (Power *et al.*1991b; Power *et al.*1991a; Denner *et al.*1990) and phosphorylation on tyrosine residue of in vitro synthesized human estrogen receptor could activate its hormone binding (Migliaccio *et al.*1989; Bagchi *et al.*1992). It is not clear how the signal from peptide membrane receptor to steroid hormone receptor is transduced in the complicated intracellular network, but phosphorylation is key to these events.

### **Function of the erb B gene family**

Growth factors are present in extracellular fluids and secretions including plasma, urine, saliva, amniotic fluid, and milk (Carpenter, 1985). However growth factors appear to be synthesized by specific cell types; for example, epithelial cells are the major source of TGF- $\alpha$  synthesis under normal conditions (Valverius *et al.*1989).

The physiological role of the erbB gene family is not well defined. Synthesis of EGF in mouse salivary glands is strongly induced by both androgens and  $\beta$  adrenergic agonists (Carpenter *et al.*1979a). It is detectable in saliva, milk and urine but, in man, circulates primarily in alpha granules in platelets (Oka *et al.*1983), suggesting a role in wound healing analogous to PDGF and TGF- $\alpha$ , which are also found in platelet granules (Ross *et al.*1986b). In vitro, EGF is a potent mitogen for many cultured cells, and in vivo, it stimulates the proliferation and differentiation of skin tissue, corneal, lung and tracheal epithelia (Catterton *et al.*1979; Sundell *et al.*1980). Daily subcutaneous injections of EGF into newborn mice accelerates incisor eruption and eyelid opening (Goustin *et*

*et al.* 1986) as well as causing a marked stunting of the animals with an inhibition of hair growth (Cohen, 1962). EGF also inhibits gastric acid and pepsin secretion and stimulates gastrointestinal cell proliferation (Dembinski *et al.* 1982). EGF may also influence the male reproductive function in mice by stimulating the meiotic phase of spermatogenesis (Tsutsumi *et al.* 1986). Recently, it has been shown that EGF antisense oligonucleotides can block the initiation of odontogenesis in murine mandibular explants (Kronmiller *et al.* 1991).

TGF- $\alpha$  was originally discovered in the medium of tumor cells (De Larco *et al.* 1978). However the normal role of the endogenous TGF- $\alpha$  is likely to drive epithelial cell proliferation. Exogenously administered EGF or TGF- $\alpha$  can influence the development of several tissue. Similar to EGF, exogenous TGF- $\alpha$  accelerates tooth eruption and eyelid opening in new born mice (Smith *et al.* 1985). Since TGF- $\alpha$  is highly expressed in fetal stages, it has been suggested that TGF- $\alpha$  may play a role in morphogenesis and organogenesis during development (Derynck, 1992). In adults, TGF- $\alpha$  mRNA and/or protein have been detected in the epithelia cells of the anterior pituitary (Samsoundar *et al.* 1986), maternal decidua (Han *et al.* 1987), skin keratinocytes (Coffey *et al.* 1987), bronchus, intestine, kidney tubules, female genital tract (Kommos *et al.* 1990), brain (Wilcox *et al.* 1988), and activated macrophages (Madtes *et al.* 1988). TGF- $\alpha$  has also been implicated in inflammation and wound healing (Barton *et al.* 1991; Schulze *et al.* 1987), cell migration (Barrandon *et al.* 1987), angiogenesis (Schreiber *et al.* 1981) and bone resorption (Stern *et al.* 1985).

Little is known of the involvement of EGF and TGF- $\alpha$  in neoplasia. Elevated

levels of EGF have been reported in 12% of pancreatic cancers (Barton *et al.*1991), 86% of pleomorphic adenomas of salivary gland (Yamahara *et al.*1988), 68% of prostatic carcinomas (Fowler *et al.*1988) and 20% of gastric carcinomas (Sugiyama *et al.*1989). The elevated expression of TGF- $\alpha$  has also been frequently associated with neoplastic transformation. Recently, research has focused on the receptors (the erbB gene family) and their correlation with cancer.

## The erb B gene family and cancer

Growth factors and their receptors are commonly expressed in normal tissue and play a role in cell proliferation and influence differentiation. The first link of growth factors and their receptors with cancer was proposed based on the evidence that neoplastically transformed cells show a decreased serum requirement for growth in culture systems compared to the normal cells in culture system (Temin, 1966; Paul *et al.* 1971; Dulbecco, 1970). It suggests that transformed cells produce their own factors to support the growth instead of the exogenous factors in serum. With the advent of serum-free-culture techniques (Barnes *et al.* 1980) and the availability of purified growth factors the altered serum requirement in transformed cells could be translated into a diminished or absent requirement for a specific exogenous growth factor. The loss of requirement for specific growth factors is a common finding in many types of cancer cells (Moses *et al.* 1981; Kaplan *et al.* 1987), which could be mediated by (1) the activation of autologous growth factor synthesis, (2) unregulated expression of growth factor and growth factor receptor or substrates of signal transduction chain, or (3) synthesis of an altered growth factor receptor or substrate. Genetic aberrations in growth factor signalling pathways are also inextricably linked to developmental abnormalities and to a variety of cancers. Some of the more convincing evidence linking growth factors and cancer has come from the function of receptors as oncogenes.

### Evidence for EGF receptor (erb B 1) and its ligands

The biological chain of EGF receptor-mediated ligand-dependant signal transduction is involved in regulating proliferation and differentiation of certain cells. As a consequence, any quantitative or qualitative change may influence the outcome of signal transduction and cause an abnormal growth response.

As proto-oncogenes, the potential of this gene family to contribute significantly to the processes of transformation in vivo is demonstrated most convincingly by the EGF receptor itself. A direct link between the EGF receptor and carcinogenesis was provided by the high structural homology between the EGF receptor and a transforming protein v-erbB of the avian erythroblastosis virus. It has been demonstrated that the amino acid sequence of the human EGF receptor is nearly identical (95% homology) to the predicted amino acid sequence of the protein encoded by the v-erb B oncogene (Downward *et al.*1984b; Ullrich *et al.*1984). The v-erb B oncogene was initially identified in avian erythroblastosis virus and was also activated as a cellular oncogene by insertional mutagenesis in chicken erythroleukemias. Further studies confirmed that the v-erb B oncogene was a truncated version of the EGF receptor that contains its tyrosine kinase and transmembrane domains but lacks the extracellular ligand binding domain (Downward *et al.*1984b). The v-erbB gene has also sustained other mutations relative to the EGF receptor, including a caboxy-terminal deletion which is a major site of self-phosphorylation and an important regulating region, and a point mutation in the kinase domain (Ullrich *et al.*1984). These mutations appear to be primarily responsible for its transforming activity. It is believed that the tyrosine kinase activity of normal EGF receptor is suppressed in the absence of EGF, and EGF binding relieves this negative

regulation, resulting in activation of the receptor tyrosine kinase. In the *erb B* oncogene, the structural consequence of the major deletion is to generate a protein which does not have the ligand binding domain. The mutation appears to abrogate the normal ligand-dependent mechanism which leads to a constitutively active protein-tyrosine kinase activity. The resulting abnormal cell proliferation contributes to neoplastic transformation.

The EGF receptor gene itself is able to become an oncogene if a viral promoter or activator is inserted into the cellular gene. This appears to be the mechanism at work in the avian leukosis virus caused transformation (Fung *et al.* 1983). In a chicken with leukosis, the virus promoter was mapped in front of the *c-erb B* gene. Additionally, increased levels of RNA which hybridize to probe against *v-erb B* were detected in the erythroblasts. Thus the inserted promoter appears to successfully induce over transcription of the EGF receptor.

Functionally, both EGF receptor and several transforming proteins which are also protein kinases, have the unusual property of being able to phosphorylate tyrosine residues (Heldin *et al.* 1984; Roussel *et al.* 1984). It has been suggested that the group of nonreceptor protein-tyrosine kinases, including *src*, *yes*, *fgr*, *luk*, *fyn*, *lyn* and *hck*, may work as substrates of the cell surface receptors, and be activated by phosphorylation at their two important tyrosine residues which are conserved through the members of the *src* protein kinase subfamily (tyrosine-416 and tyrosine-527). Phosphorylation of tyrosine 416 residue apparently increases both kinase activity and transforming potential of *src* protein. In contrast, phosphorylation of tyrosine 527 appears to down-regulate *src* kinase activity.

This indicates that the function of kinases may be regulated by phosphorylation and dephosphorylation of tyrosine residues in normal cells. Although the normal physiological pathways that mediate this regulation remain unknown, it is clear that loss of this control leads to increased kinase activity and resulting cell transformation. It has been demonstrated that the src subfamily of protein-tyrosine kinases contains amino-terminal regulatory sequences. These sequences have been divided into two domains, designated SH-2 (src-homology-2) and SH-3 (src-homology-3), which are conserved throughout members of the src subfamily (Ralston *et al.* 1985; Kypta *et al.* 1990). SH2 domains bind preferentially to tyrosine phosphorylated proteins. Mutations in SH-2 reduce both transforming potential and intracellular kinase activity, indicating that SH-2 is a positive regulatory domain. In contrast, SH-3 appears to be a negative regulatory domain since deletion mutations of SH-3 have been shown to activate src transforming potential. It has therefore been proposed that SH-3 is a site of interaction with a cellular factor that normally inhibits intracellular protein-tyrosine activity. The SH-3 domain may also be a site which could bind to plasma membrane or the cytoskeleton (Koch *et al.* 1991). Thus, the EGF receptor and src oncoproteins may both stimulate cell growth through the same or parallel phosphorylated intracellular pathways. This implies that enhancement of the signal in the middle of the transduction sequence could sufficiently induce abnormal growth without any structural changes of receptor and ligand.

Although expression of a protein resembling viral erbB in spontaneously occurring tumors has not yet been described (Goustin *et al.* 1986), the unregulated expression of kinase activity may represent a possible influence on cell growth (Yamamoto *et al.* 1983;

Gilmore *et al.*1985; Kris *et al.*1985). This quantitative change of kinase activity may account for EGF receptor-induced malignant transformation in human cancers. It has been presumed that the overexpression of EGF receptor without mutation may similarly provide an oncogenic function by increasing formation of the active ligand-receptor complex, thus enhancing the signal transduction to the nucleus and augmenting cell growth. Gene amplification and thus overexpression of EGF receptor has been found in various human neoplasms and cell lines such as A431 human epidermoid carcinoma cell line (Lin *et al.*1984; Merlino *et al.*1984; Ullrich *et al.*1984; Libermann *et al.*1985a; Yamamoto *et al.*1986a). Amplification of the EGF receptor gene appears to be a mechanism by which tumor cells improve their capacity to respond to EGF or TGF- $\alpha$ . The biological significance of amplification and enhanced expression of the EGF receptor proto-oncogene was investigated by gene transfer experiments using a vector in NIH 3T3 cells that led to expression of high levels of the normal EGF receptor. Analysis of these transfected cells indicated that their normal proliferative response to epidermal growth factor was enhanced and that, in the presence of epidermal growth factor, they displayed a transformed phenotype (Di Fiore *et al.*1987b). These results thus provided direct evidence supporting for the significance of amplification and overexpression of the normal EGF receptor in neoplastic transformation, although in this case the transformed phenotype was dependent on stimulation of the receptor by EGF.

Amplification and overexpression of EGF receptor has been found in variety of human tumors, including gliomas and meningiomas (Libermann *et al.*1985b), squamous carcinoma of the lung (Hendler *et al.*1984) and ovarian, breast, cervical and renal

carcinomas (Sainsbury *et al.*1987; Perez *et al.*1984; Mydlo *et al.*1989). This suggests that the amplification and overexpression of EGF receptor is one of the features of malignancy.

With the native EGF receptor, signal transduction is a ligand-dependent pathway. The correlation with cancer of EGF and TGF- $\alpha$ , which are the natural ligands for the EGF receptor, has been investigated. Gene transfer assays using appropriate molecular constructs indicated that constitutive overexpression of either TGF- $\alpha$  or EGF was sufficient to promote transformation of appropriate recipient cells expressing EGF receptor (Watanabe *et al.*1987; Stern *et al.*1987). In addition, EGF enhances viral transformation of cells (Fisher *et al.*1979); in rat embryo cells infected by adenovirus and then treated with EGF, the colonies appeared sooner, grew faster, and had a more diffuse morphology than did non-EGF treated cells (Fisher *et al.*1979). In another example, EGF enhanced the transformation of cultured granulosa cells infected by the Kirsten murine sarcoma virus (Harrison *et al.*1981). In the absence of EGF, infected cells formed few transformed foci, whereas in its presence, the cells formed many foci of rounded cells; removal of EGF resulted in the reversion of the majority of the infected granulosa cell to a normal phenotype. In this instance, the presence of EGF was essential for the transformed phenotype. EGF also enhances the carcinogenic potential of methylcholanthrene, a chemical carcinogen, in skin (Stoscheck *et al.*1986). Transformation of normal cells, in contrast to certain established cell lines, requires the action of at least two complementary oncogenes (Land *et al.*1983a; Land *et al.*1983b). Thus in this case, EGF is apparently substituting for one of the oncogenes. Recently, it was reported that NIH 3T3 cells

transfected with a full-length EGF precursor was sufficient to induce transformation (Heidaran *et al.*1990). This experiment indicate that EGF could act as a oncogene.

The other ligand for the EGF receptor, TGF- $\alpha$ , is highly expressed in early fetal stages and decreases in normal mature stages; this has led to the view that TGF- $\alpha$  is an embryonic form of EGF (Twardzik *et al.*1985; Lee *et al.*1985). The appearance or enhancement of TGF- $\alpha$  in a variety tumor cells implicates TGF- $\alpha$  as an important component of cellular transformation (Sporn *et al.*1985a).

The possible link between the production of TGF- $\alpha$  and the transformed phenotype was apparent from the initial discovery of the growth factor. TGF- $\alpha$  was first detected in the medium of murine sarcoma virus-transformed cells and several other retrovirus-transformed fibroblasts. Its name originated from the observation that preparations of this growth factor had the ability to induce phenotypic transformations of normal rat kidney cells, an immortalized fibroblast cell line in culture. This transforming activity, which was apparent from the acquisition of anchorage independence in soft agar, from different appearance and loss of contact inhibition in monolayer culture, was phenotypical and reversible; removal of the TGF- $\alpha$  preparations resulted in a reversal to the normal phenotype (Todaro *et al.*1980). Examination of a variety of cell lines subsequently showed that TGF- $\alpha$  was not only synthesized by the cells which are transformed by viruses, but was commonly expressed among human tumor cell lines (Bates *et al.*1988). Among human tumor types, the carcinomas are most likely to synthesize TGF- $\alpha$ . Elevated production of TGF- $\alpha$  is found in malignancies of the liver (Raymond *et al.*1989), breast (Warri *et al.*1991),stomach (Bennett *et al.*1989), lung (Liu *et al.*1990; Tateishi *et*

*al.*1991), colon (Liu *et al.*1990), kidney (Kommoss *et al.*1990), pancreas (Smith *et al.*1987), ovary (Kommoss *et al.*1990) and tumours of the thyroid (Aasland *et al.*1990).

This result suggests that TGF- $\alpha$  could be important for tumor development and/or maintenance of the transformed state.

The tumorigenesis potential of TGF- $\alpha$  has been tested indirectly by measurement of the proliferation rate of tumor cells. Exogenous TGF- $\alpha$  can strongly stimulate the proliferation of polyoma-transformed cells which have a low level of endogenous TGF- $\alpha$  (Salomon *et al.*1987a). In another experiment, the rate of skin papilloma formation in nude mice by cells that were or were not transfected with a TGF- $\alpha$  expression vector was compared; TGF- $\alpha$  expression resulted in increased tumor size and proliferation rate (Finzi *et al.*1988). Since overexpression of TGF- $\alpha$  was frequently detected in human tumor tissue, the contribution of TGF- $\alpha$  in the progression of the cell toward a fully transformed phenotype has been examined by using transgenic mice. Expression of TGF- $\alpha$  under the control of the metallothionein gene promoter in transgenic mice leads to disordered growth and differentiation in breast, liver and pancreas (Sandgren *et al.*1990; Jhappan *et al.*1990). Recent evidence that a high level of TGF- $\alpha$  influences motility and the capability of the cells to digest the extracellular matrix (Gavrilovic *et al.*1990) would indicate that TGF- $\alpha$  could contribute to a more invasive phenotype in vivo.

Examination of human tumor cell lines and biopsies revealed that expression of TGF- $\alpha$  is frequently accompanied by enhanced synthesis of the EGF receptor, at least as assessed by Northern analysis of mRNA. This is most striking in the case of squamous and renal carcinomas. The concurrent expression of TGF- $\alpha$  and EGF receptor have also

been identified as a feature of malignant tumors in both gastric and breast tissue (Bennett *et al.*1989; Mydlo *et al.*1989; Di Marco *et al.*1990a; Derynck *et al.*1987; Bates *et al.*1988). An explosion of information suggests that co-expression of EGF, TGF- $\alpha$  and EGF receptor in a variety of human neoplasms show a significant correlation with the depth of tumor invasion.

However, there is not a simple or absolute correlation of EGF receptor levels to cancer, even though many tumors do overexpress EGF receptor and EGF or TGF- $\alpha$ . In some transformed cells, including those transformed by viruses, the level of EGF receptor decreases (Todaro *et al.*1980; Ozanne *et al.*1980; Twardzik *et al.*1983b; Moses *et al.*1978; Twardzik *et al.*1983a). This decline is thought to be caused by the production of TGF- $\alpha$  and EGF which can down regulate the receptor (Carpenter *et al.*1983).

#### **c-erbB2/NEU and cancer**

The c-erbB2/NEU oncogene encodes a transmembrane protein- tyrosine kinase which is closely related to erbB1. However, the c-erbB2/NEU protein does not bind EGF and thus appears to be a receptor for a so far unidentified ligand.

The interest in c-erbB2/NEU and cancer has been focussed on the high frequency of gene amplification seen in several types of human adenocarcinomas including tumors of the breast, ovaries (Slamon *et al.*1989), stomach (Kraus *et al.*1987), salivary gland and digestive tract (Yokota *et al.*1986), kidney (Yokota *et al.*1986), skin (Maguire *et al.*1989), pancreas (Williams *et al.*1991) and lung (Kern *et al.*1990). Gene amplification has been detected in almost 25% of in breast and ovarians cancers. In breast and ovarian

carcinomas, the level of amplification and overexpression of the c-erbB2/NEU gene correlated inversely with patient survival, implying that this proto-oncogene plays a role in the clinical progression of these cancers (Slamon *et al.*1989). This conclusion awaits the results of more extensive studies.

Although the correlation of c-erbB2/NEU gene alteration and cancer is compelling, the biological significance of amplification and overexpression of the normal c-erbB2/NEU protein remains to be defined. Gene transfer experiments have demonstrated that high level expression of normal c-erbB2/NEU is indeed sufficient to induce cell transformation (Di Marco *et al.*1990b; Di Fiore *et al.*1987b; Hudziak *et al.*1987; Velu *et al.*1987; Riedel *et al.*1988). Since the ligand of c-erbB2/NEU is not well characterized, it is difficult to directly test the mechanism of c-erbB2/NEU receptor action in cancer. The structural homology between erb B1 and c-erbB2/NEU receptors indicates that c-erbB2/NEU-induced transformation may be mediated by enhanced signal transduction through phosphotyrosine kinase pathways. Experimentally, when overexpressed in NIH 3T3 cells, the carboxyl-terminal domain of c-erbB2/NEU gene product, gp185 erbB2, displays a potent transforming ability as well as constitutively elevated levels of tyrosine kinase activity in the absence of exogenously added ligand (Di Fiore *et al.*1990a; Di Fiore *et al.*1990b; Lonardo *et al.*1990). Constitutive receptor dimer formation and increased tyrosine kinase activity has also been detected in vitro and in vivo with a c-erbB2/NEU receptor with a single amino acid mutation in the transmembrane domain (Bargmann *et al.*1988a; Yarden, 1990). This mutated c-erbB2/NEU receptor has been demonstrated in a spontaneous rat neuroblastoma; no comparable mutation has been found in any human

tumor. Experimental replacement of a single valine residue by glutamic acid residue (Bargmann *et al.*1986b) in the rat NEU gene product causes elevated tyrosine kinase activity. Thus, structural mutation of receptors in the transmembrane domain could effect intracellular signal transduction chains which will lead the receptor to act as an oncogene. This observation also implies that enhancement of tyrosine kinase activity is a important event in transformation, which may occur by amplification and overexpression of c-erbB2/NEU in human tumors.

Activation of the tyrosine kinase function of the c-erbB2/NEU protein is essential for cellular transformation by the oncogenic receptor (Bargmann *et al.*1988b). Monoclonal antibodies to c-erbB2/NEU protein (Lehvaslaiho *et al.*1989; Lee *et al.*1989) demonstrated that the kinase function of the c-erbB2 receptor can be allosterically regulated by antibody or ligand binding to the extracellular domain. This result implies that the c-erbB2/NEU-mediated pathway is likely ligand-regulated. Yet c-erbB2/NEU and erbB1 receptors do not share ligands(Yarden *et al.*1989). EGF can stimulate c-erbB2/NEU receptor autophosphorylation in cells which co-express EGF and c-erbB2/NEU (Stern *et al.*1988a; King *et al.*1988; Kokai *et al.*1988; Di Fiore *et al.*1990b). This observation indicates that heterodimerization of erbB1 and c-erbB2/NEU receptors may be involved in co-functional regulation of cancer cell growth (Goldman *et al.*1990). Synergistic interaction of c-erbB2/NEU and erbB1 receptor has led to transformation of rodent fibroblasts (Kokai *et al.*1988) and may be one of the features of the erb B gene family in human tumors. Co-expression of erbB1 and erbB2 have been observed in several human tumors including breast, pancreatic, bladder and ovarian carcinomas.

### **The erb B3 and cancer**

The c-erb B3 is a relatively new member of the erb B gene family. It has been suggested that c-erb B3 is a candidate receptor for EGF-like growth factors. The significance of c-erb B3 in cancer growth is not clear. The overexpression of c-erb B3 has been observed in certain breast carcinomas (Kraus *et al.*1989) and in both primary and metastatic human colorectal cancers and colon cancer cell lines. Amphiregulin, which is structurally related to EGF, is also found to be highly expressed in colorectal carcinomas leading to the suggestion that it is the c-erb B3 ligand (Ciardiello *et al.*1991).

### **The erbB4 and cancer**

The directly relationship of erbB4 and cancer is not clear. However, the overexpression of erbB4 have been found in several mammary adenocarcinoma cell lines, neuroblastoma and pancreatic carcinoma cell lines. A potential erbB4 ligand, partially purified heparin-binding protein, is able to stimulate tyrosine phosphorylation and induce phenotypic differentiation in a human mammary tumor cell line that overexpresses erbB4 (Plowman *et al.*1993). This evidence indicates that erbB4 may play a role in cancer cell growth.

### **Erb B gene family and prostate cancer**

Development of the prostate and maintenance of adult structure and function, as well as its pathological disturbances, benign prostatic hyperplasia and prostate cancer, are strongly influenced by androgens. Androgen ablation has been established as a classical

therapy in clinical treatment for prostate cancer patients. However, a critical dilemma facing clinicians treating patients with prostate cancer is that most patients relapse following androgen ablative treatment (Mendel *et al.* 1986; Isaacs *et al.* 1987). The failure of clinical treatment indicates that other mechanisms are involved in the regulation of prostate cancer growth. The evidence clearly points to a multistep process of carcinogenesis and multifactorial contribute to the prostate cancer progression.

It has been suggested that peptide growth factors and their receptor are involved in this tumorigenic process. The suggestion that the c-erbB gene family plays a role in prostate cancer comes from the observations of other hormone-regulated cancers including breast (Mydlo *et al.* 1989; Slamon *et al.* 1989) and ovarian (Filmus *et al.* 1985), as well as other common tumors including lung, bladder, renal and colorectal carcinomas (Todaro *et al.* 1980; Yamamoto *et al.* 1986b; Yoshida *et al.* 1990; Bennett *et al.* 1989). These reports clearly implicate the c-erbB2 gene family in carcinogenesis, even though the mechanism is still under investigation.

The correlation of the c-erbB2 gene family and prostate cancer has also been suggested. The EGF-receptor has been demonstrated biochemically and immunocytochemically in human BPH and CaP tissues (Maddy *et al.* 1989; Eaton *et al.* 1988) and human prostatic cell lines (Schuurmans *et al.* 1988; Lin *et al.* 1988). In an initial study in this area, Morris & Dodd examined EGF receptor expression using RNase protection assay (Morris *et al.* 1990). EGF receptor mRNA levels were significantly higher in carcinoma (CaP) samples (N=38) than in benign hyperplasias (BPH) samples (N=35) ( $p < 0.01$ ). The highest levels were found in two prostatic

carcinoma cell lines (DUI45 and PC-3) which are derived from metastases and are androgen-independent for growth. Human EGF has been found in prostatic fluid and in extracts of prostate tissue ((Gregory *et al.*1986; Elson *et al.*1984; Shaikh *et al.*1990). EGF and TGF- $\alpha$  have been detected in human prostatic cell lines (Derynck *et al.*1987; Connolly *et al.*1989; Connolly *et al.*1990). However, a clear picture of the role EGF and EGF receptor signalling in prostate cancer has not emerged. Reports in the literature present very different views on the potential importance of EGF receptor in prostate cancer (Schuurmans *et al.*1988; MacFarlane *et al.*1985), and conflicting values on the relative levels of EGF receptor in BPH and prostatic carcinoma. Also, some literature reports address tumor cell lines while others address tissue samples. In this thesis, we have attempted to address both the presence of the ligands and receptors, and their biological relevance through an examination of both human tumor samples and cell model systems.

Interest in c-erbB2/NEU has focussed on the high frequency of gene amplification seen in several types of human adenocarcinomas including breast and ovarian tumors (King *et al.*1985; Slamon *et al.*1989). In these tumors the level of amplification and the expression of the c-erbB2/NEU gene correlated inversely with patient survival implicating this proto-oncogene in the clinical progression of these cancers. Although distinct from the EGF-receptor, the c-erbB2/NEU protein may act synergistically with EGF-receptor to transform cells (Kokai *et al.*1989). Although the prognostic value of c-erbB2/NEU gene copy number has been examined in other adenocarcinomas, no studies in human prostate tumors has been reported. Recently, overexpression of c-erbB2/NEU protein has been

demonstrated in prostate cancer patients by using histochemical analysis (Zhou *et al.*1992). Experimentally, a tumorigenic phenotype has been generated by transfecting an activated neu into the rat ventral prostate epithelial cell line (Sikes *et al.*1992a).

In this study, we have sought to establish data on prostate tumors and the expression of the erbB gene family. We have also sought to evaluate the contribution of erbB-mediated pathways to prostate tumor cell growth using cell culture models.

## HYPOTHESIS

The c-erbB genes play an important role in the proliferation of prostate cancer cells by mechanisms which include (1) enhanced expression of the c-erbB proteins, and (2) response to endogenous production of corresponding growth factor ligands, resulting in autocrine regulation of growth.

# MATERIALS AND METHODS

## Materials

### Prostate tissue

Benign prostatic hyperplasia (BPH) tissue and carcinomatous prostate (CaP) tissue were obtained at the time of surgery, and graded prior to freezing at  $-70^{\circ}\text{C}$ . Tissues containing adenocarcinoma were categorized according to their state of glandular differentiation using the Gleason system (Gleason, 1977). Material containing at least 80% carcinoma was selected for analysis. None of the patients had received hormonal or radiation therapy prior to surgery. Patients with CaP ranged from 62 to 92 years of age (mean=74.3 years, n=10). Gleason scores ranged from 5 to 9 (median=7) BPH patients were selected for age match with the CaP samples (mean=75.0 years, n=21). Prostate tissue obtained during autopsy from a 19 year old man served as control for normal diploid cells.

### Cell lines

The three established human prostate cancer cell lines used in these studies (DU145, PC3 & LNCaP) were obtained from the American Type Culture Collection (ATCC) (ATCC, Rockville, MD). DU145, PC3 and LNCaP were derived from brain, bone and lymph nodes of metastasized prostate cancer respectively (Stone *et al.* 1978; Kaighn *et al.* 1979; Horoszewicz *et al.* 1983a). Two established breast cancer cell lines (SKBR-3 & MCF-7) (Kraus *et al.* 1987) were obtained from Dr. R. P. C. Shiu. The Ishikawa cell line of endometrial cancer was obtained from Dr L. J. Murphy. Stock cultures of these cells were maintained at  $37^{\circ}\text{C}$  in 5% carbon dioxide ( $\text{CO}_2$ ) incubators and grown as monolayers in either 10% (v/v) heat-inactivated fetal bovine serum (FBS) (UBI, Upstate Biotechnology,

Inc, Lake placid, NY) in Dulbecco's modified Eagle's media (DMEM), (Gibco BRL, Burlington, Ontario) (LNCaP, SKBR-3 & MCF-7) or Minimal Essential Media (MEM) (Gibco BRL, Burlington, Ontario), with 1% antibiotic-antimycotic solution (Gibco Laboratories, Grand Island, New York) (DU145 and PC3).

#### **Antibodies, growth factors**

The following antibodies were purchased from Oncogene Science (Oncogene Sciences, Manhasset, NY) and stored at 4°C: (1) anti c-NEU Ab-2 (100ng/ml) specifically recognizes the extracellular domain of c-erbB2/NEU protein and inhibits protein-tyrosine kinase activity. (2) anti c-NEU Ab-3 (100ng/ml) recognizes the c-terminal domain of c-erbB2/NEU receptor. (3) the Ab EGF receptor (100ng/ml) recognizes the c-terminal domain of EGF receptor. The following antibodies were purchased from Upstate Biotechnology Inc (UBI, Lake placid, New York): (4) the anti phosphotyrosine (Ab-P-tyrosin) with concentration of 100ng/ml and stored at 4 °C. (5) the anti EGF receptor (Ab EGF receptor-2) with stock concentration of 5uM, is specific for the extracellular domain of EGF receptor. (6) the anti EGF (Ab EGF) with the concentration of 10uM and (7) the anti TGF- $\alpha$  (Ab TGF- $\alpha$ ) with the concentration of 500ng/ul. The Ab EGF and Ab TGF- $\alpha$  are neutralizing antibodies which specifically inhibit EGF or TGF- $\alpha$  binding to the receptor. The Ab EGF receptor-2, Ab EGF and Ab TGF- $\alpha$  antibodies were stored at -20 °C. (8) The anti mouse IgG1 antibody with a concentration of 1mg/ml purchased from Sigma (Sigma St. Louis, MO), and stored at -20 °C.

## Hormones

The hormones 5 $\alpha$ -Dihydrotestosterone (DHT) and 17 $\beta$ -Estradiol (E2) were obtained from Sigma (Sigma, St. Louis, MO), and stored at 4 °C. The antiestrogen tamoxifen was a gift from Dr. L. Murphy. A stock solution of 10<sup>-2</sup>M in ethanol was prepared and stored in the dark at -20 °C.

## Deoxyribonucleic acid (DNA) and complementary deoxyribonucleic acid (cDNA)

### probes

The probe for c-erbB2/NEU was derived from the PMAC117 genomic DNA clone purchased from American Type Culture Collection (ATCC, Rockville, MD). The Acc1-BamHI unique sequences of erb B2/NEU from PMAC 117 have been subcloned into a pGEM-3Z plasmid (Promega, madison, WI).

The probe used for the detection of c-Fos (a gift from Dr. L. Murphy) in Northern blotting is a unique sequence fragment from PC-fos (human)-1 (ATCC, Rockville, MD).

The cDNA clone for human EGF receptor (#57346, ATCC, Rockville, MD) is a ClaI fragment which was subcloned into the vector of pBR322 plasmid (Promega, Madison, WI). The cDNA clone for human EGF (received from Dr. G. I. Bell) (Murray *et al.* 1986) is an EcoRI fragment from pE7, subcloned into pGEM blue vector (Promega, Madison, WI). The cDNA clone for TGF- $\alpha$  (#59950, ATCC, Rockville, MD) is a EcoRI fragment subcloned into pGEM blue vector. These probes have been used in both Southern and Northern hybridization analysis.

The probe for human prolactin (hPRL) is a fragment from clone (#3172, ATCC, Rockvill, MD). The probe for human growth hormone (GH) (#31389, ATCC, Rockville, MD) is a Hind III fragment subcloned into pBR322 plasmid. The cDNA probe of  $\beta$ -actin was a gift from Dr. R.J.Matusik. The Pip cDNA probe was received from Dr. R.P.C.Shiu (Murphy *et al.*1987) and the D2S5 probe was obtained from ATCC (#57178, ATCC, Rockville, MD). These probes were used in Southern and Northern blots for normalization of loading of samples and to assay for aneuploidy involving specific chromosomes.

### Primers

All primers were purchased from the DNA laboratory, University of Manitoba and stored at -20 °C.

Primers specific for the estrogen receptor:

5'-GTCAAATCCACAAAGCCTGGCACCCCTCTTC-3' (upstream)

5'-CCGCCGGCATTCTACAGGCCAAATTCAGAT-3 (downstream)

Primers specific for the actin sequence:

5'-ATCATGTTTGAGACCTTCAA-3' (upstream)

5'-CATCTCTTGCTCGAAGTCCA-3' (downstream)

Primers for the EGF receptor ATP-binding site:

5'-TCTTACACCCAGTGGAGAAG-3' (upstream)

5'-GTTGGCTTTCGGAGATGTTG-3' (downstream)

Primers for the EGF receptor phosphotyrosine sites:

5'-ACCGTGGCTTGCATTGATAGAA-3' (upstream)

5'-CTGGATTTTTAGGGCTCATACT-3' (downstream)

### **Conditioned media**

Cells were grown in 10% serum media to near 90% confluence. The medium was changed to serum-free DMEM or MEM containing 2 ug/ml of Insulin (Novo Research Institute, Copenhagen, Denmark) and 2 ug/ml of transferrin (Sigma, St. Louis, MO), which is referred to as IT serum-free media. Following an overnight incubation, the conditioned media was collected, centrifuged at 3000 rpm for 10 minutes and pellets discarded. The Phenyl methyl sulfonyl fluoride (PMSF) with a final concentration 10uM (stock concentration of 100mM in ethanol) and Aprotinin with a final concentration of 0.1 TIU/ml (international units) (Sigma, St. Louis, MO) was added to the supernatant and stored at -70 °C.

## **Methods**

### **Cell cultures**

For growth studies, cells were plated at a convenient density of  $5 \times 10^3$  cells/well (DU145) or  $1 \times 10^4$  cell/well (PC-3, LNCaP) on day 0 in triplicate in 24 well plates (Flow Lab., Mississauga, Ontario) with 10% FBS in media and allowed to attach for 24 hours. Cells were grown as monolayer. The following day, cells were washed with ice cold PBS (10 mM dibasic sodium phosphate pH 7.4; 0.8% NaCl and 33 mM monobasic sodium phosphate) and changed to IT serum-free media, then experimental factors were added to each well. A triplicate wells were incubated with 1ml of trypsin-EDTA for 5 minutes at

room temperature and counted by using a electronic cell counter (Coulter electronics, INC. Hialeah, Florida). The cell counts were performed to assess plating efficiency. For all the growth experiments, the cells of control well were grown in IT serum-free media.

To study the effect of EGF, TGF- $\alpha$  and EGF receptor pathway on cell growth, the cell growth has been measured in a 5 day assay. With this time, the cells reached 90% confluence in the test well. EGF receptor antibody ranging in concentration from 0.5nM to 10nM was used in a dose response assay; and 1nM was selected as a suitable concentration for the inhibition assay. According to the results of does response, exogenous EGF (10nM), and TGF- $\alpha$  (10nM), were used in a stimulation assay. In a reverse competition assay, EGF or TGF- $\alpha$  was added to the medium in the presence of Ab EGF-R and incubated for 5 days.

To confirm if endogenous EGF or TGF- $\alpha$  are involved in regulating cell growth, conditioned media, with or without Ab EGF or Ab TGF- $\alpha$  which block EGF or TGF- $\alpha$  binding to EGF receptor, was added to the cell culture and the effect was measured by counting the cell numbers. This assay was carried out at 37 °C for 5 days.

To measure the growth effects of the c-erbB2/NEU pathway, antibody to the c-erbB2/NEU receptor ranging in concentration from 0.5 nM to 10 nM was used in a dose response assay; and 5nM was selected as a suitable concentration for the inhibition assay. Cells were harvested and counted on day 6. To examine the specific inhibition of Ab c-erbB2/NEU, an antibody of the same subclass antibody, anti-mouse IgG1, (5nM) was added in the control wells. To determine whether the decreased cell number was caused by the inhibition of anti-c-NEU or complement-mediated lysis, a reverse assay was

designed. Cells were pre-incubated with Ab c-erbB2/NEU for 6 days, then washed with cold PBS and media replaced with IT serum-free media without antibody. Growth was measured after an additional two days of incubation.

To measure the cell growth effects of DHT or E2, cells were incubated with and without DHT( $10^{-8}$  M) or E2 ( $10^{-7}$ M) in IT serum-free media. The media was changed every day to supply fresh DHT and E2 in the culture. Cells were harvested and counted on day 6.

At the end of the incubation period in all assays, cells were harvested using Trypsin-EDTA (Gibco, BRL, Burlington, Ontario). Cell numbers were determined using an electronic cell (Coulter) counter (Coulter electronics, INC. Hialeah, Florida). Each individual experiment included triplicate wells and the mean of three experiments was expressed as the result.

To study the correlation of steroid the hormone and expression of c-erbB2/NEU, Poly A<sup>+</sup> RNA was isolated from a prostate carcinoma cell line, LNCaP which remains androgen responsive. The cells were treated with  $10^{-8}$  M of DHT or  $10^{-7}$  M of E2 at variant time points (0 (control), 1h, 7hrs, 14hrs and 24hrs ). Total cellular protein was extracted from LnCaP cells which was treated with  $10^{-8}$  M of DHT or  $10^{-7}$  M of E2 at variant time points (0 (control), 1h, 4hs, 7hs, 14hs, 24hs, 48hs and 72hs). To test if the estrogen receptor is involved in the effect of E2 on the expression of c-erbB2/NEU in LNCaP, Tamoxifen, a nonsteroid chemical which specifically inhibits estrogen active estrogen receptors, was used in the inhibition assay. Poly A<sup>+</sup> RNA was isolated from LNCaP cells and treated with Tamoxifen ( $10^{-7}$  M) with or without DHT ( $10^{-8}$ M) or E2

( $10^{-7}$ M) for 14 hours. Poly A<sup>+</sup> RNA from LNCaP cells which incubated in IT serum-free media was isolated as control sample. Expression of c-erbB2/NEU was detected by using a nick translation labeled c-erbB2/NEU genomic DNA probe. For loading normalization, the Northern blots were sequentially hybridized with a nick translation labeled b-actin cDNA probe. The c-erb b2/NEU protein was detected by anti c-NEU Ab-3 antibody in Western blot.

### **Radio-receptor assay**

To study if the exogenous ligands can bind to the EGF receptor which is present on the prostate cancer cell surface, a ligand binding assay was performed. Cells were plated ( $1 \times 10^5$  cells/well) in 1 ml of 10% FBS serum methionine-free media and incubated at 37 °C for 18-24 hours. Cells were washed three times with 1 ml (each time) of pre-warmed binding buffer (20mM Hepes (4(2-hydroxyethyl)-1-piperazine ethanesulfonic acid) and 0.1% BSA (Bovin Serum Albumin) (pH 7.4) in media). The range from 0.1 to 50 nM of I<sup>125</sup>-EGF (Amersham) with and without 100-fold unlabeled EGF was added into each well in 1ml of binding buffer. Ligand binding was also tested in the presence of antibody to the EGF receptor. Wells of cells containing 10 nM of I<sup>125</sup>-EGF with and without unlabeled Ab EGF receptor with a concentration ranging from 0.1 to 50 nM, in 1 ml of binding buffer, were tested. Incubation for 1 hour at room temperature was followed by washing the cells three times with binding buffer. Cells were then removed from the wells by incubating with 1 ml of 1 M Sodium hydroxide (NaOH) for at least 1 hour at 37 °C. Radiolabel was measured using a Gamma

spectrometer (80000 Gamma Sample Counter, LKB, Wallac).

### **Genomic DNA preparation and Southern analysis**

Genomic DNA was isolated from prostate tissues (BPH and CaP) (frozen at -70 °C) and cell lines by proteinase K digestion, (stock concentration of 20mg/ml, stored at -20 °C) with final concentration of 50ug/ml, in reaction buffer (0.01M tris(hydroxymethyl) amino methane (Tris) (PH7.8), 0.005M ethylenediamine-tetraacetic acid (EDTA) and 0.5% Sodium dodecyl sulfate (SDS) at 37 °C overnight. Then incubated with addition of ribonuclease (RNase) (Stock concentration of 10mg/ml, stored at -20 °C) with final concentration of 1ug/ml for at least 2 minutes followed by phenol-chloroform (1:1) extractions and precipitation with 2.0 times the volume of 95% ethanol and 0.04 times the volume of 5M sodium chloride (NaCl) (Sambrook *et al.* 1989). Samples were stored at -70 °C.

For genomic Southern blots, 10ug of genomic DNA was digested with each restriction endonuclease, including Pst I, EcoR I, Hind III or Pvu II (1unit/ul), for at least 2 hours. Samples were mixed with loading buffer (10% glycerol, 7% sucrose and 0.025% bromophenol blue) and, separated on a 1% agarose Tris-borate-EDTA (TBE) buffered gel, then transferred to nitrocellulose membrane (Nitroplus 2000, 0.45) (Micron separations Inc. (MSI), Westboro, MA) and fixed to the nitrocellulose by baking at 80 °C with vacuum for 2 hours.

For all blots, hybridizations were performed at 42 °C overnight, in 50% deionized formamide, 5 X Denhardt's solution (50X Denhardt' solution, dissolve 5g of Fioll, 5g of

polyvinylpyrrolidone and 5g of BSA (pentax Fraction V) in 500 ml water), 0.1% SDS, 5 X standard saline phosphate-EDTA (standard 20X SSPE, dissolve 174g of NaCl; 27.6g of Sodium phosphate ( $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ ) and 7.4 g of EDTA in 1 liter water and adjust pH to 7.4)) and 200ug /ul of denatured salmon sperm DNA. Blots were washed twice for 15 minutes at room temperature in 2X standard saline citrate (20X SSC, dissolve 175.3 g of NaCl and 88.28 g of sodiaum citrate in 1 liter of water and adjust pH to 7.0), 0.1% SDS and twice for 15 minutes at 65°C in 0.1 X standard saline citrate, 0.1% SDS (Sambrook *et al.*1989). Autoradiographs were obtained at -70°C on Kodak X-omat film. The quantification of gene copy number was determined by densitometric scanning of the autoradiographs (IBM Scanplo program).

Relative gene copy number in patient samples and cell lines was determined by densitometric analysis of genomic Southern blots. The amount of DNA loaded in each lane of the gels was monitored by hybridization with a probe for the human prolactin gene (hPRL) on chromosome 6p. The degree of amplification of c-erbB2/NEU was estimated from the c-erbB2/NEU signal to the hPRL signal compared to the same ratio in normal human prostate DNA. To determine whether changes in c-erbB2/NEU gene copy number were the result of tumor aneuploidy involving chromosome 17, blots were also hybridized with a probe for the growth hormone gene locus on 17q.

To study the gene for EGF receptor, EGF, and TGF- $\alpha$ , in prostate cell lines, genomic Southern blots were sequentially incubated with EGF receptor, TGF- $\alpha$  and EGF cDNA probes. The Southern blots were also probed with prolactin, growth hormone, pip, and D5S5 cDNA probes to nomalize the loading and aneuploidy.

### RNA preparation & analysis

Total RNA was extracted from cell lines and tissues by a guanidinium isothiocyanate procedure and centrifugation through a cesium chloride gradient (Sambrook *et al.* 1989). Poly A<sup>+</sup> RNA was selected using oligo dT cellulose chromatography (Aviv *et al.* 1977).

For Northern analysis, 10ug of formamide and formaldehyde-denatured poly A<sup>+</sup> selected RNA was electrophoresed on 1% agarose/formaldehyde gels and blotted onto nitrocellulose membrane (Sambrook *et al.* 1989). For RNA dot blot analysis, total RNA was bound to nitrocellulose in aliquots containing 10ug, 5ug, 2ug, 1ug RNA. Each sample was represented on duplicate dots. All Northern blots were baked at 80°C under vacuum to fix the RNA onto the nitrocellulose paper.

Northern blots of Poly A<sup>+</sup> RNA and total RNA dot blots which included prostate carcinoma patients and BPH patients, and prostate carcinoma cell lines were sequentially incubated with EGF receptor, TGF- $\alpha$  and EGF cDNA probes labeled by nick translation with  $\alpha$ -<sup>32</sup>P-dCTP (specific activity of  $1 \times 10^8$  dpm / ug). The blots were also incubated with a 28s ribosomal RNA probe and the Northern blots were hybridized with a  $\beta$ -actin cDNA for the normalization of loading.

Expression of the c-erbB2/NEU was detected by Northern analysis. Blots were hybridized to  $\alpha$ -<sup>32</sup>P-dCTP nick-translated c-erbB2/NEU genomic DNA. The blot included three prostate carcinoma cell lines and one breast cancer cell line, SKBR-3 which has been examined as having an over expression of erbB2/NEU gene (Kraus *et*

*al.*1987).

The expression of c-fos in Ab erbB2/NEU antibody treated prostate cancer cells has been measured by using a c-fos genomic DNA probe to hybridize a Northern blot. The blot included samples which were treated with and without Ab erbB2/NEU (Ab-2) at 0, 10 minutes, 30 minutes, 1 hour and 2 hours time points.

Hybridizations for all Northern blots were performed under stringent conditions which was same as described in Southern blotting. The quantitation of gene expression was determined by densitometric scanning of the autoradiographs.

**Reverse transcription polymerase chain reaction (RT-PCR) analysis (Erlich, 1989)**

Estrogen receptor gene expression in cell lines was assayed by RT-PCR with primers specific for estrogen receptor mRNA. The primers which were used in this assay were:

5'-GTCAAATCCACAAAGCCTGGCACCCCTCTTC-3'(upstream),

5'-CCGCCGGCATTCTACAGGCCAAATTCAGAT-3' (downstream)

PCR amplification of the sequence between these primers, from position 668-1344 of cDNA sequence, was designed to produce a 676 bp fragment. Varying amounts (0.5ug, 0.05ug, 0.005ug, and 0.0005ug) of Poly A<sup>+</sup> RNA from Ishikawa cells, which has been known to express estrogen receptor, and 0.5ug of Poly A<sup>+</sup> RNA of LNCaP cells, were used as the templates for random hexamer (pd(N)<sub>6</sub>) (Pharmacia) to prime single stranded cDNA. Poly A<sup>+</sup> RNA was heat denatured at 90 °C for 5 minutes and reverse transcribed at 23 °C for 10 minutes, 42 °C for 45 minutes, and 95 °C for 10 minutes in a volume of

20ul containing 10 units AMV (Avian Myeloblastosis Virus) reverse transcriptase (Pharmacia), 4uM of pd(N)6, 1mM of dNTP and 0.5 mM MgCl<sub>2</sub> in PCR buffer (10x buffer, 500 mmol/L of KCl, 100 mmol/L of Tris-HCl, 15mmol/L of MgCL<sub>2</sub> and 1mg/ml of gelatine pH 8.3 at 20°C). The reaction was terminated by incubating at 90 °C for 10 minutes, cooled on ice, and then amplified by primers targeting the estrogen receptor cDNA sequence. PCR reactions contained entire PT reaction, 1ug of each primer, 50mM potassium chloride (KCl), 10 mM Tris, PH 8.3 and 1.5 mM Magnesium chloride (MgCl<sub>2</sub>). Taq polymerase (Boehringer, Mannheim) was added to a final concentration of 2.5 units (U). Samples were adjusted to 100ul with water and topped with light mineral oil. Amplification was performed using a thermal cycler apparatus (PTC-100, Atomic Energy of Canada limited, Chalk River, Ontario). Thirty cycles of amplification were carried out beginning with DNA denaturation at 95 °C for 1 minute, primer annealing at 55 °C for 45 seconds followed by primer extension at 72 °C for 45 seconds. The PCR product was extracted with chloroform and precipitation with 2 times the volume of 95% ethanol and 0.04 times the volume of 5M sodium chloride at -20 °C for 30 minutes, then the pellets were resuspended in 15ul water, and electrophoresed on 4% agarose gels, followed by Southern blotting. To identify a specific fragment of estrogen receptor a <sup>32</sup>P-dCTP labeled estrogen receptor cDNA probe was used (a gift from Dr. R. P. C. Shiu's laboratory). To confirm that Poly A<sup>+</sup> RNA from both LnCaP cells and Ishikawa cells was suitable for amplification, primers specific for to actin sequence were used in control PCR:

5'-ATCATGTTTGAGACCTTCAA-3' (upstream)

5'-CATCTCTTGCTCGAAGTCCA-3'(downstream)

PCR amplification of the sequence between these primers was designed to produce about a 300bp fragment. Poly A<sup>+</sup> RNA (0.5 ug) from both cell lines was used in this PCR amplification ( method as above).

RT-PCR was also used to amplify region of EGF receptor mRNA. Specific primers of EGF receptor cDNA sequence were designed to produce a 157 bp fragment from position 2278-2435 which covers the ATP binding site at amino acid 723 of the EGF receptor; a second set of primers were designed to produce a 488 bp fragment from 3343-3831 which includes the major autophosphorylation sites (Ty1068, Ty1086, Ty 1148 and Ty1173) of the EGF receptor extracellular domain. Poly A<sup>+</sup> RNA (0.5 ug) of DU145 and PC3 cells, as determined by absorbance at 260 nm, were used as the templates for the random hexamer (Pharmacia)-primed single stranded cDNA synthesis. Poly A<sup>+</sup> RNA was heat denatured at 90 °C for 5 minutes and reverse transcribed at 23 °C for 10 minutes, 42 °C for 45 minutes, and 95 °C for 10 minutes in a volume of 20ul containing 10 units AMV reverse transcriptase, 4uM of pd(N)6, 1mM of dNTP and 0.5 mM MgCl<sub>2</sub> in PCR buffer. The reaction was terminated by incubating at 90 °C for 10 minutes, cooled on ice, and then amplified by primers targeting the specific fragment of the EGF receptor sequence. PCR reactions contained 1ug of each primer, 50mM KCl, 10 mM Tris, PH 8.3, 1.5 mM MgCl<sub>2</sub>. Taq polymerase was added to a final concentration of 2.5 U. Samples were adjusted to 100ul with water. Amplification was performed using a thermal cycler apparatus. Thirty cycles of amplification were carried out beginning with DNA denaturation at 95 °C for 1 minute, primer annealing at 57 °C for 45 seconds followed by primer extension at 72 °C for 1 minute. The PCR product was extracted with chloroform

and precipitation with 2 times (v/v) of ethanol and 0.04 times (v/v) of 5M sodium chloride at -20 °C for 30 minutes, then resuspended the pellets in 30ul of water. Aliquots of 2 ul were removed of each sample to electrophorese on 4% argarose gels. The rest of sample under went the additional preparation using the Magic PCR preps DNA purification system (MPP) (Promega, Madision, WI) for Fentamol (Fmol) DNA sequencing analysis.

### **Fmol DNA sequencing**

The primer (34ng of primer for 20 mer or 37ng for 22 mer) (Fmol sequencing system technical manual, Promega, Madison, WI) were endlabeled with  $\alpha$ -<sup>32</sup>P-ATP and 4U of T4 polynucleotide kinase (PNK) (Promega, madision WI) in PNK buffer (50mM Tris-HCl pH 7.5 (at 25°C), 10mM MgCl<sub>2</sub>, 5mM dithiothreitol (DTT) and 0.1 mM spermidine) and incubated at 37 °C for 30 minutes in a volume of 5ul. The kinase was inactivated at 90 °C for 2 minutes. An aliquot of 1ul of template from the MPP preparation was mixed with the endlabeled primer (8 ng for 20 mer and 11 ng for 22 mer), 4.25 ul of Fmol sequencing buffer system (50mM Tris-HCl pH 9.0 (at 25 °C) and 2mM MgCl<sub>2</sub>) and 2.5 U of Taq polymerase. The reaction was brought up to a volume of 6 ul and distributed (4 ul) among tubes which contained G, A, T or C nucleotide mixtures. These reaction were denatured at 95 °C for 30 seconds, annealed at 58 °C for 30 seconds and extended at 70 °C for 1 minute. At total of 30 cycles were carried out and 3 ul of stopping buffer (10mM NaOH, 95% formamide, 0.05% bromophenol blue and 0.05% xylenecyanole ) was added to inactivate the reaction. Aliquots of 2 ul of each

reaction were loaded on a 5% acrylamide sequencing gel after being heated at 90 °C for 2 minutes and immediately cooled on ice. The gel were exposed to X-ray film.

### **Protein preparation & analysis**

Total cellular protein was extracted with PBSTD (10 mM dibasic sodium phosphate pH 7.2; 0.9% NaCl; 0.15% SDS; 1% Triton X-100; 30mM sodium azide; 0.5% sodium deoxycholate) from cells and pre-washed with ice cold PBS twice and from prostate tissue from surgery. The protein concentration was determined by using a Bio-Rad protein assay (Bio-Rad laboratories, Richmond, CA.).

For Immunoprecipitaion, cell were pre-labeled with <sup>35</sup>S-Methionine (100uCi/ml) in methionine free media for 12 hours. The lysates were prepared for immunoprecipitation with antibodies. Antibody (1ug/ml) and Pasorbin (staphylococcus aureus cells) (Calbiochem brand biochemicals, San Diego, CA) was mixed with 1.5ml of sample and incubated at 4 °C overnight. The samples were washed with PBSTD four times and the pellets were resuspended in 50ul of sample buffer (1.0 ml of glycerol, 0.5 ml of β-mercaptoethanol, 2.0 ml of 10% SDS, 1.25 ml of 1.0 M Tris-HCl buffer, 0.2 ml of 0.1% bromophenol blue and 0.6g of solid urea in a final volume of 10 ml in water). These samples were used in electrophoresis.

Aliquots of 30ug of total protein per sample was electrophoresed through 6% SDS-PAGE gels. The gel and nitrocellulose paper (Trans-blot transfer medium, Bio-Rad Lab. Richmond CA) were soaked in Tris buffered saline +Tween 20 (TBST) solution (10mM Tris-HCl pH 8.0, 150nM NaCl and 0.05% Tween 20) for 10-15 minutes and then

the transfer of protein from the gel to nitrocellulose were carried out by using LKB blot (LKB 227 multiphor II Nova blot, Bromma, Sweden) in a semi-dry system with blotting buffer (39 mM glycine, 48 mM Tris 0.0375% (w/v) SDS and 20% (v/v) methanol). For saturating the nonspecific protein binding sites, the nitrocellulose paper was incubated in TBST with a 1% (w/v) BSA solution for 30 minutes.

For protein detection, all nitrocellulose membranes were washed three times with TBST and incubated with 1:3000 diluted HRP (horseradish peroxidase) labeled second antibody in TBST for 1 hour at room temperature followed by three times additional washes with TBST. The proteins were detected by Enhanced Chemiluminescence Western blotting detection system (ECL) (Amersham Canada Limited, Oakville, Ontario).

To determine the content of EGF receptor, specific Western blots included three prostate carcinoma cell lines which have been examined by incubating with Ab EGF receptor antibody under the same conditions as described above.

Co-immunoprecipitation and immunoblotting has been used for the detection of the auto-phosphorylated EGF receptor. Cells were pre-labeled with <sup>35</sup>S-Methionine (100uCi/ml) in methionine free media for 12 hours. Plates were incubated for 20 minutes with or without Ab EGF-R-2 (1nM) (UBI) followed by an incubation for 30 minutes with or without EGF (10, 100 nM) or EGF (100nM) alone. Washed cells were harvested in PBSTD buffer and lysates were prepared for immunoprecipitation with Ab EGF-R-1 (Oncogene Science). Ab EGF receptor with final concentration of 1ug/ml and pansorbin (staphylococcus aureus cells) (Calbiochem brand biochemicals, San Diego, CA) was mixed with 1.5ml of sample and incubated at 4 °C overnight. The pellets mixed with

sample buffer were divided equally in two tubes and were electrophoresed on SDS-PAGE. Two identical 6% SDS-PAGE gels were run: one was autoradiographed for estimating total EGF receptor protein. The other was transferred to nitrocellulose for immunoblotting with Ab-P-tyrosine for estimating autophosphorylated EGF receptor under the same condition described above.

To measure the expression of c-erb B2/NEU in prostate carcinoma patients and prostate carcinoma cell lines, Western blots were incubated with a monoclonal antibody to human c-NEU ( Anti NEU Ab-2, 1ug/ml) in TBST for overnight at 4 °C. To determine whether overexpression of c-erb B2/NEU occurred in prostate tissue and cell lines, SKBR-3 cells which has been demonstrated that overexpress about 4 fold c-erbB2/NEU and MCF-7 breast cancer cells which express normal amounts of c-erbB2/NEU has been used as control in this study (Benz *et al.*1993; Kraus *et al.*1987).

## RESULTS AND DISCUSSIONS

## CHAPTER ONE

### ROLE OF c-erbB1 (EGF RECEPTOR) IN PROSTATE CANCER CELL GROWTH

Although it has long been recognized that the growth, development and function of the prostate gland are regulated by androgens (Cunha *et al.*1987; Eaton *et al.*1991), the clinical data on human prostate cancer indicates that tumor progression is associated with a loss of androgen dependence (Bruchovsky *et al.*1968b; Wilson *et al.*1981). This has led to the suggestion that the role of androgens is mediated by other hormones, growth factors and intracellular regulators. At present we must assume that tumor progression in the absence of androgens represents an altered ability to respond to non-androgenic growth signals and/or to ignore inhibitory controls. In the past several years, evidence has accumulated indicating that many transformed cells are able to produce and respond to their own growth factors (Goustin *et al.*1986). The EGF receptor and its ligands represent the most frequently found putative autocrine loops in human tumors. Co-expression of growth factors and their receptor, including EGF and TGF- $\alpha$ , has been identified as a feature of malignancy (Derynck *et al.*1987; Perosio *et al.*1989).

The EGF receptor is a transmembrane glycoprotein consisting of an extracellular binding domain and an intracellular domain that exhibits tyrosine kinase activity upon stimulation by epidermal growth factor (EGF) or transforming growth factor alpha (TGF- $\alpha$ ) (Carpenter, 1987; Carpenter *et al.*1979b; Winkler *et al.*1989). Binding of EGF or TGF- $\alpha$  to EGF receptor initiates a signal transduction process that results in the

stimulation of cellular proliferation (Clark *et al.*1985; Stoscheck *et al.*1986). There is evidence that the EGF receptor system is involved in oncogenic transformation of cells. Overexpression of EGF receptor can transform cells in a ligand-dependent manner. The v-erb B oncogene codes for a product homologous to the cytoplasmic domain of the EGF receptor (Downward *et al.*1984a). TGF- $\alpha$  is produced by many transformed cells and has been identified in several tumor cell lines (Todaro *et al.*1980; Mydlo *et al.*1988b; Bennett *et al.*1989). The amplification of the EGF receptor gene has been documented in some tumors, such as squamous cell carcinomas, malignant gliomas, breast, gastric and renal carcinomas (Todaro *et al.*1980; Mydlo *et al.*1988b; Bennett *et al.*1989; Yamamoto *et al.*1986b; Filmus *et al.*1985; Yoshida *et al.*1990). Recently, EGF receptor levels have been correlated with node-positive breast cancer (Spitzer *et al.*1988) and with a lack of response to endocrine therapy in recurrent tumors (Nicholson *et al.*1990). In human gastric carcinomas studied by immunohistochemistry (Yasui *et al.*1988), the expression of EGF and EGF receptors showed a significant correlation with tumor invasion. EGF receptor levels have also been correlated with the invasiveness of bladder tumors (Neal *et al.*1989). These investigations indicate that the EGF receptor and its ligands, EGF and TGF- $\alpha$ , play a prominent role in regulating many malignant cells (Sporn *et al.*1988; Sporn *et al.*1985b; Goustin *et al.*1986). This growing body of literature supports the hypothesis that increased signaling through the EGF receptor can lead to abnormal growth and transformation.

## A: EXPRESSION OF EGF RECEPTOR, EGF AND TGF- $\alpha$

### 1. INTRODUCTION

The response of human prostate cancer cells to growth factors is poorly understood, although there is evidence supporting a role for peptide growth factors and their receptors in prostate tumor cell growth. The EGF receptor has been demonstrated biochemically and immunocytochemically in human BPH and CaP tissue (Maddy *et al.*1989; Eaton *et al.*1988) and human prostatic cell lines. Human EGF has been found in prostatic fluid (Gregory *et al.*1986), extracts of prostate tissue, BPH and CaP (Elson *et al.*1984; Shaikh *et al.*1990). Both EGF and TGF- $\alpha$  have been detected in the human prostatic cell lines (DU145, PC-3 and LNCaP) (Wilson *et al.*1981; Derynck *et al.*1987; Connolly *et al.*1989; Connolly *et al.*1990). Elevated EGF receptor mRNA levels have been detected by using RNase protection assay in prostatic carcinoma tissue and cell lines (DU145 and PC-3) (Morris *et al.*1990). The findings indicate that enhanced expression of the ligands in the presence of the EGFR could contribute to growth of prostate cancer cells. Many investigators have proposed that some transformed cells are able to overcome normal growth restraints by producing growth factors which act through autocrine mechanisms (Sporn *et al.*1988; Sporn *et al.*1985b). For prostate cancer cell lines, autonomous growth involving TGF- $\alpha$  and the EGFR pathway has been reported (Hofer *et al.*1991; Connolly *et al.*1991; Wilding *et al.*1989a). However, some of the data on EGF receptor levels are contradictory regarding on the potential importance of EGF receptor in prostate cancer. Conflicting values on the relative levels of EGF receptor in BPH and CaP as measured by binding of radiolabelled EGF have been reported (Fiorelli *et al.*1991; MacFarlane *et*

*al.*1985). In addition, some studies in the literature looked at ligand(s) but not receptor, while others address tumor cell lines but not tissue samples. The evaluation of the alteration expression including ligand(s) and its receptor in both human prostate cancer cell lines and human tissue samples is still missing.

In order to address this question, we examined the structure, dosage and expression of the genes for EGF, TGF- $\alpha$  and EGFR in a series of 34 surgical samples of prostate tissue and three human prostatic carcinoma cell lines.

## 2.Results

In order to assess the potential for autocrine regulation of growth via EGF receptor-mediated pathways in human prostate tumors, we examined the expression of mRNAs encoding EGF, TGF- $\alpha$  and the EGF receptor. Total RNA was isolated from samples of prostate carcinoma (n=13), BPH (n=21) and three prostatic carcinoma cell lines. RNA from the cell lines was used in both Northern analysis and RNA dot blots to verify the specificity of the DNA probes in assessing the relative expression of each transcript. RNA from the patient samples was only sufficient for RNA dot blots.

The presence of authentic transcripts of each gene was confirmed by Northern analysis of poly A<sup>+</sup> RNA from the three cell lines. As shown in Fig. 1-1, both the 10.8 Kb and 5.6 Kb transcripts of EGF receptor mRNA were clearly detected in all three cell lines. No aberrant transcripts of any of the genes were found. The LNCaP cell line contained the highest steady state level of mRNA for EGF, while mRNAs for TGF- $\alpha$  and EGF receptor were higher in DU145 and PC-3 cell lines.

The Northern analysis of poly A<sup>+</sup> RNA was in agreement with the RNA dot blot analysis of total RNA from the cell lines (Fig. 1-2, lanes 1-3). Relative expression was determined by densitometric scanning and regression analysis of the dilution series on the dot blot. Among the cell lines, the steady state level of EGF mRNA was highest in LNCaP cells; TGF- $\alpha$  and EGF receptor mRNAs were higher in DU145 and PC-3 cells (Fig. 1-3).

Having confirmed that RNA dot blots can be used to assess the relative expression of the EGF, TGF- $\alpha$  and EGFR transcripts in total RNA, we next sought to evaluate their levels in patient samples. We examined 13 prostate carcinomas with Gleason scores ranging from 4 to 9 (higher numbers indicating greater de-differentiation). The tissues examined were 70% or more carcinoma. Only pathology assessments were available on this series of samples; clinical staging was not available. For comparison, non-malignant BPH samples from 21 patients were examined. Densitometric analysis of the RNA dot blots indicate that, relative to BPH samples, carcinoma samples in both classes (class 1: Gleason scores 4-6, class 2: Gleason scores 7-9) contain significantly higher levels of EGF mRNA and TGF- $\alpha$  mRNA (Student T analyses has performed to produce the p values) ( $p < 0.05$ ) (Fig. 1-4). There is no significant difference between the two classes of CaP samples.

In a previous study using RNase protection, we demonstrated that mRNA for EGF receptor is higher in CaP than BPH tissues (Morris *et al.* 1990). A similar trend was observed in the present study, although significance was not reached with the smaller sample size. While the carcinoma samples demonstrated enhanced expression of the

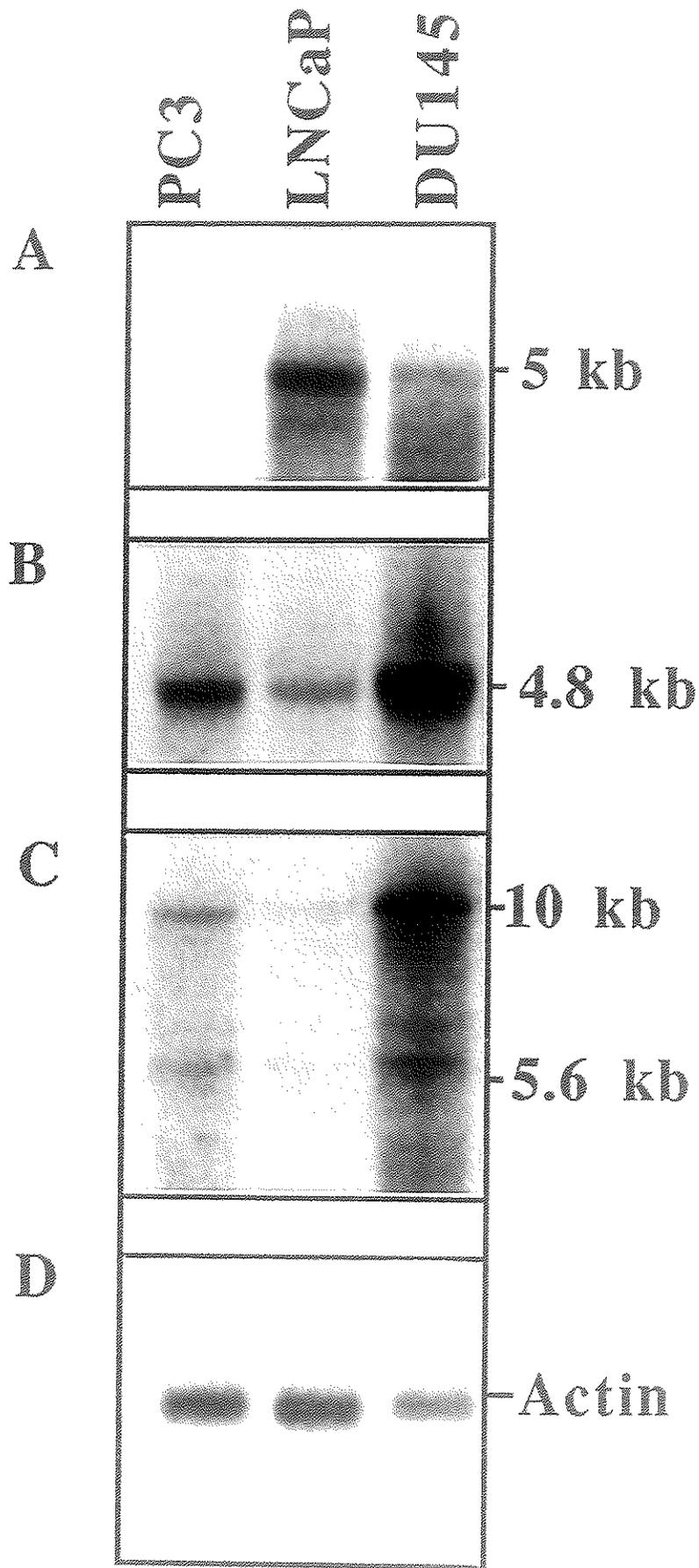
ligand transcripts and, in some cases, the receptor mRNA, the highest levels of these mRNAs were found in the cell lines.

To ascertain whether this overexpression is associated with amplification or rearrangement of TGF- $\alpha$ , EGF and EGF receptor genes in the prostate carcinoma cell lines, genomic Southern blots were performed. In addition to hybridization with TGF- $\alpha$ , EGF and EGF receptor cDNAs, blots were hybridized also with a prolactin cDNA probe to quantify loading of DNA (data not show). To control for possible aneuploidy involving any of chromosomes 2, 4 and 7, the Southern blots were also hybridized with other unique probes for each of these chromosomes which carry the EGF, TGF- $\alpha$  and EGF receptor genes, respectively. The Southern blots indicate a normal diploid complement for each of chromosomes 2, 4 and 7 in all cell lines (data not shown).

No gross rearrangement of the EGF, TGF- $\alpha$  or EGF receptor genes was observed (Fig. 1-5). The two high molecular weight fragments of the EGF receptor gene which appear in the PC-3 lane represent polymorphic alleles rather than gene rearrangement since the pattern is seen only in Pst I-restricted DNA. With other restriction enzymes (EcoRI, Hind III and Pvu I), all three cell lines show identical Southern blot bands for the EGF receptor gene (data not show). In DU145 cells, there appears to be low level amplification of the TGF- $\alpha$  gene estimated to be <5-fold (Fig. 1-5). No other gene amplification was evident.

**Fig. 1-1.**

Northern blot analysis of mRNA (15 ug/lane) from the prostatic carcinoma cell lines.  $\alpha$ -<sup>32</sup>P-labeled cDNA probes for: A, EGF; B, TGF- $\alpha$ ; C, EGF receptor and D, actin were hybridized sequentially to the blot. A, EGF; B, TGF- $\alpha$ ; C, EGF receptor and C,  $\beta$ -actin. Molecular weights of the transcripts are indicated at right in kilobases.



**Fig. 1-2.**

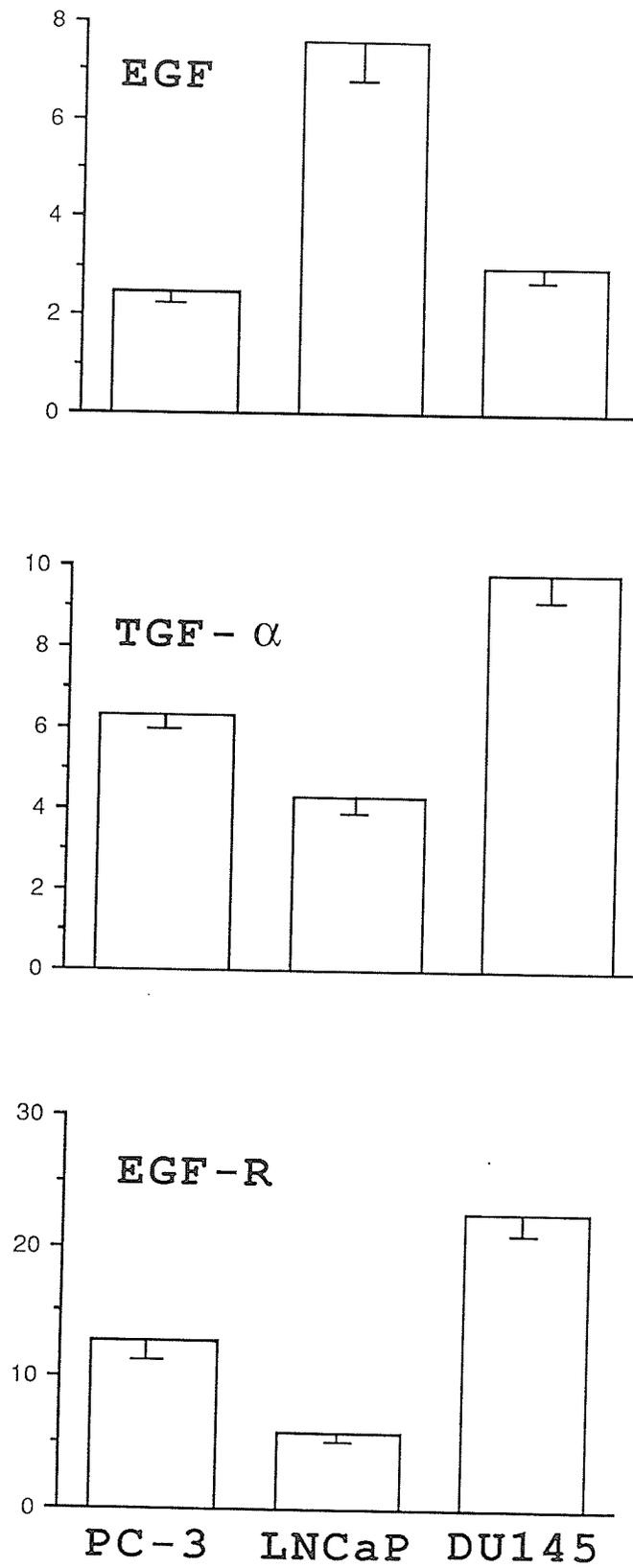
Representative dot blot analysis of RNA isolated from human prostate tissue and prostatic cell lines. Hybridization of  $\alpha$ -<sup>32</sup>P-labeled cDNA probes ( A, EGF; B, TGF- $\alpha$ ; C, EGF receptor; and D, 28s rRNA) to samples of total RNA ranging from 10ug to 2 ug (1 ug in D for the rRNA probe). Lanes contain RNA from: 1, PC-3; 2, LNCaP; 3, DU145; 4-7, BPH tissue; 8-11, CaP tissue; 12, yeast tRNA.



**Fig. 1-3.**

Relative expression of EGF, TGF- $\alpha$  and EGF receptor mRNA in total RNA from prostatic carcinoma cell lines. The relative abundance of each mRNA was determined by densitometric scanning of autoradiographs of RNA dot blot. Mean  $\pm$  S. E. for five aliquots of each cell line are represented by the bars.

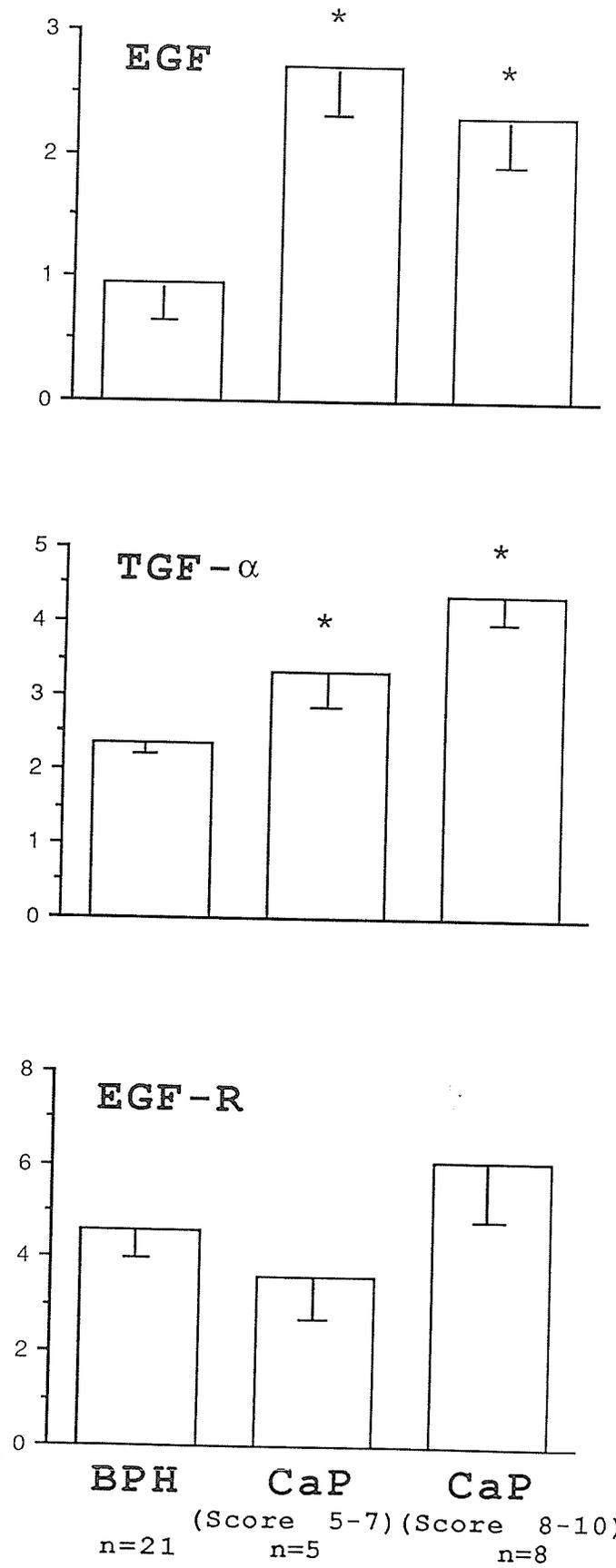
RELATIVE EXPRESSION OF mRNA



**Fig. 1-4.**

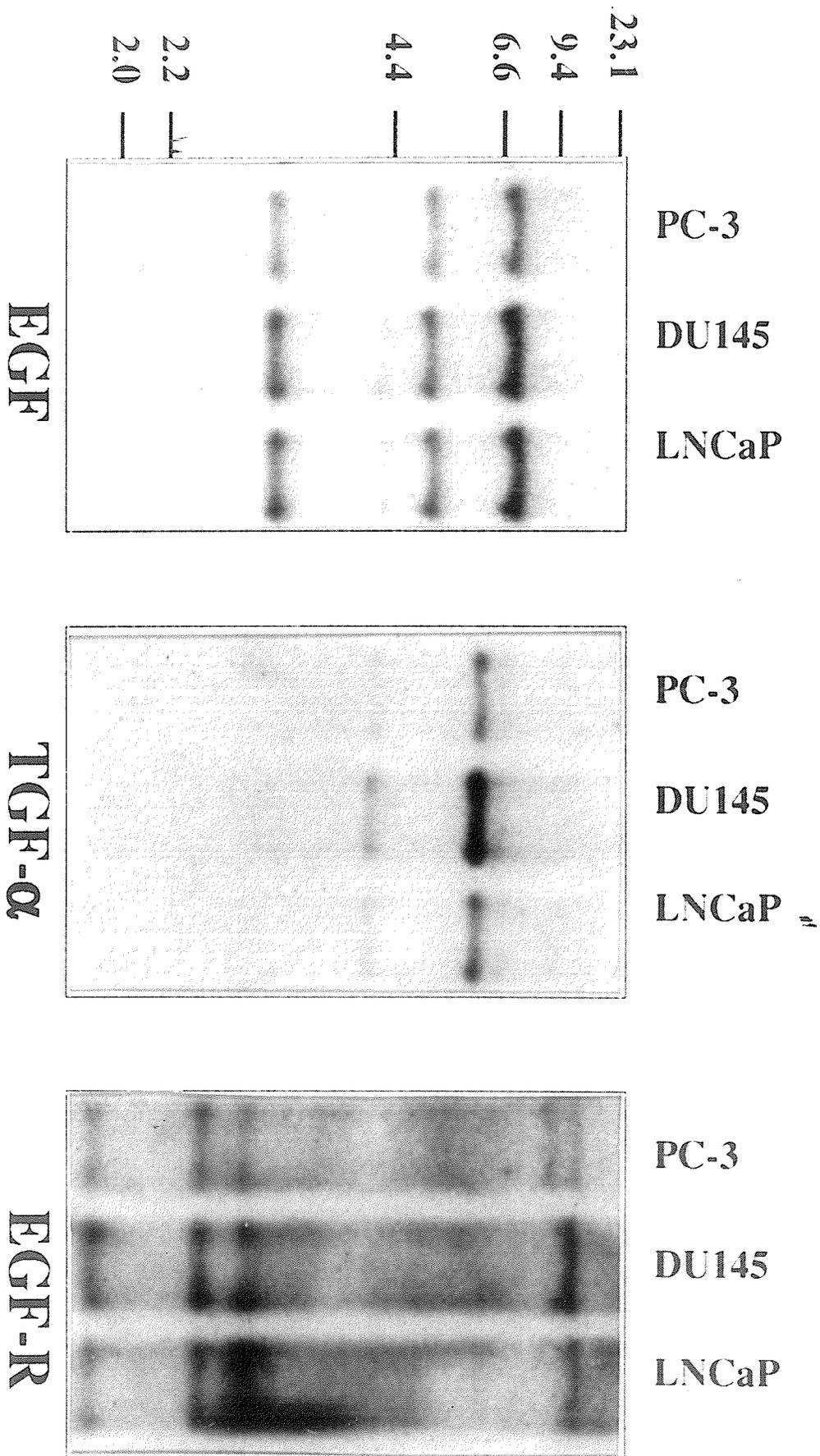
Relative expression of EGF, TGF- $\alpha$  and EGF receptor mRNA in total RNA from prostate tissue. The relative abundance of each mRNA was determined by densitometric scanning of autoradiographs of RNA dot blots. BPH, benign prostatic hyperplasia. CaP (score 5-7), carcinoma with a combined Gleason score between 5 and 7 inclusive. CaP (score 8-10), carcinoma with a combined Gleason score of 8 or greater (poorly differentiated). Means  $\pm$  S. E. are represented by the bars. Significant differences ( $p < 0.05$ ) from levels in BPH are indicated by \*.

RELATIVE EXPRESSION OF mRNA



**Fig. 1-5.**

Genomic Southern analysis of DNA from the prostatic carcinoma cell lines. Genomic DNAs (10 ug/lane) were digested with Pst I, fractionated by gel electrophoresis and transferred to nitrocellulose membrane.  $\alpha$ -<sup>32</sup>P-labeled cDNA probes for EGF, TGF- $\alpha$  and EGF receptor were hybridized sequentially to the blot. Molecular weight markers are indicated at left in kilobase pairs.



### 3. DISCUSSION

Relapse following androgen-ablation therapy in men with metastatic prostate cancer is a serious clinical problem. The mechanisms by which prostate cancer cells grow in response to non-androgenic signals is only beginning to be defined. Several groups have investigated the possible role of autocrine growth regulation involving EGF receptor-mediated signal transduction. TGF- $\alpha$  has been shown to be mitogenic in DU145 and LNCaP cells (Wilding *et al.*1989a; Connolly *et al.*1989; Connolly *et al.*1990). EGF receptor expression has also been demonstrated in prostatic cancer cell lines (Connolly *et al.*1989; Connolly *et al.*1990; Morris *et al.*1990; Derynck *et al.*1987). More recently, blockade of EGF receptor-mediated signal transduction has been shown to reduce growth of androgen-independent PC-3 cells (Hofer *et al.*1991) and DU145 cells (Connolly *et al.*1991). Although autocrine regulation via this pathway appears to contribute to the androgen-independent growth of these cell culture model systems, it is not yet clear how important such regulation is for prostate cancer in vivo.

To address this question, we evaluated the expression of EGF, TGF- $\alpha$  and EGF receptor genes in a series of prostate tissues obtained at surgery and in three prostatic carcinoma cell lines. Both pre-pro TGF- $\alpha$  and pre-pro EGF mRNAs are significantly elevated in prostate carcinomas compared to benign hyperplasias. Among the cell lines, it is interesting to note that the highest levels of TGF- $\alpha$  mRNA are seen in the two androgen-independent cell lines, DU145 and PC-3. The androgen-responsive LNCaP cell line expresses low levels of TGF- $\alpha$  but high levels of EGF mRNA. Part of the progression to hormone-independence may involve a switch in the predominant ligand

from EGF to TGF- $\alpha$ . Since all tissue were primary tumors obtained prior to treatment, all of them would likely be hormone-responsive. It would be interesting to look at TGF- $\alpha$  versus EGF expression in relapsed patient materials but these are not available for study. Among the cell lines, no gene rearrangements was evident. Only in DU145 cells was a modest level of gene amplification seen for the TGF- $\alpha$  gene (<5-fold). These data, therefore, at list in cell lines, suggest that overexpression of EGF and TGF- $\alpha$  is a frequent alteration in prostate tumor cells but likely relates to altered transcriptional or post-transcriptional control of the mRNA levels. It should noted that we have looked only at mRNA levels in the patient samples and that other factors could influence the final expressed levels of ligand. If the levels of mRNAs observed reflect the levels of processed ligands, then tumor growth stimulation by autocrine mechanisms like those seen in cell cultures are highly possible in vivo as well.

In relation to the EGF receptor, all prostate samples expressed transcripts. The elevated EGF receptor mRNA levels seen in the cell lines were not associated with either gene amplification or gross rearrangement. This is in contrast to other human cancers; amplification of EGF receptor DNA has been associated with breast, squamous cell carcinomas, gastric and renal carcinomas (Todaro *et al.*1980; Mydlo *et al.*1988b; Bennett *et al.*1989; Yamamoto *et al.*1986b; Yoshida *et al.*1990; Filmus *et al.*1985). In our study, all samples demonstrated co-expression of the EGF receptor mRNA and the ligand mRNAs. It is unclear whether overexpression of both ligand and receptor are needed for autocrine-regulated growth in vivo or whether enhanced levels of either is sufficient. In transfection expression experiments, enhanced expression of either EGF receptor (Di Fiore

*et al.*1987a) or TGF- $\alpha$  or EGF (Watanabe *et al.*1987; Stern *et al.*1987) was sufficient to induce transformation. In our study, it is clear that EGF or TGF- $\alpha$  expression appears enhanced even when the expression of the EGF receptor mRNA is modest. These results suggest that for prostate cancer, enhanced expression of the ligands may be more important than high level expression of the EGF receptor.

Our result extend the observations of others regarding autocrine regulation of androgen-independent prostate cancer cells in culture to an examination of prostate tumor tissues in vivo. The data support the involvement of EGF receptor-mediated growth in the phenotype of prostate carcinoma primarily through the quantitative modification of ligand and receptor expression.

## B: AUTOCRINE REGULATED GROWTH BY THE *erbB1* PATHWAY

### 1. INTRODUCTION

Autocrine regulation of cancer cell growth was first suggested by Sporn (Sporn *et al.*1985a) to describe the phenomenon in which transformed cells produce their own growth factors that act on the producer cells to maintain a continuous signal for proliferation. Evidence is now accumulating that many transformed cells are able to produce and respond to their own growth factors. For example, human breast and pancreatic carcinoma cell lines have been described as producing significantly higher amounts of EGF, TGF- $\alpha$  and EGF receptor and respond to EGF and TGF- $\alpha$  for growth in tissue culture; anti-EGF receptor antibody can inhibit this enhanced growth (Ennis *et al.*1989). The original hypothesis has been expanded to incorporate enhancement of receptor and post-receptor activity (Plowman *et al.*1990a). Changes to any of these critical control points in the mitogenic pathway could lead to autonomous growth and transformation.

Our previous data demonstrated that EGF receptor mRNA levels are somewhat higher in carcinoma (CaP) than in benign hyperplasias (BPH) (Morris *et al.*1990). The levels of mRNA for EGF and TGF- $\alpha$  are also significantly higher in CaP than in BPH (Ching *et al.*1993). Human prostatic carcinoma cell lines, including lines that have lost androgen response for growth, also express mRNA for EGF, TGF- $\alpha$  and EGF receptor (Wilding *et al.*1989a; Hofer *et al.*1991). These observations strongly suggest that there

is an EGF receptor-mediated autocrine loop controlling prostate cell growth. To address this possible mechanism directly, we have studied three human prostatic carcinoma cell lines for the presence of an EGF receptor-mediated autocrine pathway.

## 2. RESULTS

To demonstrate that ligands for the EGF receptor can affect prostate cancer cell growth, exogenous EGF or TGF- $\alpha$  was used in growth assays in serum-free media. We observed increased cell growth in response to exogenous ligands in two of the prostate cancer cell lines. In DU145 cells, cell number increased more than 200% with exogenous EGF and more than 150% with exogenous TGF- $\alpha$  in a 5-day assay. In LNCaP cells, cell number was 129% of control with exogenous EGF and 140% of control with exogenous TGF- $\alpha$  (Fig. 1-6).

It has been demonstrated that the function of EGF and TGF- $\alpha$  binding to EGF receptor is to initiate the EGF receptor-mediated intracellular phosphorylation cascade. To prove this pathway is involved in regulating prostate cancer cell growth, anti EGF receptor monoclonal antibody that specifically blocks the binding of EGF and TGF- $\alpha$  to the extracellular domain of EGF receptor was used in an inhibition assay. Dose response assays of anti EGF receptor antibody show that the reduction in cell growth correlates with an increased amount of anti EGF receptor antibody (Fig. 1-7) and had a similar profile in both DU145 and LNCaP cell lines; the most inhibition was seen in DU145 cells. The concentration of 1nM of anti EGF receptor antibody which blocks DU145 cell growth by 40% has been used in later inhibition assays, since the larger amounts of

antibody (5nM, 10nM) did not show greater growth inhibition in the dose-response assay. Similarly, 1nM of anti EGF receptor antibody blocks LnCaP cell growth by 30% as indicated in Fig. 1-6. To demonstrate that the reduction in cell growth was caused by the specific growth inhibition of anti EGF receptor antibody, a reverse growth assay was performed by using EGF and TGF- $\alpha$  in competition with the antibody. Although anti EGF receptor antibody had an effect on the cell growth of DU145 and LNCaP cells, excess ligand (either EGF and TGF- $\alpha$ ) was able to reverse this inhibition.

Since we have found that EGF receptor, EGF and TGF- $\alpha$  are overexpressed at the mRNA level in prostate cancer cell lines and in tissue from prostate carcinoma patients, and that prostate cancer cells in culture responded to exogenous EGF and TGF- $\alpha$  for growth, we sought to determine if the endogenous EGF and TGF- $\alpha$  can affect prostate cancer cell growth. To assess this, conditioned media was collected from the cell cultures and tested for its ability to stimulate cell growth in the presence and absence of neutralizing antibodies to EGF and TGF- $\alpha$ . In conditioned media, a significant increase in cell growth was seen in both DU145 and LNCaP cell lines; DU145 cells had an increase in growth up to 30% (Fig. 1-8); while LNCaP cells had an increase in growth up to 20% (Fig. 1-8). To confirm that the increased cell growth in conditioned media was due, at least in part, to endogenous EGF and TGF- $\alpha$  secreted by prostate cancer cells, growth assays were carried out in the presence of neutralizing anti EGF and TGF- $\alpha$  antibodies which specifically inhibit EGF and TGF- $\alpha$  from binding to the receptor. In this study, both anti EGF and anti TGF- $\alpha$  antibodies were able to reduce the growth which was stimulated by the conditioned media for both DU145 and LNCaP cell lines

compared to control. (Fig. 1-8).

A very different response to EGF and TGF- $\alpha$  was observed with the third prostate cancer cell line, PC-3. The experiment using exogenous radiolabeled EGF showed that EGF can bind to both DU145 and PC-3 cells in a similar binding pattern (Fig. 1-9-A). Similar competitive inhibition patterns also were obtained with anti EGF receptor antibody inhibition of radiolabelled EGF binding in both DU145 and PC-3 cells (Fig. 1-9-A). But under the same experimental conditions as DU145 and LNCaP cells, exogenous EGF or TGF- $\alpha$  did not stimulate cell growth of the PC-3 cell line (Fig. 1-9-B). Anti EGF receptor antibody also showed no effect on PC-3 cell growth in both the presence or absence of exogenous ligand. Conditioned medium, with or without the neutralizing anti EGF and anti TGF- $\alpha$  antibodies also failed to change the growth profile of PC-3 cells (Fig. 1-10), although the conditioned medium from PC-3 cells shown to stimulate DU145 and LNCaP cell growth in culture system (data not shown).

The influence of EGF receptor-mediated pathways on cell growth is initiated by the binding of EGF or TGF- $\alpha$  to the EGF receptor, thus inducing autophosphorylation of the receptor in the intracellular domain. To demonstrate this action in prostate cancer cells, we have measured the autophosphorylated EGF receptor by using co-immunoprecipitation and immunoblotting. In this assay, immunoprecipitation with anti EGF receptor (Oncogene Science) was used to measure the total amount of EGF receptor, and immunoblotting with anti phosphotyrosine kinase antibody (UBI) was used to detect EGF receptor phosphorylated on tyrosine residues. Shown in Fig. 1-11, DU145 cells with 10nM of EGF in serum-free medium contained detectable autophosphorylated EGF

receptor. Anti EGF receptor antibody was able to block this reaction; EGF was able to reverse the inhibition effect of the antibody in this assay (Fig. 1-11 Panel B). Panel A of Fig. 1-11 demonstrates that the total amount of EGF receptor under each condition was constant while panel B demonstrates that the phosphotyrosine content of the receptor varied. The same experiment was performed in PC-3 cells to investigate the phenomenon of the variation in EGF receptor response. Fig. 1-12 panel B shows that neither EGF nor anti EGF receptor antibody resulted in phosphorylation of tyrosine sites in the EGF receptor of PC-3 cells, even though the autophosphorylated EGF receptor of DU145 cells was detectable on the same blot. Fig. 1-12 panel A indicates that equal amounts of total EGF receptor were isolated from both DU145 and PC-3 cell lines.

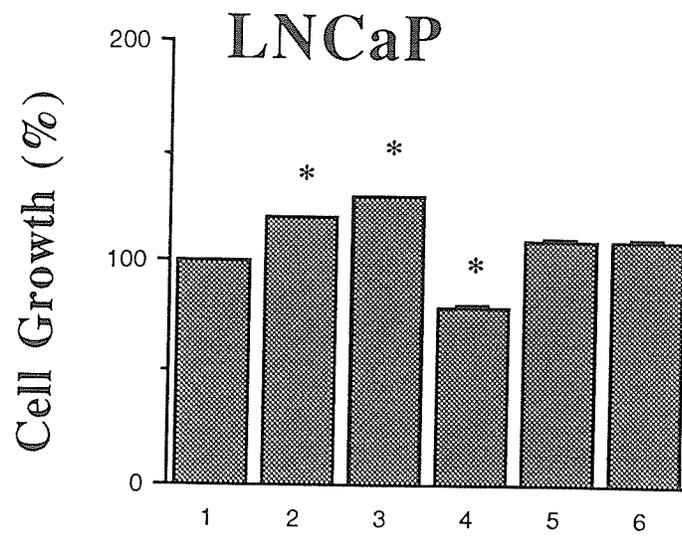
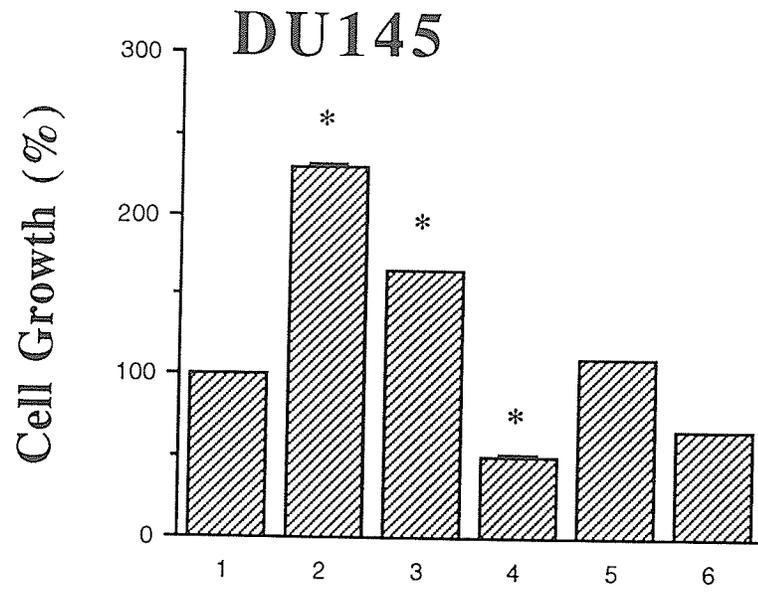
Thus, it appears as if PC-3 cells express EGF receptor and are able to bind exogenous EGF, but do not initiate the intracellular signal transduction pathway and do not proliferate in response to EGF. Since no phosphorylated EGF receptor could be detected in PC-3 cells with the anti phosphotyrosine antibody, we sought to examine the EGF receptor for possible defects in primary structure. We examined the ATP binding site and four major autophosphorylation sites in the intracellular domain of EGF receptor at the nucleotide sequence level. Fig. 1-13 is a diagram of EGF receptor which illustrates the regions we have analyzed. Since defects in the ATP binding site have been associated with a failure to undergo autophosphorylation (Moolenaar *et al.* 1988), we first examined this region of the receptor cDNA. Using RT-PCR followed by DNA sequence analysis, we determined that no point mutations had occurred in this region in PC-3 cells. Similarly, the sequence around each of the tyrosine residues which can be phosphorylated

was examined as alterations in recognition sequences might prevent kinase activity (Sorkin *et al.*1991). Again, no variation from the established EGF receptor cDNA sequence was detected in these regions.

By computer simulation, we examined the effect of frame shift mutations (either one or two nucleotides) in the cytoplasmic domain of the EGF receptor. All frameshifts occurring before the ATP binding site or before the autophosphorylation sites resulted in premature chain termination signals. Since the EGF receptor isolated from PC-3 cells is not detectably smaller than 170Kd, mutation by frameshift seems unlikely.

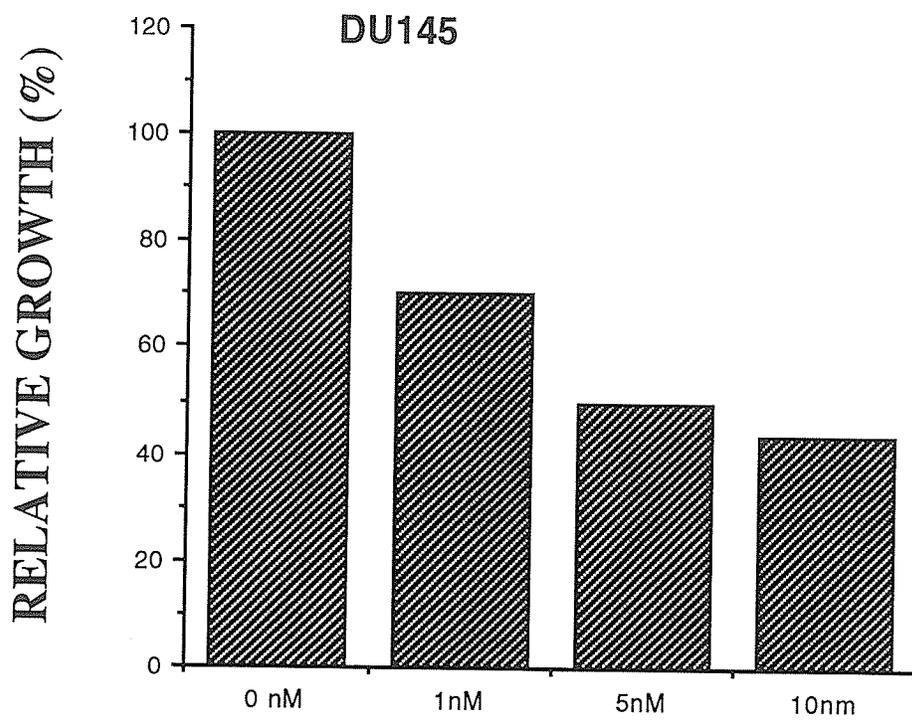
**Fig. 1-6.**

Effects of exogenous EGF, TGF- $\alpha$  (10nM) or Ab EGF receptor (1nM) on the growth of DU145 and LNCaP cells:  $5 \times 10^3$  cells/ well of DU145 (upper panel) and  $1 \times 10^4$  cells/ well of LNCaP (lower panel) were plated and cultured overnight in 10% FBS media. Media was then changed to serum-free media containing 1 $\mu$ g/ml insulin and transferrin. EGF, TGF- $\alpha$  and/or Ab EGF receptor were added into designated wells and incubated for 5 days. Columns represent 1, control; 2, 10nM EGF; 3, 10nM TGF- $\alpha$ ; 4, 1nM of anti EGF-R antibody; 5, 10nM EGF with 1nM of anti EGF receptor antibody; 6, TGF- $\alpha$  with 1nM of anti EGF-R antibody. The mean values and standard errors of three experiments are shown (\*  $P < 0.05$ ).



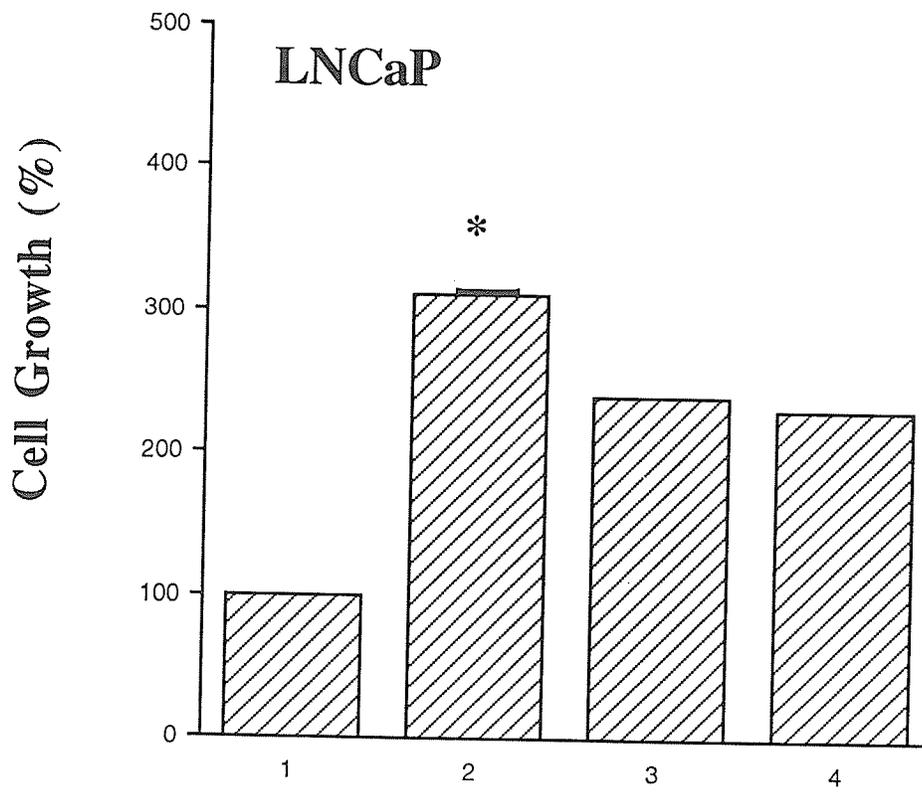
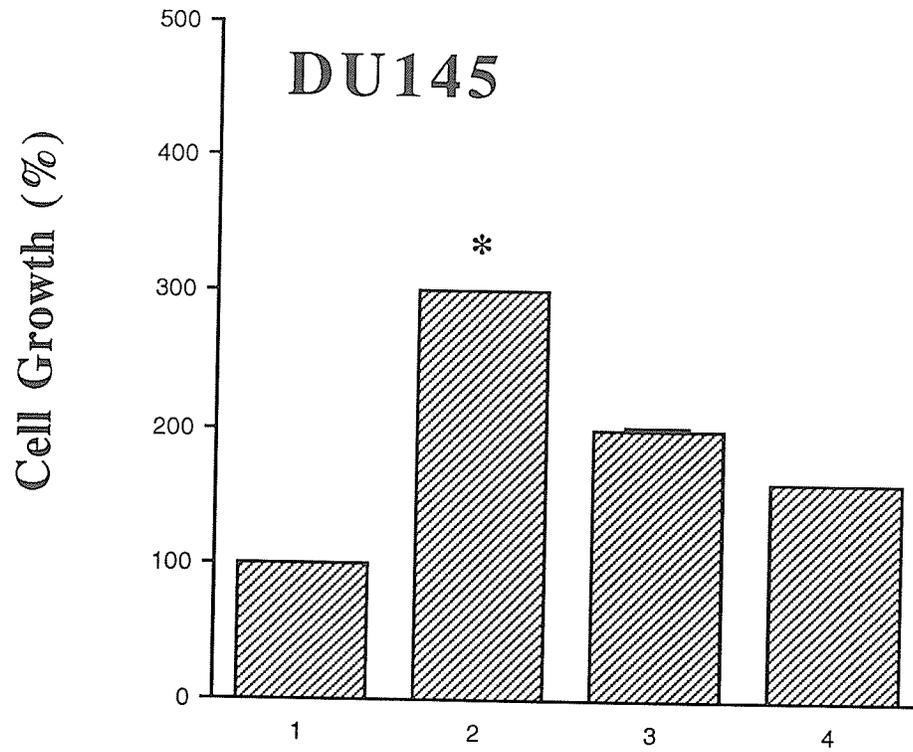
**Fig. 1-7.**

Dose response of DU145 cell growth to anti EGF receptor antibody:  $5 \times 10^3$  cells/ well of DU145 were plated and cultured overnight in 10% FBS media. Media was then changed to serum-free media containing 1ug/ml insulin and transferrin. Varying amounts of anti EGF receptor antibody were added in designated wells and incubated for 5 days. The mean values and standard errors of three experiments are shown.



**Fig. 1-8.**

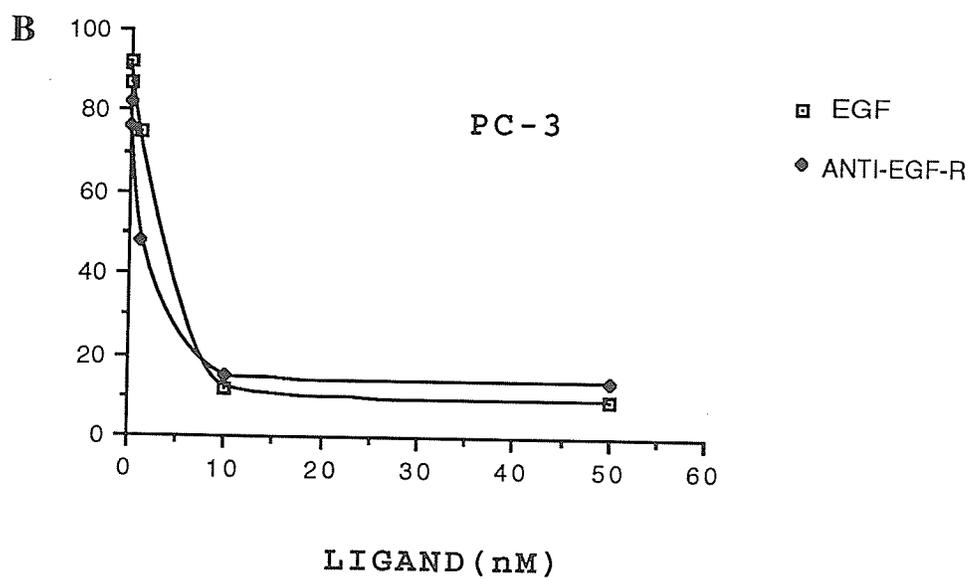
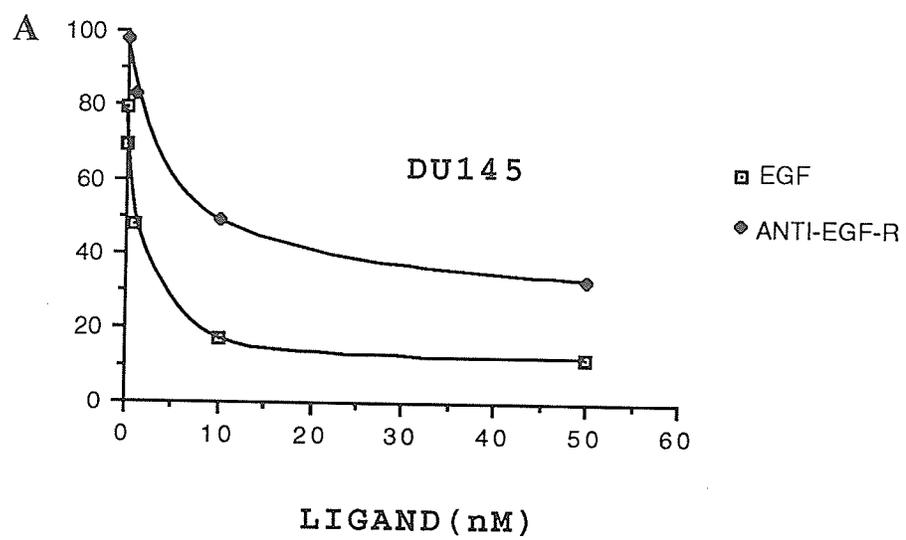
Effects of conditioned media on the growth of DU145 and LNCaP cells:  $5 \times 10^3$  cells/well of DU145 (upper panel) and  $1 \times 10^4$  cells/ well of LNCaP (lower panel) were plated and cultured overnight in 10% FBS medium. Medium was then changed to serum-free medium containing 1 $\mu$ g/ml insulin and transferrin or to conditioned medium containing 1 $\mu$ g/ml insulin and transferrin, with and without Ab EGF and Ab TGF- $\alpha$  (100nM). Cells were incubated for 5 days. Column 1, control with serum-free media; 2, conditioned medium; 3, conditioned medium with Ab EGF; 4, conditioned media with Ab TGF- $\alpha$ . The mean values and standard errors of three experiments are shown (\*  $p < 0.05$ ). The control used non-immune mouse Ig G which is no different with no-antibody.



**Fig. 1-9-A**

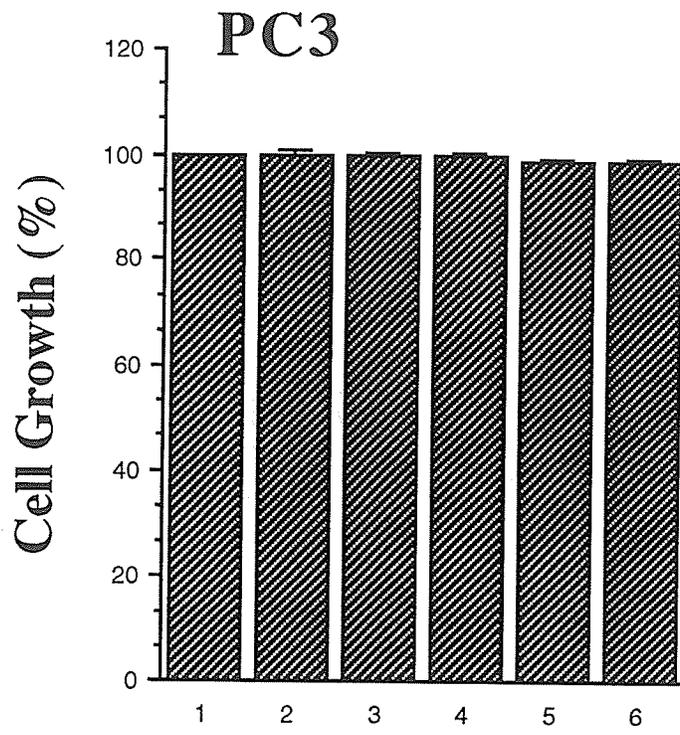
Radio-ligand binding assay. The 0.1-50 nM of  $I^{125}$  labeled EGF competed with 100-fold unlabeled EGF binding to EGF receptor. The 0.1-50 nM of anti EGF receptor antibody competed with 10 nM  $I^{125}$  labeled EGF binding to EGF receptor. Upper panel: PC-3 cell. Lower panel: DU145 cell.

# COMPETITIVE BINDING ASSAYS



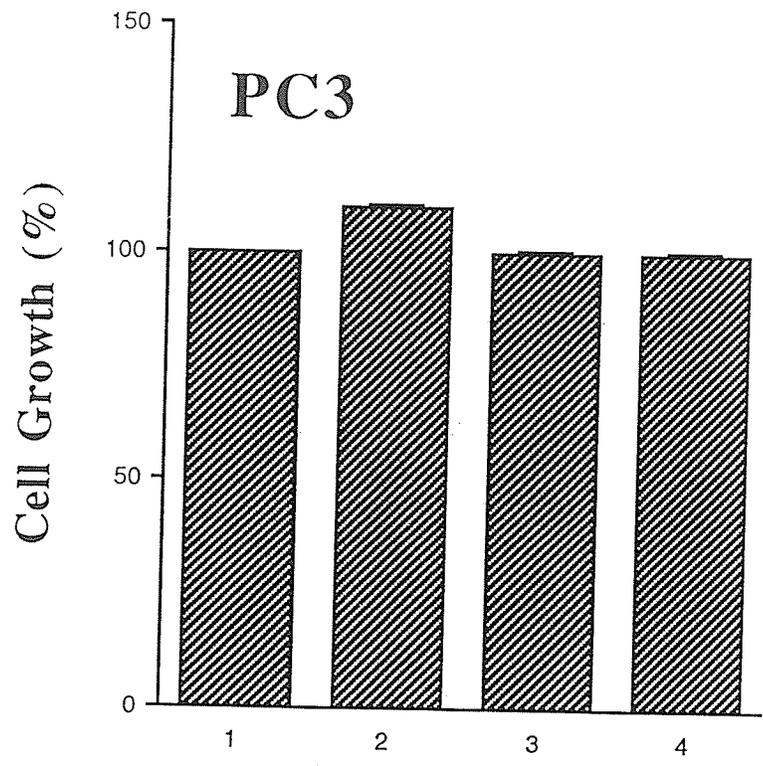
**Fig. 1-9-B.**

Effects of exogenous EGF, TGF- $\alpha$  (10nM) or Ab EGF receptor (1nM) on the growth of PC3 cells:  $1 \times 10^4$  cells/well of PC-3 cells was plated and cultured overnight in 10% FBS medium. Medium was then changed to serum free media containing 1ug/ml insulin and transferrin. EGF, TGF- $\alpha$  and/or Ab EGF receptor were added into designated wells and incubated for 5 days. Column 1, control; 2, 10nM EGF; 3, 10nM TGF- $\alpha$ ; 4, 1nM of anti EGF receptor antibody; 5, 10nM EGF with 1nM of anti EGF receptor antibody; 6, TGF- $\alpha$  with 1nM of anti EGF receptor antibody. The mean values and standard errors of three experiments are shown.



**Fig. 1-10.**

Effects of conditioned medium on the growth of PC3 cells:  $1 \times 10^4$  cells/well were plated and cultured overnight in 10% FBS medium. Medium was then changed to serum-free medium containing 1ug/ml insulin and transferrin or conditioned media containing 1ug/ml insulin and transferrin; with and without Ab EGF and Ab TGF- $\alpha$  (100nM). Cells were incubated for 5 days. Column 1, control with serum free medium; 2, conditioned medium; 3, conditioned medium with Ab EGF; 4, conditioned medium with Ab TGF- $\alpha$ . The mean values and standard errors of three experiments are shown.



**Fig. 1-11.**

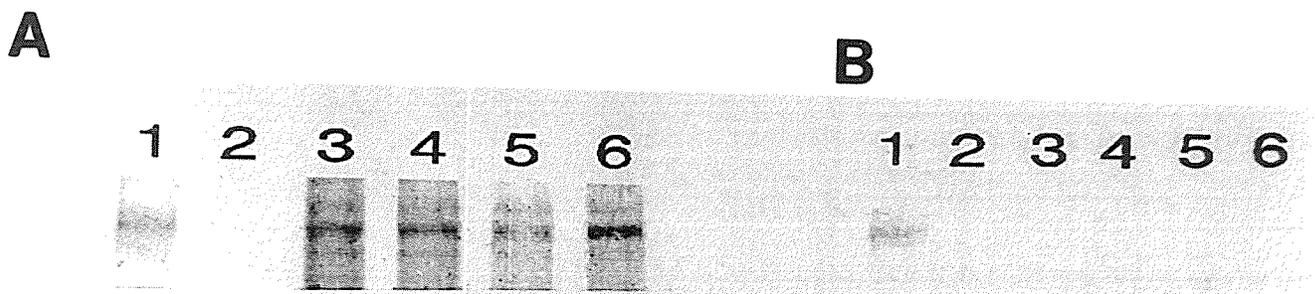
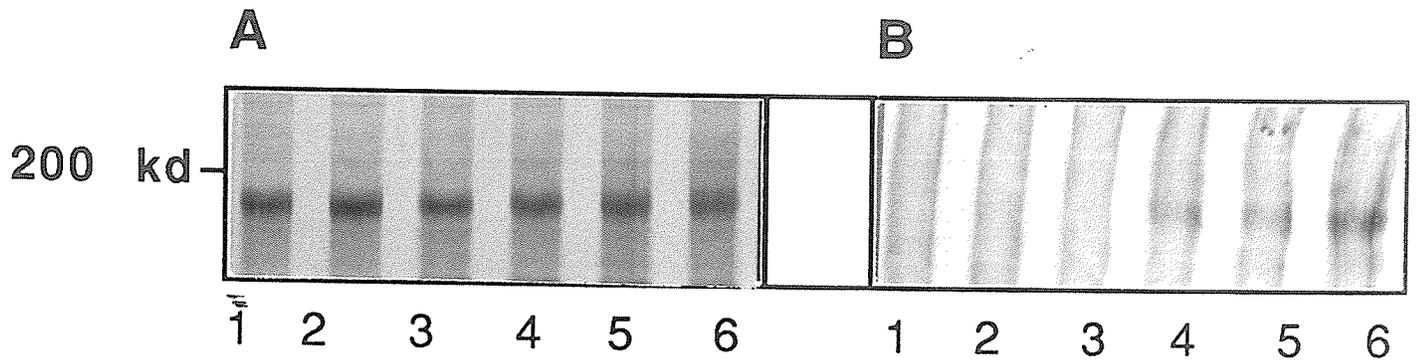
Autophosphorylation of EGF-R of DU145: Panel A. Immunoprecipitation of total EGF receptor with anti EGF receptor antibody. Panel B. Immunoblot of immunoprecipitated EGF receptor with anti-P-tyr antibody. Lane 1. 10% serum medium; Lane 2. Serum free medium; Lane 3. + Ab EGF-R; Lane 4. + Ab EGF-R + 10nM EGF; Lane 5. + Ab EGF-R + 100nM EGF; Lane 6. + 100nM EGF.

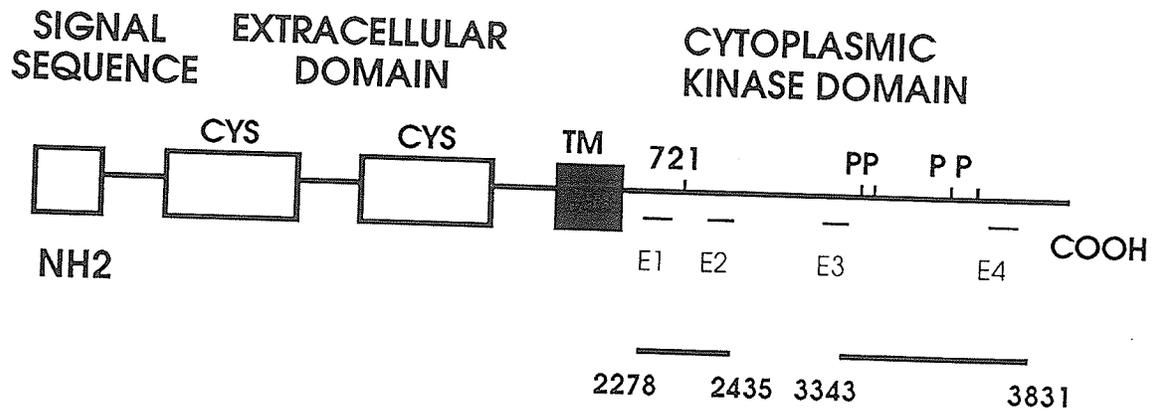
**Fig. 1-12.**

Autophosphorylation of EGF receptor of PC-3: Panel A. Immunoprecipitation of total EGF receptor with anti EGF-R antibody. Panel B. Immunoblot of immunoprecipitated EGF receptor with anti-P-tyr antibody. Lane 1. Positive control, DU145 cells + 100nM EGF; Lane 2. Blank; Lane 3, 4, 5 and 6 are PC-3 cells: 3, without EGF; 4, +Ab EGF receptor; 5, + Ab EGF receptor with 100nM EGF; 6, + 100nM EGF.

**Fig. 1-13.**

Schematic representation of the EGF receptor. Symbols are: cys, the two cysteine-rich domains, TM, the transmembrane sequence; P, the identified autophosphorylation site 1068, 1086, 1148 and 1173; 721, identified ATP binding site; E 1-4, EGF receptor primers. E1, 18 mer (2259-2278); E2, 18 mer (2435-2454); E1 and E2 generate a 157 bp fragment which covers ATP binding site. E3, 20 mer (3322-3343); E4, 20 mer (3831-3852); E3 and E4 generate a 488 bp fragment which cover the region of the autophosphorylation sites.





### 3. DISCUSSION

This investigation has focused on the hypothesis that prostate cancer cell growth is regulated, in part, by EGF receptor-mediated signals. We have shown previously that prostatic carcinoma tissue expresses high levels of EGF, TGF- $\alpha$  and EGF receptor mRNA compared to the levels expressed by BPH. Enhanced expression of mRNA for EGF, TGF- $\alpha$  and EGF receptor were detected also in three prostatic carcinoma cell lines. These observations suggested that these growth factors and their receptor play a role in controlling prostate cancer cell growth. This conclusion is supported by the present study in which a significant increase in growth of DU 145 and LNCaP cells occurred after the addition of exogenous EGF or TGF- $\alpha$  into the culture medium (Fig. 1-6). In addition, growth was reversibly inhibited by anti EGF receptor antibody, which specifically blocks the extracellular domain of EGF receptor, preventing ligand binding. This observation provides direct evidence that a specific pathway mediated by EGF and/or TGF- $\alpha$  and the EGF receptor is involved in regulation of prostate cancer cell growth.

Autocrine growth regulation has been suggested as a possible mechanism for growth factors and their receptors to control cancer cell proliferation (Sporn *et al.* 1985a). To address this possible mechanism in prostate cancer cells, assays using conditioned media were performed. We observed an elevated level of cell growth for DU145 and LNCaP cell lines in conditioned medium (Fig. 1-8) and an inhibitory effect on cell growth by the addition of anti EGF and TGF- $\alpha$  antibodies to the conditioned medium. These studies support the role of autocrine action in modulating prostate cancer cell growth.

The observation that neither antibody alone brought the growth back to control level suggests that other factors, such as FGF, growth hormone and IGF, probably also play a role in regulation prostate cancer cell growth.

In this study, an unexpected result was observed with one cell line (PC3) which did not demonstrate EGF receptor-mediated growth. Under our experimental conditions, neither exogenous EGF and TGF- $\alpha$  stimulation nor anti EGF receptor antibody were able to change the growth pattern of PC3 cells, even though both EGF and anti EGF receptor antibody show the same binding pattern in PC3 cells as in the responsive DU145 cells. Although the mRNAs of EGF, TGF- $\alpha$  and the EGF receptor were detected in PC3 cells, and the EGF receptor protein was present in PC3 cells, the conditioned medium of PC3 cells failed to influence the growth of PC3 cells. To investigate this variation in response to EGF in PC3 cells, we measured the autophosphorylated EGF receptor in the presence of the EGF in the cell culture. The initiation of autophosphorylation is a necessary step to activate the EGF receptor-mediated phosphotyrosine kinase chain to influence cell proliferation. Our studies show that PC3 cells fail to form detectable autophosphorylated EGF receptor during stimulation by EGF, and do not show a response to anti EGF receptor antibody inhibition. These results indicate that there is a functional defect in the EGF receptor in PC3 cells. The simplest explanation of a functional defect in a receptor is through a structural defect caused by an alteration in the primary amino acid sequence.

There are several reports that prove the autophosphorylation of the EGF receptor in the intracellular domain, is necessary for signal transduction (Sorkin *et al.* 1991). A single

mutation at amino acid A721 which is an ATP binding site totally abolishes the phosphorylation action of EGF receptor (Moolenaar *et al.* 1988). Since the ATP binding site and the intracellular tyrosines seemed likely sites for mutations that would block autophosphorylation, we examined the cDNA sequence of these regions. Under our conditions for RT-PCR and sequencing, we were unable to detect any mutation at the ATP binding site or the four major autophosphorylation sites. Computer simulations of the results of frame shift mutations upstream of the ATP binding site and the phosphorylation sites confirmed that truncated proteins would result. Since the EGF receptor in PC-3 cells is not detectably smaller than predicted, frame shift mutations seem unlikely.

We have not been able to determine the nature of the functional defect preventing autophosphorylation of this receptor. Alterations in the transmembrane domain or adjacent regions, or in the regions needed for receptor dimerization are other possible explanations. Lastly, there may be another, as yet uncharacterized but required, element(s) in the EGF receptor which has suffered damage in the PC-3 cell line.

## CHAPTER TWO

### ROLE OF c-erbB2/NEU IN PROSTATE CANCER CELL GROWTH

The proto-oncogene c-erbB2/NEU was originally described as a transforming gene activated in chemically induced rat neuroblastomas (Schechter *et al.*1984). The human homologue c-erbB2 or NEU, is located on chromosome 17q21 (Fukushige *et al.*1986), and encodes a messenger RNA (mRNA) of 4.8 kb and a 185 kd protein (Bargmann *et al.*1986a; Schechter *et al.*1985). The c-erbB2/NEU product is closely related to the epidermal growth factor receptor and functions as a transmembrane receptor with tyrosine kinase activity in the intracellular domain (Fukushige *et al.*1986; Downward *et al.*1984a; Nesland *et al.*1991). Expression of c-erbB2/NEU in several normal and transformed tissues (Yamamoto *et al.*1986a; Pavelic *et al.*1992) suggests c-erbB2/NEU may play a role in cell proliferation. Overexpression of c-erbB2/NEU has been correlated with tumor prognosis in human tumor and animal models (Di Fiore *et al.*1987a; Berchuck *et al.*1990). In addition, overexpression of an activated c-erbB2/NEU oncogene was sufficient to transform rat prostate epithelial cells (Sikes *et al.*1992b) suggesting that the c-erbB2/NEU may contribute to the regulation of normal growth and to malignant transformation.

The role of c-erbB2/NEU in prostate cancer is not clear, even though the expression of c-erbB2/NEU has been detected in prostate tissue by immunohistochemistry (Zhau *et al.*1992). To address this question, we have examined the gene for c-erbB2/NEU, its expression, and its mechanism of action in prostate cancer growth.

## A: ANALYSIS OF GENETIC ALTERATIONS IN c-erbB2/NEU

### 1. INTRODUCTION

Amplification and overexpression of genes encoding growth factor receptors have the potential to contribute to tumor growth and progression. Recently, the amplification of c-erbB2/NEU has been observed in several cancers (Slamon *et al.*1989; Zeillinger *et al.*1989; King *et al.*1985; Yusa *et al.*1990; Donovan Peluso *et al.*1991), and increased copy number of the c-erbB2/NEU gene was associated with poor prognosis, especially in breast and ovarian cancer (Slamon *et al.*1989; Berchuck *et al.*1990; Pavelic *et al.*1992; Winstanley *et al.*1991; Wright *et al.*1992).

We have recently demonstrated enhanced expression of EGF receptor, which is highly homologous to the c-erbB2/NEU receptor growth factor receptor, in prostate carcinomas (Morris *et al.*1990). To determine whether alterations of c-erbB2/NEU are also a feature of prostate tumors, we examined a series of high grade prostate carcinomas, established human prostate tumor cell lines (DU145, PC-3 and LNCaP) and benign prostatic hyperplasias.

### 2. RESULTS

Southern blot analyses were performed to determine the possible involvement of c-erbB2/NEU gene amplification in prostate cancer. Differences in signal intensity were seen among the three prostatic carcinoma cell lines when hybridized with the probe for human c-erbB2/NEU (Fig. 2-1 and Fig. 2-2 top panel). Fig. 2-1 illustrates the relative

gene copy number in the 3 prostate cancer cell lines. LNCaP and DU145 cells contain an amplified c-erbB/NEU gene relative to PC-3 cells. In the following figure (Fig. 2-2), the amplification is compared to that seen in SKBR-3 cells, a human breast cancer cell line, previously estimated to contain about 10 copies of the c-erbB2/NEU gene per cell. These differences were not due to aneuploidy involving chromosome 17 since rehybridization with the probe for the human growth hormone (GH) gene locus, also on 17q, showed equal signal intensity across the three cell lines (Fig. 2-1 and Fig. 2-2, bottom panel). Differences in signal intensity for c-erbB2/NEU DNA were also seen among samples obtained from 19 patients at the time of prostatectomy. Four representative samples hybridized with the c-erbB2/NEU probe are shown in Fig. 2-3 (top panel) with the corresponding rehybridization with the GH probe (lower panel). As with the cell lines, the variability in hybridization signal among the patient samples is specific for c-erbB2/NEU and does not represent aneuploidy of 17q.

Quantitation of the relative gene copy number for erbB2/NEU was achieved by densitometric analysis of the Southern blots. Blots were rehybridized with a probe for the prolactin (PRL) gene on an unrelated autosome (6p) in order to normalize for the amounts of DNA loaded. It has compared to GH locus and indicated no aneuploid of chromosome 17. The standard gene copy number ( $n=2$ ) was set using DNA from normal diploid prostate tissue obtained at autopsy from a 19 year old man and placental tissue. The relative gene copy numbers of the 19 patient samples, placental tissue and three prostate cancer cell lines are presented in Fig. 2-4. The Fig. 2-4 shows the results of the densitometric analysis of the Southern blots. What is most obvious is the consistency of

the signals among the BPH samples and the great variation of the signals among the CaP samples. The CaP samples were selected for their high proportion of tumor cells, however some degree of heterogeneity exists in the material assayed. The effect of an admixture of cells containing amplified copies of c-erbB2/NEU genes and cells containing the normal complement would be to underestimate the level of amplification present. Based on this assessment, the prevalence of c-erbB/NEU amplification in high grade prostate carcinomas is probably at least 75%. None of the 9 benign prostatic hyperplasias (BPH) analyzed showed amplification of the c-erbB2/NEU gene. In contrast, a high proportion of the prostatic carcinoma (CaP) contained amplified erbB2/NEU gene copies; 6 of 10 (60%) were amplified more than 3-fold, including 2 (20%) amplified more than 5-fold. SKBR-3, a human breast cancer cell line, was included as a positive control for erbB2/NEU amplification. It has been shown previously (Kraus *et al.*1987) to be amplified about 5-fold (ie about 10 copies of erbB2/NEU gene) per cell. Table 2-1 represents a compilation of the data relating to c-erbB2/NEU amplification in the patient samples and the cell lines.

The amplification of the erbB2/NEU gene does not represent an age-related change in the genome since it was seen only in the CaP tissue and not in the benign prostate tissue from age-matched BPH controls. Although the number of CaP samples in this study was small, the relative gene copy number of erbB2/NEU did not correlate with patient age.

To determine whether amplification of the erbB2/NEU gene is associated with overexpression of the gene product, protein analyses were performed. Western

immunoblots were used to assess the relative levels of c-erbB/NEU protein in extracts of protein from patient samples and cell lines. Two human breast cancer cell lines were used for comparison: MCF-7 cells which contain the normal complement of c-erbB2/NEU genes and express low levels of the gene product, and SKBR-3 cells which have amplified copies of the gene and express very high levels of the c-erbB2/NEU protein (Kraus *et al.*1987). Representative samples of Western blots for c-erbB2/NEU are shown in Fig. 2-5.

The Western blots (Fig. 2-5) indicated that all three prostate cancer cell lines express the c-erbB2/NEU protein. Higher levels were evident in DU145 and LNCaP cells, the lines shown to have low level amplification of the c-erbB2/NEU gene. The BPH samples contained only low levels of c-erbB2/NEU protein; only 1 of 9 BPH samples contained levels slightly higher than found in MCF-7 cells. In contrast, 5 of 10 carcinoma samples contained levels of c-erbB/NEU protein higher than found in MCF-7 cells, including 1 sample with levels equivalent to those seen in SKBR-3 cells.

The results indicate that amplification of the c-erbB2/NEU proto-oncogene is a frequent event in prostate cancer and is associated with overexpression of the c-erbB2/NEU gene product.

**Fig. 2-1.**

Genomic Southern analysis of DNA from cell lines: (lane 1, Du145; lane 2, LNCaP; lane 3, PC-3). Genomic DNA (10 ug) was digested with Pst\_1, fractionated by gel electrophoresis and transferred onto nitrocellulose membrane.  $\alpha$ -<sup>32</sup>P labelled cDNA probes for erbB2/ NEU (upper panel) and GH (human growth hormone) (lower panel) were hybridized sequentially to the blot. Molecular weight markers are indicated on the right in kilobase pairs.

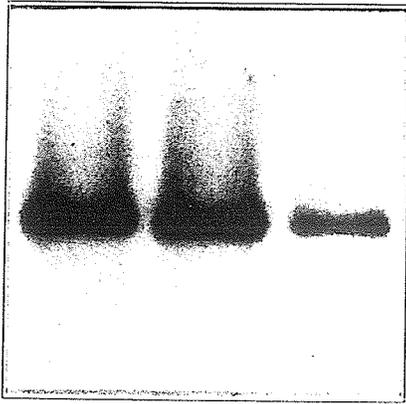
# SOUTHERN BLOT

LNCaP

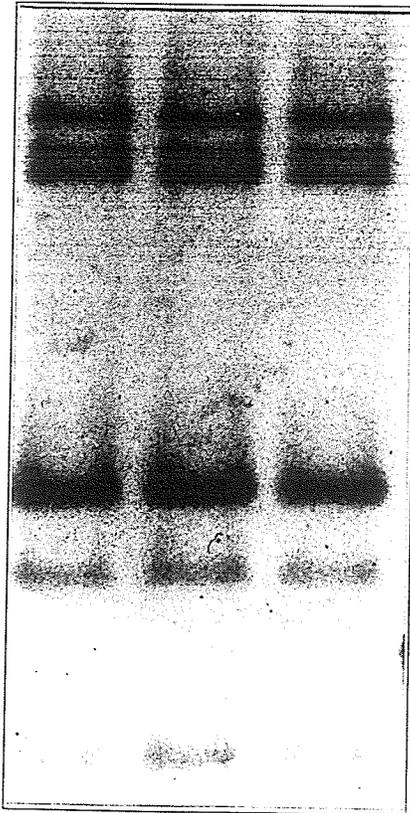
DUI45

PC-3

Erb-B2  
(neu)

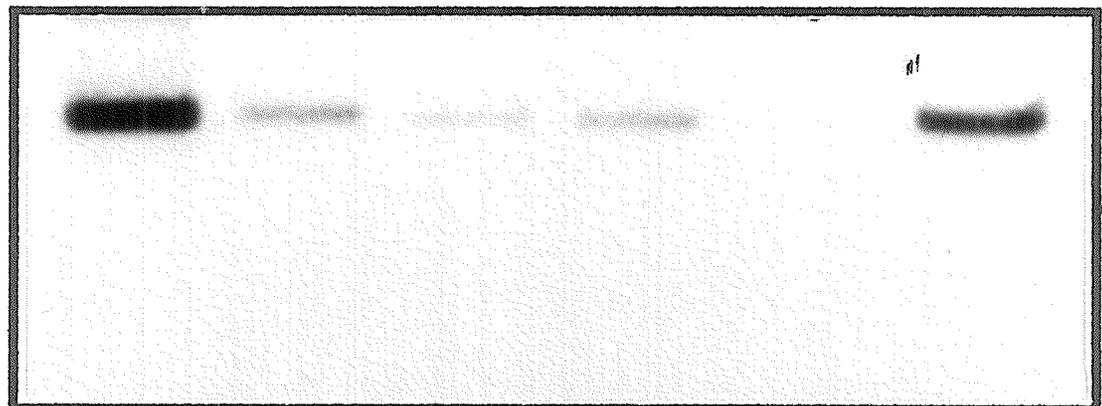


Human  
Growth  
Hormone  
cDNA



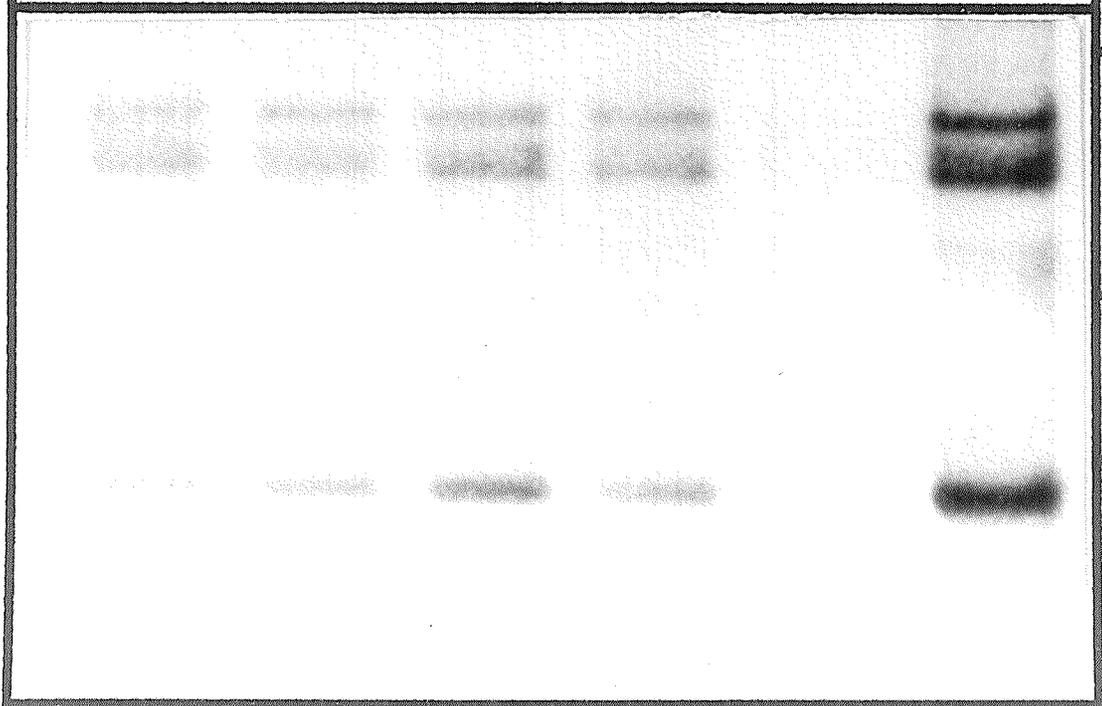
1 2 3 4 5 6

**A**



—2.3 kb

**B**



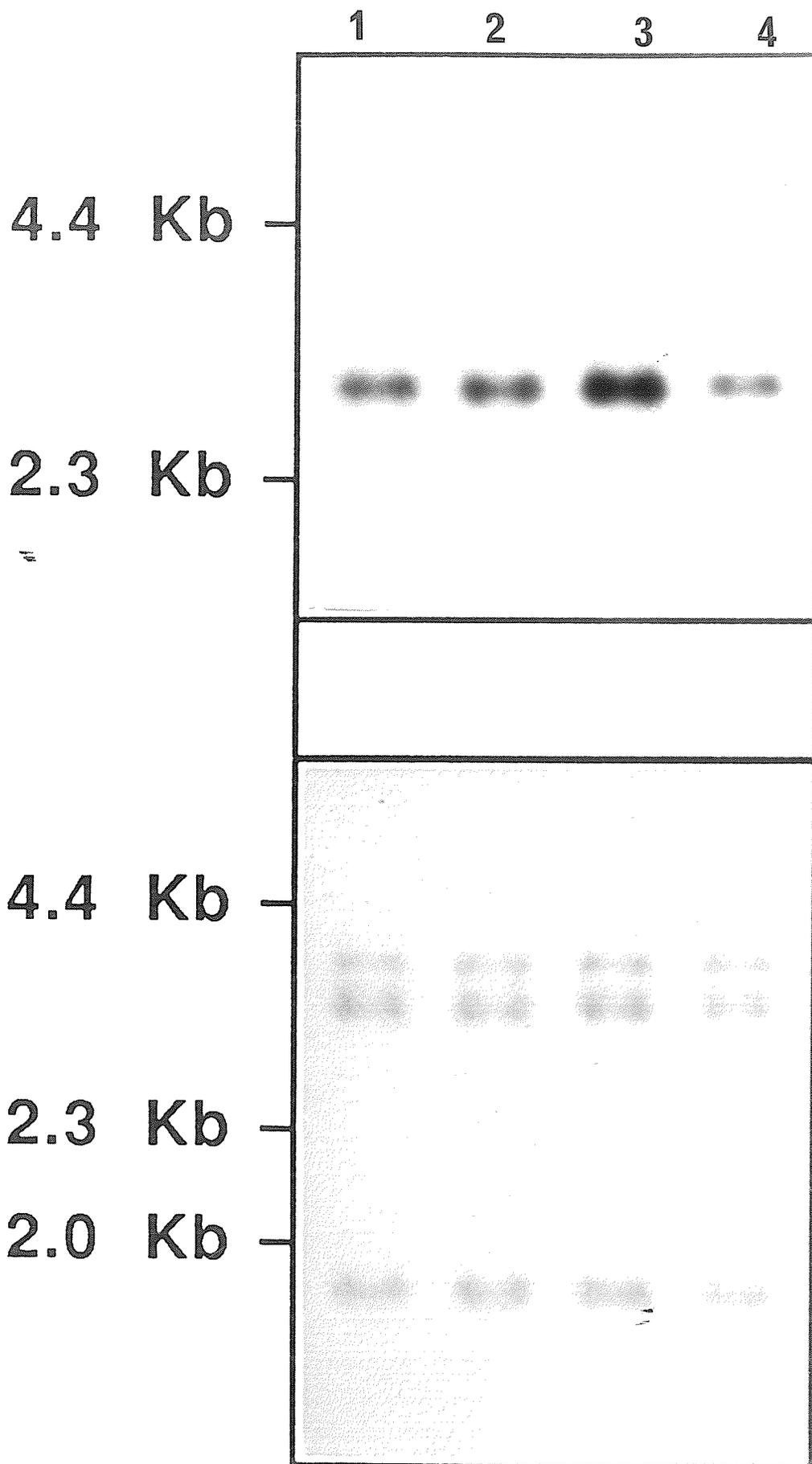
—4.4 kb

—2.3 kb

—2.0 kb

**Fig. 2-3.**

Genomic Southern analysis of DNA from representative BPH (lanes 1,2) and CaP (lanes 3, 4) patients. Genomic DNA (10ug) was digested with Pst 1, fractionated by gel electrophoresis and transferred onto nitrocellulose membrane.  $\alpha^{32}$ -P labelled cDNA probes for erbB2/NEU (upper panel) and GH (lower panel) were hybridized sequentially to the blot. Molecular weight markers are indicated on the left in kilobase pairs.

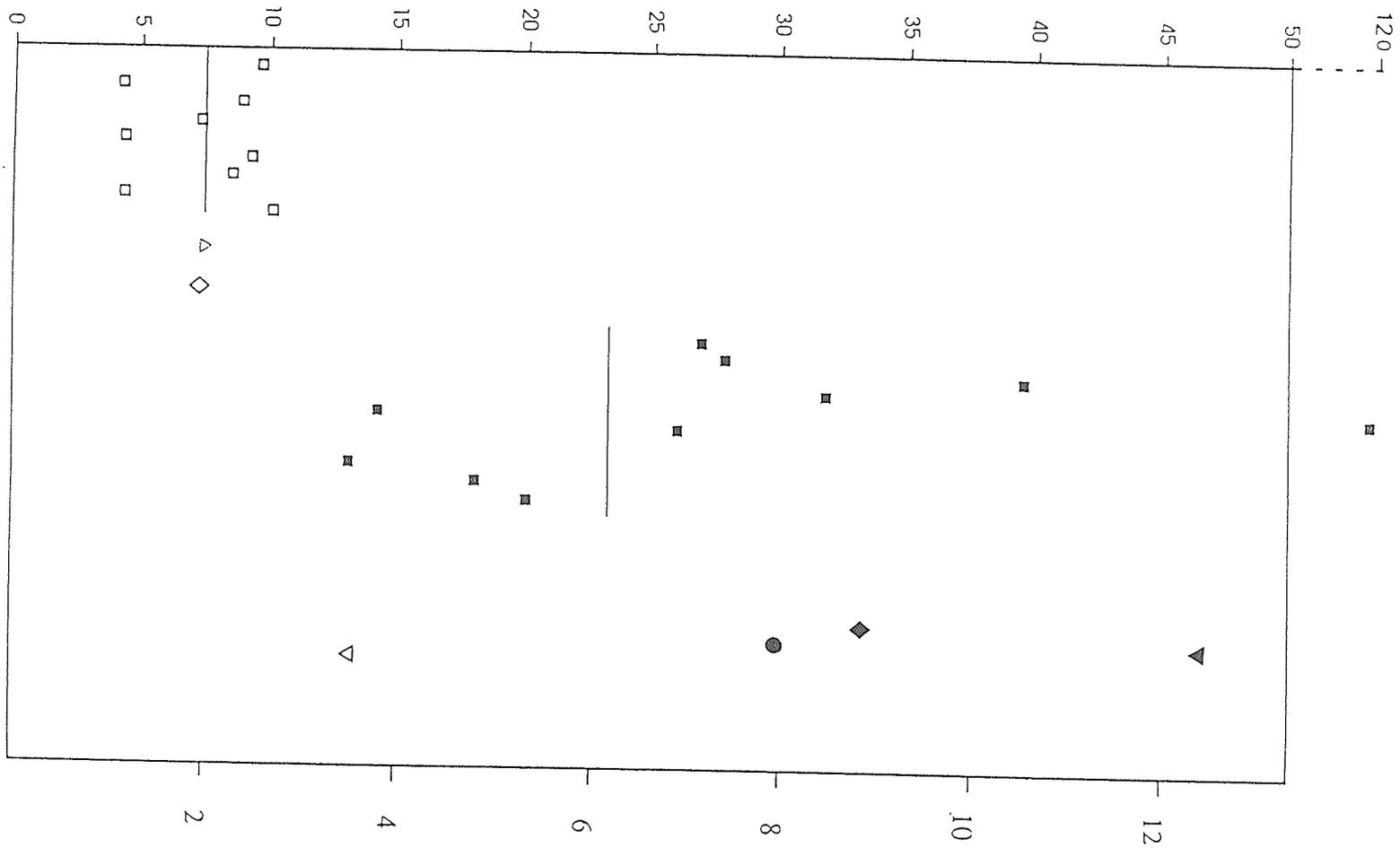


**Fig. 2-4.**

Analysis of c-erbB2/NEU gene amplification in DNA of BPH and CaP patients and three prostate carcinoma cell lines (CL). The relative gene copy number was determined by densitometric scanning of autoradiographs of Southern blots hybridized sequentially with probes for c-erbB2/NEU and PRL. DNA from normal prostate tissue and human placenta tissue served as control for the normal diploid genome. The breast cancer cell line, SKBR-3, served as a positive control for amplified c-erbB2/NEU DNA.

⊕: normal human prostate tissue, Δ: human placenta tissue, □: BPH, ■: CaP, ∇: PC-3, ◆: Du145, ●: LNCaP, ▼: SKBR-3, ○: normal prostate tissue.

# DNA RELATIVE DENSITY



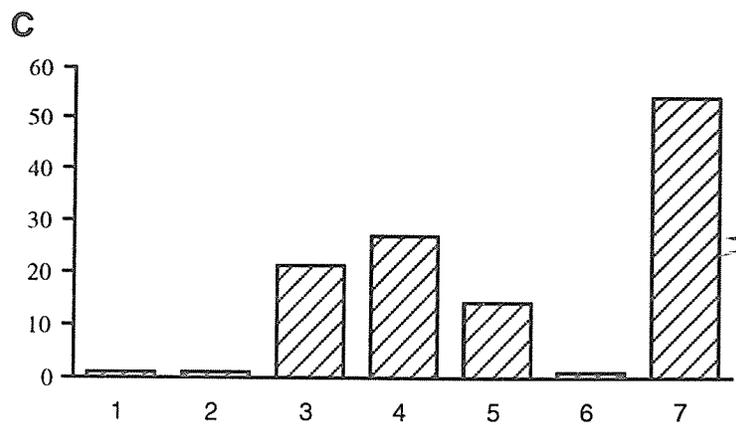
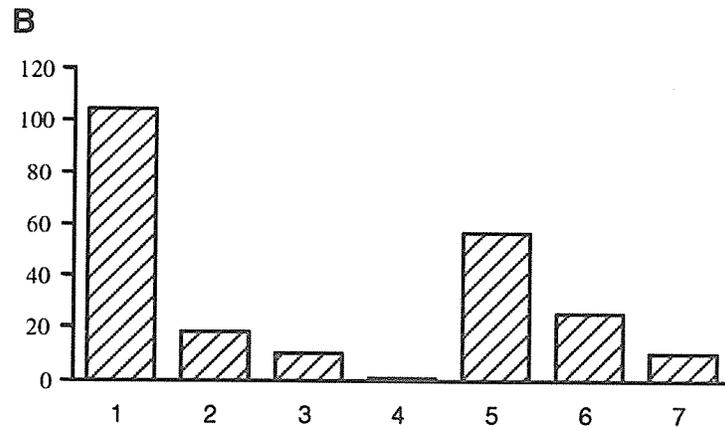
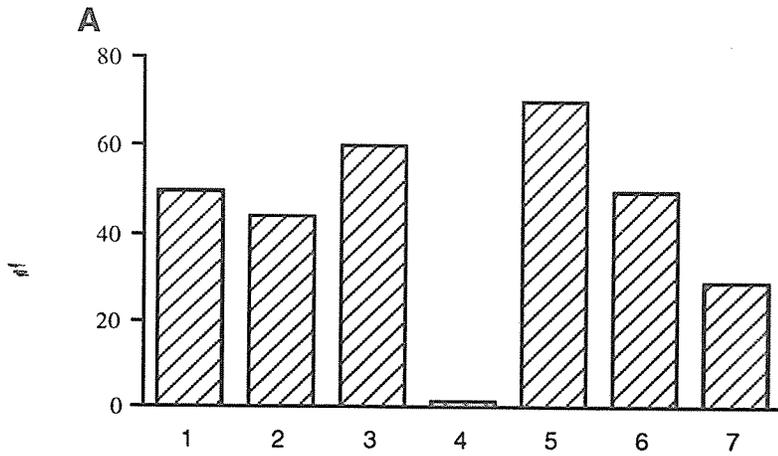
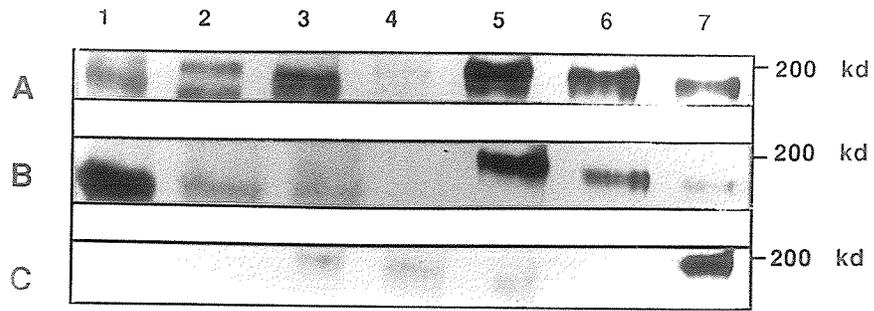
RELATIVE GENE COPY NUMBER

**Table 2-1.**

Analysis of c-erbB2/NEU gene amplification in DNA of BPH and CaP patients, and three prostate carcinoma cell lines (DU145 PC-3 and LNCaP), breast cancer cell line, SKBR-3, and normal prostate tissue (NP) and human placenta tissue. The fold of amplification was determined by densitometric scanning of autoradiographs of Southern blots hybridized sequentially with probes for c-erbB2/NEU and PRL.

Table 2-1

Sample	Fold Amplification			Total Number
	None	3-5	>5	
CaP	4	4	2	10
BPH	9	0	0	9
DU145		X		
LNCaP		X		
PC-3	X			
SKBR-3			X	
Placenta	X			
NP	X			



### 3. DISCUSSION

In this study we present evidence of c-erbB2/NEU gene amplification and overexpression in human prostate cancer. Although prostate cancer is the leading cause of new cancer cases in North American men, two previously reported surveys of human tumors for c-erbB2/NEU gene amplification included no prostate cancers (Yokota *et al.*1986; Tal *et al.*1988). ErbB2/NEU gene amplification is specific to the malignant prostate since none of the BPH samples showed amplification despite their enhanced capacity for proliferation. The amplification is also specific to the c-erbB2/NEU region of chromosome 17q since none of the samples showed any change in the growth hormone gene locus, also on 17q. Amplification was the only structural change observed in the c-erbB2/NEU gene; no gross rearrangements were evident, although more subtle alterations such as point mutations may have been present.

In our study, the frequency of c-erbB2/NEU gene amplification was 60% in selected prostatic tumors; tumors in the study were all high grade (Gleason score >5) indicating moderate to poor cellular differentiation within the tumors. Whether c-erbB2/NEU gene amplification is as frequent in low grade, well differentiated tumors will be determined in future studies. The highest frequency of c-erbB2/NEU gene amplification previously reported was 25-30% in primary breast and ovarian carcinomas (Slamon *et al.*1989; Iglehart *et al.*1990; Kury *et al.*1990). Taking the prostate, breast and ovary tumor data together, amplification of the c-erbB2/NEU gene may be one of the most common genetic events specific to adenocarcinomas.

Among breast cancer cell lines, amplification of the c-erbB2/NEU DNA is invariably

associated with overexpression of the mRNA and the protein (Kraus *et al.*1987; Tommasi *et al.*1991). This also appears to be the case for prostate cancer. Both DU145 and LNCaP cells, which contain additional copies of the c-erbB2/NEU gene, express higher levels of the p185 protein than the unamplified PC-3 cells. In patient samples, BPH tissue contains low levels of p185 protein, while a high proportion of CaP tissues contain elevated levels of p185. In breast and ovarian cancers, overexpression of c-erbB2/NEU is associated with poor prognosis and reduced survival times (Slamon *et al.*1989; Iglehart *et al.*1990; Borg *et al.*1991; Kallioniemi *et al.*1991), and is often concomitant with DNA amplification. Overexpression of c-erbB2/NEU has been associated also with advanced tumors of the urinary bladder (Moriyama *et al.*1991). We have demonstrated overexpression of c-erbB2/NEU in high grade prostatic tumors and our data are consistent with a recent immunohistochemical analysis (Zhou *et al.*1992) in which 80% (12 of 15) of high grade tumors showed positive staining for c-erbB2/NEU overexpression. However, in contrast to that study, we have demonstrated gene amplification in prostate cancer samples. Our analysis involved analysis of gene copy number relative to one gene on the same chromosome arm and one gene on an unrelated autosome. It is unclear whether their analysis involve any genes other than c-erbB2/NEU. The low level of amplification < 5-fold seen in many of our patient samples might be undetected without careful densitometric analysis. Nonetheless, even 2 to 5-fold amplification of c-erbB2/NEU had a demonstrable effect on patient survival in both breast and ovarian cancer (Slamon *et al.*1989). At present there are few prognostic markers for prostate cancer. The potential of c-erbB2/NEU as a marker of malignant potential and prognosis in human prostate

cancer warrants intensive investigation.

## **B: AUTOCRINE REGULATION GROWTH BY THE c-erbB2/NEU**

### **1. INTRODUCTION**

Alterations of c-erbB2/NEU, including amplification and overexpression have frequently been observed in several human cancers including breast, ovarian, bladder and prostate (Slamon *et al.*1989; Moriyama *et al.*1991; Zhau *et al.*1992; Ching *et al.*1993). The amplification of c-erbB2/NEU has been found to be associated with poor prognosis, particularly for breast and ovarian cancer (Slamon *et al.*1987; Berchuck *et al.*1990; Kallioniemi *et al.*1991; Winstanley *et al.*1991). This strongly suggests that the c-erbB2/NEU oncogene is one of the factors which is involved in regulation of cancer progression. Additional experiments show that the c-erbB2/NEU molecule is able to cause phenotypic transformation of the NIH3T3 cell line and is therefore implicated in the tumorigenic process (Shih *et al.*1981; Di Fiore *et al.*1987b; Hudziak *et al.*1987; Slamon *et al.*1988; Tarakhovsky *et al.*1990).

The c-erbB2/NEU protein is structurally similar to the EGF receptor in that it contains an extracellular ligand binding domain and an intracellular domain with auto-phosphorylation sites (Schechter *et al.*1985; Stern *et al.*1988a; Yamamoto *et al.*1986b; Downward *et al.*1984b). Based on the structural homology between EGF receptor and c-erbB2/NEU, it is assumed that ligand binding to the c-erbB2/NEU receptor will trigger a cascade of events from tyrosine kinase phosphorylation of cytoplasmic proteins to the nuclear transcription of genes such as c-fos (Ullrich *et al.*1990; Koskinen *et al.*1990; Scott *et al.*1991; Stancovski *et al.*1991). Although the specific ligand for c-erbB2/NEU is not fully characterized, monoclonal antibodies specific for the

extracellular domain of c-NEU provide the opportunity to study the role of c-erbB2/NEU in controlling prostate cancer cell growth. If c-erbB2/NEU-mediated signalling pathway contributes to prostate cancer cell proliferation, a blockade of the signal at the point of ligand binding should decrease cell growth. In this study, we have evaluated the expression of c-erbB2/NEU in human prostatic carcinoma cell lines and the involvement of c-erbB2/NEU in regulating prostate cancer cell growth.

## 2. RESULTS

We have demonstrated in the preceding chapter the amplification and overexpression of c-erbB2/NEU in human prostate cancer samples. To investigate the putative role of this receptor in regulating human prostate cancer cell growth, we investigated the expression of c-erbB2/NEU in human prostate cancer cell lines. The messenger RNA for the 4.4 Kb c-erbB2/NEU transcript was expressed in all three prostate cancer cell lines (DU145, LNCaP and PC-3) (Fig. 2-6). SKBR-3, a breast cancer cell line which is known to express very high levels of c-erbB2/NEU mRNA was used as a comparison (Kraus *et al.* 1987). The levels expressed by the prostate cancer cell lines were readily detectable by Northern analysis but lower than the levels expressed by SKBR-3. The assessment of c-erbB2/NEU protein levels also indicated expression the 185kd protein in all the prostate cell lines. For comparison, the p185 levels were assessed also in two breast cancer cell lines. MCF-7 cells are known to express low levels of p185 while SKBR-3 cells are known to express very high levels of the protein. All three prostate cancer cell lines contained levels of c-erbB2/NEU protein substantially higher than in MCF-7 cells

but somewhat lower than SKBR-3 cells (Fig. 2-7). The highest level of c-erbB2/NEU expression at both the mRNA and the protein level was observed in LNCaP cells (Fig. 2-7), a cell line which has retained androgen responsiveness. The multiple forms of c-erbB2/NEU protein that are evident by the Western analysis have been reported also in breast cancer (Hudziak *et al.* 1989) and represent different glycosylation patterns (Hudziak *et al.* 1989).

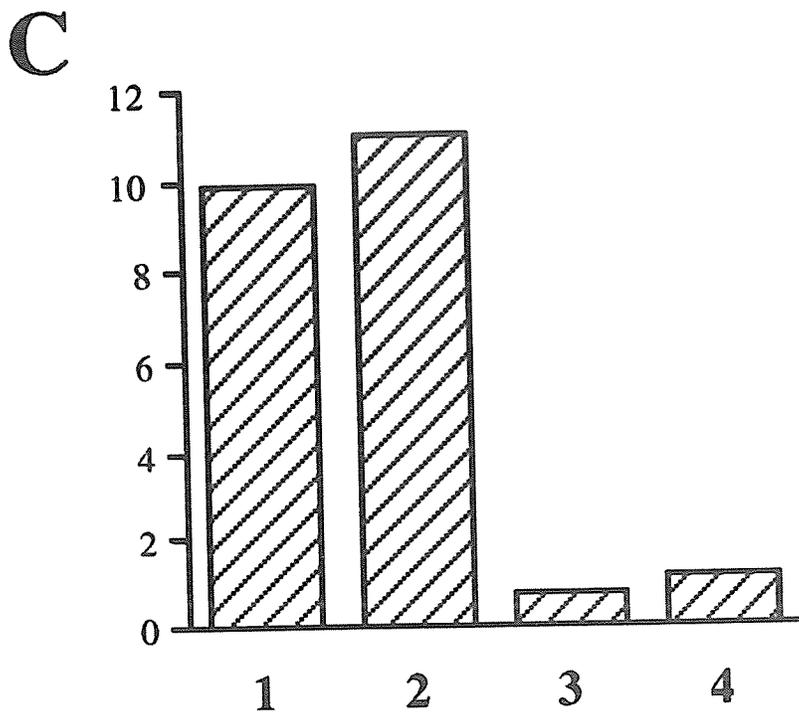
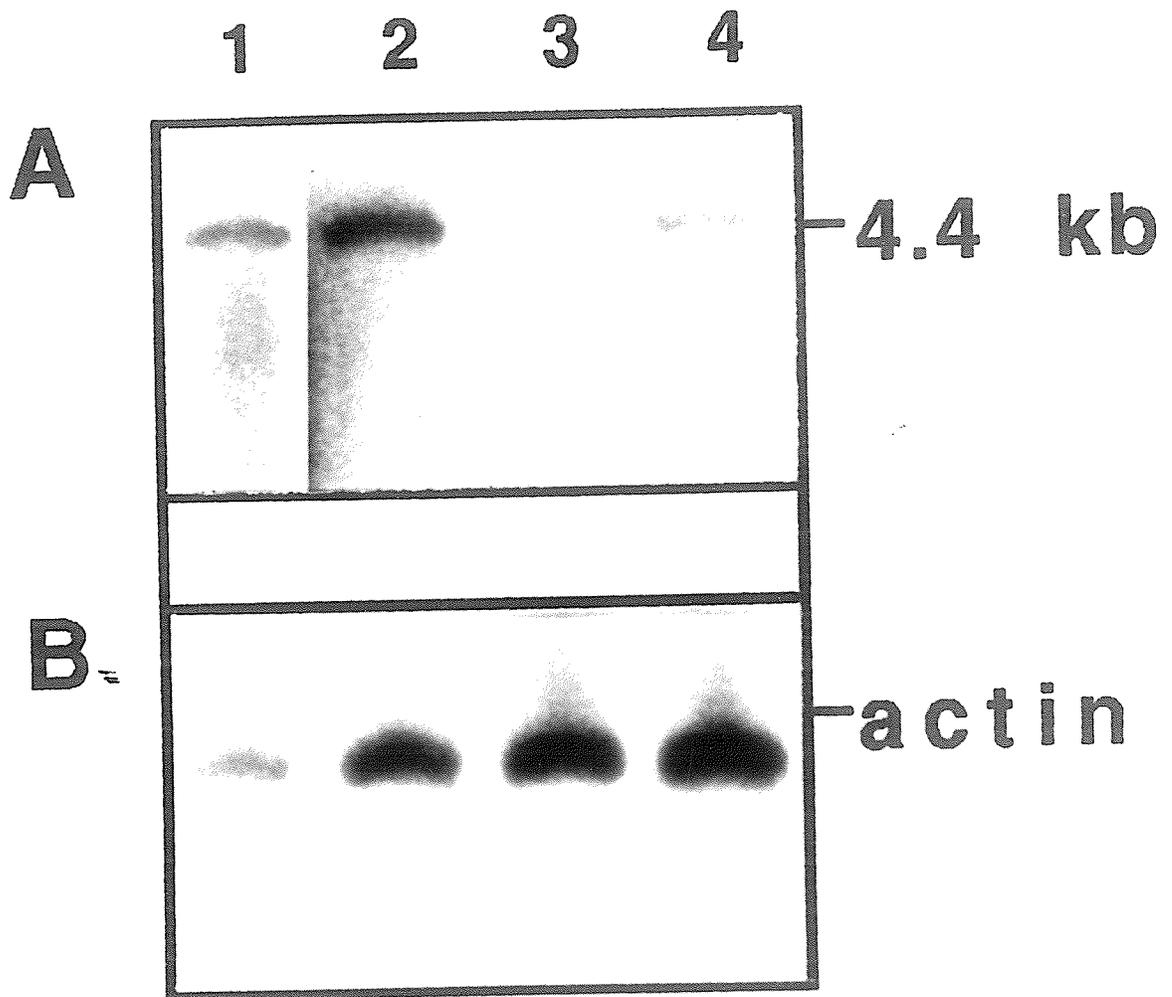
To determine the contribution of c-erbB2/NEU-mediated pathways to cell proliferation, prostate cancer cells were incubated with anti-c-NEU antibody which is specific for the extracellular domain of the protein and monitored for growth inhibition. Fig. 2-8. shows the dose-dependent reduction in proliferation of DU145 cells in the presence of anti-c-NEU antibody. Similar responses were seen for the other two cell lines. The response was specific for anti-c-NEU, as a non-specific mouse IgG did not result in the same decrease in proliferation (Fig. 2-9). The effect of anti-c-NEU antibody on cell growth is reversible, in that removal of the antibody results in a significant increase in cell number within two days (Fig. 2-10).

Transient activation of phosphotyrosine kinases by ligand induction results in a cascade of signal transduction which includes regulation of transcription of genes involved in the cell cycle. Changes in the expression of genes such as c-fos have been used as markers of signal transduction (Scott *et al.* 1991). To examine the c-erbB2/NEU-mediated signal transduction pathway in prostate cancer cells, we examined c-fos mRNA levels at various times following the addition of anti c-NEU Ab-2. The reduction in c-fos mRNA was relatively rapid, the steady state level having decreased 3-fold within 2 hours

of antibody addition (Fig. 2-11).

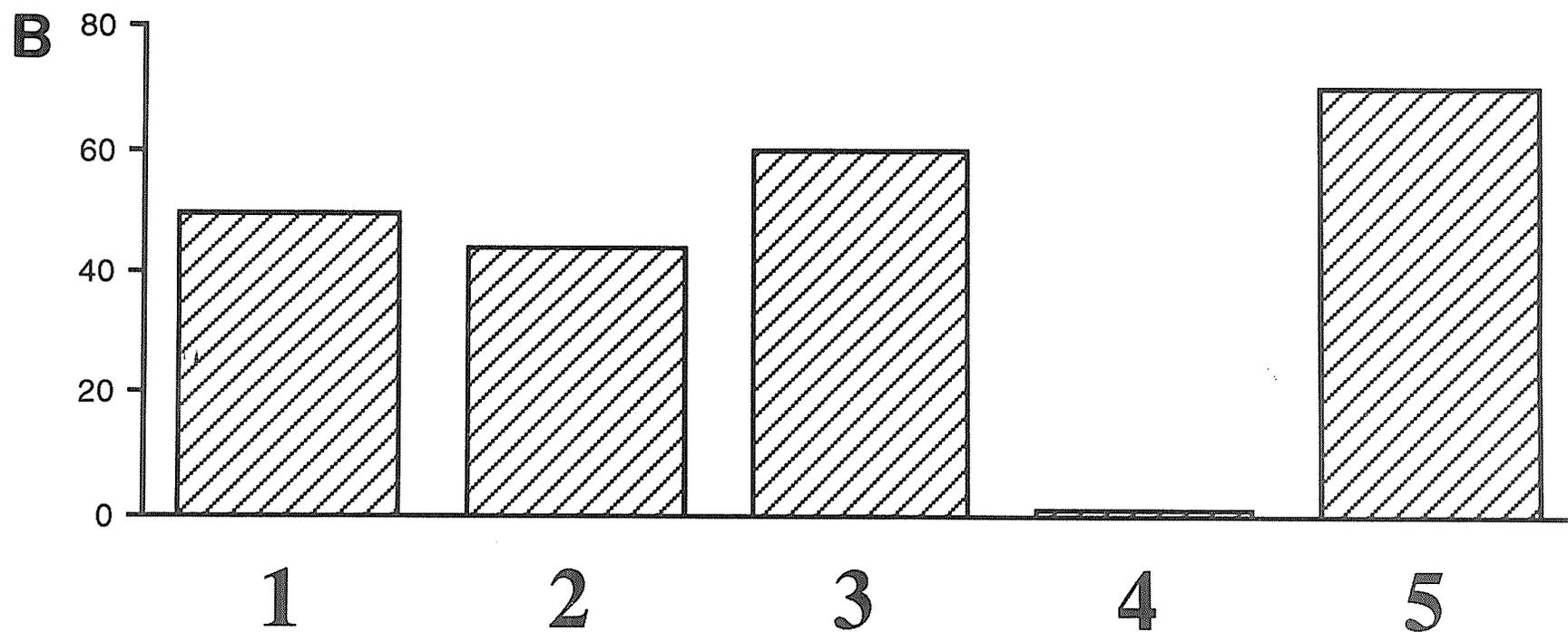
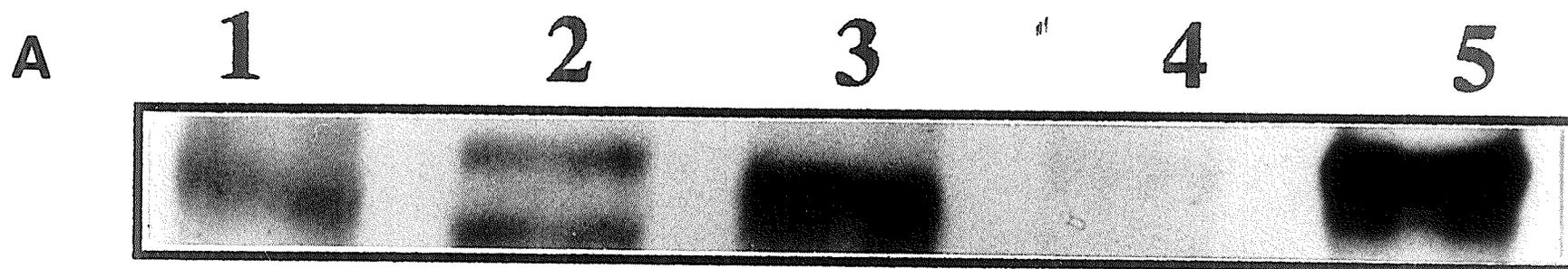
**Fig. 2-6.**

Northern blot analysis of mRNA from three prostate carcinoma cell lines, one breast cancer cell line. Poly A<sup>+</sup> RNA (10 ug) was fractionated by gel electrophoresis and transferred onto nitrocellulose membrane.  $\alpha$ -<sup>32</sup>P-dCTP labelled DNA probe for c-erbB2/NEU and actin were hybridized to the blot. Panel A: Expression of c-erbB2/NEU, lane 1: SKBR-3 cells (autoradiography for 2 hours), lane 2: LNCaP cells, lane 3: PC-3 cells and lane 4: DU145 cells (lane 2, 3 and 4 were autoradiographed for overnight). Panel B: Expression of actin shown for same blot. Panel C: Relative expression of c-erbB2/NEU mRNA assessed by densitometric analysis of panels A and B.



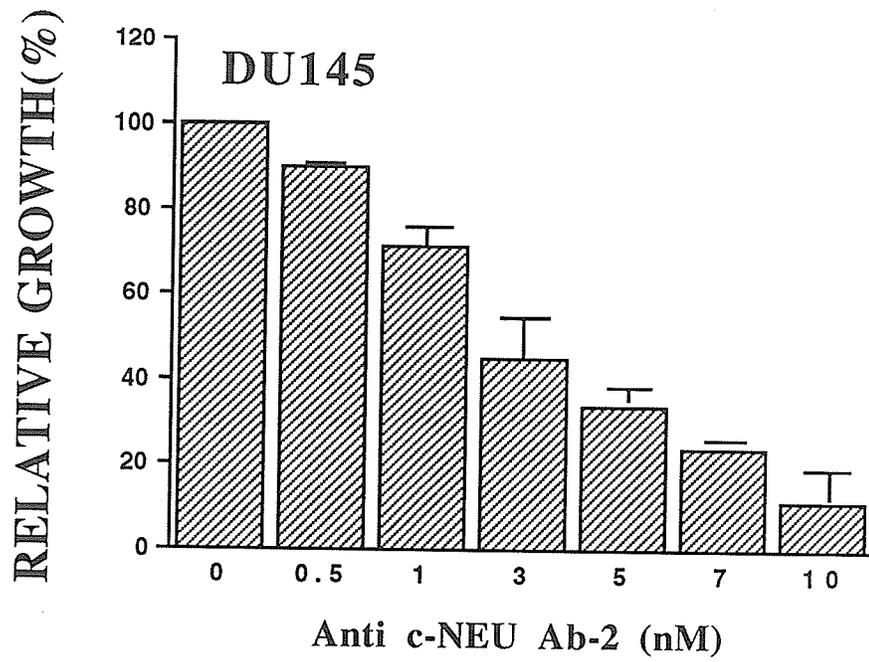
**Fig. 2-7.**

Western blot analysis of c-erbB2/NEU (p185) protein from prostate carcinoma cell lines (DU145, PC-3 and LNCaP) and breast cancer cell lines (SKBR-3 and MCF-7). Total protein extracts (30mg) were electrophoresed on a 6% SDS-PAGE gel and transferred to Nitrocellulose paper. Blots were incubated with Anti c-NEU Ab-3 and bands were detected by Enhanced Chemiluminescence Western blotting detection system. Lane 1: LNCaP; Lane 2: PC-3; lane 3: DU145; lane 4: MCF-7; and lane 5: SKBR-3. Panel A: Expression of c-erbB2/NEU protein. Panel B: Relative protein level of c-erbB2/NEU assessed by densitometric analysis of panel A.



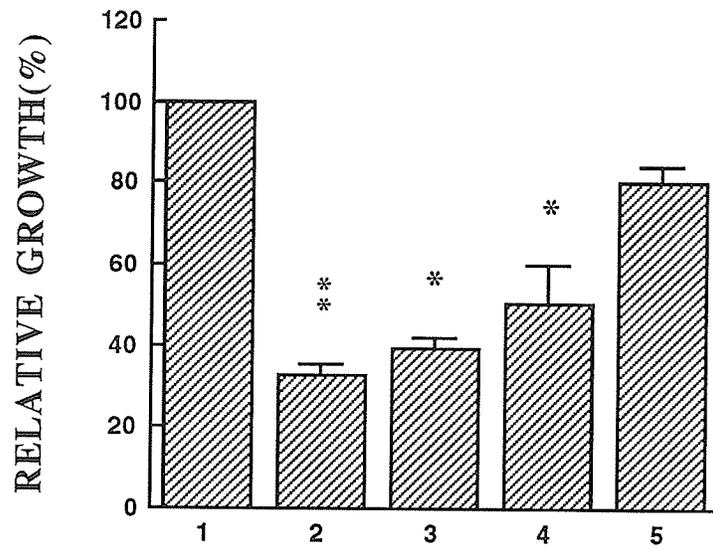
**Fig. 2-8.**

Inhibition of prostate cancer cell growth by anti c-NEU Ab-2 monoclonal antibody in vitro: Dose response. Prostate carcinoma cell line, DU145 ( $5 \times 10^3$  cells/well) were plated into triplicate wells with 10% FBS media. After one day, the medium was changed to serum-free containing 1 ug/ml of insulin and transferin. Anti c-NEU Ab-2 antibody (0, 0.5, 1, 3, 5, 7, and 10 nM) was added and at day 6, cells were counted. The mean values of relative growth and standard errors of three experiments are shown.



**Fig. 2-9.**

Inhibition of prostate cancer cell growth by anti c-NEU Ab-2 monoclonal antibody in vitro: Comparison of cell lines. Three prostate carcinoma cell lines (DU145, PC-3 and LnCaP) were compared in the same conditions as Fig. 2-8. with 5 nM of anti c-NEU Ab-2 antibody or 5 nM of mouse Ig G. Lane 1: control without antibody. Lane 2, 3, 4: are DU145, PC-3 and LNCaP cell lines with anti c-NEU Ab-2 antibody. Lane 5: LnCaP cell line with non-specific mouse Ig G1. Mean values of relative growth and standard errors of three experiments are shown(\*\* P<0.005, \* P< 0.05).



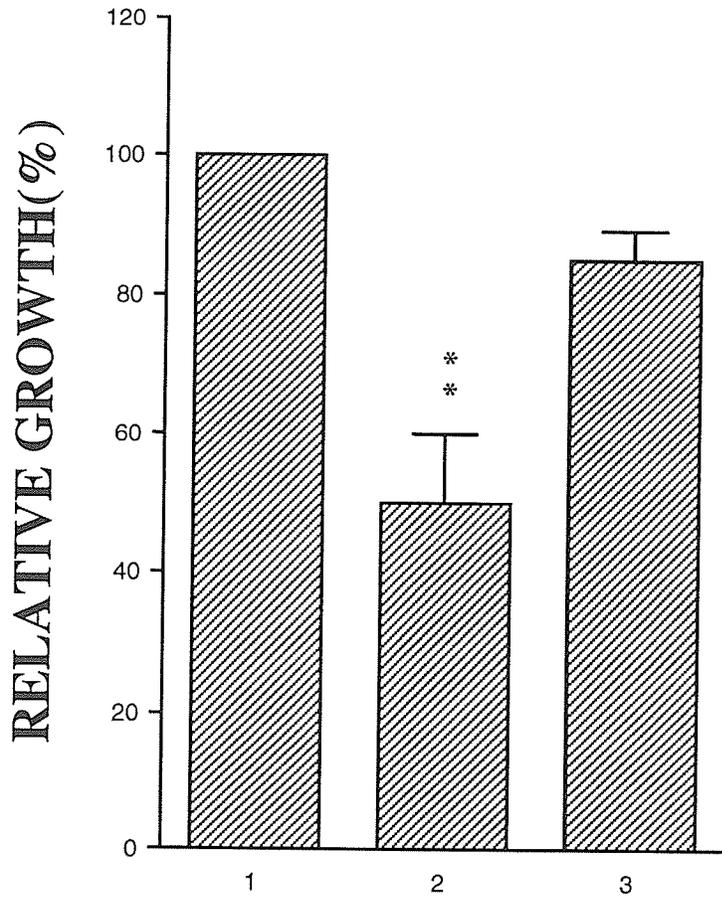
**Fig. 2-10.**

Inhibition of prostate cancer cell growth by anti c-NEU Ab-2 monoclonal antibody in vitro: Assay of reversibility. LNCaP ( $1 \times 10^4$  cells/well) were grown in the presence of 5 nM anti c-NEU Ab-2 for 6 days as in Fig. 2-8. After counting sample wells on day 6, the remaining cells were washed with ice cold PBS and supplied with serum-free medium without antibody for an additional 2 days. Mean values of relative growth and standard errors are shown ( $p^{**} < 0.005$ ).

Lane 1: without antibody

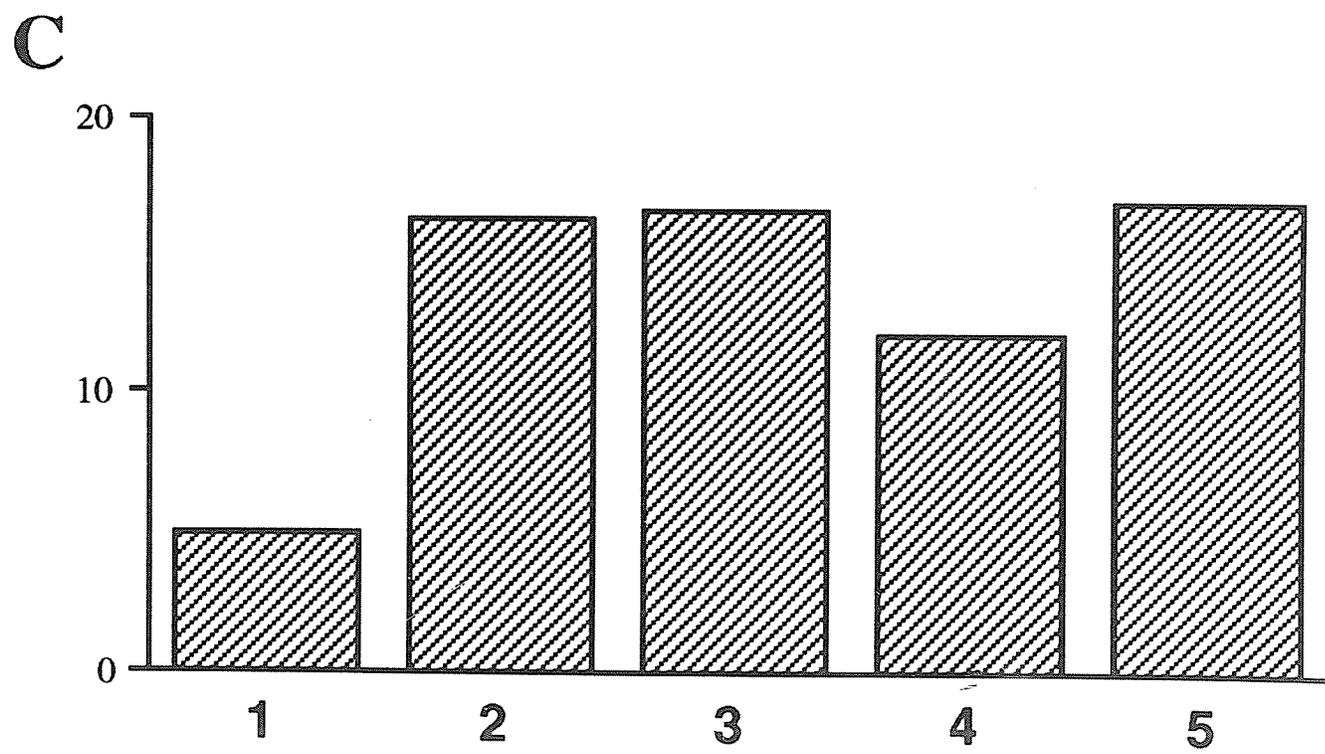
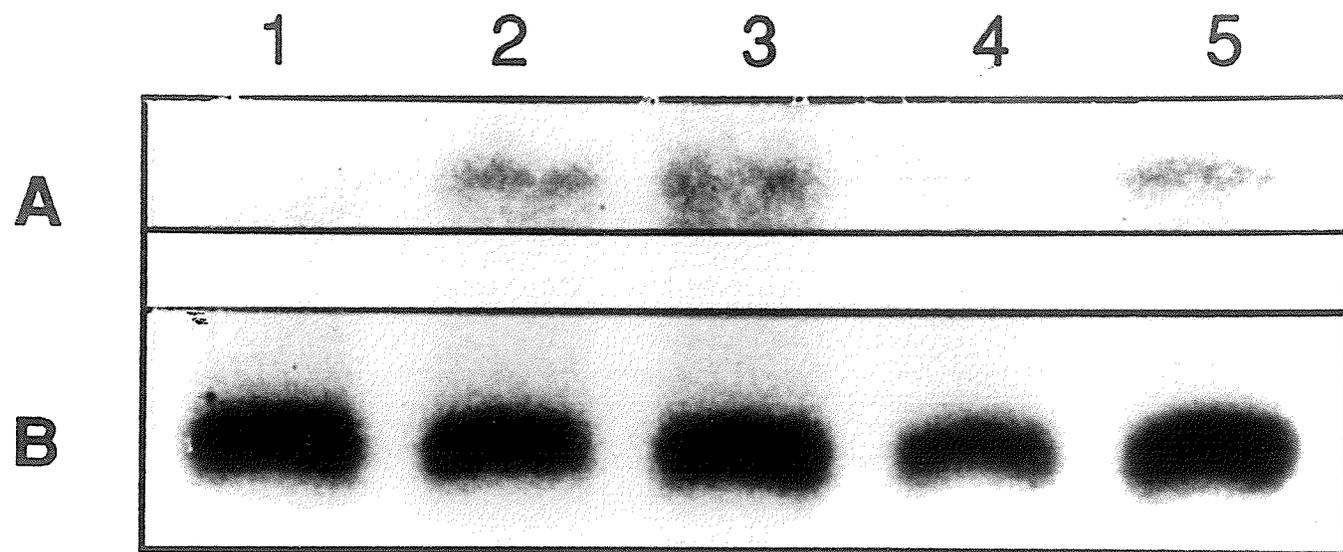
Lane 2: in the presence of antibody

Lane 3: following 2 days incubation after the removal of antibody



**Fig. 2-11.**

Expression of c-fos mRNA. Northern blot of poly A<sup>+</sup> RNA (10 ug) fractionated by gel electrophoresis and transferred onto nitrocellulose membrane.  $\alpha$ -<sup>32</sup>P-labelled cDNA probes for c-fos and actin were hybridized sequentially to the blot. Panel A: Expression of c-fos. Lane 1, 2, 3, 4 and 5 represent mRNA from cells exposed to anti c-NEU Ab-2 for 2hrs, 1hr, 0.5 hr, 0.2 hr, and 0hr respectively. Panel B: Expression of actin mRNA. Panel C: Relative expression of c-fos mRNA assessed by densitometric analysis of panels A and B.



### 3. DISCUSSION

Genetic alteration of c-erbB2/NEU has been observed in several human cancers and cell lines (Slamon *et al.* 1989; King *et al.* 1985). Amplification of c-erbB2/NEU has been correlated with poor prognosis and high probability of relapse, particularly in breast and ovarian cancer (Slamon *et al.* 1989; Berchuck *et al.* 1990). Studies demonstrating that overexpression of c-erbB2/NEU results in transformation of NIH 3T3 cell (Shih *et al.* 1981; Hudziak *et al.* 1987) suggest that erbB2/NEU contributes to malignant tumor development by modulating cell growth. Previously we observed that the amplification and overexpression of c-erbB2/NEU was also a feature in human prostatic carcinoma tissue and tumor-derived cell lines. Recently, the overexpression of c-erbB2/NEU protein has been demonstrated in prostate cancer using immunohistochemistry (Zhou *et al.* 1992).

Since the natural ligand of c-erbB2/NEU has not been fully characterized, it is difficult to assay directly the role of c-erbB2/NEU in regulating proliferation of prostate cancer cells. However, the availability of monoclonal antibodies specific for the extracellular domain of c-erbB2/NEU receptor provided the opportunity to assess the role of c-erbB2/NEU-mediated pathways in controlling cancer cell growth. A similar approach has been used to study the EGF receptor, which has structural homology with c-erbB2/NEU receptor (Downward *et al.* 1984b). Monoclonal antibodies specific for the extracellular domain of EGF receptor have been characterized and used to inhibit binding of the natural ligands, EGF or TGF- $\alpha$ . The resulting block in signal transduction and EGF receptor-mediated tyrosine kinase activity, inhibited cancer cell proliferation (Ennis *et al.* 1991; Nicholson *et al.* 1989; Bellot *et al.* 1990; Ching *et al.* 1993). By analogy, the

inhibitory effect of anti c-NEU Ab-2 on prostate cell proliferation may be due to competition for binding of the natural ligand of c-erbB2/NEU. The results we observed using prostate cancer cells are similar to those obtained by Hudziak *et al.* (Hudziak *et al.* 1987) in human breast tumor cells using other monoclonal antibodies specific for the c-erbB2/NEU extracellular domain. These results suggest a possible mechanism of autocrine action involving c-erbB2/NEU in regulating cancer cell growth.

Signal transduction involving ligand-stimulated tyrosine kinase activity starts a cascade that terminates in the transcription of nuclear factors like c-fos (Koskinen *et al.* 1990; Scott *et al.* 1991; Ullrich *et al.* 1990). In our experiment, the reduction in c-fos mRNA following treatment with anti c-NEU Ab-2 suggests that signal transduction involving c-erbB2/NEU can influence cell proliferation by regulating c-fos expression.

The discovery of the ligand-receptor interaction on the cell surface and the receptor-mediated signal transduction chain in the cytoplasm has provided new points for intervention therapeutic tools in the treatment of tumors. Monoclonal antibodies for a cell surface receptor could block the access of ligand to receptor and thus inhibit cancer cell growth. Anti-receptor antibodies coupled to toxin molecules, such as plant and bacterial toxins, isotopes and drugs, have been developed to produce an immunotoxin specific for cells expressing the receptor (Pastan *et al.* 1986; Foon, 1989). Using the monoclonal anti-EGF receptor antibody 528IgG1 conjugated to recombinant ricin A chain which inhibit protein synthesis by catalyzing the inactivation of ribosomes, *in vitro* studies have shown a potent and specific cytotoxic effect on EGF receptor-positive human cancer cell lines. These studies also suggest that the degree of immunotoxin cytotoxicity is correlated with

the number of the EGF receptor on a cell (Masui *et al.*1989; Taetle *et al.*1988). Based on our understanding of mechanisms of internalization after ligand binding to the cell surface receptor has been established (Sunada *et al.*1986), the natural ligands of the receptor also can be used as toxin conjugates. The Pseudomonas exotoxin A (PE) is made by the bacterium and acts to inhibit protein synthesis (FitzGerald *et al.*1989). Linking TGF- $\alpha$  and PE produces a conjugate in which the cytotoxicity is specifically targeted to cells expressing the EGF receptor. The TGF- $\alpha$ -PE products have been shown to exert specific cytotoxic action on a series of EGF receptor -positive cancer cell lines in vitro including cancers arising from ovary, liver, breast, kidney and colon (Siegall *et al.*1989). In our study, although the effect of anti-erbB2/NEU antibodies was cytostatic, not cytotoxic, the antibody has allowed us to demonstrate a role for c-erbB2/NEU in prostate cancer cell growth. The overexpressed c-erbB2/NEU receptors may offer a target for novel therapies in prostate cancer which is more tumor-specific than other growth factor receptor.

## C: REGULATION OF c-erbB2/NEU GENE EXPRESSION BY STEROID HORMONES IN PROSTATE CANCER CELLS

### 1. INTRODUCTION

The prostate is a natural target tissue of androgen action; androgens have long been recognized as potent stimulators of both mitotic activity and secretory functions. Since Huggins (Huggins *et al.*1940; Huggins *et al.*1941b) first introduced the concept of androgen dependency of prostate carcinoma, evidence has supported the view that prostate cancer is most likely related to cumulative exposure to androgens, perhaps in combination with estrogen. In experimental models, androgens increase the incidence of N-methylnitrosourea (NMU)-induced prostate cancer in rats (Bosland *et al.*1990; Bosland, 1988); long term administration of testosterone and oestrogen increase the incidence of prostate cancer in the Noble (Nb) rat. This evidence suggests that androgens might act as initiators or permissive agents in prostate carcinogenesis. In patients, androgen withdrawal therapy has shown a palliative effect on controlling of prostate tumor growth, reducing symptoms and increasing survival in most cases (Huggins *et al.*1941b; Turkes *et al.*1987). Unfortunately, most patients relapse following an initial response and develop androgen-independent disease (Scott *et al.*1980). Whether cells forming the androgen-independent cancer stem from cells present in the primary tumor that were never responsive to androgens, that were once responsive to androgens and subsequently lost their ability to respond, or that have an altered androgen response, remains unknown. The relapse does indicate that androgens are not the only substances capable of influencing

prostate cancer growth; the function of androgens may be mediated directly or indirectly by other factors. It is most likely that multiple factors including peptide growth factors and receptors are involved in prostate cancer cell growth.

Gene amplification and overexpression of c-erbB2/NEU have been correlated with the cancer development and patient survival in breast and ovarian cancer (Slamon *et al.*1987; Slamon *et al.*1989). The gene alteration also have been demonstrated as a feature in human prostate cancer tissue and cell lines (Zhau *et al.*1992; Ching *et al.*1993). Further, we have demonstrated that blocking c-erbB2/NEU function reduces prostate cancer cell growth in vitro. However, the effect of steroid hormones on c-erbB2/NEU expression in prostate cells has not been extensively studied.

LNCaP cell line was derived from a metastatic lesion of a human prostate carcinoma (Horszewicz *et al.*1983b). Although the cells can grow in the absence of androgens, they are responsive to androgens for growth. These cells have a point mutation in the hormone binding region of the androgen receptor (Harris *et al.*1991). The mutated androgen receptor could be activated by a range of ligands including anti-androgens and estrogen (Wilding *et al.*1989a; Harris *et al.*1991), and the cells respond with increased growth to a variety of steroids (Schuurmans *et al.*1988; Berns *et al.*1986). The LNCaP cells also show high levels of expression of EGF, TGF- $\alpha$  and EGF receptor mRNAs (Ching *et al.*1993). LNCaP cells have low level amplification and overexpression of the c-erbB2/NEU gene (Ching *et al.*1993). This cell line is a suitable model to study the relationship of steroid hormone and c-erbB2/NEU in the regulation of prostate cancer growth.

It has been demonstrated that androgen/androgen receptor complex, acting as a transcription factor, mediates its effects on a cell through transcriptional control of a specific subset of genes. In this study, we have evaluated the regulation of expression of c-erbB2/NEU by selected steroid hormones. The results suggest a potential for androgen to exert its growth effects, in part via the regulation of expression of c-erbB2/NEU receptors.

## 2. RESULTS

In this study, we have measured the growth rate of LNCaP cells by stimulation with DHT ( $10^{-8}$  M) or E2 ( $10^{-7}$  M) in culture. Both DHT and E2 were able to significantly stimulate LNCaP cell growth. The growth increase by DHT was 130% and 150% by E2 in the 6 days assay compared to the cells with no additional hormone in the serum-free culture system (Fig. 2-12) ( $p < 0.05$ ).

According to our previous observations, human prostate cancer tissue and cell lines contain extra copies of the c-erbB2/NEU gene and have overexpressed c-erbB2/NEU at both RNA and protein levels. The regulation of c-erbB2/NEU expression by DHT and E2 have been measured at the mRNA and protein levels. Poly A<sup>+</sup> RNA from LNCaP cells that were incubated with DHT or E2 for various times (0h, 1hr, 7h, 14h, and 24h) was used in Northern analysis. Increased abundance of mRNA for c-erbB2/NEU was evident at the 1h time point and reaching the maximum 14hr after stimulation with both DHT and E2 (Fig. 2-13). To evaluate the level of c-erbB2/NEU protein, a specific antibody which recognizes the extracellular domain of c-erbB2/NEU receptor was used in a Western blot. The c-erbB2/NEU protein level increases beginning at the 24 hr after

addition of DHT or E2 to the cell culture and remains elevated for at least 72 hr even though the mRNA levels have declined by 24 hours. (Fig. 2-14).

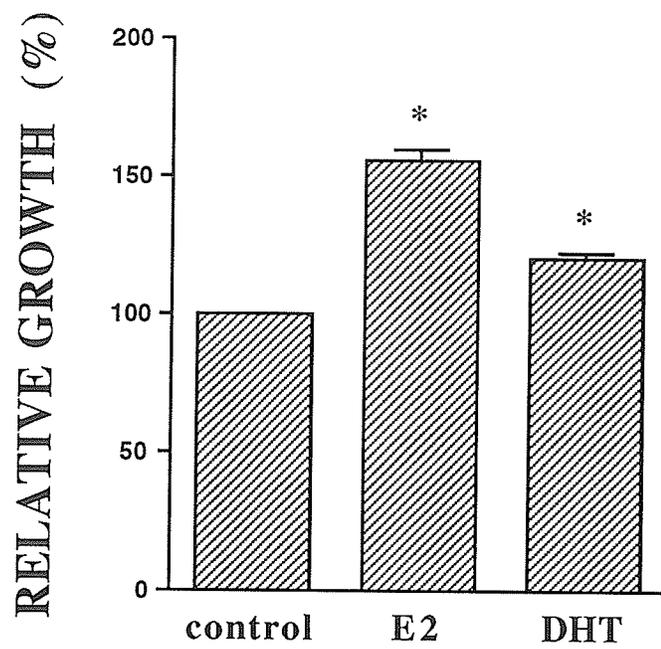
To examine whether the effect of E2 or c-erbB2/NEU gene expression is mediated through the estrogen receptor or through the mutated androgen receptor, several approaches were taken. First, LNCaP cells were incubated with Tamoxifen, one of the non-steroidal inhibitors of estrogen action at the receptor level, with E2 or DHT. The increased expression of c-erbB2/NEU mRNA seen in response to E2 or DHT did not show change significantly by incubation with Tamoxifen ( $10^{-7}$  M) (Fig. 2-15).

Next, to determine whether estrogen receptor gene expression occurs in LNCaP cells, a Northern blot containing Poly A<sup>+</sup> RNA from three human prostate cancer cell lines and from the Ishikawa human endometrial carcinoma cell line was hybridized with an estrogen receptor cDNA probe. None of the three prostate cancer cell lines demonstrated a visible band for estrogen receptor mRNA; the Ishikawa cell line, a positive control, demonstrated an intense band for estrogen receptor mRNA (Fig. 2-16). Finally, a more sensitive method for mRNA detection, RT-PCR, was used for estrogen receptor mRNA detection. Poly A<sup>+</sup> RNA (0.5 ug) from LNCaP cells and various amounts of Poly A<sup>+</sup> RNA from Ishikawa cells (0.5 ug, 0.05 ug, 0.005 ug, and 0.0005 ug) were used as templates for primer-specific targeting of estrogen receptor cDNA sequence. The Southern blot of PCR product has been hybridized with a estrogen receptor-specific cDNA probe. Although the lowest amount of template, RNA (0.0005 ug) from Ishikawa cells, yielded an intense PCR product band, the RNA template from LNCaP cells did not show a specific PCR product (Fig. 2-17-A). To confirm the quality of RNA templates,

RNA from both LNCaP cells and Ishikawa cells were used as templates for amplification of actin cDNA. Both were able to produce the expected products for actin cDNA (Fig. 2-17-B) confirming the integrity of the RNA for amplification.

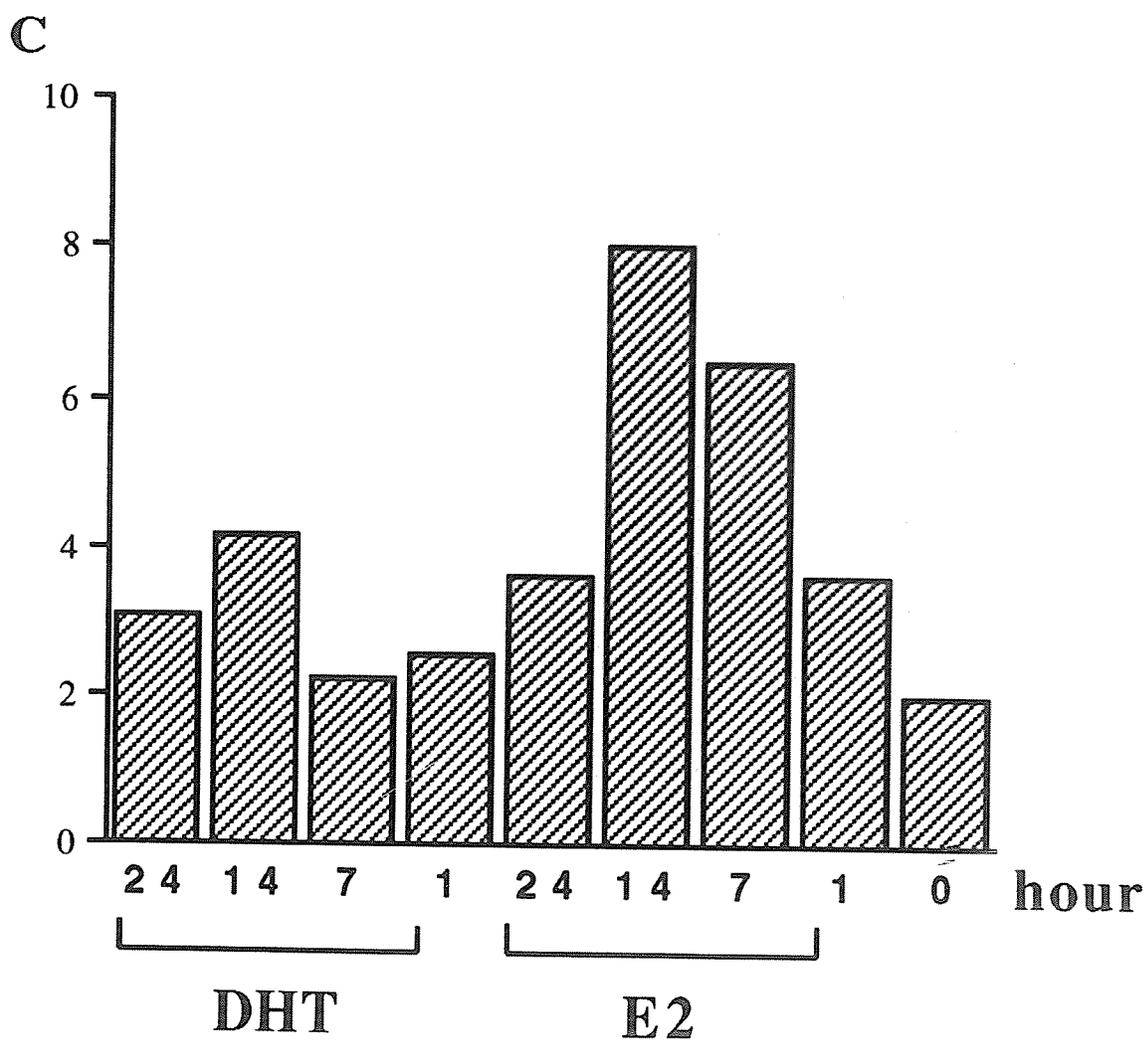
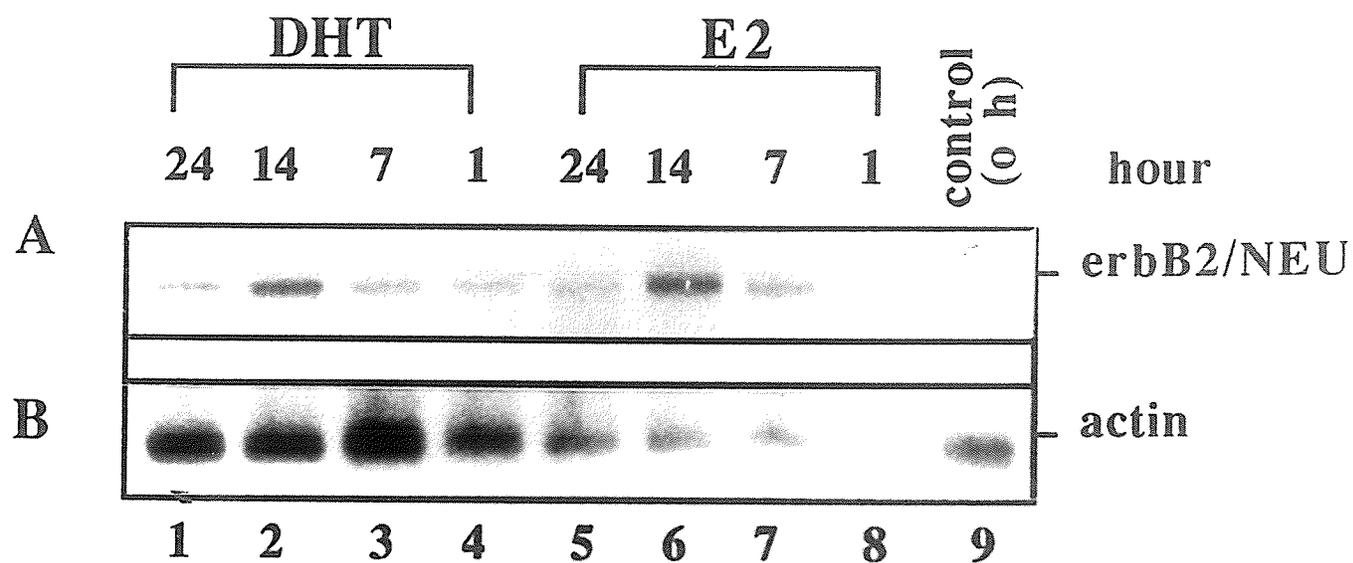
**Fig. 2-12.**

Effect of DHT or E2 on growth rate of LNCaP cells. Cell were plated ( $1 \times 10^4$  cells/well) in triplicate wells in 10% FBS media. Following overnight incubation, the medium was changed to serum-free medium containing 1 ug/ml of insulin and transferin, and either DHT ( $10^{-8}$  M) or E2 ( $10^{-7}$  M) were added in culture. At day 9, cells were counted. The mean values of growth rate and standard errors of three experiments are shown (\*  $P < 0.5$ ).



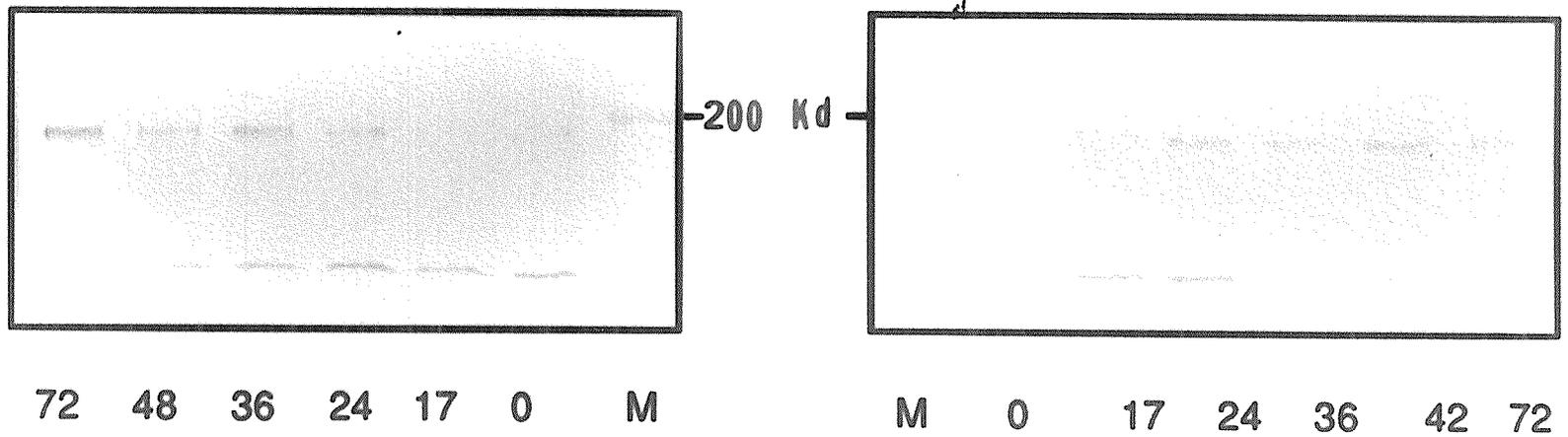
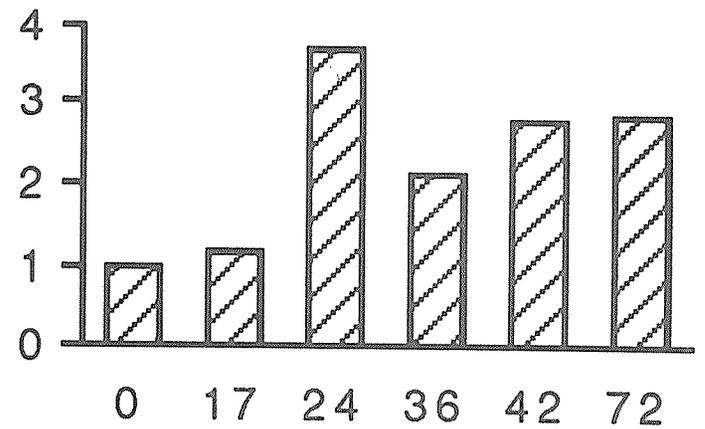
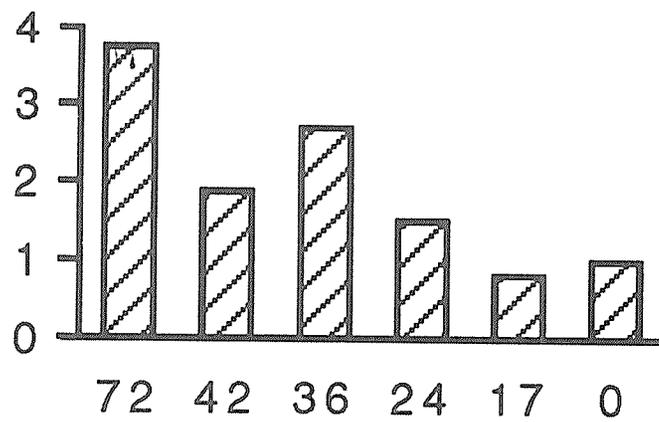
**Fig. 2-13.**

Effect of DHT or E2 on c-erbB2/NEU expression of LNCaP cells. Northern blots are shown: 10 ug of Poly A<sup>+</sup> RNA from LNCaP cells treated with DHT ( 10<sup>-8</sup> M) or E2 (10<sup>-7</sup> M) at various time points (0 h, 1h, 7h, 14h and 24h). Panel A: Expression of c-erbB2/NEU mRNA. Panel B: Expressions of actin mRNA. Panel C: Densitometric analysis of relative expression of c-erbB2/NEU mRNA using actin mRNA to normalize for gel loading.



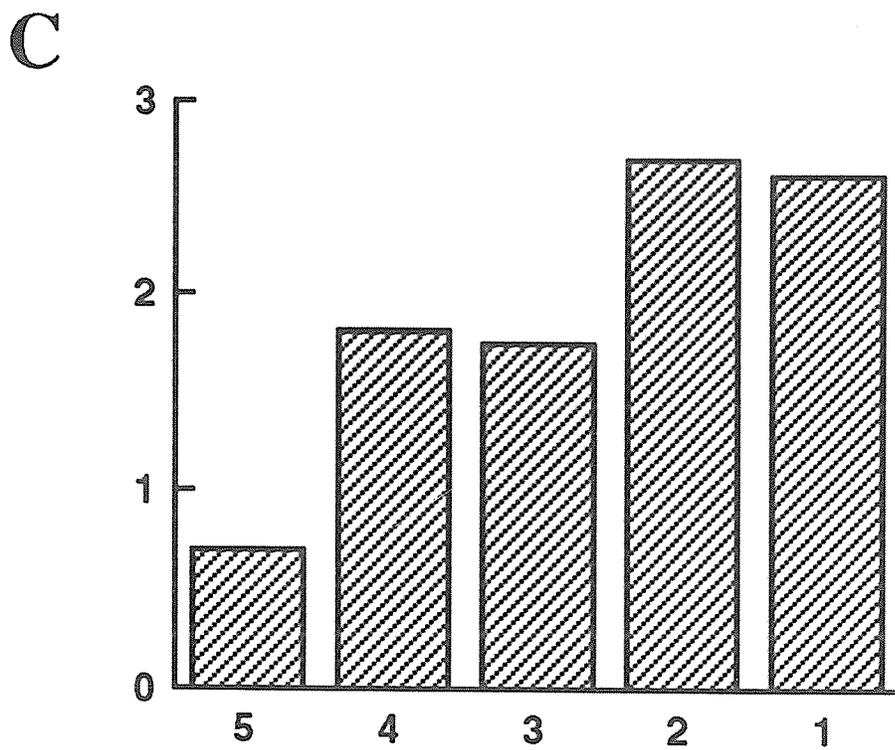
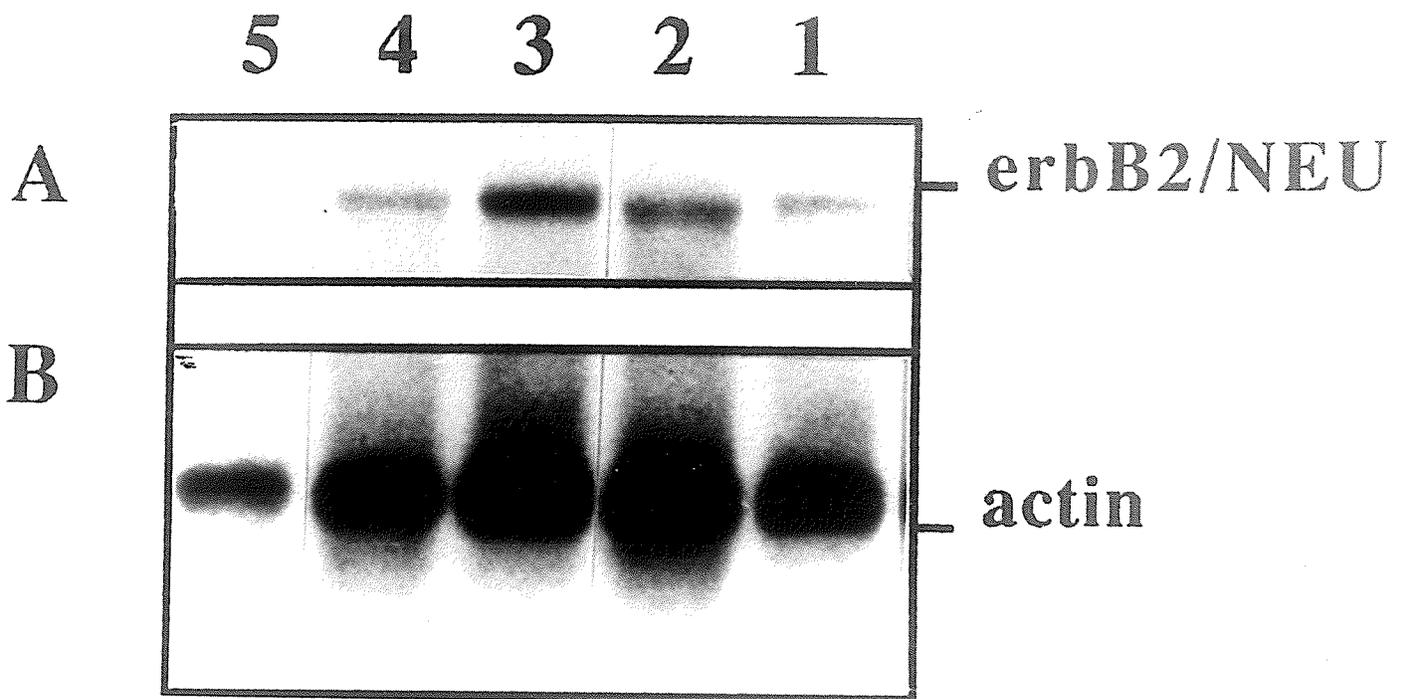
**Fig. 2-14.**

Effect of DHT or E2 on expression of c-erbB2/NEU in LNCaP cells. Western blots are shown: 30ug of total protein from cells treated with DHT ( $10^{-8}$  M) or E2 ( $10^{-7}$  M) at various time points (0h, 1h, 7h, 14h, 24h, 48h and 72h). Blots were incubated with Anti c-NEU Ab-3 and bands of c-erbB2/NEU p185 were detected by color reaction (Protolblot system, Promega). Panel A: Expression of c-erbB2/NEU protein. Panel B: Relative protein level of c-erbB2/NEU by densitometric analysis of panel A.

**A****E2****DHT****Time(hr)****B**

**Fig. 2-15.**

Effect of tamoxifen on c-erbB2/NEU expression. Northern blot analyses are shown: Poly A<sup>+</sup> RNA (10 ug) from LNCaP cells treated with DHT (10<sup>-8</sup> M) or E2 (10<sup>-7</sup> M) with or without Tamoxifen (10<sup>-8</sup> M) for 14 hours.  $\alpha$ -<sup>32</sup>P-dCTP labelled DNA probes for erbB2/NEU and actin were hybridized sequentially to the blot. Panel A: Expression of c-erbB2/NEU. Panel B: Expression of actin. C: Densitometric analysis of relative expression of c-erbB2/NEU using actin to normalize for gel loading. Lanes: 1, E2 + Tamoxifen; 2, E2; 3, DHT + Tamoxifen; 4, DHT; 5, no treatment. The Y axis shown the relative of expression of c-erbB2/NEU.



**Fig. 2-16.**

Northern analysis for measuring estrogen receptor. Poly A<sup>+</sup> RNA (10 ug) from various cell lines was fractionated by gel electrophoresis and transferred onto nitrocellulose membrane.  $\alpha$ -<sup>32</sup>P-dCTP labelled cDNA probes for estrogen receptor and actin were hybridized to the blot. Panel A: Expression of estrogen receptor mRNA. Panel B: Expression of actin mRNA. Lanes: 1, Ishikawa cells; 2, LNCaP; 3, PC-3; 4, DU145.

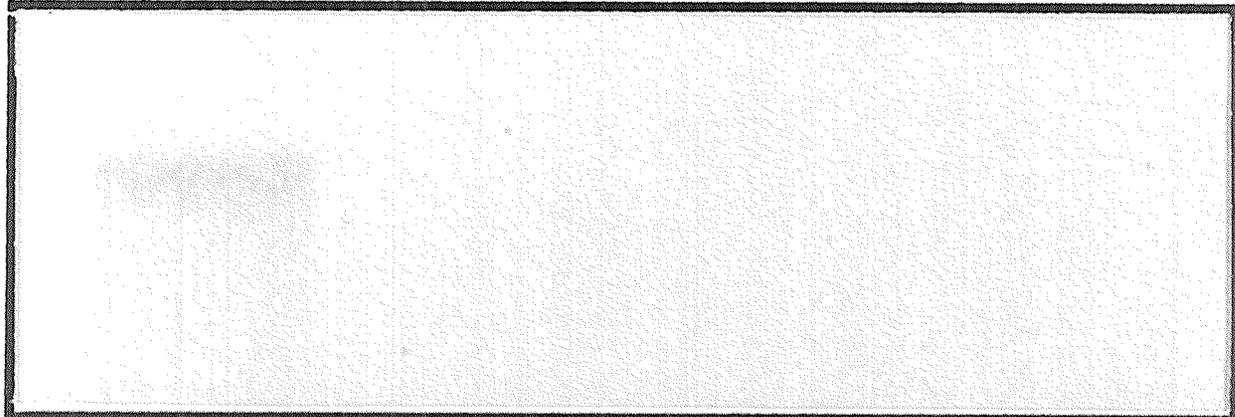
1

2

3

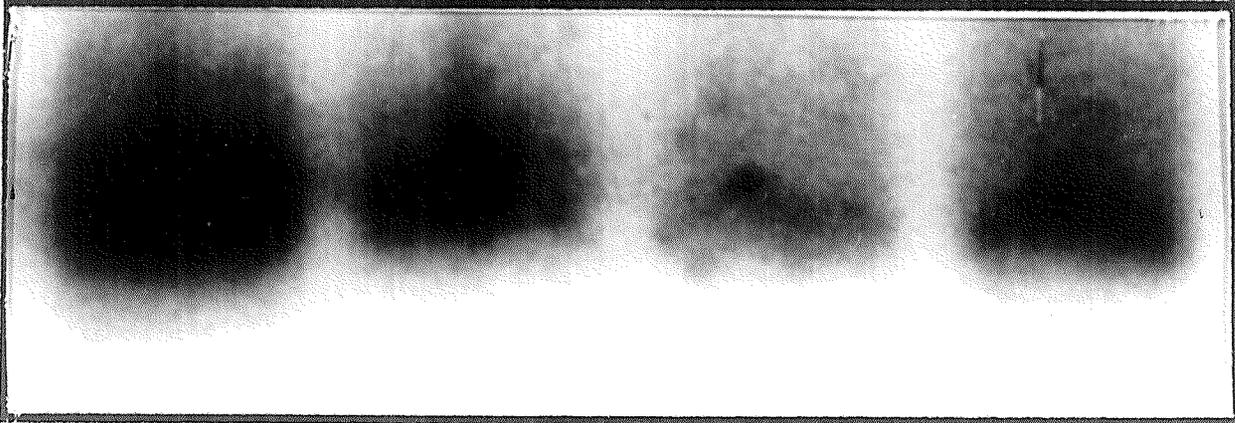
4

A



ER

B



actin

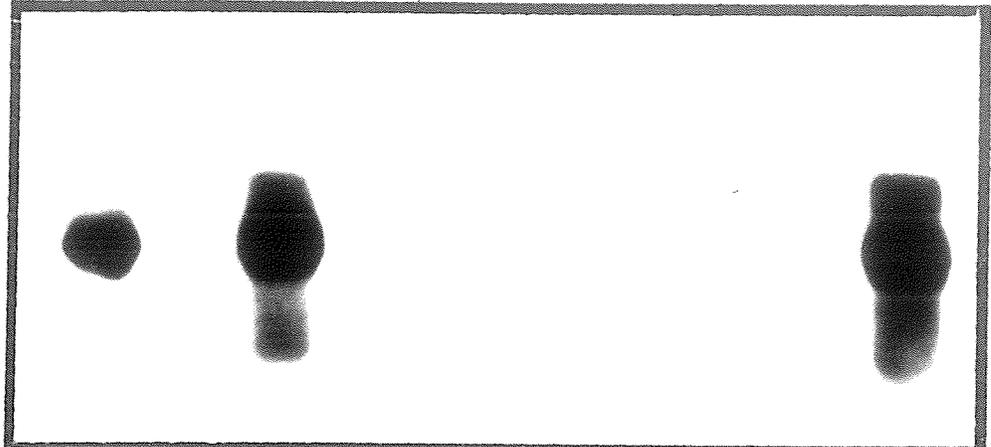
**Fig. 2-17.**

Panel A: RT-PCR analysis for detection of estrogen receptor mRNA: Southern blots of PCR products hybridized to  $\alpha$ -<sup>32</sup>P-dCTP labelled cDNA probe for estrogen receptor are shown. Poly A<sup>+</sup> RNA of Ishikawa cells (0.5, 0.05, 0.005 ug) and Poly A<sup>+</sup> RNA of LNCaP cells (0.5 ug) were used as templates for cDNA synthesis and PCR amplification of estrogen receptor cDNA. PCR products were fractionated by agarose gel electrophoresis and transferred onto nitrocellulose membrane. Panel A: Lane 1, 4 and 5; Ishikawa cells RNA template (0.5, 0.05, 0.005 and 0.0005 ug respectively); Lane 2, Ishikawa cells RNA template (0.5 ug) without addition of reverse transcriptase, Lane 3, no RNA template (Film exposed for 30 minutes). Panel B: Lane 1, Ishikawa cells RNA template (0.0005 ug); Lane 2, LNCaP cells RNA template (0.5 ug); Lane 3, LNCaP cells RNA template (0.5 ug) without adding reverse transcriptase; Lane 4, no template. (Film exposed for 4 hours).

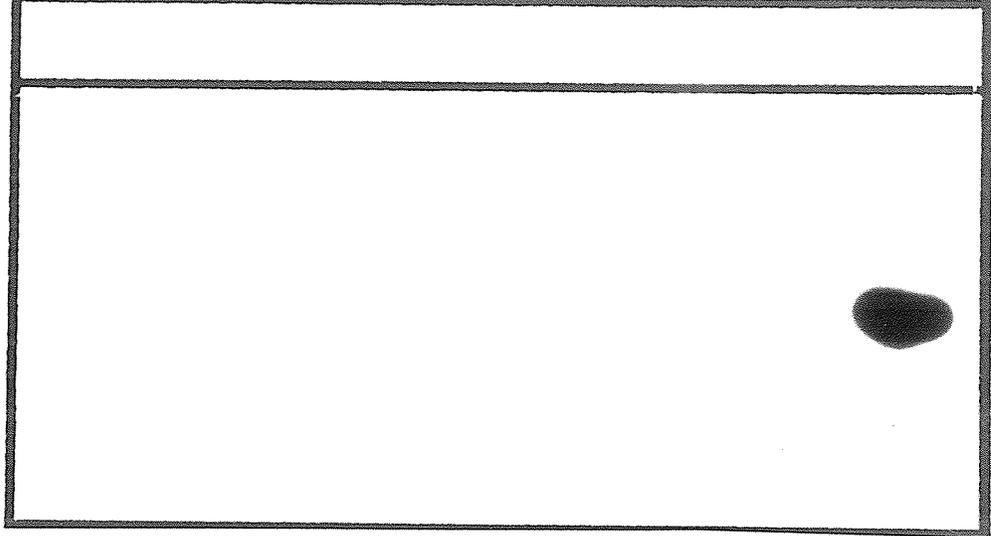
Panel B: Assay of template RNA integrity. Poly A<sup>+</sup> RNA of Ishikawa cells and LNCaP cells were used as templates for cDNA synthesis and PCR amplification of actin cDNA. PCR products were fractionated by agarose gel electrophoresis and visualized following ethidium bromide staining. Lane 1, LNCaP cells RNA template; Lane 2, Ishikawa cells RNA template.

5 4 3 2 1

A



B

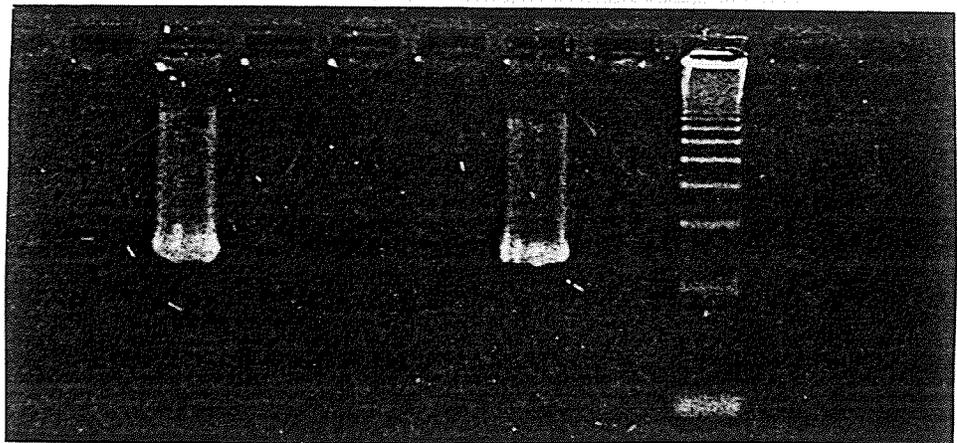


2

1

M

C



### 3. DISCUSSION

Alteration of the c-erbB2/NEU gene is associated with malignant tumor development and modulation of cancer cell growth in a variety human cancers. Overexpression of c-erbB2/NEU has been shown to contribute to transformation of NIH 3T3 cells (Hudziak *et al.*1987), which suggests that the c-erbB2/NEU gene is a potent oncogene. Our previous study showed that alteration of the c-erbB2/NEU gene is also evident in prostate cancer, and c-erbB2/NEU proteins play a role in regulating prostate cancer cell proliferation. Recently, a report from Sikes (Sikes *et al.*1992b), showed that transfection of activated c-erbB2/NEU gene into rat ventral prostate epithelial cells results in a tumorigenic phenotype. This data strongly implicate c-erbB2/NEU in regulating prostate cancer growth.

Androgens are the major steroid hormones crucial for the normal development of the prostate gland and in maintaining its functional state in the adult. The prolonged presence of androgens as an important factor in the development of prostate cancer also has long been appreciated (Huggins *et al.*1940; Huggins *et al.*1941a; Huggins *et al.*1941b). Androgens effect the expression of EGF and its receptor in human prostate cancer cell lines (Schuurmans *et al.*1988) and in mouse and rat ventral prostate (St Arnaud *et al.*1988; Hiramatsu *et al.*1988), indicating a correlation between steroid hormones and peptide growth factor expression in prostate cells.

In the present study, we have demonstrated enhanced expression of c-erbB2/NEU mRNA and protein in LNCaP cells stimulated by androgens and estrogens. This suggests that the effect of steroid hormones on prostate cancer cell growth is mediated, at least

in part, through modulation of growth factor receptor levels.

Since there was no evidence of present of estrogen receptor in LNCaP cells, it seems likely that the regulation of c-erbB2/NEU gene expression in LNCaP cells by estrogen is mediated by androgen receptor. The androgen receptor mutated in the ligand-binding domain in LNCaP cells (Tenniswood *et al.*1990; Wilding *et al.*1989a; Harris *et al.*1991) have been shown an ability to bind to estrogen and anti-androgens in vitro. It is possible likely that the estrogen-mutated androgen receptor complex, as a transcriptional factor involved in regulation of c-erbB2/NEU expression in this cells.

## CONCLUSION

At the beginning of this study, we predicted that the erbB genes play an important role in the proliferation of prostate cancer cells by mechanisms which include (1) enhanced expression of the c-erbB genes, and (2) response to endogenous production of corresponding growth factor ligands, resulting in autocrine regulation of growth. These studies have provided evidence that supports this hypothesis, although some results were obtained was not expected.

The c-erbB gene family includes four members to date: c-erbB1 (EGF receptor), c-erbB2 (HER2, NEU), c-erbB3 (HER3) and c-erbB4 (HER4). In this thesis, the focus has been on the c-erbB1 and c-erbB2 genes since these two genes have been found altered frequently in several human cancers (Todaro *et al.*1980; Mydlo *et al.*1988b; Bennett *et al.*1989; Yamamoto *et al.*1986b; Filmus *et al.*1985; Yoshida *et al.*1990). Gene amplification and overexpression of c-erbB1 and c-erbB2 have been suggested to contribute to tumor growth and progression in human and animal models (Di Fiore *et al.*1987a; Berchuck *et al.*1990; Neal *et al.*1989; Spitzer *et al.*1988). For erbB1 gene, the following observations support my hypothesis. (1) All prostate samples have been examined express the EGF receptor mRNA and mRNAs for its ligands. (2) Both pre-pro TGF- $\alpha$  and pre-pro EGF mRNA are significantly elevated in human prostate carcinomas compared to benign hyperplasias. (3) All three human prostate carcinoma cell lines express TGF- $\alpha$ , EGF and EGF receptor, although the levels of expression different among the cell lines. The link between the c-erbB1 gene and transformation was apparent from the previous studies. Gene amplification and thus overexpression of EGF receptor has

been found in various human neoplasms and cell lines (Lin *et al.*1984; Merlino *et al.*1984; Ullrich *et al.*1984; Libermann *et al.*1985a; Yamamoto *et al.*1986a). Overexpression of either TGF- $\alpha$  or EGF was sufficient to promote transformation of appropriate recipient cell expressing EGF receptor (Watanabe *et al.*1987; Stern *et al.*1987). The results of the present study indicate that enhanced expression of EGF TGF- $\alpha$  and EGF receptor mRNAs is a feature of the prostate cancer cell. Since EGF, TGF- $\alpha$  and EGF receptor are expressed in normal prostate cells (Maddy *et al.*1989; Eaton *et al.*1988), their expression in prostate cancer cells does not represent de novo synthesis. Their effects may relate to the level of expression or level of response. In this study, no evidence of gene amplification or gross rearrangement of EGF, TGF- $\alpha$  and EGF receptor genes was associated with overexpression of those genes in human prostate cancer cell lines. Interestingly, the highest levels of TGF- $\alpha$  mRNA are seen in the two cell lines which can not respond to androgens.

In the past several years, evidence has accumulated indicating that many transformed cell are able to overcome hormonal growth restraints by producing growth factors which act through autocrine mechanisms (Sporn *et al.*1988; Sporn *et al.*1985b; Goustin *et al.*1986). Human breast and pancreatic carcinoma cell lines have been described as producing significantly higher amounts of EGF, TGF- $\alpha$  and EGF receptor and responding to EGF and TGF- $\alpha$  for growth in tissue culture (Ennis *et al.*1989). The results in this thesis demonstrate that the autocrine mechanism is also present in regulation of prostate cancer cell growth. This statement is supported by the following evidence.

- (1) Exogenous EGF or TGF- $\alpha$  stimulated prostate cancer cell growth in vitro.
- (2)

response in EGF receptor in PC-3 cell remain unclear. Further study is required to examine the transmembrane domain or adjacent region, or the regions needed for receptor dimerization for possible alterations.

In summary, the results above demonstrate that the EGF receptor-mediated autocrine pathway is one of the mechanisms involved in regulation of prostate cancer cell growth. However the other factors and mechanisms are not excluded.

The second gene examined in this thesis is c-erbB2/NEU gene. This gene has been found amplified frequently in several human cancers (Slamon *et al.*1989; Zeillinger *et al.*1989; King *et al.*1985; Yusa *et al.*1990; Donovan Peluso *et al.*1991) and amplification of c-erbB2/NEU has been closely associated with poor prognosis (Slamon *et al.*1989; Berchuck *et al.*1990; Pavelic *et al.*1992; Winstanley *et al.*1991; Wright *et al.*1992). In breast and ovarian cancers, amplification and overexpression of c-erbB2/NEU is associated with reduced survival time (Slamon *et al.*1989; Iglehart *et al.*1990; Borg *et al.*1991; Kallioniemi *et al.*1991). The following observations in this thesis suggest that amplification and overexpression of c-erbB2/NEU are one of the genetic events in prostate carcinogenesis. (1) The frequency of c-erbB2/NEU gene amplification was 60% in high grade prostate tumors. (2) A high proportion of prostate carcinoma tissue contains elevated levels of c-erbB2/NEU protein compared to the level of c-erbB2/NEU in BPH. This parallels a recent report of immunohistochemical analysis of high grade prostate tumors showing 80% (12 of 15) positive staining for c-erbB2/NEU overexpression (Zhou *et al.*1992). Since the selected prostatic tumors were all high grade (Gleason score >5), the association between c-erbB2/NEU gene amplification and overexpression and poor

cellular differentiation of tumor cell has been suggested. The copy number of c-erbB2/NEU may be useful as a marker in prognosis and may predicate the survival time or response to therapy. However, whether c-erbB2/NEU gene amplification is as frequent in low grade, well differentiated tumors needs to be determined. The correlation between the degree of gene amplification and survival time or response to clinical treatment in prostate cancer patients also requires more intensive study.

The c-erbB2/NEU receptor is closely related to the EGF receptor and functions as a transmembrane receptor with tyrosine kinase activity in the intracellular domain (Stancovski *et al.*1991; Downward *et al.*1984a; Nesland *et al.*1991). The cascade of events from tyrosine kinase phosphorylation of cytoplasmic proteins triggered by ligand binding to the c-erbB2/NEU receptor is terminated at nuclear transcription factor genes, such as c-fos (Ullrich *et al.*1990; Ciardiello *et al.*1991; Scott *et al.*1991; Stancovski *et al.*1991). In this thesis, an autocrine mechanism involving c-erbB2/NEU receptor-mediated pathway has been demonstrated suggesting that c-erbB2/NEU receptor is involved in regulation of the autonomous cell growth in prostate cancer. The following observation support this conclusion. (1) The enhanced expression of c-erbB2/NEU mRNA and protein in human prostate carcinoma tissue and cell lines. (2) The ability of specific anti c-erbB/NEU antibody to reduce the expression of c-fos, which indicates ligand and c-erbB2/NEU receptor interaction is present in prostate cancer cells. (3) The ability of anti c-erbB2/NEU antibody to block human prostatic carcinoma cell growth in vitro. A method involving artificial ligands or antibodies conjugated to toxin molecules has been developed and introduced in cancer treatment (FitzGerald *et al.*1989; Pastan *et*

*al.*1986; Foon, 1989). The observation of the role for c-erbB2/NEU receptor in prostate cancer cell growth may offer a target for novel therapies for prostate cancer patients which are overexpress c-erbB2/NEU receptor, a more tumor-specific receptor than other growth factor receptor.

It has been demonstrated that steroid hormones are important factors in regulation prostate cancer cell growth. Also, it has been demonstrated that there is a correlation between steroid hormone and peptide growth factor action in human and animals (Schuurmans *et al.*1988; St Arnaud *et al.*1988; Hiramatsu *et al.*1988). In this thesis, it has been observed that (1) Both androgen and estrogen were able to stimulate LNCaP prostate cancer cell growth *in vitro*. (2) The expression of c-erbB2/NEU was regulated by androgen and estrogen in LNCaP cells. (3) The regulation by estrogen on of LNCaP cells is unlikely to occur through estrogen receptor but mutated androgen receptor. These results suggest that the effect of steroid hormones on prostate cancer cell growth is mediated, at least in part, through modulation of growth factor receptor levels.

In conclusion: multiple peptide growth factors and steroid hormones are involved in prostate cancer cell growth. The enhanced expression of growth factor and corresponding receptor is implicated as a feature of prostate cancer cells. The EGF receptor and c-erbB2/NEU receptor-mediated autocrine pathways contribute to autonomous growth of prostate cancer cells.

## REFERENCES

- Aasland R. Akslen L.A. Varhaug J.E. and Lillehaug J.R. *Co-expression of the genes encoding transforming growth factor-alpha and its receptor in papillary carcinomas of the thyroid.* Int.J.Cancer 46:382-387, 1990.
- Ablin R.J. *Immunologic studies of normal benign and malignant human prostatic tissue.* Cancer 29:1570-1575, 1972.
- Akakura K. Bruchofsky N. Goldenberg S.L. Rennie P.S. Buckley A.R. and Sullivan L.D. *Effects of intermittent androgen suppression on androgen-dependent tumors. Apoptosis and serum prostate-specific antigen.* Cancer 71:2782-2790, 1993.
- Allhoff E.P. Proppe K.H. Chapman C.M. Lin C.W. and Prout G.R.J. *Evaluation of prostate specific acid phosphatase and prostate specific antigen in identification of prostatic cancer.* J.Urol. 129:315-318, 1983.
- Aviv H. and Leder P. *Purification of biologically active globin messenger RNA by chromatography on oligothymidylic acid-cellulose.* Proc.Natl.Acad.Sci.U.S A. 67:1408-1412, 1977.
- Bagchi M.K. Tsai M.J. O'Malley B.W. and Tsai S.Y. *Analysis of the mechanism of steroid hormone receptor-dependent gene activation in cell-free systems.* Endocr.Rev. 13:525-535, 1992.
- Bagshaw M.A. Cox R.S. and Ray G.R. *Status of radiation treatment of prostate cancer at Stanford University.* NCI.Monogr. 47-60, 1988.

- Bargmann C.I. Hung M.C. and Weinberg R.A. *The neu oncogene encodes an epidermal growth factor receptor-related protein.* Nature **319**:226-230, 1986a.
- Bargmann C.I. Hung M.C. and Weinberg R.A. *Multiple independent activations of the neu oncogene by a point mutation altering the transmembrane domain of p185.* Cell **45**:649-657, 1986b.
- Bargmann C.I. and Weinberg R.A. *Oncogenic activation of the neu-encoded receptor protein by point mutation and deletion.* EMBO J. **7**:2043-2052, 1988a.
- Bargmann C.I. and Weinberg R.A. *Increased tyrosine kinase activity associated with the protein encoded by the activated neu oncogene.* Proc.Natl.Acad.Sci.U.S.A. **85**:5394-5398, 1988b.
- Barnes D. and Sato G. *Serum-free cell culture: a unifying approach.* Cell **22**:649-655, 1980.
- Barnes D.M. Meyer J.S. Gonzalez J.G. Gullick W.J. and Millis R.R. *Relationship between c-erbB-2 immunoreactivity and thymidine labelling index in breast carcinoma in situ.* Breast Cancer Res.Treat. **18**:11-17, 1991.
- Barrandon Y. and Green H. *Cell migration is essential for sustained growth of keratinocyte colonies: the roles of transforming growth factor-alpha and epidermal growth factor.* Cell **50**:1131-1137, 1987.
- Barton C.M. Hall P.A. Hughes C.M. Gullick W.J. and Lemoine N.R. *Transforming growth factor alpha and epidermal growth factor in human pancreatic cancer.* J.Pathol **163**:111-116, 1991.
- Bates S.E. Davidson N.E. Valverius E.M. Freter C.E. Dickson R.B. Tam J.P. Kudlow J.E.

- Lippman M.E. and Salomon D.S. *Expression of transforming growth factor alpha and its messenger ribonucleic acid in human breast cancer: its regulation by estrogen and its possible functional significance.* Mol.Endocrinol. 2:543-555, 1988.
- Bellot F. Moolenaar W. Kris R. Mirakhur B. Verlaan I. Ullrich A. Schlessinger J. and Felder S. *High-affinity epidermal growth factor binding is specifically reduced by a monoclonal antibody, and appears necessary for early responses.* J.Cell Biol. 110:491-502, 1990.
- Bennett C. Paterson I.M. Corbishley C.M. and Luqmani Y.A. *Expression of growth factor and epidermal growth factor receptor encoded transcripts in human gastric tissues.* Cancer Res. 49:2104-2111, 1989.
- Bentley H. Hamdy F.C. Hart K.A. Seid J.M. Williams J.L. Johnstone D. and Russell R.G. *Expression of bone morphogenetic proteins in human prostatic adenocarcinoma and benign prostatic hyperplasia.* Br.J.Cancer 66:1159-1163, 1992.
- Benz C.C. Scott G.K. Sarup J.C. Johnson R.M. Tripathy D. Coronado E. Shepard H.M. and Osborne C.K. *Estrogen-dependent, tamoxifen-resistant tumorigenic growth of MCF-7 cells transfected with HER2/neu.* Breast Cancer Res.Treat. 24:85-95, 1993.
- Berchuck A. Kamel A. Whitaker R. Kerns B. Olt G. Kinney R. Soper J.T. Dodge R. Clarke Pearson D.L. Marks P. and et al *Overexpression of HER-2/neu is associated with poor survival in advanced epithelial ovarian cancer.* Cancer Res. 50:4087-4091, 1990.
- Berger M.S. Gullick W.J. Greenfield C. Evans S. Addis B.J. and Waterfield M.D. *Epidermal growth factor receptors in lung tumours.* J.Pathol 152:297-307, 1987.
- Berns E.M. de Boer W. and Mulder E. *Androgen-dependent growth regulation of and release of specific protein(s) by the androgen receptor containing human prostate tumor*

*cell line LNCaP*. Prostate 9:247-259, 1986.

Blute M.L. Nativ O. Zincke H. Farrow G.M. Therneau T. and Lieber M.M. *Pattern of failure after radical retropubic prostatectomy for clinically and pathologically localized adenocarcinoma of the prostate: influence of tumor deoxyribonucleic acid ploidy*. J.Urol. 142:1262-1265, 1989.

Borg A. Baldetorp B. Ferno M. Killander D. Olsson H. and Sigurdsson H. *ERBB2 amplification in breast cancer with a high rate of proliferation*. Oncogene 6:137-143, 1991.

Bosland M.C. *The etiopathogenesis of prostatic cancer with special reference to environmental factors*. Adv.Cancer Res 51:1-106, 1988.

Bosland M.C. Prinsen M.K. Dirksen T.J. and Spit B.J. *Characterization of adenocarcinomas of the dorsolateral prostate induced in Wistar rats by N-methyl-N-nitrosourea, 7,12-dimethylbenz(a)anthracene, and 3,2'-dimethyl-4-aminobiphenyl, following sequential treatment with cyproterone acetate and testosterone propionate*. Cancer Res 50:700-709, 1990.

Brawer M.K. Peehl D.M. Stamey T.A. and Bostwick D.G. *Keratin immunoreactivity in the benign and neoplastic human prostate*. Cancer Res. 45:3663-3667, 1985.

Bruchovsky N. and Wilson J.D. *The conversion of testosterone to 5 $\alpha$ -androstane-17 $\beta$ -ol-3-one by rat prostate in vivo and in vitro*. J.Biol.Chem. 243:2012-2021, 1968a.

Bruchovsky N. and Wilson J.D. *The intranuclear binding of testosterone and 5 $\alpha$ -Androgen-17 $\beta$ -ol-3-one by rat prostate*. J.Biol.Chem. 243:5953-5960, 1968b.

Buhrow S.A. Cohen S. and Staros J. *affinity labeling of the protein kinase associated with the epidermal growth factor receptor in membrane vesicles from A431 cells.* J.Biol.Chem. **257**:4019-4022, 1982.

Buhrow S.A. Cohen S. Garbers D.L. and Staros J.V. *Characterization of the interaction of 5'-p-fluorosulfonylbenzoyl adenosine with the epidermal growth factor receptor/protein kinase in A431 cell membranes.* J.Biol.Chem. **258**:7824-7827, 1983.

Burgess A.W. Lloyd C.J. Smith S. Stanley E. Walker F. Fabri L. Simpson R.J. and Nice E.C. *Murine epidermal growth factor: structure and function.* Biochemistry **27**:4977-4985, 1988.

Byyny R.L. Orth D.N. and Cohen S. *Radioimmunoassay of epidermal growth factor.* Endocrinology **90**:1261-1266, 1972.

Cairns J. *Mutation selection and the natural history of cancer.* Nature **255**:197-200, 1975.

Cantley L.C. Auger K.R. Carpenter C. Duckworth B. Graziani A. Kapeller R. and Soltoff S. *Oncogenes and signal transduction [published erratum appears in Cell 1991 May 31;65(5):following 914].* Cell **64**:281-302, 1991.

Carpenter G. and Cohen S. *Epidermal growth factor.* Annu.Rev.Biochem. **48**:193-216, 1979a.

Carpenter G. King L.Jr. and Cohen S. *Rapid enhancement of protein phosphorylation in A-431 cell membrane preparations by epidermal growth factor.* J.Biol.Chem. **254**:4884-4891, 1979b.

Carpenter G. Stoscheck C.M. Preston Y.A. and DeLarco J.E. *Antibodies to the epidermal*

*growth factor receptor block the biological activities of sarcoma growth factor.* Proc.Natl.Acad.Sci.U.S.A. 80:5627-5630, 1983.

Carpenter G. *Epidermal growth factor: biology and receptor metabolism.* J.Cell Sci.Suppl. 3:1-9, 1985.

Carpenter G. *Receptors for epidermal growth factor and other polypeptide mitogens.* Annu.Rev.Biochem. 56:881-914, 1987.

Carpenter G. and Cohen S. *Epidermal growth factor.* J.Biol.Chem. 265:7709-7712, 1990.

Cassel D. and Glaser L. *Protolytic cleavage of epidermal growth factor receptor: A Ca<sup>2+</sup>-dependent, sulfhydryl-sensitive protolytic system in A431 cells.* J.Biol.Chem. 257:9845-9848, 1982.

Catalona W.J. *Prostate cancer.* Curr.Probl.Surg 27:389-461, 1990.

Catterton W.Z. Escobedo B.N. Sexson W.R. Gray M.E. Sundell H.W. and Stahlman M.T. *effect of epidermal growth factor on lung maturation in fetal rabbits.* Pediatr.Res 13:104-108, 1979.

Chaproniere D.M. and McKeehan W.L. *Serial culture of single adult human prostatic epithelial cells in serum-free medium containing low calcium and a new growth factor from bovine brain.* Cancer Res. 46:819-824, 1986.

Charhon S.A. Chapuy M.C. Delvin E.E. Valentin Opran A. Edouard C.M. and Meunier P.J. *Histomorphometric analysis of sclerotic bone metastases from prostatic carcinoma special reference to osteomalacia.* Cancer 51:918-924, 1983.

Chen W.S. Lazar C.S. Poenie M. Tsien R.Y. Gill G.N. and Rosenfeld M.G. *Requirement*

*for intrinsic protein tyrosine kinase in the immediate and late actions of the EGF receptor.* Nature 328:820-823, 1987.

Ching K.Z. Ramsey E. Pettigrew N. D'Cunha R. Jason m. and Dodd J.G. *Expression of mRNA for epidermal growth factor, transforming growth factor-alpha and their receptor in human prostate tissue and cel lines.* Molecular Cellular Biochemistry 1993.(In Press)

Chinkers M. and Brugge J.S. *Characterization of structural domains of the human epidermal growth factor receptor obtained by partial proteolysis.* J.Biol.Chem. 259:11534-11542, 1984.

Ciardello F. Kim N. Saeki T. Dono R. Persico M.G. Plowman G.D. Garrigues J. Radke S. Todaro G.J. and Salomon D.S. *Differential expression of epidermal growth factor-related proteins in human colorectal tumors.* Proc.Natl.Acad.Sci.U.S.A. 88:7792-7796, 1991.

Clark A.J. Ishii S. Richert N. Merlino G.T. and Pastan I. *Epidermal growth factor regulates the expression of its own receptor.* Proc.Natl.Acad.Sci.U.S A. 82:8374-8378, 1985.

Clark S. Cheng D.J. Hsuan J.J. Haley J.D. and Waterfield M.D. *Loss of three major auto phosphorylation sites in the EGF receptor does not block the mitogenic action of EGF.* J.Cell Physiol. 134:421-428, 1988.

Coffey R.J.J. Derynck R. Wilcox J.N. Bringman T.S. Goustin A.S. Moses H.L. and Pittelkow M.R. *Production and auto-induction of transforming growth factor-alpha in human keratinocytes.* Nature 328:817-820, 1987.

Cohen S. *Isolation of a mouse submaxillary gland protein accelerating incisor eruption and eyelid opening in the niw-bron animal.* J.Biol.Chem. 237:1555-1562, 1962.

Cohen S. and Carpenter G. *Human epidermal growth factor: isolation and chemical and biological properties*. Proc.Natl.Acad.Sci.U.S A. 72:1317-1321, 1975.

Cohen S. Carpenter G. and King L.Jr. *Epidermal growth factor-receptor-protein kinase interactions*. J.Biol.Chem. 255:4834-4842, 1980.

Cohen S. Ushiro H. Stoscheck C. and Chinkers M. *A native 17,000 epidermal growth factor receptor-kinase complex from shed membrane vesicles*. J.Biol.Chem. 257:1523-1531, 1982.

Connolly J.M. and Rose D.P. *Secretion of epidermal growth factor and related polypeptides by the DU 145 human prostate cancer cell line*. Prostate 15:177-186, 1989.

Connolly J.M. and Rose D.P. *Production of epidermal growth factor and transforming growth factor-alpha by the androgen-responsive LNCaP human prostate cancer cell line*. Prostate 16:209-218, 1990.

Connolly J.M. and Rose D.P. *Autocrine regulation of DU145 human prostate cancer cell growth by epidermal growth factor-related polypeptides*. Prostate 19:173-180, 1991.

Cooper, G.M. *Oncogene and proto-oncogene functions*. In: *Oncogenes*, Boston, Ma: Jones and Batlett Publishers Inc., 1990, p. 161-298.

Coussens L. Yang Feng T.L. Liao Y.C. Chen E. Gray A. McGrath J. Seeburg P.H. Libermann T.A. Schlessinger J. Francke U. and et al *Tyrosine kinase receptor with extensive homology to EGF receptor shares chromosomal location with neu oncogene*. Science 230:1132-1139, 1985.

Crawford E.D. Eisenberger M.A. McLeod D.G. Spaulding J.T. Benson R. Dorr F.A.

Blumenstein B.A. Davis M.A. and Goodman P.J. *A controlled trial of leuprolide with and without flutamide in prostatic carcinoma [published erratum appears in N Engl J Med 1989 Nov 16;321(20):1420]. N.Engl.J.Med. 321:419-424, 1989.*

Cummings R.D. Soderquist A.M. and Carpenter G. *The oligosaccharide moieties of the epidermal growth factor receptor in A-431 cells. Presence of complex-type N-linked chains that contain terminal N-acetylgalactosamine residues. J.Biol.Chem. 260:11944-11952, 1985.*

Cunha G.R. Donjacour A.A. Cooke P.S. Mee S. Bigsby R.M. Higgins S.J. and Sugimura Y. *The endocrinology and developmental biology of the prostate. Endocr.Rev. 8:338-362, 1987.*

Das M. Miyakawa T. Fox C.F. Pruss R.M. Ahraonov A. and Herschman H.R. *Specific radiolabeling of a cell surface receptor for epidermal growth factor. Proc.Natl.Acad.Sci.U.S.A. 74:2790-2794, 1977.*

Dati C. Muraca R. Tazartes O. Antoniotti S. Perroteau I. Giai M. Cortese P. Sismondi P. Saglio G. and De Bortoli M. *c-erbB-2 and ras expression levels in breast cancer are correlated and show a co-operative association with unfavorable clinical outcome. Int.J.Cancer 47:833-838, 1991.*

Davies P. Eaton C.L. France T.D. and Phillips M.E. *Growth factor receptors and oncogene expression in prostate cells. Am.J.Clin.Oncol. 11 Suppl 2:S1-S7, 1988.*

De Larco J.E. and Todaro G.J. *Growth factor from murine sarcoma virus-transformed cells. Proc.Natl.Acad.Sci.U.S.A. 75:4001-4005, 1978.*

de Voogt H.J. Smith P.H. Pavone Macaluso M. de Pauw M. and Suci S. *Cardiovascular side effects of diethylstilbestrol, cyproterone acetate, medroxyprogesterone acetate and*

- estramustine phosphate used for the treatment of advanced prostatic cancer: results from European Organization for Research on Treatment of Cancer trials 30761 and 30762.* J.Urol. 135:303-307, 1986.
- DeKlerk D.P. and Coffey D. *Quantitative determination of prostatic epithelial and stromal hyperplasia by a new technique biomorphometrics.* Invest.Urol. 16:240-245, 1978.
- Dembinski A. Gregory H. Konturek S.J. and Polanski M. *trophoc action of epidermal growth factor on the pancreas and gastroduodenal mucosa in rats.* J.Physiol 325:35-42, 1982.
- Denner L.A. Weigel N.L. Maxwell B.L. Schrader W.T. and O'Malley B.W. *Regulation of progesterone receptor-mediated transcription by phosphorylation.* Science 250:1740-1743, 1990.
- Dermer G.B. *Basal cell proliferation in benign prostatic hyperplasia.* Cancer 4:1857, 1978.
- Derynck R. Goeddel D.V. Ullrich A. Gutterman J.U. Williams R.D. Bringman T.S. and Berger W.H. *Synthesis of messenger RNAs for transforming growth factors alpha and beta and the epidermal growth factor receptor by human tumors.* Cancer Res. 47:707-712, 1987.
- Derynck R. *The physiology of transforming growth factor-alpha.* Adv.Cancer Res. 58:27-52, 1992.
- Di Fiore P.P. Pierce J.H. Fleming T.P. Hazan R. Ullrich A. King C.R. Schlessinger J. and Aaronson S.A. *Overexpression of the human EGF receptor confers an EGF-dependent transformed phenotype to NIH 3T3 cells.* Cell 51:1063-1070, 1987a.

Di Fiore P.P. Pierce J.H. Kraus M.H. Segatto O. King C.R. and Aaronson S.A. *erbB-2 is a potent oncogene when overexpressed in NIH/3T3 cells*. Science 237:178-182, 1987b.

Di Fiore P.P. Segatto O. Lonardo F. Fazioli F. Pierce J.H. and Aaronson S.A. *The carboxy-terminal domains of erbB-2 and epidermal growth factor receptor exert different regulatory effects on intrinsic receptor tyrosine kinase function and transforming activity*. Mol.Cell Biol. 10:2749-2756, 1990a.

Di Fiore P.P. Segatto O. Taylor W.G. Aaronson S.A. and Pierce J.H. *EGF receptor and erbB-2 tyrosine kinase domains confer cell specificity for mitogenic signaling*. Science 248:79-83, 1990b.

Di Marco E. Pierce J.H. Aaronson S.A. and Di Fiore P.P. *Mechanisms by which EGF receptor and TGF alpha contribute to malignant transformation*. Nat.Immun.Cell Growth Regul. 9:209-221, 1990a.

Di Marco E. Pierce J.H. Knicley C.L. and Di Fiore P.P. *Transformation of NIH 3T3 cells by overexpression of the normal coding sequence of the rat neu gene*. Mol.Cell Biol. 10:3247-3252, 1990b.

Dobashi K. Davis J.G. Mikami Y. Freeman J.K. Hamuro J. and Greene M.I. *Characterization of a neu/c-erbB-2 protein-specific activating factor*. Proc.Natl.Acad.Sci.U.S.A. 88:8582-8586, 1991.

Donovan Peluso M. Contento A.M. Tobon H. Ripepi B. and Locker J. *Oncogene amplification in breast cancer*. Am.J.Pathol 138:835-845, 1991.

Downward J. Parker P. and Waterfield M.D. *Autophosphorylation sites on the epidermal growth factor receptor*. Nature 311:483-485, 1984a.

Downward J. Yarden Y. Mayes E. Scrace G. Totty N. Stockwell P. Ullrich A. Schlessinger J. and Waterfield M.D. *Close similarity of epidermal growth factor receptor and v-erb-B oncogene protein sequences.* Nature **307**:521-527, 1984b.

Downward J. Waterfield M.D. and Parker P.J. *Autophosphorylation and protein kinase C phosphorylation of the epidermal growth factor receptor. Effect on tyrosine kinase activity and ligand binding affinity.* J.Biol.Chem. **260**:14538-14546, 1985.

Dulbecco R. *Topoinhibition and serum requirement of transformed and untransformed cells.* Nature **227**:802, 1970.

Eaton C.L. and Pierrepont C.G. *Growth of a spontaneous canine prostatic adenocarcinoma in vivo and in vitro: isolation and characterization of a neoplastic prostatic epithelial cell line, CPA 1.* Prostate **12**:129-143, 1988.

Eaton C.L. Davies P. Harper M. France T. Rushmere N. and Griffiths K. *Steroids and the prostate.* J.Steroid Biochem.Mol.Biol. **40**:175-183, 1991.

Eisenberger M.A. Bezerdjian L. and Kalash S. *A critical assessment of the role of chemotherapy for endocrine-resistant prostatic carcinoma.* Urol.Clin.North Am. **14**:695-706, 1987.

Elson S.D. Browne C.A. and Thorburn G.D. *Identification of epidermal growth factor-like activity in human male reproductive tissues and fluids.* J.Clin.Endocrinol.Metab. **58**:589-594, 1984.

English H.F. Drago J.R. and Santen R.J. *Cellular response to androgen depletion and repletion in the rat ventral prostate: autoradiography and morphometric analysis.* Prostate

7:41-51, 1985.

Ennis B.W. Valverius E.M. Bates S.E. Lippman M.E. Bellot F. Kris R. Schlessinger J. Masui H. Goldenberg A. Mendelsohn J. and et al *Anti-epidermal growth factor receptor antibodies inhibit the autocrine-stimulated growth of MDA-468 human breast cancer cells.* Mol.Endocrinol. 3:1830-1838, 1989.

Ennis B.W. Lippman M.E. and Dickson R.B. *The EGF receptor system as a target for antitumor therapy.* Cancer Invest 9:553-562, 1991.

Erlich, H.A.ed. *PCR Technology*, New York:Stockton press, 1989.

Fabricant R.N. DeLarco J.E. and Todaro G.J. *Nerve growth factor receptors on human melanoma cells in culture.* Proc.Natl.Acad.Sci.U.S A. 74:565-569, 1977.

Farnsworth, W.E. *The normal prostate and its endocrine control.* In: *The prostate Gland. Some Aspects of The Aetiology and Biochemistry of Prostatic Cancer*, edited by Griffiths, K. and Pierrepont, C.G. Cardiff, Wales, U.K.: Alpha Omega Alpha publishing Company, 1970, p. 3-15.

Filmus J. Pollak M.N. Cailleau R. and Buick R.N. *MDA-468, a human breast cancer cell line with a high number of epidermal growth factor (EGF) receptors, has an amplified EGF receptor gene and is growth inhibited by EGF.* Biochem.Biophys.Res.Commun. 128:898-905, 1985.

Finzi E. Kilkenny A. Strickland J.E. Balaschak M. Bringman T. Derynck R. Aaronson S. and Yuspa S.H. *TGF alpha stimulates growth of skin papillomas by autocrine and paracrine mechanisms but does not cause neoplastic progression.* Mol.Carcinog. 1:7-12, 1988.

- Fiorelli G. De Bellis A. Longo A. Pioli P. Costantini A. Giannini S. Forti G. and Serio M. *Growth factors in the human prostate*. J.Steroid Biochem.Mol.Biol. **40**:199-205, 1991.
- Fisher P.B. Bozzone J.H. and Weinstein I.B. *Tumor promoters and epidermal growth factor stimulate anchorage-independent growth of adeno-virus-transformed rat embryo cells*. Cell **18**:695-705, 1979.
- FitzGerald D. and Pastan I. *Targeted toxin therapy for the treatment of cancer*. J.Natl.Cancer Inst. **81**:1455-1463, 1989.
- Foon K.A. *Biological response modifiers: the new immunotherapy [published erratum appears in Cancer Res 1990 Jan 1;50(1):212]*. Cancer Res **49**:1621-1639, 1989.
- Fowler J.E.J. Lau J.L. Ghosh L. Mills S.E. and Mounzer A. *Epidermal growth factor and prostatic carcinoma: an immunohistochemical study*. J.Urol. **139**:857-861, 1988.
- Fukushige S. Matsubara K. Yoshida M. Sasaki M. Suzuki T. Semba K. Toyoshima K. and Yamamoto T. *Localization of a novel v-erbB-related gene, c-erbB-2, on human chromosome 17 and its amplification in a gastric cancer cell line*. Mol.Cell Biol. **6**:955-958, 1986.
- Fung Y.K. Lewis W.G. Crittenden L.B. and Kung H.J. *Activation of the cellular oncogene c-erbB by LTR insertion: molecular basis for induction of erythroblastosis by avian leukosis virus*. Cell **33**:357-368, 1983.
- Gavrilovic J. Moens G. Thiery J.P. and Jouanneau J. *Expression of transfected transforming growth factor alpha induces a motile fibroblast-like phenotype with extracellular matrix-degrading potential in a rat bladder carcinoma cell line*. Cell Regul. **1**:1003-1014, 1990.

Gibbons R.P. *Total prostatectomy for clinically localized prostate cancer: long-term surgical results and current morbidity*. NCI.Monogr. 123-126, 1988.

Gilman A.G. *G proteins: transducers of receptor-generated signals*. Annu.Rev.Biochem. 56:615-649, 1987.

Gilmore T. DeClue J.E. and Martin G.S. *Protein phosphorylation at tyrosine is induced by the v-erbB gene product in vivo and in vitro*. Cell 40:609-618, 1985.

Gittes R.F. *Carcinoma of the prostate [see comments]*. N.Engl.J.Med. 324:236-245, 1991.

Gleason, D.F. *Histologic grading and clinical staging of prostatic cancer*. In: *The Prostate*, edited by Tannenbaum, M. Philadelphia: Lea and Febiger, 1977, p. 171-198.

Gleave M. Hsieh J.T. Gao C.A. von Eschenbach A.C. and Chung L.W. *Acceleration of human prostate cancer growth in vivo by factors produced by prostate and bone fibroblasts*. Cancer Res 51:3753-3761, 1991.

Gleave M.E. Hsieh J.T. von Eschenbach A.C. and Chung L.W. *Prostate and bone fibroblasts induce human prostate cancer growth in vivo: implications for bidirectional tumor-stromal cell interaction in prostate carcinoma growth and metastasis*. J Urol 147:1151-1159, 1992a.

Gleave M.E. Hsieh J.T. von Eschenbach A.C. and Chung L.W. *Prostate and bone fibroblasts induce human prostate cancer growth in vivo: implications for bidirectional tumor-stromal cell interaction in prostate carcinoma growth and metastasis*. J.Urol 147:1151-1159, 1992b.

- Goldman R. Levy R.B. Peles E. and Yarden Y. *Heterodimerization of the erbB-1 and erbB-2 receptors in human breast carcinoma cells: a mechanism for receptor transregulation.* Biochemistry **29**:11024-11028, 1990.
- Goustin A.S. Leof E.B. Shipley G.D. and Moses H.L. *Growth factors and cancer.* Cancer Res. **46**:1015-1029, 1986.
- Gray A. Dull T.J. and Ullrich A. *Nucleotide sequence of epidermal growth factor cDNA predicts a 128,000-molecular weight protein precursor.* Nature **303**:722-725, 1983.
- Greenfield C. Patel G. Clark S. Jones N. and Waterfield M.D. *Expression of the human EGF receptor with ligand-stimulatable kinase activity in insect cells using a baculovirus vector.* EMBO J. **7**:139-146, 1988.
- Gregory H. Willshire I.R. Kavanagh J.P. Blacklock N.J. Chowdury S. and Richards R.C. *Urogastrone-epidermal growth factor concentrations in prostatic fluid of normal individuals and patients with benign prostatic hypertrophy.* Clin.Sci. **70**:359-363, 1986.
- Griffin J.E. and Wilson J.D. *Disorders of androgen receptor function.* Ann.N.Y.Acad.Sci. **438**:61-71, 1984.
- Griffiths K. Eaton C.L. and Davies P. *Prostatic cancer: aetiology and endocrinology.* Horm.Res. **32 Suppl 1**:38-43, 1989.
- Griffiths K. Eaton C.L. Harper M.E. Peeling B. and Davies P. *Steroid hormones and the pathogenesis of benign prostatic hyperplasia.* Eur.Urol **20 Suppl 1**:68-77, 1991.
- Gullick W.J. Berger M.S. Bennett P.L. Rothbard J.B. and Waterfield M.D. *Expression of the c-erbB-2 protein in normal and transformed cells.* Int.J.Cancer **40**:246-254, 1987.

- Haenzel W. and Kurihara M. *Studies of Japanese migrants I. mortality from cancer and other diseases among Japanese in the United States*. J.Natl.Cancer Inst. **40**:43-68, 1968.
- Haigler H. Ash J.F. Singer S.J. and Cohen S. *Visualization by fluorescence of the binding and internalization of epidermal growth factor in human carcinoma cells A-431*. Proc.Natl.Acad.Sci.U.S.A. **75**:3317-3321, 1978.
- Hall P.A. Hughes C.M. Staddon S.L. Richman P.I. Gullick W.J. and Lemoine N.R. *The c-erb B-2 proto-oncogene in human pancreatic cancer*. J.Pathol **161**:195-200, 1990.
- Han V.K. Hunter E.S. Pratt R.M. Zendegui J.G. and Lee D.C. *Expression of rat transforming growth factor alpha mRNA during development occurs predominantly in the maternal decidua*. Mol.Cell Biol. **7**:2335-2343, 1987.
- Hanks S.K. Quinn A.M. and Hunter T. *The protein kinase family: conserved features and deduced phylogeny of the catalytic domains*. Science **241**:42-52, 1988.
- Harris, S., harris, M.A., Rong, z., et al. *Androgen regulation of HGBF I (aFGF) and characterization of the androgen receptor mRNA in the human prostate carcinoma cell line LNCaP/A-dep*. In: *Molecular and Cellular Biology of prostate Cancer*, edited by Karr, JP., coffey, Ds., Smith, RG. and Tindall, DJ. New York: Plenum Press, 1991, p. 315-330.
- Harrison J. and Auersperg N. *Epidermal growth factor enhances viral transformation of granulosa cell*. Science **213**:218-219, 1981.
- Hasenson M. Lundh B. Stege R. Carlstrom K. and Pousette A. *PAP and PSA in prostatic carcinoma cell lines and aspiration biopsies: relation to hormone sensitivity and to cytological grading*. Prostate **14**:83-90, 1989.

Heidaran M.A. Fleming T.P. Bottaro D.P. Bell G.I. Di Fiore P.P. and Aaronson S.A. *Transformation of NIH3T3 fibroblasts by an expression vector for the human epidermal growth factor precursor.* Oncogene 5:1265-1270, 1990.

Heldin C.H. and Westermark B. *Growth factors: mechanism of action and relation to oncogenes.* Cell 37:9-20, 1984.

Helin K. Velu T. Martin P. Vass W.C. Allevato G. Lowy D.R. and Beguinot L. *The biological activity of the human epidermal growth factor receptor is positively regulated by its C-terminal tyrosines.* Oncogene 6:825-832, 1991.

Henderson B.E. Ross R.K. and Pike M.C. *Toward the primary prevention of cancer [see comments].* Science 254:1131-1138, 1991.

Hendler F.J. and Ozanne B.W. *Human squamous cell lung cancers express increased epidermal growth factor receptors.* J.Clin.Invest. 74:647-651, 1984.

Higashiyama S. Abraham J.A. Miller J. Fiddes J.C. and Klagsbrun M. *A heparin-binding growth factor secreted by macrophage-like cells that is related to EGF.* Science 251:936-939, 1991.

Hiramatsu M. Kashimata M. Minami N. Sato A. and Muraayama M. *Androgenic regulation of epidermal growth factor in the ventral prostate.* Biochem.Inter. 17:311-317, 1988.

Hoeck W. and Groner B. *Hormone-dependent phosphorylation of the glucocorticoid receptor occurs mainly in the amino-terminal transactivation domain.* J.Biol.Chem. 265:5403-5408, 1990.

Hofer D.R. Sherwood E.R. Bromberg W.D. Mendelsohn J. Lee C. and Kozlowski J.M. *Autonomous growth of androgen-independent human prostatic carcinoma cells: role of transforming growth factor alpha*. Cancer Res. **51**:2780-2785, 1991.

Holmes W.E. Sliwkowski M.X. Akita R.W. Henzel W.J. Lee J. Park J.W. Yansura D. Abadi N. Raab H. Lewis G.D. and et al *Identification of heregulin, a specific activator of p185erbB2*. Science **256**:1205-1210, 1992.

Hommel U. Dudgeon T.J. Fallon A. Edwards R.M. and Campbell I.D. *Structure-function relationships in human epidermal growth factor studied by site-directed mutagenesis and <sup>1</sup>H NMR*. Biochemistry **30**:8891-8898, 1991.

Honegger A.M. Szapary D. Schmidt A. Lyall R. Van Obberghen E. Dull T.J. Ullrich A. and Schlessinger J. *A mutant epidermal growth factor receptor with defective protein tyrosine kinase is unable to stimulate proto-oncogene expression and DNA synthesis*. Mol.Cell Biol. **7**:4568-4571, 1987.

Horoszewicz J.S. Leong S.S. Kawinski E. Karr J.P. Rosenthal H. Chu T.M. Mirand E.A. and Murphy G.P. *LNCaP model of human prostatic carcinoma*. Cancer Res **43**:1809-1818, 1983a.

Horoszewicz J.S. and Mirand E.A. *Current clinical status of interferon and management of cancer in man*. Prog.Clin.Biol.Res **132E**:315-325, 1983b.

Hricak H. *Imaging prostate carcinoma*. Radiology **169**:569-571, 1988.

Hudziak R.M. Schlessinger J. and Ullrich A. *Increased expression of the putative growth factor receptor p185HER2 causes transformation and tumorigenesis of NIH 3T3 cells*. Proc.Natl.Acad.Sci.U.S.A. **84**:7159-7163, 1987.

- Hudziak R.M. Lewis G.D. Winget M. Fendly B.M. Shepard H.M. and Ullrich A. *p185HER2 monoclonal antibody has antiproliferative effects in vitro and sensitizes human breast tumor cells to tumor necrosis factor*. Mol.Cell Biol. 9:1165-1172, 1989.
- Huggins C. and Clark F.J. *Quantitative studies of prostatic secretion.II. The effect of castration and of estrogen injection on the normal and on the hyperplastic prostatic gland of dogs*. J.Exp.Med. 72:747-762, 1940.
- Huggins C. and Hodges C.V. *Studies on prostatic cancer: The effect of castration, of estrogen and of androgen injection on serum phosphatases in metastatic carcinoma of the prostate*. Cancer Res. 1:293-297, 1941a.
- Huggins C. Stevens R.E.Jr. and Hodges C.V. *Studies on prostatic cancer II. The effects of castration on advanced carcinoma of the prostate gland*. Arch.Surg 43:209-223, 1941b.
- Hunter T. and Cooper J.A. *Protein-tyrosine kinases*. Annu.Rev.Biochem. 54:897-930, 1985.
- Ichihara I. Kawai N. Heilbronner R. and Rohr H. *Stereologic and fine-structural studies of prostatic acinar basal cells in the dog*. Cell Tissue Res. 242:519-525, 1985.
- Iglehart J.D. Kraus M.H. Langton B.C. Huper G. Kerns B.J. and Marks J.R. *Increased erbB-2 gene copies and expression in multiple stages of breast cancer*. Cancer Res. 50:6701-6707, 1990.
- Isaacs J.T. and Kyprianou N. *Development of androgen-independent tumor cells and their implication for the treatment of prostatic cancer*. Urol Res. 15:133-138, 1987.
- Iwashita S. and Fox C.F. *Epidermal growth factor and potent phorbol tumor promoters*

*induce epidermal growth factor receptor phosphorylation in a similar but distinctively different manner in human epidermoid carcinoma A431 cells.* J.Biol.Chem. **259**:2559-2567, 1984.

Jacobs S.C. Pikna D. and Lawson R.K. *Prostatic osteoblastic factor.* Invest.Urol. **17**:195-198, 1979.

Jhappan C. Stahle C. Harkins R.N. Fausto N. Smith G.H. and Merlino G.T. *TGF alpha overexpression in transgenic mice induces liver neoplasia and abnormal development of the mammary gland and pancreas.* Cell **61**:1137-1146, 1990.

Kaighn N.E. Narayna K.S. Ohnuki Y. Lechnert F. and Jones L.W. *Establishment and characterization of a human prostatic carcinoma cell line (PC3).* Invest.Urol. **17**:16-23, 1979.

Kallioniemi O.P. Holli K. Visakorpi T. Koivula T. Helin H.H. and Isola J.J. *Association of c-erbB-2 protein over-expression with high rate of cell proliferation, increased risk of visceral metastasis and poor long-term survival in breast cancer.* Int.J.Cancer **49**:650-655, 1991.

Kaplan D.R. Whitman M. Schaffhausen B. Pallas D.C. White M. Cantley L. and Roberts T.M. *Common elements in growth factor stimulation and oncogenic transformation: 85 kd phosphoprotein and phosphatidylinositol kinase activity.* Cell **50**:1021-1029, 1987.

Kashles O. Szapary D. Bellot F. Ullrich A. Schlessinger J. and Schmidt A. *Ligand-induced stimulation of epidermal growth factor receptor mutants with altered transmembrane regions.* Proc.Natl.Acad.Sci.U.S.A. **85**:9567-9571, 1988.

Kern J.A. Schwartz D.A. Nordberg J.E. Weiner D.B. Greene M.I. Torney L. and Robinson R.A. *p185neu expression in human lung adenocarcinomas predicts shortened*

*survival*. Cancer Res. **50**:5184-5187, 1990.

King C.R. Kraus M.H. and Aaronson S.A. *Amplification of a novel v-erbB-related gene in a human mammary carcinoma*. Science **229**:974-976, 1985.

King C.R. Borrello I. Bellot F. Comoglio P. and Schlessinger J. *Egf binding to its receptor triggers a rapid tyrosine phosphorylation of the erbB-2 protein in the mammary tumor cell line SK-BR-3*. EMBO J. **7**:1647-1651, 1988.

Knabbe C. Kellner U. Schmahl M. and Voigt K.D. *Growth factors in human prostate cancer cells: implications for an improved treatment of prostate cancer*. J.Steroid Biochem.Mol.Biol. **40**:185-192, 1991.

Koch C.A. Anderson D. Moran M.F. Ellis C. and Pawson T. *SH2 and SH3 domains: elements that control interactions of cytoplasmic signaling proteins*. Science **252**:668-674, 1991.

Kokai Y. Dobashi K. Weiner D.B. Myers J.N. Nowell P.C. and Greene M.I. *Phosphorylation process induced by epidermal growth factor alters the oncogenic and cellular neu (NGL) gene products*. Proc.Natl.Acad.Sci.U.S.A. **85**:5389-5393, 1988.

Kokai Y. Myers J.N. Wada T. Brown V.I. LeVea C.M. Davis J.G. Dobashi K. and Greene M.I. *Synergistic interaction of p185c-neu and the EGF receptor leads to transformation of rodent fibroblasts*. Cell **58**:287-292, 1989.

Kommos F. Wintzer H.O. Von Kleist S. Kohler M. Walker R. Langton B. Van Tran K. Pfleiderer A. and Bauknecht T. *In situ distribution of transforming growth factor alpha in normal human tissues and in malignant tumours of the ovary*. J.Pathol **162**:223-230, 1990.

Korc M. Chandrasekar B. and Shah G.N. *Differential binding and biological activities of epidermal growth factor and transforming growth factor alpha in a human pancreatic cancer cell line.* Cancer Res. 51:6243-6249, 1991.

Koskinen P. Lehtvaslaiho H. MacDonald Bravo H. Alitalo K. and Bravo R. *Similar early gene responses to ligand-activated EGFR and neu tyrosine kinases in NIH3T3 cells.* Oncogene 5:615-618, 1990.

Kraus M.H. Popescu N.C. Amsbaugh S.C. and King C.R. *Overexpression of the EGF receptor-related proto-oncogene erbB-2 in human mammary tumor cell lines by different molecular mechanisms.* EMBO J. 6:605-610, 1987.

Kraus M.H. Issing W. Miki T. Popescu N.C. and Aaronson S.A. *Isolation and characterization of ERBB3, a third member of the ERBB/epidermal growth factor receptor family: evidence for overexpression in a subset of human mammary tumors.* Proc.Natl.Acad.Sci.U.S.A. 86:9193-9197, 1989.

Kris R.M. Lax I. Gullick W. Waterfield M.D. Ullrich A. Fridkin M. and Schlessinger J. *Antibodies against a synthetic peptide as a probe for the kinase activity of the avian EGF receptor and v-erbB protein.* Cell 40:619-625, 1985.

Kroes R. and Teppema J.S. *Development and restitution of squamous metaplasia in the calf prostate after a single estrogen treatment.* Exp. Mol. Pathol 16:286-301, 1972.

Kronmiller J.E. Upholt W.B. and Kollar E.J. *EGF antisense oligodeoxynucleotides block murine odontogenesis in vitro.* Dev.Biol. 147:485-488, 1991.

Kuhn J.M. Billebaud T. Navratil H. Moulonguet A. Fiet J. Grise P. Louis J.F. Costa P. Husson J.M. Dahan R. and et al *Prevention of the transient adverse effects of a gonadotropin-releasing hormone analogue (buserelin) in metastatic prostatic carcinoma*

by administration of an antiandrogen (nilutamide). *N.Engl.J.Med.* 321:413-418, 1989.

Kury F. Sliutz G. Schemper M. Reiner G. Reiner A. Jakesz R. Wrba F. Zeillinger R. Knogler W. Huber J. and et al *HER-2 oncogene amplification and overall survival of breast carcinoma patients.* *Eur.J.Cancer* 26:946-949, 1990.

Kypta R.M. Goldberg Y. Ulug E.T. and Courtneidge S.A. *Association between the PDGF receptor and members of the src family of tyrosine kinases.* *Cell* 62:481-492, 1990.

Labrie F. Dupont A. Belanger A. Cusan L. Iacourciere Y. Monfette G. Laberge J.G. Emond J.P. Fazekas A.T.A. Paynaud J.P. and Husson J.M. *New hormonal therapy in prostatic carcinoma: combined treatment with an LHRH agonist and an antiandrogen.* *Clin. Invest. Med.* 5:267-275, 1982.

Labrie F. Dupont A. Belanger A. Lacoursiere Y. Raynaud J.P. Husson J.M. Gareau J. Fazekas A.T. Sandow J. Monfette G. and et al *New approach in the treatment of prostate cancer: complete instead of partial withdrawal of androgens.* *Prostate* 4:579-594, 1983a.

Labrie F. Dupont A. Belanger A. Lefebvre F.A. Cusan L. Monfette G. Laberge J.G. Emond J.P. Raynaud J.P. Husson J.M. and Fazekas A.T. *New hormonal treatment in cancer of the prostate: combined administration of an LHRH agonist and an antiandrogen.* *J.Steroid Biochem.* 19:999-1007, 1983b.

Land H. Parada L.F. and Weinberg R.A. *Cellular oncogenes and multistep carcinogenesis.* *Science* 222:771-778, 1983a.

Land H. Parada L.F. and Weinberg R.A. *Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes.* *Nature* 304:596-602, 1983b.

- Lasnitzki L. and Mizuno T. *Induction of rat prostate gland by androgens in organ culture.* J.Endocrinol. 74:47, 1977.
- Lax I. Burgess W.H. Bellot F. Ullrich A. Schlessinger J. and Givol D. *Localization of a major receptor-binding domain for epidermal growth factor by affinity labeling.* Mol.Cell Biol. 8:1831-1834, 1988.
- Lee D.C. Rochford R. Todaro G.J. and Villarreal L.P. *Developmental expression of rat transforming growth factor-alpha mRNA.* Mol.Cell Biol. 5:3644-3646, 1985.
- Lee J. Dull T.J. Lax I. Schlessinger J. and Ullrich A. *HER2 cytoplasmic domain generates normal mitogenic and transforming signals in a chimeric receptor.* EMBO J. 8:167-173, 1989.
- Lehvaslaiho H. Lehtola L. Sistonen L. and Alitalo K. *A chimeric EGF-R-neu proto-oncogene allows EGF to regulate neu tyrosine kinase and cell transformation.* EMBO J. 8:159-166, 1989.
- Lemoine N.R. Staddon S. Dickson C. Barnes D.M. and Gullick W.J. *Absence of activating transmembrane mutations in the c-erbB-2 proto-oncogene in human breast cancer.* Oncogene 5:237-239, 1990.
- Lemoine N.R. Barnes D.M. Hollywood D.P. Hughes C.M. Smith P. Dublin E. Prigent S.A. Gullick W.J. and Hurst H.C. *Expression of the ERBB3 gene product in breast cancer.* Br.J.Cancer 66:1116-1121, 1992.
- Lepor H. and Walsh P.C. *Long-term results of radical prostatectomy in clinically localized prostate cancer: experience at the Johns Hopkins Hospital.* NCI.Monogr. 117-122, 1988.

- Levine R.L. and wilchinsky M. *Adenocarcinoma of the prostate: a comparison of the disease in blacks versus whites.* J.Urol. **121**:761-762, 1979.
- Li H.C. Chernoff J. Chen L.B. and Kirschonbaum A. *A phosphotyrosyl-protein phosphatase activity associated with acid phosphatase from human prostate gland.* Eur.J.Biochem. **138**:45-51, 1984.
- Libermann T.A. Nusbaum H.R. Razon N. Kris R. Lax I. Soreq H. Whittle N. Waterfield M.D. Ullrich A. and Schlessinger J. *Amplification and overexpression of the EGF receptor gene in primary human glioblastomas.* J.Cell Sci.Suppl. **3**:161-172, 1985a.
- Libermann T.A. Nusbaum H.R. Razon N. Kris R. Lax I. Soreq H. Whittle N. Waterfield M.D. Ullrich A. and Schlessinger J. *Amplification, enhanced expression and possible rearrangement of EGF receptor gene in primary human brain tumours of glial origin.* Nature **313**:144-147, 1985b.
- Lilja H. and Laurell C.B. *Liquefaction of coagulated human semen.* Scand.J.Clin.Lab.Invest **44**:447-452, 1984.
- Lilja H. *A kallikrein-like serine protease in prostatic fluid cleaves the predominant seminal vesicle protein.* J.Clin.Invest **76**:1899-1903, 1985.
- Lin C.R. Chen W.S. Kruiger W. Stolarsky L.S. Weber W. Evans R.M. Verma I.M. Gill G.N. and Rosenfeld M.G. *Expression cloning of human EGF receptor complementary DNA: gene amplification and three related messenger RNA products in A431 cells.* Science **224**:843-848, 1984.
- Lin M.F. and Clinton G.M. *Human prostatic acid phosphatase has phosphotyrosyl protein phosphatase activity.* Biochem.J. **235**:351-357, 1986.

- Lin M.F. and Clinton G.M. *The epidermal growth factor receptor from prostate cells is dephosphorylated by a prostate-specific phosphotyrosyl phosphatase.* Mol.Cell Biol. 8:5477-5485, 1988.
- Lin Y.Z. Caporaso G. Chang P.Y. Ke X.H. and Tam J.P. *Synthesis of a biological active tumor growth factor from the predicted DNA sequence of Shope fibroma virus.* Biochemistry 27:5640-5645, 1988.
- Liu C. Woo A. and Tsao M.S. *Expression of transforming growth factor-alpha in primary human colon and lung carcinomas.* Br.J.Cancer 62:425-429, 1990.
- Lonardo F. Di Marco E. King C.R. Pierce J.H. Segatto O. Aaronson S.A. and Di Fiore P.P. *The normal erbB-2 product is an atypical receptor-like tyrosine kinase with constitutive activity in the absence of ligand.* New Biol. 2:992-1003, 1990.
- Lund F. and Rasmussen F. *Flutamide versus stilboestrol in the management of advanced prostatic cancer. A controlled prospective study.* Br.J.Urol. 61:140-142, 1988.
- Lupu R. Colomer R. Zugmaier G. Sarup J. Shepard M. Slamon D. and Lippman M.E. *Direct interaction of a ligand for the erbB2 oncogene product with the EGF receptor and p185erbB2.* Science 249:1552-1555, 1990.
- MacFarlane J.R. and Tolley D.A. *Flutamide therapy for advanced prostatic cancer: a phase II study.* Br.J.Urol. 57:172-174, 1985.
- Maddy S.Q. Chisholm G.D. Busuttil A. and Habib F.K. *Epidermal growth factor receptors in human prostate cancer: correlation with histological differentiation of the tumour.* Br.J.Cancer 60:41-44, 1989.

Madtes D.K. Raines E.W. Sakariassen K.S. Assoian R.K. Sporn M.B. Bell G.I. and Ross R. *Induction of transforming growth factor-alpha in activated human alveolar macrophages.* Cell 53:285-293, 1988.

Maguire H.C.J. Jaworsky C. Cohen J.A. Hellman M. Weiner D.B. and Greene M.I. *Distribution of neu (c-erbB-2) protein in human skin.* J.Invest.Dermatol. 92:786-790, 1989.

Mao P. and Angrist A. *The fine structure of the basal cell of human prostate.* Lab.Invest 15:1768-1782, 1966.

Margolis B. Rhee S.G. Felder S. Mervic M. Lyall R. Levitzki A. Ullrich A. Zilberstein A. and Schlessinger J. *EGF induces tyrosine phosphorylation of phospholipase C-II: a potential mechanism for EGF receptor signaling.* Cell 57:1101-1107, 1989.

Margolis B.L. Lax I. Kris R. Dombalagian M. Honegger A.M. Howk R. Givol D. Ullrich A. and Schlessinger J. *All autophosphorylation sites of epidermal growth factor (EGF) receptor and HER2/neu are located in their carboxyl-terminal tails. Identification of a novel site in EGF receptor.* J.Biol.Chem. 264:10667-10671, 1989.

Marquardt H. Hunkapiller M.W. Hood L.E. and Todaro G.J. *Rat transforming growth factor type I: structure and relation to epidermal growth factor.* Science 223:1079-1082, 1984.

Massague J. *Epidermal growth factor-like transforming growth factor. II. Interaction with epidermal growth factor receptors in human placenta membranes and A431 cells.* J.Biol.Chem. 258:13614-13620, 1983.

Masui H. Kamrath H. Apell G. Houston L.L. and Mendelsohn J. *Cytotoxicity against human tumor cells mediated by the conjugate of anti-epidermal growth factor receptor*

- monoclonal antibody to recombinant ricin A chain.* Cancer Res 49:3482-3488, 1989.
- Mayes E.L. and Waterfield M.D. *Biosynthesis of the epidermal growth factor receptor in A431 cells.* EMBO J. 3:531-537, 1984.
- McConnell J.D. *Androgen ablation and blockade in the treatment of benign prostatic hyperplasia.* Urol.Clin.North Am. 17:661-670, 1990.
- McDonald N. Murray Rust J. and Blundell T. *Structure-function relationships of growth factors and their receptors.* Br.Med.Bull. 45:554-569, 1989.
- McKeehan W.L. Adams P.S. and Rosser M.P. *Direct mitogenic effects of insulin, epidermal growth factor, glucocorticoid, cholera toxin, unknown pituitary factors and possibly prolactin, but not androgen, on normal rat prostate epithelial cells in serum-free, primary cell culture.* Cancer Res. 44:1998-2010, 1984.
- McKeehan W.L. *Growth factor receptors and prostate cell growth.* Cancer Surv. 11:165-175, 1991.
- McNeal J.E. *Regional morphology and pathology of the prostate.* Am.J.Clin.Path. 49:347-357, 1968.
- Mendel D.B. Bodwell J.E. and Munck A. *Glucocorticoid receptors lacking hormone-binding activity are bound in nuclei of ATP-depleted cells.* Nature 324:478-480, 1986.
- Menon, M. and Walsh, P.C. *Hormonal therapy for prostatic cancer.* In: *Prostatic Cancer*, edited by Murphy, G.P. Littleton, Mass.: PSG Publ. Co., 1979, p. 175-200.
- Merk F.B. Ofner P. Kwan P.W. Leav I. and Vena R.L. *Ultrastructural and biochemical*

*expressions of divergent differentiation in prostates of castrated dogs treated with estrogen and androgen. Lab.Invest 47:437-450, 1982.*

Merlino G.T. Xu Y.H. Ishii S. Clark A.J. Semba K. Toyoshima K. Yamamoto T. and Pastan I. *Amplification and enhanced expression of the epidermal growth factor receptor gene in A431 human carcinoma cells. Science 224:417-419, 1984.*

Migliaccio A. Di Domenico M. Green S. de Falco A. Kajtaniak E.L. Blasi F. Chambon P. and Auricchio F. *Phosphorylation on tyrosine of in vitro synthesized human estrogen receptor activates its hormone binding. Mol.Endocrinol. 3:1061-1069, 1989.*

Miller, C.J. *diagnosis of stage a prostate cancer in the People's Republic of China. In: a Multidisciplinary Snalysis of Controversies In The Management of Prostate Cancer,* edited by Coffey, D.S., Resuick, M. and Dorr, F.A. New york: Plenum press, 1988, p. 17-24.

Molloy C.J. Bottaro D.P. Fleming T.P. Marshall M.S. Gibbs J.B. and Aaronson S.A. *PDGF induction of tyrosine phosphorylation of GTPase activating protein. Nature 342:711-714, 1989.*

Moolenaar W.H. Bierman A.J. Tilly B.C. Verlaan I. Defize L.H. Honegger A.M. Ullrich A. and Schlessinger J. *A point mutation at the ATP-binding site of the EGF-receptor abolishes signal transduction. EMBO J. 7:707-710, 1988.*

Mori H. Maki M. Oishi K. Jaye M. Igarashi K. Yoshida O. and Hatanaka M. *Increased expression of genes for basic fibroblast growth factor and transforming growth factor type beta 2 in human benign prostatic hyperplasia. Prostate 16:71-80, 1990.*

Moriyama M. Akiyama T. Yamamoto T. Kawamoto T. Kato T. Sato K. Watanuki T. Hikage T. Katsuta N. and Mori S. *Expression of c-erbB-2 gene product in urinary*

- bladder cancer*. J.Urol. 145:423-427, 1991.
- Morris G.L. and Dodd J.G. *Epidermal growth factor receptor mRNA levels in human prostatic tumors and cell lines*. J.Urol 143:1272-1274, 1990.
- Morrison D.K. Kaplan D.R. Escobedo J.A. Rapp U.R. Roberts T.M. and Williams L.T. *Direct activation of the serine/threonine kinase activity of Raf-1 through tyrosine phosphorylation by the PDGF beta-receptor*. Cell 58:649-657, 1989.
- Moses H.L. Proper J.A. Volkenant M.E. Wells D.J. and Getz M.J. *Mechanism of growth arrest of chemically transformed cells in culture*. Cancer Res. 38:2807-2812, 1978.
- Moses H.L. Branum E.L. Proper J.a and Robinson R.A. *Transforming growth factor production by chemical transformed cells*. Cancer Res. 41:2842-2848, 1981.
- Mroczkowski B. Reich M. Chen K. Bell G.I. and Cohen S. *Recombinant human epidermal growth factor precursor is a glycosylated membrane protein with biological activity*. Mol.Cell Biol. 9:2771-2778, 1989.
- Murphy L.C. Tsuyuki D. Myal Y. and Shiu R.P.C. *Isolation and sequencing of a cDNA clone for a prolactin-inducible protein (PIP)*. J.Biol.Chem. 262:15236-15241, 1987.
- Murray J.C. DeHaven C.R and Bell G.I. *RFLPs for epidermal growth factor (EGF), a single copy sequence 4q25-4q27*. Nucleic Acids Res. 14:5117-5121, 1986.
- Mydlo J.H. Bulbul M.A. Richon V.M. Heston W.D. and Fair W.R. *Heparin-binding growth factor isolated from human prostatic extracts*. Prostate 12:343-355, 1988a.
- Mydlo J.H. Michaeli J. Heston W.D. and Fair W.R. *Expression of basic fibroblast*

- growth factor mRNA in benign prostatic hyperplasia and prostatic carcinoma.* Prostate 13:241-247, 1988b.
- Mydlo J.H. Michaeli J. Cordon Cardo C. Goldenberg A.S. Heston W.D. and Fair W.R. *Expression of transforming growth factor alpha and epidermal growth factor receptor messenger RNA in neoplastic and nonneoplastic human kidney tissue.* Cancer Res. 49:3407-3411, 1989.
- Nakamoto T. Chang C.S. Li A.K. and Chodak G.W. *Basic fibroblast growth factor in human prostate cancer cells.* Cancer Res. 52:571-577, 1992.
- Natali P.G. Nicotra M.R. Bigotti A. Ventura I. Slamon D.J. Fendly B.M. and Ullrich A. *Expression of the p185 encoded by HER2 oncogene in normal and transformed human tissues.* Int.J.Cancer 45:457-461, 1990.
- Neal D.E. Smith K. Fennelly J.A. Bennett M.K. Hall R.R. and Harris A.L. *Epidermal growth factor receptor in human bladder cancer: a comparison of immunohistochemistry and ligand binding.* J.Urol. 141:517-521, 1989.
- Nesland J.M. Ottestad L. Heikilla R. Holm R. Tveit K. and Borresen A.L. *C-erbB-2 protein and neuroendocrine expression in breast carcinomas.* Anticancer Res. 11:161-167, 1991.
- Nicholson S. Sainsbury J.R. Halcrow P. Chambers P. Farndon J.R. and Harris A.L. *Expression of epidermal growth factor receptors associated with lack of response to endocrine therapy in recurrent breast cancer.* Lancet 1:182-185, 1989.
- Nicholson S. Wright C. Sainsbury J.R. Halcrow P. Kelly P. Angus B. Farndon J.R. and Harris A.L. *Epidermal growth factor receptor (EGFr) as a marker for poor prognosis in node-negative breast cancer patients: neu and tamoxifen failure.* J.Steroid

Biochem.Mol.Biol. 37:811-814, 1990.

Nicolson G.L, *Cancer Metasis*. Sci.Am. 3:66-76, 1979.

Nishi N. Matuo Y. Kunitomi K. Takenaka I. Usami M. Kotake T. and Wada F. *Comparative analysis of growth factors in normal and pathologic human prostates*. Prostate 13:39-48, 1988.

Nowell P.C. *The clonal evolution of tumor cell populations*. Science 194:23-28, 1976.

Oesterling J.E. Brendler C.B. Epstein J.I. Kimball A.W.J. and Walsh P.C. *Correlation of clinical stage, serum prostatic acid phosphatase and preoperative Gleason grade with final pathological stage in 275 patients with clinically localized adenocarcinoma of the prostate*. J.Urol. 138:92-98, 1987.

Ofner, P., Morfin, R.F., Vena, R.L. and Aliapoulios, M.A. *Androgene metabolism in the human prostate*. In: *The Prostate Gland. Some Aspects of The Aetiology and Biochemistry of Prostatic Cancer*, edited by Griffiths, K. and Pierrepoint, C.G. Cardiff, Wales, U.K.: Alpha Omega Alpha publishing Company, 1970,

Oka Y. and Orth D.N. *Human plasma epidermal growth factor/beta-urogastrone is associated with blood platelets*. J.Clin.Invest 72:249-259, 1983.

Ozanne B. Fulton R.J. and Kaplan P.L. *Kirsten murine virus transforming cell lines and a spontaneous transformed rat cell line produce transforming factors*. J.Cell Physiol. 105:163-180, 1980.

Paolson D.F. and Uro-oncology research group. *The impact of current staging procedures in assessing disease extent of prostatic adinocarcinoma*. J.Urol. 121:300-302, 1979.

- Papsidero L.D. Kuriyama M. and Wang M.C. *Prostate antigens: a marker for human prostate epithelia cells.* JNCI 66:37-42, 1981.
- Pardee A.B. *G1 events and regulation of cell proliferation.* Science 246:603-608, 1989.
- Parkes H.C. Lillycrop K. Howell A. and Craig R.K. *C-erbB2 mRNA expression in human breast tumours: comparison with c-erbB2 DNA amplification and correlation with prognosis.* Br.J.Cancer 61:39-45, 1990.
- Pastan I. and Willingham M.C. *Journey to the center of the cell: role of the receptosome.* Science 214:504-509, 1981.
- Pastan I. Willingham M.C. and FitzGerald D.J. *Immunotoxins.* Cell 47:641-648, 1986.
- Paul D. Lipton A. and Klinger I. *Serum factor requirements of normal and simian virus-40-transferred 3T3 mouse fibroblasts.* Proc.Natl.Acad.Sci.U.S.A. 68:645-648, 1971.
- Pavelic Z.P. Pavelic L. Lower E.E. Gapany M. Gapany S. Barker E.A. and Preisler H.D. *c-myc, c-erbB-2, and Ki-67 expression in normal breast tissue and in invasive and noninvasive breast carcinoma.* Cancer Res. 52:2597-2602, 1992.
- Perez R. Pascual M. Macias A. and Lage A. *Epidermal growth factor receptors in human breast cancer.* Breast Cancer Res.Treat. 4:189-193, 1984.
- Perkel V.S. Mohan S. Herring S.J. Baylink D.J. and Linkhart T.A. *Human prostatic cancer cells, PC3, elaborate mitogenic activity which selectively stimulates human bone cells.* Cancer Res. 50:6902-6907, 1990.
- Perosio P.M. and Brooks J.J. *Expression of growth factors and growth factor receptors*

*in soft tissue tumors. Implications for the autocrine hypothesis.* Lab.Invest **60**:245-253, 1989.

Pfeffer S. and Ullrich A. *Epidermal growth factor. Is the precursor a receptor? [news].* Nature **313**:184, 1985.

Pledger W.J. Stiles C.D. Antoniades H.N. and Scher C.D. *Induction of DNA synthesis in BALB/C 3T3 cells by serum components: reevaluation of the commitment process.* Proc.Natl.Acad.Sci.U.S.A. **74**:4481-4485, 1977.

Pledger W.J. Stiles C.D. Antoniades H.N. and Scher C.D. *An ordered sequence of events is required before BALB/C 3T3 cells become committed to DNA synthesis.* Proc.Natl.Acad.Sci.U.S.A. **75**:2839-2843, 1978.

Plowman G.D. Green J.M. McDonald V.L. Neubauer M.G. Distche C.M. Todaro G.J. and Shoyab M. *The amphiregulin gene encodes a novel epidermal growth factor-related protein with tumor-inhibitory activity.* Mol.Cell Biol. **10**:1969-1981, 1990a.

Plowman G.D. Whitney G.S. Neubauer M.G. Green J.M. McDonald V.L. Todaro G.J. and Shoyab M. *Molecular cloning and expression of an additional epidermal growth factor receptor-related gene.* Proc.Natl.Acad.Sci.U.S.A. **87**:4905-4909, 1990b.

Plowman G.D. Culouscou J.M. Whitney G.S. Green J.M. Carlton G.W. Foy L. Neubauer M.G. and Shoyab M. *Ligand-specific activation of HER4/p180erbB4, a fourth member of the epidermal growth factor receptor family.* Proc.Natl.Acad.Sci.U.S.A. **90**:1746-1750, 1993.

Popescu N.C. King C.R. and Kraus M.H. *Localization of the human erbB-2 gene on normal and rearranged chromosomes 17 to bands q12-21.32.* Genomics **4**:362-366, 1989.

- Power R.F. Lydon J.P. Conneely O.M. and O'Malley B.W. *Dopamine activation of an orphan of the steroid receptor superfamily*. *Science* **252**:1546-1548, 1991a.
- Power R.F. Mani S.K. Codina J. Conneely O.M. and O'Malley B.W. *Dopaminergic and ligand-independent activation of steroid hormone receptors*. *Science* **254**:1636-1639, 1991b.
- Press M.F. Cordon Cardo C. and Slamon D.J. *Expression of the HER-2/neu proto-oncogene in normal human adult and fetal tissues*. *Oncogene* **5**:953-962, 1990.
- Prigent S.A. Lemoine N.R. Hughes C.M. Plowman G.D. Selden C. and Gullick W.J. *Expression of the c-erbB-3 protein in normal human adult and fetal tissues*. *Oncogene* **7**:1273-1278, 1992.
- Quirke P. Pickles A. Tuzi N.L. Mohamdee O. and Gullick W.J. *Pattern of expression of c-erbB-2 oncoprotein in human fetuses*. *Br.J.Cancer* **60**:64-69, 1989.
- Rall L.B. Scott J. Bell G.I. Crawford R.J. Penschow J.D. Niall H.D. and Coghlan J.P. *Mouse prepro-epidermal growth factor synthesis by the kidney and other tissues*. *Nature* **313**:228-231, 1985.
- Ralston R. and Bishop J.M. *The product of the protooncogene c-src is modified during the cellular response to platelet-derived growth factor*. *Proc.Natl.Acad.Sci.U.S.A.* **82**:7845-7849, 1985.
- Rasmussen F. *Treatment of advanced prostate cancer*. *Horm.Res.* **32 Suppl 1**:47-49, 1989.
- Raymond V.W. Lee D.C. Grisham J.W. and Earp H.S. *Regulation of transforming growth factor alpha messenger RNA expression in a chemically transformed rat hepatic*

*epithelial cell line by phorbol ester and hormones. Cancer Res. 49:3608-3612, 1989.*

Reddy G.P.V. and Pardee A.B. *Multienzyme complex for metabolic channeling in mammalian DNA replication. Proc.Natl.Acad.Sci.U.S A. 77:3312-3316, 1980.*

Rich A.R. *On frequency of occurrence of occult carcinoma of prostate. J.Urol. 33:215-223, 1935.*

Riedel H. Massoglia S. Schlessinger J. and Ullrich A. *Ligand activation of overexpressed epidermal growth factor receptors transforms NIH 3T3 mouse fibroblasts. Proc.Natl.Acad.Sci.U.S.A. 85:1477-1481, 1988.*

Ross R. Bernstein L. Judd H. Hanisch R. Pike M. and Henderson B. *Serum testosterone levels in healthy young black and white men. J.Natl.Cancer Inst. 76:45-48, 1986a.*

Ross R. Raines E.W. and Bowen Pope D.F. *The biology of platelet-derived growth factor. Cell 46:155-169, 1986b.*

Roussel M.F. Rettenmier C.W. Look A.T. and Sherr C.J. *Cell surface expression of v-fms-coded glycoproteins is required for transformation. Mol.Cell Biol. 4:1999-2009, 1984.*

Russo M.W. Lukas T.J. Cohen S. and Staros J.V. *Identification of residues in the nucleotide binding site of the epidermal growth factor receptor/kinase. J.Biol.Chem. 260:5205-5208, 1985.*

Sainsbury J.R. Farndon J.R. Needham G.K. Malcolm A.J. and Harris A.L. *Epidermal-growth-factor receptor status as predictor of early recurrence of and death from breast cancer. Lancet 1:1398-1402, 1987.*

Saitoh H. Hipa M. Shimbo t. Nakamura K. Kamagata J. and Satoh T. *Metastatic patterns of prostatic cancer: correlation between sites and number of organs involved.* Cancer 54:3078-3084, 1984.

Salomon D.S. Perroteau I. Kidwell W.R. Tam J. and Derynck R. *Loss of growth responsiveness to epidermal growth factor and enhanced production of alpha-transforming growth factors in ras-transformed mouse mammary epithelial cells.* J.Cell Physiol. 130:397-409, 1987a.

Salomon D.S. Perroteau I. Kidwell W.R. Tam J. and Derynck R. *Loss of growth responsiveness to epidermal growth factor and enhanced production of alpha-transforming growth factors in ras-transformed mouse mammary epithelial cells.* J.Cell Physiol 130:397-409, 1987b.

Sambrook, J., Fritsch, E.F. and Mantiatis, T. *Molecular cloning. a laboratory manual,* New York:Cold Spring Harbor Laboratory press, 1989.

Samsoondar J. Kobrin M.S. and Kudlow J.E. *Alpha-transforming growth factor secreted by untransformed bovine anterior pituitary cells in culture. I. Purification from conditioned medium.* J.Biol.Chem. 261:14408-14413, 1986.

Sandgren E.P. Luetkeke N.C. Palmiter R.D. Brinster R.L. and Lee D.C. *Overexpression of TGF alpha in transgenic mice: induction of epithelial hyperplasia, pancreatic metaplasia, and carcinoma of the breast.* Cell 61:1121-1135, 1990.

Saya H. Ara S. Lee P.S. Ro J. and Hung M.C. *Direct sequencing analysis of transmembrane region of human Neu gene by polymerase chain reaction.* Mol.Carcinog. 3:198-201, 1990.

Schechter A.L. Stern D.F. Vaidyanathan L. Decker S.J. Drebin J.A. Greene M.I. and

Weinberg R.A. *The neu oncogene: an erb-B-related gene encoding a 185,000-Mr tumour antigen*. Nature **312**:513-516, 1984.

Schechter A.L. Hung M.C. Vaidyanathan L. Weinberg R.A. Yang Feng T.L. Francke U. Ullrich A. and Coussens L. *The neu gene: an erbB-homologous gene distinct from and unlinked to the gene encoding the EGF receptor*. Science **229**:976-978, 1985.

Schlessinger J. Shechter Y. Willingham M.C. and Pastan I. *Direct visualization of binding aggregation, and internalization of insulin and epidermal growth factor on living fibroblastic cells*. Proc.Natl.Acad.Sci.U.S.A. **75**:2659-2663, 1978.

Schlessinger J. *The epidermal growth factor receptor as a multifunctional allosteric protein*. Biochemistry **27**:3119-3123, 1988.

Schreiber A.B. Lax I. Yarden Y. Eshhar Z. and Schlessinger J. *Monoclonal antibodies against receptor for epidermal growth factor induce early and delayed effects of epidermal growth factor*. Proc.Natl.Acad.Sci.U.S.A. **78**:7535-7539, 1981.

Schroeder F.H. *Treatment of localised prostatic carcinoma*. Horm.Res. **32 Suppl 1**:44-46, 1989.

Schulze, H., Isaacs, J.T. and Coffey, D.S. *Prostate cancer Part a: research endocrine treatment, and histopaathology progress in clinical and biological research*. In: *a critical Review of The Concept of Fatal Androgen Ablation In the Treatment of Prostatic Cancer*. Vol.243A, edited by Murphy, G.P., Khoury, s., Kuss, R., Chatelain, C. and Denis, L. New York: Alan,R. Liss, Inc, 1987, p. 1-19.

Schuurmans A.L. Bolt J. and Mulder E. *Androgens stimulate both growth rate and epidermal growth factor receptor activity of the human prostate tumor cell LNCaP*.

Prostate 12:55-63, 1988.

Scott G.K. Dodson J.M. Montgomery P.A. Johnson R.M. Sarup J.C. Wong W.L. Ullrich A. Shepard H.M. and Benz C.C. *p185HER2 signal transduction in breast cancer cells.* J.Biol.Chem. 266:14300-14305, 1991.

Scott J. Urdea M. Quiroga M. Sanchez Pescador R. Fong N. Selby M. Rutter W.J. and Bell G.I. *Structure of a mouse submaxillary messenger RNA encoding epidermal growth factor and seven related proteins.* Science 221:236-240, 1983.

Scott W.W Menon M. and Walsh P.C. *Hormonal therapy of prostatic cancer.* Cancer 45:1929-1936, 1980.

Segatto O. King C.R. Pierce J.H. Di Fiore P.P. and Aaronson S.A. *Different structural alterations upregulate in vitro tyrosine kinase activity and transforming potency of the erbB-2 gene.* Mol.Cell Biol. 8:5570-5574, 1988.

Segatto O. Lonardo F. Wexler D. Fazioli F. Pierce J.H. Bottaro D.P. White M.F. and Di Fiore P.P. *The juxtamembrane regions of the epidermal growth factor receptor and gp185erbB-2 determine the specificity of signal transduction.* Mol.Cell Biol. 11:3191-3202, 1991.

Seidman H. Mushinski M.H. Gelb S.K. and Silverberg E. *Probabilities of eventually developing or dying of cancer--United States, 1985.* CA. 35:36-56, 1985.

Semba K. Kamata N. Toyoshima K. and Yamamoto T. *A v-erbB-related protooncogene, c-erbB-2, is distinct from the c-erbB-1/epidermal growth factor-receptor gene and is amplified in a human salivary gland adenocarcinoma.* Proc.Natl.Acad.Sci.U.S.A. 82:6497-6501, 1985.

Shaikh N. Lai L. McLoughlin J. Clark D. and Williams G. *Quantitative analysis of epidermal growth factor in human benign prostatic hyperplasia and prostatic carcinoma and its prognostic significance.* Anticancer Res. 10:873-874, 1990.

Shalaby M.R. Shepard H.M. Presta L. Rodrigues M.L. Beverley P.C. Feldmann M. and Carter P. *Development of humanized bispecific antibodies reactive with cytotoxic lymphocytes and tumor cells overexpressing the HER2 protooncogene.* J.Exp.Med. 175:217-225, 1992.

Shannon J.M. and Cunha G.R. *Autoradiographic localization of androgen binding in the developing mouse prostate.* Prostate 4:367-373, 1983.

Shepard H.M. Lewis G.D. Sarup J.C. Fendly B.M. Maneval D. Mordenti J. Figari I. Kotts C.E. Palladino M.A.J. Ullrich A. and et al *Monoclonal antibody therapy of human cancer: taking the HER2 protooncogene to the clinic.* J.Clin.Immunol. 11:117-127, 1991.

Shi D. He G. Cao S. Pan W. Zhang H.Z. Yu D. and Hung M.C. *Overexpression of the c-erbB-2/neu-encoded p185 protein in primary lung cancer.* Mol.Carcinog. 5:213-218, 1992.

Shih C. Padhy L.C. Murray M. and Weinberg R.A. *Transforming genes of carcinomas and neuriblastomas introduced into mouse fibroblasts.* Nature 290:261-264, 1981.

Shoyab M. McDonald V.L. Bradley J.G. and Todaro G.J. *Amphiregulin: a bifunctional growth-modulating glycoprotein produced by the phorbol 12-myristate 13-acetate-treated human breast adenocarcinoma cell line MCF-7.* Proc.Natl.Acad.Sci.U.S.A. 85:6528-6532, 1988.

Shoyab M. Plowman G.D. McDonald V.L. Bradley J.G. and Todaro G.J. *Structure and*

*function of human amphiregulin: a member of the epidermal growth factor family.* Science 243:1074-1076, 1989.

Siegall C.B. Xu Y.H. Chaudhary V.K. Adhya S. FitzGerald D. and Pastan I. *Cytotoxic activities of a fusion protein comprised of TGF alpha and Pseudomonas exotoxin.* FASEB J. 3:2647-2652, 1989.

Sikes R.A. and Chung L.W. *Acquisition of a tumorigenic phenotype by a rat ventral prostate epithelial cell line expressing a transfected activated neu oncogene.* Cancer Res 52:3174-3181, 1992a.

Sikes R.A. and Chung L.W. *Acquisition of a tumorigenic phenotype by a rat ventral prostate epithelial cell line expressing a transfected activated neu oncogene.* Cancer Res. 52:3174-3181, 1992b.

Silverberg E. and Lubera J.A. *A review of American Cancer Society estimates of cancer cases and deaths.* CA. 33:2-8, 1983.

Silverberg E. and Lubera J.A. *Cancer statistics, 1989 [see comments].* CA.Cancer J.Clin. 39:3-20, 1989.

Silverberg E. Boring C.C. and Squires T.S. *Cancer statistics, 1990 [see comments].* CA.Cancer J.Clin. 40:9-26, 1990.

Simpson E. Harrod J. Eilon G. Jacobs J.W. and Mundy G.R. *Identification of a messenger ribonucleic acid fraction in human prostatic cancer cells coding for a novel osteoblast-stimulating factor.* Endocrinology 117:1615-1620, 1985.

Sistonen L. Holtta E. Lehvaslaiho H. Lehtola L. and Alitalo K. *Activation of the neu tyrosine kinase induces the fos/jun transcription factor complex, the glucose transporter*

- and ornithine decarboxylase. J.Cell Biol. 109:1911-1919, 1989.*
- Sitaras N.M. Sariban E. Bravo M. Pantazis P. and Antoniadis H.N. *Constitutive production of platelet-derived growth factor-like proteins by human prostate carcinoma cell lines. Cancer Res. 48:1930-1935, 1988.*
- Slamon D.J. Clark G.M. Wong S.G. Levin W.J. Ullrich A. and McGuire W.L. *Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science 235:177-182, 1987.*
- Slamon D.J. and Clark G.M. *Amplification of c-erbB-2 and aggressive human breast tumors? Science 240:1795-1798, 1988.*
- Slamon D.J. Godolphin W. Jones L.A. Holt J.A. Wong S.G. Keith D.E. Levin W.J. Stuart S.G. Udove J. Ullrich A. and et al *Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. Science 244:707-712, 1989.*
- Slieker L.J. and Lane M.D. *Post-translational processing of the epidermal growth factor receptor. Glycosylation-dependent acquisition of ligand-binding capacity. J.Biol.Chem. 260:687-690, 1985.*
- Smith J.A.J. Eyre H.J. Roberts T.S. and Middleton R.G. *Transphenoidal hypophysectomy in the management of carcinoma of the prostate. Cancer 53:2385-2387, 1984.*
- Smith J.J. Derynck R. and Korc M. *Production of transforming growth factor alpha in human pancreatic cancer cells: evidence for a superagonist autocrine cycle. Proc.Natl.Acad.Sci.U.S.A. 84:7567-7570, 1987.*
- Smith J.M. Sporn M.B. Roberts A.B. Derynck R. Winkler M.E. and Gregory H. *Human*

*transforming growth factor-alpha causes precocious eyelid opening in newborn mice.* Nature **315**:515-516, 1985.

Soomro S. Shousha S. Taylor P. Shepard H.M. and Feldmann M. *c-erbB-2 expression in different histological types of invasive breast carcinoma.* J.Clin.Pathol **44**:211-214, 1991.

Sorkin A. Krolenko S. Kudrjavniceva N. Lazebnik J. Teslenko L. Soderquist A.M. and Nikolsky N. *Recycling of epidermal growth factor-receptor complexes in A431 cells: identification of dual pathways.* J.Cell Biol. **112**:55-63, 1991.

Spitzer E. Koepke K. Kunde D. and Grosse R. *EGF binding is quantitatively related to growth in node-positive breast cancer.* Breast Cancer Res.Treat. **12**:45-49, 1988.

Sporn M.B. and Roberts A.B. *Autocrine growth factors and cancer.* Nature **313**:745-747, 1985a.

Sporn M.B. and Roberts A.B. *Autocrine, paracrine and endocrine mechanisms of growth control.* Cancer Surv. **4**:627-632, 1985b.

Sporn M.B. and Roberts A.B. *Peptide growth factors are multifunctional.* Nature **332**:217-219, 1988.

St Arnaud R. Poyet P. Walker P. and Labrie F. *Androgens modulate epidermal growth factor receptor levels in the rat ventral prostate.* Mol.Cell Endocrinol. **56**:21-27, 1988.

Stamey T.A. and Kabalin J.N. *Prostate specific antigen in the diagnosis and treatment of adenocarcinoma of the prostate. I. Untreated patients.* J.Urol. **141**:1070-1075, 1989.

- Stancovski I. Hurwitz E. Leitner O. Ullrich A. Yarden Y. and Sela M. *Mechanistic aspects of the opposing effects of monoclonal antibodies to the ERBB2 receptor on tumor growth*. Proc.Natl.Acad.Sci.U.S.A. **88**:8691-8695, 1991.
- Steinberg G.D. Carter B.S. Beaty T.H. Childs B. and Walsh P.C. *Family history and the risk of prostate cancer*. Prostate **17**:337-347, 1990.
- Stern D.F. Hare D.L. Cecchini M.A. and Weinberg R.A. *Construction of a novel oncogene based on synthetic sequences encoding epidermal growth factor*. Science **235**:321-324, 1987.
- Stern D.F. and Kamps M.P. *EGF-stimulated tyrosine phosphorylation of p185neu: a potential model for receptor interactions*. EMBO J. **7**:995-1001, 1988a.
- Stern D.F. Kamps M.P. and Cao H. *Oncogenic activation of p185neu stimulates tyrosine phosphorylation in vivo*. Mol.Cell Biol. **8**:3969-3973, 1988b.
- Stern P.H. Krieger N.S. Nissenson R.A. Williams R.D. Winkler M.E. Derynck R. and Strewler G.J. *Human transforming growth factor-alpha stimulates bone resorption in vitro*. J.Clin.Invest **76**:2016-2019, 1985.
- Stone K. Mickey D.D Wunderli H. Mickey G. and Paulson D.F. *Isolation of human prostate carcinoma cell line (DU145)*. Int.J.Cancer **21**:274-281, 1978.
- Story M.T. Sasse J. Jacobs S.C. and Lawson R.K. *Prostatic growth factor: purification and structural relationship to basic fibroblast growth factor*. Biochemistry **26**:3843-3849, 1987.
- Stoscheck C.M. and Carpenter G. *Biology of the A-431 cell: a useful organism for*

*hormone research. J.Cell Biochem. 23:191-202, 1983.*

Stoscheck C.M. and King L.E.J. *Role of epidermal growth factor in carcinogenesis. Cancer Res. 46:1030-1037, 1986.*

Sugiyama K. Yonemura Y. and Miyazaki I. *immunohistochemical atudy of epidermal grwoth factor and epidermal growth factor receptor in gastric carcinoma. Cancer 63:1557-1561, 1989.*

Sunada H. Magun B.E. Mendelsohn J. and MacLeod C.L. *Monoclonal antibody against epidermal growth factor receptor is internalized without stimulating receptor phosphorylation. Proc.Natl.Acad.Sci.U.S.A. 83:3825-3829, 1986.*

Sundell H.W. Gray M.E. Serenius F.S. Escobed M.B. and Stallman M.T. *Effects of epidermal growth factor on lung maturation in feta lambs. Am.J.Pathol 100:707-726, 1980.*

Taetle R. Honeysett J.M. and Houston L.L. *Effects of anti-epidermal growth factor (EGF) receptor antibodies and an anti-EGF receptor recombinant-ricin A chain immunoconjugate on growth of human cells. J.Natl.Cancer Inst. 80:1053-1059, 1988.*

Takeda H. Mizuno T. and Lasnitzki I. *Autoradiographic studies of androgen-binding sites in the rat urogenital sinus and postnatal prostate. J.Endocrinol. 104:87-92, 1985.*

Takeda H. Lasnitzki I. and Mizuno T. *Analysis of prostatic bud induction by brief androgen treatment in the fetal rat urogenital sinus. J.Endocrinol. 110:467-470, 1986.*

Tal M. Wetzler M. Josefberg Z. Deutch A. Gutman M. Assaf D. Kris R. Shiloh Y. Givol D. and Schlessinger J. *Sporadic amplification of the HER2/neu protooncogene in*

*adenocarcinomas of various tissues. Cancer Res. 48:1517-1520, 1988.*

Tarakhovskiy A.M. Resnikov M. Zaichuk T. Tugusheva M.V. Butenko Z.A. and Prassolov V.S. *Polymorphic changes of cell phenotype caused by elevated expression of an exogenous NEU proto-oncogene. Oncogene 5:405-410, 1990.*

Tateishi M. Ishida T. Mitsudomi T. and Sugimachi K. *Prognostic implication of transforming growth factor alpha in adenocarcinoma of the lung--an immunohistochemical study. Br.J.Cancer 63:130-133, 1991.*

Temin H.M. *Studies on carcinogenesis by avian sarcoma viruses, VI. the differential effect of serum and polyamions on multiplication of uninfected and converted cells. J.Natl.Cancer Inst. 37:167-175, 1966.*

Tenniswood, M.P., Montpetit, M.L., Leger, J.G., Wong, P., Pineault, J.M. and Rouleau, M. *Epithelial-stromal interactions and cell death in the prostate. In: The Prostate As An Endocrine Gland, edited by Farnsworth, W.E. and Ablin, R.J. Boca Raton, Florida: CRC press Inc., 1990, p. 187-206.*

Todaro G.J. and DeLarco J.E. *Growth factors produced by sarcoma virus-transformed cells. Cancer Res. 38:4147-4154, 1978.*

Todaro G.J. Fryling C. and DeLarco J.E. *Transforming growth factors produced by certain human tumor cells: polypeptides that interact with epidermal growth factor receptors. Proc.Natl.Acad.Sci.U.S.A. 77:5258-5262, 1980.*

Tommasi S. Paradiso A. Mangia A. Barletta A. Simone G. Slamon D.J. and De Lena M. *Biological correlation between HER-2/neu and proliferative activity in human breast cancer. Anticancer Res. 11:1395-1400, 1991.*

- Tornqvist H.E. and Avruch J. *Relationship of site-specific beta subunit tyrosine autophosphorylation to insulin activation of the insulin receptor (tyrosine) protein kinase activity.* J.Biol.Chem. 263:4593-4601, 1988.
- Traish A.M. and Wotiz H.H. *Prostatic epidermal growth factor receptors and their regulation by androgens.* Endocrinology 121:1461-1467, 1987.
- Tsutsumi O. Kurachi H. and Oka T. *A physiological role of epidermal growth factor in male reproductive function.* Science 233:975-977, 1986.
- Turkes A.O. Peeling W.B. and Griffiths K. *Treatment of patients with advanced cancer of the prostate: phase III trial, zoladex against castration; a study of the British Prostate Group.* J.Steroid Biochem. 27:543-549, 1987.
- Tuzi N.L. Venter D.J. Kumar S. Staddon S.L. Lemoine N.R. and Gullick W.J. *Expression of growth factor receptors in human brain tumours.* Br.J.Cancer 63:227-233, 1991.
- Twardzik D.R. Todaro G.J. Marquardt H. Reynolds F.H. and Stephenson J.R. *Transformation by abelson murine leukemia virus involves production of a polypeptide growth factor.* Science 216:894-897, 1983a.
- Twardzik D.R. Todaro G.J. Reynolds F.H. and Stephenson J.R. *Similar transforming growth factor (TGFs) produced by cells transformed by different isolates of feline sarcoma virus.* Virol 124:272-274, 1983b.
- Twardzik D.R. Kimball E.S. Sherwin S.A. Ranchalis J.E. and Todaro G.J. *Comparison of growth factors functionally related to epidermal growth factor in the urine of normal and human tumor-bearing athymic mice.* Cancer Res. 45:1934-1939, 1985.
- Ullrich A. Coussens L. Hayflick J.S. Dull T.J. Gray A. Tam A.W. Lee J. Yarden Y.

Libermann T.A. Schlessinger J. and et al *Human epidermal growth factor receptor cDNA sequence and aberrant expression of the amplified gene in A431 epidermoid carcinoma cells.* Nature **309**:418-425, 1984.

Ullrich A. and Schlessinger J. *Signal transduction by receptors with tyrosine kinase activity.* Cell **61**:203-212, 1990.

Urist M.R. *Bone formation by autoinduction.* Science **150**:893-899, 1965.

Valverius E.M. Bates S.E. Stampfer M.R. Clark R. McCormick F. Salomon D.S. Lippman M.E. and Dickson R.B. *Transforming growth factor alpha production and epidermal growth factor receptor expression in normal and oncogene transformed human mammary epithelial cells.* Mol.Endocrinol. **3**:203-214, 1989.

Velu T.J. Beguinot L. Vass W.C. Willingham M.C. Merlino G.T. Pastan I. and Lowy D.R. *Epidermal-growth-factor-dependent transformation by a human EGF receptor proto-oncogene.* Science **238**:1408-1410, 1987.

Velu T.J. Vass W.C. Lowy D.R. and Beguinot L. *Functional heterogeneity of proto-oncogene tyrosine kinases: the C terminus of the human epidermal growth factor receptor facilitates cell proliferation.* Mol.Cell Biol. **9**:1772-1778, 1989.

Wada T. Qian X.L. and Greene M.I. *Intermolecular association of the p185neu protein and EGF receptor modulates EGF receptor function.* Cell **61**:1339-1347, 1990.

Wahl M.I. Nishibe S. Suh P.G. Rhee S.G. and Carpenter G. *Epidermal growth factor stimulates tyrosine phosphorylation of phospholipase C-II independently of receptor internalization and extracellular calcium.* Proc.Natl.Acad.Sci.U.S.A. **86**:1568-1572, 1989.

- Walsh P.C. *Physiologic basis for hormonal therapy in carcinoma of the prostate.* Urol.Clin.North Am. 2:125-140, 1975.
- Warri A.M. Laine A.M. Majasuo K.E. Alitalo K.K. and Harkonen P.L. *Estrogen suppression of erbB2 expression is associated with increased growth rate of ZR-75-1 human breast cancer cells in vitro and in nude mice.* Int.J.Cancer 49:616-623, 1991.
- Wasner G. Hennermann I. and Kratochwil K. *Ontogeny of mesenchymal androgen receptors in the embryonic mouse mammary gland.* Endocrinology 113:1771-1780, 1983.
- Watanabe S. Lazar E. and Sporn M.B. *Transformation of normal rat kidney (NRK) cells by an infectious retrovirus carrying a synthetic rat type alpha transforming growth factor gene.* Proc.Natl.Acad.Sci.U.S.A. 84:1258-1262, 1987.
- Weiner D.B. Liu J. Cohen J.A. Williams W.V. and Greene M.I. *A point mutation in the neu oncogene mimics ligand induction of receptor aggregation.* Nature 339:230-231, 1989.
- Wells A. and Bishop J.M. *Genetic determinants of neoplastic transformation by the retroviral oncogene v-erbB.* Proc.Natl.Acad.Sci.U.S.A. 85:7597-7601, 1988.
- Wides R.J. Zak N.B. and Shilo B.Z. *Enhancement of tyrosine kinase activity of the Drosophila epidermal growth factor receptor homolog by alterations of the transmembrane domain.* Eur.J.Biochem. 189:637-645, 1990.
- Wilcox J.N. and Derynck R. *Developmental expression of transforming growth factors alpha and beta in mouse fetus.* Mol.Cell Biol. 8:3415-3422, 1988.
- Wilding G. Chen M. and Gelmann E.P. *Aberrant response in vitro of*

- hormone-responsive prostate cancer cells to antiandrogens.* Prostate 14:103-115, 1989a.
- Wilding G. Zugmeier G. Knabbe C. Flanders K. and Gelmann E. *Differential effects of transforming growth factor beta on human prostate cancer cells in vitro.* Mol.Cell Endocrinol. 62:79-87, 1989b.
- Wilding G. *The importance of steroid hormones in prostate cancer.* Cancer Surv. 14:113-130, 1992.
- Williams T.M. Weiner D.B. Greene M.I. and Maguire H.C.J. *Expression of c-erbB-2 in human pancreatic adenocarcinomas.* Pathobiology 59:46-52, 1991.
- Wilson J.D. George F.W. and Griffin J.E. *The hormone control of sexual development.* Science 211:1278-1284, 1981.
- Winkler M.E. O'Connor L. Winget M. and Fendly B. *Epidermal growth factor and transforming growth factor alpha bind differently to the epidermal growth factor receptor.* Biochemistry 28:6373-6378, 1989.
- Winstanley J. Cooke T. George W.D. Murray G. Holt S. Croton R. Griffiths K. and Nicholson R. *The long term prognostic significance of oestrogen receptor analysis in early carcinoma of the breast.* Br.J.Cancer 64:99-101, 1991.
- Wrann M.M. and Fox C.F. *Identification of epidermal growth factor receptors in a hyperproducing human epidermoid carcinoma cell line.* J.Biol.Chem. 254:8063-8068, 1979.
- Wright C. Nicholson S. Angus B. Sainsbury J.R. Farndon J. Cairns J. Harris A.L. and Horne C.H. *Relationship between c-erbB-2 protein product expression and response to endocrine therapy in advanced breast cancer.* Br.J.Cancer 65:118-121, 1992.

Wynder E.L. Mabuchi K. and Whitmore W.F. *Epidemiology of cancer of the prostate.* Cancer 28:344-360, 1971.

Yamahara M. Fujito T. Ishikawa T. Shimosato T. Yokozaki H. Yasui W. and Tahara E. *Phenotypic expression of human epidermal growth factor in foetal submandibular gland and pleomorphic adenoma of salivary gland.* pathol.Anat. 412:301-306, 1988.

Yamamoto T. Nishida T. Miyajima N. Kawai S. Ooi T. and Toyoshima K. *The erbB gene of avian erythroblastosis virus is a member of the src gene family.* Cell 35:71-78, 1983.

Yamamoto T. Ikawa S. Akiyama T. Semba K. Nomura N. Miyajima N. Saito T. and Toyoshima K. *Similarity of protein encoded by the human c-erb-B-2 gene to epidermal growth factor receptor.* Nature 319:230-234, 1986a.

Yamamoto T. Kamata N. Kawano H. Shimizu S. Kuroki T. Toyoshima K. Rikimaru K. Nomura N. Ishizaki R. Pastan I. and et al *High incidence of amplification of the epidermal growth factor receptor gene in human squamous carcinoma cell lines.* Cancer Res. 46:414-416, 1986b.

Yang Y. Chisholm G.D. and Habib F.K. *Epidermal growth factor and transforming growth factor alpha concentrations in BPH and cancer of the prostate: their relationship with tissue androgen levels.* Br.J.Cancer 67:152-155, 1993.

Yarden Y. and Ullrich A. *Molecular analysis of signal transduction by growth factors.* Biochemistry 27:3113-3119, 1988.

Yarden Y. and Weinberg R.A. *Experimental approaches to hypothetical hormones: detection of a candidate ligand of the neu protooncogene.* Proc.Natl.Acad.Sci.U.S.A.

86:3179-3183, 1989.

Yarden Y. *Agonistic antibodies stimulate the kinase encoded by the neu protooncogene in living cells but the oncogenic mutant is constitutively active.* Proc.Natl.Acad.Sci.U.S.A. 87:2569-2573, 1990.

Yarden Y. and Peles E. *Biochemical analysis of the ligand for the neu oncogenic receptor.* Biochemistry 30:3543-3550, 1991.

Yasui W. Hata J. Yokozaki H. Nakatani H. Ochiai A. Ito H. and Tahara E. *Interaction between epidermal growth factor and its receptor in progression of human gastric carcinoma.* Int.J.Cancer 41:211-217, 1988.

Yokota J. Yamamoto T. Toyoshima K. Terada M. Sugimura T. Battifora H. and Cline M.J. *Amplification of c-erbB-2 oncogene in human adenocarcinomas in vivo.* Lancet 1:765-767, 1986.

Yoshida K. Kyo E. Tsujino T. Sano T. Niimoto M. and Tahara E. *Expression of epidermal growth factor, transforming growth factor-alpha and their receptor genes in human gastric carcinomas; implication for autocrine growth.* Jpn.J.Cancer Res. 81:43-51, 1990.

Yu D.H. and Hung M.C. *Expression of activated rat neu oncogene is sufficient to induce experimental metastasis in 3T3 cells.* Oncogene 6:1991-1996, 1991.

Yusa K. Sugimoto Y. Yamori T. Yamamoto T. Toyoshima K. and Tsuruo T. *Low metastatic potential of clone from murine colon adenocarcinoma 26 increased by transfection of activated c-erbB-2 gene.* J.Natl.Cancer Inst. 82:1633-1636, 1990.

Zeillinger R. Kury F. Czerwenka K. Kubista E. Sliutz G. Knogler W. Huber J. Zielinski

C. Reiner G. Jakesz R. and et al *HER-2 amplification, steroid receptors and epidermal growth factor receptor in primary breast cancer.* Oncogene 4:109-114, 1989.

Zhau H.E. Wan D.S. Zhou J. Miller G.J. and von Eschenbach A.C. *Expression of c-erb B-2/neu proto-oncogene in human prostatic cancer tissues and cell lines.* Mol.Carcinog. 5:320-327, 1992.