REGULATION OF TGF-B SIGNALING BY Xrel3, A MEMBER OF THE Rel/NF-kB FAMILY IN HUMAN CERVICAL CANCER CELLS

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Your file Votre référence
ISBN: 0-612-89628-5
Our file Notre référence
ISBN: 0-612-89628-5

Regulation of TGF-β Signaling by *Xrel3*, a member of the Rel/NF-κB family in human cervical cancer cells

by

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A thesis submitted to the School of Graduate Studies
in partial fulfillment of the requirements for the
degree of Master of Science

Faculty of Medicine
Memorial University of Newfoundland
May, 2003

St. John's Newfoundland and Labrador

ABSTRACT

The Rel/NF-κB and TGF-β/SMAD signal transduction pathways are essential for maintaining homeostasis, controlling cell proliferation and survival in every species studied and in many different cell and tissue types. Both pathways are implicated in numerous cancers resulting from their abnormal regulation. For instance, constitutive Rel/NF-κB activity leads to continous pro-survival stimulation allowing cancer cells the opportunity to continue growing in otherwise detrimental conditions. Also, due to its role in growth suppression, TGF-β-insensitivity has been reported in a majority of tumor types. Until recently, however, very few studies have attempted to determine whether there are interactions between the signaling members of each of these pathways. Therefore, this study was designed for this purpose.

The tetracycline-inducible system (Tet-On) was chosen to control the expression of a constitutively active Rel/NF-κB member, Xrel3, in CaSki human cervical carcinoma cell lines. The responses to TGF-β stimulation were measured in Xrel3-expressing and non-expressing CaSki cells to investigate whether the presence of Xrel3 affects TGF-β signaling. This thesis provides evidence that Xrel3 is able to block the TGF-β-induced growth inhibition, and PAI-1 and p21 induction, but not SMAD2 phosphorylation or p15 induction in CaSki cervical cancer cells. A mutated form of Xrel3, mutNLS, was not able to affect TGF-β responses of growth inhibition or marker induction. These results suggest that constitutive Rel/NF-κB activity may be responsible for the loss of sensitivity to TGF-β and may provide insight for therapeutic strategies for treating tumors which display this activity.

ACKNOWLEDGMENTS

I want to thank my co-supervisors, Dr. Alan Pater and Dr. Kenneth Kao for all their support and guidance throughout this academic pursuit. Their combined efforts were fundamental for the completion of this thesis, and I am very grateful to have them as supervisors. I sincerely thank the other members of my supervisory committee, Dr. Gary Paterno and Dr. Laura Gillespie for their valuable comments and constructive criticisms during the experimental stages of this project and the evaluation of this thesis.

I want to thank Garry Chernenko for his excellent technical assistance, but also his friendship. His presence helped make the lab an enjoyable place in which to work. I also consider myself very fortunate to have worked alongside the other members of this lab, Bilan Mo, Jieying Xiong, Beth Kirby, Jason Davidge, and Dr. Jun Chen. I especially want to thank Zhihu Ding for his friendship and guidance that were essential when I started this project. I also thank Blue Lake for the construction of the mutNLS Xrel3 construct that was critical for interpreting my results and Mike Stacey for his help in creating many of the figures and diagrams that are included throughout this thesis.

I have been very fortunate to be surrounded by a large group of friends that have all played very valuable roles at numerous points along the way. I appreciate all that everyone has done for me and I will remember all the good times we have shared during the past few years. I feel lucky to say: there are just too many of you to list, so... Thanks!!

Lastly, I want to thank my parents and family for their continuous love and support, of which I'm sure I will rely on again in the not so distant future!

TABLE OF CONTENTS

Abstract i
Acknowledgments ii
Table of Contents iii
List of Figures vi
List of Tables viii
Abbreviations ix

Chapter 1. Introduction

1.1 Intracellular Signal Transduction 1

1.2 Rel/NF-κB Family of Transcriptional Regulators 2

1.2.1 Structure of Rel/NF-κB Proteins 2
1.2.2 Classification of Rel/NF-κB Family Members 3
1.2.3 The Rel/NF-κB Signaling Pathway 5
1.2.4 Rel-DNA Binding 9

1.3 The Transforming Growth Factor β (TGF-β) Superfamily and Signal Transduction 10

1.3.1 The TGF-β Superfamily 10
1.3.2 TGF-β Receptor Family 11
1.3.3 SMAD Family of Signal Transducers 16
1.3.4 SMAD Activation and Signal Transduction 17
1.3.5 Regulation of TGF-β/SMAD Signaling 19

1.4 Experimental Strategies for Regulating Gene Expression in Mammalian Cells 20

1.4.1 Inducible Gene Expression 21
1.4.2 Tetracycline-Inducible Gene Expression Systems 22

1.5 Thesis Rationale 25

Chapter 2. Materials and Methods

2.1 Cell Culture 28

2.2 Establishing Tet-Inducible *Xrel3*-Expressing CaSki Cells 28

2.2.1 Establishing Stable CaSKi-Tet Clones 28
2.2.2 Selecting Optimal CaSki-Tet Clone using Luciferase Assay 29
2.2.3 Tet-On Plasmid Construction 30
2.2.4 Stable Transfection with pTRE2-constructs 31
2.2.5 Genomic DNA Extraction 32
2.2.6 Southern Blot Probe Preparation 33
2.2.7 Southern Blot Analysis 34
2.2.8 Gene Expression Assays 35
2.2.8.1 RNA Extraction 35
2.2.8.2 Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) .. 36
2.2.8.3 Protein Extraction 36
2.2.8.4 Western Blot Analysis 37

2.3 Cell Proliferation Assay 38

2.4 Hematoxylin and Eosin Staining 39

2.5 Apoptosis Assay 39

Chapter 3. Results

3.1 Xel3 is a nuclear and constitutively active protein in CaSki cells 41

3.2 Generation of Tet-inducible, Xrel3-expressing cells 43

3.2.1 Construction of stable pTet-On transfected CaSki cells 43
3.2.2 Construction of tetracycline-responsive pTRE2-Xrel3 and pTRE2-mutNLS vectors 47
3.2.3 Stable Transfection of CaSki-Tet cells with pTRE2-Xrel3 and pTRE2-mutNLS 47
3.2.4 Confirming the inducible expression of Xrel3 plasmids in CaSki cells 49
3.2.4.1 Doxycycline induction of Xrel3 and mutNLS mRNA in CaSki-Tet cells 49
3.2.4.2 Translation of Xrel3 and mutNLS mRNA in CaSki-Tet cells .. 51

3.3 Xrel3 blocks cell morphological changes induced by TGF-β 54

3.4 Xrel3 blocks TGF-β-mediated inhibition of cell proliferation 63

3.5 Xrel3 prevents transcription of TGF-β-induced target genes 70

3.6 Xrel3 does not inhibit SMAD2 phosphorylation 73

3.7 Xrel3 alters TGF-β induced expression of cell cycle regulators 74

3.7.1 Xrel3 inhibits induction of $p21^{Cip/WAF1}$ expression by TGF-β 76
3.7.2 Xrel3 does not affect TGF-β stimulation of $p15^{INK4B}$ 78

3.8 TGF-β CaSki cell growth inhibition and the block of inhibition by Xrel3 is not due to apoptosis 80

Chapter 4. Discussion

4.1 Inducible expression system for Xrel3 was developed in CaSki cells 87

4.2 Xrel3 expression in CaSki cells 88

4.3 CaSki growth suppression by TGF-β 90

4.4 Cell cycle arrest by TGF-β is inhibited by Xrel3 in CaSki cells 93

4.5 Future experiments with inducible Xrel3 CaSki cell model 96

4.5.1 Does Xrel3 affect other TGF-β-inducible cell cycle regulators? 96
4.5.2 Does Xrel3 affect other SMAD signaling molecules? 97

4.6 Future Directions 100

Chapter 5. References 103

List of Figures

CHAPTER 1

1.1 Classification and structure of Rel/NF-κB proteins 4

1.2 Intracellular signal transduction via NF-κB 7

1.3 Transforming growth factor β signals through the SMAD proteins 18

1.4 Tet-On inducible gene expression system 24

CHAPTER 3

3.1 CaSki cellular localization of Xrel3 and its nuclear localization mutant, mutNLS 42

3.2 Selection of CaSki-Tet clones for doxycycline-responsiveness using a doxycycline-inducible luciferase assay 45

3.3 Conformation of stable insertion of pTet-On in CaSki cells 46

3.4 Construction of pTRE2-Xrel3 vectors 48

3.5 Genomic insertion of *Xrel3* constructs in transfected CaSki-Tet clones 50

3.6 Doxycycline-dependent transcription of *Xrel3* and *mutNLS* in CaSki cells 52

3.7 Doxycycline-dependent expression of Xrel3 protein in CT-Xrel3 and CT-mutNLS cells 53

3.8 Untransfected CaSki cells grown in the absence or presence of doxycycline and/or TGF-β 56

3.9 CaSki-Tet cells grown in the absence or presence of doxycycline and/or TGF-β 59

3.10 CT-Xrel3 cells grown in the absence or presence of doxycycline and/or TGF-β 60

3.11 CT-mutNLS cells grown in the absence or presence of doxycycline and/or TGF-β 62

3.12 Effects of doxycycline and/or TGF-β treatments on CaSki cell growth 64

3.13 Effects of doxycycline and/or TGF-β treatments on CaSki-Tet cells 66

3.14 Effects of doxycycline and/or TGF-β treatments on CT-Xrel3 cells 67

3.15 Effects of doxycycline and/or TGF-β treatments on CT-mutNLS cells 69

3.16 Effects of Xrel3 expression and its nuclear localization on the expression of TGF-β-responsive genes 71

3.17 Densitometric analysis of PAI-1 as compared to β-actin control levels in CaSki cell lines 72

3.18 Mechanisms of epithelial cell cycle inhibition by the activated TGF-β receptor complex 75

3.19 Densitometric analysis of $p21^{Cip/Waf1}$ as compared to β-actin control levels in CaSki cell lines 77

3.20 Densitometric analysis of $p15^{INK4B}$ as compared to β-actin control levels determined by Western blot 79

3.21 Apoptosis of CaSki cells following 48 hour treatments of either doxycycline and/or TGF-β 83

3.22 Apoptosis of CaSki-Tet cells following 48 hour treatments of either doxycycline and/or TGF-β 84

3.23 Apoptosis of CT-Xrel3 cells following 48 hour treatments of either doxycycline and/or TGF-β 85

3.24 Apoptosis of CT-mutNLS cells following 48 hour treatments of either doxycycline and/or TGF-β 86

CHAPTER 4

4.1 Potential actions of Xrel3 in preventing growth arrest by TGF-β and its effects the expression of TGF-β markers 95

List of Tables

CHAPTER 1

1.1 The transforming growth factor β (TGF-β) superfamily divisions 12

1.2 Mammalian and *Xenopus* TGF-β type I and type II receptor families 15

CHAPTER 3

3.1 Percentage of CaSki and CT-Xrel3 cells visibly undergoing mitosis in the presence of doxycycline and TGF-β 58

3.2 Percentage of apoptotic cells for each cell line following 48 hour treatments of doxycycline and TGF-β 82

Abbreviations

ATP	adenosine triphosphate
CAK	cdk-activating kinase
Cdk	cyclin-dependent kinase
CDKI	cdk inhibitor
cDNA	complementary deoxyribonucleic acid
CMV	cytomegalovirus
DMEM	Dulbecco's Modified Eagle's Medium
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide
DTT	dithiothreitol
EDTA	ethylenediamine-tetraacetic acid
FACS	fluorescence activated cell sorter
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
GFP	green fluorescence protein
H&E	hematoxylin and eosin
HPV	human papillomavirus
IκB	inhibitory kappa B
INK4	inhibitor of CDK4
IPTG	isopropyl-β-D-thiogalactopyranoside
LB	Luria-Bertani medium
NF-κB	nuclear factor- kappa B
NLS	nuclear localization signal
nm	nanometre
PAI-1	plasminogen activator inhibitor type 1
PBS	phosphate buffered saline
PI	propidium iodide
PMSF	phenylmethylsulfonyl fluoride
RHD	rel homology domain
RNA	ribonucleic acid
RT-PCR	reverse-transcriptase polymerase chain reaction
rtTA	reverse tetracycline transactivator
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
Smad	*Sma* and *mad*-related protein
SSC	saline sodium citrate
TB	Terrific broth
TβR	TGF-β receptor
TE	Tris-EDTA

TEMED	tetramethylethylenediamine
TetO	tetracycline operator
TetR	tetracycline repressor
TGF-β	transforming growth factor β
TNF	tumor necrosis factor

CHAPTER 1
INTRODUCTION

1.1 Intracellular Signal Transduction

Signaling from outside the cell surface to the nucleus in response to extracellular stimuli is a highly complex process involving a great variety of ligands and stimuli, cell surface receptors, signaling molecules, as well as all of the appropriate target genes and proteins for each situation. Intracellular signal transduction pathways are therefore assigned to cascades or families of cascades based on structure, function, ligands, targets, or a combination of these factors. The outcome of successful signal transduction could include the activation or prevention of cell division, stem cell differentiation, hormone secretion, muscle contraction, nerve conduction, or an immune reaction.

Due to the complexity of the signaling process, the coordination of transmitting the intended signal for a particular stimulus has many potential obstacles. If any of the components of the signaling mechanism become altered, such that they lose or gain responsiveness to a particular stimuli, it will often manifest consequences for the cell that directly result in disease conditions. The classic example is a cancer cell that is hyperproliferating due to constitutive activation of a particular growth-promoting signal pathway. In fact, many human diseases result from malfunctioning signal transduction pathways. For this reason, it is highly important to understand not only how each individual cascade functions, but also how they are able to communicate or cross-talk within the cell to modify and regulate the thousands of potential outcomes.

This thesis will focus on two important signal transduction pathways that involve the Rel/NF-κB family of transcriptional regulators and the transforming growth factor (TGF)-β superfamily of signaling molecules. Both pathways are implicated in various cancers and recent studies indicate interactions between them exist in order to modify and regulate the individual responses. A complete understanding of both pathways will be necessary in order to evaluate future therapeutic options for understanding and controlling carcinogenesis (Garg and Aggarwal, 2002).

1.2 Rel/NF-κB Family of Transcriptional Regulators

The Rel/NF-κB (nuclear factor-kappaB) family of ubiquitously expressed transcriptional regulators are highly conserved proteins and are activated via a well studied signaling pathway. They are involved in regulating immune responses (Grilli *et al.*, 1993; Kopp and Ghosh, 1995; Ghosh *et al.*, 1998), apoptosis (Sonenshein, 1997), neural signaling (Grilli and Memo, 1999), cell growth (Foo and Nolan, 1999), cell cycle progression (Joyce *et al.*, 2001) and potential carcinogenesis (Bours *et al.*, 1994; Luque and Gélinas, 1997).

1.2.1 Structure of Rel Proteins

All Rel proteins contain the N-terminal Rel Homology Domain (RHD) that shares 35-61% identity between family members (Baldwin, 1996). The RHD has four motifs that are responsible for the characteristic functions of all Rel members including DNA binding, dimerization, nuclear localization, and interaction with inhibitory IκB family members (Chen

and Ghosh, 1999). Rel proteins are found *in vivo* as homodimers or heterodimers and regulate transcription of target genes by binding 10 base pair (bp) promoter sequences called κB sites containing the consensus 5′-GGGGYNNCCY-3′ sequence (Kunsch *et al.*, 1992). The specific sequence of each κB site varies and determines the binding affinities of each Rel dimer thus conferring greater diversity in cellular responses (Menetski, 2000).

1.2.2 Classification of Rel Family Members

The prototype of the Rel family was identified in mature B cells as a nuclear factor necessary for κ light chain transcription and was therefore named NF-κB (Sen and Baltimore, 1986a). Since the characterization of NF-κB, several Rel family members have been identified and are divided into two classes (Figure 1.1). This dichotomy is based on the sequences of their variable C-terminal regions while the RHD domain is common to all Rel members and is found at the N-terminus.

The Class I Rel proteins include p105 and p100 which are proteolytically cleaved to give the active forms p50 and p52 respectively, and the *Drosophila* homologue Relish (Gnosh *et al.*, 1998). Although similar in structure and function, p105 and p100 differ in their regulation. The cleavage of p105 is constitutive while the processing of p100 is highly regulated (Xiao *et al.*, 2001). Class I Rel proteins contain approximately seven ankyrin repeats at the C-terminus that inhibit activation of the protein until they are cleaved at the protease site (Figure 1.1), releasing the shorter, active form. These Rel members are

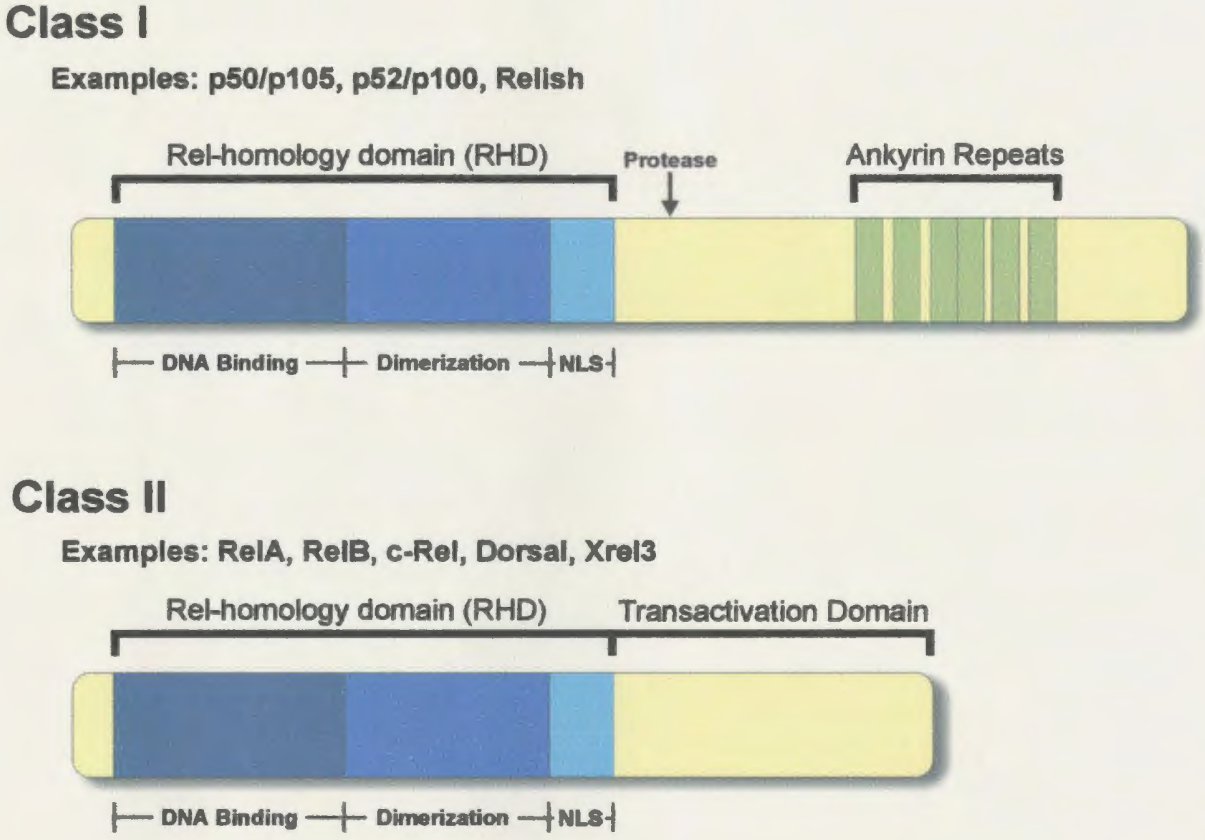

Figure 1.1 Classification and structure of Rel/NF-κB proteins. All Rel/NF-κB members contain the characteristic Rel Homology Domain containing sequences necessary for DNA-binding, interactions with IκB members, and a nuclear localization signal. Class I Rel/NF-κB members contain several inhibitory ankyrin repeat sequences that are proteolytically cleaved for activation. Class II Rel/NF-κB members contain a transactivation domain in the C-terminus that is essential for Rel activity.

therefore considered inhibitory Rel proteins unless they are heterodimerized with a member of the activating Class II Rel proteins (Gilmore, 1999).

Class II Rel/NF-κB members contain at least one transactivation domain in the C-terminus that is necessary for the activation of target gene expression (Schmitz *et al.*, 1994). They include RelA (or p65), RelB, c-Rel, Dorsal, v-rel, Xrel3, and the *Drosophila* Dif protein (Gnosh *et al.*, 1998). All of these members are able to form homodimers (with the exception of RelB) and heterodimers. The NF-κB heterodimer is composed of one RelA protein and one p50 protein bound in a complex and will be described in detail later in the text.

1.2.3 The Rel/NF-κB signaling pathway

Rel/NF-κB responds to many varied exogenous and endogenous stimuli (summarized in Pahl, 1999). NF-κB pathways can be activated by the products of infectious agents, such as bacterial outer surface lipopolysaccharide (LPS) (Sen and Baltimore, 1986b; Guha and Mackman, 2001), viral transactivating proteins such as Tax, the human T-cell leukemia viral protein (Leung and Nabel, 1988; Jeang, 2001), or double-stranded viral RNA (Visvanathan and Goodbourn, 1989; Kaufman, 1999). NF-κB can also be activated as part of the stress response by cell damaging agents such as ultraviolet (UV) light (Stein *et al.*, 1989) or reactive oxygen species, ROS (Schreck *et al.*, 1991; Mercurio and Manning, 1999). Proinflammatory cytokines such as interleukin-1 (IL-1), IL-2, and tumor necrosis factor-α (TNF-α) are potent inducers of Rel transactivation activity (Osborn *et al.*, 1989; Hazan *et al.*,

1990; Israël *et al.*, 1989). Some anti-cancer therapeutic drugs are known activators of Rel/NF-κB members including cisplatin (Nie *et al.*, 1998) and tamoxifen (Ferlini *et al.*, 1999). Follicle stimulating hormone (FSH), insulin, and TGF-α will also activate NF-κB (Delfino and Walker, 1998; Bertrand *et al.*, 1995; Lee *et al.*, 1995). The combined results highlight the ubiquitous nature of Rel/NF-κB signaling and the universal significance of Rel/NF-κB in cell growth, function, and homeostasis.

NF-κB is the most extensively studied Rel member and whose pathway is best known (Figure 1.2). NF-κB is normally found sequestered in an inactive state within the cytoplasm, bound to a member of the IκB family. This family contains seven mammalian members, IκBα, IκBβ, IκBγ, IκBϵ, Bcl-3, and the p100 and p105 Rel precursors (Ghosh *et al.*, 1998). IκB proteins contain multiple ankyrin repeats which interact with Rel proteins. The IκB ankyrin repeats each form two helices followed by a hairpin loop (Huxford *et al.*, 1998; Jacobs and Harrison, 1998) that stack on each other to form finger-like extensions (Ghosh and Karin, 2002). These extensions interact with the RHD to inhibit Rel/NF-κB activity.

The stimulation of NF-κB is initiated when an extracellular signal leads to the activation of the IκB kinase (IKK) complex (Figure 1.2). This complex is estimated to be greater than 700 kDa and contains IKKα, IKKβ, and NEMO/IKKγ along with other suspected components such as Cdc37 and Hsp90 (Chen *et al.*, 2002). IKK phosphorylates IκB proteins on serine residues at the N-terminus, an event that targets IκB for ubiquitination at lysine residues by a specific ubiquitin ligase in the Skp-1/Cul/F box (SCF) family (Ben-

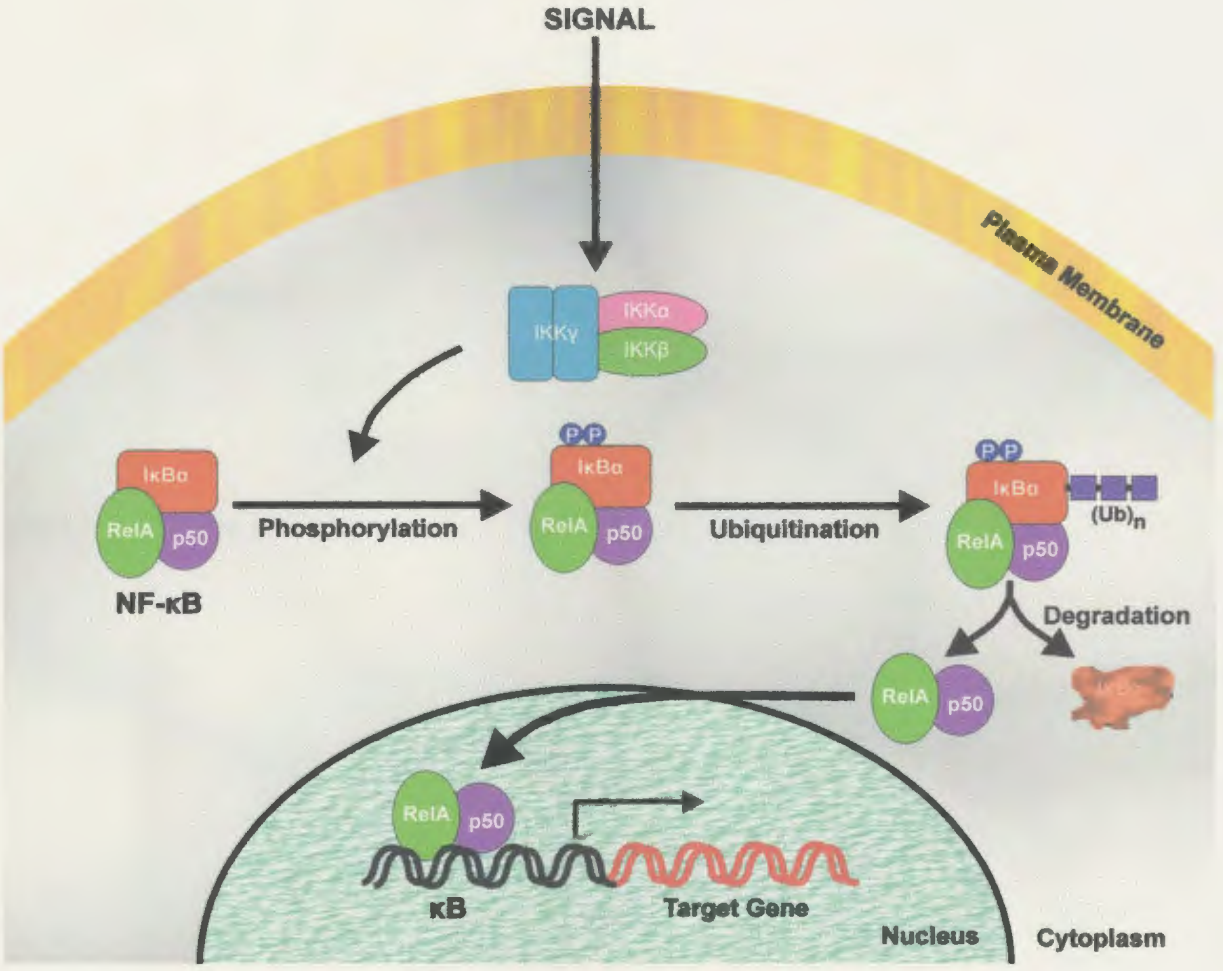

Figure 1.2 Intracellular signal transduction via NF-κB. Once stimulated, the IκB kinase complex (IKK) phosphorylates its downstream target, IκB that normally sequesters NF-κB in the cytoplasm. Following phosphorylation, IκB is targeted for polyubiquitination and subsequent degradation by the 26S proteoasome.

Neriah, 2002). Polyubiquitination of IκB leads to its rapid degradation by the 26S proteasome (Karin and Ben-Neriah, 2000), unmasking the NF-κB nuclear localization signal (NLS). NF-κB is then able to translocate to the nucleus where it can bind κB promoter sites and activate target genes (Figure 1.2). The kinetics of this process have been studied and it has been found to take approximately 16 minutes for nuclear accumulation of RelA (and therefore p50) following TNF-α stimulation (Nelson *et al.*, 2002).

While this scenario appears to be complete, there are many details that are unknown. Many regulatory mechanisms have been suggested at almost every stage of the Rel/NF-κB pathway that modify the signal to produce the ligand-specific response. For instance, NF-κB:IκB complexes have been shown to continuously shuttle in and out of the nucleus, controlled by the balancing of NLS and nuclear export signals (NES) in each molecule (Johnson *et al.*, 1999; Rodriguez *et al.*, 1999; Huang *et al.*, 2000; Tam *et al.*, 2000). The significance of this nuclear shuttling of NF-κB:IκB complexes is not clear (Ghosh and Karin, 2002). Second, Rel proteins themselves can be posttranslationally modified. Most Rel proteins contain a consensus sequence for protein kinase A (PKA) phosphorylation (Naumann and Scheidereit, 1994; Neumann *et al*, 1995). This phosphorylation enhances dimer-DNA binding (Neumann *et al.*, 1995) and enables interactions with CBP/p300 transcriptional coactivators (Zhong *et al.*, 2002). Many recent studies indicate that Rel phosphorylation is a regulator of Rel activity; however its importance is still unclear (Martin *et al.*, 2000; Wang *et al.*, 2000; Martin *et al.*, 2001).

More evidence is accumulating that Rel/NF-κB activation may alternatively involve

an IκB-independent mechanism (Lienhard Schmitz *et al.*, 2001). This model involves the phosphorylation of NF-κB at various residues by kinases other than IKK, that leads to a conformational change of NF-κB, stimulating nuclear accumulation and interactions of NF-κB with co-activators and co-repressors. Rel/NF-κB proteins have all been shown to be activated by casein kinase II (Wang *et al.*, 2000), protein kinase Cζ and Ras^{p21} (Anrather *et al.*, 1999), mitogen- and stress-activated protein kinase 1 (MSK-1) (Krause *et al.*, 1998), p38 MAPK (Carter *et al.*, 1999; Carter and Hunninghake, 2000), Akt or protein kinase B (Ozes *et al.*, 1999), and glycogen synthase kinase-3β (Hoeflich *et al.*, 2000). Therefore, much more research is needed in these areas to determine how these factors all act to regulate Rel/NF-κB pathways.

1.2.4 Rel-DNA binding

Every Rel dimer has its characteristic DNA binding site specificity and affinity (Chen and Ghosh, 1999) resulting in variable transactivation potential depending on the Rel member, cell type, growth conditions, and differentiation stage (Kang *et al.*, 1992; Plaksin *et al.*, 1993; Brown *et al.*, 1994; Hansen *et al.*, 1994a; Hansen *et al.*, 1994b). Three-dimensional structures of many Rel members binding to their κB sites have been analyzed (Ghosh *et al.*, 1995; Müller *et al.*, 1995; Cramer *et al.*, 1997; Chen *et al.*, 1998). Rel dimers straddle DNA, giving rise to a unique tertiary structure that has not been found in other transcription factors. Contained within the RHD, are two N-terminal β sheet folds which bind DNA in a base-specific and a backbone-nonspecific fashion (Coleman *et al.*, 1993;

Toledano *et al.*, 1993). Extending from these folds are ten flexible loops that mediate direct DNA contacts with κB sequences. These contacts are contained within two subsites of κB sequences that are pseudosymmetric (Chen and Ghosh, 1999) and are combinations of DNA nucleotide base-specific contacts within the major groove, (for example, arginine residues contact conserved guanines), hydrogen bonds (for example, glutamate residues bind cytosines), and van der Waals contacts (for example, positively-charged arginine interacts with pyrimidine bases) (Chen and Ghosh, 1999).

Specific DNA sequences are known to preferentially bind to certain dimers and also to be present in various complexes containing unique tertiary structures (Menetski, 2000). Due to the many existing forms of Rel dimers and the various sequences found in κB promoter sites, it is not surprising that Rel proteins are involved in so many cellular functions.

1.3 The Transforming Growth Factor β (TGF-β) Superfamily and Signal Transduction

1.3.1 The TGF-β Superfamily

The TGF-β signal transduction pathway is another ubiquitous signaling cascade that has been studied extensively due to its roles in many cellular processes. This signaling cascade is activated by a superfamily of structurally related and highly conserved growth factors whose prototype is TGF-β1 (reviewed in Massagué, 1998; Massagué *et al.*, 2000). These growth factors and their internal signaling mechanisms help regulate cell proliferation

and differentiation (Moses and Serra, 1996; Yue and Mulder, 2001; ten Dijke *et al.*, 2002), embryonic development (Wall and Hogan, 1994; Whitman, 2001), cell cycle regulation (Alexandrow and Moses, 1995; Hocevar and Howe, 1998), cell motility (Nakata *et al.*, 2002), wound healing and maintenance of the extracellular matrix (ECM) (Verrecchia and Mauviel, 2002), aging (Blumenfeld *et al.*, 2002), and cellular adhesion (Dunker and Krieglstein, 2000).

The TGF-β superfamily contains 28 known and predicted members (Venter *et al.*, 2001) and includes the activins, bone morphogenetic proteins, vegetal factors (Vg), the TGF-β subfamily members, and many other more distant members. A list of the common TGF-β family members and their known functions are listed in Table 1.1. The active form of TGF-β proteins are dimeric complexes, usually bound by a disulfide bond or hydrophobic interactions (Sun and Davies, 1995); however it does exist in a latent form as well (Lawrence, 2001). Loss of TGF-β signaling has been linked to many disorders including many cancers (Teicher, 2001; Wakefield and Roberts, 2002). Due to the physiological and pathological roles of the TGF-β superfamily, it is key to understand TGF-β signaling, its regulation, and its effects on target genes in order to elucidate where errors can possibly occur in associated disorders.

1.3.2 TGF-β Receptor Family

Signaling of TGF-β is initiated by ligand binding to its appropriate TGF-β receptor

Table 1.1. The Transforming Growth Factor β (TGF-β) Superfamily Divisions

TGF-β Subfamily	Function	References
Activin		
Activin βA Activin βB Activin βC Activin βE	Follicle-stimulating hormone production, erythroid cell differentiation, mesoderm induction	Harland, 1994; Gaddy-Kurten *et al.*, 1995
BMP2		
BMP2 BMP4	Important roles in gastrulation, neurogenesis, chondrogenesis, and mesoderm patterning	Hogan, 1996; Mehler *et al.*, 1997; Harland, 1994
BMP3		
BMP3/osteogenin GDF10	Endochondral bone formation; osteogenic differentiation; monocyte chemotaxis	Cunningham *et al.*, 1992
BMP5		
BMP5 BMP6/Vgr1 BMP7/OP1 BMP8/OP2	Necessary for the development of virtually all organs especially during neurogenesis	Hogan, 1996; Mehler *et al.*, 1997
GDF5		
GDF5/CDMP1 GDF6/CDMP2 GDF7	Chondrogenesis in embryonic development	Hogan, 1996; Kingsley, 1994
TGF-β		
TGF-β1 TGF-β2 TGF-β3	Epithelial cell cycle arrest; wound healing; immunosuppression; extracellular matrix production	Massagué, 1990; Roberts and Sporn, 1993; Alexandrow and Moses, 1995

Table 1.1. The Transforming Growth Factor β (TGF-β) Superfamily (continued)

TGF-β Subfamily	Function	References
Vg1		
GDF1/Vg1	Axial mesoderm induction	Kingsley, 1994
GDF3/Vgr2		
Intermediate Members		
Nodal/Xnr	Mesoderm induction; left-right asymetry; neural tube cell differentiation; inhibition of skeletal muscle growth	Basler, *et al.*, 1993; Beddington, 1996; Hogan, 1996; McPherron *et al.*, 1997
Dorsalin		
GDF8		
GDF9		
Distant Members		
MIS/AMH	Müllerian duct regression; inhibition of activin; dopaminergic neuron survival; kidney formation and development	Josso *et al.*, 1993; Gaddy-Kurten *et al.*, 1995; Massagué, 1996
Inhibin α		
GDNF		

BMP, bone morphogenetic protein. CDMP, cartilage-derived morphogenetic protein. GDF, growth and differentiation factor. GDNF, glial cell-derived nerotrophic factor. MIS/AMH, Müllerian inhibiting substance/anti-Müllerian hormone. OP, osteogenic protein. TGF, transforming growth factor. Vg, vegetal. Xnr, *Xenopus* nodal-related protein. (Adapted from Massagué, 1998).

(TβR) family of serine-threonine kinases. This family of receptors is divided into two groups based on structure and function (see Table 1.2). Type I receptors are unphosphorylated homodimers prior to ligand binding whereas type II receptors have a constitutive kinase activity that autophosphorylates on serine residues (Derynck and Feng, 1997; Dennler *et al.*, 2002). All TGF-β type I receptors contain three essential domains, the extracellular domain, the GS domain, and the kinase domain (Massagué, 1998; Dennler *et al.*, 2002). The extracellular domains of both type I and II receptors have approximately 150 amino acids and are *N*-glycosylated (Wells *et al.*, 1997). The TβR extracellular domain also contains a cluster of cysteines adjacent to the transmembrane sequence and up to ten or more other cysteine residues scattered about the extracellular domain (Wrana *et al.*, 1994). The arrangement of these cysteine residues influences the overall three-dimensional structure of each receptor via the intramolecular disulfide bonds formed between them (Fischer *et al.*, 1999). A feature within the intracellular domain common to only TβR-IIs is the constitutive phosphorylation of a serine residue in the non-ligand bound state (Luo and Lodish, 1997).

Type I receptors, TβR-Is, contain a glycine-serine (GS) domain with the highly conserved TTSGSGSG sequence adjacent to the protein kinase domain (Wrana *et al.*, 1994). Activation of type I receptors by ligand binding involves the phosphorylation of this sequence at the serine and threonine residues (Souchelnytskyi *et al.*, 1996). Due to its role in regulating TGF-β-induced cell responses, when the GS domain is mutated it leads to constitutive activity that occurs mostly on a threonine or glutamine residue located immediately adjacent to the kinase domain (Wieser *et al.*, 1995).

Table 1.2. Mammalian and *Xenopus* TGF-β Type I and Type II Receptor Families

TβR	References
TβRI	
ActR-1 (ALK2)	Attisano *et al.*, 1993; Yamashita *et al.*, 1995
ActR-1B (ALK4)	Cárcamo *et al.*, 1994
ALK1	Attisano *et al.*, 1993
ALK7	Tsuchida *et al.*, 1996; Rydén *et al.*, 1996
ALK8	de Caestecker *et al.*, 2002
BMPR-1A (ALK3)	Yamashita *et al.*, 1995
BMPR-1B (ALK6)	Koenig *et al.*, 1994
TβR-1 (ALK5)	Yamashita *et al.*, 1994
XTrR-II	Mahony and Grudon, 1995
TβRII	
ActR II	Mathews and Vale, 1991; Attisano *et al.*, 1992;
ActR-IIB	Mathews *et al.*, 1992
AMHR	Baarens *et al.*, 1994; di Clemente *et al.*, 1994
BMPR-II	Liu *et al.*, 1995; Nohno *et al.*, 1995; Rosenzweig *et al.*, 1995
TβR-II	Lin *et al.*, 1992

ActR, activin receptor. ALK, activin receptor-like kinase. AMHR, anti-Müllerian hormone receptor. BMPR, bone morphogenetic protein receptor. TβR, transforming growth factor β receptor. XTrR, *Xenopus* transforming growth factor β-related receptor. Adapted from Massagué, 1998.

The functional intracellular serine/threonine kinase domain is common in both TGF-β type I and type II receptors (Mathews and Vale, 1991; Frazén *et al.*, 1993). TβR-I substrates are the serine residues of *Sma*- and *Mad*-related proteins (SMADs) which function to transmit TGF-β and activin signals to the nucleus (SMADs 2 and 3) (Macias-Silva *et al.*, 1996) or BMP signals (SMADs 1, 3, and 5) (Kretzschmar *et al.*, 1997). The TβR-II autophosphorylate (Mathews and Vale, 1993) and TβR-I on serine and threonine residues (Wrana *et al.*, 1994; Wrana, 1998). Unlike the tyrosine kinase receptor family (eg: fibroblast growth factor, FGF, receptors) which have a critical C-terminal region necessary for signal transduction (McKeehan *et al.*, 1998; Powers *et al.*, 2000), TGF-β receptors do not contain functionally important C-terminal regions (Wieser *et al.*, 1993). TβR-I has negligible C-terminal region beyond the kinase domain while TβR-II has a short extension that does not appear to be important for signaling. Only BMPR-II contains a C-terminal region, but it has no known function (Kawabata *et al.*, 1995; Nohno *et al.*, 1995).

1.3.3 SMAD Family of Signal Transducers

The intracellular communication of TGF-β signals is dependent on the Sma and Mad (SMAD) related proteins. The SMADs comprise a family whose members are ubiquitously expressed throughout development and are found in all adult tissues (Flanders *et al.*, 2001; Luuko *et al.*, 2001). They were first identified in *Drosophila* (Raftery *et al.*, 1995; Sekelsky *et al.*, 1995) and *C. elegans* (Savage *et al.*, 1996) and later termed SMADs when eight members were identified in vertebrates (Riggins *et al.*, 1996).

SMAD proteins have two homologous domains, the N-terminal Mad-homology 1 (MH1) domain and the C-terminal Mad-homology 2 (MH2) domain connected by a proline-rich linker region (Yue and Mulder, 2001). In addition to these characteristic conserved motifs, SMAD proteins are functionally divided into three groups. In the first group, the receptor-activated SMADs (R-SMADs) are phosphorylated by type I receptors and are comprised of SMADs 1,2,3,5, and 8. The second group are the common-partner SMADs (co-SMADs), that dimerize with activated R-SMADs whose only mammalian homologue is SMAD4. The third group are the inhibitory SMADs (I-SMADs), which include the mammalian SMADs 6 and 7 (Moustakas *et al.*, 2001).

1.3.4 SMAD Activation and Signal Transduction

TGF-β signal transduction is activated by the formation of a heterotetrameric receptor complex of two type I receptors (TβR-I) and two type II receptors (TβR-II) bound by the dimeric TGF-β superfamily ligand (Massagué, 1998; Figure 1.3). This complex can be formed in one of two ways. First, TGF-β and activin ligands bind to TβR-II which then recruits TβR-I in a sequential manner (Attisano *et al.*, 1993; Ebner *et al.*, 1993). Alternatively, BMP ligands bind both types of receptors cooperatively with high affinity (Liu *et al.*, 1995; Nohno *et al.*, 1995). In the latter situation, both types of receptors have a low ligand affinity individually, but when present together, their affinities increase.

Following the formation of the heterotetrameric TGF-β receptor complex, the two type I receptors phosphorylate two C-terminal serine residues of the R-SMADs, SMAD2 and

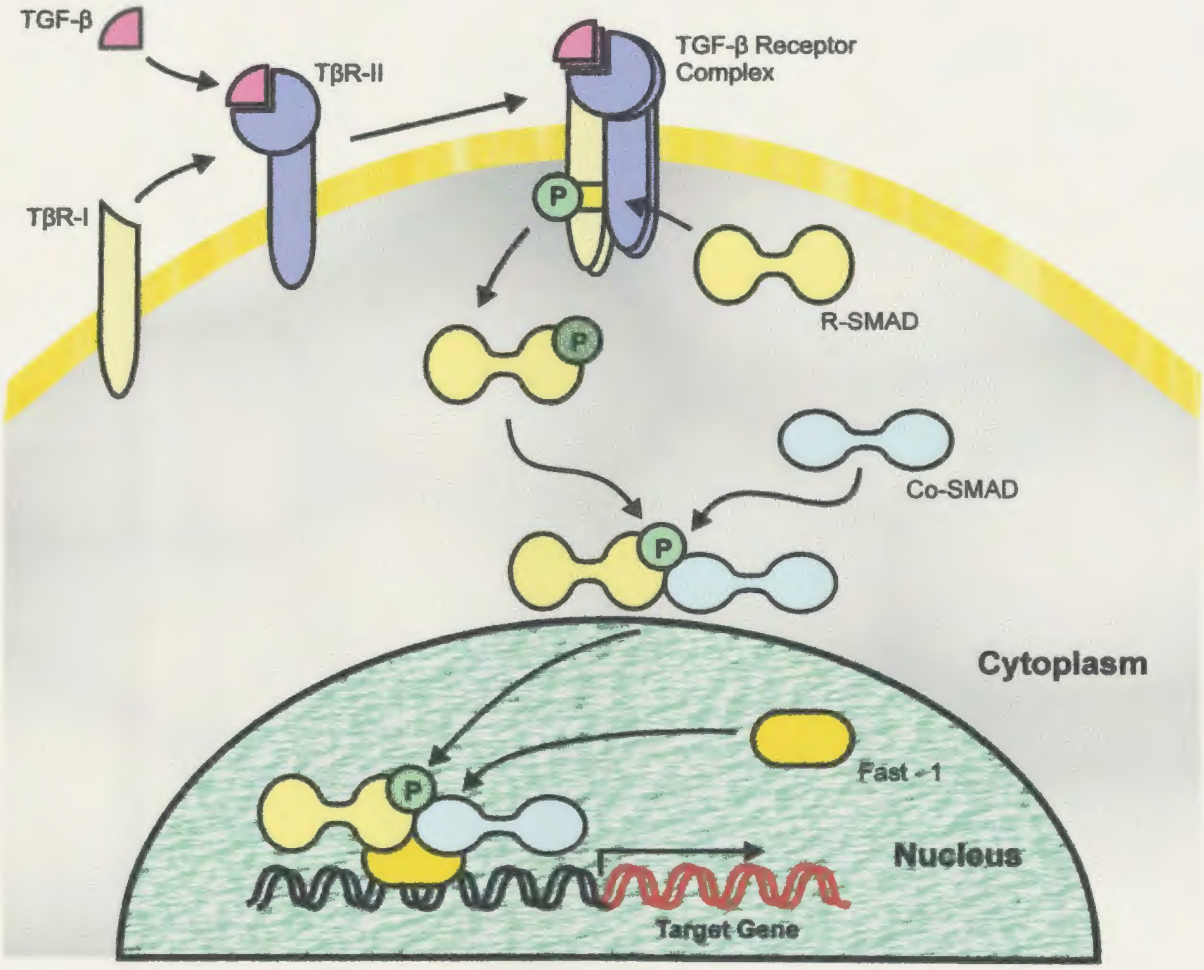

Figure 1.3 Transforming growth factor β signals through the SMAD proteins. Upon ligand binding, TβR-II phosphorylates TβR-I which then activates a R-SMAD by phosphorylation. The activated R-SMAD recruits a co-SMAD and the dimer translocates to the nucleus to regulate transcription of various target genes.

SMAD3. Activated R-SMADs then complex with the co-SMAD, SMAD4. The stoichiometry of this complex has been debated in the literature and has been reported to be heterodimeric (Wu *et al.*, 2001), heterotrimeric (Chacko *et al.*, 2001), and heterohexameric (Shi *et al.*, 1997). It is likely to be dependent upon many factors including cell conditions, specific-ligand binding, or availability of each R-SMAD and co-SMAD.

Activation of SMADs alter their conformation that lead to their accumulation in the nucleus due to exposure of a NLS-like sequences in the N-terminus (Pierreux *et al.*, 2000; Watanabe *et al.*, 2000). Nuclear SMAD complexes can bind directly to specific DNA sequences called SMAD binding elements (5'AGAC-3') or indirectly via associations with other transcriptional factors (Heldin *et al.*, 1997), such as FAST-1 (Figure 1.3).

1.3.5 Regulation of TGF-β/SMAD Signaling

As with the Rel/NF-κB signal transduction pathway, much more data has been accumulating recently regarding the regulation of SMAD-mediated activation and repression. Firstly, it is now known that the presentation of ligand to the receptor can be controlled by TGF-β-binding proteins such as betaglycan (López-Casillas *et al.*, 1994) and endoglin (Barbara *et al.*, 1999; Ma *et al.*, 2000). The data is controversial in that betaglycan has been shown to repress TGF-β1/TβR-II formation but enhances TGF-β2/TβR-II binding. It is assumed that this ability to repress TGF-β1/TβR-II formation is regulated by the glycosaminoglycan modifications (Eickelberg *et al.*, 2002). Secondly, many of the SMADs require C-terminal phosphorylation of the MH2 domain in order to bind SMAD binding

elements (Dennler *et al.*, 1998; Johnson *et al.*, 1998; Shi *et al.*, 1998). Thirdly, R-SMADs are able to recruit co-activators p300 and CBP by their MH2 domains (Feng *et al.*, 1998; Nishihara *et al.*, 1998; Pearson *et al.*, 1999). Fourthly, SMAD nuclear interacting protein 1 (SNIP1) interacts with SMAD4 preventing associations with CBP/p300 and repressing TGF-β signaling (Kim *et al.*, 2000). For a complete summary of all TGF-β/SMAD regulators, co-activators, repressors, and co-repressors, see ten Dijke *et al.* (2002).

Because of the importance of TGF-β and the complexity of regulating TGF-β signaling, it is not surprising to find examples of mutations that lead to a diseased state in its pathway. TGF-β-ligand mutations are responsible for hereditary chondrodysplasia and persistent Müllerian duct syndrome (Attisano and Wrana, 2002). Also, TGF-β is a growth suppressor and therefore mutations have been found in many cancers which become resistant to its growth inhibitory effects (Massagué *et al.*, 2000; de Caestecker *et al.*, 2000; Teicher, 2001). Clearly, there is a need to expand our knowledge for how this pathway is regulated in order to provide a basis for therapeutic advances.

1.4 Experimental Strategies for Regulating Gene Expression in Mammalian Cell Lines

The study of gene expression within a cell can be problematic because the effects are often cell-specific or are dependent on the cell culture conditions. To fully understand the impact of a gene product on a given cell requires comparison of cell behavior after modulating the level of that gene product while maintaining all other variables. The most

common technique for establishing these conditions is to remove or "knock-out" the gene of interest using homologous recombination, site-specific mutagenesis, neutralizing antibodies or more recently, anti-sense, and interference RNA technology. These methods are particularly useful in determining the necessity of the gene product of interest for certain functions such as normal growth patterns or signal transduction. However, it is often desirable to determine the effects of a gene product in a particular cell type. Stable transfections of cells with genes has been one very useful mechanism; however controlling gene expression has been difficult. The classical procedure involves inserting DNA encoding the gene of interest into the cell genome under the control of a constitutive promoter such as the cytomegalovirus promoter (CMV). The researcher has no control over the degree of transcription or the timing of transcription that may contribute to the results of the experiment. Also, the effects of unknown changes occurring during transfecting, selecting and growing clones often generate uncertainties in cell characteristics that cannot be controlled.

1.4.1 Inducible Gene Expression

The induction of gene expression by an effector is not a novel idea. Many systems have been designed and employed that use inducible promoters from heat shock proteins, metal ion activators, and steroid hormone induction (reviewed in Yarranton, 1992; Hirt *et al.*, 1994; Saez *et al.*, 1997; Rossi and Blau, 1998; Walker *et al.*, 1999). One of the more extensively studied systems involved using the *E. coli lac* operon model. The *lac* operon has

a tightly controlled operator (*lac*O)-repressor (*lac*R) complex which prevents transcription of the lactose metabolism genes by blocking the binding of RNA polymerase. In the presence of lactose or its synthetic homologue isopropyl-β-D-thiogalactopyranoside (IPTG), the *lac*R is sequestered from *lac*O by binding to IPTG allowing RNA polymerase binding and induction of the *lac* operon genes. In eukaryotic cells, however, this system has not been as fruitful as anticipated. Several problems arose when attempting to develop stable *lac*-containing cell lines (Gossen *et al.*, 1993). First, induction rarely produced levels greater than 60-fold. The chromosomal position of the *lac*O sequence can affect the properties of the promoter and the induction rates. Second, it was found that elongation factor SII can bypass the *lac*O-R complex and initiate transcription. Third, studies involving kinetics were not always possible in eukaryotic cells due to the slow action of IPTG in removing *lac*R from its operator sequence. For these reasons, the *lac* operon system is not as useful as another inducible gene system, the tetracycline-resistance operon found on the prokaryotic *Tn10* transposon (Foster *et* al., 1981).

1.4.2 Tetracycline-Inducible Gene Systems

Tetracycline-inducible conditional gene expression systems have been developed, based on the *E. coli* tetracycline repressor (TetR) (Gossen and Bujard, 1992; Gossen *et al.*, 1995). The tetracycline uninduced (Tet-Off) and induced (Tet-On) gene expression systems (Clontech Inc.) take advantage of the high specificity and affinity of TetR for its operator sequence (*tet*O) induced by tetracycline and its derivative antibiotic doxycycline. The

binding of TetR to *tet*O was also enhanced by fusing the herpes simplex virus (HSV) transactivation domain VP16 to TetR generating the potent tTA protein (Gossen and Bujard, 1992). The Tet-Off system was initially developed with two components, the regulatory pTet-Off plasmid and the tetracycline response element (TRE) plasmid pTRE. The pTet-Off plasmid encodes the tTA fusion protein that is constitutively expressed upon stable transfection. The tTA protein binds doxycycline that is added to the culture media which prevents tTA from binding *tet*O sequences in the pTRE plasmid and initiating transcription. In order to turn on the desired gene expression inserted in the stably transfected pTRE plasmid, the culture medium containing doxycycline is removed and replaced with culture medium without doxycycline (Gossen and Bujard, 1992). Without the inhibitory doxycycline present, tTA binds the pTRE promoter which initiates the transcription of the desired gene.

The Tet-On system was developed following the discovery of a mutant tTA protein. The pTet-On plasmid encodes a variant of the tTA protein, the reverse tetracycline transaction (rtTA) protein. The rtTA protein also binds doxycycline but when it is bound to doxycycline, rtTA behaves as an activator of transcription. In stably transfected cells grown in the absence of doxycycline, rtTA is unable to bind the pTRE promoter and therefore there is no gene expression from the pTRE plasmid (Figure 1.4). When doxycycline is present, it binds to rtTA and changes its conformation. rtTA then binds *tet*O in pTRE to activate transcription in a dose-dependent manner (Figure 1.4). Therefore, the control of gene expression can regulated by the addition of doxycycline.

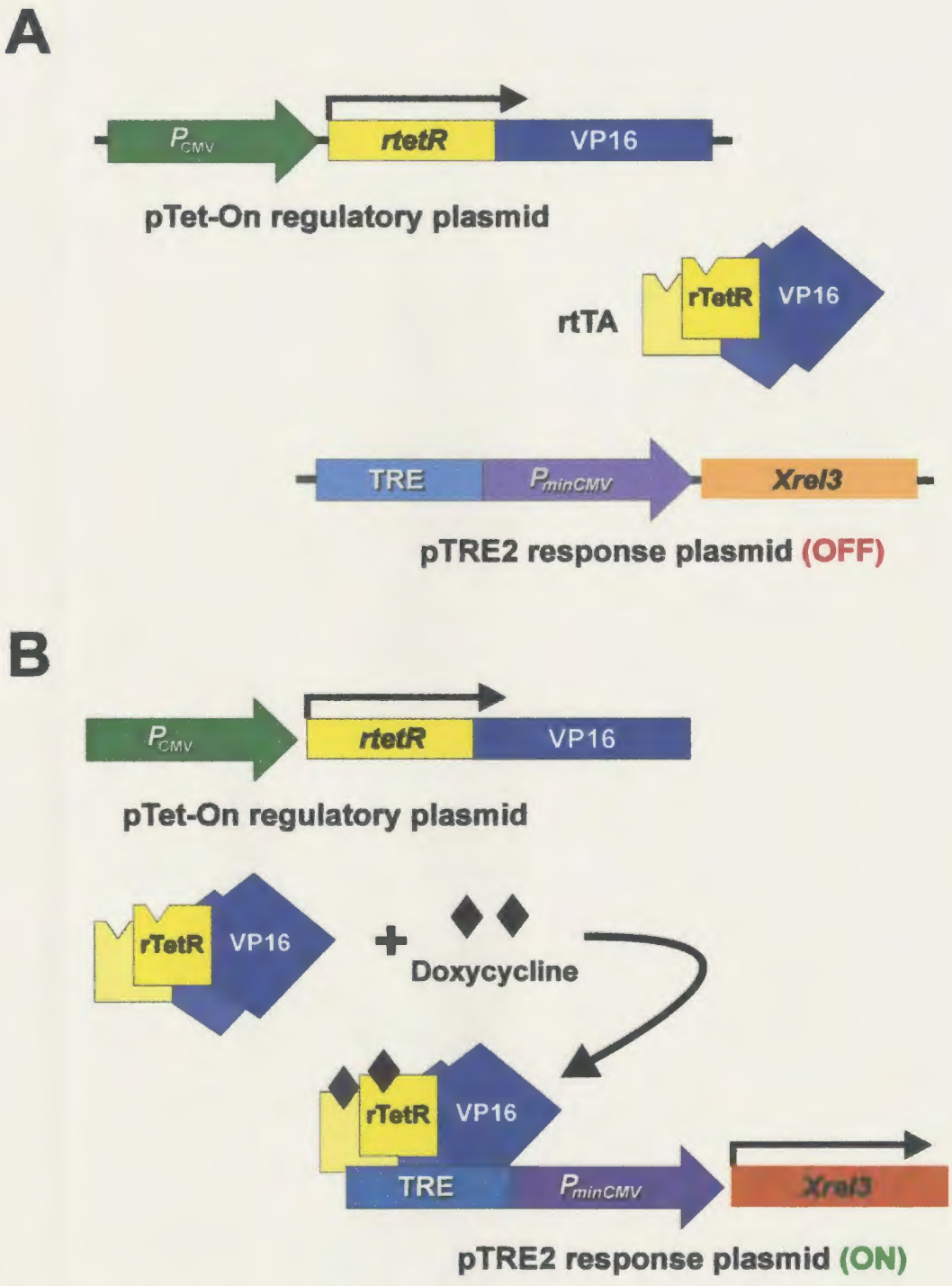

Figure 1.4 Tet-On inducible gene expression system. (A) In the absence of doxycycline, the constitutively expressed rtTA (shown as a fusion of tTetR and VP16) is unable to bind the tetracycline responsive element (TRE) found in the promoter of pTRE2, therefore no *Xrel3* is transcribed. **(B)** When bound to doxycycline, rtTA is able to bind the tetracycline responsive element (TRE) found in the promoter of pTRE2, and initiate *Xrel3* transcription. (Adapted from Tet-On user manual).

The second regulatory element of the Tet-On system is the tetracycline response element (TRE) which is contained in the response plasmid pTRE2. The TRE consists of seven direct repeats of the 42 base pair (bp) *tet*O sequence immediately upstream of a minimal CMV promoter. The minimal CMV promoter does not contain any of the enhancer elements of the typical CMV promoter. Therefore, its activity is completely dependent on the TRE. Therefore, in the presence of doxycycline, rtTA will bind the TRE to allow the assembly of transcriptional machinery. Experimentally, the Tet-On system therefore requires a double-stable transfected cell line containing both pTet-On and a pTRE2 construct in which transcription can be regulated by doxycycline.

1.5 Thesis Rationale

Rel/NF-κB proteins can act as cellular pro-survival factors (Zong *et al.*, 1999; De Smaele et al., 2001), while TGF-β is a known suppressor of epithelial cell growth (Moustakas *et al.*, 2001). It is therefore suggested that these two factors may play antagonistic roles in regulating cell proliferation. For example, TGF-β1 induces apoptosis in B cells by inhibiting NF-κB activity (Arsura *et al.*, 1996) through the induction of IκB-α expression (Azuma *et al.*, 1999). Also, the up-regulation of TGF-β in antigen-presenting cells overexpressing thrombospondin causes down-regulation of NF-κB (Masli *et al.*, 2002). Conversely, constitutively active NF-κB resulting from Ras- and Raf-transformation has been associated with TGF-β1 resistance (Arsura *et al.*, 2000). In breast cancer cells, constitutively activated Rel/NF-κB modifies TGF-β sensitivity (Sovak *et al.*, 1999). Further,

activation of NF-κB by the latent membrane protein 1 of the Epstein-Barr virus leads to an inhibition of TGF-β signaling (Prokova *et al.*, 2002).

Taken together, the accumulated data suggest that there may be a direct, mutually antagonistic relationship between the Rel/NF-κB and TGF-β signaling pathways that is commonly disrupted during tumorigenesis. For instance, many epithelial tumors are resistant to the growth suppressing effects of TGF-β (ten Dijke *et al.*, 2002). Also, NF-κB has been identified in many tumors to be constitutively active, and promote cell survival (Bours *et al.*, 1994; Luque and Gelinas, 1997). Although a reciprocal relationship has been suggested between the expression of NF-κB and SMAD proteins by others (Bitzer *et al.*, 2000; Moustakas *et al.*, 2001), it remains unclear whether there is a direct correlation between activation of NF-κB and TGF-β resistance (Gurumurthy *et al.*, 2001).

The roles of both Rel/NF-κB and TGF-β superfamily members have been studied in *Xenopus laevis* embryos. Overexpression of RelA in embryonic cells prevented mesoderm induction by activin (Kao and Lockwood, 1996). Recently, another *Xenopus* Rel member, Xrel3, was shown to prevent mesoderm induction by suppressing TGF-β-responsive embryonic patterning genes (Ford, Skhirtladze, and Kao; manuscript submitted).

My hypothesis based on the results obtained from developing *Xenopus* embryos is that Xrel3 blocks the potential growth suppression effects of TGF-β, allowing the cells to grow normally by interfering with the induction of TGF-β-responsive genes. Therefore this thesis was designed to establish a tetracycline-inducible, Xrel3-expressing human CaSki cervical carcinoma cell line for examining the effects of constitutive Rel/NF-κB on TGF-β

signaling and growth suppression. Following the successful establishment of the CaSki-Tet-On cell line, I determined the effects of both Rel/NF-κB induction and the addition of TGF-β on growth rates and morphology; the expression of appropriate markers using Western blot analysis; and used an apoptosis assay to correlate any changes in cell growth with potential changes in apoptosis.

CHAPTER 2
MATERIALS AND METHODS

2.1 Cell Culture

Human CaSki cervical carcinoma cells (ATCC) were grown in Dulbecco's modified Eagles medium (DMEM) (Invitrogen) supplemented with 10% fetal calf serum (FCS), 100 units/ml penicillin G, and 100 µg/ml streptomycin. Cells were maintained at 37°C with 5% CO_2 until reaching 70-90% confluence, washed with phosphate-buffered saline (PBS), and then were passaged using trypsin-ethylenediamine-tetraacetic acid (EDTA) (Invitrogen). Cells were counted using a hemocytometer (Fisher).

2.2 Establishing Tet-Inducible *Xrel3*-Expressing CaSki Cells

The TET-ON gene expression system (Clontech) involves the regulatory plasmid pTET-ON and the response plasmid pTRE2. To establish Tet-inducible *Xrel3* cell lines, cells are first transfected with the pTET-ON vector, colonies are selected for neomycin resistance, screened for optimal transcriptional activation using a pTRE2-luciferase construct, and then stably transfected with pTRE2-Xrel3. Hygromycin-resistant clones must then be screened for Tet-dependent induction of *Xrel3* to isolate the appropriate cell line.

2.2.1 Establishing Stable CaSki-TET Clones

Approximately 1×10^5 CaSki cells were seeded in 6 well plates and upon reaching 70% confluency the cells were transfected with 1 µg pTET-ON using Lipofectamine reagent

(Invitrogen). Plasmid DNA was diluted in 100 µl serum-free DMEM and combined with 8 µl Lipofectamine reagent diluted in 100 µl serum-free DMEM for 45 min at room temperature to form DNA-liposome complexes. Following this incubation, 800 µl serum-free DMEM were added to the complex mixture, which was then added to PBS-rinsed cells. Cells were incubated overnight with the complexes after which 1 ml of DMEM + 20% FCS was added to each well followed by further incubation overnight. Then, the medium was aspirated, cells were washed with PBS, and fresh DMEM medium + 10% FCS was added. Cells were allowed to grow for a further 24 hours and then were passaged 1:10 into selective DMEM containing 400 µg/ml G418 (Invitrogen) to isolate neomycin-resistant clones. The resulting clones were then subcultured in DMEM maintenance medium containing 200 µg/ml G418 and screened for their induction of gene expression by doxycycline and a luciferase reporter construct, pTRE2-luc.

2.2.2 Selecting Optimal CaSki-Tet Clone using Luciferase Assay

To select the appropriate clone, it should have low background gene expression in the absence of doxycycline and high gene induction when doxycycline is present. Therefore, for obtaining the CaSki-Tet clone that highly expresses the desired gene product, each cell line was transiently transfected with pTRE2-luc and luciferase activity was measured with and without the addition of doxycycline. Cell extracts were prepared for the luciferase assay using reporter lysis buffer (Promega) as per instructions and relative luciferase activity was measured. Briefly, 400 µl Reporter Lysis Buffer and were added to the pTRE2-luc

transfected cells. Cells were scraped from the dish and transferred to 1.5 ml tubes. Cell lysates were quick-frozen in liquid nitrogen, thawed in a 37°C water bath, chilled on ice for 10 min and centrifuged for 10 min at 4°C. The supernatant was stored at -70°C until luciferase activity was measured.

Relative luciferase activity was determined by mixing 20 µl cell extract with 100 µl luciferase assay reagent (Promega) and measuring luminescence using a Monolight 2010 luminometer (Analytical Luminescence Laboratory) for 10 sec following a 2 sec measurement delay.

2.2.3 Tet-On Plasmid Construction

pCS2-Xrel3 (Yang *et al.*, 1998) was digested with *Eco*RI and *Xho*I to release the full-length Xrel3 cDNA, which was purified by 1% agarose gel electrophoresis and extracted using the QIAquick Gel Extraction kit (Qiagen). This cDNA fragment was ligated into the *Eco*RI and *Xho*I restriction sites of the tetracycline responsive plasmid, pTRE2, at room temperature for two hours with 1 U T4 DNA ligase (Invitrogen) [50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 1 mM adenosine triphosphate (ATP), 1 mM dithiothreitol (DTT), 5% polyethylene glycol, 3:1 insert:vector DNA] resulting in the plasmid pTRE-Xrel3. Also cloned was an *Xrel3* cDNA containing a mutated nuclear localization signal (mutNLS) to give pTRE-mutNLS. Ligation products were incubated on ice with MAX Efficiency *DH5α* competent cells in Terrific Broth (TB) (12 g/l tryptone, 24 g/l yeast extract, 0.4% glycerol, 17 mM KH_2PO_4, 72 mM K_2HPO_4) for 30 min, heat-shocked at 42°C for 60 sec and chilled

immediately on ice for 2 min. Transformed cells were allowed to recover by adding 800 µl TB broth to each sample and incubating at 37°C with shaking for 1 hour. Cells were applied to LB (Luria-Bertani) plates containing 100 µg/ml ampicillin (Invitrogen) and incubated overnight at 37°C. Resulting colonies were selected and miniprep cultures in 2 ml TB broth containing 100 µg/ml ampicillin were prepared to identify desired plasmids by restriction digest analysis. Overnight cultures were prepared (200 ml TB broth containing 100 µg/ml ampicillin) for each positive colony and plasmids were extracted using the Maxi-Prep kit (Qiagen) as instructed and DNA concentrations were determined using a spectrophotometer. Correct insertions were further confirmed at the 5′ and 3′ ends by direct DNA sequencing (DNA Sequencing Facility, Hospital for Sick Children, Toronto) for the plasmid constructs.

2.2.4 Stable Transfection with pTRE2-constructs

The tetracycline-responsive pTRE2-constructs were transfected into CaSki-Tet cells to produce stable *Xrel3* and rtTA expressing cell lines. Briefly, approximately 2×10^5 CaSki-Tet cells were seeded in 60 mm culture dishes (Corning) 24 hours prior to transfection in 1.6 ml DMEM. Effectene transfection reagent was used to co-transfect pTRE-*Xrel3* constructs and pTK-Hyg, a hygromycin selection vector. Briefly, both plasmids were diluted in buffer EC to a total volume of 150 µl at 0.5 µg/µl concentration of both plasmids combined. Enhancer was added (8 µl) and mixtures were incubated at room temperature for 5 min before adding 25 µl Effectene reagent to form transfection complexes. Cells were washed with PBS and 4 ml of complete DMEM were added. The complex mixtures were

diluted in 1 ml DMEM and added to the culture dishes. After 24 hours incubation, cells were passed 1:5 into 100 mm dishes and selected for hygromycin-resistance with 200 μg/ml hygromycin B (Clontech). Resulting clones were isolated using plastic cloning cylinders (Fisher) and seeded in 12-well culture dishes before passing to 100 mm dishes. Double-transfectants were maintained in DMEM with 100 μg/ml hygromycin B to ensure that the cells retained the transfected plasmid.

2.2.5 Genomic DNA Extraction

To confirm the insertion of the appropriate pTRE2 construct, genomic DNA from CaSki, CaSki-Tet, and all double-transfected clones was isolated using the DNeasy Tissue Kit (Qiagen). In brief, approximately 5×10^6 cells were centrifuged for 5 min at $400 \times g$ and resuspended in 200 μl PBS. To obtain RNA-free DNA, 4 μl of RNase A (100 mg/ml) was added and the samples were incubated at room temperature for 2 min. To each sample, 20 μl Proteinase K and 200 μl lysis buffer were added and the tubes were mixed and incubated at 70°C for 10 min. After lysis, 200 μl of 100% ethanol were added and the samples were added to DNeasy spin columns and centrifuged at $6000 \times g$ for 1 min to bind the DNA. The spin columns were washed with 500 μl of buffer AW1, centrifuged, washed with 500 μl of buffer AW2, centrifuged, and then dried by further centrifugation at full speed for 3 min. DNA was eluted in 200 μl elution buffer and centrifugation at $6000 \times g$ for 1 min.

Isolated genomic DNA was precipitated by adding 1/10 volume of 3 M sodium acetate and 2.5 volumes of 100% ethanol. Tubes were placed in -70°C overnight. DNA was

pelleted at 12,000 × *g* for 25 min at room temperature and washed with 500 µl of 70% ethanol and centrifuged at 12,000 × *g* for 10 min. Supernatants were decanted and residual ethanol was removed by pipette. DNA pellets were air-dried for 5 min and resuspended in 25 µl TE buffer (10 mM Tris, 1 mM EDTA) overnight at room temperature. DNA concentrations were determined from A_{260}.

2.2.6 Southern Blot Probe Preparation

All stable Tet-On-Xrel3 cell lines were screened for the presence of pTRE2 constructs using [^{32}P]-dCTP-labeled Xrel3. Full-length Xrel3 cDNA probes were labeled using Rediprime II random primer labeling kit (Amersham). Approximately 50 ng of probe was diluted in 445 µl TE buffer, boiled for 5 min to denature, and chilled for 5 min on ice. Denatured probe was added to the Rediprime reaction mixture, and incubated at 37°C for 15 min with 5 µl of [^{32}P]-dCTP. The labeling reaction was stopped with 5 µl of 0.2 M EDTA.

Unincorporated radiolabeled nucleotides were removed using Nuc-Trap probe purification columns (Stratagene). Columns were equilibrated with 70 µl 1 × STE buffer [100 mM NaCl, 20 mM Tris-HCl (pH 7.5), 10 mM EDTA] and then labeled probe was added to the top of the column and forced through the resin. To elute the probe, 70 µl 1 × STE was added and pushed through the column into a collection tube. The incorporation of [^{32}P]-dCTP was determined from 1 µl purified probe in 10 ml scintillation fluid.

2.2.7 Southern Blot Analysis

For Southern blot analysis, 8 μg genomic DNA was digested with *Eco*RI and *XhoI* overnight at 37°C and resolved in a 0.8% agarose gel along with unlabeled Xrel3 cDNA as a control. Electrophoresis was approximately 20 hours at 30 V. DNA in the gel was depurinated for more efficient transfer of large DNA fragments in depurination solution (18 ml 12 M HCl in 1 l water) for 8-10 min with shaking. The gel was rinsed in deionized H_2O and soaked in denaturation buffer (0.5 M NaOH, 1.5 M NaCl) for 30 min with gentle agitation. After H_2O rinsing, the gel was soaked in neutralization buffer (0.5 M Tris, 1.5 M NaCl) for 30 min and rinsed again.

The Southern transfer apparatus was assembled according to standard methods. A piece of plexi-glass served as a platform that was laid across a Pyrex dish containing 20 × SSC (sodium saline citrate). The platform was covered with 3 sheets of Whatman paper soaked in 20 × SSC to serve as a wick. The gel was then placed on the wick (well-side down) and surrounded with Parafilm. Hybond-XL nylon membrane (Amersham) was cut to size and placed carefully onto the gel, ensuring air bubbles were not trapped underneath. Three sheets of Whatman paper soaked in 20 × SSC were placed on the membrane. A 5 cm stack of paper towels was placed on the top to create the capillary gradient. A glass plate and weight were added to the top and transfer proceeded overnight. The next day, the transfer apparatus was inverted and disassembled. The membrane was baked at 80°C for 2 hours under vacuum to bind the DNA to the membrane.

Hybridization of labeled probes was performed using the Rapid-Hyb buffer

(Amersham). Membranes were completely immersed in 65°C hybridization buffer and then incubated for 30 min with shaking in heat-sealed plastic bags for pre-hybridization. Xrel3 probes were denatured by boiling for 5 min and then chilled on ice. Approximately 200 000 cpm of labeled probe was added to the bags and hybridization was performed for 1 hour at 65°C. Following hybridization, membranes were washed as follows: 20 min in 2 × SSC, 0.1% SDS (sodium dodecyl sulphate) at room temperature; 15 min in 1 × SSC, 0.1% SDS at 65°C; and 10 min in 0.1 × SSC, 0.1% SDS at 65°C, until signal was localized using the Geiger counter. The washed membranes were wrapped in plastic wrap and exposed to film for autoradiography.

2.2.8 Gene Expression Assay

2.2.8.1 RNA Extraction

Total cellular RNA was isolated from non-induced and doxycycline-induced CaSki cell lines using a RNeasy kit (Qiagen). Approximately 1×10^6 cells were seeded in 60 mm culture dishes 24 hours before doxycycline and/or TGF-β1 treatments. After 48 hours, cells were trypsinized and pelleted at 400 × *g* for 5 min before lysis with 500 µl of supplied lysis buffer. Samples were homogenized by passing them through a 20-Gauge needle and 500 µl of 70% ethanol was added to each tube. All samples were centrifuged through the RNeasy spin columns to bind the RNA. After washing with the supplied wash buffers, total RNA was eluted from the column in 40 µl RNase-free water by centrifugation at 8000 × *g* for 1 min. RNA concentrations were determined from the A_{260} of the samples.

2.2.8.2 Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Clones were screened for the induction of Xrel3 RNA following treatment with doxycycline using RT-PCR analysis (Ding *et al.*, 2002). First strand complementary DNA (cDNA) was synthesized from 1.0 μg/ml total cellular RNA with 1.4 μl of 0.2 μg/ml random primers (Invitrogen) in RNase-free water, heated to 70°C for 10 min, and placed on ice. Then 5× first strand buffer, 10 mM deoxynucleotide (dNTP) mix, 0.1 M DTT, and 1 Unit of Superscript II reverse transcriptase (Invitrogen) were added to begin cDNA synthesis. Samples were heated to 42°C for 50 min, 70°C for 15 min, and held at 4°C. A 320 bp fragment of Xrel3 template was then amplified from the cDNA using the following primer pair, Xrel3-Forward (5'-CCG CGG CCC CGA ATT CGA GC-3') and Xrel3-Reverse (5'-AAC TGT GCGATC TGA ACC AA-3'). The β-actin primer pair, Actin-Forward (5' ATC TGG CAC CAC ACC TTC TAC AAT GAG CTG CG-3') and Actin-Reverse (5'-ATG GCT GGG GTG TTG AAG GTC TC-3') was used to amplify a 142 bp product. Samples were amplified in 31 cycles of 94°C for 40 sec, 55°C for 60 sec, and 72°C for 60 sec, followed by 72°C for 7 min to ensure complete extension. Products were separated on 1% agarose gels, stained with ethidium bromide, and photographed with the Eagle Eye II still video system (Stratagene).

2.2.8.3 Protein Extraction

Antibodies directed against PAI-1 (Santa Cruz Biotechnology), VP16 (Clontech), SMAD2 (Transduction Laboratories) and phospho-SMAD2 (Upstate Biotechnologies),

SMAD7 (Santa Cruz Biotechnology), β-actin (Sigma) , and p15 and p21 (Oncogene Research Products) were used for Western blot analysis to determine relative protein expressions. Cell proteins were extracted from 100 mm culture dishes as previously described (Yang *et al.*, 1997). Cells were washed with ice-cold PBS and 1 ml of ice-cold lysis buffer [50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.02% sodium azide, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate, 10 µg of 10 mg/ml phenylmethylsulfonyl fluoride (PMSF), and 5 µl of 2 mg/ml aprotinin] was added to the cells which were then scraped into a 1.5 ml tube. Cell lysates were chilled on ice for 30 minutes and centrifuged at 12,000 × *g* for 10 minutes at 4°C. The supernatants were stored at -70°C. Protein concentrations were determined using the BioRad DC Lowry protein assay according to the manufacturer.

2.2.8.4 Western Blot Analysis

Protein extracts were mixed with equal amounts of 2 × SDS gel loading buffer (200 mM Tris, pH 6.8, 4% SDS, 0.2% bromophenol blue, 20% glycerol) and boiled for 5 min. Denatured proteins were loaded onto the stacking gel [5% acrylamide, 125 mM Tris-HCl (pH 6.8), 0.1% SDS, 0.1% ammonium persulphate, 5 µl tetramethylethylenediamine (TEMED)] and separated in the resolving gel [10% acrylamide: 2.66% bis-acrylamide, 375 mM Tris-HCl, pH 8.8, 0.1% SDS, 0.1% ammonium persulphate, 8 µl TEMED] for SDS-polyacrylamide gel electrophoresis (PAGE) at 200 V in SDS-PAGE running buffer (25 mM Tris, 250 mM glycine) using a Protean II minigel apparatus (BioRad).

2.3 Cell Proliferation Assay

The CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega) was used to determine the effects of Xrel3 expression on cell growth rates. This is a colorimetric method containing a MTS tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium], which is reduced by cells in the presence of an electron coupling reagent PES (phenazine ethosulfate) into a colored formazan product that remains soluble in DMEM. The reduction reaction involves dehydrogenase enzymes found in metabolically active cells and uses NADPH or NADH as the electron acceptor. Therefore, the amount of formazan produced, measured by the A_{490nm} is directly proportional to the number of living cells in culture.

CaSki, CaSki-Tet, CT-Xrel3, and CT-NLS cell growth rates were determined in four conditions: untreated; doxycyline-induced; TGF-β1-induced; and induction by both doxycycline and TGF-β1. Approximately 5000 cells were seeded per well at 6 wells for each condition in 96-well plates. Doxycycline was added (1 μg/ml) at the time of seeding and cells were incubated at 37°C overnight. TGF-β1 was added (2 ng/ml) to the appropriate wells and cells were incubated as indicated above. Medium was aspirated and replaced every 24 hours.

To determine the number of cells present in each condition, 20 μl of MTS solution was added to each well, the 96-well plate was returned to 37°C incubator for 2 hr and the absorbance at 490 nm of each well was recorded using a 96-well plate reader (Molecular Devices Inc.).

2.4 Hematoxylin and Eosin Staining

The effects of Xrel3 expression and TGF-β1 treatment on cell morphology was determined using hematoxylin and eosin staining. Approximately 2×10^5 cells were seeded in 2-well cell culture chamber slides (Nunc) in DMEM or DMEM containing 1 μg/ml doxycycline. After 24 hours, medium was removed and replaced with DMEM, DMEM + 1 μg/ml doxycycline, DMEM + 2 ng/ml TGF-β1, or DMEM + doxycycline and TGF-β1. After 48 hrs incubation, cells were washed with PBS and fixed with -20°C methanol for 5 min. Methanol was aspirated and cells were left to air dry. The chambers were removed and the slides were immersed in PBS to wash and then stained in hematoxylin (Sigma) for 15 seconds. Slides were immediately rinsed in running tap water for 1 min and then stained in eosin (Sigma) for 30 secs. Slides were processed in a dehydration series as follows: 1 min in 70% ethanol; 1 min in 95% ethanol; 1 min in 100% ethanol; and 3 minutes in xylene. Slides were mounted with Permount (Sigma) and coverslips were applied.

2.5 Apoptosis Assay

To determine whether the growth suppression due to TGF-β treatments was due to apoptosis, the cells were stained with Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) following the manufacturer's assay protocol (Pharmingen). Cells undergoing apoptosis are positive for Annexin V-FITC and negative for PI while cells at the end stage of apoptosis or already dead was stained positive for both Annexin V-FITC and PI. The percentage of stained cells were determined using flow cytometry and a fluorescence

activated cell sorter (FACS) (Faculty of Medicine, Memorial University).

Following 48 hrs of TGF-β treatment, each cell sample was collected by trypsin digestion along with all culture medium and PBS washes in a 50 ml tube. All attached and detached cells were pelleted by centrifugation at 400 × g for 5 min. Cells were resuspended in 1× binding buffer (10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) at a concentration of 1×10^6 cells/ml. For FACS analysis, 100 µl of cell suspension was mixed with 5 µl Annexin V-FITC, 10 µl PI and incubated for 15 min at room temperature in the dark. To each sample, 400 µl of 1× binding buffer was added and samples were analyzed by the FACS Analysis Facility, Faculty of Medicine, Memorial University of Newfoundland.

CHAPTER 3
RESULTS

The objectives of these experiments were to: (1) construct the Tet-On Xrel3 induction system in CaSki cell line clones, (2) examine the effects of Xrel3 on cell morphology, (3) further determine whether Xrel3 can modify TGF-β-induced growth inhibition, and finally, (4) understand the molecular mechanisms involved.

3.1 Xrel3 is a nuclear and constitutively active protein in CaSki cells

To confirm potential interactions between Rel/NF-κB proteins and TGF-β signaling molecules, I chose to study a constitutively active Rel/NF-κB member, Xrel3. It had been shown in previous studies in *Xenopus laevis* that Xrel3 is found within the nucleus and is widely expressed (Yang *et al.*, 1998). To verify the behavior of Xrel3 protein in the human CaSki cervical cancer cell line, green fluorescent protein (GFP)-tagged Xrel3 was transiently transfected into these cells. A cell permeable fluorescent DNA stain, 4',6'-diamidino-2-phenylindole (DAPI) was used to confirm the localization of transfected proteins because it binds to DNA minor grooves within the nucleus. Empty GFP vectors produced only the green fluorescent protein that was diffusely expressed in both the cytoplasm and nuclei of transfected CaSki cells (Figure 3.1, A and B). GFP-Xrel3 on the other hand, was found in high abundance and almost exclusively in the nucleus (Figure 3.1, C and D). The NLS mutated form of Xrel3, altered by site-specific mutagenesis (mutNLS; kindly provided by Blue Lake) was also transfected into CaSki cells and used as a control. This mutNLS Xrel3

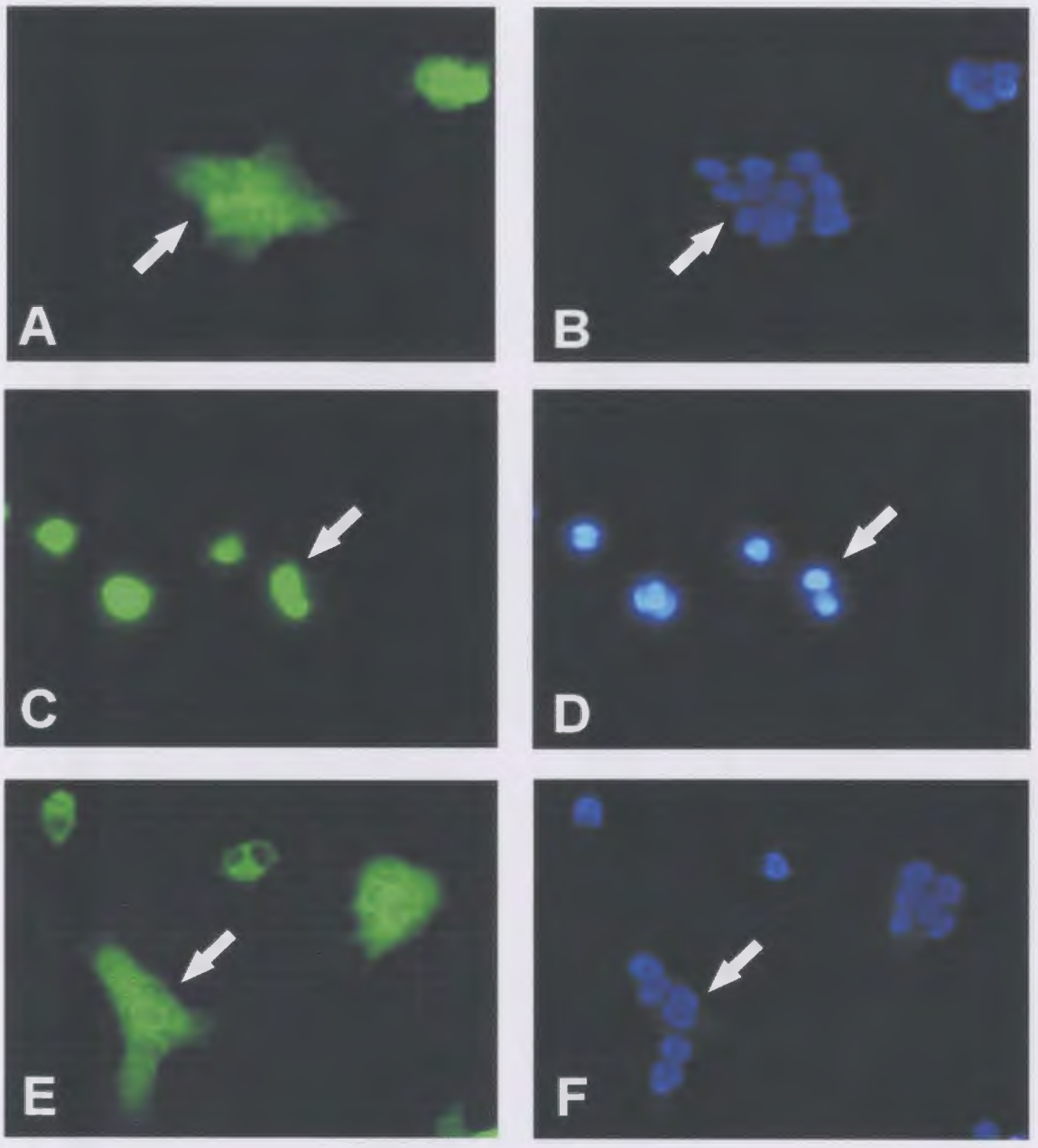

Figure 3.1 CaSki cellular localization of Xrel3 and its nuclear localization mutant, mutNLS. (A) Transiently expressed GFP is diffuse and present throughout the cell nuclei and cytoplasm when viewed under fluorescence. **(C)** GFP-tagged Xrel3 is targeted to the nucleus of CaSki cells and is not detected in the cytoplasm. **(E)** GFP-tagged mutNLS remains cytoplasmic with little to no expression within the nuclei. Panels**B**, **D**, and **F** show the position of the corresponding cell nuclei using the DAPI stain for DNA.

protein (mutNLS) was only found in the cytoplasm of CaSki cells when transiently expressed (Figure 3.1, E and F). This result suggests that the NLS motif is required for its nuclear localization and that mutNLS Xrel3 can be used as a control for further experiments.

3.2 Generation of Tet-inducible, Xrel3-expressing CaSki cells

Initial attempts at creating stably-transfected, Xrel3-expressing CaSki cells using the pcDNA3.1-Xrel3 vector failed to produce viable, neomycin-resistant clones. It was hypothesized that the presence of Xrel3 at a constitutively high level was toxic or prevented cell growth and that, if an inducible gene expression system was used, viable clones producing low Xrel3 levels could be established in which the expression of Xrel3 could then be induced at the appropriate time during an experiment. This conditional expression strategy is routinely used to examine the effects of lethal proteins or conditional oncogenes (Xie *et al.*, 1999; Aurisicchoio *et al.*, 2001; Berkovich and Efrat, 2001). In addition, this approach reduces uncertainties of unknown cell changes occurring during clonal selection. For these reasons, the Tet-On system was chosen to stably transfect Xrel3 into CaSki cells in an inactive state and then to verify expression of Xrel3 experimentally following the addition of doxycycline (a tetracycline derivative) to the growth medium.

3.2.1 Construction of stable pTet-On-transfected CaSki cervical cells

The control plasmid pTet-On contains the reverse tetracycline transactivation protein rtTA fused to the C-terminus of the herpes simplex virus VP16 activation domain (Gossen

and Bujard, 1992; Gossen *et al.*, 1995). The rtTA fusion protein binds doxycycline and is necessary for subsequent initiation of transcription at the tetracycline response element within the Xrel3-containing plasmid. The transcription of rtTA is driven by the constitutive CMV promoter to give high levels of rtTA within the cells. After transfection, the resulting cell clones that exhibited neomycin-resistance were selected and subcultured in maintenance medium for determination of transcriptional activity.

To test the integrity of the Tet-On system it was necessary to identify the clone that would have the highest transfection efficiency and have the highest ratio of induced to background expression. Therefore each clone was transiently transfected with the pTRE2-luc construct and cultured in the presence or absence of doxycycline. The clone which has the highest induction of luciferase activity, must also have a high transfection efficiency that is necessary to generate the final Xrel3-expressing clones. As shown in Figure 3.2, ten clones tested had relatively low induction of luciferase activity, in the range of 1 to 4 times background. In response to the addition of doxycycline, clone 7 had almost 12 times greater luciferase activity than in the untreated medium. Therefore clone 7 has a high transfection efficiency and has the highest rate of doxycycline-induced luciferase activity. This clone was used for all further studies and subsequently identified as CaSki-Tet.

CaSki-Tet was further analyzed by Western blot to confirm the presence of the reverse tetracycline transactivation domain rtTA using an anti-VP16 primary antibody. According to the manufacturer, the levels of VP16 found in stably transfected cell lines may be below the sensitivity of Western analysis, but as shown in Figure 3.3, the levels of rtTA

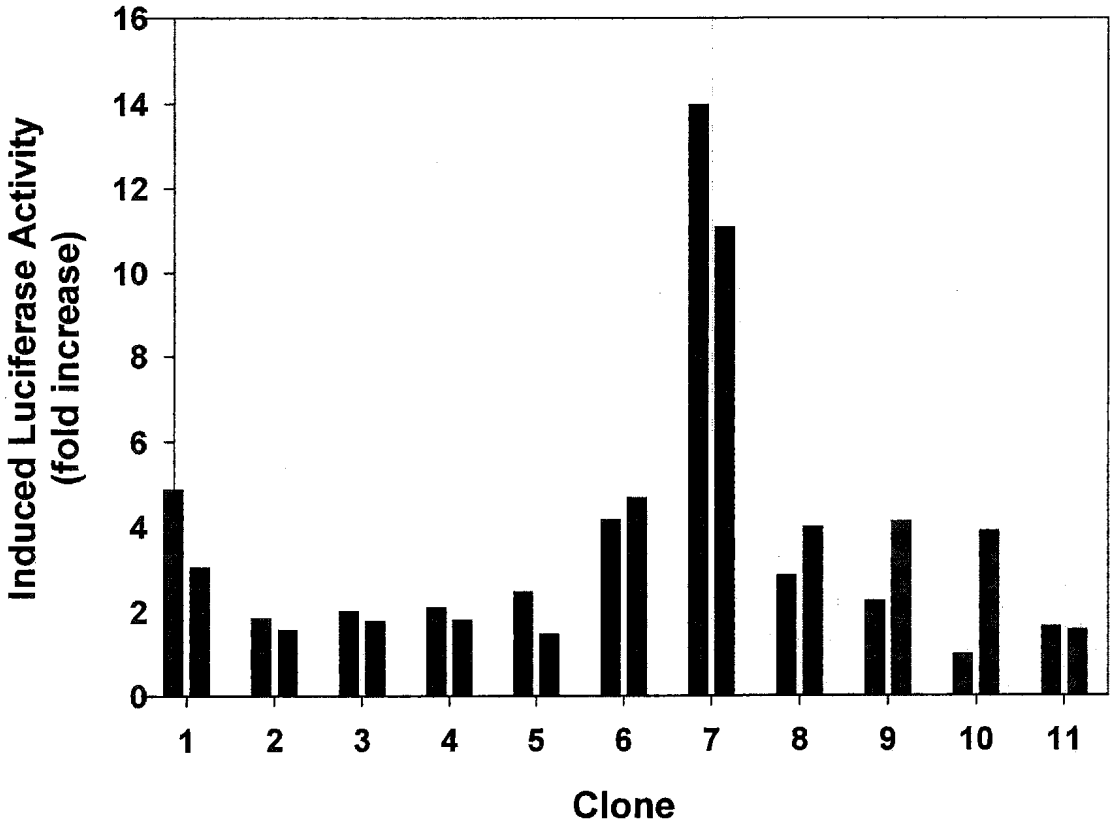

Figure 3.2 Selection of CaSki-Tet clones for doxycycline-responsiveness using a doxycycline-inducible luciferase assay. Untransfected CaSki and CaSki-Tet clones were transiently transfected with pTRE2-luc and cultured in the absence or presence of 1 μg/ml doxycycline for 48 hours. Luciferase activity was measured as relative light units (RLU). Only those clones with luciferase activity equivalent to or lower than untransfected CaSki background levels before doxycycline treatment are shown. (Two separate experiments).

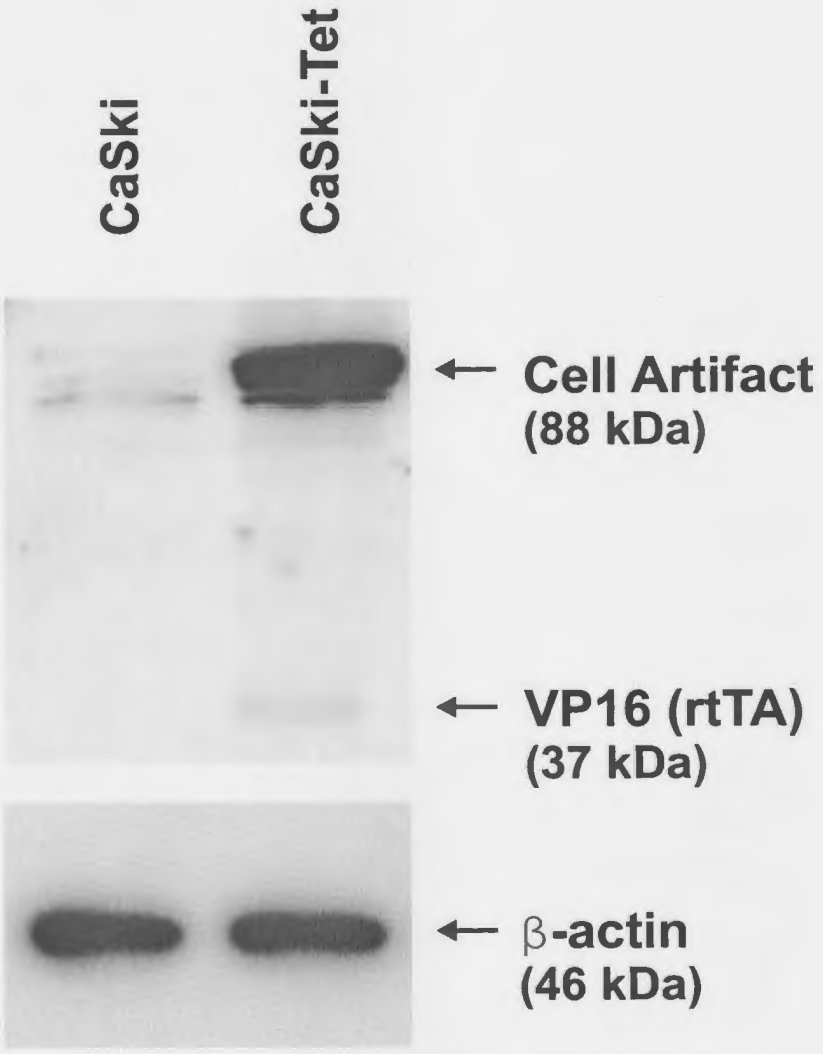

Figure 3.3 Conformation of stable insertion of pTet-On in CaSki cells. Western blot analysis showing the presence of rtTA fusion protein in the selected CaSki-Tet clone. The detection of VP16 indicates successful insertion of the pTet-On vector in the CaSki and the successful transcription/translation of the 37 kDa *rtTA* fusion protein. According to the anti-VP16 antibody manufacturer, cell artifacts (88 kDa) are detected by the anti-VP16 antibody and are not a result of the transfection. To confirm this, the same membrane was stripped and probed with anti-β-actin as a loading control whose expression was comparable in both CaSki and CaSki-Tet cells.

are detectable in CaSki-Tet cells but not in untransfected CaSki cells. The larger unidentified protein seen in both CaSki and CaSki-Tet extracts is found regardless of transfection. The manufacturer of the pTet-On system offers no explanation for the artifact therefore it is labeled "Cellular artifact". The observable difference in expression levels of this artifact between CaSki and CaSki-Tet cannot be accounted for at this time but was repeatable in three separate experiments. When the same membrane was probed with anti-β-actin, comparable levels were found in both the CaSki and CaSki-Tet cells suggesting the amount of total protein loaded on the gel were the same (Figure 3.3).

3.2.2 Construction of the tetracycline-responsive pTRE2-Xrel3 and pTRE2-mutNLS vectors

Wild-type Xrel3 and mutNLS cDNAs were inserted into the *EcoR*I and *Xho*I sites of the doxycycline-response plasmid pTRE2, downstream of the tetracycline operator sequences (Figure 3.4). The insertion of these cDNAs was confirmed initially by restriction digest analysis and ultimately by direct sequencing from the 5′ and 3′ ligation sites (Figure 3.4).

3.2.3 Stable transfection of CaSki-Tet cells with pTRE2-Xrel3 and pTRE2-mutNLS

CaSki-Tet cells were co-transfected with the pTRE2-Xrel3 and pTRE2-mutNLS plasmids and pHyg (for antibiotic selection) at a ratio of 20:1 to favor selecting clones that incorporate co-transfected pTRE2-Xrel3 plasmids. Following transfection and selection,

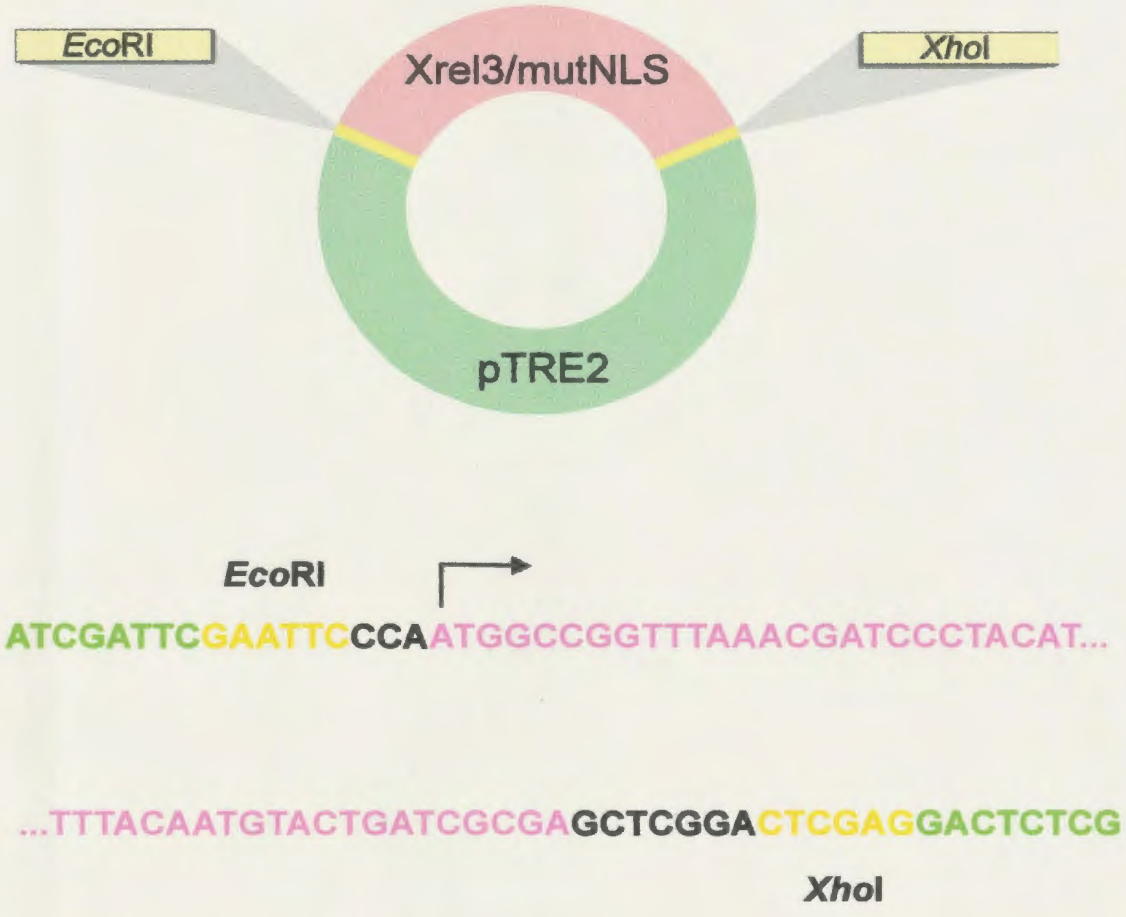

Figure 3.4 Construction of pTRE2-Xrel3 vectors. Complete copies (1.8 Kb) of wild type *Xrel3* cDNA or the NLS-mutated *Xrel3* cDNA were ligated into the *Eco*RI and *Xho*I restriction sites within the multiple cloning site of the pTRE2 plasmid. The resulting ligation products were sequenced at the 5' and 3' insertion sites to confirm proper orientation of the inserted cDNAs.

approximately 15 clones from each of the cDNA constructs were chosen and cultured in maintenance medium containing hygromycin until further selection by Southern blot, RT-PCR, and Western blot analyses.

The resulting clones were initially screened using Southern blot analysis to confirm the presence of the Xrel3 insert in the cell genomic DNA. To determine the presence of the Xrel3 insert, *Eco*RI and *Xho*I double digested genomic DNA was analyzed using [^{32}P]-labeled Xrel3 probe. Xrel3 and mutNLS cDNAs were present in the successfully double-transfected cell lines (CT-Xrel3 and CT-mutNLS) and was absent in CaSki and CaSki-Tet cell lines (Figure 3.5).

3.2.4 Confirming the inducible expression of Xrel3 plasmids in CaSki cells

In order to reveal the appropriate CT-Xrel3 and CT-mutNLS cell lines to use, further screening was necessary to prove the induction of Xrel3 and mutNLS mRNA and protein. Clones that were positive for the insertion of pTRE-Xrel3 and pTRE-mutNLS plasmids by Southern blot analysis were further screened for their ability to induce the expression of Xrel3 and mutNLS mRNA using RT-PCR semiquantitative analysis and Western blot analysis to detect the translation of these mRNA molecules.

3.2.4.1 Doxycycline induction of Xrel3 and mutNLS mRNA in CaSki-Tet cells

To test the clones for the induction of Xrel3 and mutNLS mRNA, total cellular RNA was extracted from each clone before and after doxycycline treatments and used for RT-

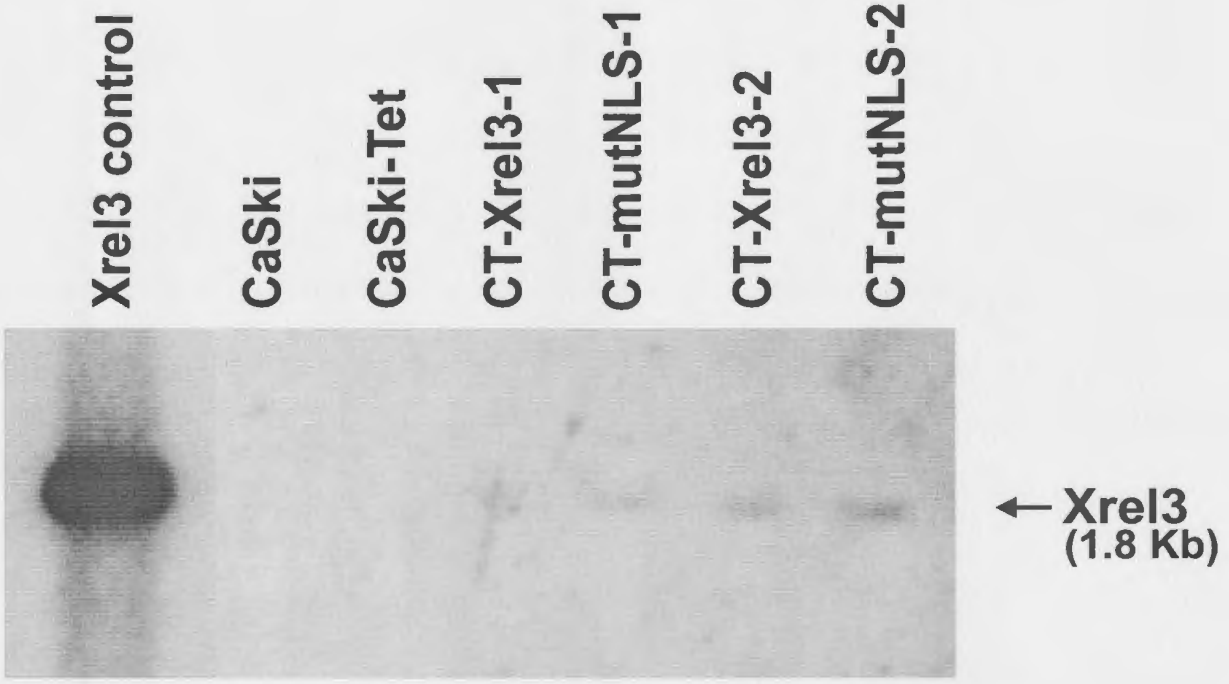

Figure 3.5 Genomic insertion of *Xrel3* constructs in transfected CaSki-Tet clones. Cellular DNA was digested with *Eco*RI and *Xho*I to release the 1.8 Kb *Xrel3* insert and probed with [^{32}P]-labeled Xrel3 cDNA using Southern blot analysis. Digested pTRE-Xrel3 was loaded as a control.

PCR. As a control, pTRE-Xrel3 plasmid was also amplified in identical conditions to detect the same 320 bp fragment. As expected, CaSki and CaSki-Tet did not express Xrel3 mRNA regardless of doxycycline treatment (Figure 3.6). CT-Xrel3 cells did not have detectable levels of Xrel3 mRNA in the absence of doxycycline. When doxycycline is added to the growth medium, there is a clear induction of Xrel3 mRNA. The same results are shown with CT-mutNLS cells; there are no detectable levels of mutNLS present in the absence of doxycycline, while there is a large increase in expression following 24 hours of doxycycline treatment. These combined results show both the tight regulation of transcriptional gene expression by the lack of detectable Xrel3 mRNA in the absence of doxycycline, and the specificity of induction by doxycycline.

3.2.4.2 Translation of Xrel3 and mutNLS mRNA in CaSki-Tet cells

In order to confirm the effectiveness of this cell model system it was necessary to further determine whether the transcribed mRNA was being translated to protein within these cell clones. Without an antibody raised against Xrel3, I decided to use an anti-serum raised against XrelA (Bearer, 1994) as a means of detecting Xrel3. Xrel3 and XrelA share homology (Yang *et al.*, 1998) and therefore it was predicted that anti-serum raised against XrelA could detect Xrel3 protein from lysates containing human proteins. Therefore, proteins were extracted from cell clones grown in DMEM with 2 μg/ml doxycycline and Xrel3 protein levels were accordingly probed using XrelA anti-serum. Figure 3.7 shows the absence of detectable proteins with a molecular weight of 65 kDa in CaSki-Tet cells

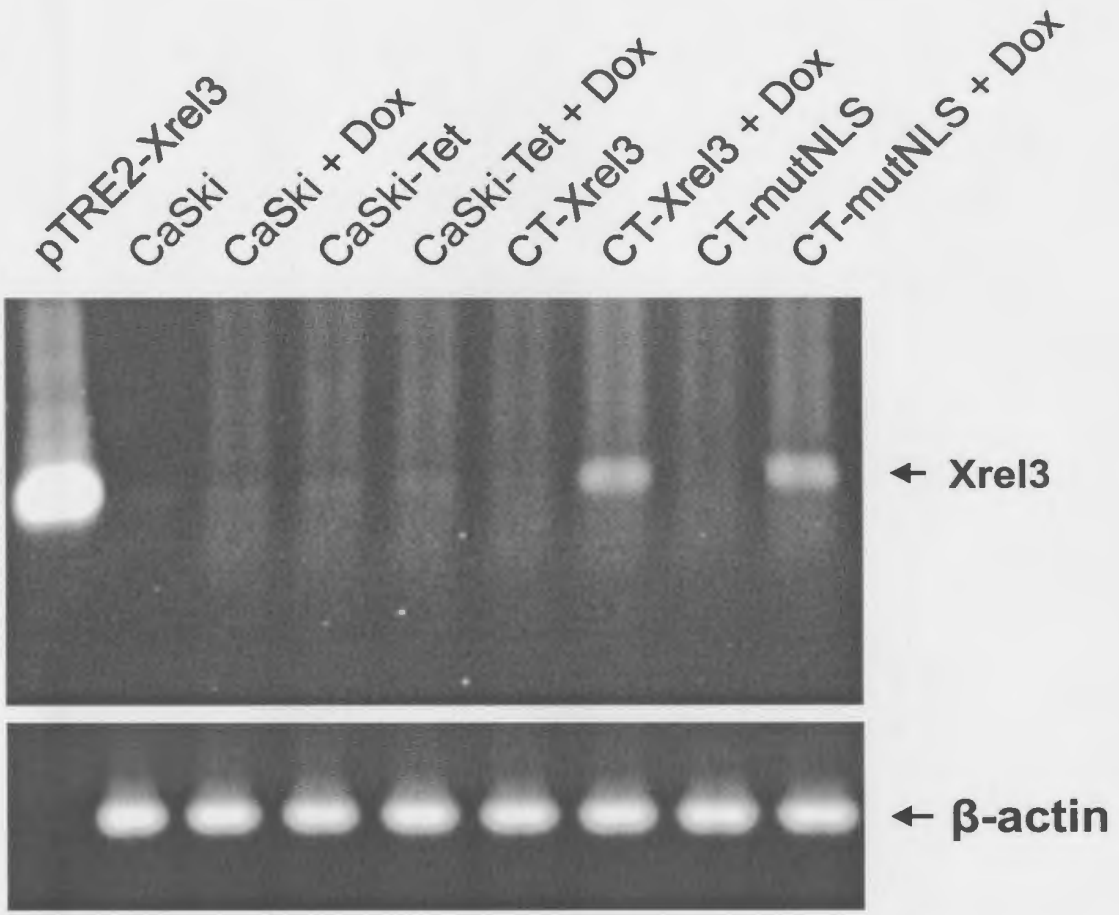

Figure 3.6 Doxycycline-dependent transcription of *Xrel3* and *mutNLS* in CaSki cells. RT-PCR analysis confirms the expression of *Xrel3* and *mutNLS* mRNAs are only present when the double-transfected cells are grown in the presence of doxycycline. The levels of β-actin were also detected to control for the PCR reaction and as a loading control. Shown are the ethidium bromide stained 1.2 % agarose gels from both experiments.

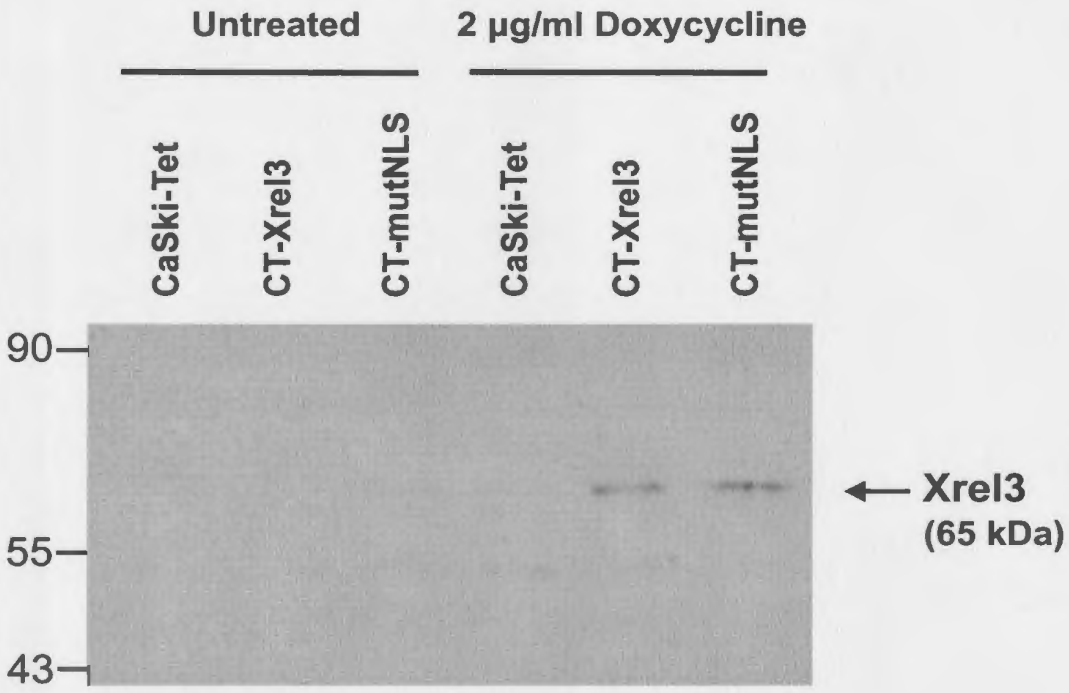

Figure 3.7 Doxycycline-dependent expression of Xrel3 protein in CT-Xrel3 cells and CT-mutNLS. Western blot analysis confirms the induction of the 65 kDa Xrel3 protein in CT-Xrel3 cells only after the addition of doxycycline as detected using anti-XrelA serum.

regardless of the presence of doxycycline. However, upon the addition of doxycycline in CT-Xrel3 and CT-mutNLS cell cultures, a band was detected by the anti-XrelA serum at the corresponding molecular weight of approximately 65 kDa for Xrel3 protein (Figure 3.7). The clones showing the greatest induction of Xrel3 and mutNLS at the protein level were chosen and subsequently termed CT-Xrel3 and CT-mutNLS cell lines, respectively. These results show the successful generation of human cervical cells with inducible expression of Xrel3, and its NLS mutant designed to be defective for nuclear translocation, which can now be further investigated to determine the effects of this gene and its functions on cell growth, morphology, and its potential roles in modifying TGF-β signaling.

3.3 Xrel3 blocks cell morphological changes induced by TGF-β

The morphological appearance of *in vitro* cervical epithelial cells has been described (Stanley and Greenfield, 1992). Under ideal *in vitro* conditions, cultured cervical epithelial cells individually have a polygonal, flattened appearance; are slightly elongated; and contain frequent cytoplasmic protrusions and extensions when viewed under light microscopy (Stanley and Greenfield, 1992). Cultures of cervical epithelial cells typically grow in sheets of cells with a clear presence of mitotic figures, commonly around the edges of the cultures where the cells are spreading out to form the sheet-like epithelial structure (Stanley and Greenfield, 1992). These descriptions of cervical epithelial cells and cervical epithelial cell cultures, can be used to assess possible changes in the morphological appearance of CaSki, CaSki-Tet, CT-Xrel3, and CT-mutNLS cell lines grown in DMEM and in DMEM containing

doxycycline and/or TGF-β.

The first experiments performed using this inducible CaSki cell system were to determine the effects of doxycycline and TGF-β on cell morphology in untransfected control CaSki cells. Untransfected CaSki cells were grown in DMEM with or without 1 μg/ml doxycycline, 2 ng/ml TGF-β, or both doxycycline and TGF-β. Figure 3.8 shows the effects of these culture conditions on cell morphology. Stained with hematoxylin and eosin, untreated CaSki cells have the predicted epithelial cell shape and appearance (Stanley and Greenfield, 1992) with long cytoplasmic protrusions and large numbers of mitotic figures. CaSki cells were seen as large sheets of epithelial cells growing closely together in the more confluent regions than in the outer edges of the culture. CaSki cells treated with doxycycline alone have a similar, epithelial appearance suggesting doxycycline does not alter the appearance of CaSki cells within these specific parameters (Figure 3.8).

TGF-β is an inhibitor of epithelial cell growth (Yue and Mulder, 2001). CaSki cells are epithelial in origin and have intact TGF-β signaling pathways (Lee *et al.*, 2001). I expected therefore, to detect signs of growth inhibition of CaSki cells upon TGF-β treatment. As can be seen in Figure 3.8, CaSki cells show atypical growth inhibition in the presence of TGF-β, agreeing with previously published results (Lee *et al.*, 2001). The cells grew in smaller regions without the characteristic cytoplasmic projections evident in the untreated or doxycycline-treated cells. The cell morphology was not as elongated or polygonal, resulting in a more rounded appearance and giving rise to the localized distribution of cells. There was a lower percentage of mitotic bodies present in these cultures as well (Figure 3.8

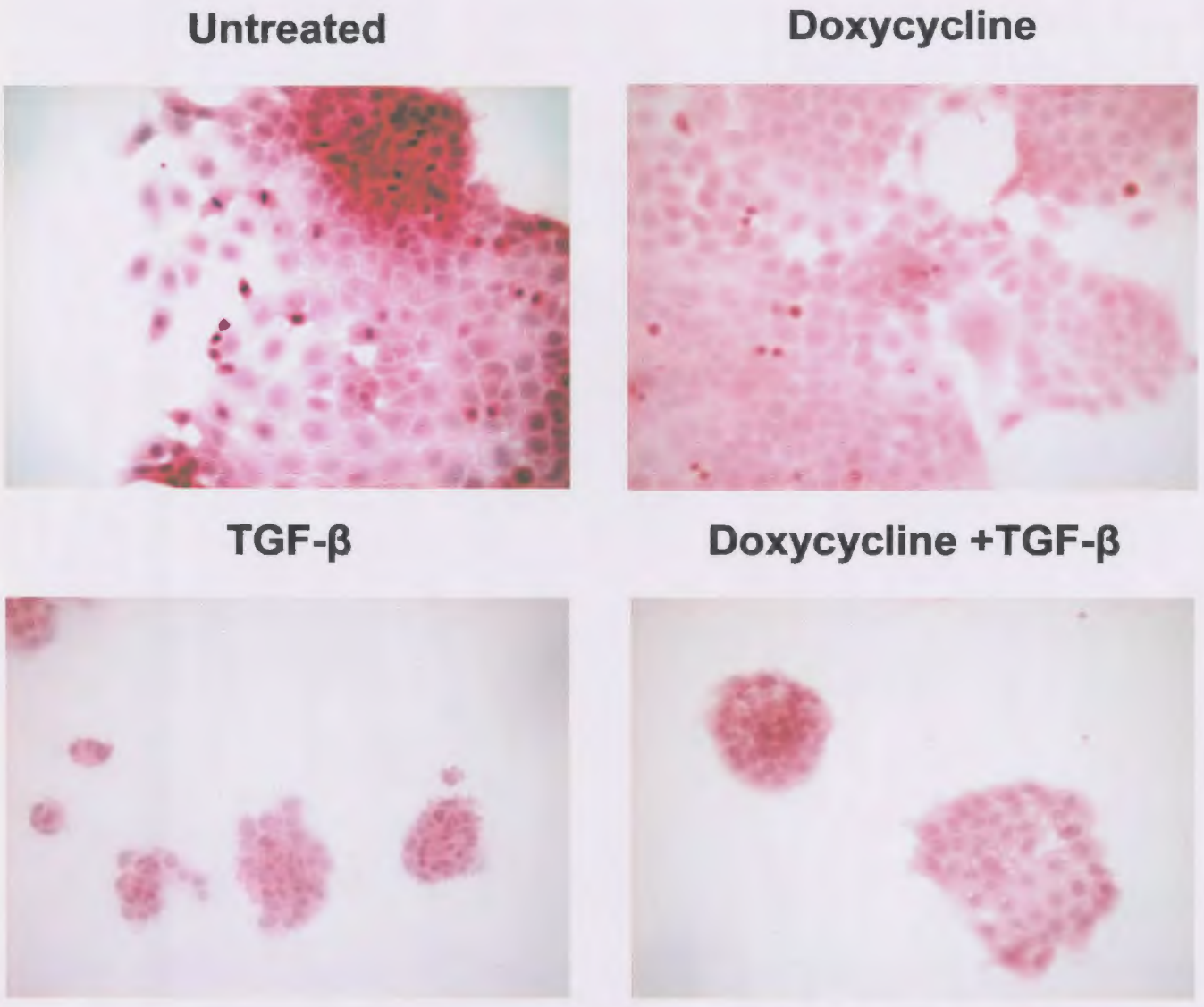

Figure 3.8 Untransfected CaSki cells grown in the absence or presence of doxycycline and/or TGF-β. Haematoxylon and eosin stained CaSki cells appeared to have characteristic epithelial growth patterns in DMEM and when treated with doxycycline. The addition of TGF-β largely suppressed the growth of CaSki cells to small colonies as depicted. This reduced gorwth pattern was also seen when TGF-β and doxycycline were added to the growth media. (400× magnification).

and Table 3.1), indicating cell division occurred at a slower rate. CaSki cells therefore were responsive, morphologically to TGF-β. The addition of TGF-β and doxycycline together give the same result as TGF-β alone, with restricted cell distribution, fewer mitotic bodies, and fewer cells with normal flattened appearance characteristic of healthy epithelial cell growth (Figure 3.8). Therefore doxycycline did not interfere with the morphological effects of TGF-β treatment on CaSki cells.

The effect of the stable transfection of the pTet-On plasmid in CaSki-Tet cells was examined in cells cultured without doxycycline or TGF-β treatments. CaSki-Tet cells have the same epithelial appearance as CaSki cells when cultured in DMEM and when doxycycline is added (Figures 3.8 and 3.9). These results confirm that the insertion the pTet-On plasmid which expresses the rtTA molecule, did not alter cellular morphology or growth characteristics in DMEM. Also as expected, no gross observable differences could be seen when doxycycline was added further suggesting that doxycycline at this concentration is unable to affect CaSki growth patterns and that the activated rtTA transcription factor did not alter the appearance of CaSki-Tet cells under light microscopy (Figure 3.9). The growth of CaSki-Tet cells was also inhibited by TGF-β, even in the presence of doxycycline indicating that these cells maintain normal responsiveness to the growth factor.

To determine the effects of Xrel3 expression on cell morphology, CT-Xrel3 cells were grown in DMEM in the absence or presence of doxycycline. Untreated CT-Xrel3 cells appeared morphologically similar to CaSki and CaSki-Tet cells, having the same polygonal shape, numerous cytoplasmic extensions (Figure 3.10), and similar numbers of mitotic

Table 3.1. Percentage of CaSki and CT-Xrel3 cells visibly undergoing mitosis in the presence of doxycycline and TGF-β.

Treatment	CaSki	CT-Xrel3
Untreated	4.06 ± 0.46	4.86 ± 0.98
Doxycycline	4.87 ± 0.99	5.01 ± 0.55
TGF-β	1.96 ± 0.76	1.29 ± 0.22
Doxycycline + TGF-β	1.96 ± 0.34	3.96 ± 0.36

Values indicate the percentage of mitotic figures counted ± standard deviation from three representative views from a single experiment.

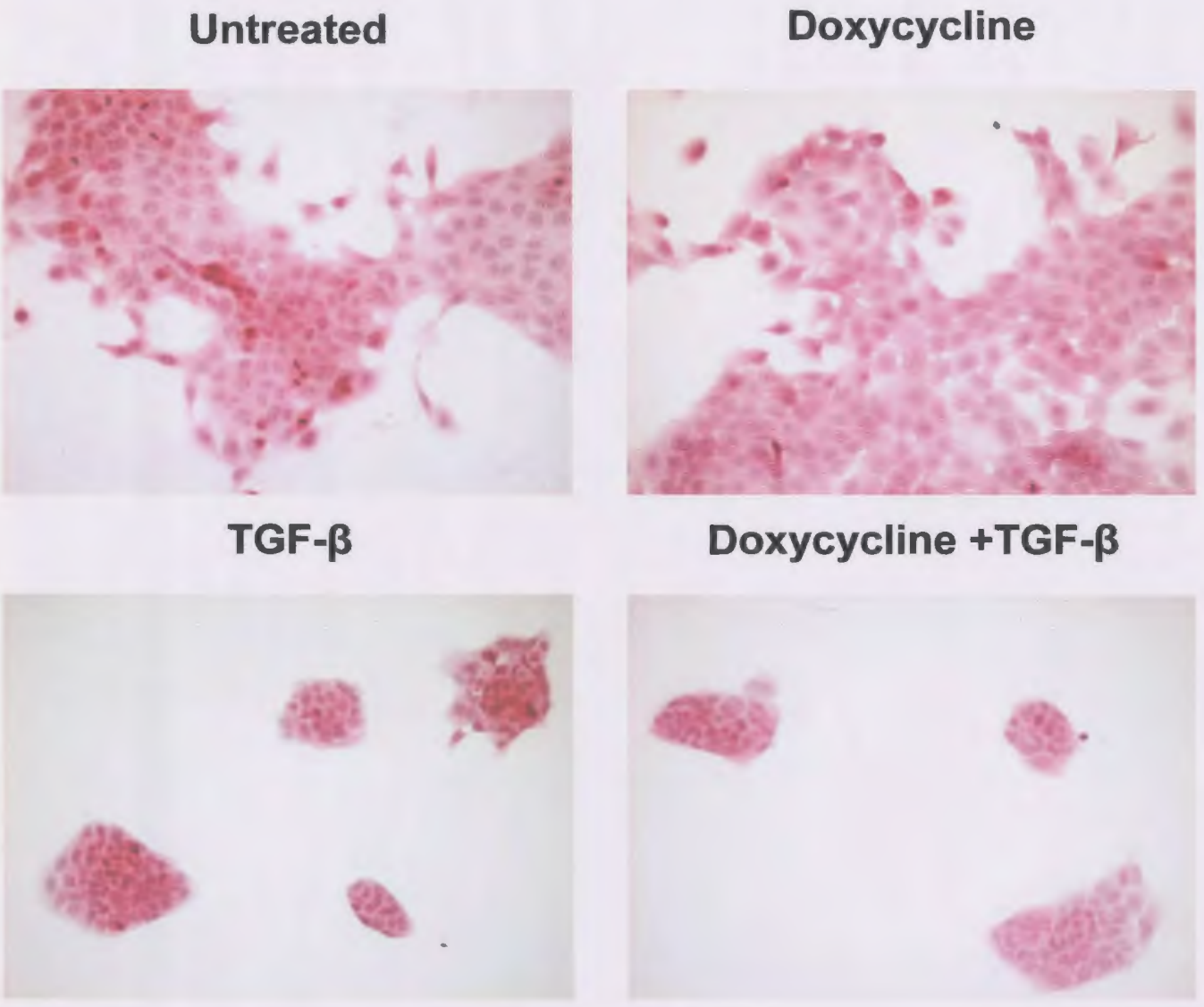

Figure 3.9 CaSki-Tet cells grown in the absence or presence of doxycycline and/or TGF-β. CaSki-Tet cells were stained with haematoxylon and eosin following 48 hrs of growth in the various culture conditions. Although stably transfected with the pTet-On plasmid, CaSki-Tet cells show characteristic growth patterns of epithelial cell cultures in DMEM or DMEM + doxycycline. Following treatments with TGF-β, the vast majority of CaSki-Tet cells appear to be restricted to growth in small colonies, in the absence or presence of doxycycline. (400× magnification).

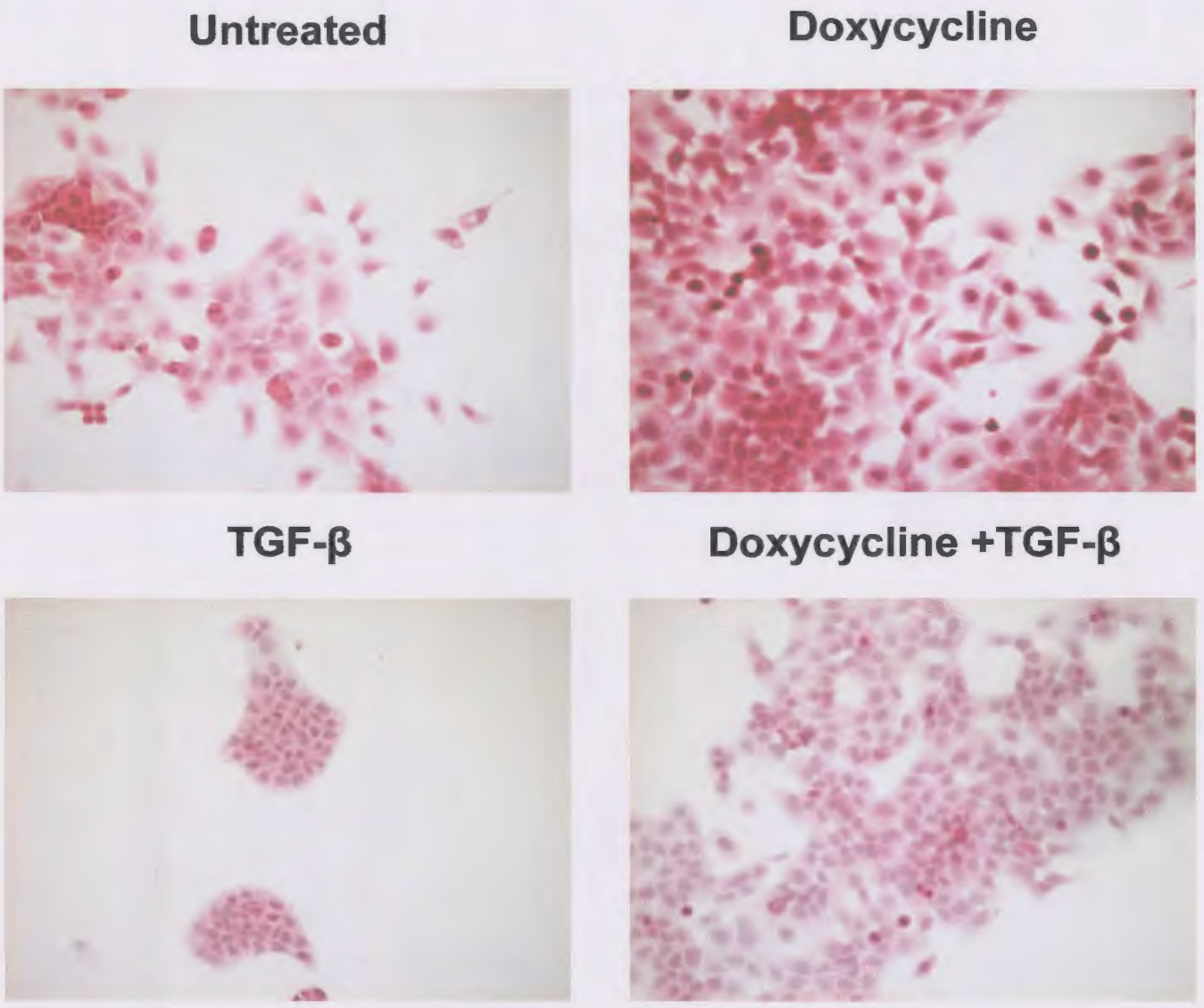

Figure 3.10 CT-Xrel3 cells grown in the absence or presence of doxycycline and/or TGF-β. CT-Xrel3 cells were stained with haematoxylon and eosin following 48 hrs of growth in the various culture conditions. CT-Xrel3 cells grown in DMEM and DMEM + doxycycline appeared flattened, elongated and contained periodic protrusions and extensions typical of epithelial cell morphology. In the presence of TGF- β however, CT-Xrel3 cell growth was restricted to smaller colonies without the typical, sheet-like pattern seen in DMEM alone. Also noted, was the reduction of mitotic figures within these colonies. Xrel3 expression by doxycycline treatment, prevented the growth pattern resulting from TGF- β alone, resulting in a typical epithelial cell morphology. (400× magnification).

figures as CaSki and CaSki-Tet control cells (Table 3.1). Importantly, the stable insertion of three plasmids, pTet-On, pTRE-Xrel3, and pTK-Hyg within CaSki cells did not noticeably alter the cellular morphology. CT-Xrel3 cells also grown in the presence of doxycycline appear morphologically like wild-type CaSki cells. Therefore, the expression of Xrel3 protein induced by doxycycline, did not appear to alter the cell's appearance or growth.

TGF-β treatments alone on CT-Xrel3 cells showed similar results to those of CaSki cells (Figures 3.8 and 3.10). There was a significant reduction in the number of cellular protrusions, and growth was contained within small clusters or colonies that lack the elongated shape of typical cervical epithelial cells (Stanley and Greenfield, 1992). There was also a reduction in the number of mitotic figures present, indicating a reduction in cell division (Table 3.1). When CT-Xrel3 cells expressing Xrel3 were treated with TGF-β, the cellular morphology was more similar to that of the untreated cells as shown in the TGF-β-treated untransfected CaSki cells (Figure 3.8 and 3.10). The cells had the appearance of untreated CaSki with mitotic levels comparable to untreated CaSki cells (Table 3.1). These results show the presence of Xrel3 is able to rescue typical cell growth patterns and morphology when treated simultaneously with TGF-β.

The requirement of nuclear localization of Xrel3 was investigated in CT-mutNLS cell morphology under the various culture conditions. Stained CT-mutNLS cells appeared similar to untreated CaSki cells when cultured in DMEM alone, or when doxycycline was added to the medium (Figure 3.11). The cells have long cytoplasmic projections and there

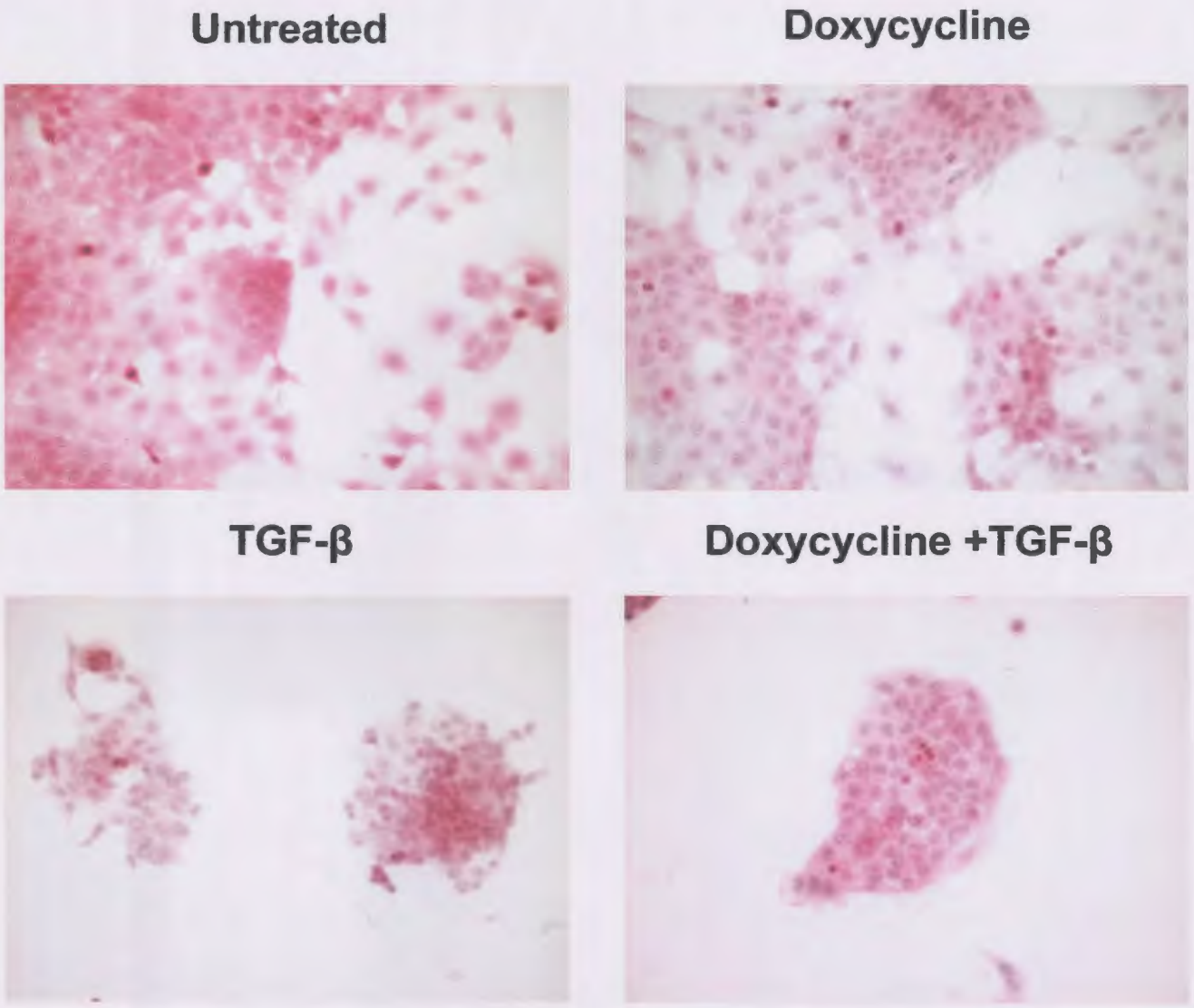

Figure 3.11 CT-mutNLS cells grown in the absence or presence of doxycycline and/or TGF-β. CT-mutNLS cells were stained with haematoxylon and eosin following 48 hrs of growth in the various culture conditions. The morphology of CT-mutNLS cells was similar to the control CaSki cells when cultured in DMEM and DMEM + doxycycline. As with the other cell lines tested in this thesis, TGF-β was able to reduce the growth of CT-mutNLS cells to smaller, tighter colonies with fewer mitotic figures and cytoplasmic projections. The presence of the NLS mutant Xrel3 did not prevent this growth suppression by TGF- β as shown in the last panel. (400× magnification).

are high numbers of cells undergoing mitosis. Upon the addition of TGF-β however, there was the characteristic growth constraint found in TGF-β-treated CaSki and CaSki-Tet cell lines. The combination of doxycycline and TGF-β treatments continued to cause the restriction of growth into smaller clusters with decreased cell numbers (Figure 3.11). These results indicate that, unlike the wild-type Xrel3, the NLS mutant is unable to prevent the growth inhibition by TGF-β. Therefore the observations suggest there is a direct interruption of TGF-β signaling by Xrel3 which is dependent on its nuclear localization.

3.4 Xrel3 blocks TGF-β-mediated cell growth inhibition

The differential responses of CaSki, CaSki-Tet, CT-Xrel3, and CT-mutNLS cell lines to TGF-β treatments suggested that the different cell lines may have different growth characteristics in response to TGF-β. Therefore, the growth rates of each of the cell lines was determined using a colorimetric cell proliferation assay in the various culture conditions. Briefly, approximately 5000 cells were seeded in DMEM or DMEM containing 1 μg/ml doxycycline, for 24 hours before treatments with 2 ng/ml TGF-β. Absorbance was measured every 12 hours while the medium was changed every 24 hours with continuous doxycycline and TGF-β treatments.

CaSki control cells appeared to grow normally in DMEM + 10% FCS with a steady increase in absorbency between days 2 and 5.5, following an initial lag phase of approximately 24 hours (Figure 3.12). Consistent with the morphological data, the addition of doxycycline to the medium did not affect the growth curve. CaSki cells cultured in the

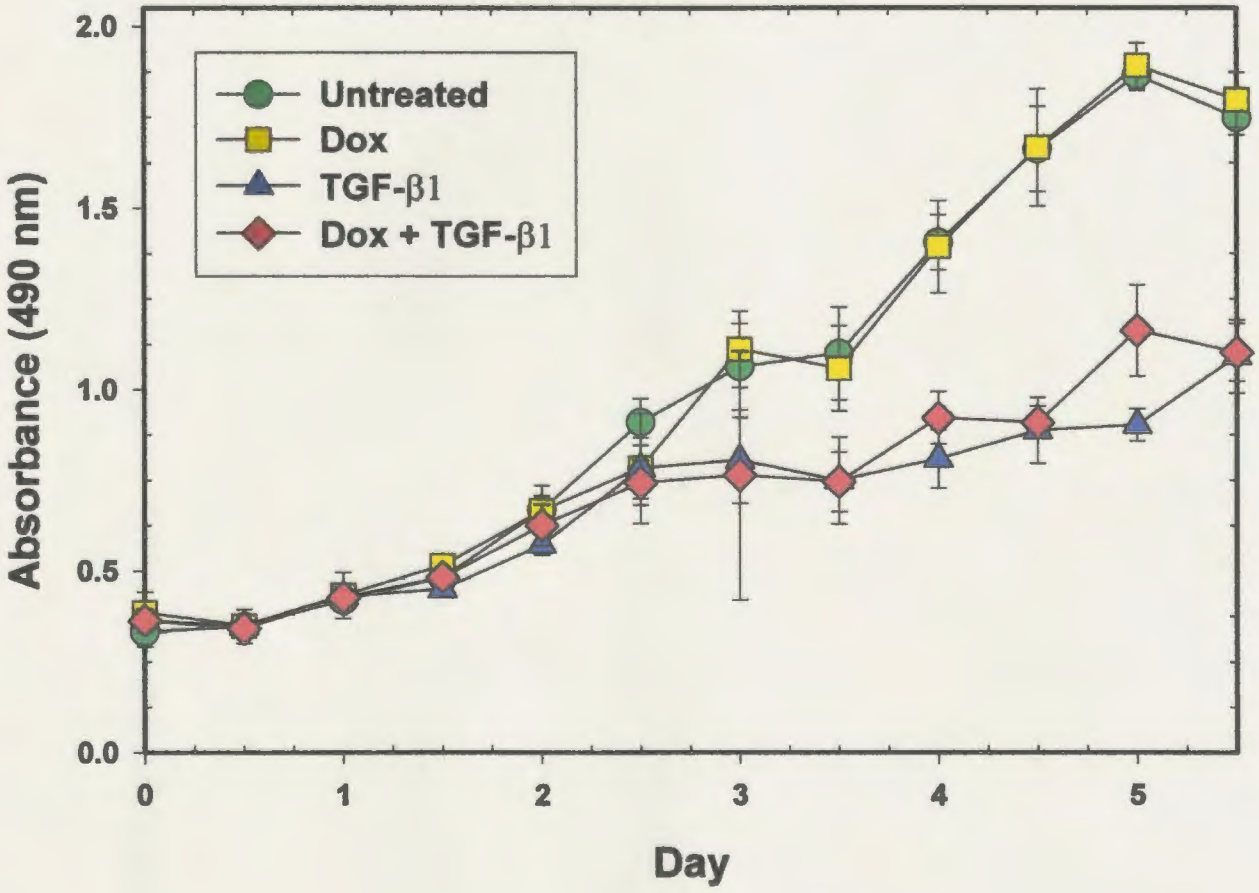

Figure 3.12 Effects of doxycycline and/or TGF-β treatments on CaSki cell growth. Cells were cultured in the above conditions and the absorbance at 490 nm was recorded following incubation with the MTS substrate which directly correlated to the number of cells in the culture. (Error bars indicate standard deviation from six experiments.)

presence of TGF-β, however, underwent significant cell growth inhibition with or without the presence of doxycycline in the culture media. After 5 days of incubation, the absorbance of the TGF-β-treated cells was approximately 55% of the untreated and doxycycline-treated cells. Thus, TGF-β as expected, inhibits CaSki epithelial cell growth, which was unaffected by the addition of doxycycline.

CaSki-Tet cells were also used as a control to ensure changes in growth in Xrel3 expressing cells were not due to the stable transfection of pTet-On plasmid or the presence of the rtTA protein. As shown in Figures 3.13, there were no apparent effects from either pTet-On or rtTA since the untreated CaSki-Tet cells grew comparably to untransfected CaSki cells (Figures 3.12 and 3.13). Doxycycline alone did not significantly change the growth curve, further indicating activated rtTA alone was not responsible for changes in cellular growth characteristics (Figure 3.13). The addition of TGF-β alone or in combination with doxycycline resulted in decreased cell numbers, confirming the morphological results seen in Section 3.3 and also confirming the stable insertion of pTet-On was not contributing to changes in cellular appearance or growth patterns.

The growth rates of CT-Xrel3 cell cultures within these same conditions were also investigated. When cultured in DMEM only, CT-Xrel3 cells grew normally as expected for untransfected cells (Figure 3.14). Therefore, the stable transfection of pTRE-Xrel3 did not change normal growth rates. This is important, because any differences in this curve from that of CaSki-Tet cells would be due to the random insertion of the pTRE-Xrel3 plasmid into a critical element necessary for normal growth. After inducing the expression of Xrel3 with

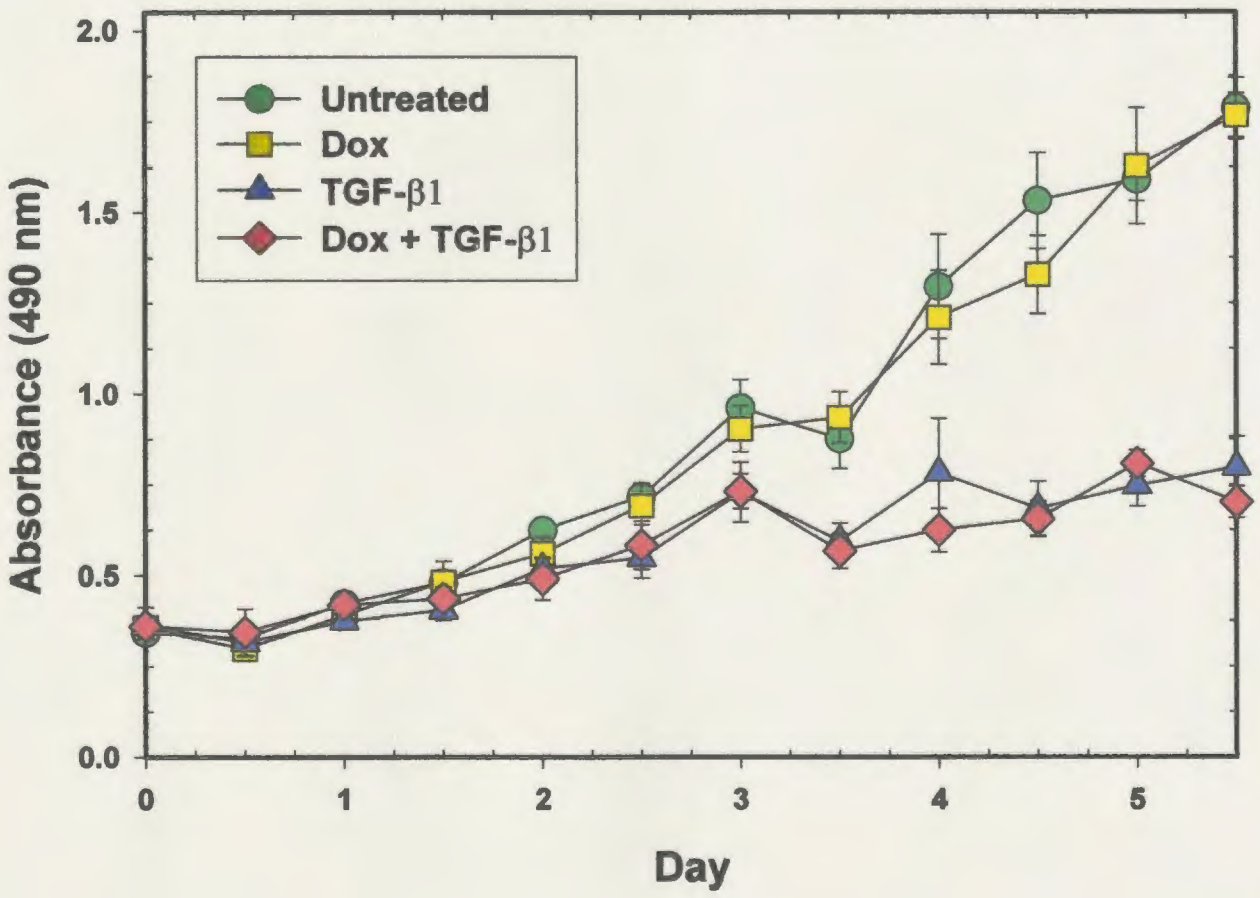

Figure 3.13 Effects of doxycycline and/or TGF-β treatments on CaSki-Tet cells. Cells were cultured in the above conditions and the absorbance at 490 nm was recorded following incubation with the MTS substrate which directly correlates to the number of cells in the culture. (Error bars indicate standard deviation from six experiments.)

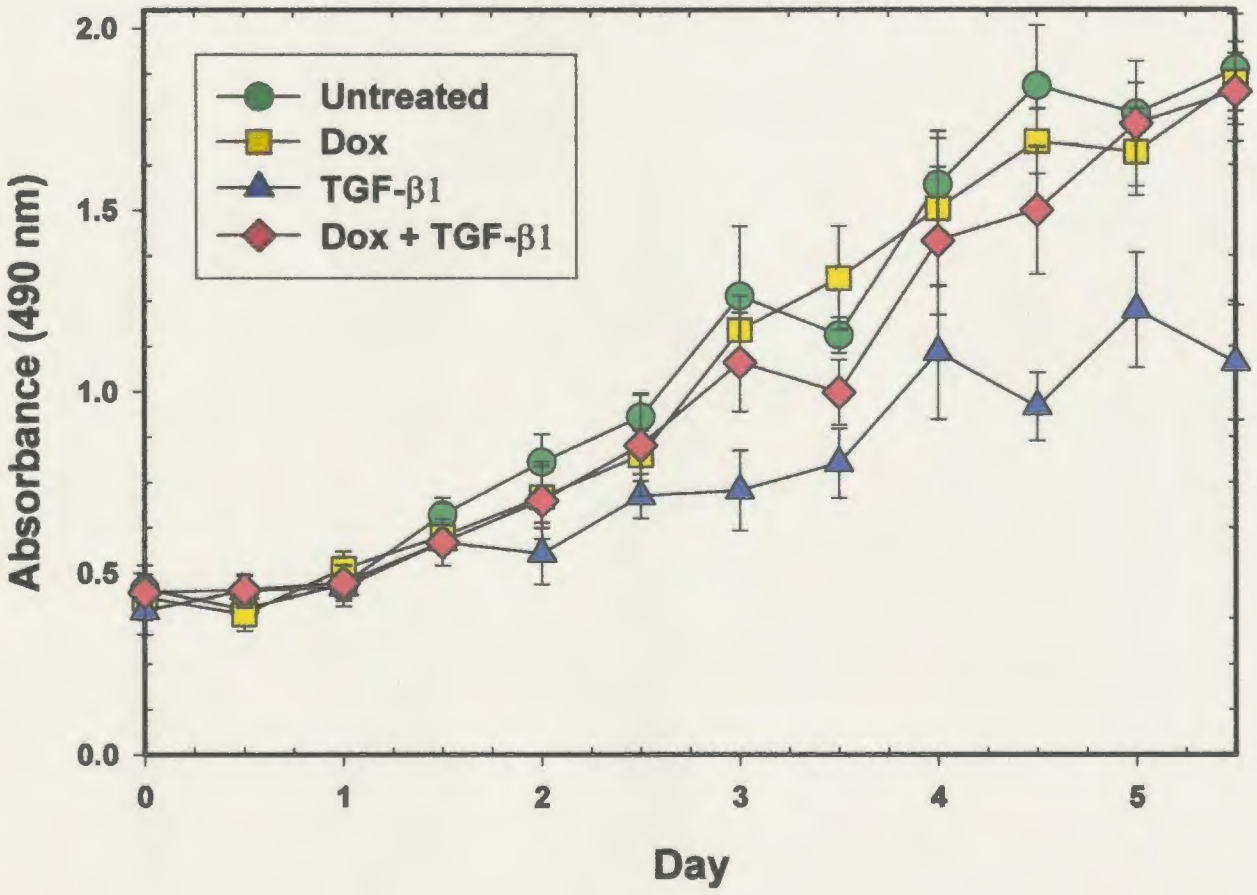

Figure 3.14 Effects of doxycycline and/or TGF-β treatments on CT-Xrel3 cells. Cells were cultured in the above conditions and the absorbance at 490 nm was recorded following incubation with the MTS substrate which directly correlates to the number of cells in the culture. (Error bars indicate standard deviation from six experiments.)

doxycycline, the CT-Xrel3 cells continued to grow at an equal rate to that of uninduced cells (Figure 3.14). Thus, the expression of Xrel3 protein does not alter the normal control of cell proliferation in either a negative or positive way. When TGF-β was added to the culture medium, uninduced CT-Xrel3 cells responded as did untransfected CaSki cells and growth rates were suppressed. After five days of culture in the presence of TGF-β, the number of cells was approximately 50% of the cells in untreated conditions. Therefore, TGF-β significantly decreases proliferation of CT-Xrel3 cells.

I next examined the effects of TGF-β on the growth of CT-Xrel3 cells when Xrel3 expression was already induced by doxycycline. As seen in Figure 3.14, the growth rate of Xrel3-induced, TGF-β-treated CT-Xrel3 cells did not appear to be growth inhibited throughout the five days of culture, and in fact, showed the same level of growth at the end of the experiment as the untreated cells in the absence and presence of doxycycline. This agrees with the morphological data of CT-Xrel3 seen in Figure 3.10. Therefore, expression of Xrel3 in CaSki cells appears to inhibit the growth suppression effects of TGF-β in CaSki cervical epithelial cells.

The growth rates of CT-mutNLS cells were determined in the same manner as the previous cell lines. These cells exhibited normal growth rates in both untreated medium and also when doxycycline was present (Figure 3.15) indicating that the stable transfection of the pTRE-mutNLS plasmid did not alter normal cell growth. TGF-β treatments on CT-mutNLS cells reduced cell numbers to approximately 55% of untreated cells following 5.5 days of treatment which is consistent with the results of TGF-β treatments on the other cell

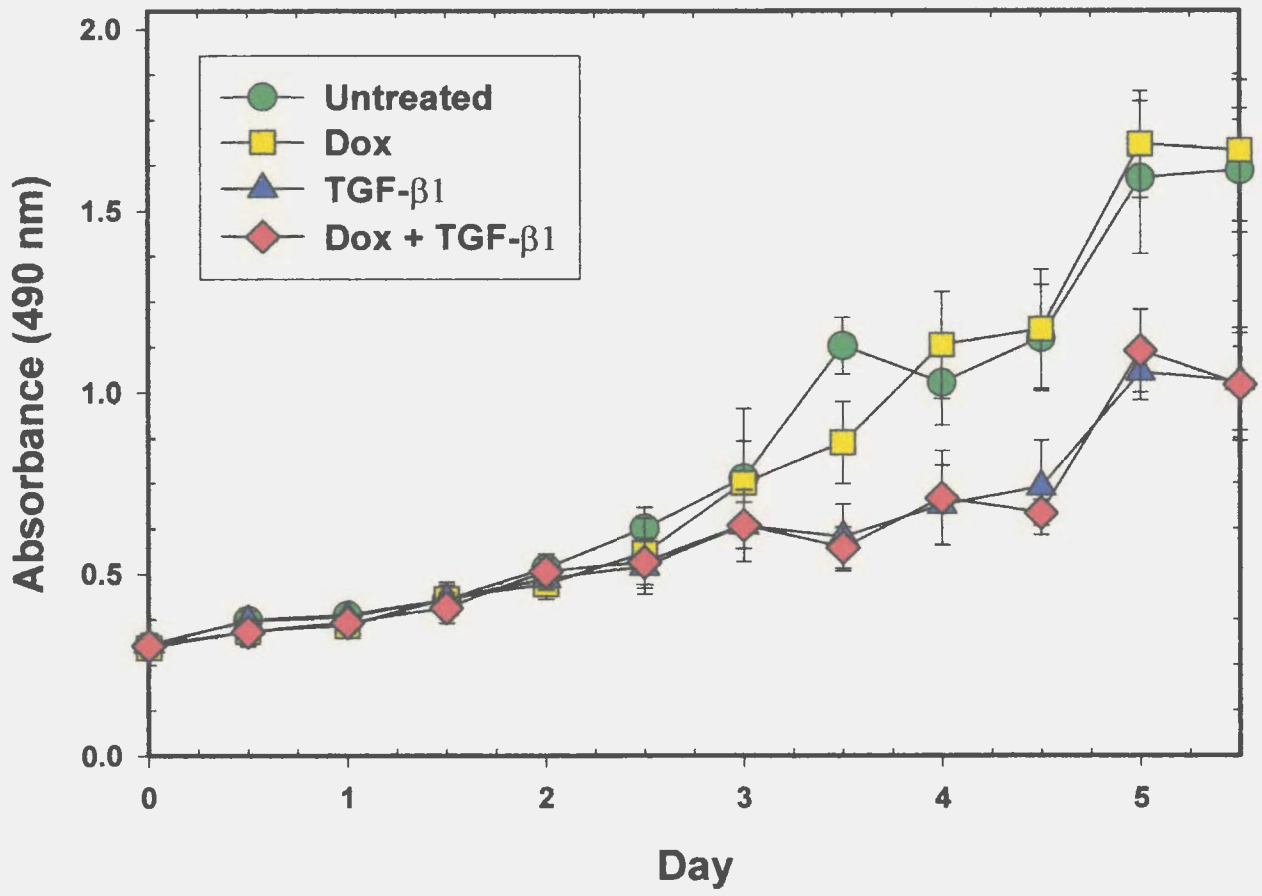

Figure 3.15 Effects of doxycycline and/or TGF-β treatments on CT-mutNLS cells. Cells were cultured in the above conditions and the absorbance at 490 nm was recorded following incubation with the MTS substrate which directly correlates to the number of cells in the culture. (Error bars indicate standard deviation from six experiments.)

types (Figure 3.15). Also shown in Figure 3.15, the mutant NLS form of Xrel3 induced by doxycycline in CT-mutNLS cells was unable to prevent the growth inhibitory effects of TGF-β as wild type Xrel3 was able to do. Thus, the NLS region of the RHD appears necessary for this particular role of Xrel3.

3.5 Xrel3 prevents transcription of TGF-β-induced target genes

Xrel3 may suppress the responses to TGF-β by interfering with either the TGF-β signaling cascade, or at the level of transcription. I initially used CT-Xrel3 cells to investigate the levels of expression of TGF-β-induced molecular targets in these cells when Xrel3 was expressed. One of the more common markers for positive TGF-β signaling is type 1 plasminogen activator inhibitor or PAI-1 (Laiho *et al.*, 1986; Lund *et al.*, 1987; Keski-Oja *et al.*, 1988; Sawdey *et al.*, 1989; and Westernhausen *et al.*, 1991). *In vivo*, TGF-β decreases cell growth by contributing to extracellular matrix (ECM) remodeling by elevating the production of fibronectin and procollagen and by increasing secretion of inhibitors of extracellular proteolytic enzymes such as PAI-1 (Datta *et al.*, 2000). For these reasons, the protein levels of PAI-1 were analyzed by Western blot to determine if TGF-β signaling was affected by the presence of Xrel3.

The levels of PAI-1 in CaSki-Tet, CT-Xrel3, and CT-mutNLS cells treated or untreated with doxycycline and/or TGF-β are shown in Figure 3.16 and quantified in Figure 3.17. In untreated and doxycycline-treated CaSki-Tet cells, very small levels of PAI-1 expression were detected. The addition of TGF-β, alone or in combination with

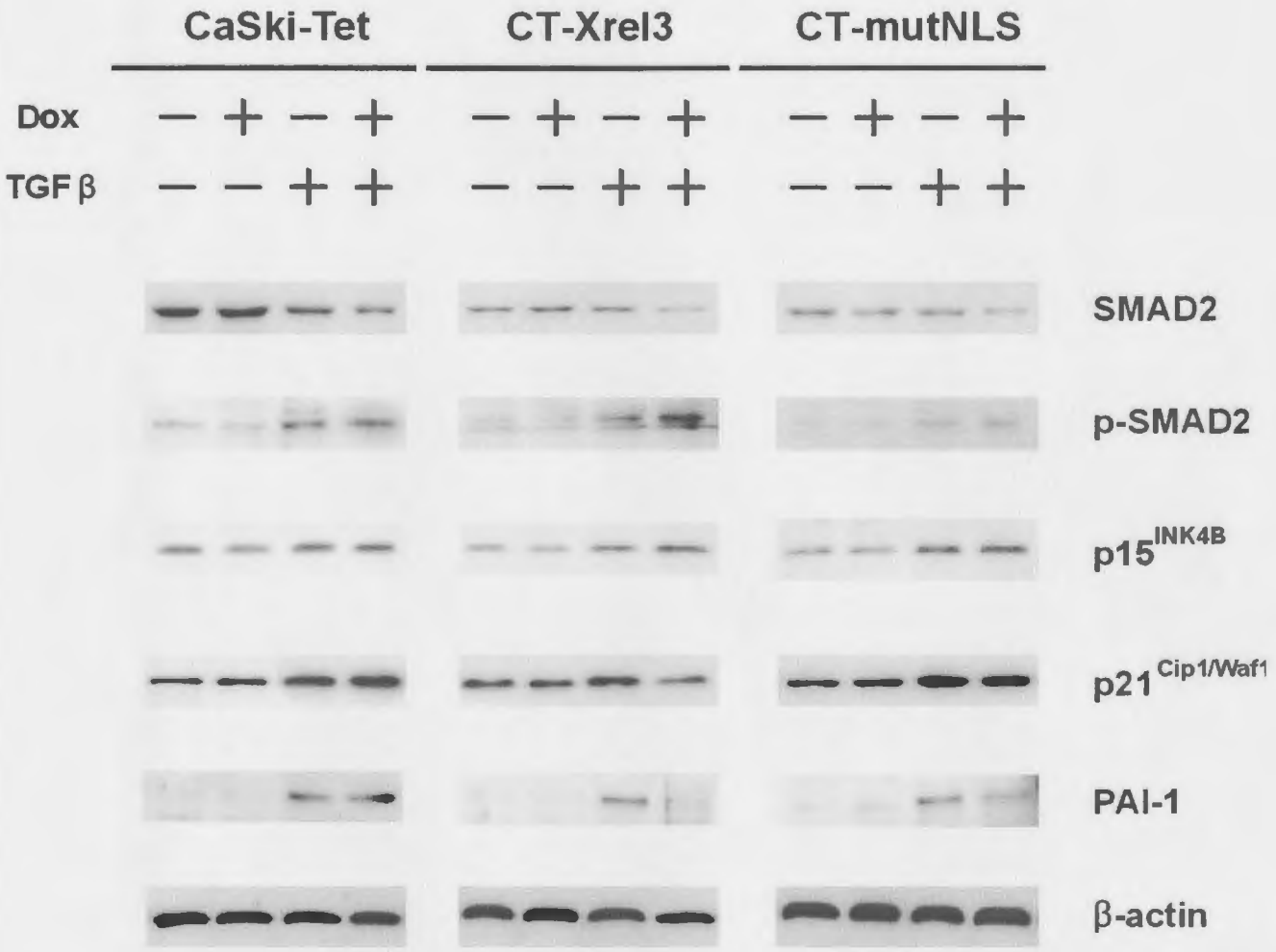

Figure 3.16 Effects of Xrel3 expression and its nuclear localization on the expression of TGF-β-responsive genes. Total cellular protein was analyzed by Western blot to determine the expression levels of TGF-β-responsive genes when Xrel3 and mutNLS were expressed. See text for description of results. (Representation of a minimum of three separate experiments for each protein detected.)

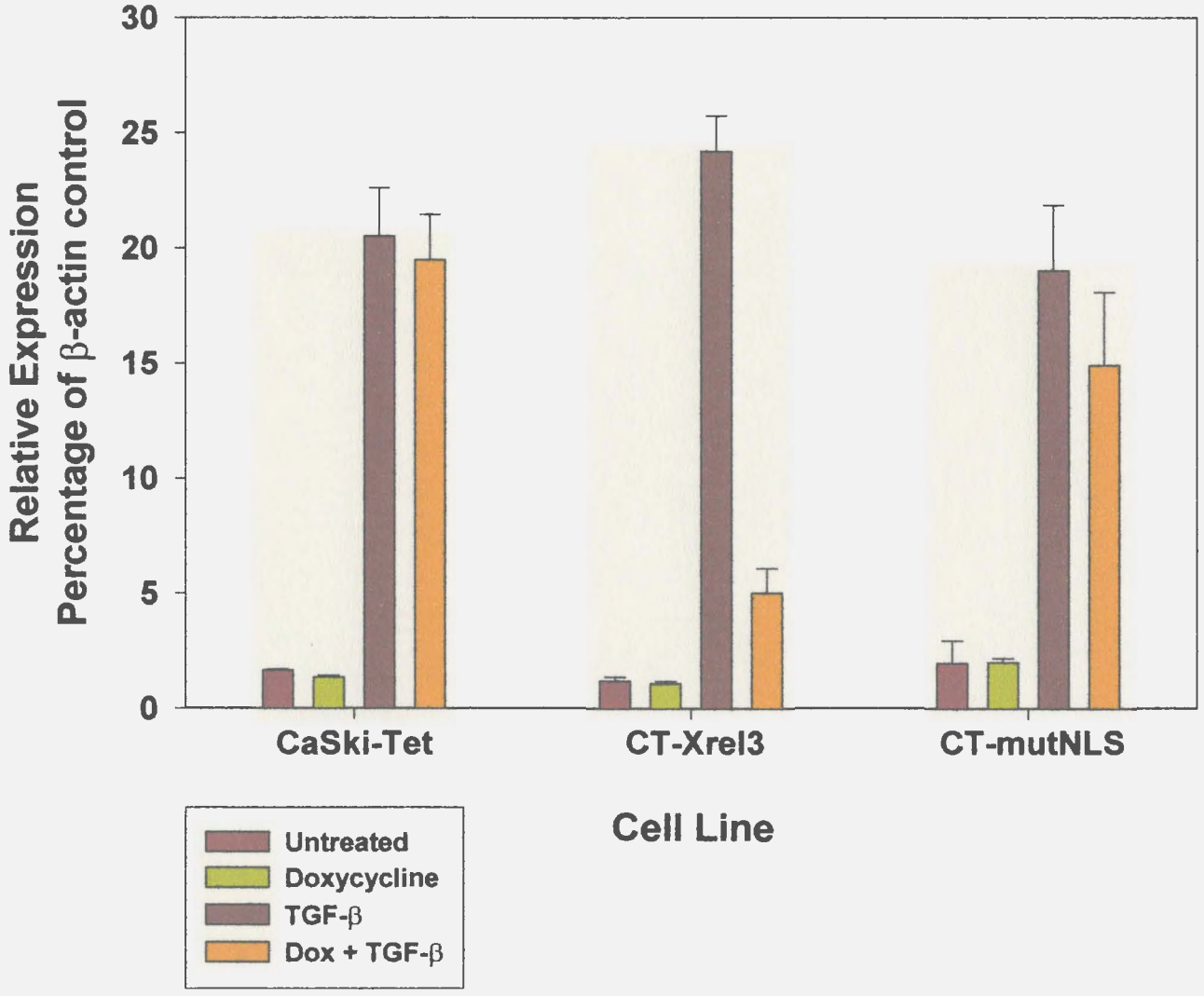

Figure 3.17 Densitometric analysis of PAI-1 as compared to β-actin control levels in CaSki cell lines. Levels of PAI-1 protein expression detected by Western blot were plotted as a percentage of β-actin control levels as recorded by densitometry. Error bars indicate standard deviation from triplicate experiments.

doxycycline, induced a significant increase in PAI-1 protein expression in CaSki-Tet cells as expected.

Untreated and doxycycline-treated CT-Xrel3 cells showed the same endogenous expression of PAI-1 that was seen in CaSki-Tet cells when cultured under the same conditions (Figure 3.16). As expected, the level of PAI-1 increased significantly when the cells were treated with TGF-β alone (Figure 3.16). However, when Xrel3 was expressed at the time of TGF-β treatment, there was a significant reduction of induced PAI-1 protein expression. This suggests that Xrel3 prevented successful transmission of the TGF-β signaling cascade leading to PAI-1 induction in CT-Xrel3 cells.

CT-mutNLS cells also had very low PAI-1 expression when cultured in DMEM or DMEM containing doxycycline (Figure 3.16). As with the previous two cell lines, TGF-β was able to significantly induce the expression of PAI-1. When the NLS mutant of Xrel3 was induced prior to TGF-β treatment, the expression of PAI-1 did increase significantly compared to the untreated and doxycycline-treated CT-mutNLS cells, as indicated in Figure 3.16.

3.6 Xrel3 does not inhibit SMAD2 phosphorylation

TGF-β signals through the SMAD family of proteins (Massagué, 1998; Whitman, 1998). In particular, SMAD2 phosphorylation has been implemented in PAI-1 activation (Goumans *et al.*, 2002) along with SMAD3 (Datta *et al.*, 2000). Therefore, tests of the phosphorylation of SMAD2 could be used to determine if the block to TGF-β signaling by

Xrel3 was occurring upstream or downstream of this key event. Phosphorylated SMAD2 (p-SMAD2) and unphosphorylated SMAD2 protein levels were detected in CaSki-Tet, CT-Xrel3, and CT-mutNLS cells (Figure 3.16). CaSki-Tet cells grown in DMEM and DMEM containing doxycycline, exhibited high levels of SMAD2 with little detectable p-SMAD2 protein expression. When treated with TGF-β, CaSki-Tet cells had significant induction of p-SMAD2 with subsequent reduction in unphosphorylated SMAD2, independent of the presence of doxycycline (Figure 3.16). Untreated CT-Xrel3 had low endogenous levels of p-SMAD2 and which remained unchanged after the induction of Xrel3 expression with doxycycline. As expected, the levels of p-SMAD2 increased upon treatment of TGF-β in non-Xrel3 expressing cells. When Xrel3 was expressed in CT-Xrel3 cells at the time of TGF-β treatment, the levels of p-SMAD2 were comparable to the TGF-β treated CaSki-Tet cells with no Xrel3 present. CT-mutNLS cells showed increased levels of p-SMAD2 protein in the presence of TGF-β, regardless of mutNLS expression induced by doxycycline (Figure 3.16). These results therefore indicate that Xrel3 did not affect SMAD2 phosphorylation in CaSki cells in response to TGF-β.

3.7 Xrel3 alters TGF-β induced expression of cell cycle regulators

The formation, activation, and subsequent inactivation of cyclin-Cdk complexes allow progression through the cell cycle. At the G1 checkpoint, the primary complexes involved are the cyclin D-Cdk4 and cyclin E-Cdk2 complexes (Hunter, 1993; Sherr, 1993). TGF-β induces cell cycle arrest in the G1 phase through several mechanisms (Figure 3.18),

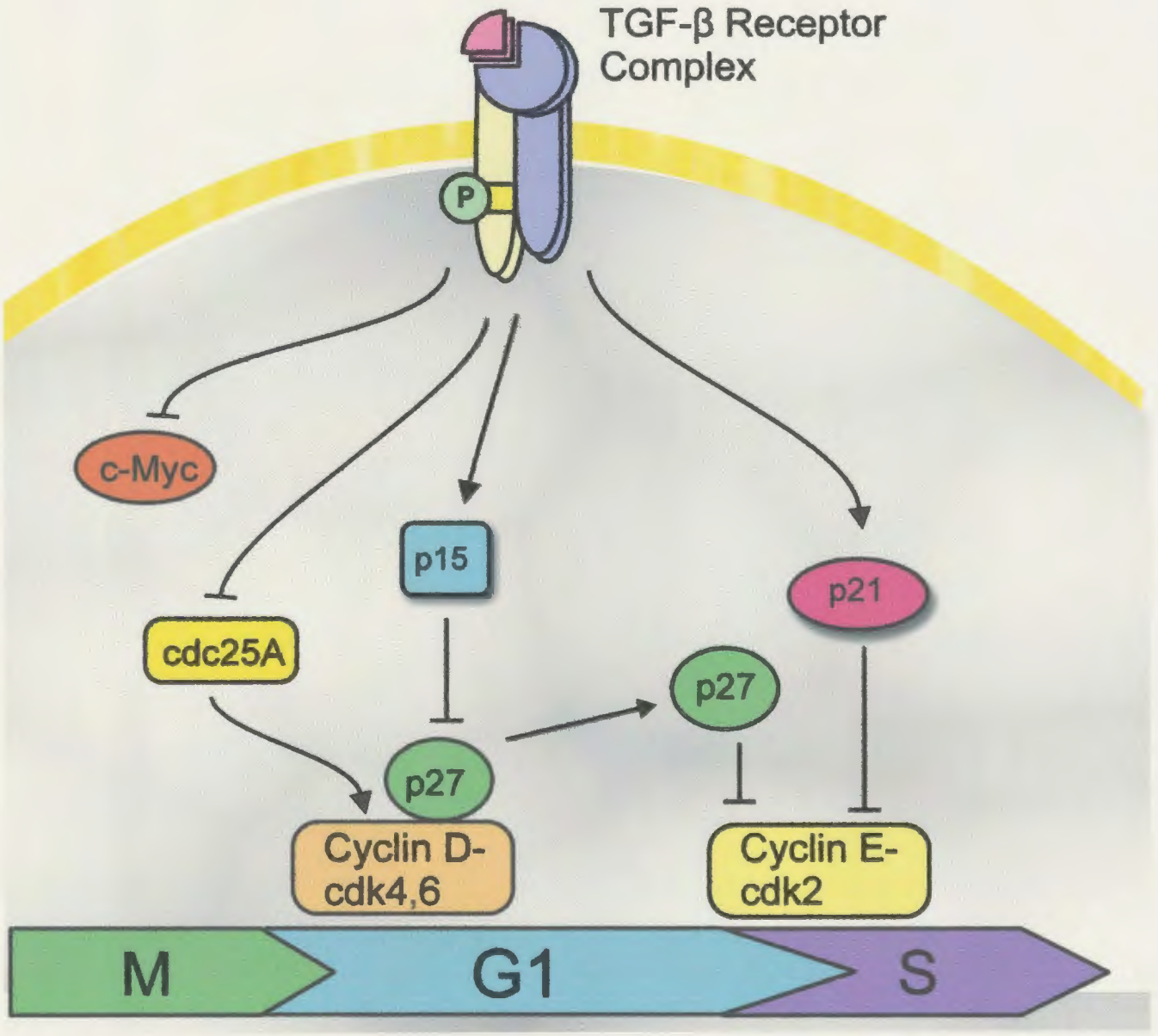

Figure 3.18 Mechanisms of epithelial cell cycle inhibition by the activated TGF-β receptor complex. TGF-β has been shown to inhibit c-Myc and cdc25A activity (cell cycle enhancers) and induce p15 and p21 expression (cell cycle inhibitors) culminating in G1 arrest.

which alter the expression of G1 cyclins, Cdks, and Cdk inhibitors, preventing the G1 to S phase transition (Geng and Weinberg, 1993; Sherr and Roberts, 1999). Two key mechanisms of TGF-β-induced cell cycle arrest are the induction of the cyclin E-Cdk2-specific inhibitor $p21^{CIP/WAF1}$ (Datto *et al.*, 1995a; Sewing *et al.*, 1997), and the induction of the Cdk4-specific inhibitor $p15^{INK4B}$ (Hannon and Beach, 1994; Ewen, 1996). Therefore, the expression levels of these proteins were investigated within this cell system.

3.7.1 Xrel3 inhibits induction of $p21^{Cip/WAF1}$ expression by TGF-β

The induction of $p21^{Cip/WAF1}$ (p21) following TGF-β treatment aids in cell cycle arrest. p21 is involved in a complex composed of cyclin D, Cdk4, and proliferating cell nuclear antigen (Xiong *et al.*, 1992). The first studies on p21 suggested that the transcription of p21 was controlled by p53 and p21 is an effector for the suppression of cell proliferation following DNA damage (Peter and Herskowitz, 1994; Waga *et al.*, 1994; Datto *et al.*, 1995b). However, it has been shown that p21 is induced by TGF-β independently of p53 status and is a potent effector in TGF-β-induced growth inhibition. CaSki cells were also reported to have fully inducible, wild-type p21 expression (Funaoka *et al.*, 1996; Narayanan *et al.*, 1999). For these reasons, the induction of p21 was examined as a marker for TGF-β signaling within the CaSki cell model system and determine if Xrel3 could prevent this specific mechanism of growth inhibition.

CaSki-Tet, CT-Xrel3, and CT-mutNLS cell lines showed relatively high endogenous levels of p21 in the absence of doxycycline or TGF-β (Figures 3.16 and 3.19). The addition

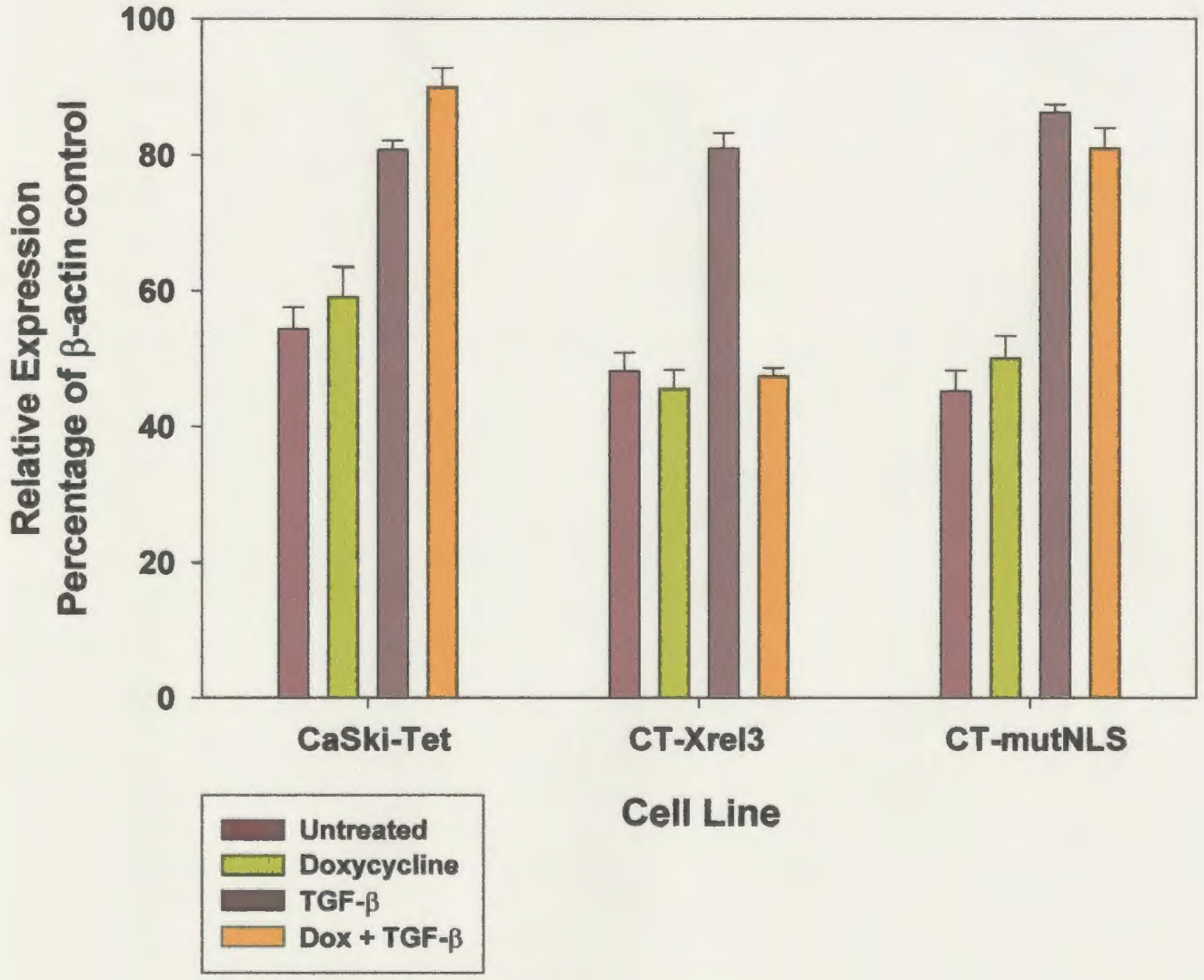

Figure 3.19 Densitometric analysis of $p21^{Cip/Waf1}$ as compared to β-actin control levels in CaSki cell lines. Levels of $p21^{Cip/Waf1}$ expression detected by Western blot were plotted as a percentage of β-actin control levels as recorded by densitometry. Error bars indicate standard deviation from triplicate experiments.

of doxycycline to the culture medium of CaSki-Tet cells, did not significantly alter the expression of p21, which indicates doxycycline is unable to activate p21 expression. The expression of either wild-type Xrel3 or mutNLS also did not affect p21 expression levels in CT-Xrel3 or CT-mutNLS cells, respectively. As expected, the addition of TGF-β lead to an increase in p21 protein in CaSki-Tet, CT-Xrel3, and CT-mutNLS cell lines. In the CaSki-Tet control cells, the combined treatment of doxycycline and TGF-β did not alter the induction of p21 as the expression is similar to that found in TGF-β treatments alone. Doxycycline-induced CT-Xrel3 cells, but not CT-mutNLS however, show p21 levels that are similar to the endogenous levels in the presence of TGF-β (Figures 3.16 and 3.19). Thus, the expression of Xrel3 inhibited p21 induction in CaSki cells. These results suggest that Xrel3 was able to prevent cell growth inhibition induced by TGF-β at least partly due to the prevention of p21 induction.

3.7.2 Xrel3 does not affect TGF-β induction of $p15^{INK4B}$ expression

TGF-β is able to arrest cells in the G1 phase also by the induction of $p15^{INK4B}$ (p15) (Hannon and Beach, 1994; Reynisdottir *et al.*, 1995; Reynisdottir and Massagué, 1997). The mechanism of action of p15 is different than p21 in that it inhibits Rb phosphorylation, maintaining it in a hypophosphorylated state via inhibition of the cdk4,6-cyclin D complex (Sherr and Roberts, 1999). Due to its alternate mode of growth inhibition, p15 expression was also used as a marker for intact TGF-β signaling. The p15 expression levels are shown in Figure 3.16 and further quantified in Figure 3.20.

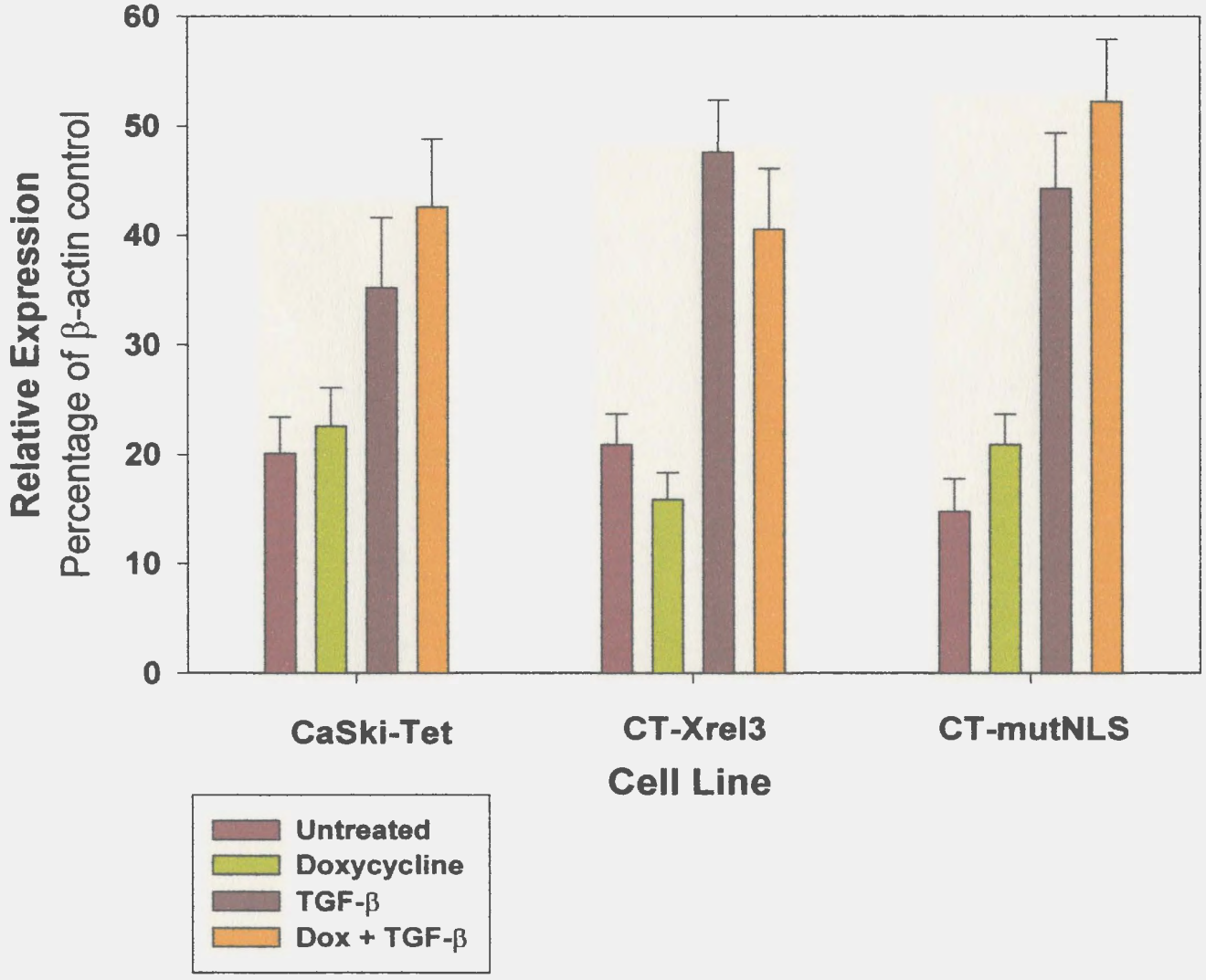

Figure 3.20 Densitometric analysis of $p15^{INK4B}$ as compared to β-actin control levels determined by Western blot. Levels of $p15^{INK4B}$ expression detected by Western blot were plotted as a percentage of β-actin control levels as recorded by densitometry. Error bars indicate standard deviation from triplicate experiments.

Untreated CaSki-Tet cells showed low, endogenous levels of p15 which did not change significantly when doxycycline was added alone. With the addition of TGF-β, there was a significant induction of p15 expression as expected. The combination of doxycycline and TGF-β, induced p15 expression to levels similar to TGF-β treatments alone. These results indicate that doxycycline neither induces p15 itself, nor interferes with the induction of p15 by TGF-β treatment. Untreated CT-Xrel3 cells express p15 at levels equivalent to untreated CaSki-Tet cells (Figures 3.16 and 3.20). As expected, the addition of doxycycline alone did not affect p15 levels. CT-Xrel3 cells treated with TGF-β show a significant increase in p15 protein, but, surprisingly, the presence of Xrel3 induced by doxycycline did not alter the induction of p15 expression (Figures 3.16 and 3.20). CT-mutNLS cells have the same endogenous levels of p15 in untreated and doxycycline-treated conditions and show equivalent induction of p15 following TGF-β treatments in the absence and presence of doxycycline. These results indicate that neither wild-type Xrel3 nor the mutated Xrel3 proteins were able to prevent the induction of p15 by TGF-β.

3.8 TGF-β-induced CaSki growth inhibition is not due to apoptosis

Various studies report various cell types respond to TGF-β by growth arrest and eventually the cells enter an apoptotic pathway (Gurumurthy *et al.*, 2001; Bottinger and Bitzer, 2002; Gorelik and Flavell, 2002; Gressner *et al.*, 2002). Studies have also reported that TGF-β treatment of HPV-16-containing cells induces apoptosis (Rorke and Jacobberger, 1995). Therefore, it was hypothesized that the reduction in cell growth seen in Figures 3.12-

3.15, may be due to increases in apoptosis and that Xrel3, a member of the anti-apoptotic proteins, may block this TGF-β response. To test this hypothesis, the percentage of cell death by apoptosis was measured for each cell line in the various culture conditions by flow cytometry using the annexin V-FITC staining kit. The percentages of apoptotic cells for each condition are shown in Table 3.2 as are the corresponding FACS analysis (Figures 3.21-3.24). As indicated, TGF-β treatments did not lead to an increase in apoptosis in either CaSki, CaSki-Tet, CT-Xrel3, or CT-mutNLS cell lines. Also noted was the induction of wild-type Xrel3 in CT-Xrel3 cells did not alter the percentage of apoptotic cells. These results suggest that in CaSki cervical cancer cells, TGF-β can inhibit growth independently of apoptosis, and more likely through the inhibition of cell cycle progression beyond the G1 phase. In addition, the presence of Xrel3 in CaSki cervical carcinoma cells did not affect apoptosis.

Table 3.2. Percentage of apoptotic cells for each cell line following 48 hour treatments.

Cell Line	Untreated	Doxycycline	TGF-β	Dox + TGF-β
CaSki	6.31 ± 1.57	4.39 ± 1.23	4.92 ± 1.43	4.80 ± 1.80
CaSki-Tet	7.51 ± 1.92	4.59 ± 1.80	7.71 ± 2.06	4.17 ± 1.18
CT-Xrel3	5.48 ± 1.47	5.83 ± 1.84	7.45 ± 1.92	7.22 ± 2.23
CT-mutNLS	4.35 ± 0.84	4.21 ± 1.52	4.94 ± 1.69	4.71 ± 1.55

Values are the mean ± standard deviation of six individual experiments.

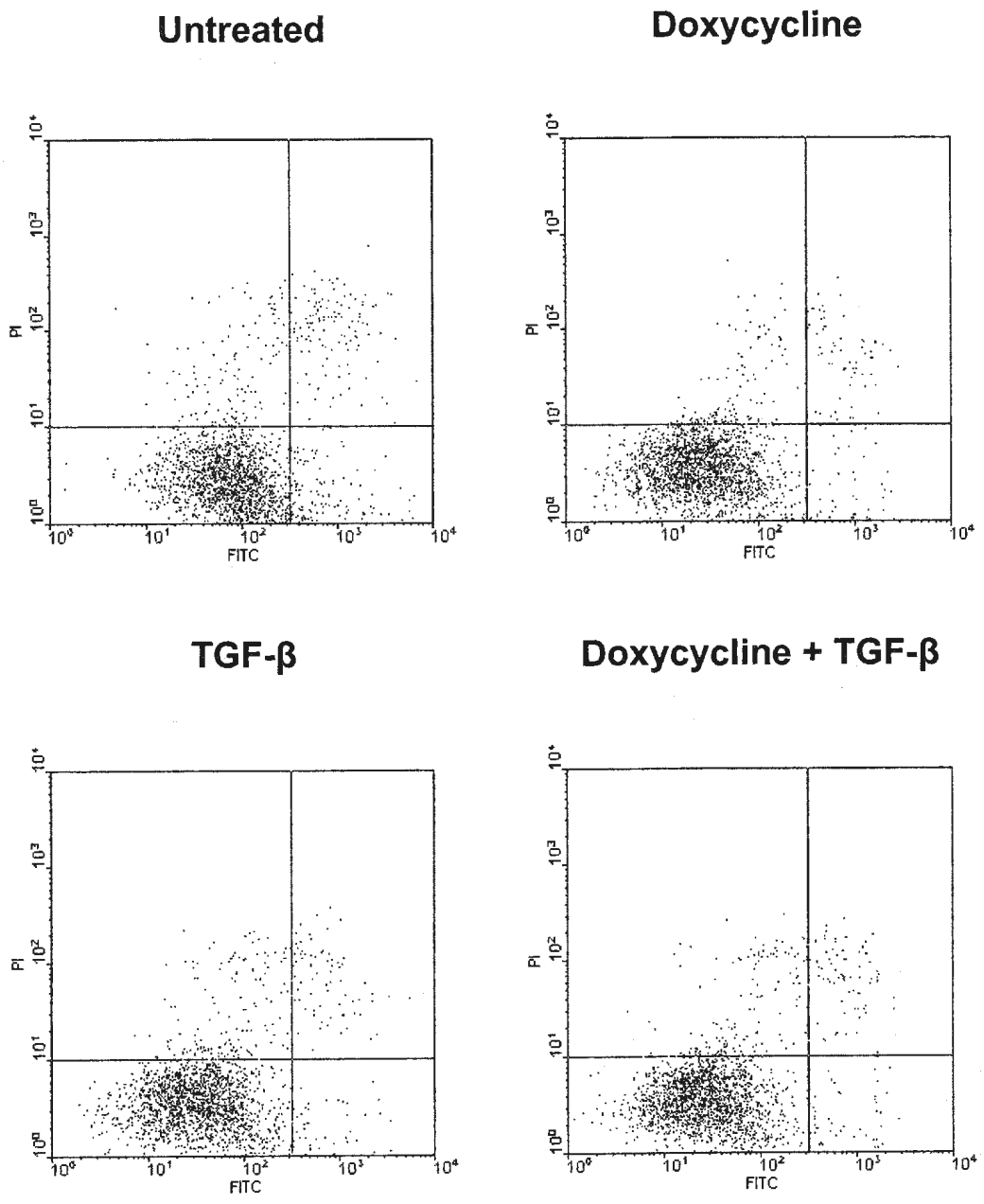

Figure 3.21 Apoptosis of CaSki cells following 48 hour treatments of either doxycycline and/or TGF-β. Apoptotic cells have high FITC and PI staining, and are shown in the upper right quadrant of each panel. Doxycycline and/or TGF-β treatments did not significantly alter the percentage of apoptotic CaSki cells.

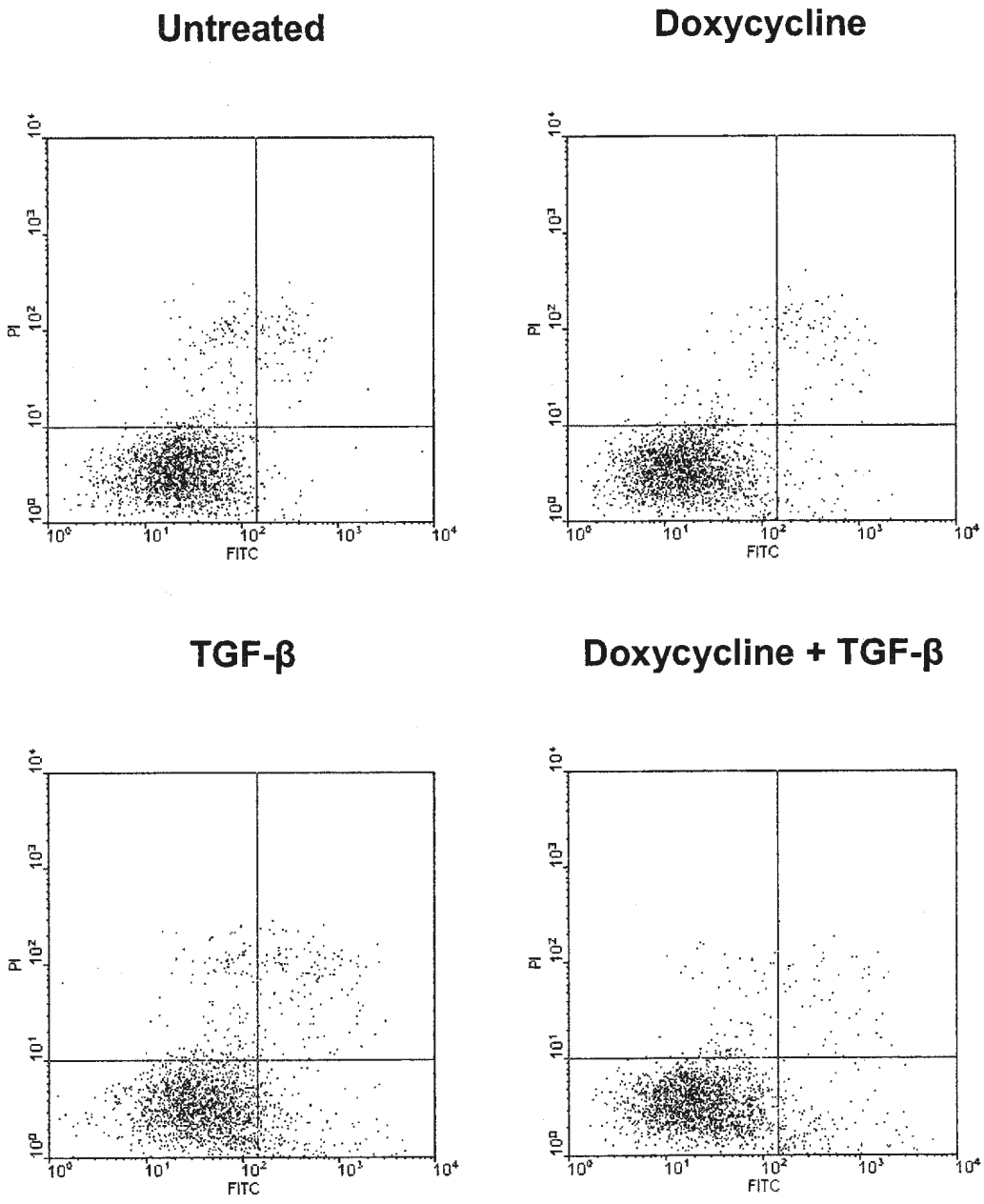

Figure 3.22 Apoptosis of CaSki-Tet cells following 48 hour treatments of either doxycycline and/or TGF-β. Apoptotic cells have high FITC and PI staining, and are shown in the upper right quadrant of each panel. Doxycycline and/or TGF-β treatments did not significantly alter the percentage of apoptotic CaSki-Tet cells.

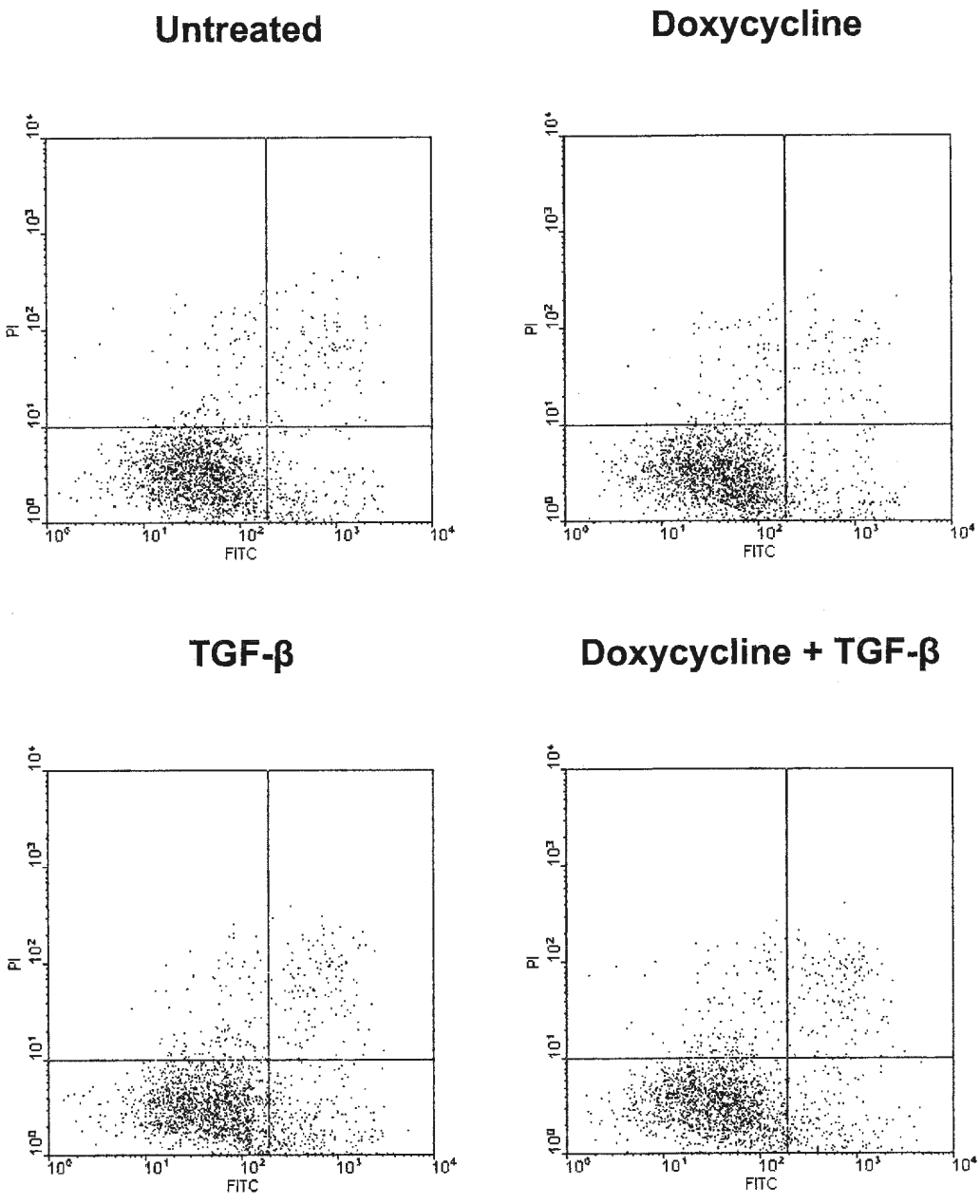

Figure 3.23 Apoptosis of CT-Xrel3 cells following 48 hour treatments of either doxycycline and/or TGF-β. Apoptotic cells have high FITC and PI staining, and are shown in the upper right quadrant of each panel. Doxycycline-induced expression of *Xrel3* and/or TGF-β treatments did not significantly alter the percentage of apoptotic CT-Xrel3 cells.

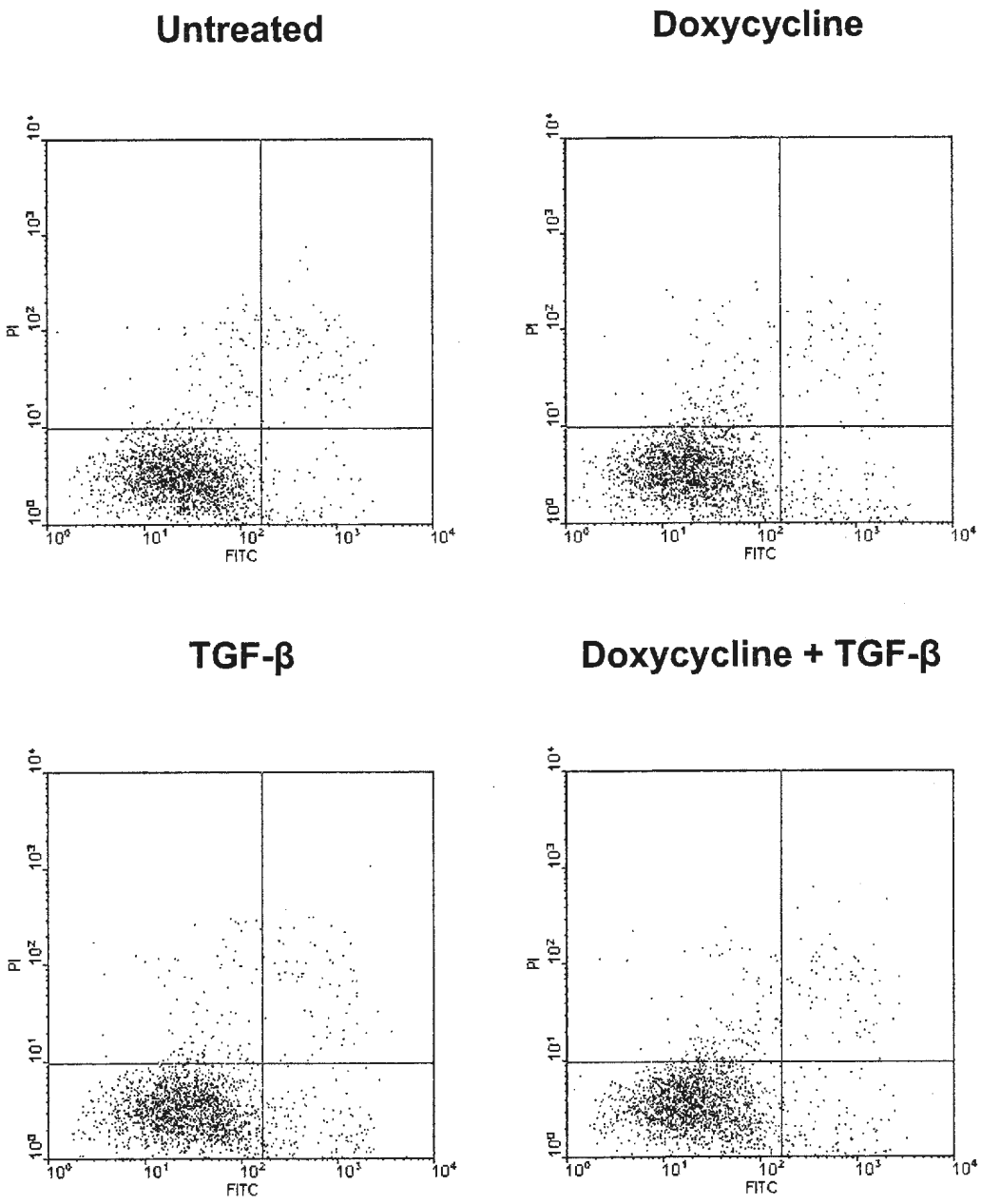

Figure 3.24 Apoptosis of CT-mutNLS cells following 48 hour treatments of either doxycycline and/or TGF-β. Apoptotic cells have high FITC and PI staining, and are shown in the upper right quadrant of each panel. Doxycycline-induced expression of *mutNLS* and/or TGF-β treatments did not significantly alter the percentage of apoptotic CT-mutNLS cells.

CHAPTER 4
DISCUSSION

4.1 Inducible expression system for Xrel3 was developed in CaSki cells

In order to study possible interactions between the Rel/NF-κB and TGF-β families of signal transduction pathways, several factors had to be considered. First, due to the complexities and ubiquitous nature of both pathways, it was paramount to choose which particular member to study. Xrel3 of the Rel/NF-κB family was chosen because its mRNA has been shown to be constitutively expressed in embryonic cells (Yang *et al.*, 1998; Lake *et al.*, 2001), and I have shown that Xrel3 localizes to the nucleus when expressed in CaSki cells (Figure 3.1). Also, recent evidence indicates that during *Xenopus* mesoderm induction, Xrel3 is involved in mediating TGF-β superfamily members such as the *Xenopus* nodal-related (Xnr) protein signaling and Xrel3 also interacts with the SMAD-associated FAST-1 co-factor (K. R. Kao, personal communication). Xrel3 contains all Rel/NF-κB motifs including the RHD, a functional NLS domain, and a potential protein kinase A phosphorylation site (Yang *et al.*, 1998). These preliminary observations suggested that Xrel3 and its NLS-mutated form (mutNLS) could be used to study potential interactions between Rel/NF-κB and TGF-β signaling mechanisms.

The cell line chosen for these experiments was the human CaSki cervical cancer cell line. From previous studies, many tumor cell lines have been found to be resistant to TGF-β treatments, commonly due to mutations in either the TGF-β receptors or mutations in SMAD signaling molecules, most notably SMAD4 (Akhurst and Derynck, 2001; Derynck *et al.*,

2001; Wakefield and Roberts, 2002). However unlike most cancer cells, CaSki cells show marked sensitivity to TGF-β treatments and have the ability to activate a number of TGF-β-responsive genes suggesting there is a complete, intact TGF-β signaling pathway (Lee *et al.*, 2001). For these reasons, CaSki cervical cancer cells were chosen for Xrel3 expression studies and its effects on TGF-β signaling pathway in my thesis.

The Tet-On system is a proven method for controlling gene expression in many model systems, including primary and transformed cell lines, and more recently in transgenic mouse models (Gossen *et al.*, 1995; Baron *et al.*, 1997; Saez *et al.*, 1997; Tremblay *et al.*, 1998; Wang *et al.*, 1999; Ahmad *et al.*, 2000; Baron and Bujard, 2000; Zhu *et al.*, 2002). A single transfected cell line can be induced to express the exogenous gene without the pleiotrophic effects that may occur by the isolation of separate cell lines for control and experimental cells. This model can therefore be used with a high degree of confidence for studying the interactions between Rel/NF-κB and TGF-β with all the benefits of an inducible gene expression system that allows for simple, straightforward interpretation of experimental results. I was able to construct an inducible CaSki cell line with both wild-type and NLS-mutated forms of Xrel3 whose expressions were regulated by the addition of doxycycline to the cell culture medium (Figures 3.6 and 3.7).

4.2 Xrel3 expression in CaSki cells

Rel/NF-κB proteins are often activated in response to antiapoptotic, pro-survival signals (Luque and Gélinas, 1997; Caamaño and Hunter, 2002). This function of Rel/NF-κB

is due to its ability to regulate the expression of several antiapoptotic genes including TRAF1, TRAF2, c-IAP1, c-IAP2, IEX-1L Bcl-x_L, and Bfl-1/A1 (Wang *et al.*, 1998; Wu *et al.*, 1998; Grumont *et al.*, 1999; Zong *et al.*, 1999; Khoshnan *et al.*, 2000). The antiapoptotic roles of Rel/NF-κB where clearly demonstrated both *in vitro* and *in vivo*. For instance, embryonic fibroblasts lacking RelA activity are significantly more susceptible to tumor necrosis factor (TNF)-induced apoptosis while $RelA^{-/-}$ mice do not survive gestation due to TNF-induced apoptosis of liver cells (Beg *et al.*, 1995; Alcamo *et al.*, 2001). Normal embryonic development is rescued when TNF is also removed from the developing mice (Doi *et al.*, 1999).

When Xrel3 was expressed in CaSki cells, I saw no apparent changes in cell morphology or growth rates when cultured in DMEM or in the presence of TGF-β (Figures 3.10 and 3.14). Further, the expression of Xrel3 did not significantly decrease the percentage of cells undergoing apoptosis (Figure 3.21 and Table 3.2). When cultured in DMEM + 10% FCS, CaSki cells are not exposed to apoptotic stimuli. Therefore, any anti-apoptotic effects due to Xrel3 expression would not normally be detected unless the cells were exposed to an apoptotic stimulus.

Following a previous report which showed HPV-16-transformed cells treated with TGF-β had increased amounts of apoptosis (Rorke and Jacobberger, 1995), I cultured all four CaSki cell lines in the presence of TGF-β in order to test whether Xrel3 had any functional antiapoptotic, pro-survival mechanisms. Contrary to this report however, all four cell lines, including the untransfected, parental CaSki cell line, did not show significant increases in

apoptosis when treated with TGF-β alone (Figures 3.8-3.15 and Table 3.2). Without significant changes in apoptosis occurring in the presence of TGF-β, any possible antiapoptotic effects of Xrel3 expression could not be determined from these experiments. In order to fully address this possible function of Xrel3, CT-Xrel3 cells should be cultured in the presence of a potent activator of apoptosis such as TNF-α before and after Xrel3 induction. The preliminary information displayed in this thesis only indicates that Xrel3 expression is not toxic to CaSki cells as suggested by the similar percentage of apoptotic cells in CT-Xrel3 cultures containing doxycycline as those that where untreated (Table 3.2).

4.3 CaSki growth suppression by TGF-β

All cell lines studied in this thesis, CaSki, CaSki-Tet, CT-Xrel3, and CT-mutNLS were susceptible to the growth suppression effects of TGF-β (Figures 3.12 to 3.15). Recently, several cervical cancer cell lines were investigated for their responsiveness to TGF-β, and those that were unresponsive, were studied to determine if this was due to a particular mutation in the TGF-β/SMAD signaling process (Lee *et al.*, 2001). In this paper, the authors state that CaSki cells are moderately responsive to the presence of TGF-β showing only a mild reduction in the rate of cell growth when using the MTS assay. My results show that all four CaSki cell lines had significantly reduced cell growth following TGF-β treatment (Figure 3.12). While both studies used the same concentration of TGF-β (2 ng/ml), the previous study only measured CaSki cell growth after three days of treatment but only presented data collected on another cervical cancer cell line, SiHa. Figures 3.12 to

3.15 show the response of CaSki, CaSki-Tet, CT-Xrel3, and CT-mutNLS for 5.5 days of continuous TGF-β treatment. It is clear from these figures that TGF-β-mediated growth suppression largely occurs in CaSki cells after the second day of treatment. Because they do not show their collected data for CaSki cell growth, it is my prediction that the authors of the previous paper simply did not measure cell growth long enough to see the full extent of the TGF-β response. After five days of TGF-β treatment, my cells did not reach confluency in the 96-well plate due to the growth suppression by TGF-β. Untreated or doxycycline-treated CaSki cells however, did reach confluency as indicated by the plateau in the growth curves of the four cell lines tested (Figures 3.12 to 3.15). CaSki cervical cells therefore, are fully responsive to TGF-β, most notable following 48 hours of treatment. It is now clear that CaSki cells have fully intact, fully functional TGF-β/SMAD signaling pathways.

CaSki cervical cells are transformed by human papillomavirus (HPV) type 16. There are currently greater than 100 types of HPV identified (Münger and Howley, 2002). HPV-16 is considered a high risk HPV as is it highly associated with cervical cancer and malignancy. HPV-16 encodes two oncoproteins, E6 and E7 that are able to transform primary cervical cells into immortalized cells (Sun *et al.*, 1992; Tsutsumi *et al.*, 1992). It has been well established that E7 proteins from high risk HPVs form complexes with several cellular proteins including the retinoblastoma protein (pRb), that is a key cell cycle regulator and tumor suppressor gene (Alani and Münger, 1998).

HPV E7 was recently reported to block TGF-β signaling and inhibition of cell

proliferation by directly inhibiting SMAD-DNA binding in HepG2 and Mv1Lu cell lines (Lee *et al.*, 2002). From the results of this paper, one would expect that CaSki cells should not be sensitive to TGF-β due to the presence of E7. In our studies and those of others (Lee *et al.*, 2001), CaSki cells were sensitive to TGF-β. It is possible that due to mutations, CaSki cells do not express E7 at adequate levels to alter SMAD-DNA binding. To date, there has been no reported evidence demonstrating E7 protein levels in CaSki cell lysates that would confirm or refute this possibility. A more likely explanation for these contradictions in TGF-β sensitivity and E7 expression is the cellular context in which these experiments were conducted. Specifically, these transfection experiments were conducted using non-cervical cells overexpressing E7, and SMADs 2,3,and 4 proteins. The authors chose to use HepG2 and Mv1Lu cells, a human liver cell line and a mink lung epithelial cell line as their *in vitro* model of HPV infection and cervical carcinoma (Lee *et al.*, 2002). The reasons for choosing these cell lines were never stated within the paper. There is also no comment on the endogenous SMAD protein levels prior to the overexpression of exogenous proteins. It is likely that the overexpression of individual SMADs alters the cellular response to TGF-β treatments. The authors conclude that their results are applicable to cervical carcinoma in that the expression of E7 is responsible for the insensitivity to TGF-β seen in many cervical tumors (Lee *et al.*, 2002). Contrary to this conclusion, Rorke *et al.* (2000) reported that cell cycle arrest of human HPV-16-immortalized cells by TGF-β was also correlated with a decrease in E6/E7 mRNA and an increase in p21 expression. My evidence leads me to conjecture that the TGF-β growth inhibition of CaSki cells, presented both in this

thesis and by others (Lee *et al.*, 2001), is a more accurate description of cervical cancer. The roles of HPV proteins in the TGF-β signaling mechanism remain unclear. It is therefore obvious that more research is required to fully understand the effects of HPV infection on TGF-β/SMAD signaling.

4.4 Cell cycle arrest by TGF-β is inhibited by Xrel3 in CaSki cells

Epithelial cell proliferation can be regulated by TGF-β. Cells respond to TGF-β ligand binding by arresting the cell in the G1 phase of the cell cycle. This is accomplished by at least four TGF-β-specific responses including the inhibition of c-myc, the induction of $p15^{INK4B}$, the induction of $p21^{CIP/WAF1}$, and the inhibition of cdc25A (ten Dijke *et al.*, 2002). These mechanisms (Figure 3.18) are ligand-specific and tissue-specific responses that are unique to each cell condition and differentiation stage. My present study examined the effects of Xrel3 on only two of these pathways, the p21 and p15 tumor suppressor protein activation pathways. The induction of both p15 and p21 occurs via SMAD-dependent signaling (ten Dijke *et al.*, 2002). The mechanism of c-Myc and cdc25A inhibition by TGF-β is still unclear however, it does involve an adapter protein, the Myc-interacting zinc-finger protein 1 (Miz-1) that negatively regulates TGF-β signaling (Seoane *et al.*, 2001; Staller *et al.*, 2000) which may not involve the SMAD signaling molecules.

As was shown in Figure 3.18, p15 is a cdk4-specific inhibitor and therefore functions early in the G1 phase of the cell cycle. The cdk2-specific inhibitor p21 functions later in the G1 phase and into the S phase. These temporal differences in activation of cdk inhibitors

may account for the variations in p15 and p21 observed in my studies in response to Xrel3 expression. As a first possibility, my data suggested that Xrel3 was able to prevent p21 induction and block growth inhibition, whereas it had no effect on p15 (Figure 3.16). This could suggest that Xrel3 may act only on transfected CaSki cells during later phases of the G1/S transition. Secondly, Xrel3 may alternatively be acting on a specific SMAD protein that is used only in the induction of p21 but not of p15. Since various combinations of SMAD proteins as dimers are used to carry signals to the nucleus, it is possible that p21 induction and p15 induction have distinct SMAD dimers and that Xrel3 is only able to interact with the p21-specific dimer (Figure 4.1). A third possible explanation is that Xrel3 blocks the binding of an obligate transcription factor to the specific SMAD dimer. As depicted in Figure 1.3, the binding of SMAD proteins requires an auxiliary protein (for example, Fast-1) to enhance DNA binding and transcriptional activation. The upregulation of p15 and p21 cyclin-dependent kinase inhibitors by TGF-β leads to the specific inhibition of cyclinD-CDK4/6 and cyclinE-CDK2-dependent phosphorylation of retinoblastoma protein (pRb), respectfully (ten Dijke *et al.*, 2002). The upregulation of both of these inhibitors (Figure 4.1) occurs via Sp1 binding sites (Datto *et al.*, 1995; Li *et al.*, 1995). It is therefore conceivable that Xrel3 either competes for SMAD binding with Sp1 or Fast-1 transcriptional regulators or is indirectly responsible for blocking the formation of these complexes.

The results of this project show that Xrel3 is able to inhibit the TGF-β-mediated

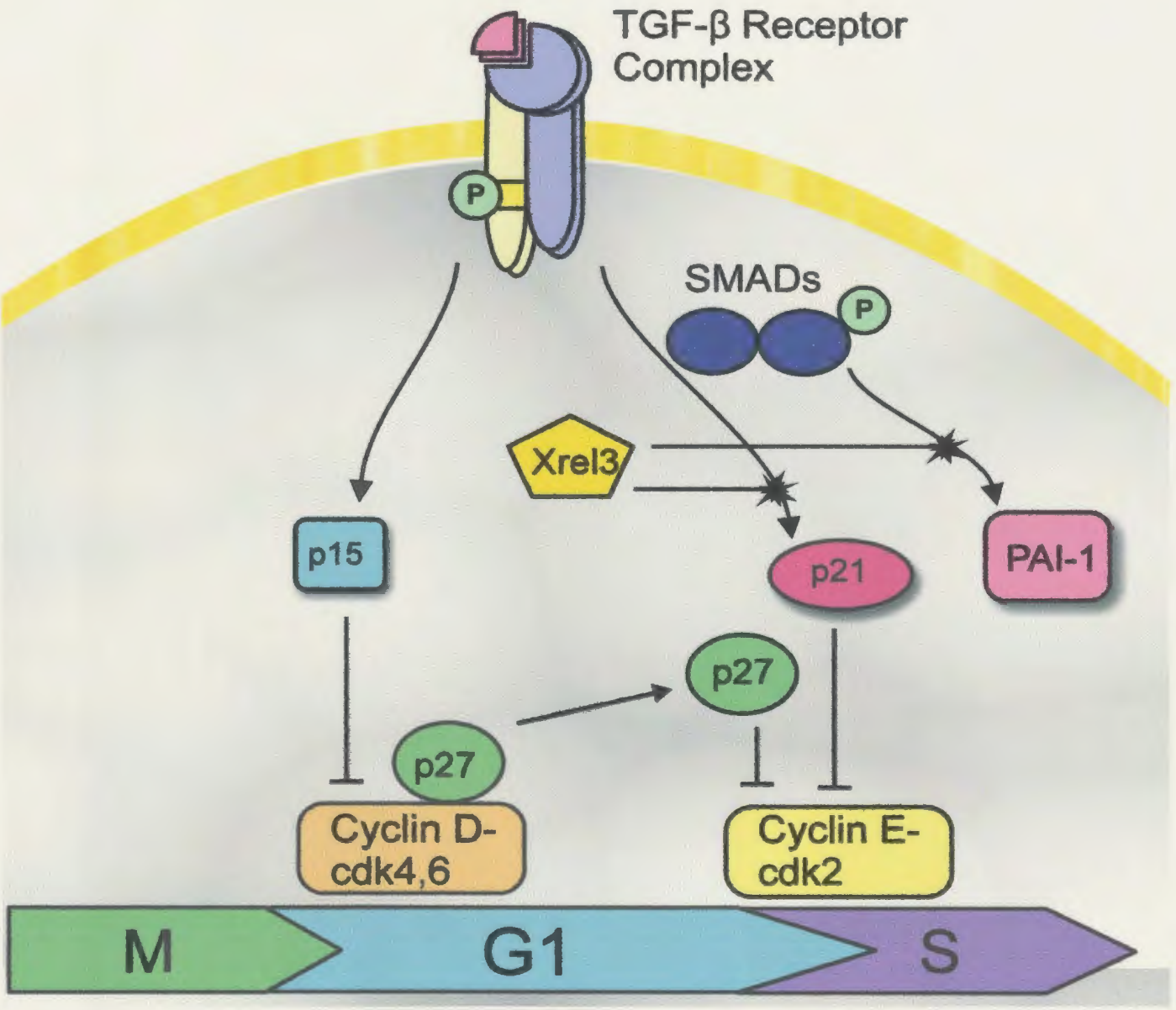

Figure 4.1 Potential actions of Xrel3 in preventing growth arrest by TGF-β and its effects the expression of TGF- β markers. Based on the results of this thesis, Xrel3 is likely functioning later in G1-S phase transition where it is able to interfere with the induction of p21, but not p15. These specific interactions may involve SMAD3/SMAD4 complexes binding to DNA directly, or may require other co-transcriptional suppressors or activators such as FAST-1.

induction of p21 but not of p15. From the growth curves of CT-Xrel3 and control cell lines, cell proliferation is halted following TGF-β treatment such that after five days of treatment, less than 50% of untreated cells are present. However, when Xrel3 was present, cell proliferation rates are restored to that of the untreated cell lines in the presence of TGF-β. Due to the quick recovery of cell growth, it was predicted that Xrel3 was able to prevent all of the TGF-β-dependent mechanisms of cell cycle arrest. However, my results indicate that $p15^{INK4B}$ induction is unaffected by the presence of Xrel3. Therefore, Xrel3 appears to act in a specific manner. The current data simplifies the possible mechanism of Xrel3 interaction to that shown in Figure 4.1. Much more research is required to further characterize the specific interactions between TGF-β signaling molecules and Rel/NF-κB and to fully understand how these antagonistic pathways are balanced within normal and cancer cells using conditions of abnormal expression. Therefore this cell model system will be useful for conducting such experiments as outlined in the following sections.

4.5 Future experiments with inducible Xrel3 CaSki cell model

4.5.1 Does Xrel3 affect other TGF-β-inducible cell cycle regulators?

Western blot analysis should be utilized to further investigate the interactions between Xrel3 and TGF-β/SMAD signaling. Based on the results shown in this thesis, it seems that Xrel3 is not able to affect all cell cycle regulators downstream of TGF-β signaling. It would therefore be advantageous to determine if Xrel3 can block the actions of TGF-β signaling on other known TGF-β responsive genes such as c-Myc, p27, and

cdc25A levels.

The cell cycle regulator c-Myc normally functions to prevent p15 activity and therefore allows regular cell cycle progression (Gartel and Shchors, 2003). Upon TGF-β treatment, c-Myc levels decrease, resulting in p15 activation which then contributes to arresting the cell in G1 phase. It could be hypothesized that Xrel3 is unable to affect c-Myc based on this function. It has been shown by others that c-Myc also functions to inhibit TGF-β-induced p21 expression (Seoane *et al.*, 2002). My results demonstrate that Xrel3 can interfere with p21, and it may also interfere with c-Myc-specific inhibition of p21 activity. Therefore it warrants further study into the effects of Xrel3 expression on c-Myc protein in the presence of TGF-β.

The cdk tyrosine phosphatase enzyme cdc25A, normally controls cell cycle regulation by maintaining p27 in an unphosphorylated state (Nagahara *et al.*, 1999). When TGF-β is present, the activated receptor complex inhibits cdc25A function possibly through the SMAD proteins. When this occurs, p27 is activated and is recruited to cyclin E-cdk2 complexes causing a reduction in cell cycle progression. It would be important to determine if Xrel3 is able to prevent the inhibition of cdc25A activity and in addition, determine if Xrel3 can block the formation of p27/cyclin E/cdk2 complexes. These experiments would be beneficial for confirming this novel function of Rel/NF-κB proteins.

4.5.2 Does Xrel3 affect other SMAD signaling molecules?

The abilities of TGF-β to control cellular functions and gene expression are largely

dependent on the SMAD family of proteins (ten Dijke *et al.*, 2002). The results displayed here show that Xrel3 cannot prevent SMAD2 phosphorylation. It is now compelling to further investigate the status of phosphorylated SMAD2 in these cells to determine if SMAD2 is still active. This experiment could be conducted using immunocytochemistry to analyze the cellular localization of phosphorylated SMAD2.

The expression levels of SMAD3, phosphorylated SMAD3, and SMAD4 should be analyzed to determine if activation of these SMAD proteins are affected by Xrel3. As noted, SMAD3 is another member of the R-SMADs that can be activated following TGF-β ligand-binding while SMAD4 is the only known co-SMAD in human cells (ten Dijke *et al.*, 2002). For a complete understanding of the actions of Xrel3 on TGF-β signaling in CaSki cells, it would be necessary to consider the expression of these other SMAD signal transducers. For instance, TGF-β activation of *PAI-1* transcription likely involves a SMAD3/SMAD4 dimer (Song *et al.*, 1998) while the activation of p21 involves SMAD3/SMAD4 in concert with the Sp1 transcription factor (Pardali *et al.*, 2000). In astrocytes, the induction of p15 by TGF-β is dependent on SMAD3 signaling and may also be responsible for its induction in cervical epithelial cells (Rich *et al.*, 1999). Cellular localization and Western blot experiments would therefore be beneficial for examining the specific SMAD activation occurring in these cell lines.

SMAD7 levels could not be detected in these CaSki cell lines by Western blot. SMAD7 is an inhibitory SMAD that is involved in a negative-feedback loop that silences the TGF-β signal transduction once the stimulus is removed (Moustakas *et al.*, 2001). It is

possible that CaSki cells simply do not express SMAD7 due to mutations within its gene sequence or that Western blot analysis is not sensitive enough to detect its expression. The other known inhibitory SMAD, SMAD6, may also be key within these cell lines due to the inability to detect SMAD7 expression. Assessing the expression levels of SMAD7 and SMAD6, and also their translocation events following Xrel3 expression, are other key experiments that would help determine the specific function that Xrel3 is performing in these cell lines and possibly in tumor specimens.

Recently, growing evidence of SMAD-independent transduction of TGF-β signals in various cell and tissue types has emerged in the literature. For instance, TGF-β1 decreases the secretion of glycoproteins in adrenocortical cells by activating c-Jun and MEKK signaling (Lafont *et al.*, 2002); activates the p38 MAPK pathway in mouse mammary epithelial cells (Yu *et al.*, 2002); and simultaneously activates JNK activity in Mv1Lu cells apart from SMAD molecules (Engel *et al.*, 1999). In addition, another TGF-β superfamily member BMP-2 can induce apoptosis in human osteoblasts via the protein kinase C pathway (Hay *et al.*, 2001). Therefore, it is conceivable that Xrel3 may also be influencing a SMAD-independent signaling mechanism within transfected CaSki cells since SMAD2 phosphorylation in Xrel3-expressing cells and the parental CaSki cells . This point emphasizes the need to further characterize precise interactions between Rel/NF-κB and TGF-β family members that may be occurring in these cells and many other, different cell lines and tissue specimens.

4.6 Future Directions

The studies described in this thesis were initiated based on observations that many tumors have constitutively active Rel/NF-κB and insensitivity to TGF-β treatments. The results shown in this cell model system now suggests that these observations may be more than just correlative. Instead, my findings provide preliminary evidence that there can be a direct association between the Rel/NF-κB and TGF-β pathways and more importantly, they provide a precedent for further studies of this relationship. To this extent, a number of studies can be proposed. Firstly, to further characterize the exact mode of interaction between Xrel3 within this CaSki cervical cell system on blocking TGF-β signaling, recent technologies including pathway-specific gene array analysis (GEArray, SuperArray Bioscience Corporation) can be used to examine changes in the expression of genes closely related to Rel/NF-κB and TGF-β signaling pathways. Secondly, various other cancer cell lines that are known to have either constitutive Rel/NF-κB activity or TGF-β insensitivity can be examined. It is my hypothesis that a correlation exists between these two events in various available cell lines. Thirdly, it will be interesting to survey various tumor specimens from clinical samples to characterize the status of Rel/NF-κB and TGF-β pathways and determine if abnormally active Rel/NF-κB is responsible for TGF-β insensitivity in tumors that develop resistance to its growth inhibitory effects.

To investigate how Xrel3 is able to prevent normal CaSki cell growth inhibition by TGF-β treatment, pathway-specific gene array kits could be used (SuperArray Bioscience Corporation). RNA from untreated and doxycycline-induced CT-Xrel3 cells grown in the

presence or absence of TGF-β is extracted and hybridized to membranes blotted with 96 cDNAs of genes closely related to either the Rel/NF-κB or the TGF-β/SMAD signaling pathways. Following autoradiography, changes in gene expression are quantified by densitometry to highlight genes that are either significantly down-regulated or up-regulated in the presence of Xrel3 protein. The identification of those genes significantly affected by the presence of Xrel3 will lead to a better understanding of the specific mechanisms of action that Xrel3 has on modifying TGF-β signaling. These key findings may eventually lead to possible areas of therapeutic intervention in various tumors that no longer respond to TGF-β treatments.

The second major research focus area arising from this thesis is to survey the status of Rel/NF-κB and TGF-β-responsive cancer cell lines. Each cell type could be first assayed for constitutive Rel activity using immunocytochemistry. Cells can be probed with primary antibodies specific for various Rel family members, then incubated with the appropriate fluorescence-labeled secondary antibody to visualize the location of the Rel protein in normal, non-stimulated culture conditions. Cell lines showing nuclear localization of Rel proteins in normal medium can be identified as having constitutively active Rel/NF-κB. The same cell lines could also be cultured with TGF-β to see if there is a correlation between constitutive Rel/NF-κB activity and insensitivity to TGF-β. The results of this thesis predict that such a correlation is highly possible. There have been no studies to date however, which can prove or disprove such an hypothesis. Cancer cell lines that prove to be insensitive to TGF-β and do not show growth inhibition can be treated with various known inhibitors of

Rel activity. It is my prediction that these cells will subsequently respond to the TGF-β treatments once Rel activity has been blocked. If this prediction holds, then more clinical studies could be initiated using common Rel inhibitors such as the non-steroidal anti-inflammatory drugs, that could be used in combination with TGF-β to enhance the therapeutic benefits of cancer treatments involving tumors of epithelial origin.

The third new focus evolving from this present study is to investigate these interactions in pathological conditions. Various tumor sections and adjacent normal tissues from various cervical cancer patients can be analyzed by immunohistochemistry, differential gene array analysis, and Western blotting to determine whether Rel/NF-κB activity blocks TGF-β mediated growth inhibition in clinical specimens. Biopsies taken from pre- and post-chemotherapy can be assessed for constitutive Rel activity and correlated to the tumor responsiveness to TGF-β. Again, our present model predicts that Rel activity is blocking normal responses to TGF-β, not only *in vitro* but also *in vivo*.

In summary, my preliminary findings suggest that Rel/NF-κB regulates TGF-β signaling in human cervical cancer cells and provide the basis for further molecular and biological studies involving these pathways.

CHAPTER 5
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