IDENTIFICATION OF PEA3 TARGET GENES IN HUMAN CELLS

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IN HUMAN CELLS

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ABSTRACT

Mouse PEA3 is the founding member of the PEA3 subfamily of ETS transcription factors that includes ERM and ER81. Numerous studies implicate PEA3 subfamily members in a diversity of human cancers, especially breast cancer. Dominant-negative PEA3 (ΔNPEA3En) effectively represses activated transcription by all three PEA3 subfamily members. When expressed under control of the MMTV promoter, ΔNPEA3En significantly delays the appearance of mammary tumors and reduces their number and size in mouse models of HER2 mediated breast cancer. In addition, ΔNPEA3En is not expressed in the mammary tumors that do develop in these mice. These findings strongly suggest a required role for PEA3 subfamily members or other ETS proteins with similar DNA binding specificity in HER2-mediated oncogenesis. The primary objective of this research was to identify the PEA3 subfamily target genes that could play a role in the initiation and progression of tumors, specifically in the breast. To achieve this, a recombinant adenovirus carrying ΔNPEA3En was constructed to express ΔNPEA3En in three human mammary tumor cell lines: MDA-MB-468, BT-549 and MDA-MB-361. Gene expression analysis using Affymetrix® GeneChip® technology identified a common set of 39 downregulated and 2 upregulated genes in cells expressing ΔNPEA3En compared to control cells in all three tumor cell lines. Differentially expressed genes included some that have been shown to play key roles in tumorigenesis such as activating transcription

factor 3, heat shock 70kD protein 1A and interleukin-8. In addition one colon carcinoma cell line, SW620, was used for gene expression analysis and 7 genes identified in the mammary tumor cell lines were also identified in the colon carcinoma cell line. The results suggest a role for PEA3 subfamily genes in a multiple human cancers mediated through a small subset of common target genes. The genes identified as being differentially expressed by ΔNPEA3En hold potential value not only as targets for therapeautic drug discovery, but also as diagnostic or prognostic markers for human cancers, specifically breast cancer.

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CHAPTER 1: INTRODUCTION

1.1 The ETS family of transcription factors

1.1.1 Protein structure and function

ETS proteins are a related family of transcription factors found exclusively in metazoan organisms. These proteins are involved in such biological processes as growth, differentiation, development, apoptosis and oncogenic transformation (reviewed in [1]). Currently, in mammals, the ETS family includes at least 27 genes homologous to *Ets-1*, the founding member. *Ets-1* was the first characterized cellular homologue of the *v-ets* viral oncogene found in the avian transforming retrovirus E26 (**E**-**t**wenty-**s**ix) [2]. Although the majority of ETS family members are transcriptional activators, several are known to repress transcription.

All ETS family members contain an evolutionarily conserved DNA binding domain of approximately 85 amino acids called the ETS domain which recognizes the core sequence motif 5'-GGAA/T-3' [3]. NMR studies have revealed the ETS domain to have a winged-helix-turn-helix structure [4-6]. While all ETS factors recognize the core motif, DNA binding specificity among ETS factors is conferred by sequences flanking the ETS domain, protein modification, expression pattern, and interactions with protein co-factors [1, 7]. ETS family members are further divided into subfamilies based on sequence similarity within the ETS domain, the position of the ETS domain, the occurrence of additional conserved domains and overall gene architecture [8]. Some ETS factors also possess domains which positively or negatively regulate DNA binding [9].

1.1.2 Normal role of ETS factors

Several ETS factors are known to be nuclear targets of signal transduction cascades, in particular, the MAPK (mitogen-activated protein kinases) signaling cascade, and are phosphorylated in response to growth factors and various cytokines [7, 10]. Modification by SUMO (small ubiquitin-related modifier) has also been shown for ETS factors TEL and ERM [11, 12]. Resulting posttranslational modifications of ETS factors may change attributes such as DNA binding, transcriptional regulatory activity, protein-protein interactions, stability and subcellular localization [7, 13]. Posttranslational modification of TEL by SUMO affects its localization into cell-cycle-specific nuclear regions termed TEL bodies [11]. The leukemia-associated fusion protein TEL/AML1, which is also modified by SUMO, is also found in TEL bodies in a pattern distinct from the pattern observed for AML1. This suggests that SUMO-modification of TEL is important for both normal and abnormal function. In the case of ERM, modification by SUMO inhibits its transcriptional activity [12]. Other posttranslational modifications such as glycosylation and acetylation have also been described for ETS factors (reviewed in [13]). Many modifications are targeted to the same site and may thus compete and act in opposing or reciprocal ways. This, combined with the potential for multiple targets on the same protein, allows for complex regulation.

The expression pattern of ETS factors ranges from some members being ubiquitously expressed to others being expressed in a tissue-specific manner [14]. Tissue-restricted and temporal-expression likely contribute to functional specificity of certain ETS factors. For example, Ets-1 is expressed in all organs of day-15 mouse

embryos [15]. At later fetal stages, Ets-1 is primarily expressed in the brain, lymphoid tissues and the mesodermal cells of organs undergoing morphogenesis. During neonatal development, Ets-1 expression is restricted to lymphoid organs and the brain. In adults, high expression of Ets-1 is further restricted to lymphoid tissues.

Biological roles for some ETS factors have been evidenced by both *in vitro* and *in vivo* techniques. Mammalian cell culture systems have indicated multiple roles for several ETS factors. Members of the TCF subfamily of ETS factors participate in the regulation of proliferation [16]. Ets-2 has been shown to inhibit apoptosis [17]. Regulation of cellular senescence has been attributed to both Ets-1 and Ets-2 [18].

The generation of knockout mice for *Ets* genes has proven to be a valuable tool to elucidate ETS function. Of the 26 murine *Ets* genes, knockouts for 13 have been described [19]. *Ets-1* knockout mice show increased apoptosis in T-cells and induced terminal differentiation in B-cells [20, 21]. *Pu.1* knockout embryos have reduced multipotential lymphoid-myeloid progenitors [22, 23]. *Fli-1* mutant mice have also been shown to have impaired hematopoiesis and reduction of T-cells [24, 25]. *Ets-2*-null mice show impaired extracellular matrix remodeling and trophoblast function, and as a consequence impaired placenta formation [26]. *Tel* knockout mice show disrupted yolk sac angiogenesis and increased apoptosis in mesenchymal and neural cells [27]. In contrast, *Elk-1* knockout mice show no phenotypic effect save for mild reduction of neuronal gene activation, suggesting redundancy or compensatory mechanisms [28]. *Pea3*-null mice present no overt phenotype, however *Pea3*-null males were unable to reproduce [29].

1.1.3 Role of *ETS* genes in cancer

ETS factors have been implicated in cancer since the first identification of Ets-1 in the E26 retrovirus. Inappropriate expression of ETS factors, either by occurrence as fusion proteins resulting from translocations/rearrangements or by misregulation, leads to the inappropriate expression of target genes, disturbing and deregulating physiological processes contributing to the development of both leukemias and solid tumors.

Nearly all leukemias are characterized by a translocation resulting in a fusion protein. More than twenty translocations involving the *ETS* gene *TEL* resulting in several different types of leukemias have been reported [30]. Fusions of *TEL* to the gene for a number of tyrosine kinases such as platelet-derived growth factor receptor β (*PDGFRβ*) [31] and fibroblast growth factor receptor 3 (*FGFR3*) [32] have been described. In this case regions of *TEL* allow self-association while regions of the dimerized tyrosine kinases become activated, contributing to transformation [30]. A second type of fusion involving *TEL* occurs by fusion with *AML1*. The resulting chimeric protein competes with endogenous AML1 for AML1 target genes, thereby acting as a transcriptional repressor [33]. Another example of *ETS* gene fusions involved in cancer is provided by EWS (**Ew**ing **s**arcoma), an RNA-binding protein. EWS is characteristically found as a fusion with ETS proteins FLI-1 or ERG in Ewing's sarcomas and neuroectodermal tumors of childhood, respectively [34, 35]. Interestingly, there is evidence that EWS/FLI-1 and FLI-1 may regulate different sets of genes [36] although the ETS domain is essential for the activity of both.

Overexpression of several ETS factors has been found in various tumors. ETS-1 is up-regulated in breast cancer [37], gastric cancer [38], ovarian cancer [39] and hepatocellular carcinoma [40]. The ETS factor ESE-1/ESX is overexpressed in breast cancer [41]. In most cases, ETS factor expression levels correlate with tumor progression. Experiments in mammalian cell culture systems have reinforced the *in vivo* observations pertaining to the role of ETS factors in human cancers. Hepatoma-derived cell lines transduced with ETS-1 show increased expression of MMP-7 [42]. Furthermore, the promoter of MMP-7 was shown be transactivated by ETS-1. Ras-MAPK-mediated activation of Ets-1 and Ets-2 transactivates the *JunB* promoter [43]. Ets-2 also activates the human *cyclin D1* promoter in human trophoblasts [44].

1.2 PEA3 subfamily

1.2.1 PEA3 subfamily structure and function

Mouse Pea3 was initially identified as an activity that bound to and activated transcription from the polyomavirus enhancer [45, 46]. PEA3 (E1A-F/ETV4) [46, 47] is the founding member of the PEA3 subfamily of *ETS* genes. The PEA3 subfamily contains three members: PEA3, ERM (ETV5) [48, 49], and ER81 (ETV1) [50, 51]. The gene encoding each PEA3 subfamily member is found on a different chromosome and each is composed of 14 exons equivalent in size and each encoding similar sequences relative to each protein [52, 53]. PEA3, ERM and ER81 are more than 95% identical in the ETS domain, more than 85% identical in the amino-terminal acidic domain, and nearly 50% identical overall [54]. Multiple domains that regulate transactivation and DNA binding of mouse and zebrafish Pea3 have been mapped [9, 55]. The amino-

terminal acidic domain of mouse pea3 acts as a strong activation domain and is flanked by regions that independently inhibit its activity. Likewise, regions flanking the ETS DNA binding domain of mouse Pea3 inhibit DNA binding. Similar regions have been found in all PEA3 subfamily members as well as other ETS proteins [56-60]. It is currently unknown how the autoinhibition of transactivation and DNA binding is alleviated to allow PEA3 to activate transcription. *In vitro* phosphorylation experiments demonstrate that the transcriptional activity of human ERM and ER81 is increased by phosphorylation via a conserved serine residue near the ETS domain [61].

1.2.2 Role of PEA3 subfamily members

During murine embryonic development, Pea3 subfamily members show both distinct and overlapping expression patterns in several developing organs with Pea3 and Erm showing significant coexpression, suggesting differential regulation and redundant as well as specific roles [49, 62]. Pea3 is predominantly expressed in the brain, testis and epithelial cells in organs undergoing branching morphogenesis during development such as lung, salivary gland, kidney, and mammary gland [46, 63]. To suggest a role in branching morphogenesis, the overexpression of Pea3 and Erm in mouse TAC-2.1 mammary epithelial cells enhances the formation of duct-like structures when grown in collagen gels [63]. In the developing chick, Pea3 and Er81 show the same expression pattern in specific neurons [64]. *Er81*-knockout mice show a lack of connections between sensory and motor neurons [65], while *Pea3*-null mice show male sexual dysfunction which may be attributable to neuronal defects [29]. Pea3 has also been shown to play a role in myogenic differentiation in adult murine myoblast cells [66].

Pea3 subfamily members are potential transcriptional targets of FGF-signaling and likely transcriptional effectors, mediating FGF-signaling [67, 68]. Pea3 and Erm are targets of FGF-signaling involved in neuronal development in zebrafish and their expression is tightly coordinated with that of Fgf3 and Fgf. FGF-signaling has been shown to be necessary and sufficient for expression of Pea3 and Erm in the developing nasal region of chicks [69]. Also, Pea3 and Erm have been shown to be necessary and sufficient for and to mediate the expression of the tendon-specific transcription factor, scleraxis (*Scx*) in response to FGF-signaling [70].

1.2.3 PEA3 subfamily members and cancer

PEA3 subfamily members have been shown to be involved in multiple cancer types. Ewing's sarcomas involving fusion to the *EWS* gene have been described for several *ETS* genes including *ER81* [71] and *PEA3* [72, 73]. *In vivo*, PEA3 expression has been correlated to breast cancer [74], gastric cancer [75], colorectal cancer [76, 77], ovarian cancer [78], non-small cell lung carcinoma [79] and oral squamous cell carcinoma [80].

A large number of reports have linked PEA3 expression to known oncogenes and matrix metalloproteinases with known involvement in cancer, both *in vitro* and *in vivo*. *In vitro*, PEA3 can induce the human MMP-3 gene in response to the tumor promoting compound 12-O-tetradecanoylphorbol-13-acetate (TPA) [81]. In transient expression assays PEA3 has been shown to activate the promoters of MMP-1, MMP-3 and MMP-9 [82]. In oral squamous carcinoma cells PEA3 expression correlates with that of MMP-1 and MMP-9 [83]. Transfection of PEA3 into the weakly invasive human breast cancer

cell line, MCF7, was able to induce an invasive phenotype and up-regulate MMP-9 expression [84]. Furthermore, antisense *PEA3* RNA reduced the invasive potential and expression of *MMP-1*, *MMP-3*, and *MMP-9* in HSC3 cells, an oral squamous-cell-carcinoma-derived cell line expressing high levels of PEA3, MMP-1, and MMP-9 [85]. In human breast tumor samples, PEA3 expression is positively correlated to MMP-2 expression and tumor aggressiveness [74]. Pea3 can transactivate the promoter for the Wilms' tumor suppressor gene 1 (*WT1*) [86], which is overexpressed in some human breast cancers [87, 88].

The involvement of PEA3 in signaling pathways relevant to cancer has been suggested. *PEA3* RNA is overexpressed in 76% of human breast tumors and in 93% of the HER2-positive subclass of these tumors, which represents 20-30% of all breast cancers [89]. The *HER2/neu* protooncogene is a prognostic marker of human breast, ovarian and gastric cancer [90]. In fact, all three Pea3 subfamily members have been shown to be coordinately overexpressed in Her2-induced mouse mammary tumors [91] as well as in a majority of human tumor cells lines [92]. Pea3 has been found to be up-regulated in response to Wnt1 signaling and to activate cyclooxygenase-2 (Cox-2) in mouse mammary tumors [93]. *Wnt1* is a mammary oncogene, signaling through which stabilizes β-catenin and subsequently leads to transcriptional activation by β-catenin/TCF. *COX-2* has been found to be overexpressed in several human cancers and its expression linked to PEA3 in early stage colorectal carcinogenesis in humans [94]. Interestingly, cyclin D1, c-myc and MMP-7 have been shown to be directly activated by β-catenin [95-97]. PEA3 subfamily members synergistically cooperate with C-JUN, β-

catenin and LEF-1 to regulate the activity of the *MMP-7* promoter [98] and the osteopontin promoter [99], both of which are involved in mammary development, oncogenic transformation and metastasis. PEA3 expression has been correlated with MMP-1 and MMP-7 in human colorectal tumors [76]. The expression of both *MMP-7* and *COX-2* mRNA correlated with that of PEA3 in early stages of human colorectal cancer [77]. The angiogenesis promoting factor inducible nitric oxide synthase (iNOS) has been shown to mediate the induction of COX-2 by PEA3 in colorectal cancer [94, 100]. Hepatocyte growth factor (HGF), a factor thought to play a role in motility and invasion, has been shown to stimulate the expression of PEA3, which correlated to an increase in *MMP-1*, *MMP-3* and *MMP-9* mRNA [79, 101]. In transient transfection assays PEA3 has been shown to activate transcription of p21$^{wafl/cipl}$, a key cell cycle regulatory protein, in response to genotoxic stress [102].

1.3 Dominant-negative PEA3 (ΔNPea3En)

The method of examining ETS factor function by interfering with endogenous ETS factor activity has been used with techniques such as antisense oligos, RNA interference, overexpression of ETS factors that endogenously act as transcriptional repressors, and the use of dominant-negative mutants (reviewed in [14]). Interference methods circumvent the potential problem that overexpressed wild-type transcription factors may not be in their active state and may lack necessary cofactors needed to function properly. The DNA-binding domain of Ets-2 along with the nuclear localization signal but lacking sequences for transactivation fused to the prokaryotic *lacZ* gene (Ets-LacZ) expressed in murine 3T3 cells suppressed a transformed phenotype induced by the

v-ras oncogene [103]. The same Ets-2 transdominant mutant expressed in BT20 breast carcinoma cells completely inhibited anchorage-independent growth and CSF-1-induced invasion [104] and induced apoptosis in human thyroid carcinoma cell lines but not in normal thyroid cells [105]. An Ets-1 mutant (Ets1-DB) lacking the activation domain was constitutively expressed in murine brain capillary endothelial cells resulting in a phenotype of increased spreading and adhesion and a greater capacity to form branched structures when cultured on Matrigel [106]. Ets1-DB was able to inhibit both normal and tumor FGF-2-induced angiogenesis in a mouse ear model [107].

Our lab has constructed a dominant-negative version of Pea3 (ΔNPea3En) consisting of the mouse Pea3 DNA-binding domain (amino acids 334-480) fused to the *Drosophila* engrailed repression domain (amino acids 2-298) [91]. The region of Pea3 present contains the ETS DNA binding domain but lacks the activation domain [9]. The region of Engrailed present acts as a strong repressor of activated transcription when fused to a heterologous DNA binding domain [108-110]. The strategy of studying transcription factor function with Engrailed fusion proteins (within the context of inducible expression systems) has been reviewed and offers benefits over alternative methods such as the ability to create stable cell lines expressing potentially cytotoxic proteins [111]. ΔNPea3En has been shown to repress the capacity of Pea3, Erm and Er81 to transactivate a Pea3-responsive reporter in transient transfection assays and, when expressed under the control of the MMTV promoter in MMTV-*neu* transgenic mice, delayed the onset and reduced the size and number of mammary tumors [91]. Interestingly, ΔNPea3En is not expressed in the mammary tumors that do form in these

transgenic mice, suggesting a requirement for Ets activity for tumorigenesis. Furthermore, ΔNPea3En was able to repress Pea3-mediated transactivation of the human *CYCLIN D1* promoter in transient transfection assays (Mesier, in preparation).

1.4 Ecdysone inducible expression system

The ecdysone-inducible system is based on the molting system of *Drosophila melanogaster*. The insect hormone ecdysone (or analogs) can activate transcription in mammalian cells that express the *Drosophila melanogaster* ecdysone receptor (EcR) and a functional binding partner together with a promoter containing an appropriate binding site [112, 113]. EcR is a member of the retinoid-X-receptor (RXR) family of nuclear receptors. EcR has three domains: an N-terminal activation domain (AD), a central DNA-binding domain (DBD), and a C-terminal ligand-binding and dimerization domain. In insect cells transcription is regulated by EcR via heterodimerization with its natural partner, ultraspiracle [112].

To avoid interaction with endogenous mammalian host pathways and to optimize induction, the EcR protein and the EcR response element (EcRE) have been altered for use in mammalian cells [113]. Cotransfecton of a reporter with EcR and ultraspiracle expression vectors resulted in only a 3-fold induction of reporter expression by the hormone [112]. Three amino acids of EcR were changed to that of the glucocortocoid receptor (GR), which normally binds to two inverted repeats separated by three nucleotides. Normally, EcR recognizes perfect inverted repeats with single nucleotide separation - unlike most RXR-type receptors - which recognize direct repeats separated by a single nucleotide. This GR-EcR fusion retains the ability to dimerize with RXR and

binds to a synthetic RE and resulted in increased induction from 3- to 11-fold [113]. Replacement of ultraspiracle with RXR yielded an induction of 34-fold. A further modification replaced the EcR AD with the more potent VP16 AD, resulting in VgEcR with induction of 212-fold. In the absence of the inducer the heterodimer binds DNA and represses transcription [114]. The system utilizes two vectors to achieve inducible expression. One vector (pVgRXR) constitutively expresses the heterodimeric receptor, VgEcR and RXR. A second vector (pIND) contains a 5x repeat of the synthetic response element driving expression of the gene of interest. This system provides two modes for modulating expression. The time of induction can be altered, as can the concentration of the inducer.

One of the chief benefits of an inducible system is that it does not require physiological stimulation and is therefore uncomplicated by upstream signaling pathways. Inducible systems offer other potential benefits over constitutive systems. Clonal heterogeneity often requires that several clones of constitutively expressing cells be assayed for statistically meaningful results. For inducible systems however, the experimental samples and the control samples are derived from the same clone, eliminating the concern of clonal heterogeneity. Furthermore, the ability to modulate the level and duration of expression allows for the approximation of physiologically relevant expression, circumventing potential cytotoxic effects related to constitutive gross overexpression and counterselection. When compared with other widely used inducible systems, the ecdysone system ranks well in terms of leakiness, induction rates, and maximal expression [115, 116].

1.5 Experimental rationale

ETS factors, including PEA3 subfamily members, have been implicated in a number of human cancers and in pathways relevant to oncogenic transformation and tumor progression, as well as in tumors arising in various transgenic mouse models of cancer. ∆NPea3En can specifically repress Pea3 subfamily function *in vitro* in transient transfection reporter assays and *in vivo* by delaying the onset and size of tumors in transgenic mice [91]. Despite the identification of as many as 200 putative ETS target genes very few *bona fide* target genes for specific ETS factors have been reported [117]. The complications include the potential for overlapping endogenous function of various ETS factors *in vivo* as well as the promiscuity of ETS factor binding displayed *in vitro*.

The primary objective of this study was to identify PEA3-regulated genes relevant to cancer in a human cell line. The experimental system employed was an inducible expression system to control the expression of both Pea3 and ∆NPea3En in the human embryonic kidney (HEK)293 cell line. Phenotypic changes associated with inducible expression of either Pea3 or ∆NPea3En were monitored. In the case of inducible Pea3 expression, representational difference analysis (RDA) and glass microarrays were used to identify genes whose expression was altered by Pea3. In the case of ∆NPea3En expression, Affymetrix GeneChip analysis was used to identify differential gene expression. The identification of such genes may provide insight into potential therapeutic anti-cancer strategies.

CHAPTER 2: MATERIALS AND METHODS

2.1 Cell culture

All cells were maintained at 37˚C and 5% CO_2 in a humid chamber in Dulbeco's

modified Eagle's medium (DMEM) (Gibco BRL) supplemented with 10% fetal bovine

serum and antibiotics (penicillin, streptomycin, and fungizone). Human embryonic

kidney (HEK293) cells (and all derivative cells lines) were additionally supplemented

with L-glutamine.

2.2 DNA constructs

The vectors for inducible expression (pIND and pVgRXR) (Figure 1) were

purchased from Invitrogen as part of the Ecdysone Inducible Expression kit. The

pCANmyc expression vector used to express factors for transient transfection, contains

the human cytomegalovirus promoter upstream of sequences encoding an epitope of c-

myc protein. The construction of pCANmyc/Pea3 and pCANmyc/ΔNPea3En have been

previously described [91]. For inducible expression, mouse Pea3 and ΔNPea3En were

excised from their respective pCANmyc vectors and separately cloned into the multiple

cloning site of the pIND vector, to create pIND/Pea3 and pIND/ΔNPea3En. The 5xPea3-

luc reporter construct, which contains five tandem repeats of an optimal synthetic Pea3

binding site upstream of the adenovirus major late promoter TATA sequence and the

luciferase gene of the pGL3-Basic vector (Promega), has been described [9]. The HMB-

luc, SNM-luc, and XNM-luc reporters (a gift from Dr. Linda Penn), which contain

fragments of the human *c-myc* promoter upstream of luciferase, have also been described

Figure 1. Plasmids used in the ecdysone inducible expression system. (A) pIND contains a multiple cloning site (MCS) into which Pea3 or ΔNPea3En was cloned using the *Hind* III and *Xba* I restriction enzyme sites. (B) pVgRXR includes both components of the constitutively expressed heterodimeric receptor complex which binds the inducer, PonA, to drive expression of a cloned gene. The parental cell line (EcR-293) used for the generation of inducible clones was purchased stably transfected with pVgRXR.

A

B
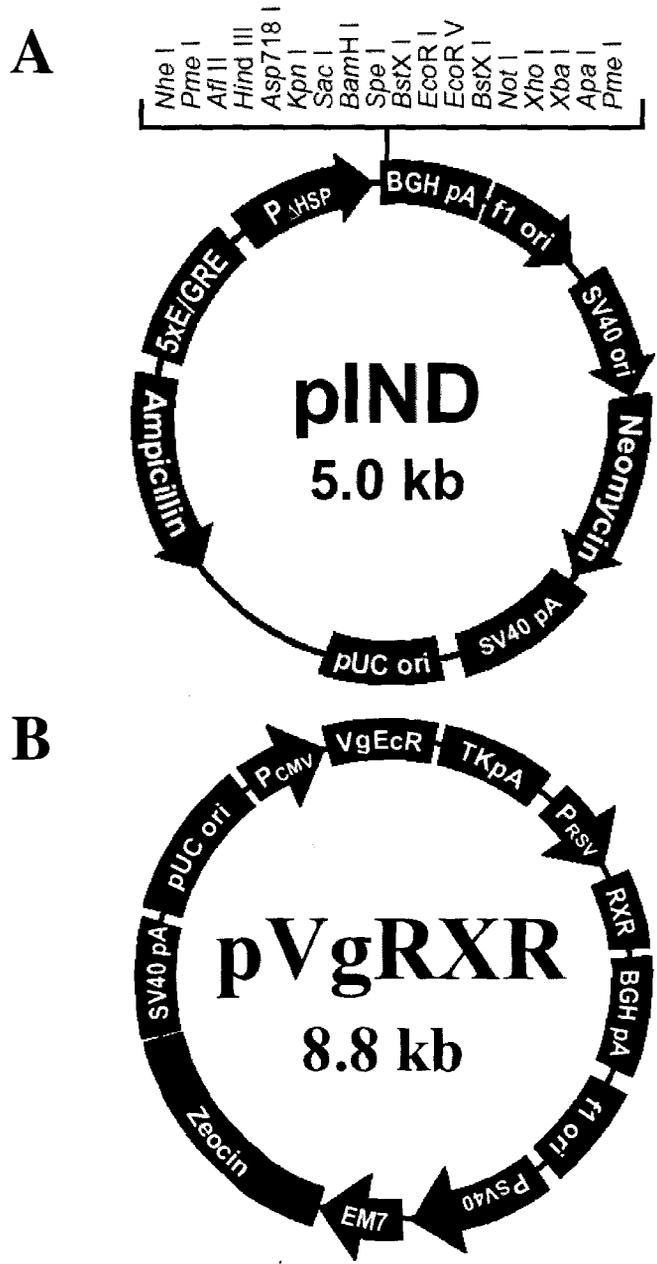
[118]. Adenoviral vectors Ad-ΔNPea3En and Ad-STOP-ΔNPea3En were constructed and prepared by Darryl Vaz (McMaster University, Hamilton, Ontario)

2.3 Cloning

Vector (pIND) and insert (pCANmyc/Pea3 or pCANmyc/ΔNPea3En) DNA was separately digested for 3 hours at 37°C with *Hind*III and *Xba*I restriction enzymes (Gibco BRL) in a volume of 20μL in 1x digestion buffer. Digested DNA was electrophoresed in a 1% agarose gel and visualized by ethidium bromide staining. Appropriate size bands corresponding to the vector and insert DNA were excised from the gel and the DNA extracted using the QIAquick Gel Extraction Kit according to the manufacturers protocol (Qiagen). Vector DNA was subsequently dephosphorylated with calf intestinal alkaline phosphatase (Roche). Vector and insert DNA was ligated in a 4:1 ratio at 4°C overnight in a 39μL reaction volume with 1 unit of T4 DNA ligase (Invitrogen), 10mM ATP and 1X Ligation Buffer. Ligation reactions were used to transform MAX Efficiency DH5α™ competent cells (Gibco BRL) and plated on LB agar plates containing 100μg/ml ampicillin for selection. Plates were incubated overnight at 37°C and formed colonies were screened for appropriate plasmid (pIND/PEA3 and pIND/ΔNPea3En) DNA by restriction analysis.

2.4 Generation of stable cell lines for inducible expression

The Ecdysone Inducible System (Invitrogen) utilizes two plasmids to achieve inducible expression – pIND and pVgRXR. pIND contains a multiple cloning site (MCS) into which either full-length Pea3 or ΔNPea3En was cloned and allows selection with Neomycin. pVgRXR encodes a heterodimeric receptor complex. The EcR-293 cell

line was purchased from Invitrogen having already been stably transfected with the pVgRXR plasmid, which bears the Zeocin™ resistance gene and allows for selection with Bleocin (Calbiochem). pIND/Pea3 and pIND/ΔNPea3En were each separately transfected into 293-EcR cells using Lipofectamine™ (Invitrogen) following the manufacturer's protocol. Briefly, 1 x 10⁶ cells were seeded on to 100mm plates the day before transfection. 8 μg of pIND/mPea3 or pIND/ΔNPea3En was incubated with 64μL of transfection reagent in 800 μL of serum-free DMEM for 45 minutes before addition to the plates with complete media. 24 hours after transfection, the cells were passaged 1:3 into selective media. Stable transfectants were selected in 300 μg/mL G418 (Gibco-BRL) and 30 μg/mL Bleocin (Calbiochem). Induction was achieved by the addition of Ponasterone A (Invitrogen) to the growth media at a concentration of 5 μM in ethanol (5 μL stock / mL media). Mock induced samples were treated with an equivalent volume of ethanol alone. The clones selected for studying induction of Pea3 and ΔNPea3En were termed the 1C clone and the DN3 clone, respectively.

2.5 Protein isolation and Western blot analysis

In order to examine protein levels, either whole cell lysates or nuclear extracts were used. Plates (100 mm) bearing sub-confluent (50-90%) cultures of cells were rinsed with PBS and harvested by scraping. To prepare whole cell lysates ice-cold lysis buffer (1% NP40, 50 mM Tris-HCl-pH 7.4, 5 mM EDTA, 400 mM NaCl, plus protease inhibitors) was used. Cellular debris was cleared by centrifugation at 12000 r.p.m. in a microcentrifuge and the supernatant was stored at -80°C. Nuclear extracts were prepared as follows. Cells were rinsed with PBS, scraped into 1.5 mL Eppendorf tubes and

centrifuged at 4°C for 3 minutes at 7000 rpm. Pellets were resuspended in cold buffer A (10 mM HEPES pH 7.6, 10 mM KCl, 0.1 mM EDTA pH 8.0 and protease inhibitors) and incubated on ice for 15 minutes. IGEPAL detergent was added and the samples vortexed for 15 seconds. Nuclei were pelleted by centrifugation for 30 seconds at 13000 rpm at 4°C. Pellets were resuspended in ice-cold buffer C (20 mM HEPES pH 7.6, 0.4 mM NaCl, 1 mM EDTA pH 8.0 and protease inhibitors) and rocked at 4°C for 1-2 hours. Samples were then centrifuged at 13000 rpm at 4°C and the supernatants stored at -80°C. Protein concentration was determined by Bio Rad Protein assay using bovine serum albumin (Sigma) as the standard. Protein lysates were boiled for 3 minutes in 1X SDS loading buffer (50 mM Tric-Cl pH 6.8, 10% glycerol, 2% SDS, 0.29 M β-mercaptoethanol, 0.0005% bromophenol blue) and separated on an 8% SDS-polyacrylamide gel prior to transfer to a polyvinylidene difluoride membrane by electroblotting. Membranes were blocked overnight at 4°C in Blocking Buffer (5% skim milk in TBS-T [10 mM Tris-HCl pH7.3, 150 mM NaCl, 0.05% Tween20]) on a rotating platform. All antibodies were diluted in blocking buffer before incubation. To detect mouse Pea3 either the Pea3-specific monoclonal antibody MP16 or a combination of MP16 with MP13, also a Pea3-specific monoclonal antibody, or PN1, a rabbit polyclonal antibody, was used. To detect ΔNPea3En a Pea3-specific rabbit polyclonal antibody PC2 was used. Goat anti-mouse horseradish peroxidase-conjugated secondary antibody (KPN; 474-1806) was used followed by visualization with chemiluminescence (NEN Life Sciences Products) and exposure to X-ray film (Kodak Scientific Imaging).

2.6 RNA isolation and Northern analysis

Total RNA was harvested from sub-confluent cultures using Trizol (Invitrogen) or RNeasy mini kit (Qiagen) reagent according to the manufacturer's protocol. PolyA$^+$ RNA was purified from total RNA using kits from Stratagene (Poly(A)Quick) or from Ambion (Poly(A)Pure) according to the manufacturer's protocol. RNA concentration was measured by absorbance at 260nm (A_{260}) and purity was assessed by A_{260} / A_{280}. RNA was resolved on a 1% agarose-formaldehyde gel and transferred to a membrane (GeneScreen Plus, Dupont). Prehybridizations and hybridizations were done with UltraHyb solution (Ambion). Probes were made from DNA excised from plasmids using appropriate restriction enzymes and gel purified. Labelling was accomplished using the random priming method with ^{32}P-dCTP or StripEZ DNA kits (Ambion) following the manufacturer's protocol. Most membranes were used at least twice by addition of boiling 0.1% SDS and agitating for one half hour or using the StripEZ DNA protocol for stripping (Ambion). In between multiple hybridizations blots were exposed to film to confirm successful stripping. Quantification of signals was done using PhosphorImager analysis (Molecular Dynamics) and normalization to glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

2.7 Assessment of transcriptional activity

The functionality of the expressed Pea3 and ΔNPea3En was assessed by use of the Luciferase Reporter system (Promega) using reporter constructs derived from the pGL3-Basic vector (Promega). Transient transfections were carried out using SuperFect reagent (Qiagen) according to the manufacturer's recommended protocol. Cells were seeded at

1×10^5 cells per well of a 6-well plate the day prior to transfection. For transient transfection of both reporter and effector, 0.1 μg of reporter and varying amounts of effector (0.005 μg, 0.025 μg, 0.05 μg, 0.1 μg, 0.25 μg or 0.5 μg) or empty vector were transfected. Total amount of transfected DNA was kept constant at 1.5 μg per well with calf thymus DNA (Sigma) as carrier. One-day post transfection each well was replated into four wells of a 12-well plate. In the case of induction experiments, two of the four wells were induced (and the other two mock induced). 24 hours after replating (and induction) the cells were washed with PBS and lysed with Reporter Lysis Buffer (Promega) for 20 minutes at room temperature. Lysed cells were scraped into microcentrifuge tubes and centrifuged for 1 minute at 13,000 rpm at $4^{\circ}C$. The supernatant was transferred to fresh tubes and stored at $-70^{\circ}C$ or assayed immediately. 20μL of lysate with 100μL of Luciferase Assay Reagent (Promega) was assayed in Sarstedt 12X75 mm polystyrene tubes for luciferase activity using either a lumat 9501 (Berthold) or a Luminoskan Ascent (Labsystems) luminometer. Luciferase activity was normalized to the total protein concentration of the lysates, as determined by the Bradford assay kit (Bio-Rad) and expressed as relative luciferase units (RLU).

2.8 Representational difference analysis (RDA)

Representational difference analysis of cDNA was performed according to the protocol of Hubank and Schatz [119] with minor alterations. Briefly, total RNA was isolated from 1C cells induced ('tester') or mock induced ('driver') for 6 hours. Twice-selected polyA+ RNA was purified from the total RNA using the Poly(A)Quick kit (Stratagene). Double-stranded cDNA was synthesized with the SuperScript

Preamplification system (Life Technologies) using oligo(dT) primer for first strand synthesis followed by the Choice system (Life Technologies) for second strand synthesis according to the manufacturer's protocol. The tester:driver ratios used to generate DP1, DP2 and DP3 were 1:100, 1:800 and 1:40,000, respectively. DP2 and DP3 subtraction products were directly cloned using the TA-cloning kit from Invitrogen. The cDNA inserts were then used for DNA sequencing and as probes for Northern analysis. Sequence identity was determined by BLAST search.

2.9 Growth curves

1×10^5 cells were seeded on 35mm plates and were either induced (PonA in ethanol) or mock induced (ethanol alone) at the time of seeding or at some later time, typically the day after seeding. Media including PonA or ethanol was replenished every second day. Cells were trypsinized and counted with a hemocytometer at one or two day intervals. Values are presented as the mean number of cells counted from duplicate wells (+/- average deviation) normalized to unit area (cm^2).

2.10 cDNA microarray analysis

Glass microarrays were purchased from the Toronto Microarray Consortium. The microarrays used consisted of PCR amplified plasmid DNA spotted on coated glass slides and covalently linked to the surface. A single slide representing approximately 1700 cDNAs or a set of two slides representing approximately 19000 cDNAs was used. Total RNA from each of the samples (1C cells induced or mock induced for various times) was fluorescently labelled with a different dye by a reverse transcription reaction. Essentially, 50 µg of total RNA was reverse transcribed with 5x first strand buffer (GIBCO), 20 mM

dNTP (6.7 mM each dATP, dTTP, dGTP), 2 mM dCTP, 0.1 mM DTT, and 1 mM either Cy5-dCTP or Cy3-dCTP (Amersham Pharmacia) at 65°C for 5 minutes followed by 42°C for 5 minutes. Superscript II RNase H- (GIBCO) was added and the reaction was incubated at 42°C for 2 hours. The reaction was stopped with 2 uL 0.5 M EDTA (pH 8.0) and RNA removed by incubating at 65°C for 20 minutes with 2 uL of 10 N NaOH. RNA hydrolysis was stopped by the addition of 4 uL of 5 M acetic acid and the probe precipitated with isopropanol, washed with 70% ethanol and re-suspended in 5 of uL water. During labeling the DNA microarray was prehybridized with 40 uL DIG Easy Hyb (Boehringer Mannheim) with 1 ul yeast tRNA (10 mg/mL) and 1uL sonicated salmon sperm single stranded DNA (10 mg/mL) under a cover slip (single slide) or face-to-face/head-to-tail (two slide set) in a humid chamber at 37°C for 1 hour. 2.5 uL of each of the Cy3 and Cy5 probe reactions was added to 40 uL of prehybridization solution and added to the array(s). The hybridization mixture was incubated in a humid chamber at 37°C overnight. Post-hybridization washes were as follows: arrays were rinsed in 0.1X SSC to remove the cover slip, 3 washes in 0.1X SSC / 0.1% SDS at 50°C 10 minutes each with occasional agitation, one final rinse in 0.1X SSC. Prior to scanning the arrays were spun dry at 700 rpm for 8 minutes. Image acquisition utilized the ScanArray 4000 (GSI Lumonics) or the GenePix 4000B (Axon Instruments). Image analysis to obtain signal intensities was done with GSI Lumonics software (Quantarray), GenePix Pro 4.0 software (Axon), or with software freely available from Stanford University (ScanAlyze). Signal ratios were obtained and normalized to the total signal for each dye on a slide-to-slide basis.

2.11 Affymetrix GeneChip analysis

DN3 cells were grown to approximately 80% confluence on 100mm plates and induced or mock induced for 2, 6, 12 or 24 hours. Total RNA was isolated as described for Northern analysis. Samples were processed at the Gene Expression Facility, Ottawa Health Research Institute, Ottawa, ON for analysis on Affymetrix GeneChip Human Genome HG95A (24h) or HG-U133A arrays (2, 6 and 12h) according to standard protocols (Affymetrix). Images were analyzed and processed using Microarray Suite (MAS) 5.0 and Data Mining Tool (DMT) 3.0. Probe set intensity was calculated after subtracting mismatch intensity from perfect match intensity and is proportional to mRNA abundance. Fold change was calculated by pairwise comparisons as well as by averaging all control and all experimental replicates. Signal intensities of all different probe sets on all arrays were scaled to the same arbitrary value before calculating fold change. All adjustable analysis and report parameters were left at default values.

2.12 Real-time quantitative RT-PCR

A two-step RT-PCR method was employed to measure the transcript levels of several genes in cells induced or mock induced for varying amounts of time. Total RNA was isolated as described for Northern analysis from sub-confluent cultures grown on 10cm plates. The same RNA that was used for Affymetrix GeneChip analysis as well as independant RNA was used as template for oligo-dT primed reverse transcription (RT). 5μg of RNA was used for each RT reaction using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen) according to the manufacturer's recommended protocol. In most cases replicate RT reactions were run in parallel and the resultant cDNA pooled

to eliminate reaction-to-reaction variation. Most RT reactions were diluted 3- to 10-fold for subsequent quantitative PCR.

Standard unmodified primers were designed using the web-based primer design tool Primer3 using default parameters. Primers selected were biased to amplify sequences toward the 3' end of the mRNA and to span intron/exon boundaries, where possible. All primers used are shown in Table 1.

Real-time quantitative RT-PCR was performed using the LightCycler instrument with LightCycler DNA master SYBR Green I (Roche). All samples were normalized to the amount of GAPDH. 10 μL reaction volumes contained 1 μL cDNA template, 1 μL LightCycler DNA master SYBR Green I, 3-5 mM $MgCl_2$, and 0.5-1.0 μM each primer. A step-down method of PCR was employed in most cases to increase the fidelity of the amplification. Melting curve analysis was performed with each reaction to examine the specificity of product formation. In most cases either a sharp product peak alone or a sharp product peak with a discernable primer-dimer peak at a lower melting temperature was observed. The PCR reactions of all primer sets were initially examined on a 1% agarose gel with ethidium bromide staining to confirm amplification of products of expected length. Relative quantitation was achieved with a standard curve generated by a dilution series of one of the samples per reaction. No template and –RT controls were also included in reactions.

2.13 Phalloidin staining

Cells were plated on 4-well Lab-Tek II glass chamber slides (Nalge Nunc International) and either induced with PonA or mock-induced with ethanol alone for

Table 1. Primer sets used for RT-PCR quantitation. Primers are listed forward 5'-3', reverse 5'-3'. Product sizes are also shown.

Gene	Forward/Reverse Primers 5'-3'	Product Size
murine PEA3 and ΔNPEA3En	CGGGGTGCCTTACAACTGTGG GTAAGAATATCCACCTCTGTG	421
DNPEA3En	CGGGGTGCCTTACAACTGTGG CGGGGTGCCTTACAACTGTGG	508
c-myc	ACCACCAGCAGCGACTCTGA TCCAGCAGAAGGTGATCCAGACT	116
fibroblast growth factor receptor 2	GTTAACACCACGGACAAAGAGATTGAG AACACGGTTAATGTCATAGG	831
cytokeratin 10 (set 1)	GCAAAATCAAGGAGCGGTATGA AACTGTTCTTCCAGAGCGGA	726
cytokeratin 10 (set 2)	GGCTCTGGAAGAATCAAACTATGAGC GGATGTTGGCATTATCAGTTGTTAAGG	166
multidrug resistance-associated	GCAAATCCAGGAGACAGCTC TTGATCCGAAATAAGCCCAG	350
midline 1 (set 1)	ATCATTCTCCTTCAGAGAGTGTT GAAATGTGTTTATTGATAGTGGC	668
midline 1 (set 2)	CATTTTGCCCTGCTTCTCTC GGAGCGGGAAATAGGAGAAC	238
purinoreceptor 2x4	CTCTGCTTGCCCAGGTACTC ATGATGTCACACAGCACGGT	263
stanniocalcin (set 1)	TCCACATCTTCACTCAAGCC CCTTTGGAAAGTGGAGCAC	194
insulin receptor substrate 4	GTCAAGGAGAGCGGTTTCAG CCGAGCTATGGCTACTGGAG	290
protease, serine, 23	GGCAATTTGGTGTATCGCTT TGCCAATAATTTTTCGCTCC	154
Hox2G (B3), homeobox transcription factor	TATGGCCTCAACCACCTTTC AAGCCTGGGTACCACCTTCT	299
MDM2-like p53-binding protein (MDM4) (set 1)	GAGTGGCAGTGTACTGAATGC GCATCTTTAGGTCTAACGACAG	221
3-hydroxy-3-methylglutaryl coenzyme A synthase	GCTTCTCCCCGTGAATCATA	162

ACCATAAGCCCAGGACAGTG

Table 1 continued

insulin growth factor binding protein 5	TCTGCTCACCCAGAGACCTT GAAGGGGCATGATCAGAAAA	368
autotaxin	ACAACGAGGAGAGCTGCAAT AGAAGTCCAGGCTGGTGAGA	127
hairy (Drosophila-homolog)	CGTTTTCCCTCCTGTTGAAA ACCAGGACTGTTAAGGGGAT	127
8-oxoguanine DNA glycosylase	AAGAAATTCCCCAAGCACCT TTAGCGCTGTCTCCCTCAAT	176
selenoprotein P	GATGGAGCAACTGAAAGGTG CCCCTAGGTCATAGTTTACG	112
mitogen-activated protein kinase kinase 5	ACGTGAAGCCCTCCAATATG ACTGCTCCCCTGAAATCCTT	150
prothymosin alpha	CGGGAATGCTAATGAGGAAA TCGGTCTTCTGCTTCTTGGT	213
ras-related C3 botulinum toxin substrate (rac)	ATGCAGGCCATCAAGTGTGTGGTG TTACAACAGCAGGCATTTTCTCTTCC	578
cytochrome P-1-450	CTTGGACCTCTTTGGAGCTG CGAAGGAAGAGTGTCGGAAG	212
clones 23667 and 23775 zinc finger protein	CTGGGAGTCAAGAGCAGGAG GATCCTTCAGCAAGCACCTC	187

varying amounts of time. Actin filaments were examined using Texas Red®-X phalloidin (Molecular Probes) according to the manufacturer's recommended protocol. Cells were washed with warm PBS and fixed with 3.7% formaldehyde in PBS for 10 min at room temperature. Cells were washed again and permeabilised with 0.1% Triton X-100 in PBS for 3 min on ice. Blocking solution consisting of 1% BSA in PBS was added for 20 min at room temperature. One unit of fluorescent phalloidin in 200 uL of 1%BSA/PBS was used to stain each slide for 20 min at room temperature in the dark. After final rinsing with PBS, Vectashield mounting medium containing DAPI DNA counterstain (Vector Laboratories, Inc.) was applied under a coverslip and the slides sealed with nail polish. A Leica DM IRB fluorescent microscope was used to examine the cells and capture images.

2.14 TUNEL assay

Cells were plated on 4-well Lab-Tek II glass chamber slides (Nalge Nunc International) and either induced with PonA or mock-induced with ethanol alone for varying amounts of time. TUNEL assays were performed using the *In Situ* Cell Death Detection Kit (Roche) according to the manufacturer's recommended protocol. Cells were fixed for 1 h at room temperature with 4% paraformaldehyde. After rinsing with PBS, cells were permeabilised with 0.1% Triton X-100 in 0.1% sodium citrate for 2 min on ice. Cells were rinsed with PBS and incubated with TUNEL reaction mixture for 1 h at 37°C in the dark. After final rinsing with PBS, Vectashield mounting medium containing DAPI DNA counterstain (Vector Laboratories, Inc.) was applied under a coverslip and the slides sealed with nail polish. A Leica DM IRB fluorescent microscope was used to examine the cells and capture images.

2.15 Cell cycle analysis

Cells were plated on 10 cm plates and either induced with PonA or mock-induced with ethanol alone for varying periods. Both adherent and cells in suspension were collected by centrifugation at 1000 rpm for 5 min. Cells were resuspended in 1 mL cold PBS and fixed by adding 4 mL of -20°C ethanol drop-wise with gentle vortexing. Cells were re-collected by centrifugation as above and resuspended in 1 mL PBS. 20 μg of DNAse-free RNAse was added to the cells and incubated at 37°C for 30 min. Cells were stained with 40 μg/μL of propidium iodide and analyzed on a flow cytometer (Coultier EPICS XL).

2.16 Virus infection

Adenoviral vectors Ad-ΔNPea3En and Ad-STOP-ΔNPea3En, containing ΔNPea3En (Darryl Vaz, McMaster University, Hamilton, Ontario) were used as controls for expression of ΔNPea3En. High titre CsCl purified stocks of virus were used in experiments. The concentration of viral particles was estimated from measurement of optical density at a wavelength of 260nm where 1 OD_{260} unit equals 1.1×10^{12} viral particles/mL (Ng et al., 2002). It was assumed that 1 in 100 viral particles were infectious (Mitani et al., 1995; Morissey et al., 2002). Therefore, reports of moi (multiplicity of infection) during experiments are estimates. Viral stocks were diluted in PBS and added directly to the cells in regular growth media and incubated for the indicated time.

2.17 Immunocytochemistry

Cells were plated on 4-well Lab-Tek II glass chamber slides (Nalge Nunc International) and were treated as indicated for varying amounts of time. Cells were rinsed in PBS and fixed for 10 min in 4% paraformaldehyde at room temperature. After washing with PBS, the cells were permeabilised with 0.1% Triton X-100 in 0.1% sodium citrate for 2 min on ice. Slides were blocked for 30 min with 3% normal goat serum before incubation with primary antibody for 1 h at room temperature. Slides were rinsed with PBS before incubation with secondary antibody for 30 min at room temperature. A Leica DM IRB fluorescent microscope was used to examine the cells and capture images. For staining of Pea3, the mouse monoclonal Pea3-specific antibody, MP16 was used. ΔNPea3En, which lacks the determinants detected by MP16, was stained with one of two different antibodies. The rabbit polyclonal antibody PC2 recognizes determinants still present in ΔNPea3En. ΔNPea3En also contains a myc-epitope tag, which facilitates the use of the anti-myc antibody 9E10. All primary antibodies were used at a dilution of 1:100. Secondary antibodies used were Alexa Fluor 594 (Molecular Probes) for PC2 and Alexa Fluor 488 (Molecular Probes) for MP16 and 9E10, both used at 1:300.

CHAPTER 3: RESULTS

3.1 Inducible Pea3 expression

3.1.1 Clone derivation and selection

The ability to induce Pea3 expression using the ecdysone inducible system was first tested for functionality in a mammalian cell system using transiently transfected COS-1 cells followed by induction and analysis by Western blotting. Full-length mouse *Pea3* cDNA was cloned into the multiple cloning site of pIND resulting in the pIND/Pea3 plasmid. Transfections were done with equal amounts of the pVgRXR vector (1 μg) and the pIND/Pea3 (1 μg). Cells were left un-transfected (negative control), transfected with equal amounts of both vectors (pVgRXR and pIND/Pea3) without induction, or transfected with both vectors and induced with PonA. The manufacturer as an initial induction time recommended 20h. Figure 2 indicates that the ecdysone inducible system can be used to express Pea3 protein in COS-1 cells. Figure 2A shows the initial induction of Pea3 in transiently transfected COS-1 cells. GST-Pea3, which ran more slowly than normal Pea3, displayed significant degradation. The experiment was repeated with additional induction times to examine the induction kinetics. Figure 2B shows the induction of Pea3 expression in COS-1 cells over several time points. Pea3 protein is absent in un-induced and untransfected samples, increased from 6h to 24h and decreased thereafter. The reduction of Pea3 over time may be a consequence of transient transfection and subsequent loss of expression vectors with time. It may also be that the inducer (PonA) is not stable over the course of the induction. Protein degradation may also reduce net protein levels at longer times. Purified control GST-Pea3 protein, which

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is often at higher concentration than experimental samples in experiments, often shows degradation. However, the observed degradation of control protein varies from experiment to experiment, suggesting that handling of the protein may be the principal cause of degradation.

Having established that the ecdysone inducible system could be used to express Pea3 in mammalian cells, we next sought a suitable human cell line in which we could inducibly express Pea3. In order to examine the role of PEA3 in cancer and to induce changes in our chosen cell line – in terms of gene expression and/or phenotype – we required a cell line in which PEA3 could function but was expressed at low levels. Also, based on the expression pattern of Pea3 we sought an epithelial cell line [46, 63]. Therefore, our initial selection criteria were an epithelial cell line with low endogenous PEA3 and a non-transformed phenotype. Our first choice was the MCF7 cell line, a breast tumor line that is phenotypically non-invasive and non-metastatic. The MCF7 cell line has also been shown to be endowed with a transformed phenotype by exogenous human PEA3 [84]. Initial attempts at stably transfecting MCF7 cells with one or both of the required plasmids for inducible expression failed. The human HEK293 cell line stably transfected with the receptor-encoding plasmid (pVgRXR), which was commercially available, was used to expedite work with the inducible system.

Figure 2. Testing the ecdysone inducible expression system. Both plasmids for the ecdysone inducible expression system were transiently transfected into COS-1 cells with and without addition of PonA. Western analysis using the anti-Pea3 monoclonal antibody MP16 with whole cell lysate was used to check for inducible expression. Purified GST-Pea3, which runs higher than normal Pea3, was used as a positive control. Un-transfected cells were used as a negative control. (A) A single induction time of 20h was used to check for Pea3 expression. (B) Induction of Pea3 over several times was examined. Arrows indicate the position of both GST-Pea3 and Pea3.

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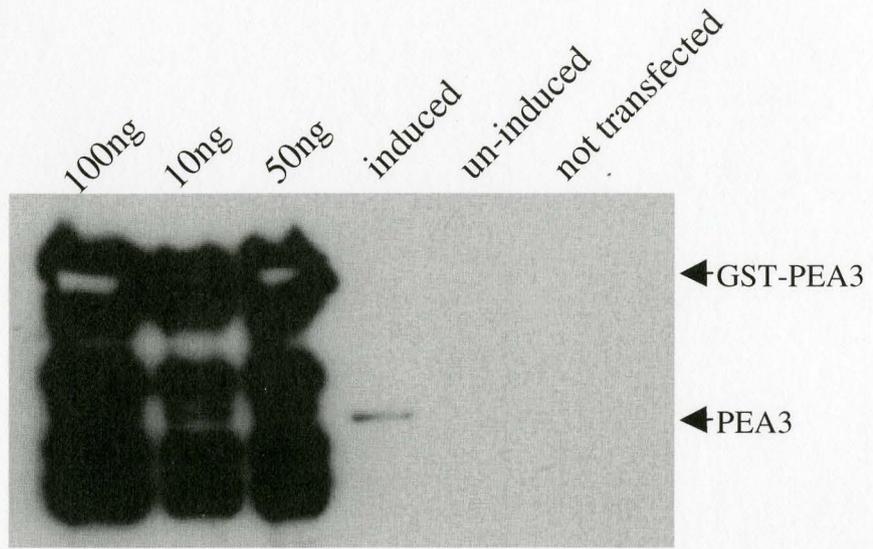
B
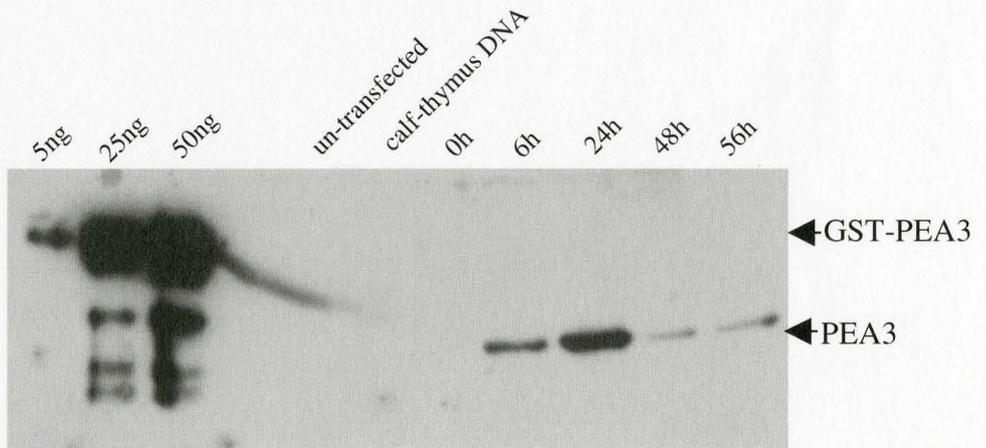
The HEK293 cell line was originally derived by transforming primary HEK cells with sheared adenovirus type 5 (Ad5) DNA and was generally thought to be epithelial [120]. Subsequent studies have shown that HEK293 cells express markers thought to be either epithelial or neuronal, exclusively, and may therefore be of a rare neuronal lineage [121]. Regardless of the actual lineage of HEK293 cells, published microarray data (http://www.mbi.ufl.edu/~shaw/293.html) suggest that, for the purpose of this work, the use of HEK293 cells should be appropriate. In terms of ETS factor expression data from array experiments conducted in this lab, the PEA3 subfamily is apparently not expressed in HEK293 cells with the exception of ERM, which may be expressed at low levels. Of the other ETS factors represented on the arrays used, ETS-2, ERF, GABP and NERF-1 are expressed while ETS-1, PEP1, ERT, ERG, PDEF and TEL are absent. PCR primers designed to amplify the human PEA3 subfamily members apparently failed to specifically detect these genes. For all three genes the primers amplified product but the product did not appear specific. Northern analyses using the full-length mouse *Pea3* cDNA as a probe often detected a species migrating above *Pea3* (see Figure 5A) and Western analyses using the PC2 antibody also detected a band migrating above PEA3 or ΔNPea3En (see Figure 13C) which may represent an ETS family member. Other published data suggests that HEK293 cells express little or no PEA3 subfamily member RNA and protein [98]. In terms of expression pattern, the Pea3 subfamily members are expressed in the kidney as are several other Ets factors [52, 63]. Furthermore, Pea3 subfamily members are also expressed in neural tissue [46, 62]. Therefore, the use of

HEK293 cells as an experimental model system in which ETS activity is altered is likely biologically relevant in terms of ETS activity if not PEA3 activity.

To isolate stable transfectants, the parental cell line (EcR-293) was transfected with pIND/Pea3 and clones were isolated in selective media. Clones were screened for the inducible expression of Pea3 protein by Western analysis. Again, 20h was used as the initial induction time. 45 clones were initially selected and batch-screened. Of the 12 clones first screened, 8 produced detectable Pea3 protein upon induction (Figure 3). Based on the levels of Pea3 in both the induced samples and the un-induced samples, 2 clones (designated 9B and 1C) were chosen for further characterization. Time course analyses indicated that unlike transient transfection in COS-1, Pea3 levels appear stable over time when induced in HEK293 cells (Figure 4). Also unlike the case in transient transfection in COS-1 cells was apparent lack of Pea3 protein degradation in the stable clones. This may be due to differences in the amount of protein expressed, the rate at which it is expressed, and differences related to the cell in which Pea3 is expressed. It is also possible that different handling of the samples resulted in increased degradation. The amount of GST-Pea3 control protein used among experiments differed and cannot be used as a direct reference to compare various experiments. Both clones seemed capable of rapid Pea3 expression that was sustained over time. However, the 9B clone exhibited some 'leakiness' with detectable Pea3 in the un-induced sample (time 0h) in one out of three replicate experiments, which if real may hamper subsequent experiments by affecting Pea3-dependent changes prior to induction. Therefore, the 1C clone was chosen as the experimental clone for further analyses.

Figure 3. Screening stable clones for inducible Pea3 expression. EcR-293 cells were transfected with pIND/Pea3 and picked as colonies after growth in selective media. Western analysis of whole cell lysates was used to screen individual clones for inducible expression of Pea3 with MP16 monoclonal antibodies. Shown are 12 of the 45 clones that were isolated as potential stable transfectants capable of inducible Pea3 expression.
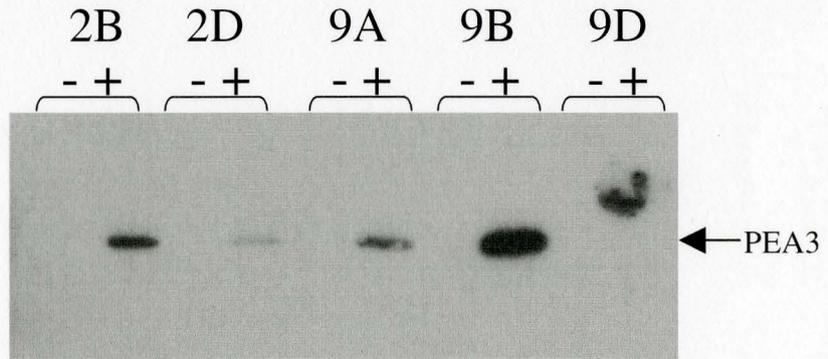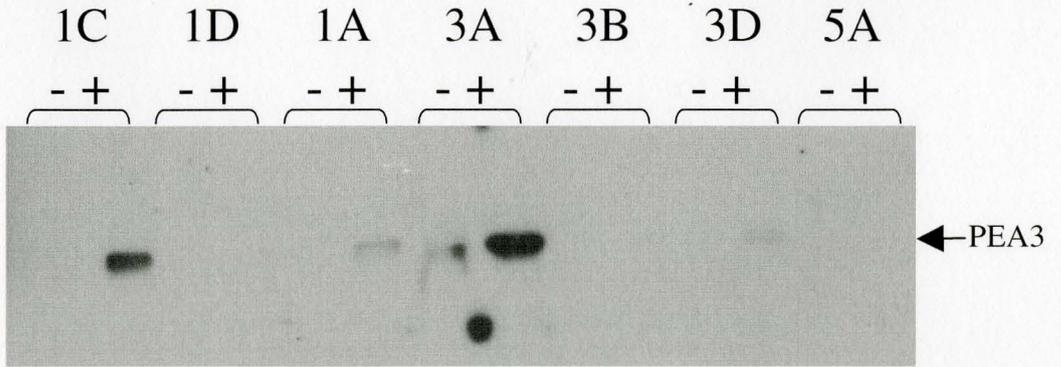
Figure 4. Comparing Pea3 protein induction kinetics of two candidate clones. Pea3 protein induction kinetics of two clones selected for further study was examined by Western analysis. The Pea3-specific monoclonal antibody MP16 was used with whole cell lysates from cells mock-induced (0h) or induced for various times (6, 12, 18, 24 and 30 hours).

3.1.2 Inducible Pea3 clone characterization

Having selected a clone for further analysis, the Pea3 induction kinetics at both the RNA and protein levels were examined in more detail (Figure 5). Northern analysis indicates that Pea3 RNA was detected as early as 2h post induction (Figure 5A), while the protein is detected faintly at 1h post induction and distinctly at 2h (Figure 5B). Both Pea3 RNA and protein continue to increase throughout the course of the induction. This data demonstrates that the 1C clone is capable of tightly controlled Pea3 expression with rapid induction kinetics at both the RNA and protein levels.

Examination of the inducible expression of Pea3 on a cellular level by immunocytochemistry suggests that only a subset of induced cells expresses high levels of Pea3 (Figure 6). The data also suggests that another subset of cells expresses Pea3 at levels marginally above the background observed in mock-induced cells. Control cells transiently transfected with a Pea3 expression vector display more positive cells with little variation in intensity, likely reflecting transfection efficiency, which was not rigorously optimized in this case. It is possible that all induced cells express Pea3 at varying levels. However, we would expect that since the 1C line is clonal, all induced cells should uniformly express Pea3. It may be that cell-to-cell signaling differentially effects the inducible expression by some mechanism.

Figure 5. Induction of Pea3 in 1C cells. The Pea3 inducible clone selected as the experimental cell line (1C) was examined for both RNA and protein induction kinetics at early time points. Cells were either mock-induced or induced for various times. (A) Northern blot illustrating the induction kinetics of *Pea3* RNA (top panel) in the 1C cell line (0 to 8h) with corresponding levels of *GAPDH* (lower panel). The "0 hour" sample represents "8 hour" mock-induced. (B) Western blot illustrating the induction kinetics of Pea3 protein in the 1C cell line (0 to 12h). The "0 hour" sample represents "12 hour" mock-induced. A truncated GST-Pea3, which runs at the same size as full-length Pea3 and includes the MP16 epitope, was included as a positive control (first 3 lanes). For comparison levels of Pea3 resulting from transient transfection were included (last 2 lanes). Arrows indicate the position of Pea3. Different regions of the same autoradiagram have been juxtaposed to facilitate visualization of the data – this is indicated by a thin white line.

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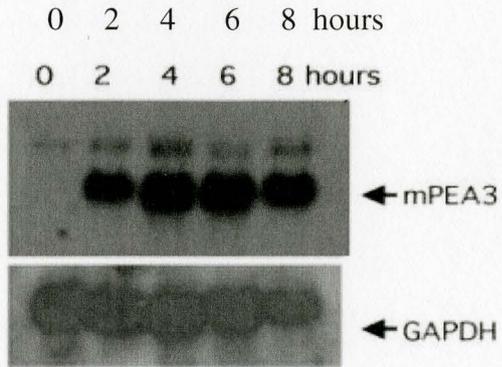
B
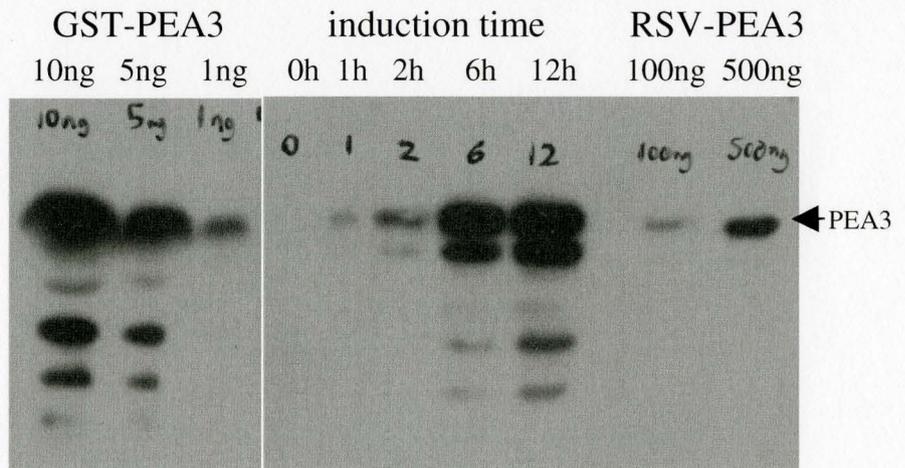
Figure 6. Immunocytochemical analysis of induced Pea3. The expression of induced Pea3 protein at the cellular level was examined with the MP16 mouse monoclonal Pea3 antibody. 1C cells were seeded and grown in 4-well chamber glass slides. Wells on the same slide were either mock-induced or induced for various times (only 24 hours is shown). Separate slides were stained with different secondary antibodies (Alexa Fluor 594/red or Alexa Fluor 488/green) and all were counter-stained with DAPI (blue). Images of the same field showing the Pea3 signal alone and the Pea3 signal merged with the DAPI signal are shown. Images taken at 400X magnification.

	mock	induced
Alexa Fluor488 (PEA3)		
merged with DAPI		
Alexa Fluor594 (PEA3)		
merged with DAPI		
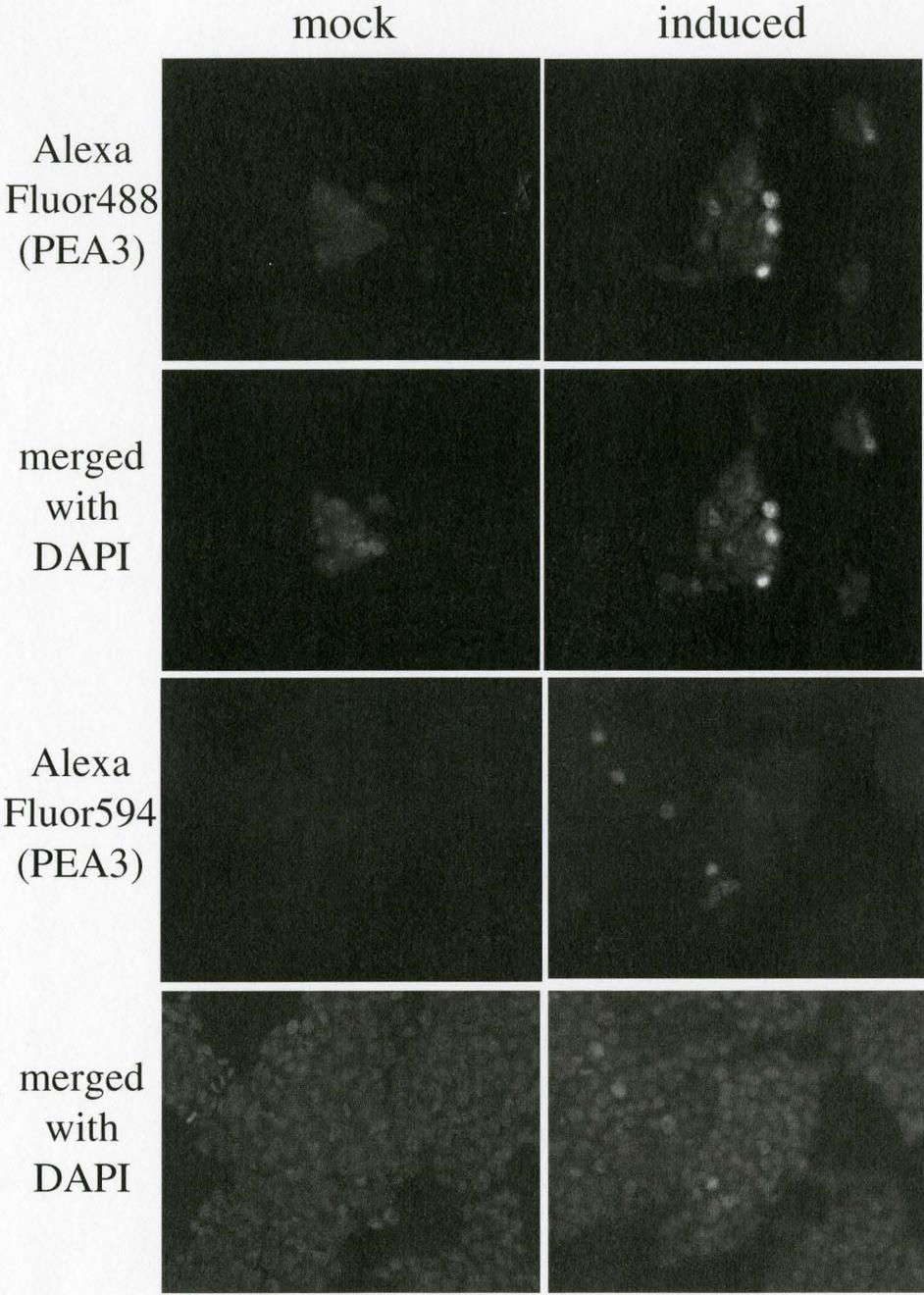
There is evidence that even clonally selected cells expand to produce heterogeneous populations of cells. These cells may respond differently in terms of protein induction and stability. Regardless, the cause of the apparent differential induction is unknown. As a consequence any measures from biochemical analyses done with cell populations may not be representative of the original clone and may therefore be underestimated.

3.1.3 Effects of Pea3 induction on phenotype and transactivation

We next examined the effects of induced Pea3 in the HEK293 cell line. There was no overt phenotype associated with prolonged induction in the 1C line. Cell morphology did not noticeably change in response to Pea3 induction. In both the 1C line and the parental EcR-293 line the growth rate remained equivalent between induced and mock-induced samples.

The ability of induced Pea3 to function as a transcriptional activator was assessed with a luciferase reporter assay. An optimal synthetic Pea3 binding site repeated upstream of the luciferase gene (5xPea3-luc) was transfected into 1C cells with or without induction and the luciferase activity was measured. The results varied with the amount of reporter transfected and the induction time. Furthermore, the assessment of induced Pea3 transcriptional activity was characterized by a marked lack of reproducibility. Instances of induced Pea3 repressing reporter gene expression were as common as instances of induced Pea3 increasing reporter gene expression and equally inconsistent in terms of induction time and amount of reporter vector. There was no instance of any dose-response in terms of induction time. Figure 7 shows representative results for two different strategies attempted to measure the transcriptional activity of

induced Pea3. For the first set of experiments, each induction was started after reporter vector transfection and staggered such that all samples would be harvested at the same fixed time (24h post-transfection) (Figure 7A). It should be noted that while the values shown in the graph (Figure 7A) are normalized based on un-induced samples being set to a value of 1, the non-normalized relative luciferase units per unit protein are approximately 20-times higher with 500ng reporter than with 100ng. Longer induction times were also examined by inducing cells prior to reporter vector transfection (Figure 7B). In this case induction times correspond to 14h, 18h, 24h and 36h by inducing cells 12h prior to transfection and re-inducing cells post-transfection as for 7A. Done in this way repression rather than transactivation was observed. The RLU of both induced and un-induced samples increased with time. Induced samples had RLU values marginally lower than corresponding induced samples with the greatest difference occurring at the longest induction time. The increase in luciferase expression is likely the result of increased accumulation of reporter plasmid and reporter plasmid product expression driven by endogenous factors. The half-life of the reporter product (luciferase) is approximately 3-hours and has been designed to be sufficiently short to be able to monitor transcriptional changes within the time frame of these experiments. It should be noted that in nearly all experiments using the Pea3-responsive reporter in HEK293 cells a relatively high basal level of luciferase was expressed, presumably from endogenous ETS factors expressed in these cells.

Figure 7. Transcriptional activity of induced and transiently transfected Pea3. Transiently transfectd Pea3 increases reporter gene transcription in a dose-dependent manner, while induced Pea3 is unable to consistently effect transcription. Transcriptional activity was assessed by measuring luciferase expression from 5xPea3-luc, an artificial promoter bearing repeats of an optimal Pea3 binding site. (A) Two different amounts of reporter plasmid were transfected into 1C cells and samples induced (post transfection) for increasing times. (B) Cells were transfected with reporter plasmid and increasing amounts of effector plasmid or equivalent amounts of empty control vector. For panel A, all values are relative to the un-induced samples, which were set to a value of 1. For panel C, luciferase values for Pea3 transfected samples were divided by the corresponding empty vector control samples. Data shown are from representative experiments performed in triplicate (mean ± S.E.).

A

Transcriptional activity of induced Pea3

B

Effects of pre-induction on transcriptional activity of Pea3

C

Transcriptional activity of transfected Pea3
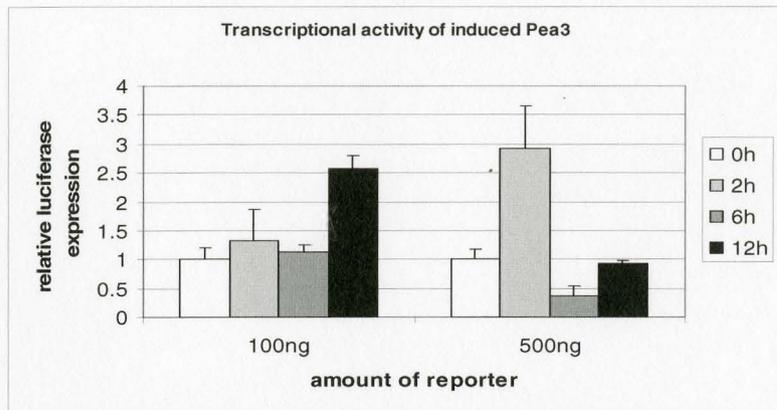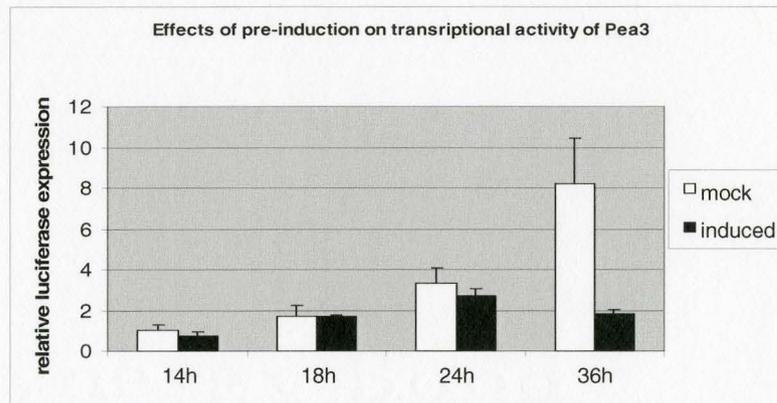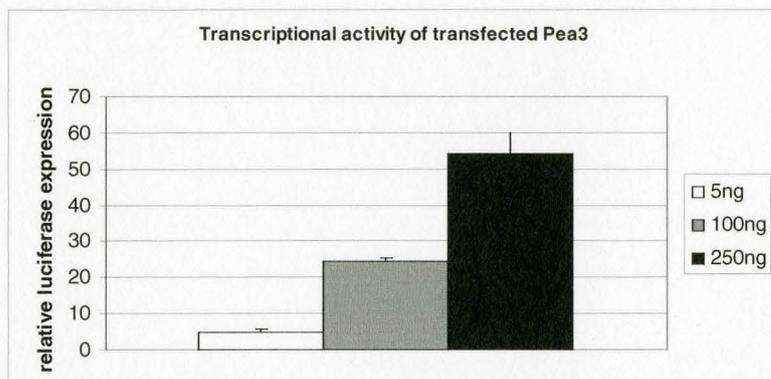
For comparison, the ability of transiently transfected Pea3 to effect transcription was examined in the same cell line without induction (Figure 7C). Transiently transfected Pea3 was able to increase luciferase expression in a dose-dependent manner to a higher degree than that observed in any induction experiment. For comparison to induction times, transiently transfected Pea3 roughly corresponds to an induction time of 24h since the cells were harvested 24h post-transfection. In order to further examine this discrepancy we compared the levels of Pea3 protein expressed from transfection and induction (Figure 5B). The amount of Pea3 present in whole cell lysate harvested from cells 24h post-transfection (100ng Pea3 vector) is approximately equivalent to the amount present in lysate harvested from cells induced for between 1-2h. Transfection with 500ng of Pea3 vector results in levels that approximate those of cells induced for between 2-6h. Based on the protein expression data (Figure 5B) the highest levels of Pea3 would be expected to be in the samples in Figure 7B and the lowest in samples in Figure 7C. This would suggest that low levels of Pea3 are more efficient at transcriptional activation and that high levels of Pea3 may be inhibitory. The phenomena of a transcriptional activator inhibiting transcription when expressed at high levels has been described and termed 'squelching' [122]. The mechanism of squelching is thought to be through the titration of components of the transcriptional machinery by excess protein to which these components bind. Another important factor is the possible relative differences in the kinetics of protein expression between induction and transient transfection. In the case of induction it has been shown that Pea3 levels increase over the course of several hours to a maximum that is sustained. In the case of transient

transfection it is possible that the expression kinetics are different as has been suggested by transient transfection followed by induction in COS-1 cells. It is possible that the transcriptional assay is biased towards an experimental system where both the reporter and effector are supplied simultaneously in the same mode (i.e. both transfected as plasmid). Another possibility is that transfection may be interfering with the induction of Pea3 to an extent. It is known that cationic-lipid transfection reagents such as those used in this study can be toxic to cells. In support of this, a specific stress-response due to cationic-lipid transfection reagent alone has been documented [123]. It has been estimated that the approximate half-life of the inducer (PonA) in solution at room temperature is on the order of 30-hours. Therefore, over the course of the experiments conducted the availability of PonA should not be a limitation. The cellular expression data (Figure 6) suggests the possibility that only a subset of induced cells highly expresses Pea3. If this is accurate, this could also explain the lack of consistent transactivation by induced Pea3, whereas transient transfection of Pea3 results in more cells expressing Pea3, allowing the measurement of transactivation. This would suggest that any measurable changes might be diluted-out.

3.1.4 RDA of cells induced to express Pea3

The main technique used to identify PEA3 target genes was representational difference analysis (RDA), originally developed as a method to discover differences between genomes [124] and later adapted for the comparison of cDNA [119]. Essentially, RDA uses a combination of PCR amplification and subtractive hybridization to preferentially amplify messenger RNA (mRNA) species whose expression level differs

between an experimental sample (induced/tester) and a control sample (un-induced/driver). A schematic representation of the technique is shown in Figure 8. The final output is a population of cDNA that should, theoretically, represent genes whose expression is up-regulated by induction of Pea3. Based on the kinetics of Pea3 induction and transactivation experiments, a short induction time were Pea3 protein was clearly expressed was chosen (6h). It was also hoped that a short induction time would allow the preferential identification of direct- or primary Pea3 target genes rather than downstream target genes.

Poly(A)$^+$-RNA was extracted from 1C cells induced to express Pea3 for 6h and from mock-induced cells. The major steps involved in generating the difference products are shown in Figure 9. The RNA was used to generate double-stranded cDNA, which was then digested with the restriction enzyme *Dpn*II. This particular enzyme is used since, as a four-cutter, it should produce fragments from most of the expressed genome, within a limited size range suitable for subsequent PCR and subtractive hybridization reactions [125]. Figure 9A shows the 0h and 6h cDNA samples before and after *Dpn*II digestion. A shift to a smaller size distribution can be observed after digestion, indicating successful digestion. Approximately equivalent size distribution and concentration can

Figure 8. Schematic representation of cDNA RDA to the first difference product. Solid boxes represent oligonucleotides used to generate the representations. Cross-hatched boxes represent oligonucleotides used to generate the difference products. (A) Generation of tester and driver. Zigzag arrows depict DNA synthesis. (B) RDA to generate the first difference product. Subsequent difference products are generated by reintroducing a generated difference product as tester in the scheme.

A

Total RNA

cDNA

Restriction digest
(*Dpn*II)

Ligate R12/24 linker

Melt 12-mer; fill in

PCR

Representative amplicon
(Tester and Drivers)

B

Tester

Driver 1

Driver 2
(16S and 23S rRNA-derived)

Digest;
Ligate J12/24 linker

Digest

Digest

Mix 1:50:50; Melt; Hybridize

tester:driver

tester:tester

driver:driver

Melt 12-mer; Fill in;
PCR (10 cycles);
Mung Bean Nuclease;
PCR (18 cycles)

Linear amplification

Exponential
amplification

No amplification

FIRST DIFFERENCE PRODUCT

Figure 9. Generation of RDA representations. Twice purified poly(A)$^+$-RNA from 1C cells induced to express Pea3 for 6h and mock-induced cells was used to generate the representations used for RDA. (A) Generation of cDNA from mRNA. Double-stranded cDNA generated from the poly(A)$^+$-RNA was digested with *Dpn*II. (B) Generation of 'representations'. *Dpn*II-digested cDNA was PCR-amplified to generate representations of the induced and un-induced samples. Control DNA (*Dpn*II-digested salmon sperm DNA) was used to estimate the concentration of the representations.

A
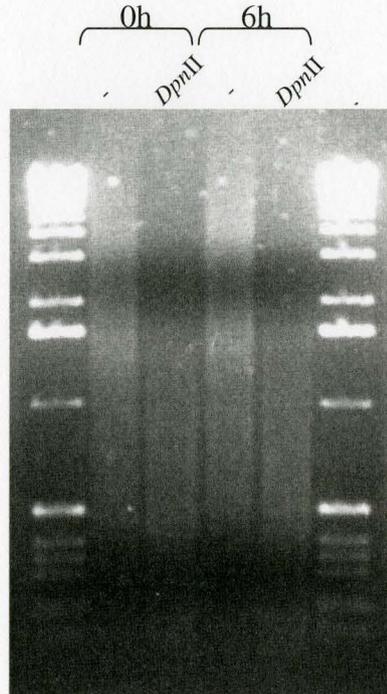
B
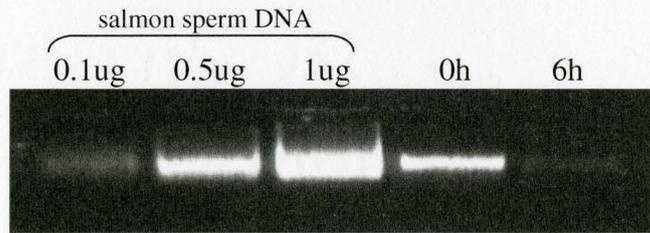
be confirmed by inspection of the gel. Adaptors were then ligated to the digested cDNA to allow PCR amplification to generate the representations. Salmon sperm DNA at a known starting concentration and digested with *Dpn*II was used to approximate the concentration of the representations (Figure 9B). The gel was only run for a short time to facilitate visualization of the DNA as a band rather than a smear. The fact that the 0h sample (driver) is significantly more concentrated than the 6h sample (tester) is not a concern, since the driver is always hybridized in excess. The difference products generated from iterative subtractive hybridization are shown in Figure 10. DP1 was generated with a tester:driver ratio of 1:100 (Figure 10A). Comparison of all three difference products is shown in Figure 10B. The adaptors removed from DP2 and DP3 are visible on the gel. Comparison of the difference products (Figure 10B) with the tester and driver (Figure 9A) reveals discernable bands in the difference product samples, which correspond to differentially expressed genes flanked by *Dpn*II restriction sites, rather than the smear observed with the original representation cDNA (Figure 9A).

DP2 and DP3 inserts were cloned into vectors for propagation in bacteria. Individual colonies were randomly picked, the plasmids isolated and the inserts sequenced. NCBI BLASTN searches were used to identify specific differentially expressed genes. In total, 17 inserts from DP2 and 20 from DP3 were sequenced. Table 2 lists the genes identified as putative Pea3 target genes up-regulated by Pea3. Also shown are the insert size and the degree of insert homology to target gene sequence. As can be seen from Table 2 four genes (mouse Pea3, cytochrome oxidase subunit I, cytochrome *b* and enolase 1) were identified in multiple colonies from both DP2 and

Figure 10. Generation of RDA difference products. Iterative subtractive hybridization reactions were performed to produce the difference products. DP1 was used to generate DP2, DP2 was used to generate DP3. (A) Concentration standards (*Dpn*II-digested salmon sperm DNA) were used to estimate the concentration of DP1 product. (B) Comparison of all three difference products. Note: adaptors/primers are visible in the DP2 and DP3 samples.

A

salmon sperm DNA

0.1ug 0.5ug 1ug   DP1

B

DP1   DP2   DP3
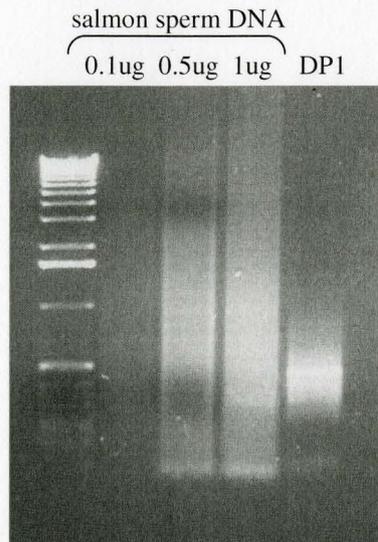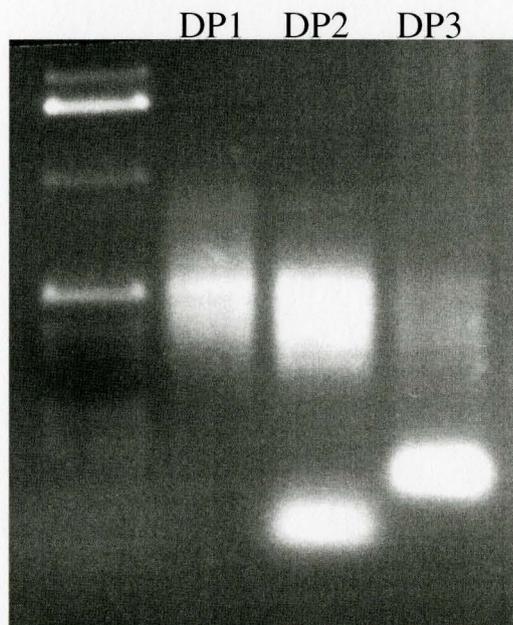
Table 2. Putative PEA3 target genes identified by RDA.

VECTOR indicates cloning plasmid sequence with no gene insert.

NO MATCH indicates good quality sequence data with no BLASTN identity.

N/D indicates poor quality sequence data.

[A] RDA clone identity as determined by insert sequencing followed by NCBI BLASTN search.

[B] Percent homology as given by NCBI BLASTN search 'Identities' result for the specific gene identified.

[C] Gene previously independently identified under different conditions or in a different model system.

RDA CLONE	CLONE IDENTITYA	INSERT SIZE	HOMOLOGYB	PREVIOUSLY IDENTIFIEDC
DP2-1	novel gene mapping to chromosome 1; similar to hypothetical protein MGC8902	503	98%	
DP2-2	ribosomal protein L9	344	99%	yes
DP2-3	enolase 1 (alpha)	377	96%	
DP2-4	v-akt	334	99%	
DP2-5	cytochrome oxidase subunit I	449	98%	yes
DP2-6	cytochrome oxidase subunit I			
DP2-7	VECTOR			
DP2-8	mouse PEA3	647	99%	
DP2-9	mouse PEA3			
DP2-10	cytochrome oxidase subunit I			
DP2-11	cytochrome oxidase subunit I			
DP2-12	cytochrome b	301	100%	
DP2-13	RNA polymerase I associated factor 53	414	79%	
DP2-14	cytochrome oxidase subunit I			
DP2-15	leucine rich repeat containing 5	408	100%	
DP2-16	cytochrome oxidase subunit I			
DP2-17	NO MATCH	217		
DP3-1	cytochrome oxidase subunit I			
DP3-2	cytochrome oxidase subunit I			
DP3-3	cytochrome oxidase subunit I			
DP3-4	mouse PEA3			
DP3-5	N/D			
DP3-6	guanine nucleotide binding protein (G protein), beta polypeptide 2-like 1	247	99%	yes
DP3-7	chromosome 21 open reading frame 108; KIAA0539	349	99%	
DP3-8	serologically defined colon cancer antigen 3; NY-CO-3	205	99%	
DP3-9	cytochrome oxidase subunit I			
DP3-10	cytochrome oxidase subunit I			
DP3-11	similar to nuclear autoantigenic sperm protein isoform 3; histone H1-binding protein	315	99%	
DP3-12	thioredoxin domain containing 5	371		
DP3-13	N/D	289		
DP3-14	mouse PEA3	485	99%	
DP3-15	cytochrome b			
DP3-16	calcium binding protein P22	359	99%	
DP3-17	ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit, isoform 1	344	99%	
DP3-18	cytochrome oxidase subunit I			
DP3-19	cytochrome b			
DP3-20	enolase 1 (alpha)			

DP3. This would suggest that those genes identified multiple times may be differentially expressed to a higher degree than genes identified only once. Alternatively, such genes may be more amenable to detection by RDA for such reasons as transcript size and sequence. In some cases no gene sequence match was found. There were three different types of such negative results. In one instance vector DNA was identified (denoted VECTOR in Table 2), likely indicating the lack of an insert. There were two cases in which the sequencing reaction returned low quality data, likely due to low quality DNA from the miniprep used for sequencing (denoted as N/D in Table 2). In one instance (NO MATCH in Table 2) a good sequencing reaction yielded no BLASTN match, likely representing an unidentified gene. In all, 16 unique genes were identified as putative Pea3 target genes. Other researchers in our lab have previously performed RDA using different conditions or model systems. One such model system utilized mouse embryo fibroblasts (MEF) isolated from wild-type mice and *Pea3*-null mice (Xin, unpublished). Another system employed RAT1 cells engineered to inducibly express Pea3. Three of the genes identified in this study have also been identified in these other RDA experiments and are indicated on Table 2.

A particularly important result was the identification of mouse Pea3 as a differentially expressed gene. This suggests that the RDA was successfully applied as Pea3 is the only gene that we know is up-regulated *a priori*. Examination of the mouse Pea3 cDNA sequence confirms that the inserts retrieved from DP2 and DP3 correspond to *Dpn*II-flanked regions.

The same inserts were used as probes for Northern analysis to confirm differential expression in independent samples for several of the RDA-identified putative PEA3 target genes. Table 3 lists the fold-change calculated by Northern analysis for the genes examined.

3.1.5. cDNA microarray analysis of 1C cells induced to express Pea3

Several global gene expression analysis experiments were conducted on 1C cells induced to express Pea3 for times ranging from 1h to 24h. The microarrays used consisted of PCR amplified plasmid DNA spotted on coated glass slides and covalently linked to the surface. Single slides consisting of approximately 1,700 genes or a set of two slides representing approximately 19,000 genes were used. Individual experiments produced lists of putative PEA3 target genes. However, repeated attempts failed to identify any reproducibly differentially expressed genes. Considering the transcriptional activity of induced Pea3 and the lack of a phenotype associated with induction of Pea3, it may be that the microarray experiments were not sensitive enough to detect any changes in gene expression. In contrast, the greater sensitivity of the RDA may have allowed the identification of small or few expression changes. Another potential problem may be evidenced by the relatively high 'ETS' activity observed in HEK293-derived cells. This coupled with the transactivation and phenotype results suggested the possibility that PEA3-regulated genes may already be in a state of activation and that further increases in ETS activity by overexpression of Pea3 may be limited in effect. Therefore, this particular line of research was modified to better reflect the experimental model system being used.

Table 3. Northern blot confirmation of PEA3 target genes identified by RDA. Fold-change was calculated by dividing *GAPDH*-normalized mock-induced samples by 6h-induced samples. Multiple values indicate multiple blots with independent RNA preparations. * indicates a Northern band not corresponding to alpha enolase.

GENE	FOLD-CHANGE
alpha enolase	0.8, 1.5, 1.2, 1.2, 1.1
alpha enolase - related*	14.2, 9.4
v-akt	1.2, 1.2, 1.2
cytochrome oxidase subunit I	2.16, 3.0, 6.0
cytochrome *b*	2.0, 2.6
GNB2L1	1.2, 1.0, 0.9
NY-CO-3	no signal
ATP synthase alpha subunit	1.6

3.2 Inducible dominant-negative Pea3 (ΔNPea3En) expression

3.2.1 Clone derivation and selection

As an alternative to the overexpression of Pea3 we next sought to examine the effects of reducing or inhibiting specific PEA3 or general ETS function in HEK293 cells. This was achieved with the use of a dominant-negative version of Pea3 (ΔNPea3En). It was expected that this fusion protein would retain the DNA-binding properties of wild-type Pea3 while repressing transcription of all target genes [91]. However it was also shown that ΔNPea3En inhibits transactivation mediated by all three Pea3 subfamily members. By extension it is possible that ΔNPea3En is capable of inhibiting the activity of other ETS factors as well. In this way we examined the effects of reducing ETS activity, specifically or generally, in a transformed human cell line that had apparently high endogenous ETS activity.

The various successes and problems encountered with deriving a Pea3 inducible cell line influenced the generation, characterization and selection of a ΔNPea3En inducible cell line. Cloning of ΔNPea3En into the pIND vector and subsequent transfection into the EcR-293 cell line was carried-out essentially as it was for the generation of the Pea3-inducible 1C clone. However, rather than initially screening clones for inducible expression of the transgene protein product, the effects on transactivation were examined first. It had proved relatively straightforward to derive several cell lines in which Pea3 protein could be inducibly expressed. The problem became showing that the induced Pea3 could effect transcription as expected for a transcriptional activator. Therefore, an initial panel of six colonies was screened for

inducible ΔNPea3En expression (Figure 11). The ability to reduce reporter gene expression from a Pea3-responsive promoter was examined (Figure 11A). Clone 1 data is absent due to poor growth and low yield of protein. ΔNPea3En protein expression of each clone induced for 24h and mock-induced is also shown (Figure 11B). 3/5 of the remaining clones showed a reduction in luciferase activity after 24h of induction (Figure 11A, clone 2, 3 and 4). The protein expression data for these three clones confirms that ΔNPea3En is inducibly expressed. Clone 5 and 6 show no or little reduction in luciferase activity upon induction, respectively. Western analysis confirms that clone 5 expresses very low levels of ΔNPea3En, if any, upon induction. Clone 6 is apparently leaky, expressing ΔNPea3En without induction and upon induction expression of reduced. While these findings with respect to clone 6 are interesting they do not likely represent anything pertinent to ETS factor function. Clone 3 was chosen as the experimental cell line for further analysis and designated clone DN3.

3.2.2 Inducible ΔNPea3En clone characterization

The ability of ΔNPea3En to effect transcription in the DN3 was examined more closely (Figure 12). The effect of transiently transfected ΔNPea3En on the 5xPea3-luc reporter was included for comparison to induced ΔNPea3En (Figure 12A and 12B). To ensure that the repressive effects of ΔNPea3En were not somehow general to all luciferase reporters, a human cyclin D1 reporter was also examined. This reporter has been shown to be responsive to both transiently transfected Pea3 and ΔNPea3En in COS-1 cells (Messier, in preparation). As can be seen, ΔNPea3En failed to reduce luciferase

Figure 11. Screening stable clones for inducible ΔNPea3En expression. EcR-293 cells were transfected with pIND/ΔNPea3En and picked as colonies after growth in selective media and subsequently screen for inducible ΔNPea3En expression. (A) The effect on transcriptional activity was assessed by measuring luciferase expression from 5xPea3-luc, an artificial promoter bearing repeats of an optimal Pea3 binding site. (B) Western analysis of whole cell lysates was used to screen clones for inducible expression of ΔNPea3En with PC2 polyclonal antibodies. Arrow indicates the position of ΔNPea3En. A "-" indicates mock-induced, a "+" indicates induced for 24h.

A

SCREENING OF pIND/ΔNPea3En EcR-293 CLONES

B
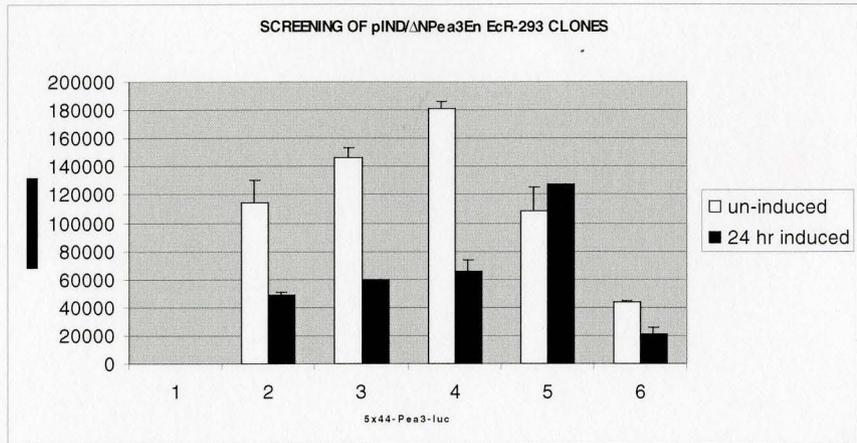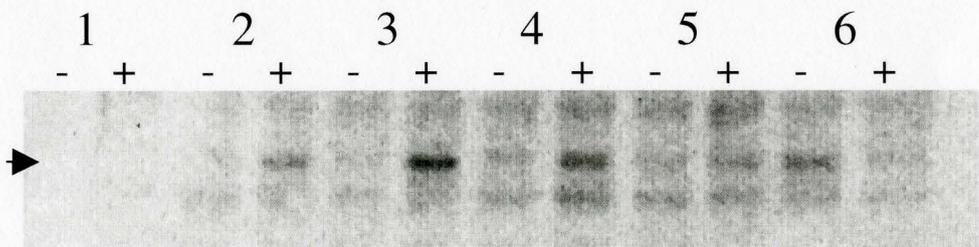
Figure 12. ΔNPea3En represses transcriptional activity of a Pea3-responsive promoter. Both transiently transfected and induced ΔNPea3En repress transactivation by endogenous factors on a Pea3-responsive reporter. Transcriptional activity was assessed by measuring luciferase expression from 5xPea3-luc, an artificial promoter bearing repeats of an optimal Pea3 binding site. (A) Increasing amounts of ΔNPea3En were transiently transfected into DN3 cells with constant amounts of the 5xPea3-luc reporter. (B) DN3 cells were either un-induced or induced for 24h. The values from the mock induced samples were set to 100. (C) Increasing amounts of ΔNPea3En were transiently transfected into DN3 cells with constant amounts of the -963 cyclin D1-luc reporter. Data shown are from a representative experiment performed in triplicate (mean ± S.E.).

A

Effect of ΔNPea3En on the basal activity of 5xPea3-luc (250ng)

B

Effect of ΔNPea3En induction on the basal activity of 5xPea3-luc (250ng)

C

Effect of ΔNPea3En on the basal activity of -963 Cyclin D1-luc (250ng)

71
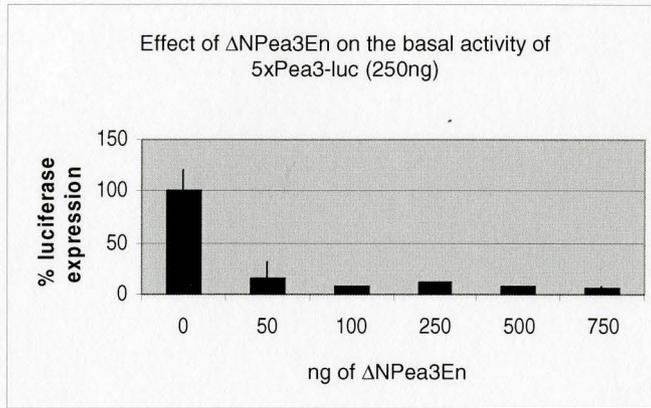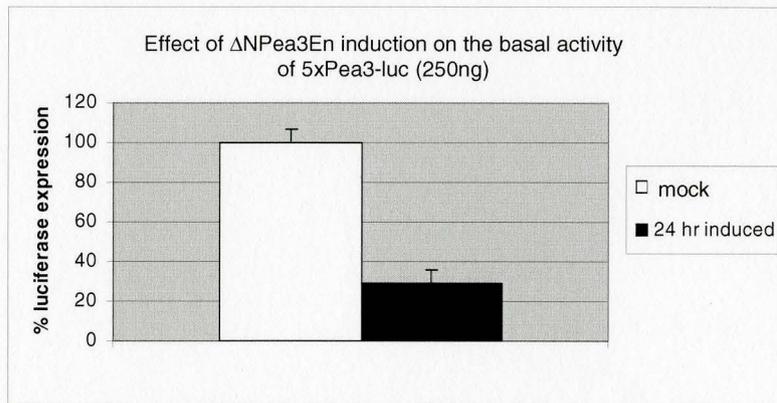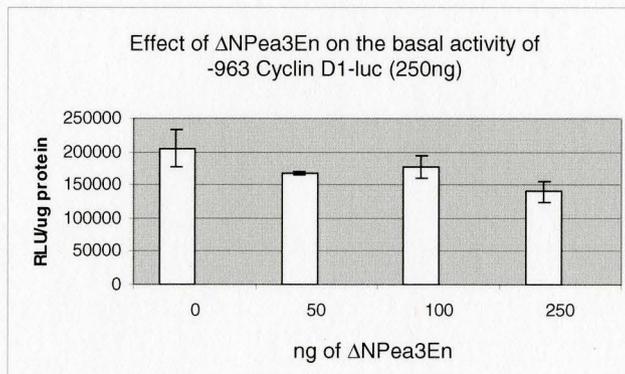
expression in the DN3 cell line suggesting that the effects are not general (Figure 12C). The magnitude of the relative transcriptional effects of induced ΔNPea3En compared to transfected ΔNPea3En may be similar to the results of induced and transfected Pea3 with the main difference being that the characteristics of the parental cell line are more amenable to repression by ΔNPea3En than activation by Pea3.

The induction kinetics of ΔNPea3En in the DN3 clone were examined in more detail and the results are shown in Figure 13. Induction at the RNA level was monitored by both RT-PCR (Figure 13A) and Northern analysis (Figure 13B). Protein induction was examined by Western analysis (Figure 13C). As was the case with the characterization of the Pea3-inducible clone, induction of ΔNPea3En was tightly controlled and rapid with no indication of leaky expression. In terms of relative induction of Pea3 in the 1C line (Figure 5) and ΔNPea3En, RNA induction was approximately equivalent, while induction of Pea3 protein is apparently higher than that of ΔNPea3En. This could be due to differences in antibody affinity or specificity. The epitope recognized by the MP16 monoclonal antibody, our Pea3-specific antibody of choice, is absent from ΔNPea3En, which required the use of the PC2 polyclonal Pea3-specific antibody. The level of ΔNPea3En resulting from induction is less than that from infection with virus encoding ΔNPea3En. However, the DN3 clone is still seemingly well-suited for continued experiments.

We next examined the expression of induced ΔNPea3En at the cellular level with immunocytochemistry (Figure 14). Again, as was observed with the 1C clone, only a small subset of induced DN3 cells expresses high levels of ΔNPea3En while a larger

Figure 13. Induction of ΔNP3a3En in DN3 cells. Characterization of ΔNPea3En induction in the DN3 clone. (A) Levels of ΔNPea3En RNA during induction were monitored by real-time quantitative PCR using a LightCycler instrument (Roche) with two-step PCR. The amount of cDNA in each of the samples has been normalized to *GAPDH* cDNA levels. (B) Northern blot showing the induction of dominant-negative *Pea3* transcript after 24h induction (top panel) and corresponding gel photo showing the 18S and 28S ribosomal bands (bottom panel) to confirm equal loading and RNA integrity. (C) Immunoblot illustrating the induction of ΔNPea3En protein. Un-induced (24h mock induced), 2h induced, 6h induced, 24h induced, and adenoviral-mediated ΔNPea3En expression as a positive control. Induction samples were 100 μg nuclear lysate and the positive control was 30 μg whole cell lysate. The abundance of ΔNPea3En was detected using a polyclonal antibody (PC2) that recognizes an epitope in the carboxyl terminus of Pea3. Arrows indicate the position of ΔNPea3En. Images shown are representative of experiments performed at least three times.

A
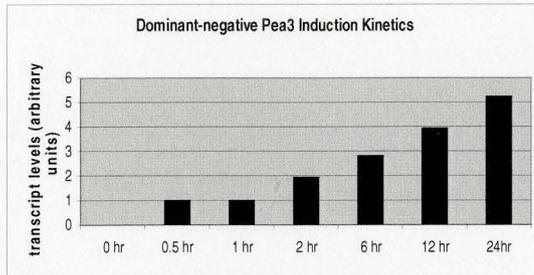
B
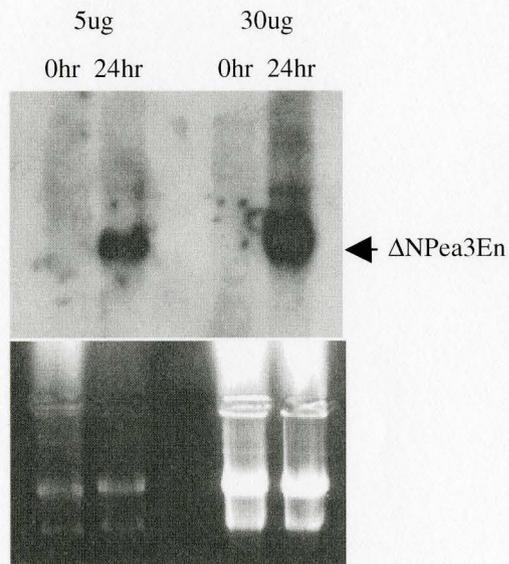
C
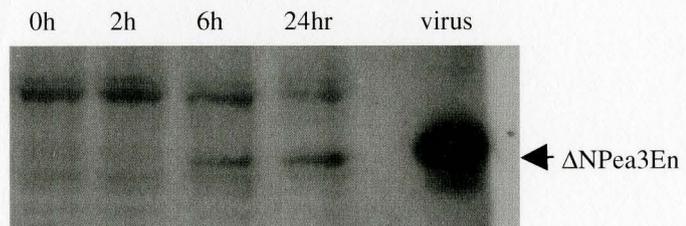
Figure 14. Immunocytochemical analysis of induced ΔNPea3En in DN3 cells. The expression of induced ΔNPea3En in DN3 cells was examined at the cellular level. DN3 cells were seeded and grown in 4-well chamber glass slides. (A) Different wells on the same slide were either induced to express ΔNPea3En for 20h or mock-induced. (B) As a positive control for the expression of ΔNPea3En, cells were infected by adenovirus containing ΔNPea3En or adenovirus containing STOP-ΔNPea3En for 20h. Cells were fixed and stained with polyclonal Pea3 anti-body PC2 (red) and counter-stained with DAPI (blue). Images of the same field with ΔNPea3En signal merged with the DAPI signal are shown. Images taken at 400X magnification. Images shown are from experiments performed at least three times.

A

mock   induced

B

Ad-STOP-ΔNPea3En   Ad-ΔNPea3En

76

subset expresses a modest level of ΔNPea3En slightly above background. The positive control cells infected with virus encoding ΔNPea3En show significantly more cells expressing higher levels of ΔNPea3En.

3.2.3 Effects of ΔNPea3En induction on phenotype and transactivation

The effects of induced ΔNPea3En in DN3 cells are shown in Figure 15. The first phenotypic observation of cells induced to express ΔNPea3En was a reduction in cell number relative to un-induced or mock-induced cells (Figure 15A). This was not observed in EcR-293 cells, the parental cell line (Figure 15B). This effect is typically first evident at 2- to 3-days post induction and continues until the mock-induced samples reach confluence. Examination of cell morphology throughout several days of induction induction reveals remaining cells as dense tightly packed colonies (Figure 15C). Interestingly, adenoviral-mediated overexpression of ΔNPea3En in human mammary tumor cell lines (BT-549 and MDA-MB-468) results in a complete loss of cells by 48h post infection, although the cause was not determined (Vaz, in preparation).

In order to more closely examine the growth deficit and to determine whether suppression of ETS transcriptional activity by ΔNPea3En induced growth arrest or programmed cell death, we examined apoptosis by TUNEL assay and DNA content and cell cycle distribution by flow cytometry (Figure 16). DN3 cells induced to express ΔNPea3En for 24h and 48h displayed a modest increase in the number of apoptotic cells relative to mock-induced (Figure 16A). Examination of cells under fluorescent microscope revealed a low incidence of apoptotic cells in mock-induced samples (approx. 0.2%). Induction of ΔNPea3En increased the portion of apoptotic cells (approx. 1%)

Figure 15. Effect of induced ΔNPea3En on cell growth. Growth of cells expressing ΔNPea3En show a reproducible growth defect, while the parental cells carrying no inducible gene are unaffected by induction. (A) Growth curve of cells induced to express ΔNPea3En or mock-induced. 1x10⁵ cells were seeded per 35 mm plate in DMEM supplemented with 10% FBS. Induction with 5 µM PonA (1 µL in 100% ethanol per mL media – red line) or mock induction (1 µL 100% ethanol per mL media – black line) was started 3 hours after seeding. Media was changed and inducer/mock inducer replenished every two days. Cells were counted with a hemocytometer. (B) Growth curve of parental cells exposed to inducer or mock inducer performed as in *A*. (C) Fixing and staining of cells. Cells were grown and treated as described for the growth curves. On the indicated days, cells were fixed with 10% buffered formalin and stained with Giemsa. Images taken at 100X magnification.

A

INDUCED DN3 CELLS

B

INDUCED EcR-293 CELLS

C

Day
0

mock   induced

1

3

5
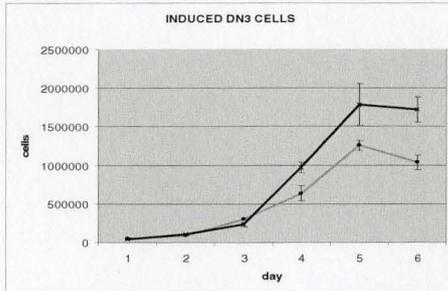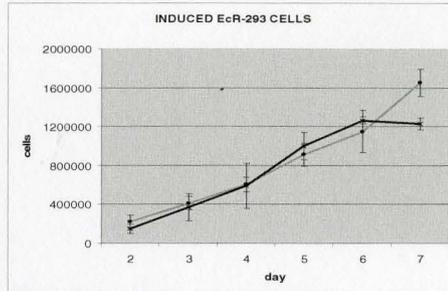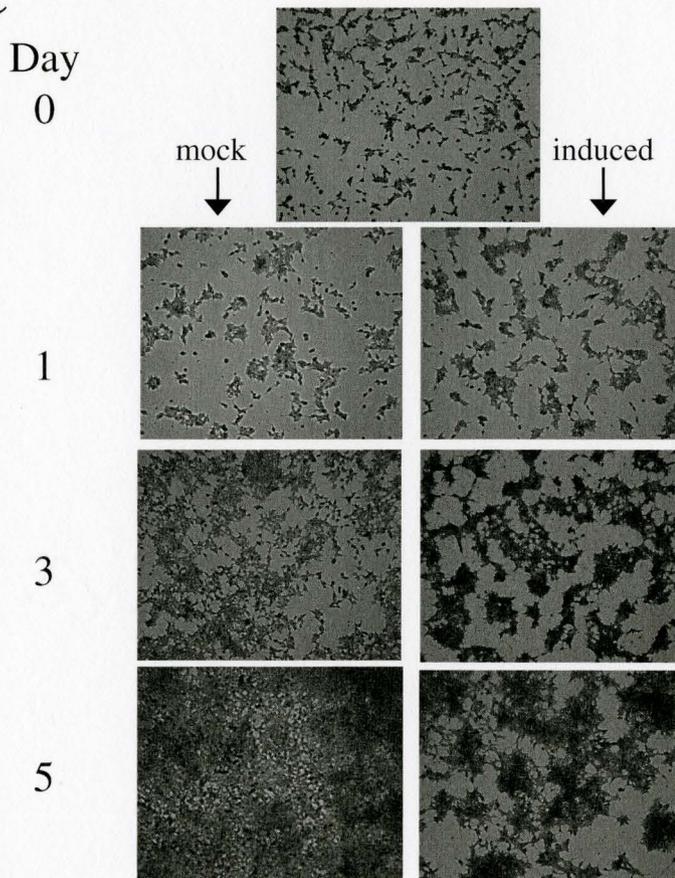
Figure 16. Examination of apoptosis in DN3 cells induced to express ΔNPea3En. DN3 cells were induced with PonA for the indicated times and either analyzed by TUNEL assay or flow cytometry. (A) Apoptotic cells after 24h and 48h of induction were detected by TUNEL assay. *Top row* shows only the FITC channel signal for DNA-nicked positive cells, *bottom row* shows merged image for FITC and DAPI (for nuclei). Images taken at 100X magnification. (B) Representative DNA content analysis by flow cytometry for cells mock-induced, induced for 24h and induced for 48h. (C) Average values for flow cytometry analysis of induced DN3 cells. Fraction of sub-G_1 representing apoptotic cells is shown in bold. Data shown are from representative experiments performed in triplicate.

A

FITC

| mock | 24h | 48h |

| mock | 24h | 48h |

MERG

B

| mock | 24h | 48h |
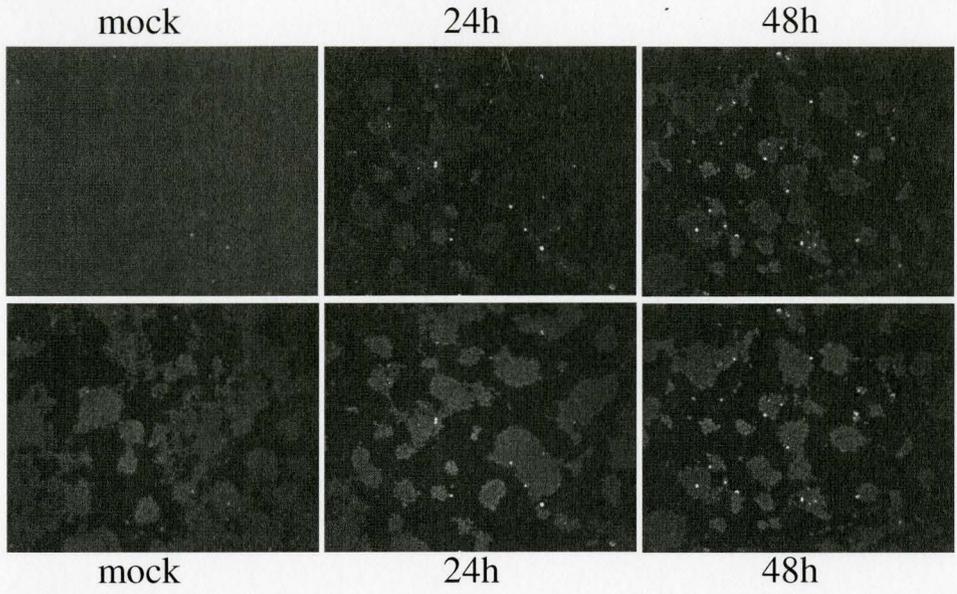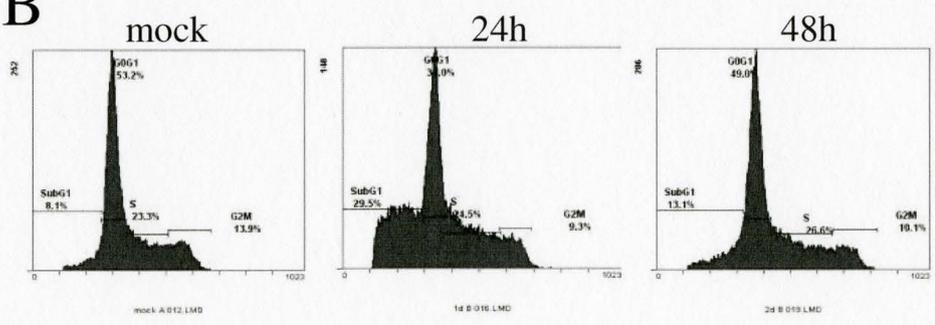
C

	mock	12h	24h	48h	72h
subG1	**10**	**9**	**32**	**17**	**18**
G0G1	47	46	34	45	43
S	27	28	19	24	22
G2M	13	13	12	12	11

representing an increase of 5-fold. It was noted that the total number of DAPI-stained nuclei counted per unit area was consistently lower for induced samples than for mock-induced samples suggesting that cells may have detached from the surface of the culture dishes of induced cells. Flow cytometry revealed the greatest increase in sub-G_1 cells to be approximately 3-fold after 24h of induction in DN3 cells (Figure 16B, 16C). After 48h and 72h induction the portion of sub-G_1 cells remained higher than mock-induced cells but reduced relative to 24h at approximately 1.7-fold. There was no change in sub-G_1 after 12h induction of ΔNPea3En. No other stage of the cell cycle was reproducibly affected by induction of ΔNPea3En. The number of apoptotic cells detected by TUNEL assay was lower than by flow cytometry. However, the fold-increase in apoptosis as determined by TUNEL was higher than by flow cytometry. Additionally, there was an induction time component revealed by flow cytometry that was not evident by TUNEL assay – a greater increase in apoptotic cells at 24h than any other time examined. This discrepancy is likely due to the loss of apoptotic cells during the fixing and washing steps of the TUNEL protocol that have no equivalent in the flow cytometry protocol. DNA fragmentation as an indicator of apoptosis is thought to be a rapid and irreversible process [126, 127]. This is consistent with the reduction in cell number observed after induction during the TUNEL assay. Thus it appears as though induction of ΔNPea3En in DN3 cells induces apoptosis between 12h and 24h post induction at an initial rate, which gradually lowers, with prolonged induction.

3.2.4 Expression profiling of genes transcriptionally responsive to ΔNPea3En

In order to examine the transcriptional effects of ΔNPea3En expression in DN3 cells on a global scale we carried-out expression analysis using Affymetrix GeneChips. DN3 cells were induced or mock-induced for 24h and RNA harvested for analysis. A relatively long time point was chosen to increase the likelihood of inducing measurable changes in target gene transcription. Three independent inductions were used, with one being assayed twice as a technical replicate, representing four total replicates. Affymetrix software was used for all statistical analyses. Two statistical measures were examined for consistent differential expression across replicates: t-test with 95% confidence and individual pairwise comparisons. Four induced replicates were compared to four mock-induced replicates using a t-test with 95% confidence. Additionally, each replicate experiment was subjected to pairwise analysis (individual mock vs. induced). We then grouped each differentially expressed gene into one of three categories, depending on whether it passed one or both of the statistical tests: t-test only, three out of four pairwise comparisons only, or both. The most stringent category contained 44 genes, 8 up-regulated and 36 down-regulated. The category consisting of genes passing only the t-test had 258 genes, 123 up-regulated and 135 down-regulated. The third category containing genes passing three out of four pairwise comparisons had 52 genes, 9 up-regulated and 43 down-regulated. We sought to confirm the differential expression of a subset with RT-PCR as an independent method. 20 genes from each of the three categories were chosen with the predominance coming from the most stringent category. In all, the differential expression of 14 genes was confirmed (Table 4). Six of the 20

genes chosen for confirmation either failed to show differential expression as measured by RT-PCR or working primers were not found. The direction and magnitude of the change of expression as measured by RT-PCR was similar to the GeneChip data for most of the 14 genes, with only two showing a smaller fold change and one showing a larger fold change. These 14 genes along with all genes passing both statistical tests are shown in Table 4 and represent those genes with the highest confidence of true differential expression. Another possible criterion to increase confidence in difference expression is the occurrence of multiple independent probe sets passing the statistical tests. This occurs for several genes in all three categories.

Table 4. Genes with statistically significant differential expression due to induction of ΔNPea3En for 24h. Fold-change reported is expressed as the ratio of the average signal intensity of all induced samples (n=4) over the average of all mock samples (n=4). Negative values indicate down-regulation relative to overexpression of ΔNPea3En. * indicates RT-PCR confirmation, + indicates multiple probe sets presenting the same trend.

Gene	probe ID	fold-change 24h / 0h
Cytochrome P-1-450 (TCDD-inducible)*+	36767_at	9.5
Stanniocalcin*	41354_at	4.1
8-hydroxyguanine glycosylase*	38335_at	3.5
selenoprotein P*	34363_at	2.9
P2X4 purinoreceptor*	38332_at	2.3
Autotaxin*	41123_s_at	1.9
IgG Fc receptor hFcRn	31431_at	1.6
Keratin 18	35766_at	1.6
Retinoic acid receptor-like protein	405_at	-1.3
transmembrane 4 superfamily member 8	38612_at	-1.3
VDAC1	31608_g_at	-1.3
Mrp17	39812_at	-1.3
ADA3-like protein	35749_at	-1.3
E2F-related transcription factor (DP-1)	37757_at	-1.4
Midline 1 fetal kidney isoform 3*	34296_at	-1.4
Lactate dehydrogenase-A	41485_at	-1.5
cysteine and glycine-rich protein 2	41401_at	-1.5
CpG island-associated gene	36209_at	-1.5
SOX-4	33131_at	-1.5
5-AMP-activated protein kinase, gamma-1 subunit	34346_at	-1.5
pre-mRNA splicing factor SF2p32	37668_at	-1.5
cyclin D1	38418_at	-1.5
S-adenosylmethionine decarboxylase	262_at	-1.5
glutathione peroxidase	37033_s_at	-1.5
Proto-Oncogene C-Myc, Alt. Splice 3, Orf 114	1936_s_at	-1.5
clone 24538	39381_at	-1.6
Keratin 19	40899_at	-1.6
ras-related C3 botulinum toxin substrate (rac)*	2050_s_at	-1.6
ZIC2	35554_f_at	-1.6
MEK5*	513_at	-1.7
Insulin-like growth factor binding protein 5*	38650_at	-1.7
Translation initiation factor eIF-2 gamma subunit	35934_at	-1.7
D9 splice variant A	35710_s_at	-1.7
forkhead/winged helix-like transcription factor 7	41027_at	-1.7

Translin associated protein X	41051_at	-1.7
Prothymosin alpha*+	38589_i_at	-1.8
c-myc*+	37724_at	-1.9
CAGH1 alternate open reading frame	38648_at	-1.9
hairy and enhancer of split 1	37393_at	-2.1
clone 23870	41672_at	-2.1
fibroblast growth factor receptor 2*	1970_s_at	-2.4
Acidic (type I) cytokeratin 10	38610_s_at	-2.5
Insulin receptor substrate 4*	33604_at	-5.2

3.2.5 Transcriptional effects on the human *MYC* promoter

One of the putative PEA3 target genes was chosen for further study. The *MYC* oncogene passed the most stringent criteria applied to our expression profiling and has previously been shown to be regulated, directly or indirectly, by ETS factors [104, 128, 129]. Luciferase reporter constructs containing fragments of the human *C-MYC* promoter were used to examine the transcriptional effects of Pea3 and ΔNPea3En (Figure 17). ΔNPea3En repressed luciferase activity from both *MYC* promoter reporters tested with the largest effect being on HMB-luc, which contains the largest *MYC* promoter fragment, while induced Pea3 had no effect (Figure 17A). We next tested whether, as was the case with the optimal synthetic Pea3-responsive reporter, transiently transfected Pea3 was capable of inducing transcription of the *MYC* promoter. Transiently transfected Pea3 was able to transactivate the *MYC* promoter (Figure 17B). Unlike induced ΔNPea3En, transiently transfected Pea3 had a greater effect on the shorter *MYC* promoter fragment, XNM-luc.

Figure 17. Effects of Pea3 and ΔNPea3En on transcription of the human *C-MYC P2* promoter. Transcriptional activity was assessed by measuring luciferase expression from HMB-luc and XNM-luc, reporters consisting of fragments of the human *C-MYC* promoter which contains at least one ETS binding site. (A) ΔNPea3En or Pea3 expression was mock-induced or induced in DN3 or 1C cells, respectively, for 24h. The values from the mock-induced samples were set to 100. (B) The pCANmyc expression vector was used to express Pea3 in DN3 cells (without induction of ΔNPea3En) at varying doses (25 ng, 100 ng, 250 ng); pCANmyc empty vector was independently transfected at amounts corresponding to those of pCANmyc/Pea3 as a control. The basal luciferase activity from the transfection of the lowest amount of empty vector control with reporter was set to 1; luciferase values for transfected Pea3 were divided by the corresponding luciferase values obtained from empty vector/reporter transfections. Numbers above the bars indicate fold transactivation. Data shown are from a representative experiment performed in triplicate (mean ± S.E.).

A

Effect of induced Pea3 or ΔNPea3En on the *MYC* P2 promoter

B

Effect of transfected Pea3 on the *MYC* P2 promoter
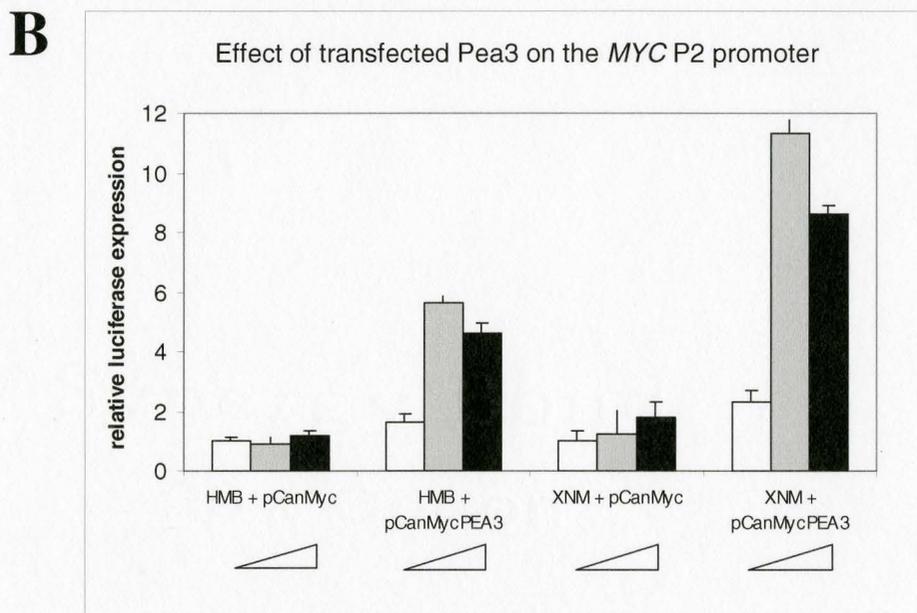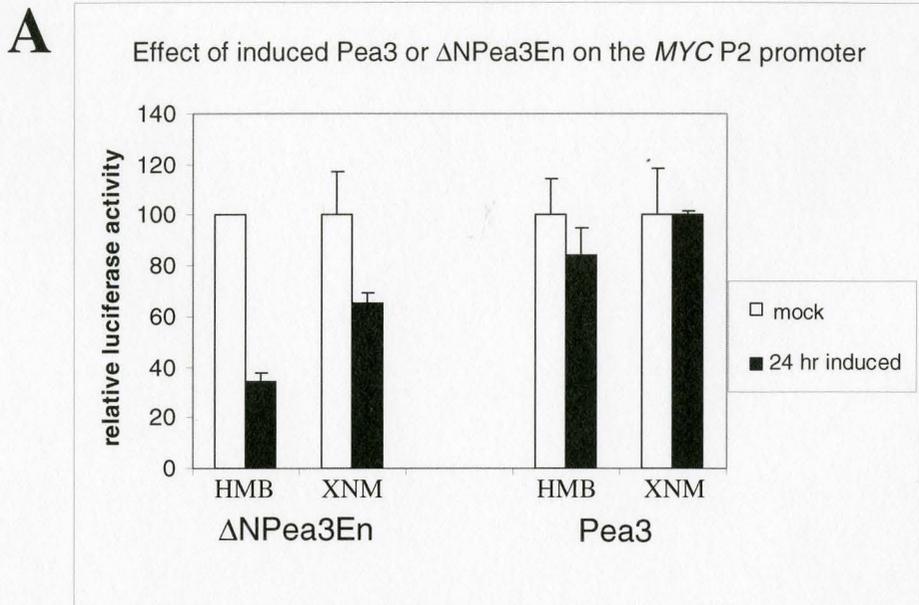
CHAPTER 4: DISCUSSION

ETS factors are involved in a wide range of cellular processes and misregulation of many ETS factors including PEA3 have been implicated in several types of cancers in humans and their importance in cancer is becoming more evident (reviewed in [130]). For example PEA3 is upregulated in human breast cancer [89], colorectal cancer [76, 94], gastric cancer [75]; ETS-1 is upregulated in human breast cancer [131] and gastric cancer [132]; ERG is upregulated in acute myeloid leukemia [133] and prostate cancer [134]. Misregulation of ETS factors would lead to misregulation of their targets, which, in turn, would potentially lead to phenotypic changes. Currently little is known regarding the transcriptional targets of specific ETS factors but the list of genes whose expression has been shown to be regulated by ETS factors is growing. PEA3 [75, 82, 83, 135] and ETS-1 [136, 137] have been shown to regulate multiple matrix metalloproteinases; PEA3 regulates beta1,4-galactosyltransferase I which is upregulated in lung cancer cells [138] and the mammaglobin gene involved in breast cancer [139]; ETS-1 regulates the interferon-regulated genes STAT1 and NMI [140]; unspecified ETS factors have been shown to regulate the extracellular matrix protein adhesion receptor, alpha3beta1 integrin [141]. We chose to employ an inducible expression system to overexpress both Pea3 and a dominant-negative Pea3 fusion protein consisting of the Pea3 DNA-binding domain fused to the *Drosophila* engrailed repression domain (ΔNPea3En). We then used either RDA or DNA microarrays to attempt to identify specific PEA3-regulated genes.

Through the use of ΔNPea3En we sought to identify PEA3 target genes by interfering with endogenous ETS activity in a human cell line and monitor for phenotype

and gene expression profile changes. Several researchers have successfully employed a similar strategy. The DNA-binding domain of Ets-2 fused to the prokaryotic *lacZ* gene (Ets-LacZ) expressed in murine 3T3 cells suppressed a transformed phenotype induced by the *v-Ras* oncogene or colony-stimulating factor 1 receptor (Csf-1R) with colony-stimulating factor 1 (Csf-1) stimulation [103]. Csf-1 along with its receptor, controls proliferation, differentiation and survival or certain cell types and has been shown to increase tumorigenicity and invasiveness of normal and transformed cells [142]. It was found that Ets-LacZ expression impaired the induction of Myc and that enforced expression of Myc countered the effects of Ets-LacZ [103]. Furthermore, an earlier study had demonstrated that 3T3 cells expressing a mutant Csf-1R could not form Csf-1-dependant colonies and impaired Myc response, which was restored by exogenous Myc expression [143]. An ETS-2 transdominant mutant expressed in BT20 breast carcinoma cells completely inhibited anchorage-independent growth and CSF-1-induced invasion [104]. An Ets-1 mutant (Ets1-DB) lacking the activation domain was constitutively expressed in murine brain capillary endothelial cells resulting in a phenotype of increased spreading and adhesion and a greater capacity to form branched structures when cultured on Matrigel [106]. Contradictory results were obtained by another group with a similar transdominant-negative Ets-1 mutant (TMEts1) expressed in murine spleen endothelial cells [144]. The discrepancies may be explained by differences in experimental design, expression levels and the cell lines used. Ets1-DB was able to inhibit both normal and tumor Fgf-2-induced angiogenesis in a mouse ear model [107]. Another Ets dominant-negative construct (Ets-Z) induced apoptosis in human thyroid carcinoma cell lines but

not in normal thyroid cells [105]. Interestingly, this induced apoptosis was dependent on a reduction of MYC protein and was prevented by MYC overexpression. Interference strategies other than dominant-negative mutants have also been used to examine the role of various ETS factors. Small interfering RNAs (small interfering RNAs) were used in conjunction with microarrays to identify genes regulated by EWS/FLI-1 in Ewing cells [145, 146]. Triplex-forming oligonucleotides have been developed to specifically inhibit ETS-2 in human prostate cancer cells resulting in both anchorage-dependent and – independent growth and increased apoptosis, concomitant with an reduction in BCL-X_L, cyclin-D1 and MYC [147, 148]. Our particular dominant-negative construct (ΔNPea3En) has been shown to repress the capacity of Pea3, Erm and Er81 to transactivate several Pea3-responsive reporters in transient transfection assays and, when expressed under the control of the MMTV promoter in MMTV-*neu* transgenic mice, delayed the onset and reduced the size and number of mammary tumors [91]. Interestingly, ΔNPea3En is not expressed in the mammary tumors that do form in these transgenic mice, indicating a requirement for Ets activity. Furthermore, ΔNPea3En was able to repress Pea3-mediated transactivation of the human *CYCLIN D1* promoter in transient transfection assays in Cos-1 cells (Messier, in preparation), although this was not observed in the 1C cells.

A potential caveat to the use of such a dominant-negative construct is the potential to broadly interfere with ETS function [149]. Thus, any effects or differentially expressed genes identified due to the induction of dominant-negative Pea3 construct may in fact be related to other ETS factors or the combined activity of several ETS factors. However, at least *in vitro*, there is a large overlap in the binding specificity of particular

ETS binding sites [7, 150-152] and this may reflect the activity of endogenous ETS factors *in vivo* rather than being a purely *in vitro* artifact. It should also be noted that the putative PEA3 target genes identified through ΔNPea3En would be expected to have an inverse relationship in terms of differential expression relative to Pea3 *in vivo*. Thus genes upregulated by ΔNPea3En would be expected to be downregulated by PEA3 if they were *bona fide* target genes. Likewise, genes downregulated by ΔNPea3En would be expected to by upregulated by PEA3. This was however not found among the experimental systems employed. It may be that no specific PEA3 target genes were identified and that the target genes of different ETS factors were identified in different experimental systems. In the case of ΔNPea3En this could very well be true since it may affect many ETS-regulated genes in addition to Pea3 subfamily members. It is less likely for the case of induced Pea3 in the 1C line since it has been shown that Pea3 was significantly overexpressed and that the subsequent changes in gene expression are due to Pea3.

Inducible expression of Pea3 and ΔNPea3En was achieved with the ecdysone-inducible system [113]. In this system expression is controlled by an ecdysone-responsive promoter upstream of the gene-of-interest and a constitutively expressed receptor which is activated by the addition of an inducing synthetic steroid analog. One of the chief benefits of an inducible system is that it does not require physiological stimulation and is therefore uncomplicated by upstream signaling pathways. Inducible systems offer other potential benefits over constitutive systems. Clonal heterogeneity often requires that several clones of constitutively expressing cells be assayed for

statistically meaningful results. For inducible systems however, the experimental samples and the control samples are derived from the same clone, largely eliminating the concern of clonal heterogeneity. Furthermore, the ability to modulate the level and duration of expression allows for the approximation of physiologically relevant expression, circumventing potential cytotoxic effects related gross overexpression and counterselection. When compared with other widely used inducible systems, the ecdysone system ranks well in terms of leakiness, induction rates, and maximal expression [116]. Coupling engrailed fusion proteins with inducible expression systems for the study of eukaryotic transcription factors has become a widely used approach and has been reviewed in [111].

Induction of Pea3 expression in HEK-293 cells yielded no phenotype. The effects of induced Pea3 on transcription were not consistent or expected (Figure 7A and 7B), although transiently transfected Pea3 produced the expected result of increasing reporter gene transcription (Figure 7C). It was believed that this discrepancy was likely due differences in the expression kinetics and levels of protein expression resulting from induction and transfection (Figure 5B). This combined with the observation that the HEK-293 cell line has a high endogenous ETS activity prior to increasing Pea3 expression suggests potential difficulties associated with measuring Pea3 activity in these cells.

Despite these potential problems RDA was performed. It was hoped that the technique would be sensitive enough to identify differentially expressed genes related to Pea3 induction. Several candidate PEA3 target genes were identified (Table 2 and 3).

The identification of mouse Pea3 was an encouraging result as was the identification of three genes that were previously identified by independent RDA experiments. The most interesting result was the frequent recovery of the gene for cytochrome oxidase subunit I (*COI*), a mitochondrial-encoded gene. This particular gene was also one of the genes previously identified by RDA in our lab. This was complemented by the recovery of two additional genes with roles in the mitochondria: cytochrome *b* (mitochondrial-encoded) and ATP synthase *alpha* subunit (nuclear-encoded).

These results suggested that PEA3 may somehow be involved in regulation of mitochondrial factors – both mitochondria-encoded and nuclear-encoded. The result that PEA3, a nuclear transcription factor, may regulate genes in the mitochondria seemed problematic. However, examination of literature regarding mitochondrial genetics and biology revealed possible models. A review of mitochondrial genetics and biology is provided by [153]. Mitochondria are thought to have arisen from the incorporation of α-purple bacteria by a prototypic eukaryotic organism resulting in an endosymbiotic relationship. Over time many but not all essential genes of the mitochondria were transferred to nuclear chromosomes. Mitochondria play a major role in regulation of apoptosis; however the main function of the mitochondria is the production of energy in the form of ATP via oxidative phosphorylation. The human mitochondrial genome (mtDNA) is a circular, double-stranded molecule of about 16.6 kb that utilizes a genetic code different from that of the nucleus. Present day mammalian mitochondria code for 13 polypeptides, 2 rRNA molecules, and 22 tRNAs. All other mitochondrial factors,

such as metabolic enzymes, DNA/RNA polymerases and mtDNA regulatory factors are encoded by the nucleus and exported to the mitochondria.

Substantial work has been done on the regulation of nuclear-encoded mitochondrial genes. ETS factors have been associated with the mitochondria since the Ets factor GABP (GA-binding protein) was shown to have identity with NRF-2 (nuclear respiratory factor-2), a key transcription factor that regulates genes involved in mitochondrial respiration [154]. NRF-2 binding sites contain an essential GGAA recognition motif – the core of the ETS binding site. NRF-2 was found to regulate a number of mitochondrial genes. Subsequently, human NRF-2 was found to be the human homologue of mouse GABP. The promoters of murine cytochrome c oxidase subunit IV and subunit Vb were found to contain Ets binding sites which bound Ets-related factors [155], [156]. An ETS binding site in the human ATP synthase β-subunit promoter is essential for ETS-1- and ETS-2-mediated transactivation [157]. The gene for bovine cytochrome c oxidase subunit VIIa contains functional GABP sites [158]. Two Ets binding sites are found in the promoter for rat muscle-specific cytochrome c oxidase subunit VIII [159]. A bi-directional promoter was found directing the expression of mouse GABPα (a subunit of functional GABP) and ATP synthase coupling factor 6, a nuclear-encoded mitochondrial factor [160]. One of the most important elements connecting ETS factors to the mitochondria is mitochondrial transcription factor A (mtTFA, human or Tfam, mouse), a key factor in mitochondrial biology. Human *mtTFA* was shown to be activated by NRF-2 [161].

mtTFA has been proposed to coordinate transcription from two separate genomes – that of the nucleus and that of the mitochondria. Each of the five multi-subunit enzyme complexes responsible for oxidative phosphorylation in the mitochondria is composed of both mitochondrial- and nuclear-encoded polypeptides. Furthermore, each of the subunits operates in stoichiometric relationships that have been solved and it is not known if there is any significance to unbalanced expression. Therefore, mechanisms likely exist to coordinate expression from these two genomes (reviewed in [162]). mtTFA is essential for both mtDNA maintenance and transcription. Since it has been shown to regulate *mtTFA*, GABP has been theorized to function within the context of a bi-genomic regulator. Therefore, increases in GABP activity would upregulate several genes destined for the mitochondria including mtTFA, which is required for transcription of the mitochondrial genome. The differential expression of mtTFA in response to Pea3 induction was investigated with RT-PCR, however primer sets used failed to amplify the expected fragment.

Both NRF-1 and GABP have been theorized to be involved in the transduction of proliferation signals (in review by [163]). In this role proliferation signals equate to increased energy requirements, which would require coordinated upregulation of both nuclear- and mitochondrial-encoded factors. This coordination has been experimentally observed. Zhang and Wong-Riley [164] utilized the fact that in the dendrites of neurons mitochondria may be spatially separated from the nucleus by far greater distances than in other cell types. It was found that stimulation of activity in cultured rat neurons resulted in increased expression of GABP. This was followed by an upregulation of both

mitochondrial- and nuclear-encoded mitochondrial proteins. This, in turn, was followed by increased mitochondrial enzyme activity. In a similar fashion both mitochondrial- and nuclear-encoded mitochondrial mRNAs increased in rat hearts when energy metabolism was disrupted [165]. ATP synthase β-subunit levels have been shown to increase in response to proliferative signals in several cultured cell lines [166]. Also of interest is that during fetal liver development and liver regeneration when *ets-2* expression is high ATP synthase β-subunit mRNA levels are increased [15]. *COI* has been identified as being upregulated by p53 [167]. In this work, researchers used a human colon cancer cell line that lacked functional p53 and inducibly expressed wild-type p53. The other gene identified as upregulated by p53 was isozyme 6 of aldehyde dehydrogenase, a gene found to be upregulated in cancer cell lines resistant to anticancer drugs.

Mitochondrial involvement has been linked to several disease states, including cancer and aging (reviewed by [168]). Mitochondrial diseases involve base mutations and rearrangement mutations (deletions and insertions) in mtDNA. Diseases in which mitochondria are involved in may also result from nuclear mutations that impinge on mitochondrial maintenance and function. Viral hepatitis or repeated necrosis and regeneration may increase mtDNA transcription [169]. Mitochondrial-encoded genes (including *COI*) were found to be upregulated in diabetic human skeletal muscle compared to normal [170]. *COI* was also found to be upregulated in 5/6 nephrectomized mouse kidneys compared to normal kidneys [171].

Mitochondria have also been implicated prominently in aging. Indeed, a widely held theory of aging involves the accumulation of reactive oxygen species (a toxic by-

product of cellular respiration) leading to the accumulation of DNA damage resulting in an overall decrease in energy production. The findings concerning mtDNA levels, transcript levels, protein levels, and functional enzyme activity do not necessarily show a positive correlation. Most findings indicate that mitochondrial activity decreases with age [172]. This contrasts with the finding that protein levels (both mitochondrial- and nuclear-encoded) [173], and subunit transcript levels (both mitochondrial- and nuclear-encoded) [174] all increase with age. The likely explanation is a compensatory increase in mitochondrial assembly to counter decreased energy production due to the accumulation of mutations.

An interesting correlation exists between mitochondria, Pea3, the effects of age and calorie restriction. In mice, calorie restriction counters the age-associated loss of mitochondrial function [175], [176]. It has been shown that Pea3 is involved in myogenic differentiation [66] and is upregulated in damaged muscle and the muscle of aged mice [177]. Importantly, it has also been shown that calorie restriction completely counters this age-associated increase in *Pea3* [178]. Together, these studies show that both mitochondrial content (mtDNA and transcripts) are elevated under the same conditions as is *Pea3* (age-related) and that the same treatment (calorie restriction) counters both.

The use of glass cDNA microarrays was explored as an additional method to expand and correlate the RDA findings and identify additional PEA3 target genes. Several experiments were carried-out with Pea3 induced in the 1C cell line with no degree of reproducible results. This lack of results coupled to the small fold-change of

differential gene expression measured by Northern analysis used to confirm the RDA findings along with the other limitations encountered with induction of Pea3 in the 1C line prompted a change in methodology. Two main changes were made to the experimental scheme. First, rather than increase ETS activity by increasing Pea3 expression, endogenous ETS activity was reduced with ΔNPea3En. Concomitant with this was the use of oligonucleotide-based arrays (Affymetrix GeneChips) instead of glass cDNA microarrays.

The use of ΔNPea3En seemingly overcame most of the initial limitations encountered while characterizing the Pea3-inducible line. The expected effect of induced ΔNPea3En on transcription was observed (Figure 11A and 12B). A phenotype was also readily observed; ΔNPea3En induction reduced cell number and increased apoptosis (Figure 15 and 16). Thus, by extension it is attractive to speculate that PEA3 or any number of ETS factors endogenously expressed in the DN3 cell line may have an anti-apoptotic role. In support of this our lab has observed that virally transduced ΔNPea3En, which is expressed at significantly higher levels than induced ΔNPea3En (Figure 13C), causes a drastic reduction in cell density in two mammary tumor cell lines, BT-549 and MDA-MB-468 (Vaz, in preparation). An anti-apoptotic role for ETS factors has been identified in several reports. Ets-1 deficiency has been shown to negatively affect the survival of T-cells by increasing apoptosis [20, 21]. Both Erg and Fli-1 inhibit apoptosis in fibroblasts under serum-deprived conditions [179]. Furthermore, Ewing sarcoma cells expressing EWS fusions of Erg or Fli-1, which are resistant to apoptosis induced by chemotherapeutic agents, are sensitized to apoptosis by targeted disruption of the fusion

proteins [179]. Fli-1 has been shown to inhibit apoptosis in primary erythroblasts [180]. Ets-2 has been shown to protect macrophages from apoptosis induced by Csf-1 deprivation through a through a Bcl-xL-dependent mechanism [17]. TEL knockout mice are embryonic lethal with apoptotic mesenchymal and neural cells [27]. E2F1-dependent apoptosis has been shown to be suppressed by the Ets factor GABP*gamma*1 [181]. Thromboxane synthase, a factor promoting invasion and resistance to apoptosis is transcriptionally activated by ETS-1, while tumor suppressor p53 inhibits ETS-1-dependent transcription [182]. Ets-1 has been found to protect vascular smooth muscle cells from apoptosis by activating both basal and inducible transcription of the cyclin-dependent kinase inhibitor p21$^{WAF1/Cip1}$ [183]. Several reports have cited cases where Ets factors have been linked with pro-apoptotic events. PU.1 in combination with DMSO induces growth arrest and apoptosis in murine erythroleukemia cells [184]. Oxidative stress induces Ets-2 which sensitizes cells to apoptosis and is thought to play a role in the increased rate of apoptosis in fibroblasts and Down syndrome neurons [185, 186].

The complicated and contradictory situation of the role of ETS factors in apoptosis can be demonstrated by Ets-1. Ets-1 has been shown to be anti-apoptotic in T-cells [20, 21]. A splice-variant of ETS-1 (p42-ETS-1), which lacks a domain that inhibits DNA-binding, induces apoptosis in human colon carcinoma cells [187]. In endothelial cells VEGF has been shown to be anti-apoptotic [188] and has been shown to induce ETS-1 [189]. However, overexpression of ETS-1 increases the rate of apoptosis in endothelial cells under conditions of serum-deprivation [190]. Dominant-negative ETS-1 increased the anti-apoptotic effects of VEGF [190]. Thus, ETS-1 seems to act to counter

the anti-apoptotic effects of VEGF. DNA array analysis identified a number of pro-apoptotic genes upregulated and a number of anti-apoptotic genes downregulated by ETS-1 suggesting a pro-apoptotic role. Surveying the relevant literature does not readily identify any predominant factor that may dictate whether a particular ETS factor will be pro- or anti-apoptotic. The cellular context likely determines the effects of ETS factor misregulation. The expression level of the particular ETS factor being studied as well as the expression of all the ETS factors in the cell may influence the outcome of dysregulation of a single ETS factor.

A number of the differentially expressed genes identified through induction of ΔNPea3En have a documented role in apoptosis and/or cancer. The MYC proto-oncogene has a well-documented role in important biological activities such as cell cylce progression, differentiation, and apoptosis. *MYC* is most often overexpressed or amplified, however rare rearrangements have been reported [191]. Activation of *MYC* has been identified in many human cancers (reviewed in [192]). The transcriptional changes induced by MYC consist of thousands of genes, few however, have actually been shown to be direct target genes [193]. In general, upregulation of *MYC* is associated with mitogenic signals and increased proliferation and growth, and in some cases, induction of or sensitization to apoptosis. Downregulation of *MYC*, in general, is associated with reduced growth and anti-apoptotic pathways. However, several contradictory cases have been reported of *MYC* having an anti-apoptotic role. MYC has been associated with anti-apoptosis in human hematopoietic cells [194]. In human leukemia cells, downregulation of *MYC* has been linked to increased p53-mediated

apoptosis [195] and with apoptosis resulting from treatment with protein phosphatase inhibitors [196]. The combination treatment of *MYC* antisense oligo and a farnesyltransferase inhibitor in several human cancer cell lines induced apoptosis through increased caspase activity and was correlated with high levels of endogenous *MYC* and mutations in other oncogenes, particularly *RAS* [197]. In human melanoma cells *MYC* downregulation has been shown to mediate glutathione depletion which induces apoptosis after treatment with anti-cancer alkylating agents [198] as well as sensitization to radiation-induced apoptosis [199] and has been shown to involve increased levels of the cyclin-dependent kinase inhibitor p27^{kip1}, which has been shown to promoter apoptosis in multiple human tumor cell lines [200] . Reduction of *MYC* expression in MCF-7 cells with interfering RNA (RNAi) results in reduced tumor growth in nude mice increased apoptosis upon serum withdrawal [201]. Treatment of MCF7 cells with the antitumor drug salvicine decreases *MYC* transcription and increases apoptosis [202]. Examination of *MYC* mutations that occur frequently in Burkitt's lymphomas revealed only one of several mutations to endow MYC with anti-apoptotic properties, while most resulted in modest increases in transforming activity and one, the most frequently actually resulting in substantially decreased transforming activity [203]. The authors suggest that there is no strong selection for anti-apoptotic *MYC* alleles. Interestingly, analysis of *Myc* deletion mutants revealed that Myc induction of cell cycle progression and acceleration of apoptosis are separable functions and that acceleration of apoptosis was correlated with Myc repression of transcription [204]. Also, Myc and Bcl-2 have been shown to influence the type of cell death that occurs in hypoxic rat-1 fibroblasts –

apoptosis, necrosis, or an intermediate form (aponecrosis) [205]. It is noteworthy that reports where reduction of MYC is linked to increased apoptosis often utilize cancer cells and tumor cell lines. Thus, it may be that a cellular environment that contains multiple misregulated genes and has an established transformed phenotype is more conducive to a MYC having an ant--apoptotic role. ETS factor involvement in regulation of *MYC* has previously been established. It was shown that Ets-1 can transactivate mouse and human *MYC* promoter-reporter constructs [128]. An ETS binding site conserved (mouse and human) in the P2 region of the *MYC* promoter binds Ets-1 and Ets-2 and is essential for Ets-1-mediated transactivation.

Many of the other genes downregulated by ΔNPea3En have been reported to have involvement in cancer and anti-apoptotic function. Mitogen/extracellular signal regulated kinase kinase-5 (MEK5) has been reported to be a survival factor in apoptotic-resistant MCF-7 breast carcinoma cells [206]. Targeted deletion of *mek5* in mice results in embryonic death marked by increased apoptosis [207]. MEK5 overexpression has been associated with metastatic prostate cancer and, like PEA3, with the expression of MMP-9 and increased invasion [208].

Oncoprotein prothymosin-α (PTMA) is required for cellular growth and survival and related to the proliferative state of the cell. PTMA is able to transform rodent fibroblast cells and has been reported to be a prognostic marker in neuroblastoma, lung and breast cancer in humans [209-212]. Interestingly, PTMA is a c-MYC target gene [213, 214] and reduction of PTMA with antisense oligonucleotides induces or sensitizes human cancer cells to apoptosis [215, 216].

Insulin receptor substrate proteins play an important role in signal transduction. Insulin receptor substrate 4 (IRS4) has both insulin-dependent and insulin-independent function [217]. While it is well-established that IRS4 is involved in proliferation, it may also regulate apoptosis [218]. Irs4 is able to prevent apoptosis associated with decreased Irs2 in a rat pancreatic beta-cell line [219].

Insulin-like growth factor binding protein-5 (IGFBP5) is a component of signaling through the insulin-like growth factor (IGF) system, as well as insulin-independent functions and is the most conserved IGFBP across species. IGFBP5 has been shown to promote survival and proliferation in both normal and tumor cells while inhibiting apoptosis [220]. Silencing of IGFBP5 in human neuroblastoma cells with interfering RNA inhibits growth, impairs differentiation and increases apoptosis [221]. IGFBP5 has been shown to be overexpressed in human thyroid carcinomas [222]. In the human breast cancer cell line Hs578T, IGFBP5 promotes cell attachment and survival against specific apoptotic stimuli [223].

RAC1, a member of the RAS superfamily of small guanosine triphosphatases (GTPases), control cytoskeletal rearrangements and cell growth. RAC1 has been reported to have and anti-apoptotic effect in human cells [224, 225]. The anti-apoptotic role of Rac1 has been associated with interaction with Akt [226], the cellular homologue of a putative PEA3 target gene identified by RDA in the 1C cell line (Table 2). RAC1 has also been shown to be essential for the motility of human melanoma cells induced by autotoxin [227] which was upregulated by ΔNPea3En (Table 4).

Fibroblast growth factors (FGFs) are a family of secreted ligands that signal through cell-surface tyrosine-kinase receptors. In mammals there are more than twenty FGF family members. FGF receptors (FGFRs) are encoded by four genes, which due to alternative splicing produce seven receptors. The FGFRs have differing affinities for specific FGFs and have varying expression patterns (reviewed in [228] and [229]). FGFs stimulate proliferation of many cells types and are involved in control of differentiation, angiogenesis, and embryogenesis. Downregulation of FGFR2 implicates ETS factors as potential participants in FGF signaling. Disregulation of FGF signaling has been associated with breast cancer (reviewed in [230]). FGFR-2 is overexpressed in human breast cancer cell lines and tumors [231], [232]. Research has shown that signaling through Fgfr-2 stimulates migration of murine endothelial cells via mitogen-activated protein kinase (MAPK) [233]. In murine glioma cells, expression of dominant negative Fgfr-1 or Fgfr-2 results in inhibition of both anchorage-independent and –dependant growth, reduction in tumor development in immunodeficient mice accompanied by reduced tumor density, and a decrease in angiogenic response [234]. Specific isoforms of FGF-8, which can activate FGFR-2, have been shown to be elevated in human breast cancer [235], ovarian cancer [236], and prostate cancer [237]. MCF-7 cells overexpressing FGF-8 have increased invasiveness and tumor growth [238]. A link between ETS factors and FGF signaling has been previously established. An ETS-AP1 binding site in the promoter of human interstitial collagenase (MMP1) is important for FGF signaling impinging on the promoter [239]. Interestingly, some data suggests that FGF signaling modulates the expression of some ETS factors. Xenopus Er81 levels are

increased by Fgf signaling and dominant negative Fgfr blocks Er81 expression [240]. Both zebrafish Erm and Pea3 have been shown to be targets of Fgf signaling [67], [68]. Downregulation of FGFR2 has also been associated with progression of malignant prostate cancer [241, 242].

Mutations in the MID1 gene cause Opitz syndrome in humans, a malformation disorder. MID1 associates with microtubules and targets microtubule-associated phosphatase 2A for ubiquitin-dependent degradation [243, 244]. Unlike most of the other putative target genes discussed, MID1 does not have a documented role in cancer or apoptosis.

Several of the putative target genes upregulated by ΔNPea3En also have reported roles in cancer and apoptosis. There are examples of both pro-apoptotic genes being upregulated and anti-apoptotic genes being downregulated. Cytochrome P450 (CYP1A1) is an enzyme that metabolizes polycyclic aromatic hydrocarbons into reactive metabolites that can induce oxidative DNA damage, causing mutations and apoptosis. Deregulation of CYP1A1 has been linked to cancer in humans [245]. Reports have linked increased CYP1A1 expression with induction of apoptosis and reduced expression with resistance to apoptosis [246-248]. Expression of CYP1A1 in medulloblastomas has been associated with positive prognosis [249]. Thus, upregulation of CYP1A1 may be involved in the induction of apoptosis resulting from ΔNPea3En expression. Alternatively, the increase in CYP1A1 may be the result the differential expression of other genes regulated by ΔNPea3En.

DNA-repair enzyme 8-oxoguanine glycosylase 1 (OGG1) is induced in response to specific DNA mutations and stimuli that cause mutations such as oxidative stress and radiation, which may lead to transformation or apoptosis, and is reduced in many tumors (reviewed in [250]). The OGG1 gene encodes two splice variants; one targeted to the nucleus and one targeted to the mitochondria [250]. Specific mutations in the *OGG1* gene affect the repair activity of OGG1 protein contributing to transformation [251]. The increase in OGG1 due to expression of ΔNPea3En suggests that ETS activity may be involved in repression of DNA-repair mechanisms, thereby contributing to oncogenic transformation.

Purinergic receptors are membrane ion channels that have been shown to mediate induction of apoptosis in epithelial cells in response to high extracellular ATP levels (reviewed in[252]). In the apoptotic cells of aging rat prostates four of the examined purinergic receptors examined were downregulated while three, including P2X4, were upregulated [253]. Again, the increase in the purinergic receptor, P2X4, by ΔNPea3En may be an indirect consequence of ΔNPea3En expression.

Selenoprotein P (SELP) binds selenium and prevents oxidative stress and has been shown to be downregulated in mouse and human prostate cancers [254]. Upregulation of SELP has been positively correlated to survival in human hepatocyte cells [255] and with sensitization of mouse hepatocytes to tumor necrosis factor-α-induced apoptosis [256]. Human hepatitis B virus X protein induces oxidative stress in the HepG2 human hepatoma cell line by downregulating SELP, which upregulates TNF*alpha* [257].

Stanniocalcin is a hormone implicated in calcium homeostasis and resistance to hypoxia and may be a marker for multiple cancers in humans [258, 259]. Stanniocalcin and its receptor are targeted to the mitochondria where it stimulates electron transfer and therefore, energy production [260]. RDA results with inducible Pea3 suggest that PEA3 may be involved in upregulation of mitochondrial factors. Extrapolation of the results with ΔNPea3En and stanniocalcin suggests that PEA3 downregulates energy production. One of these apparent contradictory findings could be the result of a cellular response countering an induced change. Thus, several of the genes upregulated by ΔNPea3En are indicative of a cell that has been sensitized to apoptosis or is attempting to mount an anti-apoptotic response to cytotoxic stress.

Some of the putative PEA3 target genes identified by ΔNPea3En seem counter-intuitive based on the expected effect of ΔNPea3En, which is also supported by the majority of genes examined and confirmed by RT-PCR. In our model using the ΔNPea3En-inducible cell line it is expected that, since most of the literature suggests that PEA3 overexpression is associated with transformation and increased oncogenesis, induction of ΔNPea3En in a transformed cell line would result in a downregulation of genes associated with oncogenesis. Autotaxin is a tumor cell motility- and angiogenesis-stimulating factor that has been shown to greatly augment the invasive and metastatic potential of Ras-transformed NIH3T3 cells [261, 262]. Autotaxin has been linked to the invasiveness of human breast and thyroid cancer cells [263, 264]. It has also been found to protect NIH3T3 cells from apoptosis [265]. ΔNPea3En apparently upregulates autotaxin despite increasing apoptosis. The upregulation of autotaxin may be the result

of an unidentified function. Alternatively, it may be an indirect result of the differential expression of one or more other genes. For example, RAC1 has been shown to be essential for autotoxin-induced motility in human melanoma cells [227].

Utilizing the same inducible system, in the same parental cell line, researchers identified BRCA1 target genes [266]. It was found that BRCA1, a tumor-suppressor, regulated genes involved in breast tumorigenesis. Among the 62 BRCA1-regulated genes were four genes identified in our system: c-myc, stanniocalcin, selenoprotein P, and autotaxin – all showing the same trend of expression. The coordinated expression of BRCA1 and stanniocalcin was further confirmed in primary breast and ovarian tumors. It is interesting that our strategy of interfering with PEA3 function apparently has similarities to tumor suppression. This is intuitively reasonable, as PEA3 is thought to promote oncogenic transformation. Another study using the same system and cell line identified mitogen-activated protein kinase kinase 7 (MKK7) target genes [267]. Other than activating JNK family kinases, little is known about MKK7. Two of the nine genes identified were identified in our system: c-myc was down-regulated and autotaxin was up-regulated. Although it is possible that the commonly identified genes may reflect a bi-product specific only to the ecdysone inducible system, it is unlikely. While the control used in the present study was solely mock-induced cells, which does not account for the possible effects of the inducing agent, PonA. Welcsh et al included EcR293 cells transfected with empty vector as a control and thus, account for PonA [266].

Discrepancies in the differential expression of putative target genes could be due to a number of factors. The effect of particular genes likely depends greatly on cellular

context. For example, introduction of *EWS-Ets* fusions into different cellular models leads to different outcomes including cell cycle arrest, apoptosis, transformation, differentiation, trans-differentiation, or blocked differentiation (reviewed in [268]). Similarly, Rho-GTPases have been shown to have either a pro- or anti-apoptotic role, depending on cellular context [226]. Another consideration related to cellular context, particularly *in vivo*, is the temporal and spatial requirement for different expression profiles and the interactions of distinct cell types or tissues. It has been demonstrated in transgenic mouse models that the genes required for tumor initiation may be different than the genes required for tumor progression. In the hyperplastic mammary gland and primary tumors or *c-Myc* transgenic mice, expression of cyclin A2 and E2F-1 co-localized with c-Myc [269]. However, the highly proliferating, low apoptotic foci of these tumors were characterized by low c-Myc expression and high cyclin D1 and cyclin E expression. It was suggested by the authors that c-Myc/cyclin A2/E2F-1 are required for the onset of mammary cancer, whereas once a tumor has formed reduction of c-Myc protects the cells from apoptosis and releases c-Myc-mediated cyclin D1 repression, allowing progression through the cell cycle. Also, different levels of expression of a given gene may elicit different responses, particularly when outside the range of physiological relevance.

The discussion of specific putative PEA3 target genes has been confined to those genes whose differential expression has been confirmed by a method other than the method used to originally identify them. They only represent a subset of the putative target genes. Many more interesting genes remain to be confirmed and studied. What

also remains is the identification and discrimination of primary target genes whose promoters are directly bound by ΔNPea3En or PEA3 from indirect target genes.

The ecdysone inducible system has allowed for the tightly controlled expression of Pea3 and ΔNPea3En. The combined results suggest that PEA3 or other ETS factors are involved in regulating apoptosis and mitochondrial factors. Both of the findings support the role of PEA3 in cancer. Furthermore, the functional significance of any of the putative target genes remains to be examined both within the context of the inducible model system used and in human tumor samples. The validation of PEA3 target genes may provide insight into potential therapeutic anti-cancer strategies.

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