



# The targeting of the cyclin D1 oncogene by an Epstein-Barr virus promoter in transgenic mice causes dysplasia in the tongue, esophagus and forestomach

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**Cyclin D1 in cooperation with its major catalytic partners, cyclin-dependent kinases cdk4 and cdk6, facilitates progression through the G<sub>1</sub> phase of the eukaryotic cell cycle, in part through phosphorylation of the retinoblastoma protein. Cyclin D1's oncogenic properties have been suggested by its cooperation with *ras* or adenovirus *E1a* to transform cultured cells, as well its overexpression in transgenic mice that leads to breast cancer. Activated by a number of different mechanisms in human cancers, the cyclin D1 gene is frequently amplified in squamous epithelial cancers derived from the head/neck and esophageal regions. In order to study the functional consequences of cyclin D1 overexpression in these squamous epithelial specific sites, we have linked the Epstein-Barr virus ED-L2 promoter to the human cyclin D1 cDNA and utilized this transgene to generate founder lines. This transgene is transcribed specifically in the tongue, esophagus and forestomach, all sharing a stratified squamous epithelium. The transgene protein product localizes to the basal and suprabasal compartments of these squamous epithelial tissues, and mice from different lines develop dysplasia, a prominent precursor to carcinoma, by 16 months of age in contrast to age-matched wild-type mice. This transgenic model is useful in demonstrating cyclin D1 may be a tumor initiating event in aero-upper digestive squamous epithelial tissues.**

**Keywords:** cyclin D1; oncogene; squamous epithelial cells; transgenic mice

## Introduction

The eukaryotic cell cycle is regulated in part by the coordinated action of cyclins and cyclin-dependent kinases (cdks). The G<sub>1</sub> specific cyclins include members of the cyclin D family (Sherr, 1995). In particular, cyclin D1 has been shown to form a physical complex with certain cyclin-dependent kinases, such as cdk4 or cdk6, proliferating cell nuclear antigen (PCNA), and cdk inhibitors, namely p21 and p16 (Xiong *et al.*, 1992; Serrano *et al.*, 1993). Overexpression of cyclin D1 by

stable transfection in cultured cells will cause a shortened G<sub>1</sub> phase and more rapid entry into S phase (Quelle *et al.*, 1993; Jiang *et al.*, 1993a). Microinjection of cyclin D1 antibody or antisense oligonucleotide will cause cells to arrest in G<sub>1</sub> phase (Baldin *et al.*, 1993).

Cyclin D1 has been associated with a number of malignancies. The gene may be amplified to a variable extent in cancers originating from the head and neck (Bartkova *et al.*, 1995), esophagus (Jiang *et al.*, 1993a,b; Nakagawa *et al.*, 1995), larynx (Jares *et al.*, 1994), and breast (Buckley *et al.*, 1993). Interestingly enough, these cancers have a stratified squamous epithelial origin with the exception of breast cancer. Chromosomal rearrangement as another cause of cyclin D1 overexpression may also be found in centrocytic lymphomas (Williams *et al.*, 1992) and parathyroid adenomas (Motokura *et al.*, 1991), among other tumors. Cyclin D1's association with cancer has led to the investigation of its oncogenic properties *in vitro* and *in vivo*. Indeed, cyclin D1 can cooperate with either the *ras* oncogene (Lovec *et al.*, 1994b) or complement a defective adenovirus *E1a* oncogene (Hinds *et al.*, 1994) to transform cultured cells. Additionally, the MMTV promoter when fused to the human cyclin D1 gene, results in mammary hyperplasia and carcinomas in transgenic mice (Wang *et al.*, 1994). Conversely, targeted disruption of the cyclin D1 in embryonic stem cells of mice leads to abnormal mammary and retinal development (Sicinski *et al.*, 1995), although the effects on certain squamous epithelial tissues such as oral cavity (tongue), esophagus, and forestomach are not cited.

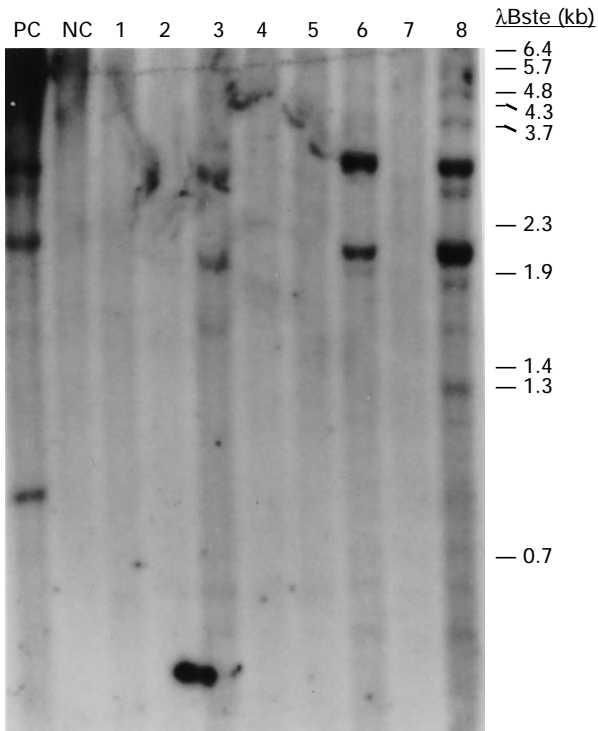
Cyclin D1 is frequently overexpressed in squamous cell carcinomas of the esophagus and of the head and neck, both sharing a stratified squamous epithelium. Targeting of cyclin D1 to the esophagus as well as the head and neck in transgenic mice has many attractions, including that of providing a novel genetic model to study multistage oncogenesis and delineating cyclin D1's role in site-specific squamous epithelial tissues.

In a report by Wilson *et al.* (1990), where expression of an Epstein-Barr virus (EBV) gene encoding the latent membrane protein-1 (LMP-1), was targeted to the skin epidermis and resulted in hyperplasia, it was observed that a 0.6 kb EBV specific transcript was expressed at high levels in tongue and esophageal tissues, independently from LMP-1 gene transcription. This 0.6 kb transcript is derived from an EBV lytic promoter (ED-L2) which resides within the 3' non-

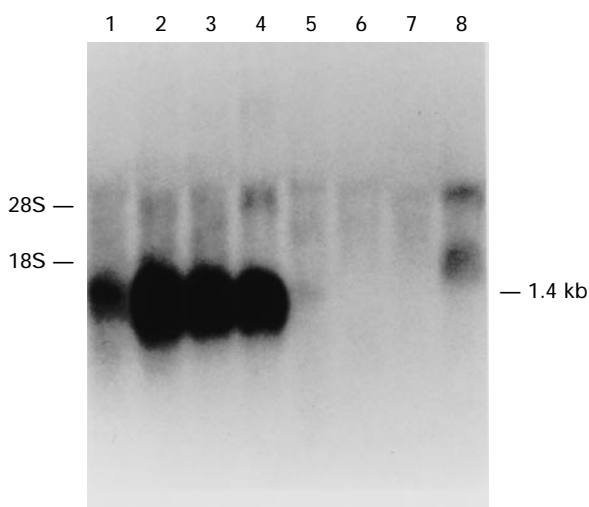
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**Figure 1** Southern blot analysis of eight sets of tail DNA from potential L2hD founder lines (#1-#8), digested with *Pst*I and probed with random-primed labeled  $\alpha$ -<sup>32</sup>PdCTP human cyclin D1. NC represents 'negative control' indicating a normal mouse tail DNA, and PC represents 'positive control' indicating MMTV-cyclin D1 tail DNA (gift of T Wang). Founders 3, 6 and 8 are positive for human cyclin D1 transgene expression. The digestion pattern for founders 3, 6 and 8 was confirmed by independent digestion with *Bam*HI/*Hind*III (data not shown)

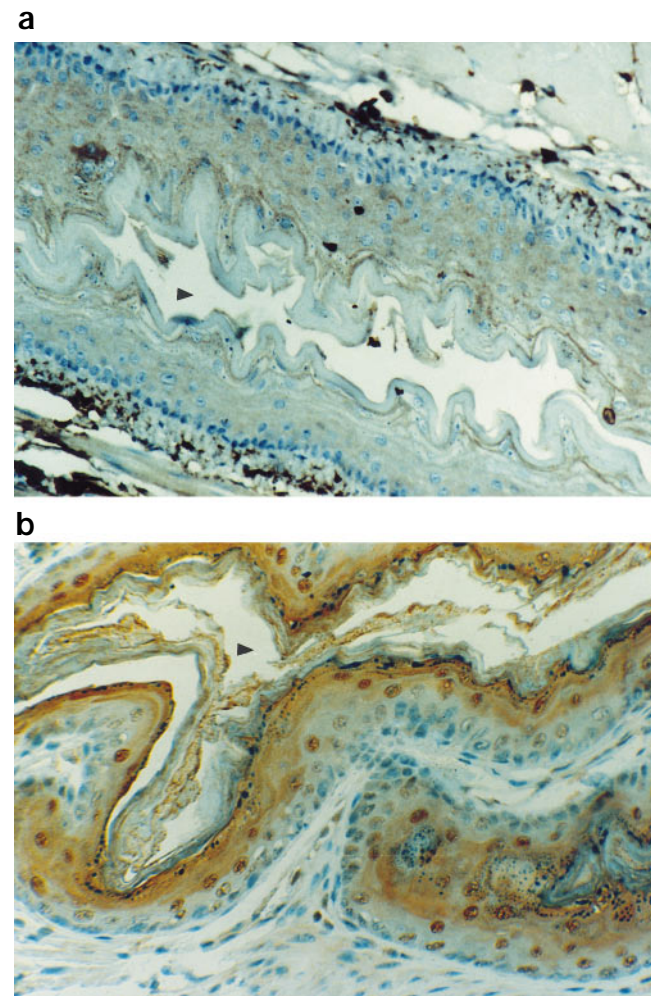


**Figure 2** Northern blot analysis. Total RNA was extracted from various tissues, electrophoresed on a 1% formaldehyde agarose gel, and hybridized with a random-primed  $\alpha$ -<sup>32</sup>PdCTP labeled human cyclin D1 cDNA probe. Also observed are 28S rRNA and 18S rRNA. Equal loading of RNA was confirmed by ethidium bromide staining and  $\beta$ -actin hybridization. Lanes 1: skin; 2: tongue; 3: esophagus; 4: forestomach; 5: distal stomach; 6: liver; 7: kidney; 8: spleen. (Although not shown, there was no detectable human cyclin D1 RNA expression in the brain, heart, lung, pancreas, small and large intestine, testis, ovary)

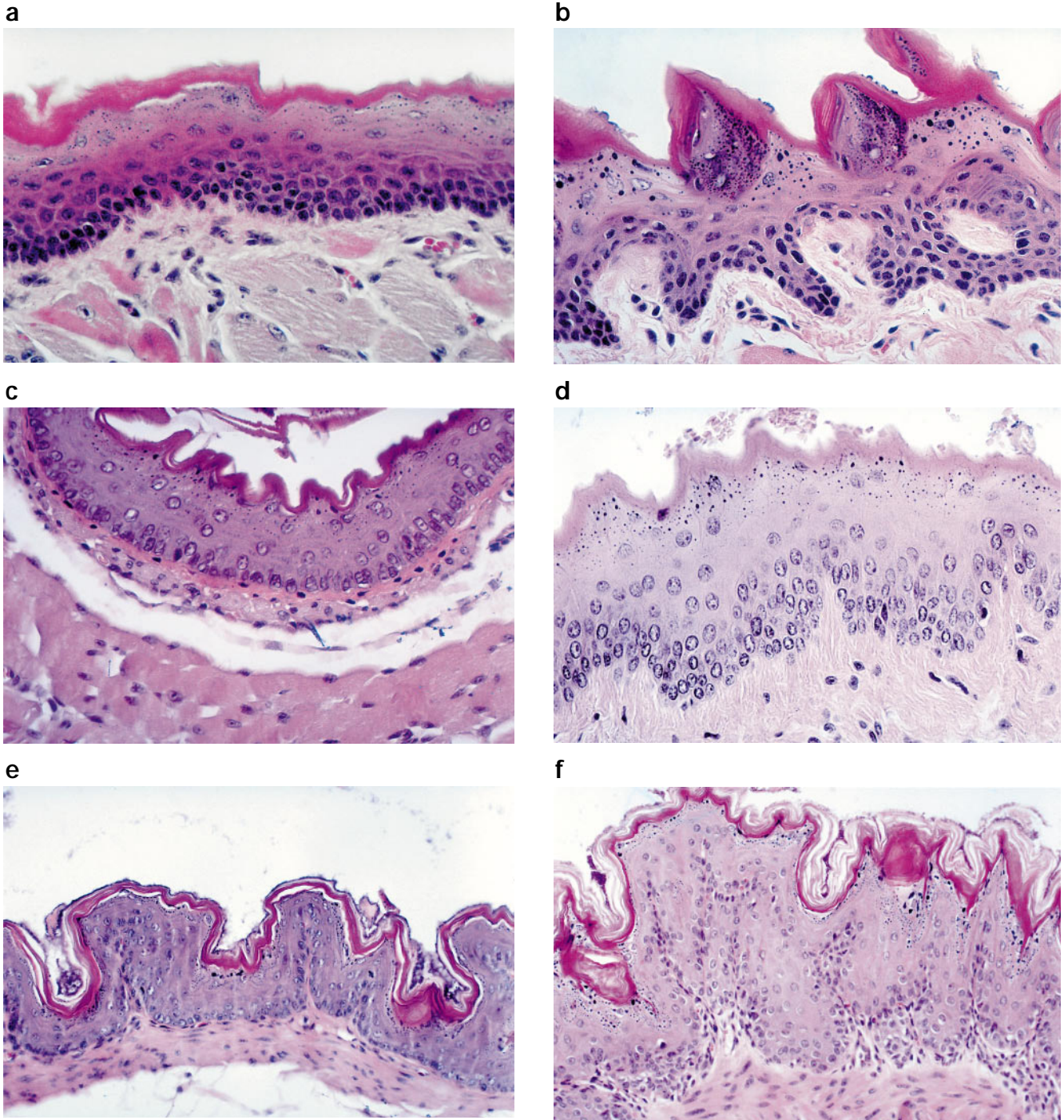
coding sequences of the LMP-1 gene. Taking advantage of these findings, together with the tropism of EBV for oropharyngeal squamous epithelial cells, we have generated transgenic mouse lines targeting the human cyclin D1 oncogene to the tongue (representative of head and neck), esophagus and forestomach tissues using the EBV ED-L2 promoter. The resultant transgenic expression of human cyclin D1 leads to a phenotype of dysplasia, a premalignant stage, in the tongue, esophagus and forestomach.

**Results**

Potential founders were screened for the integration of the L2hD transgene by Southern blot analysis of the genomic tail DNA. Figure 1 shows that founders 3, 6 and 8 are positive for the L2hD transgene. The MMTV-cyclin D1 genomic DNA serves as a positive control. These founders were bred further with wild-type FVB/N mice for the generation of G<sub>1</sub> mice.



**Figure 3** Cyclin D1 immunohistochemical staining (100 ×) in the esophageal squamous epithelium of wild-type FVB/N mouse (a) compared with G<sub>1</sub> mouse (b), both at 4 weeks of age. Note the brown nuclear staining (cyclin D1) in the basal and suprabasal layers in (b) (Although not shown, a similar pattern was observed in the tongue and forestomach of the cyclin D1 mice, but not the wild-type FVB mice).  $\blacktriangleright$  = Lumen



**Figure 4** Hematoxylin and eosin staining. Tongue: (a) wild-type FVB/N mouse at 16 months (400 $\times$ ); (b) cyclin D1 transgenic mouse at 16 months. Evidence of nuclear atypia, enlargement and hyperchromasia. Elongation and bridging of epithelial pegs (400 $\times$ ). Esophagus: (c) wild-type FVB/N mouse at 16 months (400 $\times$ ); (d) cyclin D1 transgenic mouse at 16 months. Evidence of nuclear atypia and enlargement and loss of polarity with migration of atypical cells into intermediate and superficial layers (400 $\times$ ). Forestomach: (e) wild-type FVB/N mouse at 16 months (400 $\times$ ); (f) cyclin D1 transgenic mouse at 16 months. Evidence of nuclear atypia, enlargement and hyperchromasia as well as loss of polarity (400 $\times$ )

In order to assess whether the human cyclin D1 transgene was transcribed,  $G_1$  mice aged 4 weeks were sacrificed. RNA was extracted from a panel of different tissues for Northern blot hybridization with the human cyclin D1 cDNA as illustrated in Figure 2. The 1.4 kb human cyclin D1 transcript is expressed in the tongue, esophagus and forestomach, all of stratified squamous epithelial origin. It should be noted that the murine

forestomach is squamous and embryologically is similar to the esophagus, in contrast to the distal glandular stomach. Figure 2 also shows expression of the human cyclin D1 transcript in the mouse skin, however, its expression was absent in other lines. It is possible that the polyoma promoter/enhancer elements described by Wilson *et al.* (1990) along with the EBV promoter are required for stronger expression of

transgenes in the skin, but that the EBV ED-L2 promoter is sufficient for expression of cyclin D1 in the tongue, esophagus, and forestomach. Importantly, there is no detectable human cyclin D1 transcript in other mouse tissues such as the brain, heart, lung, liver, pancreas, distal glandular stomach, large and small intestine, kidney, spleen, and gonads (ovary or testis).

To determine cellular localization of the human cyclin D1 transgene protein product, human cyclin D1 immunohistochemical staining was performed in tissues from the different founder lines in G<sub>1</sub> mice aged 4 weeks. Figure 3 demonstrates that the human cyclin D1 protein localizes within basal and suprabasal cells of the squamous epithelium as evidenced by increased nuclear (brown) staining. This antibody does not cross-react with any members of the mouse cyclin D family of proteins. The stratified squamous epithelium has three compartments: basal cells/suprabasal cells, intermediate cells, and superficial squamous cells. Importantly, basal cells are undifferentiated or proliferating cells which also contain yet to be identified stem cells and it is within the suprabasal cells that differentiation is initiated. Eventually, cells become terminally differentiated in the superficial squamous compartment prior to sloughing off in the lumen. Immunohistochemical staining did not demonstrate human cyclin D1 staining in other cell types within the stratified squamous epithelium or nonsquamous cells in the lamina propria, submucosa or muscularis mucosae.

G<sub>1</sub> mice from each of the founder lines were then followed for development of histopathologic changes in the tongue, esophagus and forestomach. Mice sacrificed at ages 5–6 months showed evidence of nuclear atypia and some increased mitotic figures in the basal cell layers (three mice analysed in each of the founder lines). This progressed to mild dysplasia at ages 8–10 months manifest by nuclear enlargement, nucleolar regeneration, nuclear hyperchromasia, and some loss of polarity in the tongue, esophagus and forestomach (three mice analysed in each of the founder lines). By 15–16 months, there was evidence of severe dysplasia with abnormal nuclear: cytoplasmic ratio, marked nuclear hyperchromasia, migration of abnormal cells into the middle-third and top-third of the squamous epithelium, and loss of polarity (nine mice analysed in each of the founder lines) (Figure 4). Interestingly, the tongue also revealed bridging of the basal layer with invasion into the lamina propria. Furthermore, these changes were present in all transgenic mice tested and of a diffuse nature in the dorsal and ventral tongue, all parts of the esophagus and throughout the forestomach. Such changes were not present in age-matched wild-type FVB/N mice at any of the time points tested. While analysis to date has not revealed carcinoma in the tongue, esophagus, or forestomach, cyclin D1 overexpression has resulted nonetheless in dysplasia, a prominent precursor to cancer.

## Discussion

Cyclin D1 plays a critical role in the progression of the normal cell cycle from mid G<sub>1</sub> phase to the G<sub>1</sub>/S phase transition. It has also become clear that cyclin D1 may be overexpressed by a number of different mechanisms,

including gene amplification, chromosomal translocation, transcriptional activation by proliferative growth factors (Matsushime *et al.*, 1991; Won *et al.*, 1992), and posttranscriptional stabilization of cyclin D1 mRNA (Rosenwald *et al.*, 1995).

An understanding of the functional consequences of cyclin D1 overexpression can in part be appreciated by stable transfection studies, but in even greater measure by animal models as exemplified by transgenic mouse approaches. In this context, cyclin D1 has been linked to breast cancer development where MMTV-cyclin D1 transgenic mice develop mammary hyperplasia by 4–8 months and mammary carcinoma by 16 months (Wang *et al.*, 1994). In order to study the effects of cyclin D1 overexpression in malignant transformation of squamous epithelial cells of the head/neck and esophagus, we have successfully established transgenic mouse lines where the EBV ED-L2 promoter targets the human cyclin D1 cDNA in a tissue specific fashion to the tongue (representative of the head/neck region), esophagus, and forestomach, underscoring the utility of this promoter. The EBV ED-L2 promoter lies within the 3' untranslated region of the EBV latent membrane protein (LMP-1) transcript and transcripts initiated at this site utilize the same efficient polyadenylation signal as LMP-1 transcripts which is included in the pL2hD transgene construct. The EBV ED-L2 promoter is classified as a lytic promoter since transcription from the promoter is low in the EBV positive B-cell line B95-8 but dramatically upregulated upon induction of the EBV lytic cycle by 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) and more when viral replication is not blocked by treatment of cells with phosphonoacetic acid (PAA) (Hudson *et al.*, 1985). Given the etiology of EBV, a lytic promoter might be predicted to be active in certain squamous epithelia. This is supported by the observation that in transgenic mice harboring the LMP-1 transcription unit, the ED-L2 promoter was demonstrated to be highly active in specific squamous epithelial tissues, namely the tongue and esophagus (forestomach was not tested), with some activity in the skin, and no activity in other epithelial tissues such as kidney, small intestine, testis or in any other tissue types (Wilson *et al.*, 1990). This also suggests that cellular transcriptional factors in the stratified squamous epithelia of the tongue and esophagus interact with cis-regulatory elements of the EBV ED-L2 promoter to regulate its activity (Nakagawa and Rustgi, submitted for publication). Other promoters, such as human papillomavirus E6 or cytokeratins, if linked to cyclin D1, likely would not have yielded the desired tissue specificity in transgenic mice.

The net effect of cyclin D1 transgenic overexpression in the tongue, esophagus, and forestomach is the onset of nuclear atypia in the first 5–6 months, mild dysplasia by 10–12 months, and severe dysplasia by 15–16 months. These changes progressed from focal areas to diffuse involvement in the tongue, esophagus and forestomach of the cyclin D1 transgenic mice. Notably, nuclear atypia or dysplasia were not evident in age-matched control wild-type FVB/N mice. Also, these changes were not observed in the skin of any of the founder lines, suggesting either the ED-L2 promoter does not yield sufficient cyclin D1 expression in the skin or potentially there are posttranscriptional mechanisms rendering the cyclin mRNA unstable.

Our studies indicate that cyclin D1 overexpression in stratified squamous epithelia of the tongue and the esophagus will lead to development of dysplasia. Progression to cancer may potentially involve other factors, either environmental or genetic. Human head and neck and esophageal squamous cell cancers share environmental and genetic causes in addition to cyclin D1 overexpression. For example, a number of environmental factors, such as tobacco, ethanol and nitrosamines have been linked epidemiologically to head and neck as well as esophageal squamous cell cancers. Perhaps, these agents may act cooperatively with cyclin D1 overexpression to cause cancer. Alternatively, although not exclusively, other genetic factors may be necessary for the development of cancer. These include p53 mutation or p16 mutation which are common events to head and neck and esophageal squamous cell cancers (Hollstein *et al.*, 1997; Mori *et al.*, 1994; Liu *et al.*, 1995; Chung *et al.*, 1993; van der Riet *et al.*, 1994). To that end, breeding of our cyclin D1 transgenic mice with mice in which the p53 or p16 genes have been disrupted in embryonic stem cells (Donehower *et al.*, 1992; Serrano *et al.*, 1996) may be feasible, and lead to the development of cancer in the tongue and esophagus.

Cyclin D1 may be necessary but not sufficient to cause cancer in the esophagus and head/neck squamous regions. This may also hold true in other animal models in which cyclin D1 is overexpressed. The immunoglobulin (Ig) enhancer when fused to cyclin D1 leads to fewer mature B and T cells in young transgenic mice, but lymphocytes are normal in cell activity, size, and mitogen responsiveness. However, when the Ig enhancer-cyclin D1 mice are crossed with Ig enhancer *c-myc* mice, the hybrid mice develop lymphomas more rapidly (Bodrug *et al.*, 1994). Similarly, transgenic mice in which the Ig heavy chain enhancer E mu is fused to cyclin D1 show only very subtle alterations in cycling behavior of B-cell populations in bone marrow without lymphoid tumors (Lovec *et al.*, 1994a). E mu directed coexpression of cyclin D1 and N-myc or L-myc in double transgenic mice reveal a strong cooperative effect, namely rapid development of clonal preB and B cell lymphomas. E mu L-myc transgenic mice express the transgene in B and T cells, and go onto develop mostly T cell tumors. However, when crossed with E mu D1 transgenic mice, the double transgenics develop exclusively B cell lymphomas (Lovec *et al.*, 1994). Furthermore, breast cancers appear only in MMTV-cyclin D1 transgenic mice that have lactated, suggesting the possibility that co-existing hormonal factor(s) may be necessary in addition to the overexpression of cyclin D1 for breast cancer development (Wang *et al.*, 1994).

The EBV ED-L2-cyclin D1 transgenic mouse model is the first genetic animal model of head/neck and esophageal oncogenesis. This model will help to provide important insights into how cyclin D1 contributes to malignant transformation in stratified squamous epithelial tissues from the aero-upper digestive tract.

## Materials and methods

### *pL2hD transgene plasmid and generation of transgenic mice*

Polyoma promoter/enhancer and LMP-1 encoding sequences were deleted from the plasmid pPyLMP up to

the *NcoI* site at EBV nucleotide 168,265 lying 100 basepairs 5' of the LMP-1 stop codon, leaving the EBV ED-L2 promoter and gene in tact (denoted plasmid pL2). Potential ED-L2 coding sequences were replaced with the human cyclin cDNA (EcoRI fragment; gift of A Arnold and EV Schmidt) by digestion of pL2 with *XhoI* (New England BioLabs) which cleaves between the RNA start sites for the L2 promoter and the first ATG, and *MluI* deleting the ED-L2 open reading frames but leaving the LMP-1 polyadenylation signal intact. The *BamHI-ClaI* 2.6 kb linear form (denoted L2hD) was isolated from plasmid sequences, purified from an agarose gel, followed by CsCl centrifugation and extensive dialysis. This transgene, at a concentration of 2 ng/ $\mu$ l, was microinjected into the male pronucleus of one cell embryos isolated from pregnant, superovulated FVB/N females (Taconic Labs). Fertilized eggs were reimplanted in the oviducts of Sw (fbr) foster females that had been previously mated with vasectomized Sw (fbr) males. Potential founders were screened by Southern blot analysis and founder lines were identified and maintained.

### *Southern blot analysis*

Potential founders were analysed for transgene incorporation by Southern blot analysis of *PstI* digested genomic (tail) DNA, using as a probe the random primed human cyclin D1 cDNA (1.4 kb). In brief, genomic DNA was extracted and purified from mouse tails as previously described (Nakagawa *et al.*, 1995). 10  $\mu$ g of genomic tail DNA was digested with *PstI* at 37°C for 12 h. Following fractionation in a 0.8% agarose gel, the gel was denatured and neutralized, and transferred onto a Hybond-N membrane by capillary action. After u.v. cross-linking, the membrane was hybridized (Rapid hybridization kit, Amersham) with a random-primed  $\alpha^{32}$ PdCTP labeled human cyclin D1 cDNA probe (Megaprime labeling kit, Amersham), washed under high stringency conditions (Nakagawa *et al.*, 1995), and exposed to X-ray film (Kodak) at -70°C.

### *Northern blot analysis*

Total RNA was extracted from various mouse tissues as previously described (Nakagawa *et al.*, 1995), electrophoresed on an ethidium bromide stained 1% formaldehyde agarose-gel, and transferred to a Hybond-N membrane by capillary action. After u.v. cross-linking, the membrane was hybridized (Rapid hybridization kit, Amersham) with a random-primed  $\alpha^{32}$ dCTP labeled human cyclin D1 cDNA probe (Megaprime labeling kit, Amersham), washed under high stringency conditions (Nakagawa *et al.*, 1995), and exposed to X-ray film (Kodak) at -70°C. Equal loading of RNA was confirmed by ethidium bromide staining and  $\beta$ -actin hybridization.

### *Histopathology and immunohistochemical staining*

Mouse tissues were fixed in 10% formalin, embedded in paraffin, and 7–10 micron sections were stained with hematoxylin and eosin. Immunohistochemical staining was performed by the avidin-biotin peroxidase complex (ABC) method using Vectastain Elite ABC kit (Vector laboratories) on the tissue sections (Nakagawa *et al.*, 1995). The affinity purified cyclin D1 rabbit polyclonal antibody #19 was used as the primary antibody and is specific for human cyclin D1, and does not cross-react with mouse cyclin D family of proteins. After incubation with biotinylated goat anti-rabbit immunoglobulin, sections were incubated with ABC Elite reagent and reaction products were developed using diaminobenzidine tetra hydrochloride (Sigma)

Chemicals) as chromogen. Interpretation of histology and immunohistochemical staining was performed without knowledge of mouse genotype.

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