CHAPTER FIVE

Immortalized Mouse Epithelial Cell Models to Study the Role of Apoptosis in Cancer

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Abstract

Human cancer cell lines are widely used to model cancer but also have serious limitations. As an alternate approach, we have developed immortalized mouse epithelial cell model systems that are applicable to different tissue types and involve generation of immortalized cell lines that are genetically defined. By applying these model systems to mutant mice, we have extended the powerful approach of mouse genetics to *in vitro* analysis. By use of this model we have generated immortal epithelial cells that are either competent or deficient for apoptosis by different gain- and loss-of-function mutations that have revealed important mechanisms of tumor progression and treatment resistance. Furthermore, we have derived immortalized, isogenic mouse kidney, mammary, prostate, and ovarian epithelial cell lines to address the issues of tissue specificity. One of the major advantages of these immortalized mouse epithelial cell lines is the ability to perform biochemical analysis, screening, and further genetic manipulations. Moreover, the ability to generate tumor allografts in mice allows the integration of *in vitro* and *in vivo* approaches to delineate the mechanistic aspects of tumorigenesis. These model systems can be used effectively to determine the molecular requirements of epithelial tumorigenesis and tumor-promoting functions. This approach provides an efficient way to study the role of apoptosis in cancer and also enables the interrogation and identification of potential chemotherapeutic targets involving this pathway. Applying this technology to other mouse models can provide insight into additional aspects of oncogenesis.

1. Introduction

Tumorigenesis is a multistep process characterized by step-wise and sequential mutational events that cooperate in a compounding manner resulting in resistance to apoptosis, uncontrolled proliferation, and invasiveness (Hanahan and Weinberg, 2000). Ideally, the best system to study the evolution of a tumor is the human body itself, where the physiology and the tumor–host interaction represent the most relevant situation. Understandably, this approach has limitations and raises ethical issues, as well as practical impediments. Moreover, there can be person-to-person variations that influence tumor physiology, therapeutic response, immunity, and other factors that may complicate assessment. Therefore, understanding the molecular mechanisms underlying tumorigenesis necessitates the use of appropriate model systems that can recapitulate this transition *in vitro* as well as *in vivo*. Animal models, where individual steps of tumorigenesis can

be assessed in a more simplified setting, represent an excellent option. Alternatively, a large number of cell lines have been generated from surgically resected human tumor samples, which have been widely used for *in vitro* experiments, as well as in generating human tumor xenografts in immune deficient mice (Hahn and Weinberg, 2002; Masters, 2000).

Despite immense convenience because of easy availability, human cancer cell lines and xenograft models suffer from limitations. First, excised human tumors are difficult to establish in tissue culture because of altered demands for growth *in vitro*. Although cancer is characterized by unlimited growth potential, establishment of human cancer cell lines in culture is far from simple. Even among cancers that are relatively easy to grow *in vitro*, it is often the metastatic cancers that are most amenable to establishment as continuous cultures (Hsu, 1999). Tumors evolve to grow in conjunction with the neighboring matrix-associated stroma and vasculature, which may partially explain their poor growth *in vitro*. Moreover, the capacity of normal cells to grow beyond a small number of divisions is often limited by insufficient culture conditions and replicative senescence imposed by telomerase attrition, and cell lines that pass this stage are likely to have accompanying compensatory mutations (Hahn and Weinberg, 2002). All of these and other factors limit the availability of biologically pure, defined, and renewable sources of both normal and cancer cells for biochemical assays and functional analysis *in vitro*.

Although established human cancer cell lines have been greatly instrumental in improving our understanding of the tumorigenic process, they suffer from the lack of genetic definition, and human systems that represent progression from normal to metastatic stages are limited. The complex mutational history of established human cancer cell lines makes systematic analysis of the stepwise transition from normalcy to neoplasia and assessment of treatment response difficult. Furthermore, human tumor xenografts in the altered host environment of immune compromised mice suffer from the lack of physiologic relevance from the disease perspective.

It is advantageous for a cancer model to use isogenic cell lines with well-defined genetic identity and the flexibility of further manipulation. Recent developments in mutant mouse technology have resulted in the generation of numerous transgenic and knockout mice with loss- or gain-of-function mutations in genes involved in cancer. Despite several morphologic and genetic differences, most of the important pathways implicated in cancer are conserved between mice and humans. Mouse models have long played a key role in explaining the molecular characteristics associated with the development of tumors. The availability of several permutations and combinations of knockout and transgenic mice renders it possible to derive multiple, immortalized, and independent cell lines from different mouse tissues of defined genetic background (Degenhardt and White, 2006). This extends the usefulness of these mouse models where compounding different combinations of mutations by conventional genetic approaches has been increasingly difficult.

Mouse cell models offer the advantage of being manipulable, reproducible, and self-replenishing, thus offering a powerful strategy to not only tease out the molecular events that lead to cancer but also to identify novel therapeutic strategies to fight the disease (Degenhardt and White, 2006; Mathew *et al.*, 2007a; Tan *et al.*, 2005). Mouse embryonic fibroblasts (MEFs) are the most commonly used mouse-derived cell lines. However, fibroblasts are not the most suitable cell type to model the physiology of human cancers, because most human tumors are epithelial in origin. Therefore, among various mouse cell types to model cancer, immortalized mouse epithelial cells with a defined genetic background are a superior alternative to MEFs.

To bridge the gap between mouse models and human cancers, we have developed the technical means to isolate primary mouse epithelial cells from multiple tissue types, to introduce genetic mutations to immortalize them, and to establish them as stable cell lines. By use of these techniques, we have generated immortalized baby mouse kidney (iBMK) epithelial cells, mouse mammary epithelial cells (iMMECs), mouse prostate epithelial cells (iMPECs), and mouse ovarian surface epithelial cells (iMOSECs) that retain their epithelial characteristics (Tables 5.1 to 5.8) (Bray *et al.*, 2008; Degenhardt and White, 2006; Degenhardt *et al.*, 2006; Karantza-Wadsworth and White, 2008; Karantza-Wadsworth *et al.*, 2007a; Karp and White, 2008; Mathew *et al.*, 2007b; Nelson *et al.*, 2004; Shimazu *et al.*, 2007; Tan *et al.*, 2005).

Major advantages of genetically defined, immortalized mouse epithelial cell lines are the ability to perform biochemical analysis, screening, and further genetic manipulations, thereby extending the utility of existing mutant mouse models. These cells are suitable for RNAi-mediated knockdown of specific gene expression using siRNA oligos targeting the gene of interest (Degenhardt, *et al.*, 2006). Stable gene knockdown using shRNA constructs or gain-of-function expression of transgenes are also possible in these cells using retrovirus-mediated gene transfer. These cells can be cultured and implanted back in mice after defined and controlled genetic manipulations for their potential use in tumorigenicity and other assays. Because these cells are nontumorigenic when immortalized, they are ideal for examining the molecular signatures involved in the oncogenic transformation into their tumorigenic counterparts. Furthermore, because these cells can be generated from any mutant mouse that survives to near birth, it is possible to combine multiple gene alterations to examine the compounding effect of multiple mutations and to test for genetic epistasis (Degenhardt *et al.*, 2002b; Nelson *et al.*, 2004). This advantage allows the evaluation of the means by which multiple genetic defects cooperate to promote epithelial tumorigenesis that is a fundamental aspect of oncogenesis (Hanahan and Weinberg, 2000). It also provides a renewable source of immortalized cells that can be used for comparison of tumorigenicity and chemotherapeutic response between different genetic backgrounds (Tan *et al.*, 2005). More importantly, the possibility of introducing compound mutations in immortalized cell lines overcomes the limitations of early postnatal mortality. Tumor allografts of

Table 5.1 Immortalized mouse (C57BL/6, wild-type or mutant) epithelial cell lines, E1A or myc and p53DD or *p53*$^{-/-}$ derived

Cell lines	Tissue type	Genotype	Reporter/vector	Reference
iBMK: C57B/6 neonatal kidney epithelia, E1A, p53DD derived:				
WTB/6.1-3	Kidney	+/+		Unpublished
iBMK: C57B/6, c-myc, p53DD derived				
WTB/6-myc.1-3	Kidney	+/+	LTR.H-myc	Unpublished
WTB/6-myc.1, BCL-2	Kidney	*Bcl-2*	LTR.H-myc, pcDNA3hBcl-2	Unpublished
WTB/6-myc.1, H-ras	Kidney	*H-ras*	LTR.H-myc, pcDNA3HrasV12	Unpublished
p53$^{-/-}$ iBMK cell lines, E1A derived				
W3, W4 (E1A, p53DD controls)	Kidney	*p53*$^{+/+}$;		Degenhardt, 2002a
P53$^{-/-}$1, p53$^{-/-}$2	Kidney	*p53*$^{-/-}$		Degenhardt, 2002a
P53$^{-/-}$A	Kidney	*p53*$^{-/-}$		Tan, 2005

these cells in mice also allow the study of *in vivo* tumorigenic processes in a more physiologically relevant microenvironment. Tumors generated from immortalized epithelial cells can be excised and used to derive tumor-derived cell lines (TDCL). This allows the direct comparison of these cell lines with the unselected, nontumorigenic, parental cell lines to facilitate *in vitro* biochemical and functional analysis to identify *in vivo* tumor-promoting functions (Karp *et al.*, 2008). This chapter describes the main procedures and assays to study the role of apoptosis in cancer by use of the iBMK, iMPEC, and iMOSEC cell models. A detailed description of the iMMEC model can be found in the accompanying chapter (Karantza-Wadsworth and White, 2008).

2. Immortalization of Baby Mouse Kidney, Mammary, Prostate, and Ovarian Surface Epithelial Cells

Immortalization of rat and mouse epithelial cells requires simultaneous inactivation of the retinoblastoma (Rb) and p53 pathways (White, 2001; 2006). The Rb pathway regulates the G_1/S transition, which is essential for

Table 5.2 Immortalized mouse (C57BL/6, wild-type or mutant) kidney (iBMK) epithelial cell lines, E1A, and p53DD derived. W2 and D3 cells were used to derive vector controls and the corresponding activated oncogene expressing cell lines (below)

Cell lines	Tissue type	Genotype	Reporter/ vector	Reference
iBMK: Bax/Bak deficient iBMK cell lines, E1A, p53DD derived				
W1, W2, W3	Kidney	$bak^{+/+}$, $bax^{+/-}$		Degenhardt *et al.*, 2002a,b
X1	Kidney	$bak^{+/+}$, $bax^{-/-}$		Degenhardt *et al.*, 2002a,b
X2, X3	Kidney	$bak^{+/-}$, $bax^{-/-}$		Degenhardt *et al.*, 2002a,b
K1, K2, K3	Kidney	$bak^{-/-}$, $bax^{+/-}$		Degenhardt *et al.*, 2002a,b
D1, D2, D3	Kidney	$bak^{-/-}$, $bax^{-/-}$		Degenhardt *et al.*, 2002a,b
W2.3.1-2, -5, -6 Control	Kidney	WT	pcDNA.3.1	Nelson; 2004; Tan, 2005
W2.Hras-2, -3, -7	Kidney	*H-ras V12*	pcDNA1. HrasV12	Degenhardt *et al.*, 2002a,b
W2.Raf-13, -15, -16	Kidney	*Raf-CAAX*	pcDNA3.Raf. CAAX	Degenhardt, 2006; Tan, 2005
W2-A1, -B1, -D2 Control	Kidney	WT	pcDNA3	
W2.AKT-C1. -D1, -E4	Kidney	*Myr-AKT*	pcDNA3. Myr-AKT	Degenhardt, 2006; Tan, 2005
W2.Bcl2-3, -14, -15	Kidney	*Bcl-2*	pcDNA3hBcl-2	Nelson, 2004
W2.19K-4, -7, -8	Kidney	*E1B 19K*	pcDNA3.1/V5/ His-TOPO19K	Nelson, 2004
D3.zeo-1, -2, -3 Control	Kidney	$bak^{-/--}$, $bax^{-/-}$	pcDNA3.1zeo	Nelson, 2004; Tan, 2005

Table 5.2 (*continued*)

Cell lines	Tissue type	Genotype	Reporter/ vector	Reference
D3.Hras-1, -3, -6	Kidney	*bak*$^{-/-}$, *bax*$^{-/-}$; *H-ras V12*	pcDNA3.1zeo	Degenhardt, 2006
D3-pcDNA3.1 Control	Kidney	*bak*$^{-/-}$, *bax*$^{-/-}$	pcDNA3.1	
D3.Raf-CAAX-1, -2, -3	Kidney	*bak*$^{-/-}$, *bax*$^{-/-}$; *Raf-CAAX*	pcDNA3.Raf-CAAX	
D3.-B4, -C4 Control	Kidney	*bak*$^{-/-}$, *bax*$^{-/-}$	pcDNA3zeo	
D3.Akt-D5, -F7, -G1	Kidney	*bak*$^{-/-}$, *bax*$^{-/-}$; *myr-AKT*	pcDNA3. Myr-AKT	Degenhardt, 2006
D3.Bcl2-3, -4, -6	Kidney	*bak*$^{-/-}$, *bax*$^{-/-}$; *Bcl-2*	pcDNA3hBcl-2 pcDNA3zeo	Nelson, 2004
D3.19K-4, -7, -8	Kidney	*bak*$^{-/-}$, *bax*$^{-/-}$; *E1B 19K*	pcDNA3.1/V5/ His-TOPO19K pcDNA3zeo	Nelson, 2004
iBMK cells expressing EGFP and the autophagy marker EGFP-LC3				
D3 EGFP	Kidney	*bak*$^{-/-}$, *bax*$^{-/-}$	GFP/pEGFP	Unpublished
D3 EGFP-LC3	Kidney	*bak*$^{-/-}$, *bax*$^{-/-}$	GFP/pEGFP-LC3	Degenhardt, 2006
iBMK cells expressing pDsRED-C1				
W2RED	Kidney	*RFP*	pDsRed-C1	Nelson, 2004; Tan, 2005
W2.3.1-5RED-4, -6,-11	Kidney	*RFP*	pDsRed-C1	Nelson *et al.*, 2004
W2.Bcl2-3RED	Kidney	*RFP, Bcl-2*	pDsRed-C1	Nelson *et al.*, 2004
W2.HRAS-3RED-3, -6,-8	Kidney	*RFP, Bcl-2*	pDsRed-C1	

(*continued*)

Table 5.2 (*continued*)

Cell lines	Tissue type	Genotype	Reporter/ vector	Reference
D3.zeo-2RED 1,2,4,10	Kidney	*RFP*, $bak^{-/-}$, $bax^{-/-}$	pDsRed-C1 pPUR	Nelson, 2004
D3.Hras-1RED-2, -7,-10	Kidney	*RFP*, $bak^{-/-}$, $bax^{-/-}$ *H-ras*,	pDsRed-C1	
D3.Bcl2-4RED-5, -9,-14	Kidney	*RFP*, $bak^{-/-}$, $bax^{-/-}$; *Bcl-2*	pDsRed-C1	

the cell proliferation, whereas p53 pathway activates the G_1-S checkpoint to prevent a premature entry into S phase because of pRb inactivation (White, 2001; 2006). The most common methods of inactivation of the Rb pathway are the direct inhibition of Rb by viral oncoproteins such as E1A or SV40 T antigen that promote epithelial proliferation (Berk, 2005; Helt and Galloway, 2003; White, 2001). In cells where p53 is functional, this Rb inactivation results in p53 mediated–growth arrest and apoptosis, which necessitates the simultaneous inactivation of both the pathways for epithelial cell immortalization (White, 2001; 2006). The p53 pathway can be inactivated by expression of a dominant negative p53 mutant (p53DD [Shaulian *et al.*, 1992]) (Degenhardt *et al.*, 2002b), or by use of epithelial cells from $p53^{-/-}$ mice (Degenhardt *et al.*, 2002a). We adapted this strategy to mutant mouse models, generating primary mouse epithelial cells where numerous apoptotic and tumor suppressor genes have been targeted for gene disruption (Tables 5.1 to 5.8) (Degenhardt and White, 2006; Degenhardt *et al.*, 2002a,b; Karantza-Wadsworth *et al.*, 2007a; Mathew *et al.*, 2007b; Shimazu *et al.*, 2007; Tan *et al.*, 2005). Coexpression of E1A or c-myc and p53DD successfully immortalizes epithelial cells from rat or mouse kidney, and mouse mammary, ovarian, and prostate tissues to form colonies (Fig. 5.1) from which immortalized cell lines are generated that retain their epithelial characteristics while remaining nontumorigenic (Degenhardt *et al.*, 2002b; Sakamuro *et al.*, 1995).

2.1. Protocol for the generation of iBMK cells

2.1.1. Solutions and reagents required

1. Collagenase/dispase: collagenase (125 mg) and dispase (1.25 g) (Sigma-Aldrich Co., St. Louis, MO) are dissolved in 500 ml sterile

Table 5.3 Control and BH-3-only (*bim*$^{-/-}$, *puma*$^{-/-}$, *noxa*$^{-/-}$, *nbk*$^{-/-}$/*bik*$^{-/-}$) deficient iBMK (C57BL/6) epithelial cell lines, E1A, and p53DD derived

Cell lines	Tissue type	Genotype	Reporter/vector	Reference
Bim-deficient iBMK cell lines, E1A, p53DD derived:				
BIM$^{+/+}$ iBMK cell lines				
BIM$^{+/+}$ A, B1,B2, C1, C2,C3	Kidney	+/+		Tan, 2005
BIM$^{+/+}$A derived vector controls				
BIM$^{+/+}$ A3.1A1, 1A2,1B1, 1D1,1D2, 1E,1F	Kidney	+/+	pcDNA3.1	
BIM$^{+/+}$A derived H-rasV12 expressing cell lines				
Bim$^{+/+}$A. Hras-B, -C, -D	Kidney	+/+; *H-ras V12*	pcDNA3. HrasV12	Tan, 2005
Bim$^{+/-}$ cells iBMK cell lines, E1A, and p53DD derived				
Bim$^{+/-}$A, B, C	Kidney	*bim*$^{+/-}$		Tan, 2005
BIM$^{-/-}$ iBMK cell lines, E1A, and p53DD derived				
Bim-/-A, B. C Control	Kidney	*bim*$^{-/-}$		Tan, 2005
Bim-/-A.3.1-C1,-C2	Kidney	*bim*$^{-/-}$	pcDNA3.1	Tan, 2005
M5-A1,-B2, -B3	Kidney	*bim*$^{-/-}$	pcDNA3.1	Tan, 2005
M2-A2,-B1, -D1	Kidney	*bim*$^{-/-}$	pcDNA3.1	Tan, 2005
M5-A1 (Bim$^{-/-}$) vector control				
M5A1C1-E4, -G6,-F5, M5A1C2-A1,-F2,-G3	Kidney	*bim*$^{-/-}$	pcDNA3.1	Tan, 2005
M5-A1 (BIM$^{-/-}$) derived H-ras expressing cell lines				
M5-A1Hras -A,-B,-C, -D,-F,	Kidney	*bim*$^{-/-}$; *H-ras V12*	pcDNA3HrasV12	Tan, 2005

(*continued*)

Table 5.3 (*continued*)

Cell lines	Tissue type	Genotype	Reporter/vector	Reference
M5-A1 (BIM$^{-/-}$A) derived Raf expressing cell lines				
M5A1.Raf-1H4,-C18,-2B5, -D	Kidney	*bim*$^{-/-}$; *Raf-CAAX*	pcDNA3.Raf-CAAX	Tan, 2005
Puma $^{+/+}$ and $^{-/-}$ iBMK cell lines, E1A, p53DD derived				
Puma$^{+/+}$A, B	Kidney	*puma*$^{+/+}$		Tan, 2005
Puma$^{-/-}$A, B	Kidney	*puma*$^{-/-}$		Tan, 2005
Noxa $^{+/+}$ and $^{-/-}$ iBMK cell lines, E1A, p53DD derived				
Noxa$^{+/+}$A, B, C	Kidney	*noxa*$^{+/+}$		Tan, 2005
Noxa$^{-/-}$A, B, C	Kidney	*noxa*$^{-/-}$		Tan, 2005
Nbk/Bik $^{+/+}$ and $^{-/-}$ iBMK cell lines				
Nbk$^{+/+}$A, B, C	Kidney	*nbk*$^{+/+}$		Shimazu, 2007
Nbk$^{-/-}$A, B. C	Kidney	*nbk*$^{-/-}$		Shimazu, 2007

phosphate-buffered saline (PBS) in a 2-L conical flask by gentle mixing. Filter sterilized collagenase/dispase solution is frozen as 50-ml aliquots at −20 °C.

2. Linearized E1A and p53DD plasmid DNA: adenoviral E1A (pCMVE1A [White *et al.*, 1991]) and dominant negative mouse p53 expression vectors (p53DD [Degenhardt *et al.*, 2002a; Shaulian *et al.*, 1992]) (10 μg each) are separately linearized by restriction digestion by use of Sca1 (1 μl; 10 U) in 50-μl reactions by incubating for 3 to 4 h at 37 °C. The restriction digestion is stopped by either heat inactivation at 65 °C or by the addition of 2 μl of 0.2 *M* EDTA, pH 8.0.

2.1.2. Protocol for the isolation and immortalization of baby mouse kidney epithelial cells

1. Primary epithelial cells are isolated from wild-type baby C57B/6 mouse kidneys: Five-day-old mouse litter is sacrificed by asphyxiation in a CO_2 chamber followed by cervical dislocation according to an IACUC-approved protocol.

Table 5.4 Control and caspase-3/caspase-7 deficient iBMK (C57BL/6) cell lines, E1A, and p53DD derived

Cell lines	Tissue type	Genotype	Reporter/ vector	Reference
Caspase-3/caspase-7 mutant iBMK cell lines, E1A, p53DD derived				
Caspase-$3^{+/+}$/ Caspase-$7^{-/-}$ -1, -6, -8	Kidney	*caspase-*$3^{+/+}$ *caspase-*$7^{-/-}$		Karp, 2008
Caspase-$3^{-/-}$/ Caspase-$7^{-/-}$ -3	Kidney	*caspase-*$3^{-/-}$ *caspase-*$7^{-/-}$		Karp, 2008
Caspase-$3^{+/+}$/ Caspase-$7^{+/-}$ -7, -10	Kidney	*caspase-*$3^{+/+}$ *caspase-*$7^{+/-}$		Karp, 2008

2. The litter is then doused with ethanol, and the skin is carefully removed from neck down to the feet to expose the dorsal surface.
3. Both kidneys are extracted by carefully running a fine-point curved forceps down along both sides of the spinal column. When the genotypes of the mice are known, kidneys of the same genotypes are pooled and transferred into a 15-ml conical tube containing sterile PBS with 1% Pen Strep (Invitrogen, Carlsbad, CA) and placed on ice. Epithelial cells are extracted from the pooled kidneys and immortalized with E1A and p53DD as per the protocols described for kidneys from single mouse in sections 2.1.3 and 2.1.4, respectively. Volumes are scaled up, depending on the total number of pairs of kidneys used. If the genotypes of the mice are not known at this point, care is taken to process each pair of kidneys from a single mouse pup separately as described below in sections 2.1.3 and 2.1.4.

2.1.3. Protocol for the isolation of kidney epithelial cells

1. Both kidneys from the same baby mouse are transferred into a petri dish containing sterile PBS and washed twice with chilled sterile PBS containing antibiotics (1% Pen Strep) with gentle shaking.
2. The washed single pair of kidneys is then transferred into another petri dish containing 5 ml collagenase/dispase solution and mechanically minced into small pieces of approximately 1 × 1 mm size using two sets of sterile forceps (pieces should be small enough to pass through the mouth of a 10-ml pipette).
3. All of the minced tissue from a pair of kidneys from a single mouse along with the collagenase/dispase solution (5 ml) is then transferred into 20 ml

Table 5.5 Control and autophagy-deficient iBMK (C57BL/6) epithelial cell lines, E1A and p53DD derived

Cell lines	Tissue type	Genotype	Reporter/ vector	Reference
Beclin1 $^{+/+}$ and $^{-/-}$ iBMK cell lines, E1A, p53DD derived:				
Beclin1 wild-type iBMK cell lines				
W4-1, -2, -A1, -B1, -C1, -E1	Kidney	+/+	pCEP-4	Mathew, 2007b
Beclin 1 $^{+/-}$ iBMK cell lines				
BLN-4-1, -34-3, -A3, -B2, -C3, -D1	Kidney	*Beclin1*$^{+/-}$	pCEP-4	Mathew, 2007b
Beclin1 $^{+/+}$ iBMK cell lines expressing Bcl-2				
WB-3, -5, -10, -13, -A2, -B1, -D1, -D3	Kidney	+/+; *Bcl-2*	pCEP-4. Bcl-2	Mathew, 2007b
Beclin1$^{+/-}$ iBMK cell lines expressing Bcl-2				
BLNB1, -4, -12, -13, -A4, -B1, -C2, -D1	Kidney	*Beclin1*$^{+/-}$*; Bcl-2*	pCEP-4. Bcl-2	Mathew, 2007b
Beclin1 iBMK cell lines expressing EGFP-LC3				
WB-13-EGFP-LC3	Kidney	+/+, *Bcl-2; EGFP-LC3*	pCEP-4. Bcl-2, pEGFP-LC3	Mathew, 2007b
BLNB-13-EGFP-LC3	Kidney	*Beclin1*$^{+/+}$*; EGFP-LC3*	pCEP-4. Bcl-2 pEGFP-LC3	Mathew, 2007b
ATG5$^{+/+}$ iBMK cell lines, E1A, and p53DD derived				
6.1, 6.2, 6.3, 6.4	Kidney	+/+		Mathew, 2007b
ATG5$^{+/-}$ iBMK cell lines, E1A, and p53DD derived				
5.1, 5.2, 5.3, 5.4	Kidney	*atg5*$^{+/-}$		Mathew, 2007b
ATG5$^{-/-}$ iBMK cell lines, E1A, and p53DD derived				
7.1, 7.2, 7.3, 7.4	Kidney	*atg5*$^{-/-}$		Mathew, 2007b
ATG5$^{+/+}$ iBMK cell lines, E1A, and p53DD derived expressing Bcl-2				
6.1/pcDNA3 Cl.1-2, 6.2/ pcDNA3 Cl.1-4	Kidney	+/+	pcDNA3	Mathew, 2007b

Table 5.5 (*continued*)

Cell lines	Tissue type	Genotype	Reporter/ vector	Reference
6.1B2, -B3, -B5, 6.2B1, -B5, -B8, -B10	Kidney	+/+; *Bcl-2*	pcDNA3. Bcl-2	Mathew, 2007b
$ATG5^{+/-}$ iBMK cell lines, E1A, and p53 DD derived expressing Bcl-2				
5.1/pcDNA3 Cl.1-2 Control	Kidney	$atg5^{+/-}$	pcDNA3	
5.1B2	Kidney	$atg5^{+/-}$; *Bcl-2*	pcDNA3. Bcl-2	
$ATG5^{-/-}$ iBMK cell lines E1A and p53 DD derived, expressing Bcl-2				
7.1/pcDNA3 Cl.1-2, 7.2/ pcDNA3 Cl.1-4	Kidney	$atg5^{-/-}$	pcDNA3	Mathew, 2007b
7.1B1,-B2, -B4, -B5, 7.2B2, -B3, -B6, -B9	Kidney	$atg5^{-/-}$; *Bcl-2*		Mathew, 2007b

of collagenase/dispase solution in a sterile 100-ml bottle equipped with a sterile magnetic stirring bar (total volume is 25 ml). The mixture is then stirred vigorously in a warm room at 37 °C for 30 to 40 min or until cells form a homogenous suspension. The completeness of digestion is checked by aseptically removing small aliquots onto a glass slide and then mounted with a glass coverslip for low-power microscopic examination (40× magnification).

4. Tissue debris is allowed to settle, and the cell suspension containing single cells is transferred into a 50-ml centrifuge tube (polypropylene), and cells are pelleted for 5 min at 1000 rpm (~300 × g).
5. The cell pellet is then resuspended in 1 ml (per one pair of kidneys) of prewarmed tissue culture medium (DMEM/5%FBS) supplemented with 1% Pen Strep (0.5 ml per kidney) and subjected to electroporation as described in section 2.1.4.

2.1.4. Protocol for the immortalization of kidney epithelial cells

1. For immortalization with E1A and p53DD, 500 μl of the preceding cell suspension, after gentle mixing, is transferred into a sterile Eppendorf tube containing linearized DNA (10 μg E1A [pCMVE1A] and 10 μg p53DD [p53DD] linearized with Sca I as described in section 2.1.1). Total amount of DNA in each transfection is adjusted to 120 μg by

Table 5.6 Immortalized mouse (C57BL/6; wild-type or mutant) mammary epithelial cell lines (iMMECs), E1A, and p53DD derived

Cell lines	Tissue type	Genotype	Reporter/vector	Reference
iMMEC: C57B/6 mammary epithelia E1A, p53DD derived				
WT-A (21), -B, -C, -D, WT-3, -5	Mammary	+/+		Karantza-Wadsworth, 2007a
WTA.V, WT3.V Control	Mammary	+/+, *pcDNA3*	pcDNA3.1	Karantza-Wadsworth, 2007a
Beclin1$^{+/-}$ iMMECs: C57B/6 mammary epithelia E1A, p53DD derived				
BLN2 (2.1), BLN4	Mammary	*Beclin1*$^{+/-}$		Karantza-Wadsworth, 2007a
BLN2.V	Mammary	*Beclin1*$^{+/-}$; *pcDNA3.1*	pcDNA3.1	Karantza-Wadsworth, 2007a
Beclin1$^{+/-}$ iMMECs: C57B/6 mammary epithelia E1A, p53DD derived, expressing Bcl-2				
BLN2.B2, .B4, .B5, .B8	Mammary	*Beclin1*$^{+/-}$, *Bcl-2*	pcDNA3.1.Bcl-2	Karantza-Wadsworth, 2007a
iMMEC: C57B/6 mammary epithelia E1A, p53DD derived expressing Bcl-2				
WTA.B1, .B4	Mammary	+/+, *Bcl-2*	pcDNA3.1.Bcl-2	Karantza-Wadsworth, 2007a
WT3.B1, .B2, .B3, .B8	Mammary	+/+, *Bcl-2*	pcDNA3.1.Bcl-2	Karantza-Wadsworth, 2007a
iMMEC: C57B/6 mammary epithelia E1A, p53DD derived expressing autophagy marker EGFP-LC3				
WTA-LC3, WT3-LC3	Mammary	+/+; *EGFP-LC3*	pEGFP-LC3	Karantza-Wadsworth, 2007a
WTA.B4-LC3 WT3.B3-LC3	Mammary	+/+; *Bcl-2; EGFP-LC3*	pcDNA3.1.Bcl-2, pEGFP-LC3	Karantza-Wadsworth, 2007a

BLN2-LC3	Mammary	*Beclin1*$^{+/-}$; *EGFP-LC3*	pEGFP-LC3	Karantza-Wadsworth, 2007a
BLN2.B4-LC3.5	Mammary	*Beclin1*$^{+/-}$, *Bcl-2 EGFP-LC3*	pcDNA3.1.Bcl-2, pEGFP-LC3	Karantza-Wadsworth, 2007a
iMMEC: C57B/6 mammary epithelia E1A, p53DD derived expressing HER2/neu				
WTA.H2, WTA.H3	Mammary	+/+, *HER2/neu*	pcDNA3.1Her2	Karantza-Wadsworth, 2007a
iMMEC: C57B/6 mammary epithelia E1A, p53DD derived expressing Myr-AKT				
WTA.A5, WTA.A7	Mammary	+/+, *AKT*	Myr-AKT	Karantza-Wadsworth, 2007a
iMMEC: C57B/6 mammary epithelia E1A, p53DD derived expressing H-Ras				
WTA.R3, WTA.R5	Mammary	+/+, *H-rasV12*	pcDNA3HrasV12	Karantza-Wadsworth, 2007a

Table 5.7 Immortalized mouse (C57BL/6) ovarian surface epithelial (iMOSECs) cell lines, E1A and p53DD derived

Cell lines	Tissue type	Genotype	Reporter	Reference
iMOSEC: C57B/6; ovarian surface epithelia E1A, p53DD derived				
MOSEC-1, -2, -3, -4, -5, -6, -7, -8	Ovary	+/+		Karp, 2008
iMOSEC: C57B/6; ovarian surface epithelia E1A, p53DD derived, expressing Bcl-2				
MOSEC-1, -3, -6 Control	Ovary	+/+	pcDNA3. Bcl-2	Karp, 2008
MOSEC-1, -3, -6/ BCL-2	Ovary	Bcl-2	pcDNA3. Bcl-2	Karp, 2008
iMOSEC: C57B/6; ovarian surface epithelia E1A, p53DD derived, expressing H-Ras				
MOSEC-1/H-Ras, -6/ H-Ras	Ovary	H-rasV12	pcDNA3. H-ras	Karp, 2008

Table 5.8 Immortalized mouse (C57BL/6) prostate epithelial (iMPECs) cell lines, E1A and p53DD derived

Cell lines	Tissue type	Genotype	Reporter	Reference
iMPEC: C57B/6; prostate epithelia E1A, p53DD derived				
MPEC-1, -2, -3, -4, 5, -6, -7, -8	Prostate	+/+		Bray, 2008

adding 100 μg salmon sperm DNA to the mixture, and the cells are transfected by electroporation as described in the following.

2. For electroporation, 250 μl of the preceding suspension is transferred into a 0.4 cm electroporation cuvette (BIO-RAD, Hercules, CA) and pulsed at 0.22 V and 950 μF by use of Gene Pulser II (BIO-RAD, Hercules, CA). The electroporated mixture is allowed to sit for 10 min and is resuspended in 40 ml of culture medium (DMEM/5%FBS/1% Pen Strep) and 5 ml each is plated in eight 6-cm plates. A duplicate electroporation is also performed using the remaining 250 μl cell suspension from step 1 (section 2.1.4) above, and plated in parallel to ensure sufficient independent immortalized clones that can be cloned and expanded.
3. The remaining 500 μl cell suspension from section 2.1.3, step 5 is divided into two equal halves of 250 ls and each is transfected separately with E1A and p53DD respectively, as negative controls for immortalization.

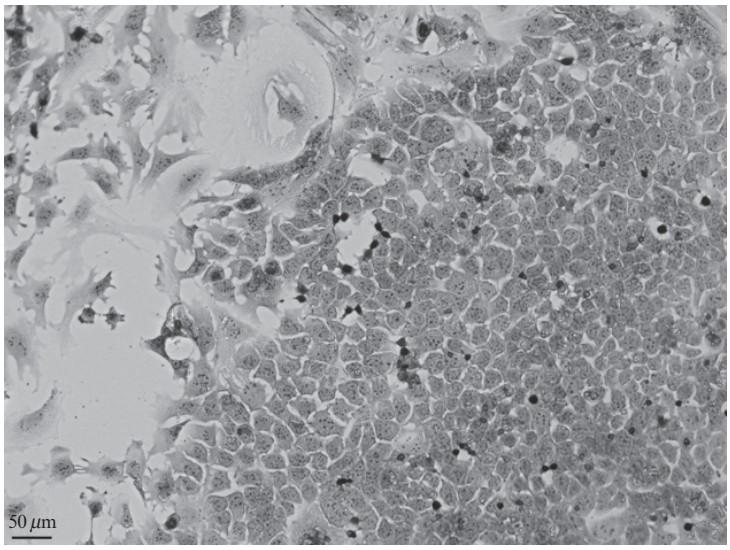

Figure 5.1 Morphology of an emerging colony of an E1A and p53DD transfection of primary baby mouse kidney cells after approximately 3 weeks of growth i*n vitro*. These colonies can be recognized by their distinctive epithelial morphology, typified by smooth, round edges and a characteristic cobblestone appearance. Colonies can be cloned and expanded to generate stable iBMK cell lines (magnification 100×).

4. No drug selections are necessary, because this is a functional selection for immortalization. This provides the additional advantage of being able to use any selectable marker for introduction of genes at a later point. Doubly transfected epithelial cells overcome p53 mediated growth arrest and grow into colonies giving rise to approximately 5 to 50 colonies per pair of kidneys per plate, which are visible at approximately 7 to 10 days (Fig. 5.1). Colonies are composed of tightly packed, cuboidal cells with typical epithelial morphology. They can be distinguished from surrounding fibroblasts by their compact appearance and sharply defined boundaries (Fig. 5.1).
5. Multiple, independent colonies are recovered from independent plates and expanded after 3 to 4 weeks. Typically, up to 20 colonies are picked and expanded to obtain approximately 5 to 10 viable colonies. Multiple early passage stocks are cryopreserved in the vapor-phase of a liquid nitrogen freezer as described in section 2.1.5.

2.1.5. Protocol for cloning, expansion, and preservation of iBMK cells

1. When each colony is approximate 0.6 to 0.8 cm in diameter, tissue culture plates are marked for ring cloning. Multiple plates for the same genotype are selected to ensure that truly independent colonies are derived.
2. Plates are washed in PBS, and well-separated colonies are marked on the bottom of the plates. Sterile cloning rings (6 × 8 mm to 10 × 10 mm;

Belco Biotechnology, Vineland, NJ) are carefully placed around the colonies with the support of autoclaved vacuum grease (VWR), to hold them in place.

3. Cells are then carefully trypsinized by use of a small amount of 0.05% trypsin-EDTA (Invitrogen, Carlsbad, CA) from the cloning rings and transferred into a 96-well plate to expand the colonies that are sequentially expanded into 24- and 12-well plates and then to 6-cm and finally into a 10-cm plate. Protein lysates are made from each clone to confirm that the clones express E1A, and p53DD by Western blotting by use of anti-E1A (Oncogene/Calbiochem Immunochemicals, San Diego, CA) and anti-p53 (Ab-1; Oncogene/Calbiochem Immunochemicals, San Diego, CA) antibodies, respectively. iBMK cells are also evaluated by genotyping and for the expression of the transgene or the loss of expression of a particular protein when the cells are derived from mutant mice.
4. For long-term storage, cells are trypsinized from five 10-cm plates at 90% confluency (approximately 10×10^6 cell/10-cm plate), resuspended in 2 ml freezing medium (90% FBS and 10% sterile tissue culture grade dimethyl sulfoxide [DMSO]) per each 10-cm plate, and placed in −70 °C freezer wrapped in paper towels overnight and then cryopreserved in vapor phase nitrogen.

3. Development of iBMK Cell Lines for Identifying the Role of Apoptosis in Cancer

Apoptosis is an effective tumor suppressor mechanism that is inactivated in many human cancers (Adams and Cory, 2007; Hanahan and Weinberg, 2000; Karantza-Wadsworth, 2007b). Identifying gain- or loss-of-function mutations capable of targeting this pathway for inactivation is extremely important to gain insight into the molecular mechanisms of tumor growth and to identify novel therapeutic targets for cancer. A common obstacle to achieving this goal is the lack of availability of genetically defined epithelial cell lines that have an intact apoptotic pathway and are nontumorigenic, but rendered tumorigenic by defects in apoptosis.

The Bcl-2 family members Bax and Bak are two functionally redundant, universal downstream regulators of apoptosis, the simultaneous deletion of both of which is one of the most efficient ways to inactivate apoptosis (Degenhardt *et al.*, 2002b; Gelinas and White, 2005; Wei *et al.*, 2001). The proapoptotic BH3-only Bcl-2 family members (e.g., Bim, Puma, Noxa, Nbk/Bik) promote apoptosis by activating Bax and Bak or antagonizing the survival activity of Bcl-2 (Gelinas and White, 2005). However, more than 95% of the $bax^{-/-}/bak^{-/-}$ mice die shortly after birth (Wei *et al.*, 2001), making it difficult to study a cancer phenotype associated with the double

deletion of *bax* and *bak*. Our method offers an alternative by allowing the generation of iBMK cells from *bax/bak* double knockout mutant mice, BH3-only mutant mice, and mice with deletions of the executioner caspases (−9 and −3) down stream of Bax and Bak (Table 5.4), providing an apoptosis-deficient epithelial cell model system to study the role of apoptosis in tumorigenesis. To this end, we have generated $Bax^{-/-}/Bak^{-/-}$, BH3-only mutants $Bim^{-/-}$, $Puma^{-/-}$, $Noxa^{-/-}$, and $Nbk^{-/-}/Bik^{-/-}$ (Shimazu *et al.*, 2007; Tan *et al.*, 2005), as well as *caspase-*$3^{+/+}$*/caspase-*$7^{-/-}$, *caspase-*$3^{-/-}$*/caspase-*$7^{+/-}$, and *caspase-*$3^{+/+}$*/caspase-*$7^{+/-}$ (Karp and White, 2008) iBMK cell lines (Tables 5.3 and 5.4).

3.1. Protocol for the immortalization of baby mouse kidney epithelial cells from $bax^{-/-}$, $bak^{-/-}$, and $bax^{-/-}/bak^{-/-}$ Mice

Breeding pairs are set up, crossing various *bax* and *bak* mutant mice ($bax^{+/-}/bak^{-/-}$ with $bax^{+/-}/bak^{-/-}$; $bax^{+/-}/bak^{+/-}$ with $bax^{+/-}/bak^{-/-}$; and $bax^{+/-}/bak^{+/+}$ with $bax^{+/-}/bak^{+/+}$). Newly born litters of pups are collected and numbered. Both kidneys from each pup are removed separately under sterile conditions, washed, and processed by use of the general immortalization protocol described in section 2.1 to generate iBMK cell lines that are either $bax^{+/+}/bak^{+/-}$, $bax^{+/-}/bak^{-/-}$, or $bax^{-/-}/bak^{-/-}$. Special care is taken to process each pair of kidneys from each animal separately, because the genotypes of the pups are not known at this stage. Colonies are expanded and cryopreserved as described in section 2.1.5. Tail snips from each pup are collected and processed for DNA isolation and PCR genotyping as described elsewhere (Lindsten *et al.*, 2000; Shindler *et al.*, 1997). Genotyping is verified on the DNA isolated from the iBMK cell lines themselves (Degenhardt *et al.*, 2002a).

4. Aposcreen: An iBMK Cell-Based Screen for the Identification of Apoptosis-Inducing Compounds as Potential Anti-Cancer Agents

In addition to being an effective tumor-suppressor mechanism, apoptosis is also an important determinant of treatment response. Compounds that specifically and irreversibly activate apoptotic pathway have the potential for use as anti-cancer agents (Fesik, 2005). Therefore, iBMK cell lines with defined and differential capacities for apoptosis, such as the apoptosis competent wild-type (W2 cells) and $bax^{-/-}/bak^{-/-}$ apoptosis-deficient (D3 cells) are extremely useful to identify novel and specific

apoptosis inducers as potential anti-cancer agents (Andrianasolo *et al.*, 2007; Degenhardt *et al.*, 2002b).

Secondary metabolites produced by marine invertebrates are a rich source of yet unknown natural products with potential proapoptotic activity. We adapted the iBMK cell system for a bioassay-guided screen (ApoScreen) for the identification, isolation, and purification of novel compounds with potential anticancer activity (Andrianasolo *et al.*, 2007). Because W2 cells are competent, and D3 cells are deficient for apoptosis, compounds that selectively kill W2 but not D3 cells are specific inducers of apoptosis and, therefore, potential anti-cancer agents. Those compounds that kill both W2 and D3 cells can be excluded as nonspecifically toxic (Andrianasolo *et al.*, 2007). As a simple and effective screening method useful for crude extracts, this assay can identify potential fractions isolated from natural sources before compound purification. Fractions showing potent bioactivity can be subjected to further chemical purification, structural elucidation, and functional validation in the ApoScreen bioassay (Andrianasolo *et al.*, 2007).

By use of this ApoScreen-guided purification we have screened and identified four novel diterpenes from the soft coral *Xenia elongata* with specific proapoptotic and, therefore, potential anti-cancer activities (Andrianasolo *et al.*, 2007). All four compounds are capable of differentially inducing cell death in the apoptosis competent W2 cells and not in the apoptosis deficient D3 cells (Andrianasolo *et al.*, 2007). Elucidation of the mechanism of apoptosis induction by these novel diterpenes, as well as further screening for proapoptotic natural products, is currently underway.

4.1. Protocol for the iBMK cell-based aposcreen bioassay

1. Apoptosis-competent W2 cells (7500 cells in 100 μl per well) and apoptosis-deficient *bax* and *bak* double knockout (D3 cells) (5000 cells in 100 μl per well) are plated in 96-well plates to achieve 50% confluency in 24 h.
2. Compounds are dissolved in DMSO and diluted in growth medium (DMEM) to make 2× suspensions of serial concentrations. 100 μl each of the preceding suspensions are added to cells at various concentrations to achieve final concentrations in the range of 100 μg/ml to 10 μg/ml, keeping the DMSO concentration uniform at 0.5% in all the wells. In separate wells, Staurosporine (0.1 μM), a strong apoptosis inducer, is used as the positive control, and DMSO (0.5%) is used as the vehicle control.
3. Cells are incubated at 37 °C for 24, 48, or 72 h at which time cell viability is assayed by use of a modification of a 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay (Mosmann, 1983). MTT solution (50 μl of 2.5 mg/ml) (Sigma-Aldrich Co., St. Louis, MO) is added to each well to a final concentration of 0.5 mg/ml, and final volume of 250 μl. The plate is then incubated at 37 °C for 3 h.

4. After 3 h of incubation in MTT, the supernatant is aspirated, and 150 μl of 100% DMSO is added to dissolve the formazan crystals, the reduction product of MTT in mitochondria. After an additional 30-min incubation at 37 °C on a shaker, absorbance is measured at 570 nm by use of a spectrophotometer. Absorbances from five wells are averaged for each concentration point as a measure of cell viability, along with standard deviation from the mean. Relative cell viability for each concentration of compound is calculated as a difference in absorbance values between time 0 (addition of the compound) and 48 h. Apoptosis induction is defined as at least 20% cell death of W2 (apoptosis competent) cells and a 10% or higher growth in D3 (apoptosis deficient) cells.

5. Adaptation of the iBMK Cell Model System for the Evaluation of Tissue-Specific Tumor-Promoting Functions

One of the strengths of the iBMK model system is its adaptability to multiple tissue types and both transgenic and knockout mouse models. By deriving immortalized epithelial cells from different tissues from an appropriate mouse model it is possible to recapitulate the tissue-specific characteristics *in vitro* with several advantages. For example, adaptation of iBMK model to mouse mammary epithelia enabled us to derive immortalized mouse mammary epithelial cells (iMMEC) that express surface markers and demonstrate functional properties that are characteristic of mammary epithelia (Karantza-Wadsworth *et al.*, 2007a). Importantly, iMMEC undergo three-dimensional ductal morphogenesis when cultured in the presence of extracellular matrix (Karantza-Wadsworth *et al.*, 2007a). iMMEC are nontumorigenic, but become tumorigenic by the activation of HER-2/*neu,* consistent with the findings in human breast cancer (Karantza-Wadsworth *et al.*, 2007a; Karantza-Wadsworth and White, 2008). For a detailed description of the iMMEC model, please see the accompanying chapter (Karantza-Wadsworth and White, 2008).

We have established similar isogenic and immortalized but nontumorigenic epithelial models from mouse ovarian surface (iMOSEC) and prostate (iMPEC) epithelia (Tables 5.7 and 5.8). Establishment of immortalized mouse hepatocyte cell lines (iHEP) is currently in progress. All these models mimic their own corresponding tissue-specific phenotype, including marked tropism in tumorigenic assays to specific anatomic locations resembling corresponding tumors in humans, thus allowing the evaluation of tissue-specific requirements for cancer formation.

5.1. Immortalization and establishment of iMMEC, iMOSEC, and iMPEC models

Generation of iMMEC cell lines (Table 5.6) are described in detail in the accompanying chapter (Karantza-Wadsworth and White, 2008). Mouse ovarian surface and prostate epithelial cells are immortalized as described in the following.

5.1.1. Protocol for the generation of iMOSEC cell lines: Isolation of primary mouse ovarian surface epithelial cells

1. Twenty adult female C57B/6 mice (11 weeks old) are sacrificed by asphyxiation in a CO_2 chamber and cervical dislocation according to an IACUC-approved protocol.
2. Both ovaries are extracted from each mouse by use of antiseptic techniques and placed in a dish with ice-cold Hank's balanced salt solution (HBSS; Invitrogen, Carlsbad, CA) (Roby *et al.*, 2000).
3. After rinsing with HBSS, the ovaries are placed in a 15-ml conical tube containing 10 ml of 0.2% trypsin in HBSS, incubated at 37 °C under humidified atmosphere (5% CO_2) for 30 min. The tube containing the ovaries is placed in a horizontal position with the ovaries distributed over the length of the tube, taking care not to agitate the ovaries.
4. After 30 min, medium containing epithelial cells is transferred to a fresh tube with 5 ml DMEM supplemented with 4% FBS, 1% Pen Strep, 5 μg/ml insulin (Sigma-Aldrich Co., St. Louis, MO), 5 μg/ml transferrin (Sigma-Aldrich Co., St. Louis, MO), and 5 ng/ml sodium selenite (Sigma-Aldrich Co., St. Louis, MO), and centrifuged at 1000 rpm ($\sim$300 g) for 10 min at room temperature and resuspended in 2 ml complete medium.
5. The cells are then plated in special CellBIND 6-well plate (Corning CellBIND Surface; Corning Incorporated Life Sciences, Lowell, MA) for facilitating cell attachment. Cells reach 95% confluency in approximately 4 days and typically provide approximately 1×10^5 cells per 10 ovaries.

5.1.2. Protocol for the generation of iMOSEC cell lines: Immortalization of primary mouse ovarian surface epithelial cells

Primary mouse ovarian epithelial cells obtained from step 5 (section 5.1.1) are transfected with 2.5 μg each of E1A and p53DD expression vectors (linearized with Sca1 as described in section 2.1.1) by use of the Amaxa nucleofection protocol (Amaxa Inc., Gaithersburg, MD) as described in the following.

1. Primary mouse ovarian surface epithelial cells growing in log-phase are harvested by trypsinization after three passages and resuspended in PBS at a density of 3×10^6 cells/ml. For transfection, 1×10^6 cells are transferred into a 15-ml conical tube and centrifuged at ~1000 rpm (~300 g).
2. The cell pellet is resuspended in 100 μl of prewarmed nucleofector solution, mixed with Sca1 linearized, E1A, and p53DD expression plasmids (2.5 μg/3×10^6 cells/transfection), transferred into Amaxa cuvettes, and transfected by use of a preset program optimized for iBMK cells (G-16). The nucleofection program and the appropriate transfection reagent need to be optimized for each cell type.
3. Transfected cells are plated immediately in prewarmed DMEM supplemented with 10% FBS, 1% Pen Strep, 5 μg/ml insulin, 5 μg/ml transferrin, and 5 ng/ml sodium selenite and incubated at 37 °C.
4. Transfected cells are plated into special CellBIND petri dishes and allowed to grow into colonies. Round colonies with smooth edges, composed of cells with cobblestone morphology characteristic to epithelial cells, become visible in 7 to 10 days and are very similar in appearance to colonies formed by iBMK cells (Fig. 5.1).
5. Well-separated colonies are cloned and expanded separately in special 12-well CellBIND tissue culture plates and eventually transferred into uncoated 6-cm dishes by use of the general protocol described in section 2.1.5.
6. Cells are allowed to grow into monolayer for a week, after which cells are cultured in DMEM containing 10% FBS and 1% Pen Strep.
7. iMOSECs cell lines (Table 5.7) are trypsinized from three 10-cm plates at 90% confluency (approximately 10×10^6 cell/15-cm plate), harvested, and cryopreserved (one 10-cm plate per vial) as described in section 2.1.5. Protein lysates are made from each clone and analyzed for expression of E1A, p53DD, and epithelial markers by use of Western blot analysis (section 2.1.5).

5.1.3. Protocol for the generation of iMPEC cell lines: Isolation of primary mouse prostate epithelial cells

1. Ten to fifteen adult male C57B/6 mice (6 weeks old) are sacrificed by asphyxiation in a CO_2 chamber and cervical dislocation according to an IACUC-approved protocol.
2. Mouse prostate glands (Abate-Shen and Shen, 2002) are carefully isolated under an inverted dissection microscope, pooled, and transferred into petri dishes containing sterile PBS and washed twice with chilled sterile PBS with gentle shaking.
3. Washed prostate glands are transferred to 25 ml of collagenase/dispase solution into sterile 100-ml bottles equipped with magnetic stirrer and

agitated on an orbital shaker at 125 rpm for 10 min 37 °C until cells have formed a homogenous suspension and examined under the microscope.
4. The suspension is allowed to settle, the supernatant is discarded, and the residue is resuspended in 25 ml of fresh collagenase/dispase solution by pipetting several times.
5. The suspension is incubated on an orbital shaker at 37 °C at 150 rpm with agitation for 40 min. After digestion, the cells are mixed by pipetting and centrifuged at 1000 rpm (~300 g) for 6 min at room temperature. A total of approximately 3 to 5 × 10^6 cells are recovered by this procedure from 10 to 15 mice.

5.1.4. Protocol for the generation of iMPEC cell lines: Immortalization of primary mouse prostate epithelial cells

1. The cell pellet obtained is resuspended in 500 μl of DMEM with 5% FBS. Primary mouse prostate epithelial cells are transfected with 10 μg each of E1A and p53DD expression vectors (linearized with Sca1; section 2.1.1) by electroporation as described in section 2.1.4.
2. After transfection, the cells are plated at a 1:3 ratio into 10-cm dishes in DMEM containing 5% FBS and 1% Pen Strep and allowed to grow into colonies for 2 weeks. Colonies appear round, with smooth edges composed of cells with a cobblestone morphology similar to iBMK cell colonies (Fig. 5.1).
3. Well-separated colonies are cloned and expanded separately in 96-well tissue culture plates and eventually transferred into 10-cm dishes by use of the general protocol described in section 2.1.5.
4. Multiple vials of iMPEC cell lines (Table 5.8) are harvested and cryopreserved as described in section 2.1.5. Protein lysates are made from each clone and analyzed for E1A and p53DD expression (section 2.1.1) and epithelial markers such as E-cadherin and cytokeratin by Western blot analysis (Bray *et al.*, 2008).

6. Tumorigenicity Assay

Immortalized primary iBMK cell, iMMEC, iMOSEC, and iMPEC lines from wild-type (C57B/6) or from adult mouse are nontumorigenic (Bray *et al.*, 2008; Degenhardt *et al.*, 2002a; Karantza-Wadsworth *et al.*, 2007a). However, they are rendered tumorigenic by cooperating functions such as blockade of apoptosis or oncogene activation (HER-2/*neu*, myr-Akt, RAF-CAAX, H-ras, Bcl-2) (Tables 5.1 to 5.8) (Bray *et al.*, 2008; Degenhardt *et al.*, 2002a; 2006; Karantza-Wadsworth *et al.*, 2007a; Nelson *et al.*, 2004; Tan *et al.*, 2005). Thus, tumorigenicity resulting from specific

genetic manipulations can be directly assessed to isogenic and nontumorigenic cell lines for comparison.

6.1. Protocol for tumor formation in mice

1. Isogenic iBMK cells of the genotype to be studied, along with their controls, growing in log-phase in normal culture condition are trypsinized, harvested.
2. Cells are grown to 90% confluency in approximately five 15-cm tissue culture plates (roughly one 15-cm plate per animal to be injected). Cells are pooled, washed twice with PBS, and viable cell number is determined by trypan blue exclusion.
3. Cells are then resuspended to a cell density to 1×10^8 cells/ml in sterile PBS.
4. 10^6 to 10^7 cells are injected into the subcutaneous space in the abdominal flank of five athymic nude mice per cell line (5 weeks old, male, NCR Nu/Nu; Taconic, German Town, NY) as per an IACUC-approved protocol.
5. Tumor growth is monitored twice a week by measurement with a 6-inch dial caliper (General Tools mfg. Co., New York, NY), and tumor growth rates are compared by calculating tumor volumes with the following formula (Streit *et al.*, 1999):

$$Volume\ (mm^3 = [(4\pi/3(0.5 \times smaller\ diameter)^2) \times (0.5 \times larger\ diameter)]$$

6. We use cell lines stably expressing fluorescence or chemiluminescence markers to assess the role of apoptosis regulators in tumorigenicity and treatment response (Tan *et al.*, 2005). In addition, tumors from cells stably expressing fluorescence reporter genes such as RFP may be monitored for tumorigenicity and treatment response noninvasively by use of Illumitool imaging and camera system (see section 7) (Lightool Research, Encinitas, CA) equipped with an RFP filter (Nelson *et al.*, 2004; Tan *et al.*, 2005).
7. When tumors reach a volume of 1000 mm^3, animals are sacrificed and tumors are excised under sterile conditions, divided into three sections, and preserved for immunohistochemistry, Western blotting, and DNA/RNA isolation, respectively.

6.2. Protocol for tail vein injections for tumor formation in mice

1. Isogenic iBMK cells, growing in log-phase in tissue culture plates are trypsinized, harvested, and resuspended in PBS at a density of 2.5×10^6 cells/ml.

2. The mice are weighed and transferred to a mouse restrainer (PLASLABS, Lansing, MI), which restrains the mouse while allowing access to the tail vein.
3. The lateral tail vein is identified on either side of the tail nerve. Start the injection at the tip of the tail and move closer to the body if you need to inject the mouse more then once. The injection site is disinfected with 70% ethanol and a needle (30G) containing the cell suspension is inserted into the vein at a slight angle. The cell suspension (0.5×10^6 cells in 200 μl volume) is injected slowly and carefully into the vein starting at the tip of the tail keeping the needle as flat and parallel to the vein as possible. If incorrect positioning occurs (which results in bulging in the tail), the process is repeated proximal to the previous site. Cells systemically migrate to various organs and anatomic sites and colonize as tumors that are visible in approximately 5 to 6 weeks. Animals are monitored for apparent tumor formation and are sacrificed after approximately 5 to 6 weeks for the assessment of tumor formations at various anatomical sites. Cell lines stably expressing fluorescence or chemiluminescence markers (Tables 5.2, 5.5, and 5.6) (also see section 7) can be tracked and monitor tumor growth noninvasively *in vivo*.

7. Noninvasive *In Vivo* Monitoring of Tumor Growth by Expression of Fluorescence and Chemiluminescence Markers

Because these immortal mouse epithelial cells are genetically manipulable, they can be easily engineered to express fluorescence or luminescence reporters such as green or red fluorescent protein (GFP or RFP respectively) to noninvasively monitor tumor growth and chemotherapeutic response *in vivo* (Table 5.2) (Nelson *et al.*, 2004; Tan *et al.*, 2005) or GFP or RFP fusions of proteins of interest (Table 5.2) (Degenhardt *et al.*, 2006; Karantza-Wadsworth *et al.*, 2007a; Mathew *et al.*, 2007b). Immortalized epithelial cell lines can also be engineered to stably express luciferase reporter genes under the control of a specific promoter element for *in vivo* imaging and examination of activation of transcription factors such as NF-κB in tumors (Degenhardt *et al.*, 2006).

We have successfully used immortalized epithelial cell lines expressing the fluorescence protein RFP to demonstrate that loss of Bax and Bak or Bim or gain of Bcl-2 blocks cell death and promotes tumor growth in nude mice (Nelson *et al.*, 2004; Tan *et al.*, 2005). iBMK cells expressing RFP that are either wild-type (W2.3.1-5), double deficient in Bax and Bak (D3.zeo-2), or expressing Bcl-2 (W2.Bcl-2-3) injected subcutaneously into nude mice. Each mouse is ear-tagged and monitored individually for tumor growth. For monitoring tumor growth *in vivo* noninvasively, animals are individually imaged by use of an Illumitool imaging and camera system

(Lightools Research, Encinitas, CA) equipped with an RFP and GFP filters (Nelson *et al.*, 2004). Only those cells with defects in apoptosis formed tumors within 20 to 60 days.

8. Tumor-Derived Cell Lines (TDCLs) as a Model System for Epithelial Tumor-Promoting Functions

Another powerful application of our immortalized mouse epithelial cell models is the ability to select and screen for *in vivo* genetic and epigenetic events that promote tumor growth by rederiving cell lines from tumors. As described earlier, immortalized epithelial cell lines (iBMKs, iMMECs, iMOSECs, and iMPECs) are nontumorigenic. However, tumors emerge with long latency (>3 months) because of clonal outgrowth caused by tumor-promoting mutations (Karp *et al.*, 2008; Nelson *et al.*, 2004). Because these cells are initially adapted for growth *in vitro*, they are capable of returning efficiently to growth *in vitro* when removed from the tumor after *in vivo* selection. These tumor cells have acquired stable genetic and epigenetic alterations that render them highly tumorigenic in subsequent tumor formation assays (Karp *et al.*, 2008). Because these tumors are generated from immortalized, nontumorigenic epithelial cells, when cells are isolated from these tumors and established as cultures, these tumor-derived cell lines (TDCLs) show remarkable phenotypic alterations consistent with their enhanced tumorigenicity acquired *in vivo* (Karp *et al.*, 2008). Clonal selection for tumor-promoting functions provides a powerful tool to analyze the molecular events regulating epithelial tumor progression.

iBMK cells are selected *in vivo* for tumorigenicity and tumors are extracted to generate TDCLs. By use of this TDCL-assisted *in vivo* selection we have identified a mammalian ortholog of the *Drosophila* tumor suppressor and polarity regulator, *crumbs* as a gene whose loss of expression disrupted tight junction formation, apicobasal polarity, and contact inhibition and promoted tumor progression in mice. Restoration of *crumbs* expression restored junctions, polarity, and contact inhibition and suppressed migration and metastasis (Karp *et al.*, 2008). Additional and different genetic events regulate tumor growth in distinct organs and anatomic sites after metastasis, which remains to be investigated.

8.1. Protocol for the generation of TDCLs

1. Isogenic iBMK cells of the genotype to be studied, along with their controls, growing in log-phase in normal culture condition, are trypsinized and harvested.

2. Cells are pooled, washed twice with PBS, and cells are then resuspended to a cell density to 1×10^7 cells/ml in PBS and allowed to form tumors in appropriate animal models and locations to generate tumor allografts as described in section 8.1.
3. Once the tumor reaches a volume of 1000 mm^3, the tumor is excised and collected in a petri dish in cold sterile PBS.
4. The excised tumor is divided into three equal pieces. One piece is immediately fixed in 10% buffered formalin solution (Buffered Formalde-Fresh; Fisher Scientific, Fair Lawn, NJ) at 4 °C over night, paraffin embedded, and sliced into 5-μm sections for immunohistochemical (IHC) analysis. The second tumor piece is weighed and snap-frozen in liquid nitrogen for DNA/RNA isolation and analysis.
5. The third tumor piece is chopped into several small pieces of approximately 1 × 1-mm sizes and transferred into a 50-ml conical tube with sterile PBS.
6. Minced tumor pieces from step 5 are washed several times in sterile PBS and broken into smaller pieces in 10 ml tissue culture medium by pipetting up and down by use of a 10-ml pipette.
7. The preceding suspension is diluted 1:4 in tissue culture medium and is transferred into four 10-cm tissue culture dishes in appropriate growth medium containing antibiotics (DMEM/10%FBS/1% Pen Strep).
8. Dissociated tissue pieces are incubated in at 37 °C (5% CO_2) for a week without any disturbance until proliferative cells emerge from small tumor fragments.
9. Cells derived from tumors are allowed to develop into monolayer, at which point cells are harvested from each plate by trypsinization, expanded, and frozen down in multiple aliquots in the vapor phase liquid nitrogen as described in section 2.1.5.
10. TDCLs are then subjected to further phenotypic characterization. Gene expression profiling is also performed by comparing the RNA isolated from the TDCLs, with that from parental cells, for the elucidation of any gain of oncogenic activations or loss of tumor suppressor functions by use of microarray analysis (Karp *et al.*, 2008).

9. Concluding Remarks and Future Perspectives

Derivation of these immortalized mouse epithelial cell line model systems described here combines the power of mouse genetics and convenience of genetically defined nontumorigenic cell systems to address the molecular requirements in epithelial tumorigenesis. The availability of mutant mice with specific gene manipulations makes it possible to derive immortalized mouse epithelial cancer cell lines from many tissues and genotypes of interest with the inherent advantage of having comparable

isogenically-derived wild-type cells. The use of these cell lines have been instrumental in the demonstration of the role of a large number of key apoptosis regulators and have provided necessary insight into how this pathway is regulated in cancer (Tables 5.1 to 5.8) (Karp *et al.*, 2008; Mathew *et al.*, 2007a; Nelson *et al.*, 2004; Shimazu *et al.*, 2007; Tan *et al.*, 2005). We are in the process of expanding our cell line panels to include mutants of an increasing number of essential genes implicated in novel pathways such as autophagy (Tables 5.5 and 5.6) (Karantza-Wadsworth *et al.*, 2007a; Mathew *et al.*, 2007a,b). This will provide the necessary impetus in establishing the role of these pathways in cancer progression.

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