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# Tumor progression and metastasis in murine D2 hyperplastic alveolar nodule mammary tumor cell lines

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**We have examined tumor progression and metastatic properties of three clonal murine mammary tumor cell lines of recent origin (D2A1, D2.OR and D2.1). These lines were derived from spontaneous mammary tumors which originated from a D2 hyperplastic alveolar nodule (HAN) line. D2A1 cells were more malignant than D2.OR or D2.1 cells, whether measured by experimental metastasis assays after intravenous injection in nude mice or chick embryos, *in vivo* growth rate of primary tumors following mammary fat pad injection in nude mice, or spontaneous metastasis assay from primary tumors growing in mammary fat pads. D2A1 cells also were more invasive *in vitro* in a Matrigel invasion assay than D2.1 cells, while the D2.OR cells were non-invasive in this assay. The increased invasiveness and malignancy of D2A1 cells were associated with increased levels of mRNA for the cysteine proteinase cathepsin L. Levels of osteopontin (OPN), nm23, int-1 and int-2 mRNAs were also examined. Nm23 levels were highest in the most malignant cell line. These cell lines provide a model for studying the tumorigenic and metastatic ability of mammary tumor cells and offer several advantages: they were cloned from mammary tumors that originate from a common source of preneoplastic cells (D2HAN); they are of relatively recent origin; and they have spontaneously arrived at different stages of tumor progression.**

**Keywords:** cathepsin L, mammary tumors, metastasis, osteopontin, tumor progression.

## Introduction

Established cell lines have been used frequently to study mammary tumor progression. While valuable information can be obtained from these studies, concerns have been raised about use of tumor lines long removed from their origins [1]. Also, when parental mammary tumor cell lines are not metastatic (i.e. SP1 cells), then metastatic variants may have to be selected from chemically treated cells [2] or cells that are transfected with oncogenes [3].

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We are using mammary tumor lines of recent origin and low passage number to study the process of mammary tumor metastasis. These clonal murine mammary tumor lines (D2A1 [4], D2.1 [5] and D2.OR [5]) were derived in the laboratory of Dr F. Miller (Michigan Cancer Foundation, Detroit, MI, USA), from spontaneous mammary tumors, which in turn originated from a D2 hyperplastic alveolar nodule (HAN) line. HANs have properties that resemble both normal and malignant cells. The morphology of the hyperplastic cells is indistinguishable from that of normal

prelactating mammary tissue [6–9]. However, HAN cells have a higher risk for mammary tumor development than corresponding normal mammary alveolar cells when injected into 'cleared' mammary fat pads [10]. HANs are thus referred to as 'premalignant' [11] or 'protoneoplastic' [12]. This system thus permits study of early steps of tumor progression in mammary cells. The three cell lines (D2A1, D2.1 and D2.OR) appear to represent mammary tumor cells at different stages of tumor progression. Their differences have developed naturally and have not been induced artificially by chemicals or exogenously added oncogenes. A previous study, which included D2A1, D2.1 and D2.OR cells, revealed an association between mammary tumor metastasis (in an experimental metastasis assay in syngeneic BALB/c mice) and a decreased binding of peanut agglutinin [5].

We have characterized the *in vivo* properties of these three cell lines, using both experimental metastasis assays (after i.v. injection into two immunodeficient hosts: nude mice and chick embryos) and spontaneous metastasis assays (after injection into mammary fat pads of nude mice). We assayed for invasive ability into Matrigel basement membrane, and for expression of three genes that have been associated with tumor progression and metastasis: cathepsin L, osteopontin (OPN), and nm23. We also tested for expression of two loci (int-1 and int-2) which often contain exogenous mouse mammary tumor virus integrations in mouse mammary tumors [13, 14].

## Materials and methods

*Cell lines*

The tumor cell line D2A1 was cloned from a spontaneous mammary tumor which arose in a syngeneic BALB/c female mouse implanted with a D2 hyperplastic alveolar nodule [4]. The D2.OR cell line was cloned from a separate spontaneous mammary tumor (ST5) which also arose in a syngeneic BALB/c female mouse implanted with a D2 hyperplastic alveolar nodule [5]. D2.OR cells were then injected subcutaneously into a BALB/c mouse, and one of the few resulting tumors was recloned to give the D2.1 cell line [5]. The D2A1, D2.1 and D2.OR cell lines were isolated and cloned in the laboratory of F. Miller (Michigan Cancer Foundation). In our laboratory, the cells were grown in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal calf serum (Bocknek, Toronto, Canada), L-glutamine, penicillin, and streptomycin [15]. The DMEM and all supplements except the fetal calf serum were obtained from Gibco Laboratories (Grand Island, NY, USA). The cell lines were all used before they had undergone 20 passages.

*Invasion of basement membrane*

We tested the ability of D2A1, D2.1 and D2.OR cells to invade reconstituted basement membrane ("Matrigel") in a transwell assay as described previously [16], using either fibronectin or laminin as the chemoattractant.

*Experimental metastasis assays*

The experimental metastatic ability of the cell lines was determined as described previously [17] by intravenous injection (lateral tail vein) of $5 \times 10^5$ cells/mouse in a volume of 0.2 ml of medium, into female BALB/c nude mice (4–5 weeks old). The mice were killed after 5–12 weeks; lungs, spleen, liver, heart, and kidneys were removed, fixed in neutral buffered formaldehyde (pH 7.6), and the number of macroscopic lung tumors was counted. The tissues were then prepared for histopathology as described previously [15].

The results of the tail vein metastasis assay were confirmed with an experimental metastasis assay in the chick embryo by injecting $5 \times 10^5$ cells/embryo into chorioallantoic veins of 11-day-old chick embryos and determining the number of viable tumor cells in chick livers 7 days later, as described previously [18, 19].

*Assays for spontaneous metastasis from mammary fat pad injection*

Mammary tumor cells ($5 \times 10^6$/mouse) were injected into the mammary fat pads of 4- to 5-week-old BALB/c nude mice as described previously [10]. Tumors were measured (length and width) with calipers every 2–5 days. Growth curves were graphed and the growth of the primary tumors was monitored from these curves. Mice were killed after the tumor reached a cross-sectional area of approximately 4 $cm^2$ or when the mice began to succumb to the burden of the tumor. Lungs, spleen, heart, liver and kidneys were removed and treated as above. In addition, the site of injection (mammary fat pad) was removed and examined by histopathology.

*RNA analysis*

RNA was extracted from BALB/c virgin mammary glands, lactating mammary glands, and the three mammary tumor cell lines. Tissues were dispersed

using a Virtis homogenizer (Gardiner, NY, USA) into a urea–LiCl buffer (6 M urea, 3 M LiCl). Tissue culture cells were scraped into the same buffer. RNA was then extracted as described previously [20].

The Northern transfer procedure has been described previously [21]. RNA was hybridized with denatured, oligolabeled ([$^{32}$P]dCTP) cDNA probes, under moderately stringent conditions (42°C, 50% formamide, 5 × SSC). The cathepsin L probe was the 1.19 kb cassette of plasmid pSP65-MEPA [22]. The OPN probe was a murine 2ar/osteopontin cDNA probe [21]. The nm23 probe was a human nm23 cDNA insert from plasmid pNM23-1 [23], and was a kind gift of Dr P. S. Steeg (NIH, Bethesda, MD, USA). The int-1 "C" plasmid was supplied by Drs H. E. Varmus (University of California at San Francisco, San Francisco, CA, USA) and R. Nusse (Netherlands Cancer Institute, Amsterdam, The Netherlands) [13]. The int-2 "C" plasmid was provided by Drs C. Dickson (Imperial Cancer Research Fund Laboratories, Lincoln's Inn Fields, London, UK) and G. Peters (Imperial Cancer Research Fund Laboratories, St. Bartholomew's Hospital, London, UK) [14]. Ribosomal cDNA prepared from virgin mammary gland RNA [15, 24] was used as a control. For comparison, an actin probe was also used as a secondary control (murine 2.1-kb $\beta$-actin cDNA in pBR322) [25].

## Results

*Invasion of basement membrane*

We determined the ability of D2.OR, D2.1 and D2A1 cells to invade into Matrigel basement membrane matrix (Figure 1). D2A1 cells were the most invasive in this assay. D2.1 cells were poorly invasive, and D2.OR cells were essentially non-invasive. These results were found whether fibronectin (Figure 1a) or laminin (Figure 1b) was used in the lower chamber as a chemoattractant.

*Experimental metastasis assays*

We determined the ability of D2A1, D2.1 and D2.OR cells to survive in the circulation and colonize at a secondary site after i.v. injection in nude mice. At 5 weeks post inoculation (p.i.), four of five mice injected with D2A1 cells had macroscopic lung metastases, ranging in number from six to 21 per mouse (Table 1). No visible tumor nodules were visible at 5 weeks p.i. in lungs of mice injected with D2.1 cells. However, by 10–12 weeks p.i., four of four mice injected with D2.1 cells had visible lung metastases (1–8 per mouse). Metastases were not seen in mice injected with D2.OR cells at 5–12 weeks p.i.

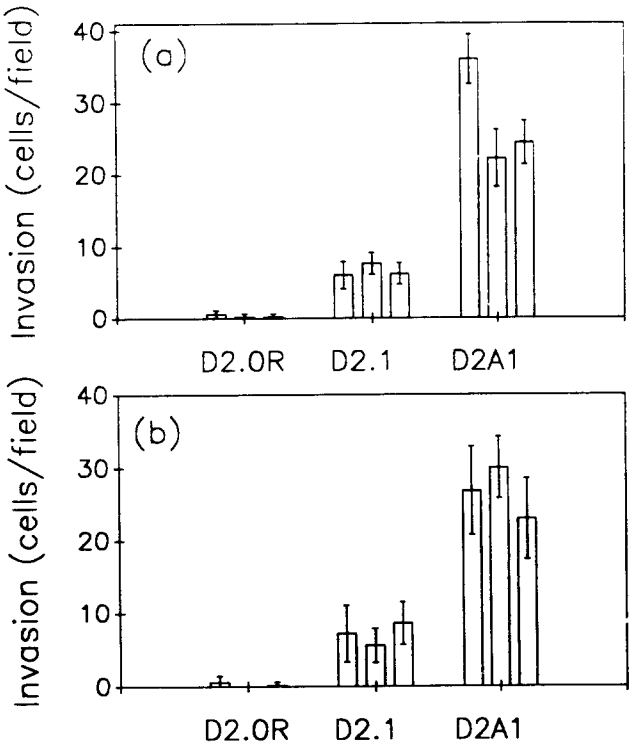


**Figure 1.** *In vitro* invasion assay. A Matrigel basement membrane (12.5 $\mu$g/well) was reconstituted in the upper compartment of a transwell chamber [16]. The lower chamber contained either (a) fibronectin (5 $\mu$g/ml) or (b) laminin (8 $\mu$g/ml). D2A1, D2.OR or D2.1 cells ($5 \times 10^4$) were applied to the upper compartment of the transwell chamber and incubated for 24 h at 37°C. The cells that migrated through the gel were fixed, stained and counted, as described [16]. Each bar represents the mean number of cells (± S.D.) that had invaded in a single experiment, using triplicate samples. In each case, results from three separate experiments per cell line are shown.

Histopathology was performed on lungs, heart, liver, spleen and kidneys of all injected animals to confirm and expand on visual observations (Table 1, Figure 2). By 5 weeks, all D2A1-injected animals had moderate to extensive metastasis to the lungs. Two of five D2A1-injected mice also had extensive metastasis to the heart. No metastases were detected in other organs examined. In the D2A1-injected mice, lung metastases consisted of perivascular, subpleural and subendothelial infiltration. In addition, multiple thrombi composed of neoplastic cells were present in many of the large

**Table 1**. Experimental metastatic ability in nude mice

| Mouse | Cells[a] injected | Time[b] (weeks) | No. of macroscopic lung metastases | Histopathology[c] | |
|---|---|---|---|---|---|
| | | | | Lung | Heart |
| A1 | D2A1 | 5 | 13 | ++ | +++ |
| A2 | D2A1 | 5 | 21 | +++ | +++ |
| A3 | D2A1 | 5 | 0 | ++ | – |
| A4 | D2A1 | 5 | 6 | ++ | – |
| A5 | D2A1 | 5 | 9 | ++ | – |
| D11 | D2.1 | 5 | 0 | – | – |
| D12 | D2.1 | 5 | 0 | – | – |
| D13 | D2.1 | 5 | 0 | – | – |
| D14 | D2.1 | 5 | 0 | – | – |
| D15 | D2.1 | 5 | 0 | – | – |
| D1 | D2.1 | 5 | 0 | – | – |
| D2 | D2.1 | 10 | 3 | +++ | – |
| D3 | D2.1 | 10 | 1 | +++ | – |
| D4 | D2.1 | 12 | 8 | +++ | +++ |
| D5 | D2.1 | 12 | 1 | ++ | – |
| C1 | D2.OR | 5 | 0 | – | – |
| C2 | D2.OR | 10 | 0 | – | – |
| C3 | D2.OR | 10 | 0 | – | – |
| C4 | D2.OR | 12 | 0 | – | – |
| C5 | D2.OR | 12 | 0 | – | – |

[a]Cells ($5 \times 10^5$) were injected in the tail veins of 4- to 5-week-old female nude BALB/c mice.
[b]Time post inoculation.
[c]–, Normal; +, minimal neoplasia; ++, moderate neoplasia; +++, extensive neoplasia. No metastasis was observed in the kidney, liver or spleen.

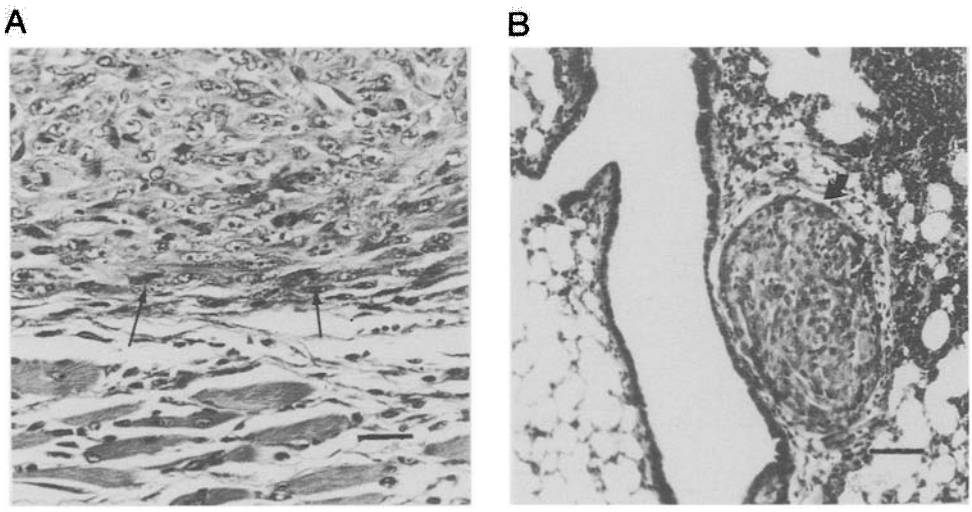


**Figure 2.** Histopathology of metastatic mammary tumors. (A) Section of intercostal muscle from D2A1-injected mouse A5 (see Table 1) illustrating marked displacement of muscle tissue, with separation and obliteration of the architecture by the infiltrating neoplastic cells. Note the identifiable myofibers (arrows) adjacent to the malignant cells. H&E. Bar = 43 $\mu$m. (B) Section of lung from D2A1-injected mouse A103 (see Table 2). Intravascular metastasis has completely occluded a large peribronchial vessel (arrow). H&E. Bar = 61 $\mu$m.

pulmonary vessels. In some cases, there was vascular occlusion by neoplastic cells. In addition, multiple nodules of neoplastic cells were observed with cellular infiltration into mediastinum and intercostal muscles (Figure 2A). In the heart, neoplastic infiltrates were most commonly observed attached to the endocardium in the auricles. The metastatic foci were characterized by the formation of sheets and palisading patterns.

No metastatic cells were observed in sections of lungs, liver, kidney, heart, or spleen from six mice inoculated with D2.1 cells and examined at 5 weeks p.i. However, by 10–12 weeks p.i., all four of the D2.1-inoculated mice had moderate to extensive lung metastases (Table 1). The histopathology of the lung and heart metastases were similar to that described above. Mice injected with D2.OR cells were examined microscopically for signs of micrometastases, and none were observed in the organs examined from 5 to 12 weeks p.i.

The experimental metastasis results from nude mice were confirmed in an experimental metastasis assay in chick embryos (Figure 3). The results of this assay have been shown generally to predict experimental metastatic ability in assays in mice [19]. With this assay, we confirmed that D2A1 cells were the most metastatic, followed in order by D2.1 and D2.OR cells.

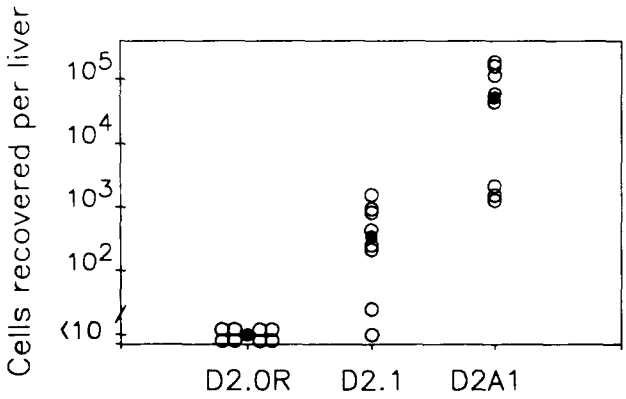


**Figure 3.** Experimental metastatic ability in chick embryos. D2A1, D2.1 or D2.OR cells ($5 \times 10^5$) were injected intravenously into the chorioallantoic membrane veins of 11-day-old chick embryos [18, 19]. After 7 days, the chick livers were removed, the cells dispersed and cultured in a ouabain-containing medium, which kills the chick embryo cells [18]. The numbers of mouse tumor cells that had metastasized to the liver were quantified and are shown for each cell line. Each point represents the number of tumor cells present in the liver of one chick embryo; the solid circle represents the median value.

*Assays for tumorigenicity and spontaneous metastases from mammary fat pad injections*

We also determined the ability of the D2A1, D2.1 and D2.OR cells to form primary tumors and spontaneously metastasize when injected into the mammary fat pads of BALB/c nude mice. We used fat pad injections instead of subcutaneous injections of tumor cells to provide a more natural environment for tumor cell growth. It has been reported that mammary tumors grow preferentially in mammary fat pads [26] and are more metastatic when implanted in mammary fat pads [27]. Primary mammary tumors in D2A1-injected mice grew most quickly (14–18 days to reach 2 $cm^2$ length $\times$ width) followed in order by D2.1 (44–52 days) and D2.OR cells (60–>90 days), the same rank ordering as was seen for invasive and experimental metastatic abilities (Table 1; Figure 3). Histopathology of the primary tumors revealed that the neoplastic infiltrates in the mammary glands consisted of masses of fusiform cells arranged in a loose to tight network, and forming sheets, palisading and whorl patterns. Histopathology confirmed the presence of a primary mammary tumor at the site of injection in all but one mouse (Table 2). The tumor cells seen in the primary tumors were similar to those observed in the metastases to other organs.

At 4 weeks p.i. in the mammary fat pads, macroscopic lung metastases were apparent in two of five mice injected with D2A1 cells (Table 2). However, histopathology revealed that all five of the D2A1-inoculated mice had minimal to moderate lung metastasis at 4 weeks p.i. None of the 10 mice injected with D2.1 cells had macroscopic metastases at 4–11 weeks p.i., a result that was confirmed by histopathology (Table 2). None of the nine mice injected with D2.OR cells had macroscopic metastases at 4–13 weeks p.i. in the lungs, and histopathology revealed minimal metastases in only two mice at 13 weeks p.i. (one in the lung and another in the spleen) and extensive metastasis to the liver in one of these mice (Table 2).

Lung metastases in D2A1- and D2.OR-injected mice contained intravascular neoplastic aggregates in major peribronchial vessels (Figure 2B). Infiltration in subpleural areas was also seen. In animal C8 (Table 2), there was a single splenic metastasis present. In animal C7 (Table 2), there were large areas within the liver that were infiltrated with neoplastic cells, with complete replacement of the parenchyma, and obliteration of the normal architecture.

**Table 2.** Spontaneous metastatic ability

| Mouse | Cells[a] injected | Time[b] (weeks) | No. of macroscopic lung metastases | Histopathology[c] | | | |
|---|---|---|---|---|---|---|---|
| | | | | Lung | Spleen | Liver | Primary mammary tumor |
| A101 | D2A1 | 4 | 0 | + | − | − | +++ |
| A102 | D2A1 | 4 | 2 | + | − | − | +++ |
| A103 | D2A1 | 4 | 0 | ++ | − | − | +++ |
| A104 | D2A1 | 4 | 0 | + | − | − | +++ |
| A105 | D2A1 | 4 | 8 | ++ | − | − | +++ |
| D101 | D2.1 | 4 | 0 | − | − | − | + |
| D102 | D2.1 | 4 | 0 | − | − | − | +++ |
| D103 | D2.1 | 4 | 0 | − | − | − | ++ |
| D104 | D2.1 | 4 | 0 | − | − | − | +++ |
| D105 | D2.1 | 4 | 0 | − | − | − | ++ |
| D6 | D2.1 | 6 | 0 | − | − | − | ++ |
| D7 | D2.1 | 6 | 0 | − | − | − | ++ |
| D8 | D2.1 | 11 | 0 | − | − | − | +++ |
| D9 | D2.1 | 11 | 0 | − | − | − | +++ |
| D10 | D2.1 | 11 | 0 | − | − | − | +++ |
| C101 | D2.OR | 4 | 0 | − | − | − | + |
| C102 | D2.OR | 4 | 0 | − | − | − | + |
| C103 | D2.OR | 4 | 0 | − | − | − | + |
| C104 | D2.OR | 4 | 0 | − | − | − | + |
| C105 | D2.OR | 4 | 0 | − | − | − | − |
| C6 | D2.OR | 12 | 0 | − | − | − | +++ |
| C7 | D2.OR | 13 | 0 | + | − | +++ | +++ |
| C8 | D2.OR | 13 | 0 | − | + | − | +++ |
| C9 | D2.OR | 13 | 0 | − | − | − | +++ |

[a]Cells ($5 \times 10^6$) were injected in the mammary fat pad of 4- to 5-week-old female nude BALB/c mice.
[b]Time post inoculation.
[c]−, Normal; +, minimal neoplasia; ++, moderate neoplasia; +++, extensive neoplasia. No heart or kidney metastases were observed.

Thus, all three lines are tumorigenic after mammary fat pad injection, with D2A1 cells growing most quickly, followed in order by D2.1 and D2.OR cells. D2A1 cells were spontaneously metastatic, while D2.1 cells were non-metastatic over the time period examined, and D2.OR cells were only minimally metastatic from primary tumors growing in mammary fat pads after prolonged growth intervals (13 weeks).

*RNA expression*

We extracted RNA from D2A1, D2.OR and D2.1 cells as well as from BALB/c lactating mammary gland and virgin mammary gland tissue. These RNAs were used in Northern transfer experiments. The expression of cathepsin L in D2A1 cells was approximately 5- to 6-fold higher than in D2.OR and D2.1 cells. In addition, expression of cathepsin L in D2A1 cells was approximately 26-fold higher than in virgin mammary glands and 17-fold higher than in lactating mammary glands (Figure 4). These results were consistent with the fact that D2A1 cells were the most invasive in the *in vitro* invasion assay and the most metastatic in the experimental and spontaneous metastasis assays. The range of values for nm23 expression in the mammary tumor cells was approximately 2- to 7-fold higher than the nm23 expression in virgin or lactating mammary glands; the expression in D2A1 cells was approximately 3-fold higher than in D2.1 or D2.OR cells. No significant difference was observed between expression of nm23 in lactating and virgin mammary gland tissue. The expression

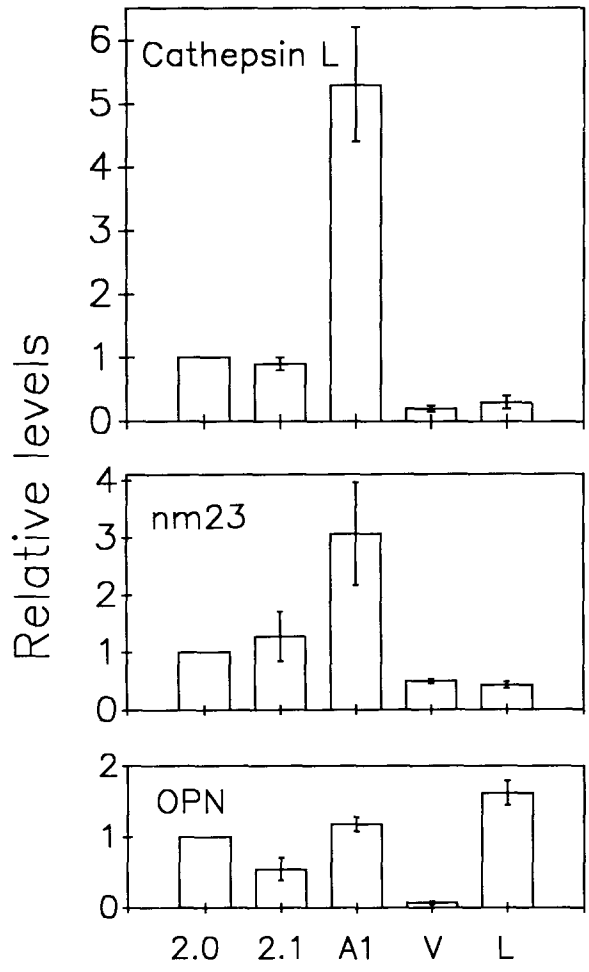


**Figure 4.** Relative expression of cathepsin L, nm23, and osteopontin (OPN) RNA in mammary tumor cell lines and in normal mammary tissue. Northern transfer experiments were performed, and the RNA on the blots annealed with gene probes (see Materials and methods). The murine cathepsin L transcript is approximately 1.8 kb [22], nm23 is approximately 1.0 kb [23] and OPN is approximately 1.6 kb [21]. The intensity of the appropriate band was determined with a laser densitometer. The $^{32}P$ radioactivity bound to the blots was allowed to decay until it was no longer detectable, and the blots were reannealed with a ribosomal cDNA probe; the intensity of annealing to the ribosomal probe was also determined with a densitometer and used to correct for any differences in amount of RNA present in each blot. For each gene, the value of the ratio (intensity gene mRNA/intensity 18S ribosomal RNA) for the D2.OR sample has been normalized to 1.0. The following abbreviations for RNA samples are used: 2.0 = D2.OR mammary tumor cells; 2.1 = D2.1 mammary tumor cells; A1 = D2A1 mammary tumor cells; V = BALB/c mouse virgin mammary gland; L = BALB/c mouse lactating mammary gland. Average values from at least three Northern transfers are shown and error bars indicate standard errors.

of OPN was approximately 23-fold higher in lactating mammary glands than in virgin glands (Figure 4). In addition, the range of values for OPN expression in the three mammary tumor cell lines was 8- to 17-fold higher than the OPN expression in virgin glands (Figure 4). No detectable RNA expression was observed with int-1 or int-2 probes (data not shown) in D2A1, D2.1, or D2.OR cells. These results were calculated relative to levels of 18S ribosomal RNA as a control. When actin mRNA levels were used as a control instead, similar results were obtained for the D2.OR, D2.1, D2A1 and the virgin mammary gland RNA samples (data not shown). However, the corresponding results for the lactating mammary gland RNA were reduced as much as 4-fold when actin was used as a control compared to values obtained with the 18S ribosomal control. This finding implies that the relative levels of actin mRNA may be reduced during mammary gland development.

## Discussion

There is a need for models to examine early stages in mammary tumor progression. Tumors that arise from murine D2 HANs provide one such model [10]. We have used three low-passage cell lines (<20 passages), D2A1, D2.1 and D2.OR cells, that were isolated from mammary tumors. These tumors all originated from the same D2 HAN line that was implanted in BALB/c mammary fat pads. In spite of the parallel origin of these three cell lines, we found that they exhibited different *in vitro* and *in vivo* properties, suggesting that they represent cells at different stages of mammary tumor progression. *In vitro* D2A1 cells were most invasive, followed in order by D2.1 and D2.OR cells. The same rank ordering was found for experimental metastatic ability after i.v. injection into two immunodeficient hosts, nude mice and chick embryos. Growth rate *in vivo* after injection into mammary fat pads of nude mice also followed the same rank order. D2A1 cells were spontaneously metastatic to lung from primary mammary fat pad tumors. Spontaneous metastases were observed rarely for D2.OR cells and not at all for D2.1 cells. Thus, these cells may represent mammary tumors at different stages of progression, with D2A1 cells being most malignant by all assays used, and D2.1 and D2.OR cells being less malignant.

We have used these cell lines to look for associations between metastatic ability and expression of genes that have been implicated in tumor progression and spread. Cathepsin L, a cysteine proteinase, has been shown to be associated with invasion and metastatic properties of *ras*-transformed tumor cells of fibroblastic origin [16, 28, 29]. We have extended these observations to mammary tumor cells of epithelial origin. The expression of cathepsin L in D2A1 cells was 5- to 6-fold higher than in D2.OR cells and in D2.1 cells. Increased expression of this cysteine protease may contribute to the increased invasiveness and malignancy of D2A1 cells relative to D2.OR or D2.1 cells.

Low levels of nm23 have been reported to be associated with metastatic ability and poor patient prognosis in human breast cancer [23, 30]. We did not find this inverse relationship between nm23 expression and metastatic potential among the three murine mammary cell lines examined here. Instead, we found that the most malignant of the three lines, D2A1, had approximately 6-fold higher expression of nm23 mRNA than did virgin mammary glands. In addition, expression of nm23 in D2A1 cells was approximately 3-fold higher than was observed in the less metastatic D2.OR and D2.1 cells. Other studies have also suggested that high levels of nm23 can be associated with increased malignancy in various tumors, including colon and breast cancer [31, 32]. Homology of nm23 to an NDP kinase [33, 34] has suggested a signal transduction function for nm23, but the role of this protein in tumor progression and metastasis remains to be clarified [35].

OPN is a secreted phosphoprotein produced by a limited set of normal tissues, including bone, kidney and milk [36–39]. Several studies have shown the OPN gene to be regulated developmentally [36, 40–42]. Structural features of the OPN protein (e.g. an RGD cell binding site, a calcium-binding domain) suggest possible functions, but the role of this protein in either normal or tumor cells remains poorly understood. We found high levels of OPN mRNA in murine lactating mammary glands when compared with virgin glands, consistent with the presence of OPN protein in milk. This result also is consistent with that of Craig and Denhardt [36], who found high OPN mRNA levels in skin and ventral fatty tissue of lactating mice, moderate levels in tissues from pregnant mice, and low levels in non-pregnant mice. OPN is also a transformation-associated protein whose expression can be induced by *ras* and tumor promoters [21, 37, 43–45]. We found that all three mammary tumor cell lines had increased levels of OPN mRNA, relative to levels in virgin mouse tissues, which were similar to the OPN levels in lactating tissues. Our results are consistent with OPN being a transformation-related protein.

While int-1 and int-2 expression is often observed in mammary tumors, our inability to detect expression of these genes in D2A1, D2.OR or D2.1 cells is consistent with observations that expression of these genes is not detected in all mammary tumors [13, 14].

The cell lines we have characterized here provide a useful model for studying tumor progression in mammary tumors, and for identifying genes whose expression may contribute to malignancy in these cells. Since the three cell lines differ in their invasive, tumorigenic and metastatic properties, they may differ in their abilities to fulfil different steps in the metastatic process. These cells thus provide the opportunity to study steps in tumor progression and metastasis in a series of closely related, but phenotypically distinct, mammary tumor cells.

## Acknowledgements

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