

# Targeted Disruption of the Tissue Inhibitor of Metalloproteinases Gene Increases the Invasive Behavior of Primitive Mesenchymal Cells Derived from Embryonic Stem Cells In Vitro

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**Abstract.** The metalloproteinase family of proteolytic enzymes can degrade extracellular matrix and facilitate invasive migration. This class of enzymes is specifically inhibited by the tissue inhibitor of metalloproteinases (TIMP-1). Using homologous recombination, we have disrupted the gene encoding TIMP-1 in pluripotent embryonic stem cells. Because the TIMP-1 gene is X linked and is hemizygous in embryonic stem cells, we have been able to study the effect of this mutation in culture. Using a basement membrane invasion

assay, we found that the mutant cells, differentiated in low concentrations of serum with retinoic acid, were more invasive than their normal cell counterparts, and that this was specifically reversed by adding exogenous TIMP-1 protein. The invasive cell population had characteristics of an early population of primitive mesenchymal cells, including expression of vimentin and a transient period of invasiveness from 4–8 d after initiation of differentiation. Therefore, metalloproteinase activity can be rate limiting for cell invasion.

**E**XTRACELLULAR matrix degradation is an important phase of biological remodeling processes, including growth of cell populations and the invasion of matrices that serve as barriers to prevent indiscriminate cell migration. Metalloproteinases are secreted enzymes that degrade macromolecules of the extracellular matrix (reviewed by Alexander and Werb, 1991; Woessner 1991), and their role in invasive and migratory processes is implied by the effect of specific inhibitors on the invasiveness of cells in culture. Expression of metalloproteinase activities has been identified in many cell types that participate in physiologic remodeling processes, such as pregnant and involuting mammary epithelium (Talhouk et al., 1992), the osteoblasts and osteoclasts of bone (Huffer, 1988), and macrophages (Welgus et al., 1990). Characterized enzymes in this family are collagenase (two isotypes), stromelysin (two isotypes), "stromelysin 3," matrilysin (72 kD gelatinase), invadolysin (92 kD gelatinase), and PUMP (punctate metalloproteinase). There are at least two inhibitors of this class of enzyme, the tissue inhibitor of metalloproteinases (TIMP-1)<sup>1</sup> and a second, more recently characterized inhibitor, TIMP-2. These inhibitors have different expression patterns, different but overlapping inhibitory profiles, and very different accessory

functions (Howard and Banda, 1991). The properties of metalloproteinases and their inhibitors, and their expression in a variety of biological systems has been reviewed in detail elsewhere (Matrisian and Hogan, 1990; Werb and Alexander, 1992).

The ratio of metalloproteinase activities to their inhibitors may be critical to the regulation of invasive processes. TIMP protein has been shown to have anti-invasive properties both in culture and in vivo. For example, melanoma cells, either in an amnion invasion assay in vitro or injected into the tail vein of mice, show a decreased invasive ability in the presence of exogenous TIMP (Schultz et al., 1988). Trophoblast, a normal but highly invasive cell type responsible for mammalian embryo implantation, is also noninvasive in the presence of TIMP (Librach et al., 1991; Behrendtsen et al., 1992). Enzymes and inhibitors apparently exist together in the pericellular milieu, creating a net invasive:anti-invasive equilibrium. To provide a critical test of this hypothesis we ablated the expression of the TIMP-1 gene by targeted gene disruption. Since the TIMP-1 gene is X linked (Jackson et al., 1987), and therefore hemizygous in embryonic stem (ES) cells (typically with an XO or XY karyotype), a single round of homologous recombination leads to complete loss of TIMP expression, allowing us to analyze the effect of this mutation in culture. ES cells were also chosen for their differentiation properties, which can be exploited in vitro to study the invasive properties of many different cell types (Robertson, 1987).

1. *Abbreviations used in this paper:* APMA, 4-aminophenylmercuric acetate; EHS, Engelbreth-Holm-Schwarm; ES, embryonic stem; PCR, polymerase chain reaction; RA, retinoic acid; TIMP, tissue inhibitor of metalloproteinases.

Grown in the presence of leukemia inhibitory factor, an inhibitor of differentiation (reviewed by Hilton and Gough, 1991), ES cells can be maintained in the undifferentiated state in culture apparently indefinitely. Their totipotency is illustrated by their ability to contribute to any tissue of a chimera of stem cells and normal preimplantation embryo. Very little information is available that compares the differentiation of ES cells in culture with the normal gastrulation process in the mouse. ES cells can be differentiated by two main protocols: in monolayer culture in the absence of leukemia inhibitory factor or in suspension cultures of cell aggregates. These aggregates, or embryoid bodies, clearly develop an organization of the classic germ layers on a time scale parallel to the evolution that would be observed in the embryo *in vivo*. During gastrulation and organogenesis there are many processes that involve invasive migration and remodeling: classic examples are the ingression of primary mesoderm during gastrulation and the highly specific migratory processes associated with neural crest and with primordial germ cells. Clearly, the value of the ES cell culture technique would be enhanced by the ability to use them as a mass culture of cells undergoing developmentally relevant reactions. Using an assay that measures cell invasion through a reconstituted basement membrane, we have studied the invasive properties of ES cells that either do or do not express TIMP-1. Our aim was to determine whether the expression of the inhibitor serves to limit a cellular invasive process.

## Materials and Methods

### Materials

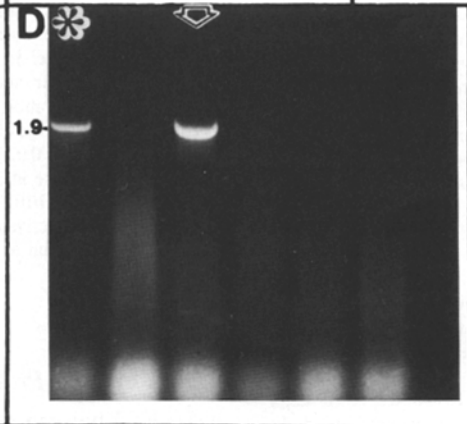
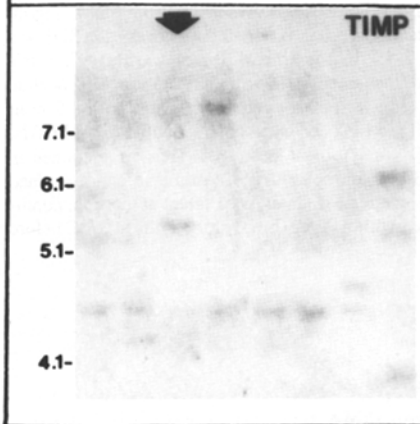
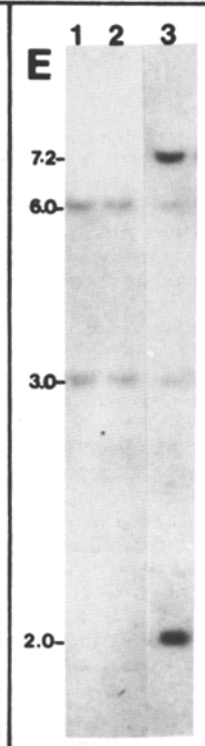
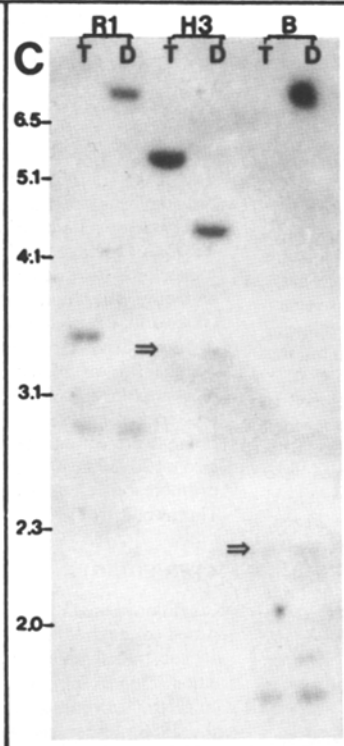
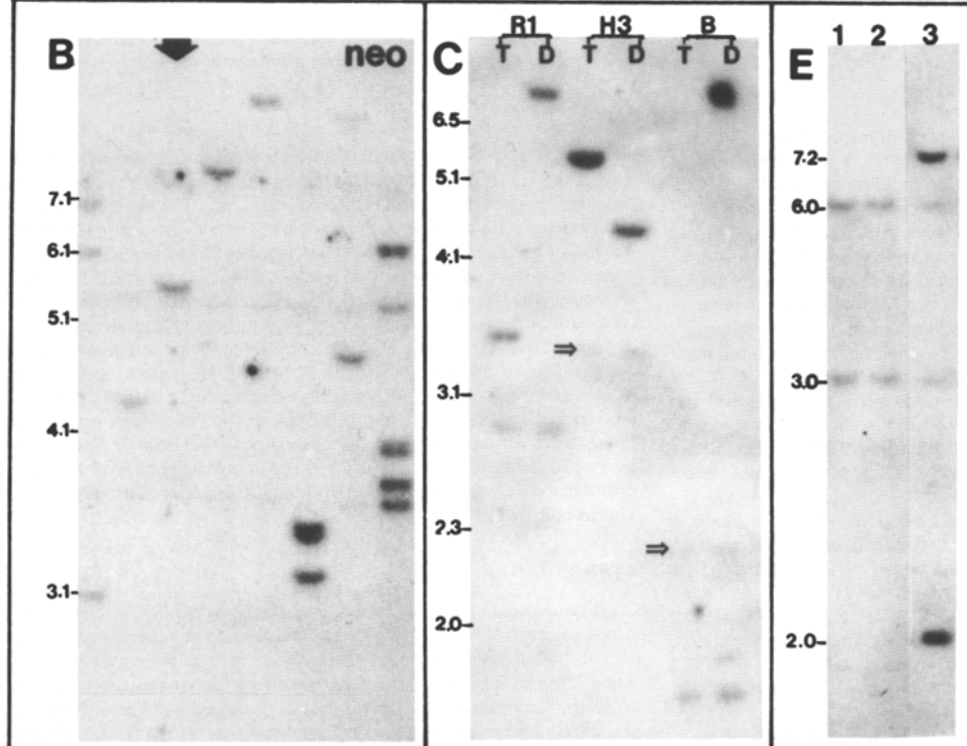
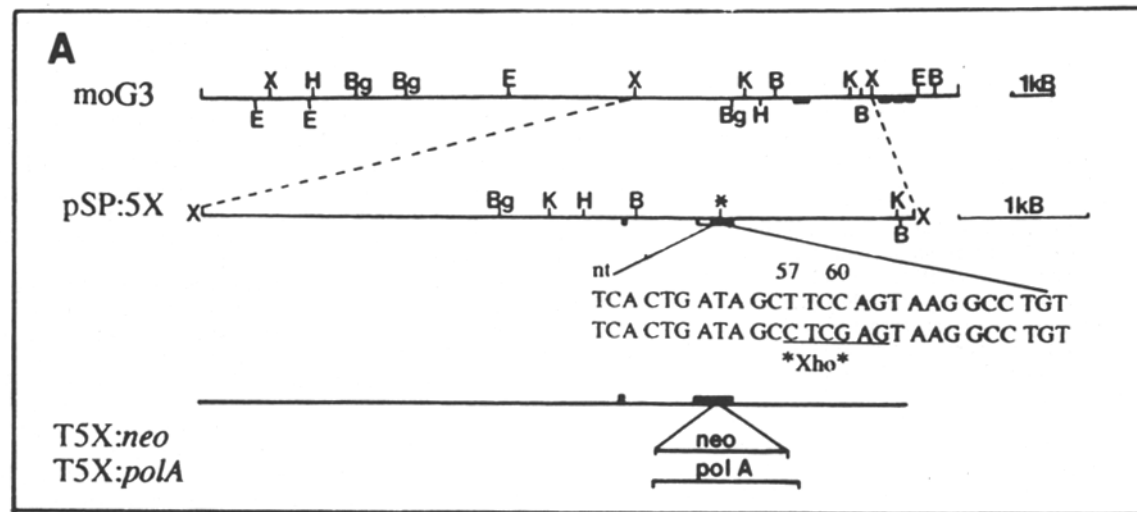
The probe used to isolate a genomic clone for use in targeting experiments was a cDNA clone for mouse TIMP (pTIMP8), a generous gift of B. Coulombe (University of Montreal, Canada) (Gewert et al., 1987). We used a Clontech mouse genomic library for screening purposes (BALB/c adult mouse liver constructed in EMBL3). Site-directed mutagenesis was performed with the Mutagene kit from BioRad Laboratories (Cambridge, MA). Stratagene (La Jolla, CA) provided the *PMCneo* and *polA*-gene cassettes (unrepaired version) and Bluescript plasmids. Promega Corp. (Madison, WI) supplied the plasmid pSP64. Buffalo Rat Liver (BRL) cells were the Glasgow isolate from J. Pitts (Glasgow University). D3 ES cells (derived from the 129 mouse strain; Doetschman et al., 1985) and TROMA-1 antibody were the kind gifts of R. Kemler (Max Planck Institute, Tübingen, Germany). Other antibodies used were a monoclonal anti-human vimentin (V5255) from Sigma Chemical Co. (St. Louis, MO), and a secondary antibody consisting of FITC-conjugated rabbit anti-mouse from DAKO-

PATTS (Copenhagen). Mice for making embryonic fibroblasts were CD-1 from Charles River; syngeneic 129/SvJ mice for ES cell injections were from Jackson Immunoresearch Laboratories, Inc. (West Grove, PA). Sera for ES cell culture were FCS from Hyclone Laboratories Inc. (Logan, UT) and iron-supplemented calf serum from Flow Labs, Inc. (McLean, VA). Tissue culture plasticware was from Corning Glass Inc. (Corning, NY), and bacteriologic grade plasticware was from Fisher Scientific Co. (Pittsburgh, PA). G418-sulfate (Geneticin), nonessential amino acids, formamide, lactalbumin hydrolysate, and Penstrep antibiotic concentrate were all from Gibco Laboratories (Grand Island, NY). Ultraser HY serum-free culture medium additive was from IBF Biotechnics (France). The all-trans-retinoic acid was from Sigma Chemical Co.; a stock solution was made at 1 mM in ethanol at  $-20^{\circ}\text{C}$ . Nucleosides and  $\beta$ -mercaptoethanol were tissue culture grade from Sigma Chemical Co. Other tissue culture media and additives were prepared by the Cell Culture Facility (University of California, San Francisco). Electroporation was performed with the Cell-Porator from Gibco Laboratories. Gelvatol mounting medium was prepared with Monsanto 20–30-grade polyvinyl alcohol. Transwell filters, 6.5-mm inserts of polycarbonate membrane with 8- $\mu\text{m}$  pores, were from Costar Corp. (Cambridge, MA). Glutaraldehyde was from Ted Pella Inc., Redding, CA (EM grade, 8% stock). Polymerase chain reaction (PCR) analysis was performed with a Thermocycler from Perkin-Elmer Cetus Instruments (Norwalk, CT). Basement membrane matrix (from the Engelbreth-Holm-Schwarm [EHS] sarcoma grown in mouse ascites) was prepared as described by Streuli et al. (1991) (modified from Kleinman et al., 1986) and was a very generous gift from the laboratory of M. J. Bissell (Lawrence Berkeley Laboratory, Berkeley, CA). Recombinant human TIMP-1 protein (rTIMP), produced in *Escherichia coli*, was a gift from D. Carmichael, Synergen (Boulder, CO). The Y chromosome-specific *Zfy-1* probe was a 1.28-kb EcoRI fragment kindly provided by C. Nagamine (University of California, San Francisco) (Nagamine et al., 1989). A mouse TIMP-2 cDNA probe was prepared by PCR from mouse 3T3 mRNA after reverse transcription. PCR primers used were GGT CTC GCT GGA CAT TGG AGG AAA G (5') and GGG TCC TCG ATG TCC AGA AAC TCC TG (3'); amplification yielded a 363-bp fragment which was subcloned to Bluescript for use as a probe.

### Constructs for Gene Targeting

A 5.5-kb XbaI fragment extending 5' from the noncoding first exon, together with the coding second exon and 70% of the second intron (Fig. 1A) was cut out of an 18-kb genomic clone (moG3) and subcloned to pSP64 (pSP:5X). A 2.5-kb KpnI fragment internal to this fragment was also subcloned from moG3 into Bluescript plasmid, and an XhoI site was introduced at nucleotide 57 by site-directed mutagenesis (numbering starts from the initiating ATG codon). This fragment was used to replace the endogenous fragment in pSP:5X. The *PMCneo* cassettes were inserted into the XhoI site to create the *polA*<sup>-</sup> (*TSXneo*) and the *polA*<sup>+</sup> (*TSXpolA*) versions of the construct. The internal restriction sites of the construct used for electroporation were compared with host DNA to check for polymorphisms or mutations. For electroporation, the 6.5-kb fragment was cut out with XbaI, gel-purified, and cleaned up with phenol-chloroform extraction followed by ethanol precipitation. The DNA was resuspended in Hepes-buffered saline for electroporation (21 mM Hepes, pH 7.05, 137 mM NaCl, 5 mM KCl, 0.7 mM Na<sub>2</sub>HPO<sub>4</sub>, 6 mM glucose).

**Figure 1.** Gene targeting protocol and characterization of TIMP-mutant (TNIL) cell line. (A) An 18-kb EMBL3 genomic clone (*moG3*) was isolated by means of a mouse cDNA probe and mapped with restriction enzymes. A 5.5-kb XbaI fragment was excised and subcloned to plasmid pSP64 for further manipulation (*pSP:5X*). The first coding exon of the gene was specifically mutated to introduce an XhoI site (changes at nucleotides 57 and 60, numbered from the initiating ATG codon). The 1-kb *PMCneo* cassette, either with or without a polyadenylation signal (*TSXpolA* and *TSXneo*, respectively), was cloned into this site. (B) Southern blots of HindIII-digested gDNA samples isolated from random individual clones that resulted from transfection of D3 ES cells with the *TSXneo* insert. (Top) Blot hybridized with a *neo* probe; (bottom) the same blot hybridized with a TIMP probe. Arrows mark the relevant lane that shows the targeting event. A single 5.3-kb *neo* band overlays a 5.3-kb TIMP band. The endogenous TIMP signal at 4.3 kb is eliminated in the DNA from this cell clone. (C) Further characterization of the parent (D) and mutant (T) cell lines by Southern blot analysis of DNA digested with EcoRI (RI), HindIII (H3), or Bam HI (B). The expected shifts in band sizes confirm the mutation as a simple gene targeting event. (D) PCR was used to confirm that the insert was integrated at the homologous gene locus. The positive control (asterisk) (10 fgs of plasmid control added to genomic DNA); the TNIL cell line (arrow). (E) A Southern blot of HindIII-digested gDNA from both D3 and TNIL cell lines was hybridized with a mouse probe for the sex-linked *Zfy* gene. Both parent (lane 1) and mutant (lane 2) cell lines contained the X-linked copy of the gene (3.0- and 6.0-kb fragment sizes), and neither contained the Y-linked copy that generates HindIII bands at 7.2 kb and 2.0 kb (as in control gDNA sample from a male mouse, lane 3).



## Culture of ES Cells

D3 ES cells were grown in ES complete medium on gelatinized plasticware and transferred onto mitomycin C-treated embryonic feeder layer cells every 6 d (every third passage). Passage number was kept as low as possible, and many small samples of cells were frozen at early passage numbers to ensure that each experiment used a parallel population. ES cells were grown in 60% BRL cell-conditioned medium supplemented with FCS and iron-supplemented calf serum (1:1) to make a total of 20% serum in ES complete medium. Other additives were nonessential amino acids,  $\beta$ -mercaptoethanol (7  $\mu$ l/liter), nucleosides (30  $\mu$ M each of cytosine, uracil, adenosine, and guanosine and 10  $\mu$ M thymidine), and DME-H21 to volume. Details of methods are described by Robertson (1987), including gelatinization of plastic dishes and testing of serum lots for maintenance of maximum growth rate and minimum toxicity. BRL-conditioned medium was prepared according to Doetschman et al. (1985): Briefly, BRL cells were grown to confluence in roller bottles in BRL medium (7.5% FCS in DME-H21 and Hams F12, 1:1), and 100 ml of BRL medium was allowed to condition for 3–4 d for five collections. Embryonic fibroblasts were grown as described by Doetschman et al. (1988) and were used at passage 4. Confluent monolayers were treated with mitomycin C (10  $\mu$ g/ml) in DME-H21/10% FCS for 3 h and allowed to recover before use.

## Gene Targeting and Selection of Homologous Recombinants

Flasks (75 cm<sup>2</sup>) of 80% confluent ES cells were trypsinized ( $\sim 2.5 \times 10^7$  cells) and washed in Hepes-buffered saline with 15  $\mu$ g of targeting insert in an electroporation cuvette. Electroporation conditions of 280 V and 330  $\mu$ F were found to be optimal. Cells were diluted into ES complete medium and plated out into at least eight 48-well dishes, with dilutions, for a final number of 10–20 colonies per plate. Colonies were selected at 24 h by the addition of 0.1–0.2 mg/ml G418. Killing with G418 was effective only at the correct cell density; too high a cell density slowed growth rate and selection efficiency declined, producing spurious background colonies and allowing the G418-resistant (G418<sup>R</sup>) colonies to be crowded out. The medium was changed every 2 d, and no feeder cells were used at this stage. After 10–12 d, colonies were clearly visible; these were trypsinized and subcloned to new 48-well clusters. Trypsinization in 48-well dishes was found to be much less traumatic to colonies than picking with a tip from 10-cm dishes: cells were established more quickly and with higher efficiency. Colonies were grown to 2 cm<sup>2</sup> (24-well dishes) and harvested. 80% of the cells were used for genomic DNA (gDNA) preparation and the remainder were frozen. gDNA was prepared by standard techniques. For PCR analysis we used a 5' primer within the *neo* gene 1 kb from the 3' end of the T5X clones (ATC GCC TTC TAT CGC CTT CT) and a 3' primer at the 5' end of the third exon of the endogenous TIMP gene (AAC CCA TGA ATT TAG CCC TTA T). The expected product size was 1.9 kb. PCR conditions were standard, with the following exceptions: SJ buffer (166 mM NH<sub>4</sub>SO<sub>4</sub>, 67 mM MgCl<sub>2</sub>, 100 mM  $\beta$ -mercaptoethanol, 670 mM Tris, pH 8.8, 10% dimethyl sulfoxide), 0.6 mM dNTPs and PCR cycle program of 45 cycles of 40 sec at 95°C, 40 sec at 52°C, and 10 min at 72°C. For Southern blot analysis, gDNA preparations were digested with HindIII, separated on 0.8% agarose gels, and blotted by standard techniques. Blots were probed with pTIMP8 (mouse TIMP cDNA) in a hybridization solution with 50% formamide at 50°C. Blots were subsequently reprobed with the *neo* gene probe. Targeted integrations should show a loss of the endogenous 4.3-kb HindIII TIMP band and the appearance of a band at 5.3 kb. The *neo* gene probe hybridizes to this band and any other nonhomologous integration sites. Positive clones, stored in liquid nitrogen, were thawed onto feeder layers and expanded for analysis.

## Differentiation of ES Cells

Embryonic feeder fibroblasts were completely eliminated from ES cell cultures by depleting twice by selective adhesion to tissue culture plastic, followed by growth for at least one passage before use. For differentiation in monolayer, ES cells were trypsinized to a single cell suspension and seeded in medium with 10% FCS and 1  $\mu$ M retinoic acid (RA) at  $0.5 \times 10^7$  cells per gelatinized 75-cm<sup>2</sup> tissue culture flask. Medium was changed at 2 d. At 3 d, the cells were changed into ES complete medium, and were used in the invasion assay after 4 d.

## Invasion Assay

The invasion assay was modified from procedures already described

(Librach et al., 1992; Fisher and Werb, 1992). The working stock of EHS matrix was stored at exactly 4°C. It was tested for protein concentration and discarded on appearance of any precipitate. For pouring of matrix membranes, EHS matrix was diluted on ice to about 10 mg/ml with serum-free medium (DME-H21 with 0.5% UltroSer HY, 0.1% lactalbumin hydrolysate, 10 mM Hepes, pH 7.4, penicillin and streptomycin) with or without additions. EHS matrix (10  $\mu$ l) was coated onto Transwell polycarbonate filters and spread to coat evenly. The gels were set for  $\sim 10$  min at ambient temperature and tested for leaks by adding 1 ml of medium to the outside of the wells. Serum-free medium (200  $\mu$ l) was added to the wells, and the whole dish was transferred to the incubator.

Cells were trypsinized, tested for viability, and counted. They were washed twice in serum-free medium and resuspended to  $10^6$  cells/ml. Medium was removed from inside the wells and replaced with 200  $\mu$ l of the cell suspension ( $2 \times 10^5$  cells). Dishes were incubated for 16–20 h. Supernatants were removed for zymography; they were almost totally cell free, indicating that plating efficiency was high and cells were not superconfluent. Filters were washed and fixed in one of three ways: (a) in freshly made glutaraldehyde (2.9% in 0.1 M sodium cacodylate) for 30–60 min for scanning EM (stored in 0.1 M sodium cacodylate, 4°C if required); (b) in methanol-acetone (1:1) at  $-20^\circ\text{C}$  for 5 min (followed by air drying and storage at  $-80^\circ\text{C}$ ) for cytoskeletal staining; (c) in acid alcohol (methanol-acetic acid [3:1] for 10 min), rinsed and incubated with bisbenzamide (Hoechst 33258, 25  $\mu$ g/ml in PBS) for 10 min for staining of nuclei.

## Cytoskeletal Staining

For plastic dishes, 5–20- $\mu$ l drops of anti-intermediate filament antisera were used; fixed Transwell filters were turned upside down in a humidified chamber and incubated with 50  $\mu$ l of primary antibody. The methanol-acetone fix is water repellent, reducing the amount of antibody required for staining. TROMA-1 (cytokeratin 8 antibody; described by Boller and Kemler, 1983) monoclonal supernatant was used without dilution, and V5255 (vimentin antibody) was diluted 1:40 into 10% FCS/DME-H21. Filters were incubated for 45–60 min and antibodies were washed off in water for a total of 5 min. Secondary antibody (FITC-conjugated rabbit anti-mouse immunoglobulin) was diluted 1:40 in 10% FCS/DME-H21, added to filters and incubated for 45–60 min. These filters were washed briefly in water and postblocked in PBS containing 0.1% BSA and 0.05% Tween for 15 min. Filters were cut out and put under coverslips in Gelvatol (16% polyvinyl alcohol 20–30 in 30% glycerol in PBS) and immediately observed by using epifluorescence optics on a Zeiss Photomicroscope III (Carl Zeiss, Inc., Thornwood, NY).

## Zymography

Conditioned medium was collected, centrifuged to remove cell debris, and stored (if required) at  $-80^\circ\text{C}$ . Medium was analyzed either by addition of 4 $\times$  substrate sample buffer or after concentration by quinine sulfate precipitation. Zymography, which identifies proteinases and proteinase inhibitors, on gelatin substrate gels was performed as described elsewhere (Herron et al., 1986; Fisher and Werb, 1992; Behrendtsen et al., 1992). Briefly, 7–22% gradient SDS-polyacrylamide gels which incorporated 1 mg/ml gelatin were poured and stacked as usual. After electrophoresis the SDS was soaked out of the gel in 2.5% Triton (three 20-min washes). The gels were rinsed in water and incubated in 4-aminophenylmercuric acetate (APMA)-activated rabbit skin fibroblast-conditioned medium for 5 min. The medium was poured off and replaced with substrate buffer (50 mM Tris, pH 8, 5 mM CaCl<sub>2</sub>, 0.02% NaN<sub>3</sub>) for at least 16 h. Gels were stained in 0.5% Coomassie blue in 30% isopropanol/10% acetic acid and destained in 12.5% isopropanol/10% acetic acid. To activate latent zymogens, conditioned medium was activated with APMA (1 mM) for 30 min at 37°C before loading; gels were run at 4°C.

## Results

### Gene Targeting Protocol

A 5.5-kb XbaI genomic DNA fragment (containing the untranslated first exon, the coding second exon, and part of the second intron of the mouse TIMP-1 gene) was used to disrupt the single copy of the TIMP-1 gene in mouse D3 ES cells (Fig. 1). A gene that confers G418 resistance (*neo*), either with (T5XpolA) or without (T5Xneo) a polyadenylation sig-

nal, was introduced into this construct at an engineered XhoI restriction enzyme site in the second exon of the gene. This mutation ablates the expression of mRNA that encodes a viable TIMP-1 protein product. Lack of a polyadenylation signal on the construct acts as an enrichment protocol, a "gene trap," enriching for G418<sup>R</sup> transfectants that have DNA inserted into sites that contain a 3' flanking polyadenylation signal for effective processing of the chimeric RNA species. We would anticipate that integration at the site of the homologous gene would be represented at a higher frequency among the final pool of G418<sup>R</sup> colonies.

The total number of G418<sup>R</sup> colonies for T5XpolA (pol A<sup>+</sup>) was 1/500 of the total cell population surviving electroporation; this transfection frequency was lowered to 1/1.5 × 10<sup>4</sup> total cells for the T5Xneo (polA<sup>-</sup>) construct. The ratio of numbers of colonies transfected with T5Xneo to numbers of colonies transfected with T5XpolA was therefore 1:30, which was considered to be the enrichment factor for this protocol.

Southern blotting was used as the primary analysis of G418<sup>R</sup> colonies (Fig. 1 B), and results were confirmed with PCR (Fig. 1 D). The endogenous 4.3-kb HindIII TIMP-1 fragment was replaced by one at 5.3 kb in clones in which the homologous gene locus had recombined with the mutated gene construct (Fig. 1 B). A clone (the TNIL cell line) that contained a single *neo* integration site was confirmed by further restriction enzyme analysis to be the result of a simple replacement reaction (Fig. 1 C). All the observed bands were accounted for by the insertion of the *PMCneo* cassette into the disrupted gene. The targeting frequency in the T5Xneo transfections was approximately 1:30 homologous/nonhomologous G418<sup>R</sup> clones.

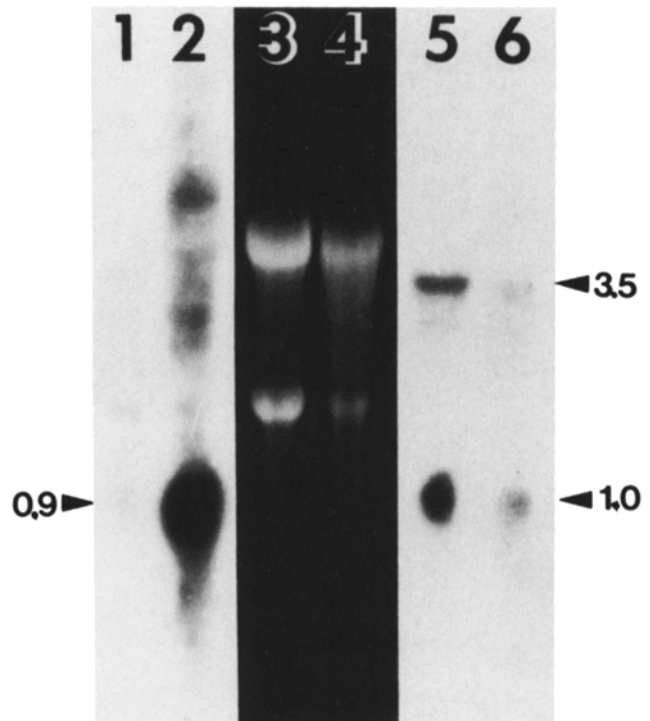
The karyotype of the cells was analyzed by chromosome counting (modal count of 39) and by using a probe for the XY chromosomes. This pair of chromosomes appears to be less stable than other chromosome pairs in ES cells. Both the parent D3 and derivative TNIL cells contained the same complement of chromosomes (XO). A probe for the mouse homolog of the sex-linked *Zfy* gene (Nagamine et al., 1989) hybridized to two bands on a Southern blot of HindIII-digested genomic DNA with sizes typical of the X chromosome locus (Fig. 1 E) and gave no detectable bands diagnostic of the Y-linked copy.

Functional disruption of the gene was confirmed by RNA blot analysis (Fig. 2). Expression of the TIMP-1 gene was missing from the TNIL cells (lane 1), whereas expression of the TIMP-2 gene was unaffected (lane 5). The TIMP-1 gene was clearly expressed in the parent D3 cells (lane 2).

#### **Disruption of the TIMP Gene Does Not Affect Morphology of Differentiated ES Cells**

There were no apparent differences between the wild-type D3 and mutant TNIL cells by various criteria, including the morphology and some functional studies of a range of differentiated cell types produced both in culture and in vivo. A line of G418<sup>R</sup> cells which did not contain a targeted gene disruption event was also compared with D3 and TNIL cells and found to have identical properties to the parent cell line.

We studied the effect of the TIMP mutation on the adhesion of ES cells, reasoning that since the adhesion of these cells is gelatin dependent, and gelatin is a substrate for metalloproteinases, the presence or absence of TIMP could



**Figure 2.** Expression of TIMP-1 mRNA is ablated in mutant TNIL cells. Total RNA from both cell lines was separated on an RNA blot and probed with the cDNA for TIMP-1. Undifferentiated D3 cells (lane 2) expressed a 0.9-kb mRNA that was absent from the TNIL cells (lane 1). The RNA on the blot was visualized by ethidium bromide staining (lanes 3 and 4) before transfer (the TNIL track was overloaded to show any residual activity). The same blot was rehybridized with a probe for TIMP-2, showing that both cell lines (TNIL, lane 5; D3, lane 6) express both the 3.5- and 1.0-kb mRNA species for this inhibitor (Stetler-Stevenson et al., 1990).

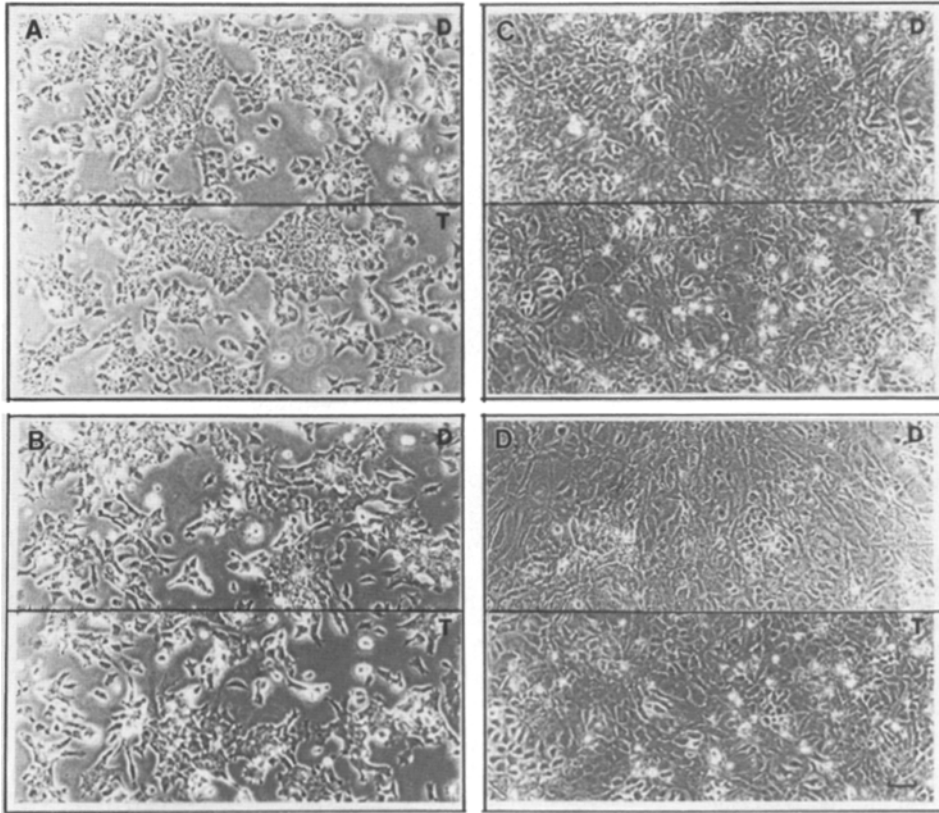
be significant. However, the presence of serum was critical to the adhesive process, and under these circumstances we could not discriminate between the parent and mutant cell lines.

Cells injected intravenously into syngeneic 129/SvJ mice (via the tail vein) did not establish teratomas at any site in the host. The TIMP mutation therefore did not lead to an increase in the metastatic capability of ES cells (data not shown), in contrast to the results of melanoma cell injection by tail vein described by Schultz et al. (1988).

#### **Disruption of the TIMP-1 Gene Increases Invasiveness**

Undifferentiated ES cells are not an invasive cell type. However, by differentiating these cells it is possible to establish derivative phenotypes that show various invasive behaviors. The pluripotentiality of ES cells in culture results in heterogeneity that tends to thwart their analysis. We chose differentiation protocols that minimized the obvious morphologic heterogeneity of the resulting cultures, in the hope of simplifying the study.

Wild-type D3 cells and mutant TNIL cells were differentiated by plating a suspension of ES cells onto gelatinized plastic to form a low-density monolayer in medium containing 10% FCS and 1 μM RA. For 2 d cell morphology was apparently unchanged (Fig. 3 B), but after 3 d cells were overtly differentiated (Fig. 3 C). At this time, the medium



**Figure 3.** Morphology of ES cells differentiating for 4 d in 10% serum with  $10^{-6}$  RA. Monolayers of ES cells were seeded at  $0.5 \times 10^7$  cells per 75-cm<sup>2</sup> flask in 10% FCS with 1  $\mu$ M RA, and the morphology of cells was observed for 4 d: (A) day 1, (B) day 2, (C) day 3, (D) day 4. The cell lines are identified on the right as parent D3 (D), or TNIL (T) cells. Medium was replaced with ES complete medium at 3 d. After 4 d, the cells were harvested and seeded onto filters for the invasion assay.

was replaced with ES complete medium, cells were grown to confluence and were harvested at 4 d (Fig. 3 D). Addition of ES complete medium at 3 d stimulated cell growth and viability considerably. This population was used to assess the invasive potential of parent and mutant cells. The invasion assay consisted of plating the cells onto a thin coat of reconstituted basement membrane from EHS matrix on a porous Transwell polycarbonate support filter (Librach et al., 1991). Cells were added to the top of the filter in serum-free medium, and invasiveness was indicated by the appearance of cells on the underside of the filter. After 16–20 h, cells were fixed in glutaraldehyde and prepared for scanning EM, or fixed with acid alcohol and stained with Hoechst 33258 nuclear stain. Mutant cells were clearly more invasive than wild-type cells (compare Fig. 4, B and E with A and D). Cell counts of 1-mm<sup>2</sup> areas are shown in Fig. 5 to illustrate the striking difference in invasive behavior.

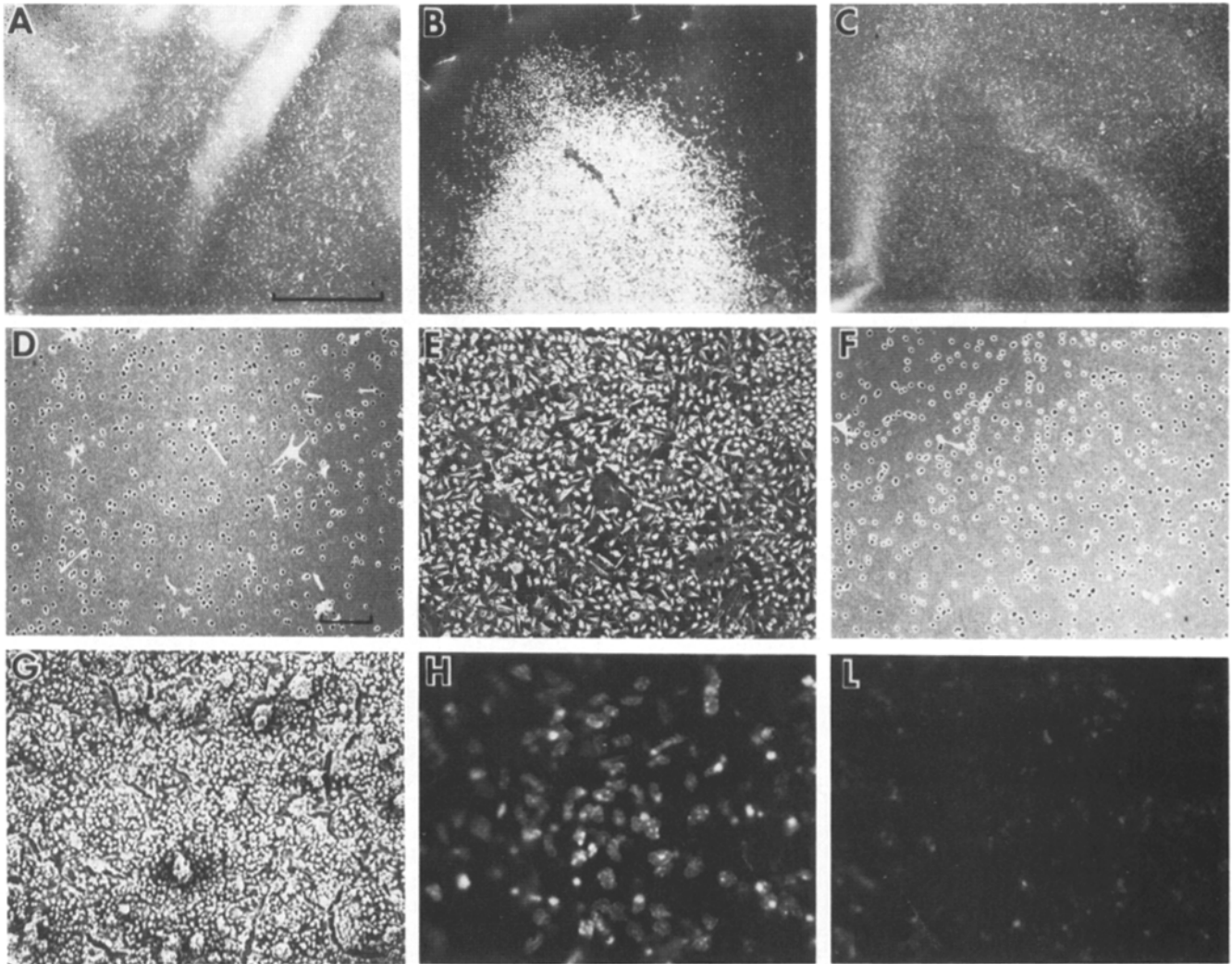
Two lines of evidence suggest that the invasive cell population also existed in the parent cell line. First, after sustained incubation (28–36 h) of D3 cells in the invasion assay, a population of cells did emerge on the undersides of the filters (Fig. 5), suggesting that the invasiveness of the TNIL cells was merely accelerated by the absence of TIMP-1. Second, the addition of medium containing 10% FCS to the underside of the Transwell filter enhanced cellular invasiveness so that the undersides of the filters were almost confluent for D3 cells after 16 h (Fig. 5). The enhancement of directional invasiveness towards serum-containing medium suggests we may be able to discern serum components that are attractants for this cell type. (By gel electrophoretic analysis, there was no generalized equilibration of serum proteins into the upper Transwell compartment). Medium containing serum was not

used routinely because serum contains metalloproteinase enzymes and inhibitors that may confuse the interpretation of the results.

Using different protocols, other cell types can be generated that do not express TIMP-1 but express other enzyme-inhibitor pairs that confer invasive potential. Furthermore, a fibroblastic derivative cell type that does synthesize large quantities of TIMP-1 in the wild-type strain does not show any invasive ability in this assay (data not shown). These data show that expression of metalloproteinase activity is only rate determining for invasive potential in specific cellular backgrounds.

#### ***Exogenous TIMP-1 Protein Results in Specific Reversion of Invasive Phenotype to Wild Type***

The property of enhanced invasiveness specifically reverted after the addition of recombinant TIMP-1 protein (Fig. 4, C and F). Exogenous rTIMP-1 was used at a concentration of 500 nM (10  $\mu$ g/ml) and added only to the EHS matrix (and not to the culture medium). TIMP-1 is a heparin-binding protein (Bunning et al., 1984) and is likely to remain bound to the heparan-sulfate proteoglycans of the basement membrane. No inhibitor was observed in the assay supernatants by zymography, suggesting little diffusion out of the EHS matrix. The addition of 500 nM rTIMP-1 resulted in the complete inhibition of invasiveness; lower concentrations produced a decrease in the reversion of the phenotype observed (Fig. 6). The morphology of the cells on the tops of the filters is shown in Fig. 5 G. We simplified the analysis of the assay by using a simple acid alcohol fix and a Hoechst nuclear stain. Cells processed in this way are shown in Fig. 5, H and L.



**Figure 4.** Enhanced invasive property of the differentiated mutant TNIL cells, and reversion with addition of TIMP-1 protein. All panels except *G* show the undersides of filters in representative invasion assays for D3 cells (*A* and *D*), TNIL cells (*B* and *E*), and TNIL cells with 10  $\mu$ M recombinant TIMP protein (*C* and *F*) after 16–20 h of incubation. Cells were seeded to confluence on the top of the EHS matrix in a Transwell insert (*G*). The assay can be visualized in either of two ways: with scanning EM (*A–G*) or by Hoechst nuclear staining of the tops and undersides of the filters at different planes of focus (*H*, TNIL cells; *L*, D3 cells). Fluorescence apparent in *L* is focused at the top of the filters. (*A–C*), Low-power magnifications of the EHS matrix; (*D–F*), more detailed micrographs of the emerging cells. Bars: (*A–C*) 1 mm; (*D–F*) 100  $\mu$ m.

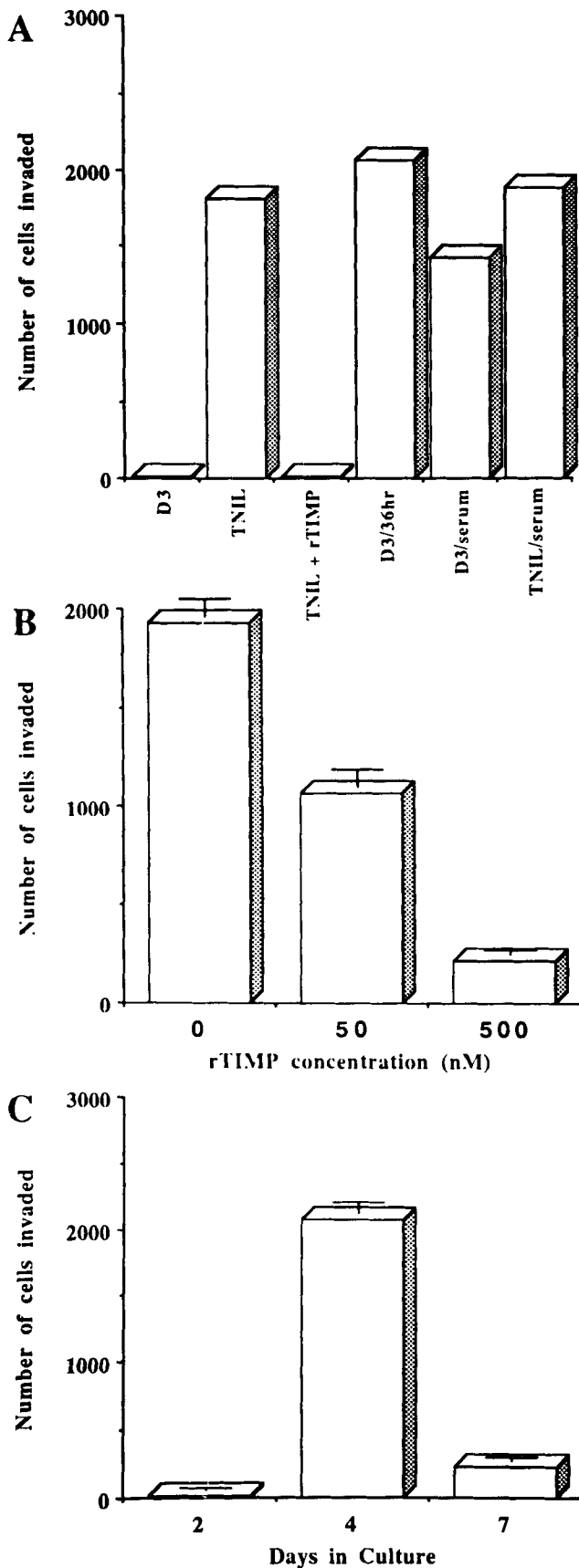
#### **Enhanced Invasive Behavior Is Temporally Controlled**

Mutant TNIL cells and their parent D3 cells did not show invasive behavior when undifferentiated, after 2 d of differentiation, or after 7 or 8 d of differentiation (Fig. 5). Undifferentiated D3 cells clearly express significant quantities of TIMP-1 mRNA (Fig. 2), but were not invasive. The invasive phenomenon is therefore transient, occurring between 2 and 7 d after initiation of differentiation in culture. There are various explanations for this period of invasiveness: either the invasive cell type no longer exists in more differentiated cultures, or the invasive property is transient in a particular cell type. Cells grown in low concentrations of serum with RA for 7 d showed a decline in viability and reduced plating efficiency, behaving like primary cultures with a short lifespan. Continued growth of differentiated cells beyond 7 d in medium containing serum with or without RA caused a continuous reduction in cell viability. Refeeding

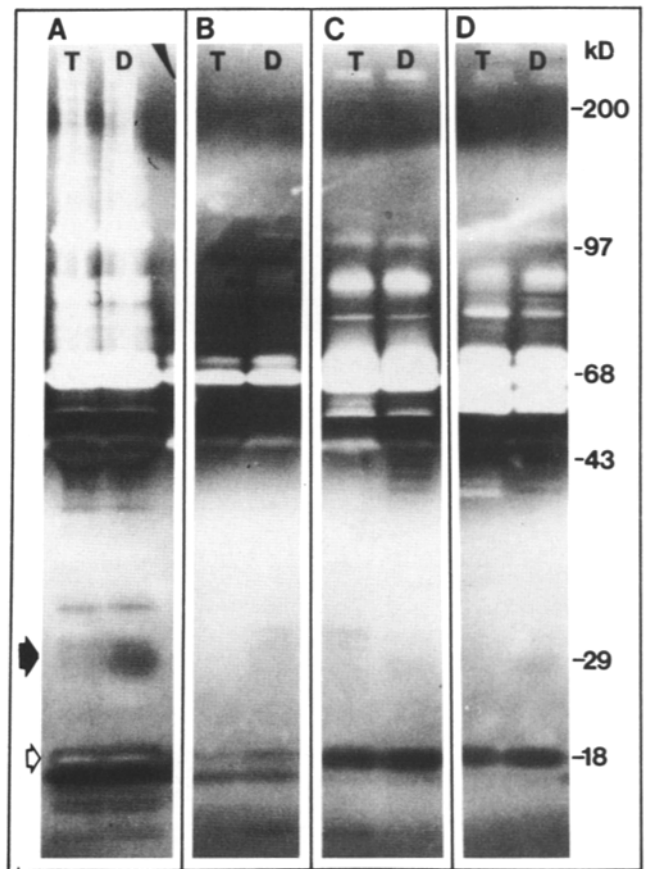
with ES complete medium maintained better cell viability and selected for the survival of a population of cells that resembled undifferentiated ES cells.

#### **Characterization of Secreted Metalloproteinases and Inhibitors**

We used substrate gel electrophoresis (zymography) to determine whether the cells were secreting metalloproteinases that could facilitate the invasive process, and to establish that both cell lines secreted similar enzymes. The usual zymographic process (which includes the metalloproteinase substrate gelatin in the polyacrylamide gel) was modified so that we could visualize both the enzymes and the inhibitors in the same gel. After renaturation of enzyme activities in the gel after separation in the presence of SDS, gels were incubated in APMA-activated rabbit skin fibroblast-conditioned medium (which contains a variety of active metalloproteinases).



**Figure 5.** Quantification of invasive potential of parent D3 and mutant TNIL cell lines. The cells visualized by scanning electron microscopy were counted in 1-mm<sup>2</sup> fields of the underside of Trans-



**Figure 6.** Expression of metalloproteinases and inhibitors in parent D3 and mutant TNIL cell lines. Reverse gelatin zymography was used to identify metalloproteinases and their inhibitors in the culture supernatants of cells plated on EHS matrix for the invasion assay. The technique is described in detail in Materials and Methods. Briefly, dark bands indicate inhibitor activities (and protein background), and clear bands indicate metalloproteinase activities (in a background of gelatin). *T*, TNIL-conditioned medium; *D*, D3-conditioned medium. (A) 200 μl of conditioned medium, concentrated with quinine sulfate, overloaded to show the production of the TIMP-1 protein in differentiated D3 cells (solid arrow). The open arrow marks an inhibitor band at 18–20 kD that is tentatively identified as TIMP-2. The dark band underneath is a distinct inhibitor activity. (B) 30 μl of conditioned medium, the same samples as A, underloaded to resolve the bands migrating at 68 kD. (C and D) Samples of conditioned medium from the same populations of differentiated cells plated on tissue culture plastic instead of EHS matrix, showing a shift of inhibitor species in the 18-kD range. The samples in D, which were briefly treated with APMA (30 min) to activate latent metalloproteinases, reveal a characteristic shift to a lower molecular weight. A significant proportion of the enzyme secreted by these cells is therefore in latent form.

well filter invasion assays. (A) D3, wild-type cells incubated in serum-free invasion assay for 16–20 h; TNIL, mutant cells under the same conditions; TNIL + rTIMP, addition of recombinant TIMP protein (500 nM) to basement membrane matrix; D3/36 hr, extended timepoint for wild-type cells; D3/serum, wild-type cells incubated in medium with 10% FCS for 16–20 h; TNIL/serum, mutant cells under the same conditions. (B) Dose-response curve for the addition of rTIMP protein to invasion assays of TNIL cells. (C) Time course of invasive properties of differentiating TNIL cells.



This incubation produced a limited degradation of the substrate gelatin except in bands containing inhibitor activity.

Fig. 6 A shows a representative profile of supernatant medium conditioned by the differentiated cells in the invasion assay. With the exception of the minor band at 18 kD all the bands were identified as metalloproteinases, based on their inhibition in zymograms with 1 mM 1,10-phenanthroline. The enzyme profiles look very similar in parent and mutant cell lines. Many activities are visualized: some bands are likely to be active proteolytic fragments of the main activities which migrate at about 72 and 92 kD. These are the molecular masses of the principal type IV collagenases characterized so far, namely, matrilysin (72 kD) and invadolysin (92 kD).

TIMP-1 protein is expressed in wild type differentiated cells (Fig. 6 A). The limit of sensitivity for visualizing TIMP-1 by reverse gelatin zymography depends on the band spread resulting from glycosylation; for the unglycosylated recombinant protein the limit is  $\sim 1$  ng. By comparison with standards, the TIMP-1 protein secreted by the differentiated D3 cells, visualized in Fig. 6 A, is assumed to be  $\sim 5$  ng per lane, equivalent to a protein secretion rate exceeding 1.5 ng/ $10^6$  cells/h. Conclusions about the quantity of TIMP secreted are limited by the facts that TIMP secretion may be heterogeneous within the cell population, that it may be vectorial, and that TIMP has matrix-binding properties. A distinct inhibitor band at 18 kD, also seen in Fig. 6 A, is likely to be TIMP-2. The other sharp dark bands represent proteins either synthesized by the cells or contained in the serum-free medium (identified by Coomassie staining of polyacrylamide gels run in the absence of gelatin).

The metalloproteinases synthesized by differentiated cells can be activated by the organomercurial agent APMA (Fig. 6 D). This reaction is diagnostic of metalloproteinases and generates a lower molecular weight active enzyme from an inactive precursor (reviewed by van Wart and Birkedal-Hansen, 1990). Both zymogens and active forms of the metalloproteinase are visible on zymograms due to their activation by the chaotrope SDS. APMA activation can also reveal the relative amounts of active and inactive enzyme in the supernatants; however, because the stability of these gelatinases after APMA activation is variable, this process needs to be very carefully controlled to be informative. The predominant metalloproteinase band at 68 kD was secreted by these cells principally in inactive form.

#### ***The Invasive Cell Population Has a Distinctive Expression for Intermediate Filament Phenotype***

We stained both invasive and total cell populations for intermediate filament proteins because vimentin expression in the absence of cytokeratin expression is a marker of mesenchymal cell type. The invasive population was analyzed by immunostaining the underside of the Transwell filters with either a vimentin antibody (Fig. 7 A) or a cytokeratin antibody (Fig. 7 D) and counterstaining with Hoechst nuclear stain. Pores in the filters could be visualized with phase-contrast microscopy (Fig. 7 B), and the nuclei on the tops of the filters were clearly in a different plane of focus (Fig. 7 C). Representative stains of the total cell population are shown in Fig. 8, A–D.

The expression of cytokeratin and vimentin was quantified by determining the number of cells positive for either stain

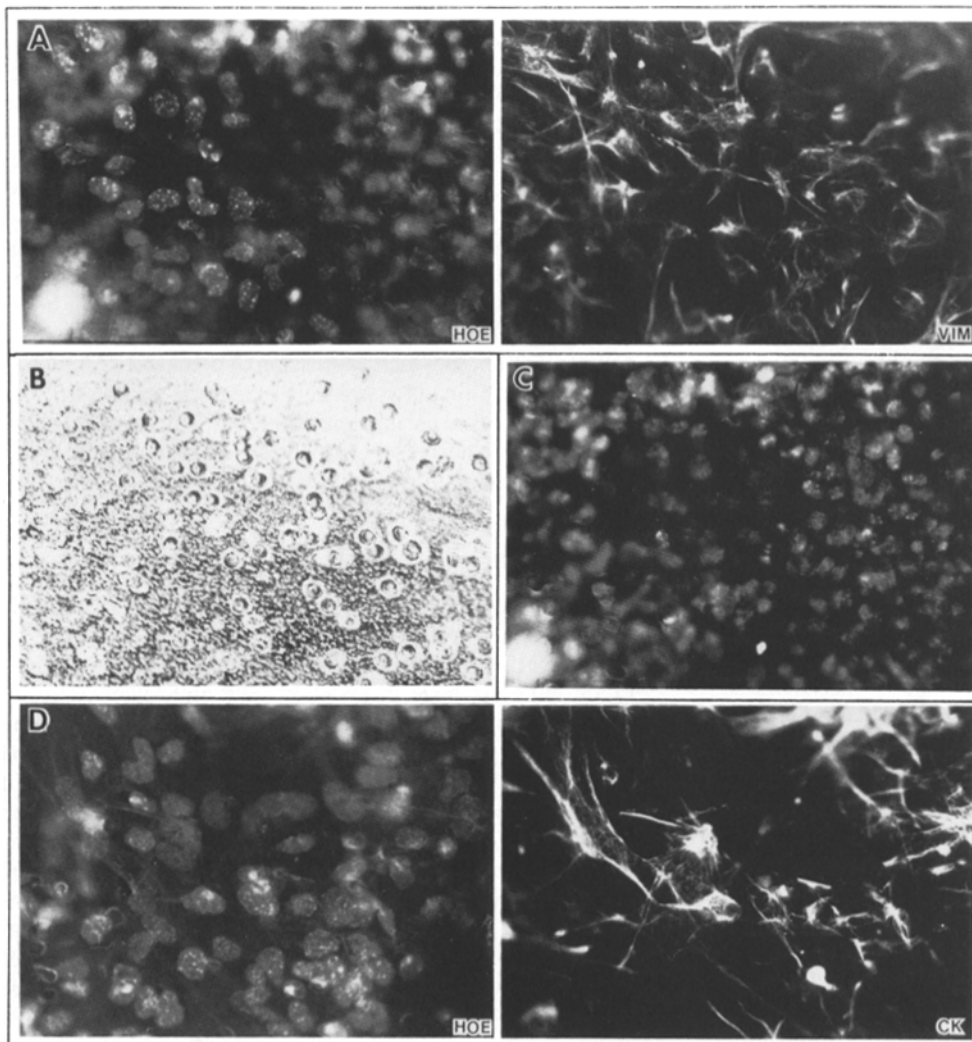
as a percentage of total number of cells. 45% of the total cell population was vimentin positive, and 90% of the invasive cell population was vimentin positive. In contrast, 91% of the total population was cytokeratin positive, but only 33% of the invasive population was cytokeratin positive. These data indicate that in the invasive cell population, a significant proportion of the vimentin-positive cells (63–75%) expressed only vimentin and almost all the remaining invasive cells co-expressed vimentin and cytokeratin. The cytokeratin networks also appeared to be diminished in quantity and extent in these cells, as if they were in the process of being dismantled or diluted out. This was in contrast to the total cell population, in which expression of cytokeratin predominated, cytokeratin networks were extensive, and 36–45% of cells co-expressed perinuclear whorls of vimentin. Thus, the expression of the mesenchymal cell marker vimentin was clearly enhanced in the invasive cells.

## ***Discussion***

In this study we changed the invasive phenotype of ES cells in culture by introducing a mutation in the TIMP gene by gene targeting. Because the TIMP gene is X linked, the mutation is hemizygous in ES cells. The only other X-linked gene targeting experiments reported to date are those at the HPRT locus, in which direct selection procedures were used (Hooper et al., 1987; Doetschman et al., 1987). Studies in which autosomal genes have been mutated (both alleles targeted) have not yet demonstrated a significant shift of phenotype in mutant cells with respect to cell proliferation or differentiation (*pim-1* oncogene, te Riele et al., 1990; G protein subunit, Mortensen et al., 1991). TIMP has been suggested to be an important player in the invasive reaction of pathologic cells such as melanoma (Schultz et al., 1988; Liotta et al., 1991), but there has so far been only correlative evidence for a role for this proteinase inhibitor in normal adult and developmental processes. The present report describes the effect of ablating expression of TIMP on the invasive behavior of cells. Because the pluripotentiality of ES cells allowed us to produce a variety of cell types from the parent lines, we were able to study this mutation in various cellular backgrounds. The generation of several differentiated cell types is of interest both for their specific enzyme/inhibitor profiles and for the possibility of identifying embryonic counterparts with analogous invasive and migratory properties.

#### ***The Invasive Reaction: TIMP as a Counterbalance***

The assay selected to test the invasive properties of cells was based on the ability of cells to invade and move through a thin gel composed of a reconstituted basement membrane matrix, appearing on the underside of the filter. The invasive reaction comprises various elements including adhesion (cell surface interaction), motility (cytoskeletal organization), and chemotaxis (perception of directionality). In matrices such as basement membrane, that normally constitute a barrier to cell migration, these properties must be supplemented by the ability to destroy the structural integrity of the membrane. Our assay measured invasive reactions that depend on enzymes capable of degrading basement components (type IV gelatinases and stromelysin) but did not measure motile

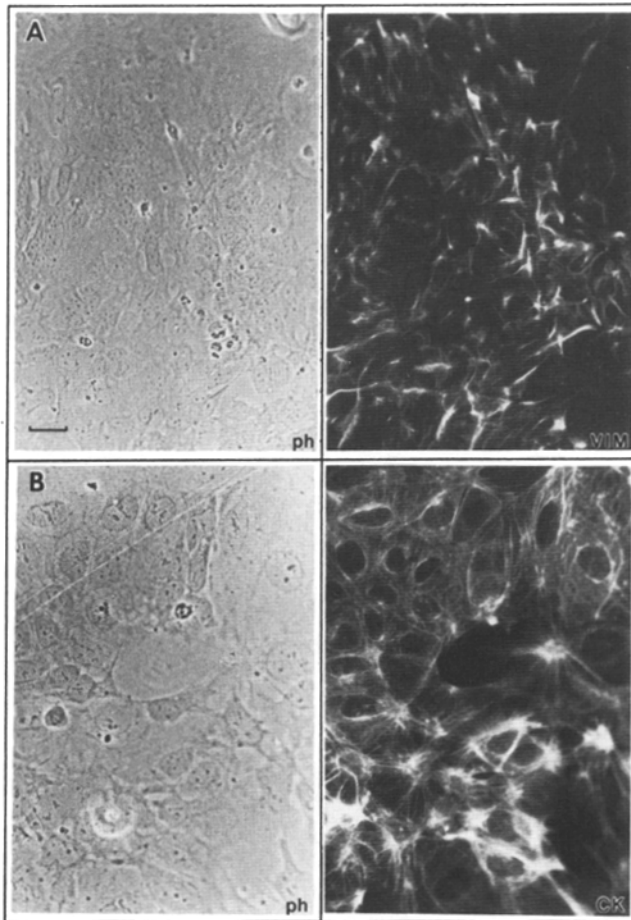


**Figure 7.** Expression of intermediate filament proteins in the invasive cell populations. Cells seeded on Transwell filters were incubated in the presence of serum for 16–20 h and fixed and stained as described in Materials and Methods. Cells shown are TNIL cells; results with D3 cells were identical. The undersides of filters were stained with antibodies to the intermediate filament proteins vimentin (A, VIM) or cytokeratin (D, CK). Cells were counterstained with Hoechst nuclear stain (HOE) so that we could determine the total number of emergent cells. Only part of the total cell field is typically in focus at this magnification. (B) Phase-contrast micrograph of the 8- $\mu$ m pores in the polycarbonate filter for the same field as A. (C) Hoechst-stained nuclei on the top of the filter, in a different plane of focus from A.

events in the interstitium that may be dependent on other enzymes such as collagenase. Cells challenged with different matrices presumably would show different invasive and motile properties.

As expected, undifferentiated cells of either the parent or the mutant cell line were not invasive in this assay. These are epithelioid cells that show no propensity to break cell-cell junctions or to migrate individually. We found a clear difference between the invasiveness of parent and mutant cells after 4 d of differentiation in the presence of RA in monolayer culture. After 3 d of incubation without leukemia inhibitor factor in medium containing a low concentration of serum (10% FCS) and RA (1  $\mu$ M), followed by 1 d of refeeding with ES complete medium, the ES cells had differentiated and the culture morphology was entirely different. Cells bearing the TIMP mutation (TNIL cells) were much more invasive than their normal cell counterparts. After prolonged incubation the parent cells generated a similar population of emergent invasive cells to the underside of the Transwell filters. The addition of serum to the underside of the filters accelerated the invasion of wild-type cells, producing a saturation density of cells after just 16 h. We have not identified the cause of this acceleration; it may be a cell-mediated effect elicited by serum growth or attractant factors, or serum may directly provide some facilitating cofactor for the reaction.

The property of enhanced invasion specifically reverted after the addition of TIMP protein. We deduce that TIMP is critical to the determination of invasive behavior for this cell type. The parent and mutant cells expressed similar zymographic profiles of metalloproteinase enzymes. The absence of TIMP allows the expression of metalloproteinase activity to predominate, and the invasive behavior is enhanced. Enzymes and inhibitors are frequently observed to be synthesized by the same cell population. Co-expression of these molecules would appear to be a futile process unless they constitute components of an equilibrium that can be tilted in either direction. Others have also suggested that TIMP is a component of an enzyme/inhibitor equilibrium. For example, in developing salivary gland explants in culture, the cleft formation was decreased or increased by the addition of collagenase or TIMP, respectively, suggesting that the remodeling of collagen is critical to gland morphology (Nakaniski et al., 1986). Transformation of endothelial cells with middle T oncogene changes the morphology of cultures from capillary-like structures to large cystic structures in fibrin gels; this process can be directly related to the balance of expression of the serine proteinase urokinase-type plasminogen activator and its inhibitors (Montesano et al., 1990). The eruption of the oocyte is facilitated by metalloproteinases, and the subsequent formation of the corpus luteum is



**Figure 8.** Expression of intermediate filament proteins in the total cell population. The total population of ES cells, differentiated for 4 d, was stained with antibodies to the intermediate filament proteins for comparison with the invasive cell population. Cells shown are TNIL cells; results with D3 cells were identical. The phase-contrast (*ph*) morphology and an intermediate filament stain are shown for (A) vimentin (*VIM*) and (B) cytokeratin (*CK*).

accompanied by the production of large amounts of TIMP protein, probably to protect the ovarian tissue from further lysis (Nomura et al., 1989; Smith and Moore, 1991; Alexander, C. M., M. Flannery, and Z. Werb, unpublished data). The invasive process of mammalian implantation, which depends on the metalloproteinase invadolysin (92 kD) (Librach et al., 1991; Behrendtsen et al., 1992), is probably limited by the expression of TIMP in decidual tissue (Nomura et al., 1989; Behrendtsen, O., M. Flannery, C. A. Alexander, and Z. Werb, unpublished observations). Mammary gland involution is dependent on metalloproteinase expression, and the newly weaned gland can be prevented from involuting using slow-release implants containing rTIMP protein (Talhok et al., 1992). Both enzymes and inhibitors have been shown to be under exquisite transcriptional control from growth factors, tumor promoters, and stress stimuli (Alexander and Werb, 1989; Matrisian and Hogan, 1990). Any of these reagents may change the balance to favor invasion of a specific cell type and lysis of the extracellular matrix, or to generate an environment that decreases cell motility and invasiveness or indeed angiogenesis, metastatic tumorigenesis (Albini et al., 1991; deClerck et al., 1991) or some other invasive process. Transcriptional control and inhibition are not the only

mechanisms of metalloproteinase control: also critical are the metalloproteinase activators. The physiologic source of enzyme activation remains to be established, especially with respect to the gelatinases. The urokinase-type plasminogen activator (or proteinase-dependent) cascade established for stromelysin and collagenase *in vitro* may or may not be relevant *in vivo* (Reich et al., 1988; for discussion, see Behrendtsen et al., 1992).

We have not yet identified which is the invasive metalloproteinase(s) which is controlled by TIMP inhibition for these cells in this invasion assay. One specific enzyme may be responsible for the invasion phenotype, or the lytic capacity may reflect the total enzyme quotient in the enzyme/inhibitor equilibrium.

### *The Invasive Cell Population: What Are They?*

The cells that show the TIMP-1-dependent invasive reaction described here underwent an ordered and reproducible transition of phenotype. The appearance of the three germ layers in the embryoid bodies derived by differentiating ES cells in suspension would suggest that the differentiation process *in vitro* is not a random cellular determination but does in fact mimic many of the changes that occur during normal development. Monolayer tissue culture conditions in the presence of a high concentration of RA would be unlikely to recapitulate the physiologic inductive stimuli in the normal embryo. However, the cellular phenotype evolving *in vitro* can be characterized by some molecular markers.

Intermediate filament expression is a powerful marker of differentiated phenotype (Lane and Alexander, 1990). The 4-d-old undifferentiated inner cell mass, or indeed the equivalent ES cells in culture, do not express any of the filament proteins so far identified. All of the early cell types with restricted potential (trophoblast, and then endoderm and ectoderm) synthesize cytokeratin networks (Jackson et al., 1980, 1981). As endoderm delaminates from trophoblast to generate parietal endoderm, vimentin is induced and is co-expressed with the simple cytokeratins (Lane et al., 1983). Primitive mesenchyme expresses only vimentin (Franke et al., 1982); there may be a temporary phase of co-expression with cytokeratin until the keratin network is diluted out. Intermediate filament proteins can be clearly and unambiguously stained by immunofluorescent techniques. This is important for ES cells, which contain high background levels of biotin and peroxidase that can confound the use of *in situ* localization techniques.

The overt morphologic differentiation of ES cells is accompanied by the elaboration of a cytokeratin network. Our results showed that almost all of the total 4-d-old differentiated cell population had extensive cytokeratin networks. Using morphologic criteria, we suggest that the remaining 9% of the population of cytokeratin-negative cells were residual undifferentiated ES cells. One half of the total cells expressed vimentin; therefore, a significant proportion of cells co-expressed both filament subtypes. The invasive population was stained *in situ* on the undersides of the Transwell filters; for these cells, most expressed vimentin and only one third expressed cytokeratin. These cytokeratin networks were also less extensive than those typical of the total cell population, suggesting their ongoing dissolution. There was thus a clear shift to a mesenchymal phenotype in these cells.

The invasive reaction was transient. At 2 or 3 d after initiation of differentiation, the cells were not invasive in our as-

say. At 8 d after, the cells were also not invasive; therefore, there is a window of invasiveness within this period that covers the 4-d timepoint. The primary mesoderm that ingresses in initiate gastrulation in the mouse is also transiently invasive. Beginning at day 6.5 post coitum, these mesodermal cells invade and migrate through the basement membrane that divides the ectodermal and endodermal compartments. This is a typical basement membrane that contains many of the characterized components of the EHS matrix (e.g., laminin, type IV collagen). Mesoderm continues to be formed until day 11 of mouse embryogenesis. The primary mesoderm, which ingresses during primitive-streak formation in chick, has been shown to be invasive in an assay that closely resembles the one used in this study (Sanders and Prasad, 1989). Medial explants of epiblast invaded a gel of reconstituted basement membrane, migrating as individual mesenchymal cells and leaving a trail of gel disruption. Explants of other regions of epiblast not evolving into mesoderm did not show the same invasive characteristics. These strands of evidence are suggestive of the evolution of a population of primary mesodermal cells in cultures differentiated by this protocol. We are investigating further the functional and molecular characteristics of this population in vitro.

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