American Journal of Pathology, Vol. 157, No. 6, December 2000

Short Communication

Matrix Metalloproteinase 9 Promoter Activity Is Induced Coincident with Invasion during Tumor Progression

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Matrix metalloproteinase 9 (MMP-9, also known as gelatinase B or 92-kd Type IV collagenase) is overexpressed in many human and murine cancers. We induced carcinomas in mice carrying a transgene that links the MMP-9 promoter to the reporter β-galactosidase so that activation of the MMP-9 promoter would be indicated by β-galactosidase. Mammary carcinomas were induced by mating the MMP-9 promoter reporter transgenic mice with mice carrying a transgene for murine mammary tumor virus promoter linked to polyoma middle T antigen, a transgene that leads to rapid development of mammary tumors in female mice. None of the hyperplastic mammary glands and none of the carcinomas *in situ* expressed β-galactosidase. However, all invasive tumors had evidence of β-galactosidase expression. In addition to the breast carcinomas, a malignant teratoma in a female and a papillary adenocarcinoma in the pelvic region of a male arose and were also β-galactosidase positive. We also induced skin tumors in the mice with the MMP-9 reporter transgene with 7, 12-dimethylbenz[*a*]anthracene (DMBA) treatment followed by phorbol 12 myristate 13-acetate (TPA). None of the papillomas or *in situ* carcinomas showed any β-galactosidase expression, but expression was seen in invasive carcinoma. Although normal skin epithelial cells did not express β-galactosidase, we did find staining in a few cells at the duct of the sebaceous gland at the base of the hair follicles. The MMP-9 reporter transgene did not lead to expression in the alveolar macrophages, confirming that additional upstream sequences are required for expression in macrophages. These experiments have revealed that MMP-9 promoter activity is induced coincident with invasion during tumor progression. Furthermore, this indicates that the more proximal upstream elements of the promoter are sufficient for MMP-9 transcription during tumor progression. ***(Am J Pathol 2000, 157:1777–1783)***

Carcinogenesis is a multistep process, often with early proliferative lesions characterized by distinctive histological patterns. With time there is often increased proliferation accompanied by altered morphology. Those lesions with nuclear dysplasia, but without evidence of invasion, sometimes termed carcinoma *in situ*, precede the development of frank carcinoma in which invasion can be detected. Tumor progression can be modeled in mice by the introduction of oncogenes into specific tissues using transgenes technology and/or recombinant knockout technology, and also by carcinogenesis. For example, expression of the polyoma middle T gene, the transforming gene of polyoma virus specifically induced in breast tissue by the murine mammary tumor virus promoter, leads to widespread proliferation or hyperplasia of the mammary epithelia early in development and is later accompanied by frequent development of malignant, invasive mammary adenocarcinomas. When these carcinomas reach a large size, macroscopic metastases can develop. Thus, during the neonatal period, benign hyperplasia of the mammary ducts is seen, followed by progression to malignancy.[1–3] Similarly, in skin carcinogenesis using the initiator-promoter methods, a mutagen is first applied to the skin, followed by frequent application of a

Supported by National Institutes of Health grant RO1 NCI CA-46830 (to R. J. M). W. J. M. is supported by a Medical Research Council of Canada Scientist Award.

Accepted for publication September 8, 2000.

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tumor promoter. Either treatment in itself is insufficient to lead to carcinoma formation, but together, in the order of mutagen followed by promoter, they lead to the frequent generation of benign skin proliferations or papillomas. Some of these papillomas then progress to invasive carcinoma.[4] We have used these models to assess alterations in the promoter activity for MMP-9.

Matrix metalloproteinase 9 (MMP-9) is a member of the family of matrix metalloproteinases.[5] Like other metalloproteinases, MMP-9 has a catalytic region that includes a putative metal binding domain. It is secreted as a latent proenzyme and is frequently isolated in a complex with its natural inhibitor, tissue inhibitor of metalloproteinase-1 (TIMP-1). An inhibitory propeptide must be cleaved to release an active enzyme. There is some evidence that the secreted molecule can bind to the surface of cells via CD44 or a chain of Type IV collagen.[6,7] This binding may be critical for its biological action. MMP-9 has been shown to be required for metastasis by both murine prostate carcinoma cells and transformed rat embryo fibroblasts.[8,9]

MMP-9 is induced in tissue culture cells by a variety of cytokines and oncogenes.[10] This induction is strongly based on transcriptional mechanisms. The 5′ upstream region of MMP-9 has been cloned from the human, rabbit, and murine genome and in each case directs transcription both constitutively and after induction by TPA or other cytokines.[10–13] The MMP-9 region upstream from each of these species is highly similar with identical transcriptional factor binding consensus sites including a proximal activated protein-1 (AP-1) site that is necessary, but not sufficient, for consitituitive expression or for TPA or tumor necrosis factor-α (TNF-α) induction.[11] This element is also required for v-src-activated transcription, but, surprisingly, is not required for transcription in SK-N-SH cells.[14,15] There is a GT-rich region that also makes a significant contribution to transcriptional control upregulated by src, ras, or in SK-N-SH cells.[11,14–19] The MMP-9 promoter from each species also contains a more distal activator protein-1 (AP-1) site, a nuclear factor κB (NFκB), an ets, and SP-1 recognition sites, all of which contribute to the activity of this promoter and its stimulation by the ras oncogene or TPA. The NFκB site is required for stimulation of MMP-9 transcription by cytokines in fibroblasts.[20] The ets site was also required for maximal stimulation by ras, TNF, cell contact, and epidermal growth factor (EGF).[11,16–19] Fini et al have identified activator protein-2 (AP-2) as a critical motif in the rat promoter for induction in wound healing.[13]

The MMP-9 promoter linked to the reporter gene β-galactosidase has been used to generate transgenic mice.[12,21] Fini et al used a 510-bp upstream fragment from the rabbit promoter to generate such a mouse. The promoter fragment they used was sufficient to drive β-galactosidase expression during development, with strong staining at the endochondrial plates during bone development and in the ventricular lining and developing neuroblasts of the central nervous system in a temporal pattern similar to that seen with *in situ* hybridization. This transgenic mouse also showed the expected pattern of MMP-9 expression during wound healing, with the migrating epithelium showing significant β-galactosidase expression.[21] This fragment of the promoter may not be sufficient to direct all expression during development. Munaut et al[12] found that the murine region homologous to that of the rabbit used by Fini et al failed to stimulate β-galactosidase expression in alveolar macrophages, cells of which a subset usually show very strong MMP-9 expression. Munaut et al also failed to see expression in osteoclasts. A 2.8-kb upstream fragment, however, led to expression in alveolar macrophages and osteoclasts. Thus, the rabbit promoter recapitulates much, but perhaps not all, of the expression expected to be directed by an MMP-9 promoter.

Materials and Methods

Mice, Breeding, and Tumor Development

Mice bearing the rabbit MMP-9 promoter (from −522 to +12) linked to the reporter β-galactosidase were developed and described by Fini et al (derived from founder 3445).[21] This founder had identical patterns of expession to a second founder, but overall levels of expression were higher. Hence, we chose to use this strain for these experiments.[21] Mice bearing the transgene linking the promoter from the mouse mammary tumor virus to the oncogene polyoma virus middle T antigen were developed and characterized by Muller's lab.[1–3] Female mice homozygous for the rabbit MMP-9 promoter (from −522 to +12) linked to the reporter β-galactosidase were mated to males homozygous for murine mammary tumor virus (MMTV) polyoma middle T antigen. All of the resultant offspring were expected to carry both transgenes. Presence of the β-galactosidase transgene was confirmed in all offspring by polymerase chain reaction of DNA extracted from the tails of 2-week-old mice. (Primers were 5′ACTCGGCGTTTCATCTGTGG and 5′ AGCGACATCCAGAGGCACTT, which yield a characteristic 1579-bp band.) All animals were sacrificed as soon as or before they developed tumors 3 cm in diameter.

Mice with the MMP-9 promoter linked to the β-galactosidase reporter were maintained by mating the homozygous mice to each other. Presence of the transgene was confirmed by polymerase chain reaction analysis for β-galactosidase and was found in all offspring as expected.

Carcinogenesis

Skin carcinogenesis by application of initiator and promoter was performed on 7 mice with the MMP-9 β-galactosidase transgene.[22] Fifty micrograms of 7,12-dimethylbenz[*a*]anthracene (DMBA) in 50 μl acetone were applied to the shaved skin of each 4- to 8-week-old mouse. Phorbol 12 myristate 13-acetate (TPA) (20 nmol) in 200 μl acetone was then applied after 10 days and twice weekly for 20 to 25 weeks. One animal died from no apparent cause without any visible tumors. Multiple papillomas were observed on 3 mice after 8 to 12 weeks and a carcinoma was observed in a fourth mouse. The remaining 2 animals had no visible tumors. Animals were sacrificed at 16 weeks.

Table 1. Histological Analysis of Breast Tissue from Mice Transgenic for MMTV-Polyoma Middle T and MMP-9 Promoter-β-Galactosidase

Tissue type	Number with β-gal staining/number of tumors examined	Number of mice
Normal ducts	0/2	2
Hyperplasia	0/3	2
Carcinoma *in situ* (without invasion)	0/1	1
Carcinoma *in situ* (in tumors with invasion)	0/3	2
Carcinoma (invasive)	12/12	3

Breast tissue from the indicated number of mice transgenic for MMTV-polyoma middle T and MMP-9 promoter-β-galactosidase were examined for β-galactosidase expression. Representative sections are shown in Figure 1. The normal ducts were from 2-week-old male mice and the hyperplastic ducts from 2-week-old females. The carcinomas were taken from mice after at least one tumor reached 3 cm in size. All mice with tumors had multiple tumors.

Tissue Evaluation

After sacrifice, mice were immediately dissected. Tissues were harvested and frozen in OCT (Fisher, Trenton, NJ) on dry ice. Frozen sections (10 μm) were cut on a cryostat. β-Galactosidase was visualized using the staining kit and protocol from Specialty Media (Phillipsburg, NJ). In brief, air-dried sections were washed in phosphate buffered saline including Ca^{2+} and Mg^{2+}, and fixed in ice-cold 2% (v/v) formaldehyde and 0.2% (v/v) glutaraldehyde for 1 hour on ice. After washing, X-gal solution was placed on the section at 37°C in the dark overnight. Sections were then fixed in 10% formalin on ice for 10 minutes, washed, and counterstained in 2% hematoxylin for approximately 10 seconds. After washing in H_2O, the sections were mounted in Permount. Skin sections were included in each batch for positive controls.

Results

Tumors Induced in MMTV-Polyoma Middle T Transgenic Mice

Female mice homozygous for the MMP-9 promoter β-galactosidase transgene were mated to male mice bearing the MMTV polyoma middle T trangene. Mice resulting from this cross were sacrificed at 2 weeks of age or after development of tumors from 1 to 3 cm in diameter. Tissues were frozen and stained for β-galactosidase activity using an X-gal-based method that results in a light blue stain. Females have hyperplasia of the mammary ducts at birth. Despite extensive hyperplasia, no β-galactosidase was evident in these ducts or the adjacent fat and stromal tissue (Table 1 and Figure 1B). Male mice at birth have histologically normal ducts; these were also negative for β-galactosidase (Table 1 and Figure 1A). The tumors were tested for β-galactosidase activity. Only one carcinoma *in situ* without any evidence of invasion was found. There was no staining in this tumor. In addition, 12 adenocarcinomas were sectioned. Three of these tumors had distinct areas of intraductal carcinoma as well as areas with invasive carcinoma. These tumors had histologies similar to those previously described for MMTV-polyoma middle T-induced breast carcinomas.[2] The areas of intraductal carcinoma or carcinoma *in situ* had no staining (Figure 1C). In all 12, the areas of invasive tumor had β-galactosidase staining. Only a fraction of the cells in the invasive areas were positive; not all tumor cells were positive. Thus, early neoplastic lesions of the breast, hyperplasia, and carcinoma *in situ* did not show evidence of activation of the MMP-9 promoter, but invasive lesions all contained some cells with MMP-9 promoter activity (Figure 1D).

In addition to breast carcinoma, other types of neoplasms were found in these transgenic mice. One female had bilateral ovarian carcinomas with histopathology consistent with malignant teratocarcinoma. This mouse did not have breast carcinoma. Many of the malignant cells in these teratocarcinomas were positive for β-galactosidase. Interestingly, the bony and cartilaginous areas were negative. One male had a carcinoma of the submandibular gland with histology consistent with a mucoepidermoid carcinoma. There was sporadic staining in this tumor as well (not shown). Another male mouse had a papillary serous cystadenocarcinoma in the testicular region that also stained positive (Figure 1I).

Skin Carcinogenesis

To extend these observations indicating that the MMP-9 promoter is activated during the stages of tumor progression associated with invasion, we examined the expression of β-galactosidase in skin tumors induced by intitiation-promotion with DMBA and TPA in mice bearing the MMP-9 β-galactosidase transgene. Skin from an untreated area is shown in Figure 1E. The epithelium and subcutaneous tissue were negative. Cells at the neck of the duct connecting the sebaceous glands associated with hair follicles to the hair shaft consistently stained. Skin from mice treated with TPA was also negative, with the exception of cells in the sebaceous glands (not shown). Seven papillomas were examined. Four showed dysplasia suggestive of carcinoma *in situ*. None of these papillomas or carcinomas *in situ* had β-galactosidase staining (Figure 1, F and G, and Table 2). Only 1 animal developed invasive carcinoma. This carcinoma had extensive staining, but again, only some, not all, of the tumor cells were positive (Figure 1H).

Discussion

In this study activation of the 510-b rabbit MMP-9 promoter was found to occur coincident with invasion during mammary carcinogenesis. Similarly, the MMP-9 promoter was inactive in benign papilloma and in dysplastic papillomas, but was activated in a squamous cell carcinoma. These results indicate that a new pattern of transcriptional activation emerges during conversion from benign to malignant lesions. One gene whose expression is activated by this switch is MMP-9.

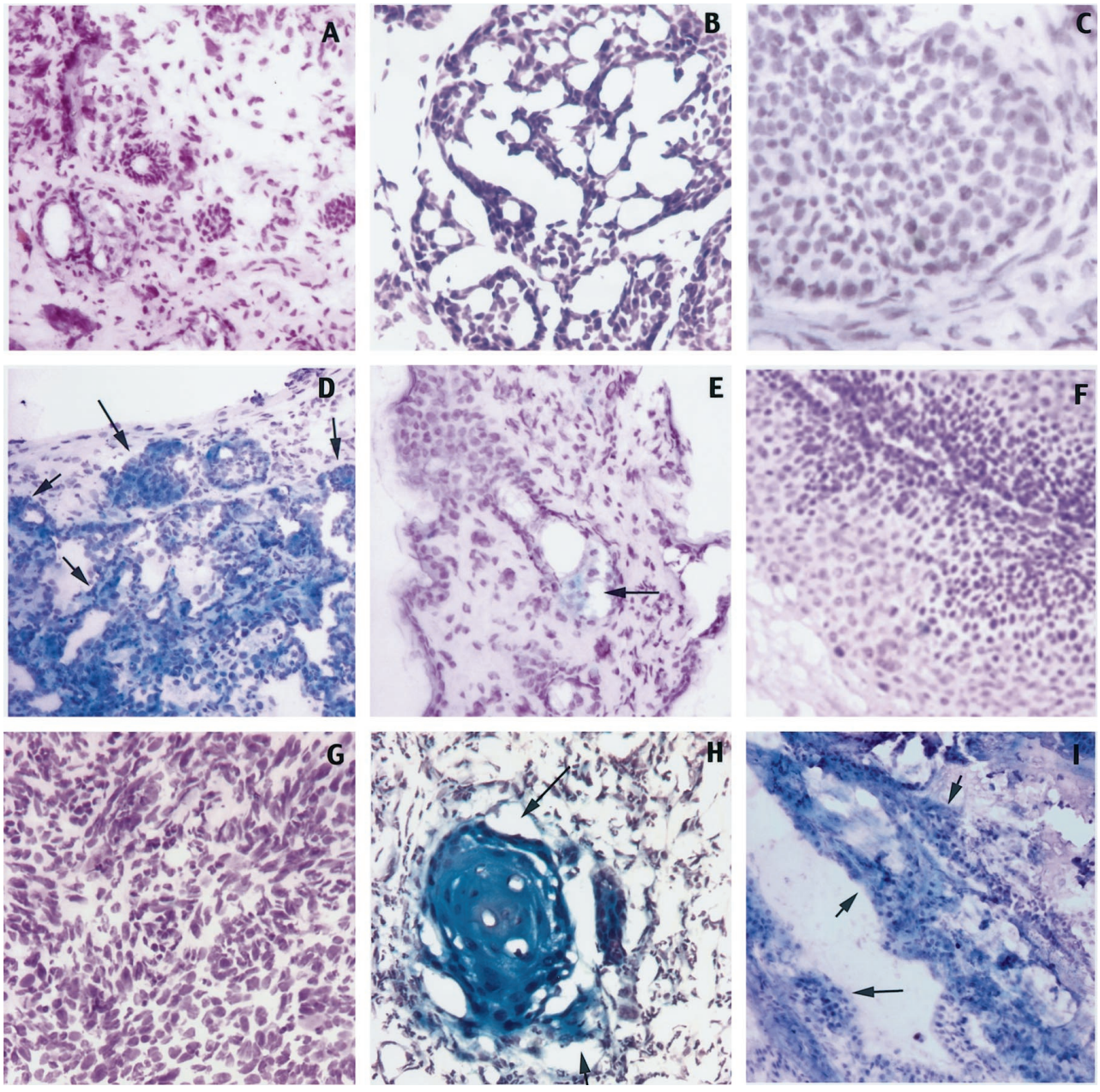

Figure 1. β-Galactosidase staining in tissues from MMP-9 promoter-reporter mice. All section shown are stained for β-galactosidase activity and counterstained with hematoxylin. **A-D:** Sections from breast tissue of mice transgenic for MMTV-polyoma middle T and MMP-9 promoter-β-galactosidase. **A:** A section from a 2-week-old male mouse breast. **B:** A section from a 2-week-old female mouse. **C:** A higher power view of a section from an area of *in situ* carcinoma from a female mouse breast tumor. **D:** An area of invasive carcinoma from a female breast carcinoma. **E-H:** Sections of skin. **E:** Normal untreated skin. **F:** A section from a papilloma induced by skin carcinogenesis. **G:** Higher power view of a dysplastic area from a papilloma. **H:** Invasive squamous cell carcinoma. **I:** Section from adenocarcinoma that developed spontaneously in the pelvic region of a male mouse.

In this study, we used two murine models for tumor progression. One, a model of squamous cell carcinoma of the skin based on carcinogenesis, results in activity of the MMP-9 promoter at the stage when invasive carcinoma can first be seen. Squamous cell carcinomas of the skin as well as at other sites in patients can be shown to express MMP-9 mRNA by *in situ* hybridization.[23–25] MMP-9 expression is also correlated with invasion and poorer prognosis in squamous cell carcinoma.[26–28] Its expression tends to be elevated at the invasive fronts, and this can be modeled in three-dimensional tissue culture.[29] In melanoma, a tumor type with a clear histological distinction between different stages of tumor progression, cells from vertical growth phase melanomas, the most invasive and aggressive stage of melanoma, express MMP-9 in culture and after stimulation with transforming growth factor-β, but cells from the earlier stage radial growth phase do not.[30] We also used a model of breast cancer. The literature is more contradictory regarding expression patterns of MMP-9 in human breast carcinoma. Although some studies find increased expression in breast carcinonomas, others have failed to

Table 2. β-Galactosidase in Skin from Mice Transgenic for MMP-9 Promoter-β-Galactosidase after Skin Carcinogenesis

Tissue type	Number with β-gal staining/number of tumors examined	Number of mice
Papilloma	0/3	2
Papilloma with dysplasia	0/4	2
Squamous cell carcinoma	1/1	1

After skin carcinogenesis in mice with the MMP-9 promoter-β-galactosidase transgene, animals were sacrificed and tumors examined for β-galactosidase. Details of the carcinogenesis are in Materials and Methods.

find any difference.[31–38] However, in several instances, manipulations that led to increased invasiveness or metastasis by breast carcinoma cells were associated with increased expression of MMP-9.[39–42] Studies in patient material tend to be difficult because immunohistochemical identification of secreted proteins may not be reliable and the sensitivity of *in situ* hybridization is variable. Nonetheless, the increase in promoter activity found here may not translate into increased MMP-9 in actual tumors. These experiments would suggest that there is a consistent change in transcriptional activity of an MMP-9 promoter during tumor progression.

The upstream elements of the MMP-9 promoter include two AP-1 sites, one adjacent to the MMP-9 transcript start site and the other more distal. In addition to AP-1, the MMP-9 promoters all contain AP-2, ets, NFκB, and Sp-1 recognition consensus elements. Each of these elements adds incrementally to MMP-9 expression in cells constitutively expressing MMP-9.[11,14,17,18] There is some evidence of the potential involvement of many of these transcription factors in tumor progression. Saez et al found that treatment of the skin of mice genetically deficient in c-*fos* with an initiator-promoter protocol for induction of skin carcinogenesis resulted in papilloma formation that failed to progress to invasion or carcinoma.[43] Since AP-1 is formed by heterodimers of *fos* and *jun* or *jun* homodimers, the absence of c-*fos* would disrupt many AP-1 complexes. This result is more complicated, however, because transgenic mice overexpressing a dominant negative inhibitor of *jun* in the skin fail to develop even papillomas after initiation and promotion.[44] Ets-1, one of a large family of proteins binding to the ets consensus binding site, has been associated with transformation and is required for transformation. Mice with a single targeted deficient allele of ets-2 show decreased progression in MMTV-polyoma middle T-induced tumors.[45] PEA-3, another transcription factor that binds to the ets consensus binding site, has been shown by Trimble et al to be overexpressed in the mammary carcinomas that result from expression of the MMTV-polyoma middle T transgene, making it an attractive candidate for involvement in the induction of MMP-9 promoter activity during tumor progression.[46] There are several reports implicating enhanced NFκB expression in carcinomas including breast carcinoma and squamous cell carcinoma of the skin.[47,48] In contrast, there is evidence that AP-2 is down-regulated during tumor progression in melanaoma and that the presence of AP-2 activity facilitates the expression of E-cadherin, a gene whose down-regulation is frequently required for invasion and metastasis. Furthermore, down-regulation of E-cadherin results in up-regulation of MMP-9 in squamous carcinoma cell lines.[49] Interestingly, up-regulation of AP-2 has been suggested to be the critical factor regulating transcriptional activity of MMP-9 during wound healing in the cornea.[21] Whether any of these transcriptional elements are activated during tumor progression and whether their enhanced activity leads to MMP-9 expression remain to be determined.

In addition to the elements indicated above, additional upstream regions have important roles in regulation of MMP-9 activity. The region we have used, 510 bp upstream from the start site of the rabbit gene, does not direct expression in alveolar macrophages, cells that frequently express MMP-9. Munaut et al obtained expression in macrophages with a 2.8kb upstream fragment, but not with a smaller fragment homologous to the promoter used here.[12] This smaller fragment directs other normal expression, in macrophages in the spleen, during wound healing in murine corneas, and in the bone, cartilage, and nervous system during development.[21]

We have also observed expression in the cells at the junction of the sebaceous gland and the hair shaft in the hair follicles, recalling the interesting association between decreased MMP-9 expression and aberrant hair formation in mice engineered to have a genetic deficiency of ets-2.[50]

In this study, we found that the smaller fragment was activated coincident with tumors developing the ability to invade. This finding indicates that there is an alteration in the transcriptional regulation of cells as they become invasive and that the shorter MMP-9 promoter can monitor this change. Whether other elements further upstream will alter this expression pattern remains to be determined. Nonetheless, the requirement of MMP-9 for metastasis in other systems is consistent with the temporal pattern of MMP-9 expression during tumor progression.

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