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# Paracrine growth regulation of human colon carcinoma organ-specific metastasis

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**Summary**

The process of cancer metastasis consists of a series of steps resulting in the spread of malignant cells beyond the site of origin and formation of metastases in distant organs. The outcome of this nonrandom process depends, in part, on the interaction of unique tumor cells with a compatible organ microenvironment. The molecular basis of the intrinsic capacity of distinct malignant cells to colonize specific organs and the degree to which host factors influence this process is under intense investigation. Biological analyses of human colon carcinoma tumors obtained from surgical specimens and implanted orthotopically into athymic nude mice revealed that these tumors are heterogeneous for metastatic properties. Moreover, recent evidence using this model suggest that whereas nonmetastatic and highly metastatic cells can grow at local sites, growth in the secondary liver-specific site was associated only with highly metastatic HCC cells. These cells also respond to mitogenic signals produced by damaged normal tissues, suggesting that physiological signals can be utilized by neoplastic cells. Molecular characterization of highly metastatic HCC cells selected in the nude mouse model as well as *in situ* mRNA hybridization of archival HCC surgical specimens for specific growth factor receptors correlated with the malignant cell's ability to respond to organ-specific growth factors. This article will focus on biological and molecular evidence supporting the hypothesis that organ-derived, paracrine growth factors regulate the site-specific growth of receptive malignant cells that possess the appropriate receptors.

## Introduction

To produce clinically relevant distant metastases, the metastatic cell must exhibit a complex phenotype favorable to the survival of subpopulations of metastatic cells preexisting in the primary tumor [1–4]. Initiation of metastasis begins with the invasion of the surrounding normal stroma either by single tumor cells with increased motility or by groups of cells from the primary tumor. Once the invading cells penetrate the vascular or lymphatic channels, they may grow there, or a single cell or clumps of cells may detach and be transported within the circulatory system. As an embolus, it must survive the host's immune and nonimmune defenses and the turbulence of the circulation, arrest in the capillary bed of compatible organs, extravasate into the organ parenchyma, proliferate, and establish a micrometastasis. Growth of these small tumor lesions requires the development of a vascular supply and continuous evasion of host defense cells. Failure to complete one or more steps of the process eliminates the cells (Fig. 1). Examples include failure to arrest in the capillary bed or failure to grow progressively in a distant organ's parenchyma possibly due to host factor transcriptional or translational

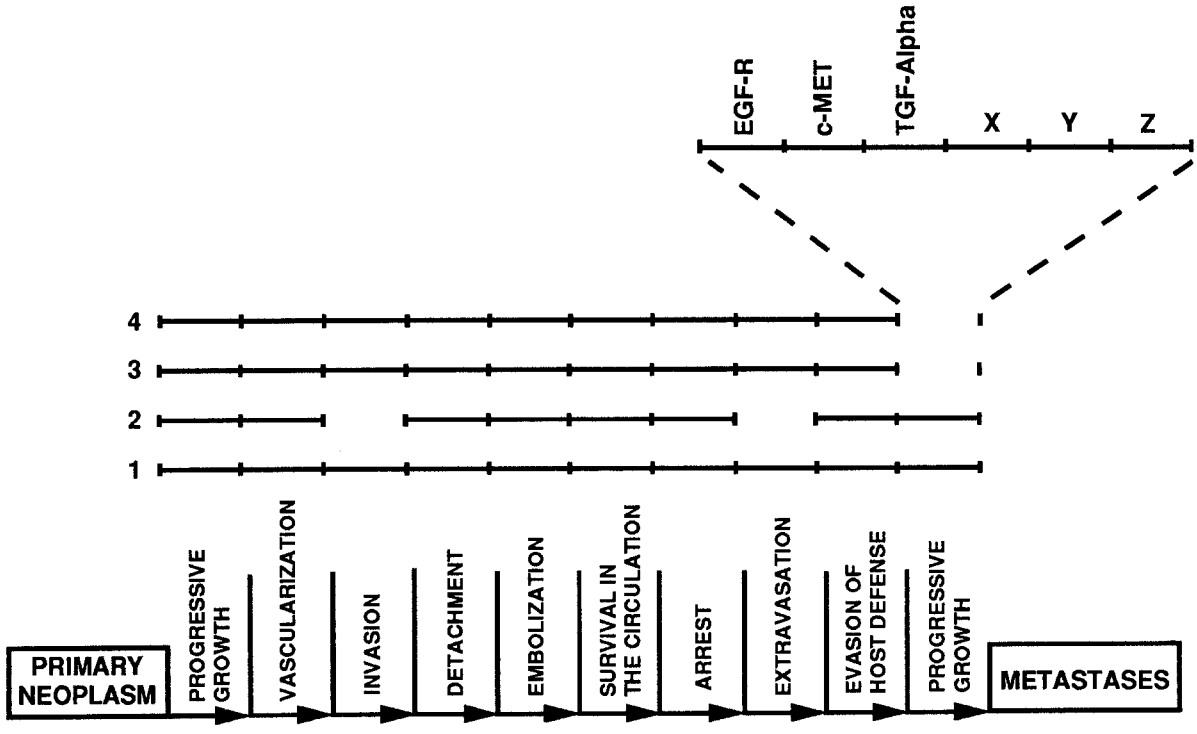


*Fig. 1.* Serial steps in the pathogenesis of tumor cell metastasis. Tumor cells must complete every step in the process to produce clinically relevant metastases. These steps include the progressive growth and vascularization of the primary neoplasm, tumor cell invasion of the surrounding tissue and detachment from the primary tumor, embolization and survival in the circulation, arrest and extravasation in the target tissue, evasion of host defenses, and finally proliferation at the secondary site of implantation [1–8]. Failure to complete even one step eliminates the cell. Examples are 1) metastasis-competent cell, 2) deficiency in invasion or extravasation step, 3) deficiency in progressive growth ability at the metastatic site due to, 4) putative alterations in the transcriptional or translational activation or deactivation of multiple growth factor receptor, growth factor, or unknown genes [X, Y, & Z]. Epidermal growth factor receptor (EGF-R), c-met (hepatocyte growth factor receptor), transforming growth factor alpha (TGF-α). (Modified from Fidler and Radinsky [5].)

gene regulation of tumor cell growth factor receptors (GF-Rs), growth factors (GFs), or uncharacterized gene products [2, 5, 6] (Fig. 1).

There is now wide acceptance that many malignant tumors contain heterogeneous subpopulations of cells [1–8]. This heterogeneity is exhibited in a wide range of genetic, biochemical, immunological, and biological characteristics such as growth rate, antigenic and immunogenic status, cell surface receptors and products, enzymes, karyotypes, cell morphologies, invasiveness, and metastatic potential. Molecular marker analyses have shown a gradual overgrowth or 'clonal dominance' of particular primary tumors by subpopulations of cells that are the forerunners of metastases [8]. This clonal evolution and progression to malignancy is influenced by both the generation of tumor cell variants and host selection pressures such as those observed in experimental studies where implantation of heterogeneous human neoplasms into the anatomically correct (orthotopic) organ influences the selection of subpopulations and their metastatic capacity [2, 9, 10]. An increased understanding of the mechanisms mediating the development of biologic heterogeneity in primary cancers and in the process of metastasis is a primary goal of cancer research. From it more effective therapies for metastasis can be developed.

In humans and in experimental systems, numerous examples exist in which particular malignant tumors metastasize to specific organs [3, 4, 11]. As early as 1889, Paget, having studied the autopsy records of breast cancer patients, proposed that the growth of metastases is due to the specific interac-

tion of particular tumor cells (the 'seed') with particular organ environments (the 'soil') [12]. This hypothesis, supported both experimentally [3] and clinically [13], may explain metastatic colonization patterns that cannot be due solely to mechanical lodgement and anatomical considerations [14]. In a review of secondary site preferences of malignant neoplasms, Sugarbaker concluded that common regional metastatic involvements could be attributed to anatomical or mechanical considerations such as efferent venous circulation or lymphatic drainage to regional lymph nodes, but that distant organ colonization by metastatic cells from numerous types of cancers established their own patterns of site specifically that depend less on anatomic and mechanical mechanisms [15]. The microenvironment of each organ can influence the implantation, invasion, survival, and growth of particular tumor cells [2, 10]. The individual importance of each step of the metastatic process can vary among different tumor systems, with the outcome of metastasis influenced by both the intrinsic properties of the tumor cell and the influence of host-specific factors. Therefore, the successful metastatic cell must be viewed as a cell receptive to its environment [2].

The mechanistic basis of a metastatic cell's ability to proliferate in the parenchyma of some organs and develop organ-specific metastases remains largely unknown. Signals from paracrine or autocrine pathways, alone or in combination, could regulate tumor cell proliferation with the eventual outcome dependent on the net balance of stimulatory and inhibitory factors. This article will focus on biological and molecular mechanisms involved in the interaction between specific tumor cells and a compatible organ environment in terms of growth factors and their receptors participating in paracrine and autocrine signal transduction pathways. I describe recent experimental evidence obtained from metastatic models of human colon carcinoma (HCC) and from analysis of archival HCC surgical specimens supporting the hypothesis that site-specific metastasis involves the proliferation of tumor cells differentially expressing GF receptors and that local paracrine GFs or organ-repair factors may regulate the growth of receptive metastatic cells.

## Environmental influences on the organ-specific metastasis of HCC

Two criteria must be met in the design of an appropriate model for human cancer metastasis. It must use metastatic cells (the 'seed'), and these cells must grow in a relevant organ environment (the 'soil') [2]. Early studies from Fidler's laboratory of four HCC's derived from surgical specimens, three from hepatic metastases and one from a lymph node metastasis, showed that subcutaneous inoculaton in nude mice resulted in local tumor growth, but in only one of more than 200 mice was distant metastasis observed [16, 17]. Other investigators have reported similar findings following the implantation of different human tumor types into the subcutis of nude mice [18]. Furthermore, two other human tumor model systems, an HCC and a renal carcinoma, both derived from surgical specimens, were found to contain heterogeneous populations of cells with different metastatic properties. This demonstration of heterogeneity required that the cells be implanted into the anatomically correct sites in athymic nude mice (orthotopic implantation) [for reviews see 2, 17, 19]. For example, HCC cells implanted into the spleen or cecum of nude mice produced exclusively liver and lymph node metastases, whereas implantation of these same tumor cells at ectopic sites (e.g. subcutaneous or intramuscular) resulted in slow growth of primary tumors and only rarely in formation of metastases [16, 17, 19–22]. Thus, if a human tumor is biologically heterogeneous, some of its cells may possess a growth advantage, depending on whether it is transplanted to the skin, the cecum, the liver, or the kidney of nude mice. Molecular genetic tagging experiments support this statement [8, 10]. Tumors of human renal carcinoma cells grown in the orthotopic site (kidney) were all populated by the same dominant clones, and each distant metastasis retained this clonality; in contrast, renal cell tumors growing subcutaneously showed a random pattern of clonal dominance [10]. The importance of orthotopic implantation of human neoplasms is also supported by results in other human tumor model systems [for review see 2, 17] including melanoma (into the skin) [23], mammary carcinoma (into the mammary fatpad) [24], prostatic carci-

noma (into the prostate) [25], and lung cancer (into the bronchi) [26]. These studies demonstrate how important anatomical compatibility of tumor cells with the organ environment is to the design of a correct *in vivo* model for human spontaneous metastasis. These results imply that environmental factors can have a strong influence in selecting specific clonal subpopulations in a growing tumor and indicate that local organ factors produce selection pressures on tumors which results in the outgrowth of different clones with varied growth potential [2, 8, 10, 27, 28].

## Isolation of metastatic cell subpopulations from primary HCC

To distinguish the malignant potential of different stage HCC's, Fidler and colleagues studied their growth in the liver parenchyma, the most common site of HCC metastasis [29]. A reproducible bioassay of hepatic metastasis was developed whereby tumor cells from HCC surgical specimens were inoculated into the spleens of nude mice [16, 17, 20–22, 30]. From this site, tumor cells gain access to the bloodstream and then reach the liver where they proliferate into tumor colonies. The growth of HCC in the liver directly correlated with the metastatic potential of the cells, i.e., cells from surgical specimens of primary HCC classified as either modified Dukes' stage D or liver metastases produced significantly more colonies in the liver of nude mice than cells from a Dukes' stage B tumor [17, 21, 22]. These results are consistent with the hypothesis that Dukes' stage B tumors are an earlier manifestation of HCC than Dukes' stage D tumors [2]. If valid, Dukes' stage B tumors should contain few metastatic cells, whereas Dukes' stage D tumors should contain large numbers of metastatic cells capable of growth in the liver. The biological characterization of these HCC surgical specimens support this premise. Radioactive distribution analyses of both Dukes' stage B and D HCC cells demonstrated that shortly after intrasplenic injection the tumor cells reached the liver [20, 30]. Therefore, the production of HCC tumors in the livers of nude mice was determined by the ability of the HCC to *proliferate* in the liver parenchyma rather than by the ability of the cells to *reach* the liver [17, 20].

To select and isolate metastatic subpopulations of HCC cells with increasing growth potential in the liver parenchyma from heterogeneous primary HCC's, cells were derived from a surgical specimen of a Dukes' stage B2 primary HCC. These HCC cells were established in culture or injected into the subcutis, cecum, and spleen of nude mice [21]. Progressively growing tumors were then isolated and established in culture. Implantation of these four culture adapted cell lines into the cecum or spleen of nude mice produced few metastatic foci in the liver. HCC cells from these few liver metastases were expanded into culture and reinjected into the spleen of nude mice to provide a source for further cycles of selection. Importantly, with each successive *in vivo* selection cycle, the metastatic ability of the isolated and propagated cells increased. Four cycles of intrasplenic selection yielded cell lines with a very high metastatic efficiency in nude mice [21]. In analogous studies of a Dukes' stage D primary HCC, highly metastatic cell lines were isolated, but successive selection cycles for growth in the liver only slightly increased their metastatic properties [17, 21, 22]. These results demonstrate that highly metastatic cells can be selected from HCC and that orthotopic implantation of HCC cells in nude mice is a valid model for determining metastatic potential [for reviews see 2, 17, 19].

## Considerations of genetic and phenotypic instability in metastatic cells

Metastatic tumors appear to be dynamic assemblages of unstable malignant cells [6]. Nicolson recently postulated that the acquisition of the metastatic phenotype may depend on the inherent phenotypic instabilities of the tumor cells and less on the selection of rare metastatic cells composing the primary neoplasm [6]. Tumor cell instability could be due, in part, to regulation by the host environment of quantitative transcriptional or translational changes in gene expression resulting in transient alterations in the amounts of specific protein products [5]. Individual cells may therefore acquire a re-

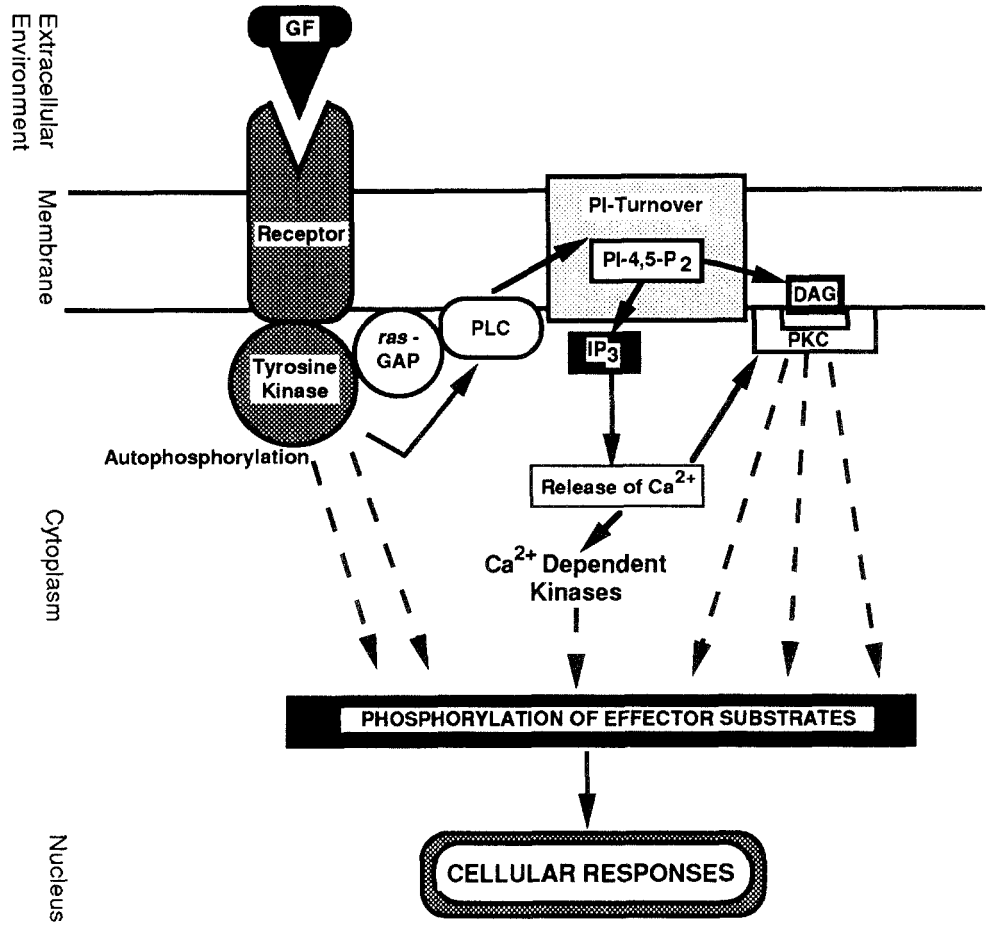


*Fig. 2.* Tyrosine kinase receptor-mediated signal transduction. The receptor diagrammed is representative of growth factor receptors with tyrosine protein kinase activity, e.g., the EGF and c-met receptors. Each receptor contains a glycosylated extracellular ligand-binding domain, a single hydrophobic transmembrane region, and a cytoplasmic tyrosine kinase catalytic domain [37, 38, 69, 72]. Following growth factor ligand binding and receptor oligomerization [69], this class of receptor autophosphorylates itself and phosphorylates and subsequently activates phospholipase C (PLC), which leads to the generation of phosphotidylinositol (PI) metabolites. Metabolites, such as inositol 1,4,5-trisphosphate (IP3), cause the release of $Ca^{2+}$ from intracellular compartments and the generation of diacylglycerol, a natural activator of $Ca^{2+}$- and phospholipid-dependent protein kinase (PKC). Interaction between the ligand and receptor also stimulates the phosphorylation of multiple effector substrates on serine and threonine residues. PKC also phosphorylates these substrates. Phosphorylation of these effector substrates, together with alterations in the ionic content of the cell, provide the signal for the specific cellular responses such as proliferation, specialized cellular functions (e.g. motility), or differentiation. (Modified from Radinsky [11].)

versible acquisition of the metastatic phenotype and once metastasis has occurred the biochemical properties that define the cell as metastatic are lost [6]. In contrast, malignant cell properties required for growth proliferation at the secondary organ-specific site (see Fig. 1) may be retained in order for survival and production of overt, clinically relevant metastases. Nowell hypothesized that cellular acquisition of successive irreversible genetic changes could result in a selective growth advantage [9], leading to the clonal expansion of more aggressive clones in the neoplasm which may ultimately metastasize [8–10, 27]. Direct experimental evidence for clonal tumor cell expansion of a rare cell carrying a specific change in a critical gene during tumor progression was demonstrated by mutation of the p53 gene which resulted in a selective growth advantage critical in the progression from low-grade to high-grade human brain tumors [31]. Thus, both irreversible genetic alterations and putative tran-

sient phenotypic properties associated with the metastatic phenotype can be feasibly studied in rare highly malignant cells sequentially selected from human neoplasms in nude mice, but rigorous comparative analyses of these cells (preferably multiple distinct clones [32, 33]) growing in culture versus at ectopic and orthotopic sites in nude mice must be accomplished. These data can then be compared with the original fresh surgical specimen (if available) or archival paraffin-embedded samples from the same patient using sensitive molecular analyses (see below). Only with stringent comparisons, can a clear association of the genetic and phenotypic properties intrinsic to the metastatic cell and the putative role of environmental regulation (*in vitro* culture, orthotopic and ectopic sites in nude mice, or the original human site) become clear.

## Organ-derived paracrine growth factors and their receptors

A modern interpretation of Paget's 'seed and soil' hypothesis for site-specific tumor growth must include interactions between receptive metastatic cells and the target organ in terms of responses to local GFs. A GF is a substance that stimulates cell proliferation and depending on the specific target cell often also promotes differentitation. A large number of growth promoting as well as growth inhibitory factors, have been identified and are known to mediate the growth of normal and neoplastic cells [11, 37]. In general, these are low molecular weight polypeptides with specific high-affinity and saturable cell surface receptors with endogenous protein tyrosine kinase activity (Fig. 2). The ubiquitous presence of GFs in a wide variety of tissues suggests that each organ may have its own growth-modulating substances. During development and tissue differentiation, organ-derived GFs act locally, either as paracrine- or autocrine-regulatory chemical messengers [37]. They continue to act as needed during adult life for tissue renewal and wound repair.

The role of particular peptide GFs produced by normal cells on the growth of organ-specific tumor cells is unclear [37]. The normal cell cycle proceeds through an orderly sequence of steps with many different control points, at each of which specific factors must be present for the cell to complete division. Evidence supporting an association between organ-derived GFs and receptive metastatic cells has been obtained, in part, from experiments on the effects of organ-conditioned medium on the growth of particular neoplastic cells. The presence of stimulatory or inhibitory factors in a particular tissue correlated with the site-specific pattern of metastasis [2–4, 6, 11]. Purification of new factors to homogeneity should lead to more definitive analysis and their putative roles in the growth regulation of organ-specific metastases. Metastatic cells may therefore proliferate in secondary organs which produce compatible GFs; that is, GFs similar to those involved in the cellular regulation of the normal tissue from which the primary tumor originated. For example, HCC tumors utilize and respond to specific GFs which regulate normal colonic epithelium. Some of these identical factors also regulate tissue renewal and repair in the liver (see below). Hence, do these same factors participate in the regulation of HCC growth at the metastatic liver-specific site?

Colonic epithelium is a rapidly proliferating organ that completely renews itself on a weekly basis [34]. Two recent reports have detected expression of transforming growth factor alpha (TGF-α) in normal colon [35, 36]. TGF-α is a secreted polypeptide that binds to the epidermal growth factor receptor (EGF-R) to produce a mitogenic stimulus [37, 38] (Fig. 2). In colonic epithelium autocrine growth stimulation by TGF-α can occur independently of malignant transformation. Markowitz and colleagues demonstrated that TGF-α and EGF-R are coexpressed in both normal and in adenomatous human colonic epithelium [36]. These results are consistent with experimental studies demonstrating that *in vivo* intravenous epidermal growth factor (EGF) is a potent mitogen for rat colonic epithelium [39]. TGF-α is therefore likely an important colonic mitogen which *in vivo* acts to stimulate proliferation of the colonic epithelium. Numerous studies have implicated TGF-α as an autocrine growth factor in solid tumors and in HCC [37, 40–42]. However, TGF-α autocrine stimulation of EGF-R in immortalized colonic epithelial cells is

*not* transforming and induces neither growth in soft agar nor tumorigenicity in nude mice [36]. Similarly, TGF-α is an autocrine growth factor in nontransformed cultured normal breast epithelium [43]. The authors of these studies suggest that normal versus malignant epithelia differ not in the existence of an autocrine loop, but in the cellular responses to such stimulation [36, 43]. Precedent exists for such altered responses to GFs and cytokines, whereby growth inhibitory polypeptides switch to growth stimulators in cells from advanced-stages of tumor progression (i.e., metastatically competent cells) [8, 28].

Hepatocyte growth factor (HGF) is another mitogen that may regulate turnover of colonic epithelium. Although HGF is the most potent *in vitro* mitogen for hepatocytes yet described, it is not only a liver-specific GF [44, 45]. This molecule has been shown to be a GF with broad specificity, stimulating a number of cells of epithelial origin [44, 45]. HGF mRNA was found in a variety of human organs and HGF protein has been located histochemically in all squamous epithelia, lining glandular epithelia, and throughout the gastrointestinal tract mucosa [44–46]. HGF was shown to be produced by stromal fibroblasts from organs including adult skin, lung, prostate, and the gastrointestinal tract [44, 45, 47, 48]. This form of HGF may represent a paracrine mediator of cellular proliferation including colonic and liver epithelium [44, 45, 48]. No mitogenic effect was observed for fibroblasts [44, 45, 48]. HGF is also identical to scatter factor, a factor named for its 'scattering' or induction of cell dissociation in tightly packed mammary epithelial cells and in its ability to increase cell mobility of a variety of epithelial cells including some cancer cells [44, 45, 49, 50]. HGF-scatter factor has also been described to be a potent chemotactic factor and has been associated with progression of carcinoma cells to the invasive phenotype [44, 51]. Collectively, these properties confer to HGF/scatter factor a potential critical role in tumor growth and metastasis.

## Wound- and liver-specific repair factors and metastasis

A mechanism for site-specific tumor growth involves the interaction between receptive metastatic cells and the organ environment, possibly modulated by local growth factors. Host factors (autocrine or paracrine) that control the processes of organ repair and regeneration are known to be organ-specific. As early as 1914, Jones and Rous observed that trauma enhanced the ability of a tissue to support the growth of cancer cells [52]. Several reports have since described the wound site as a preferred location for malignant cell proliferation in a variety of tissues and in a number of species [53–57]. For example, when MC28 sarcoma cells were injected intra-arterially into rats, tumors grew preferentially in the parts of tissue that were traumatized (i.e., colon and muscle) compared to control untraumatized areas of the same tissues [56, 58]. Furthermore, a number of investigators have reported that tumor cells inoculated intraportally showed a dramatic increase in tumor growth and incidence in mechanically traumatized or partially hepatectomized rat liver as compared to sham-operated controls [59–61]. Also, data from our laboratory indicate that liver regeneration in the nude mouse, but not nephrectomy or control surgery, stimulates the growth of HCC cells implanted subcutaneously [2]. Consistent with these observations is the appearance of factors in the peripheral blood that stimulate DNA synthesis in grafted hepatic parenchyma concomitant with the synthesis of liver DNA *in situ* [62]. These findings suggest that metastatic cells that either originate from or have affinities for growth in a particular organ can respond to physiological signals produced when homeostasis is disturbed, presumably by response mechanisms similar to those of normal cells.

Following partial hepatectomy, the liver undergoes rapid cell division, i.e., hyperplasia. This process of liver regeneration involves quantitative changes in hepatocyte gene expression [62, 63]. Recently, for example, TGF-α was described as a physiological regulator of liver regeneration by means of an autocrine mechanism [64, 65]. TGF-α production by hepatocytes may also have a paracrine role,

stimulating the proliferation of adjacent nonparenchymal cells through the EGF-R [62]. HGF is another candidate mitogen involved in liver homeostasis. This hepatic trophic factor has been demonstrated to be synthesized and secreted from liver nonparenchymal cells (Ito, endothelial and Kupffer cells), subsequent to liver damage, a rapid increase is observed [44, 45, 66]. This increase is paralleled by the down-regulation of its receptor, the c-met proto-oncogene, in hepatocytes [44, 67, 68]. The c-met receptor belongs to the tyrosine kinase family of receptors, e.g., the EGF-R [69]. These observations are consistent with the paracrine action of HGF in the growth regulation of liver (and colonic) epithelium [44]. Furthermore, TGF-β may be a component of the paracrine regulatory loop, controlling hepatocyte replication at the late stages of liver regeneration [65]. Therefore, when normal tissues such as the liver are damaged (possibly by invading tumor cells [4]), growth factors are released to stimulate normal organ tissue repair, and these known factors may also stimulate the proliferation of receptive malignant tumor cells, i.e., those that possess the appropriate receptors.

## A model for the paracrine growth regulation of HCC liver-specific metastases

A model for the paracrine growth regulation of HCC liver metastases is shown in Fig. 3. The expansion of the HCC cell into a metastatic colony may require tumor cell autocrine GFs or the ability to respond to external host cell-derived stimuli such as organ growth and differentiation factors, hormones, cell to cell contact, and interaction between cells and extracellular matrix proteins [70, 71]. By definition, HCC cells receptive to candidate stimulatory ligands such as TGF-α and HGF must express the corresponding receptors (EGF-R and c-met, respectively). Altered or overexpression of EGF-R has been reported for a variety of human carcinomas including HCC [72–74]. The exact role of EGF-R is unclear, but the more malignant the tumor the more cell surface EGF-Rs (see below) [73]. The role of c-met, the receptor for HGF is less clear. Transfection of c-met into fibroblasts conferred the competence to respond to HGF only with the activation of the mitogenic and invasive phenotypes [75]. No change in growth rate was observed [75]. Cells expressing endogenous c-met receptors such as endothelial cells, keratinocytes, and melanocytes respond to HGF by proliferation and scattering, whereas hepatocytes only proliferate [44, 45, 75]. HGF also acts as a negative growth regulator in some carcinoma cells [76]. The mechanism for the different responses to HGF is unclear. Possibilities include multiple receptor isoforms with structural differences, proteolytic processing of a protein precursor, or that the c-met receptor is linked to distinct signaling pathways dependent on the cell type or the growth and metabolic conditions of the cells [44, 45]. Nevertheless, we do not have sufficient data to establish roles for HGF and c-met in tumor progression and, specifically, in the formation and proliferation of HCC metastases. It is also very likely that the proliferation of implanted metastatic HCC cells requires the concerted action of several other GFs. Furthermore, tumor cells can release factors that the host cells (Kupffer and endothelial cells, hepatocytes or fibroblasts) respond to, resulting in a reciprocal relationship between the HCC metastatic cells and the liver microenvironment (Fig. 3) [6, 77–79]. Hence, as depicted in this model, normal homeostatic processes and those that follow damage to an organ facilitate the proliferation of normal and, in some cases, tumor cells possessing the appropriate receptors.

## Receptors on HCC cells correlating with liver-specific growth ability

The model presented in Fig. 3 for the paracrine growth regulation of metastatic HCC cells in the liver is the end result of a complex series of steps (Fig. 1). The final step is represented by the ability of the implanted cells to proliferate as a colony and to respond to GFs through the appropriate cell surface GF-Rs. We, therefore, assessed the genes encoding for GF-Rs of low- and high-metastatic HCC variants. Analyses of HCC cells from surgical specimens that differed in malignant potential (Dukes' stage B, D, or liver metastases) and adapted to *in vitro*

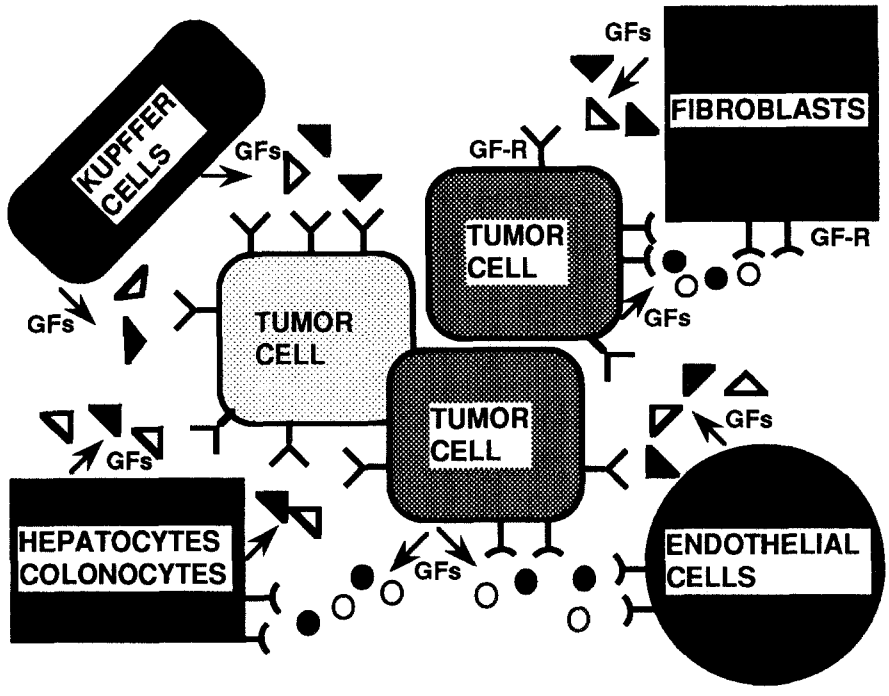


*Fig. 3.* A model for the paracrine regulation of HCC tumor cell growth at liver-specific metastases. A modern view of Paget's 'seed and soil' hypothesis [12] includes the paracrine regulation of tumor cell growth at organ-specific sites. Paracrine regulation of tumor cells can involve stimulation or inhibition by growth factors in the extracellular environment. Candidate stimulatory ligands include TGF-α, which is produced by hepatocytes in response to trauma. Recent data indicate that this physiological regulator of liver regeneration works through an autocrine loop in hepatocytes and through a paracrine mechanism in adjacent nonparenchymal cells through the epidermal growth factor receptor (EGF-R). Another candidate mitogen, hepatocyte growth factor (HGF), is synthesized and secreted from liver endothelial, Kupffer, and Ito cells, which is consistent with its paracrine action in the growth regulation of liver as well as colonic epithelium [44, 45, 66]. Furthermore after liver damage, a rapid increase in HGF production is observed in the Ito and Kupffer cells in parallel to the down-regulation of its receptor, c-met, in hepatocytes [44, 45, 66]. Hence, homeostatic processes such as inflammation and repair that follow damage to an organ facilitate the proliferation of normal, and, in some cases, tumor cells possessing the appropriate receptors. Tumor cells can also release factors that can affect the host cells resulting in a reciprocal relationship between the tumor cells and host cells in the tumor microenvironment [6, 77–79]. Growth factors (GFs), growth factor receptor (GF-R).

growth showed no amplification or rearrangement in the genes coding for the tyrosine kinase receptors EGF-R and c-*erb* B2. Similar results were observed for genes encoding specific GFs or proteins involved in intracellular signal transduction pathways. In contrast, northern blot analyses demonstrated that highly metastatic HCC variants (either Dukes' stage D or variant cells selected in nude mice from a Dukes' stage B2 tumor) expressed significantly more EGF-R mRNA transcripts than poorly metastatic HCC cell types (Radinsky *et al.*, manuscript in preparation). Scatchard analyses of the type and number of EGF-Rs on these cell types confirmed these results. The *in vitro* growth response of cells with high or low metastatic potential to picogram levels of TGF-α demonstrated the functional significance of increased EGF-R numbers on specific cell types. EGF-R specific protein-tyrosine kinase activity was also elevated in the highly metastatic HCC cells compared with the low metastatic cell types. Fluorescent-activated cell-sorting for high EGF-R expressing HCC cell types and EGF-R transfection experiments confirmed the correlation between growth ability in the liver environment and expression of functional EGF-Rs (Radinsky *et al.*, manuscript in preparation). EGF-R levels also correlated with advanced stage disease in a number of human malignancies, including non-small cell lung, bladder, and gastric cancers [72–74, 80], as well as with metastatic potential and,

hence, poor prognosis in breast cancer [81]. The results demonstrate the physiological significance of inappropriate expression of the EGF-R tyrosine kinase in normal and abnormal cellular growth control.

Related to, but distinct from the EGF-R is the c-met proto-oncogene. The protein encoded by c-met is the receptor for HGF [67, 68]. Numerous studies indicate a role for HGF and c-met in the growth and turnover of epithelial tissues and in the progression of carcinoma cells to the invasive phenotype [44, 45, 51]. Preliminary studies from our laboratory indicate high levels of c-met expression in *in vitro* adapted HCC cell types of either Dukes' stage B2, D, or liver metastases (Radinsky *et al.*, manuscript in preparation). Expression analyses of mRNA isolated directly from HCC tumor specimens versus normal colon or liver tissue suggest increased c-met transcripts in the tumor tissues (Ellis, Radinsky, and Fidler, manuscript in preparation). The significance (if any) of increased expression of c-met in HCC organ-specific metastasis and its ability to bind the HGF ligand and elicit a response (mitogenic and/or motility) is currently under investigation. Further studies of EGF-R, c-met, and other tyrosine kinase receptors and their intracellular pathways may have application in the study of cellular proliferation, possibly in terms of modifying a tumor cell's response to specific factors during metastasis.

### *In situ* mRNA hybridization for EGF-R transcripts in archival HCC surgical specimens

We recently reported the development of a rapid colorimetric *in situ* mRNA hybridization (ISH) technique using hyperbiotinylated oligonucleotide probes for analysis of EGF-R transcripts in formalin-fixed paraffin-embedded HCC surgical specimens [82] or multidrug resistance gene 1 (*mdr1*) transcripts in cultured mouse colon carcinoma cells and frozen tissue sections [83]. This 5 hour procedure is a modification of the ISH procedure developed by Brigati and co-workers for DNA [84], using specific oligonucleotides labeled with six biotin molecules at the 3' end and an avidin-alkaline phosphatase detection system [85]. This ISH technique, in conjunction with immunohistochemistry, can produce valuable information on determining the exact source of mRNA and a protein such as EGF-R in a given tumor specimen. Unlike Northern analyses, ISH can determine intratumoral heterogeneity in gene expression and identify particular cells that contain a specific mRNA transcript in formalin-fixed paraffin-embedded archival tissues. Since normal colonic epithelium and hepatocytes express EGF-R mRNA transcripts (see above), ISH analyses can determine whether the EGF-R originates in the HCC cells or normal cells or both. This ISH technique should allow analyses of the host tissue (hepatocytes, Kupffer, fibroblast, and endothelial cells, see Fig. 3) surrounding the metastatic lesion for possible upregulation and enhanced expression of candidate GF transcripts. Data obtained from the highly metastatic cells sequentially selected from HCC neoplasms in nude mice can be rigorously compared using ISH to the original archival paraffin-embedded sample from the same patient as well as to a panel of HCC primary and metastatic specimens. Thus, analyses of this kind may determine a clear association of the genetic and phenotypic characteristics intrinsic to the metastatic HCC cell and the putative role of the liver 'soil' in its growth regulation.

Based on our findings that HCC cells isolated from metastases (Dukes' stage D) expressed significantly increased EGF-R transcripts and functional protein receptors as compared to HCC cells with low metastatic potential (Dukes' stage A or B), we analyzed by ISH archival HCC primary and metastatic surgical specimens for cell specific EGF-R mRNA expression. We first determined whether paraformaldehyde fixation of cultured A431 epidermoid carcinoma cells [86] or formalin fixation and paraffin-embedding of tissues interfered with ISH for mRNA. A hyperbiotinylated oligonucleotide $d(T)_{30}$ complementary to all polyadenylated mRNA transcripts was used to confirm the integrity of the mRNA (i.e. lack of degradation) in all samples (Fig. 4A and D). A431 cells growing in culture and in solid tumors showed intense alkaline phosphatase reactivity in the cytoplasm consistent with the cellular distribution of polyadenylated mRNA

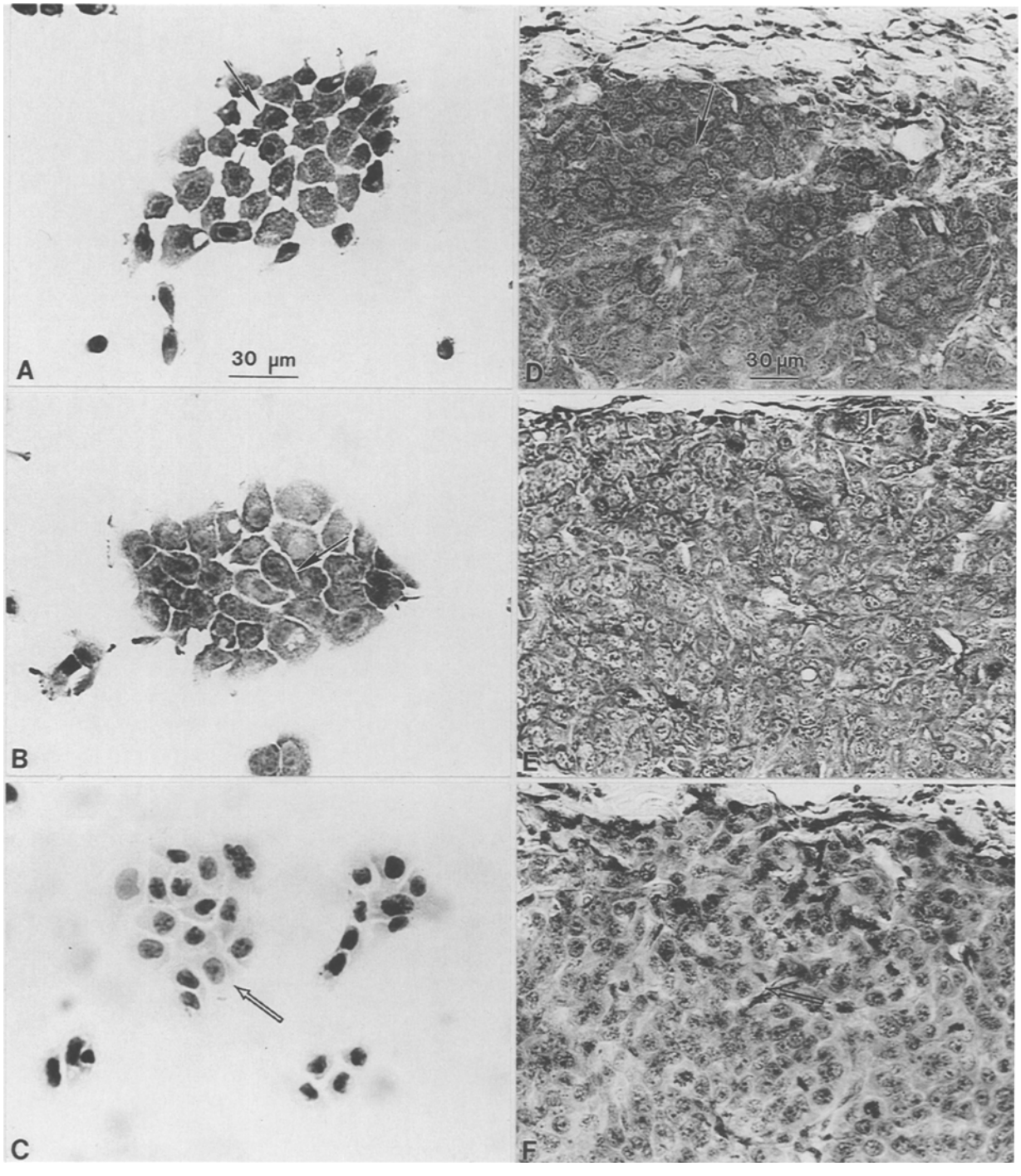


*Fig. 4. In situ* mRNA hybridization for EGF-R transcripts in human A431 epidermoid carcinoma cells. Cells were grown *in vitro* [A–C] or as tumors in nude mice [D–F]. mRNA integrity was verified using a hyperbiotinylated oligonucleotide $d(T)_{30}$ probe which showed strong cytoplasmic staining in both fixed cultured cells and formalin-fixed paraffin-embedded sections of solid tumor tissue (black arrows, A and D, respectively). A 24-base hyperbiotinylated antisense EGF-R oligonucleotide probe produced intense cytoplasmic staining (B and E, black arrows); in contrast to a control hyperbiotinylated EGF-R sense oligonucleotide probe, which showed no cytoplasmic reaction (C and F, open arrows). Avidin-alkaline phosphatase detection techniques were used [85]. All samples were counterstained with hematoxylin. (Adapted from Radinsky *et al.* [82].)

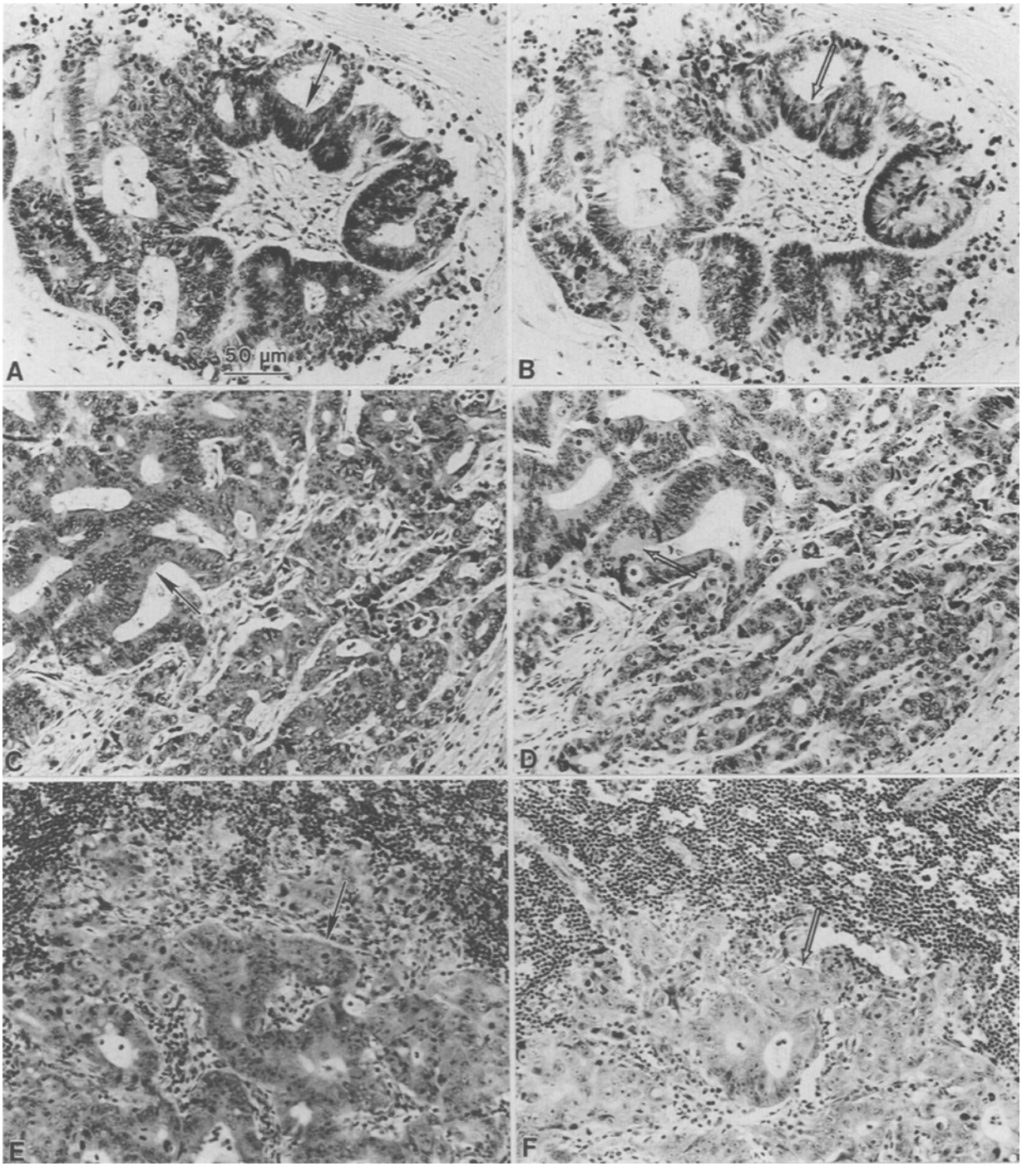


*Fig. 5. In situ* mRNA hybridization for EGF-R transcripts in formalin-fixed paraffin-embedded surgical specimens of primary and metastatic HCC. Hybridization with a hyperbiotinylated oligonucleotide $d(T)_{30}$ probe confirmed the mRNA integrity in each sample (not shown). Hybridization with the antisense EGF-R oligonucleotide probe revealed positive cytoplasmic staining of primary HCC (black arrow, A) and HCC metastases in the liver and lymph node (black arrows, C and E, respectively). Normal hepatoctyes demonstrated weak cytoplasmic reactivity (C), whereas normal lymphoid tissue demonstrated no reaction (E). EGF-R control sense probes showed no cytoplasmic reactivity with endogenous transcripts (open arrows, B, D, and F). All sections were counterstained with hematoxylin. A, B, primary HCC; C, D, HCC liver metastasis; E, F, HCC lymph node metastasis. (Adapted from Radinsky *et al.* [82].)

in the cytoplasm (black arrows, Fig. 4A and D, respectively). Proper fixation is thus mandatory for ISH results and control staining with oligonucleotide $d(T)_{30}$ is necessary to interpret negative results [82]. Most cell types display average EGF-R numbers from 20,000 to 200,000 [74]; whereas, A431 cells express approximately $2 \times 10^6$ EGF-Rs per cell [86]. The hyperbiotinylated antisense EGF-R oligonucleotide probe (complementary to EGF-R mRNA) produced dark cytoplasmic staining (Fig. 4B and E). Control ISH analyses using the corresponding hyperbiotinylated sense EGF-R probe showed minimal cytoplasmic staining (open arrows, Fig. 4C and F). Northern blot analyses and immunohistochemical analyses for EGF-R confirmed these results and provided evidence for the specificity and sensitivity of this ISH technique to detect EGF-R transcripts.

We next analyzed by ISH EGF-R transcripts in formalin-fixed paraffin-embedded primary HCC and liver and lymph node metastases (Fig. 5). Analysis of a primary Dukes' C2 HCC with the hyperbiotinylated oligonucleotide $d(T)_{30}$ confirmed that the mRNA was intact in this sample (not shown). ISH with the antisense EGF-R probe produced distinct cytochemical staining in the cytoplasm of the HCC cells (black arrow, Fig. 5A). Neither a control EGF-R sense probe (open arrow, Fig. 5B) nor a reagent control hybridization (not shown) produced staining. Immunohistochemical analysis of this paraffin-embedded primary HCC with a mouse monoclonal anti-EGF-R antibody and alkaline phosphatase detection showed positively stained tumor cells thus confirming the ISH results in this sample. We also performed ISH on paraffin-sections of HCC liver and lymph node metastases from two different patients (Fig. 5C–F). Metastatic HCC in the liver and lymph node demonstrated cytochemical reaction with the hyperbiotinylated antisense EGF-R probe (black arrows, Fig. 5C and E, respectively). Hepatocytes, which are also known to express EGF-R [64, 65] reacted with this probe albeit at a lower level (Fig. 5C). Hybridization with the control sense EGF-R probe showed no reaction (open arrows, Fig. 5D and F). Northern blot analyses of these same samples correlated directly with the ISH data (not shown). These results indicate that retrospective analyses are feasible using archival human surgical specimens. Further work is required to assess how quantitative this ISH technique is. A procedure to quantify histochemically detected alkaline phosphatase activity is currently being explored [87]. This method should allow the detection of other mRNA species involved in HCC metastasis utilizing archival specimens. The expression of GFs produced by the host environment (i.e., surrounding the HCC metastases) could also be analyzed using ISH in combination with bacterial *lacZ*-tagging of the metastatic cells [88, 89] to facilitate studies during the early formulative stages of metastatic cell growth in the secondary organ-specific environment.

## Conclusions

The Paget 'seed and soil' hypothesis proposed that the successful production of metastasis depends in part on the interaction of favored tumor cells with a compatible milieu provided by a particular organ environment. Studies with HCC support this hypothesis and show that the production of site-specific metastasis is dependent on the proliferation of tumor cells differentially expressing GF-Rs and that tissue-specific paracrine GFs or organ-repair factors may stimulate the growth of receptive metastatic cells. It is clear from the experiments presented here that organ microenvironments can influence the implantation, invasion, survival, and growth of receptive tumor cells. Thus, the successful metastatic cell, which exhibits a complex phenotype, must be viewed as a cell receptive to its environment [2].

- Anatomical compatability of tumor cells with the organ environment is critical to the design of a correct *in vivo* model for human spontaneous metastatis [2, 17, 19].
- Local organ factors may produce selection pressures on tumors which result in the outgrowth of specific clonal subpopulations in a growing tumor [2, 8, 10, 27, 28[.
- The production of HCC tumors in the livers of nude mice is determined by the ability of HCC cells to *proliferate* in the liver parenchyma rather

than by the ability of cells to *reach* the liver [2, 17, 20].
- HCC cells isolated from metastases or Dukes' stage D tumors expressed significantly increased EGF-R transcripts and functional protein receptors as compared to HCC cells with low metastatic potential to Dukes' stage A and B tumors (Radinsky *et al.*, manuscript in preparation).
- Data obtained from highly metastastic HCC cells in a nude mouse model can be rigorously compared using ISH techniques with the original archival surgical specimen as well as to a panel of HCC samples in order to determine a clear association of the intrinsic properties of the metastatic HCC cell and the role of the liver environment in its growth regulation [82, 83].

## Key unanswered questions

- Apart from known markers in HCC tumor progression, what other markers are associated with different stages of HCC progression and metastasis?
- What is the role of host factors on the selection of different high metastastic HCC clones from HCC grown at orthotopic versus ectopic sites?
- Are EGF-R and c-met directly involved in the growth regulation of HCC at the primary and/or liver-specific sites?
- EGF-R and c-met knock-out experiments using antisense, antibody, or drug technologies may determine the direct involvement of these receptors in the growth regulation of HCC liver metastases.
- What is the role of HGF in early versus late stage HCC cells?
- Do host cells adjacent to the metastatic lesion upregulate their GF production? ISH experiments may uncover this phenomenon.

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