

Cancer Invasion and Metastasis

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INTRODUCTION

- “Neoplasia” (*new growth* in Greek) is an abnormal proliferation of cells, the growth of which exceeds and is uncoordinated with that of the normal tissues and persists in the same excessive manner after cessation of the stimuli which evoked the change.
- “Neoplasm”—an abnormal mass of tissue produced.
- Neoplasms can be benign, pre-malignant, or malignant (cancer).
- There are eight fundamental changes in cell physiology, which are considered the hallmarks of cancer:
 1. Self-sufficiency in growth signals
 2. Insensitivity to growth-inhibitory signals
 3. Altered cellular metabolism
 4. Evasion of apoptosis
 5. Limitless replicative potential (immortality)
 6. Sustained angiogenesis
 7. Ability to invade and metastasize
 8. Ability to evade the host immune response.

Overview of Carcinogenesis

- Carcinogenesis is a multistep process at both the phenotypic and the genetic levels, resulting from the accumulation of multiple mutations involving multiple genes.
- Phenotypically, malignancy is characterized by excessive growth, local invasiveness and ability to form distant metastases.
- The principal targets of genetic damage include:
 - ♦ Genes promoting cell growth (proto-oncogenes)
 - ♦ Genes inhibiting cell growth (tumor suppressor genes)
 - ♦ Genes regulating apoptosis
 - ♦ Genes regulating DNA repair.
- As the tumor ages, multiple mutations accumulate independently in different cells, generating subclones with varying abilities to grow, invade, metastasize, resist/respond to therapy.
- Most malignant tumors are monoclonal.

- However, by the time they become clinically evident their constituent cells are extremely heterogeneous.
- The ability of the tumor cells to metastasize is largely dependent on the ability of these cells to migrate.
- Hence an important step in carcinogenesis is epithelial-mesenchymal transformation that bestows the tumor cells with the abilities like invasion and metastasis.
- Fundamentally, the epithelial cells and mesenchymal cells have their own characteristics that allow them to behave differently.

Characteristics of Epithelial Cells

- **Epithelial cells** are cells that line the inner and outer surfaces of the body in continuous cell layers, as a single layer or multilayered structure.
- The epithelial cell layer is attached to the underlying connective tissue by a basement membrane.
- Epithelial cells are joined together by strong intercellular cell junctions that help in the maintenance of continuous cell layers.
- Epithelial cells are characterized by specialized membrane domains:
 - ♦ Basal domain
 - ♦ Lateral domain
 - ♦ Apical domain: Characteristics of this domain depend on the functional needs of the cell.
- **Lateral domain:** Keeps the epithelial layer impenetrable and consists of desmosomes, gap junctions, tight junctions and adherens junctions.
 - ♦ *Desmosomes:* Adhesion belts connected to intermediate filaments
 - ♦ *Gap junctions:* Intercellular channels 1.5–2 nm in size that permit free passage of ions and small molecules. Also, gap junctions permit changes in membrane potential.
 - ♦ *Tight junctions:* Bind cells with claudin, occludin proteins.
 - ♦ *Adherens junctions:* These are built from cadherins.
- **Basal domain:** Allows interaction between the basement membrane and the cell. The cells adhere to extracellular matrix (ECM) by:
 - A. *Hemidesmosomes:* Bind the ECM to intermediate filaments via integrins.
 - B. *Focal adhesions:* Bind the ECM to actin microfilaments via integrins.

Characteristics of Mesenchymal Cells

- **Mesenchymal cells** have the following characteristics:
 - ♦ They do not have cytoplasmic polarity.
 - ♦ They do not form a continuous sheet.
 - ♦ There are no specific membrane domains.
 - ♦ Intercellular adhesions are less strong which accounts for the migratory capacity of the mesenchymal cells.
 - ♦ Cells are mobile with a cytoskeleton comprised by vimentin.
 - ♦ The cells have filopodia as cytoplasmic extensions when mobile, which contain active cytoskeleton.
 - ♦ They express matrix adhesion proteins and matrix metalloproteinases (MMPs).
- Mesenchymal cells contribute to the ECM by synthesizing and organizing new components by remodeling the ECM through the production of matrix-degrading MMPs.

- Mesenchymal migration is mechanistically different from epithelial movement.
- Epithelial cells move as a sheet en block, whereas mesenchymal cells move individually.

INVASION AND METASTASIS

- Invasion and metastasis are hallmarks of malignant tumors.
- They are a major cause of cancer-related morbidity and mortality.
- The causes of metastasis can be explained using four models as follows:
 - A. Metastasis is caused by rare variant clones that develop in the primary tumor.
 - B. Metastasis is caused by the gene expression pattern of most cells of the primary tumor, referred to as a metastatic signature.
 - C. Combination of A and B, in which metastatic variants appear in a tumor with a metastatic gene signature.
 - D. Metastasis development is greatly influenced by the tumor stroma, which regulates angiogenesis, local invasiveness, and resistance to immune elimination, allowing cells of the primary tumor to become metastatic.

METASTATIC CASCADE

There are two phases of metastasis:

1. Invasion of the extracellular matrix (ECM).
2. Vascular dissemination, homing of tumor cells and colonization.

INVASION OF THE ECM

- Normally cells are glued to each other due to the presence of cell adhesion molecules, e.g. cadherins.
- Loss of E-cadherin is important in the development of almost all epithelial cancers
- The tumor cells and tumor stromal cells secrete proteolytic enzymes like—MMPs, cathepsins, urokinase and plasminogen activator.
- These proteases degrade basement membrane and interstitial connective tissue.
- Cleavage products of collagen and proteoglycans, thus formed, have chemotactic, angiogenic and growth-promoting effects.
- The loosened cancer cells bind to proteolytically generated binding sites, mainly laminin and fibronectin.
- The loss of integrins favors invasion.
- **Locomotion** is the final step of invasion.
- Cytokines released by tumor cells and cleavage products of matrix facilitate migration of tumor cells.

Vascular Dissemination, Homing of Tumor Cells and Colonization

- Once the cancer cells detach from neighboring cells, they migrate to surrounding tissue.
- The cancer cells then pass through blood vessels and enter the systemic circulation (intravasation).
- On reaching the metastatic site the tumor cells exit the bloodstream (extravasation).
- In this new site the tumor cells reconstitute the tissue environment which resembles that of a primary site and is favorable for the growth and survival of tumor.

- To develop migratory behavior, epithelial cancer cells acquire properties close to mesenchymal cells and this phenomenon is called epithelial-mesenchymal transition (EMT).
- EMT facilitates cell invasion and metastasis.
- However, these mesenchymal-like tumor cells gain migratory capacity at the expense of proliferative potential.
- To establish metastasis, the metastatic tumor cells undergo inverse process of mesenchymal-epithelial transition (MET).
- MET is required to regenerate a proliferative state and form macrometastases resembling the primary tumor at distant sites.

CONCEPT OF EMT

Historical Background

- Two main cell types—epithelial and mesenchymal were recognized in the late 19th century based on their shape and organization during embryonic development and interconversion between the two states was described by Frank Lillie in 1908.
- In the late 1960s, Elisabeth Hay provided a detailed description of the formation of chick primitive streak—a structure that requires the conversion of epithelial to mesenchymal cells.
- EMT was recognized as a distinct process in 1982 by the work of Garry Greenburg and Elisabeth Hay on the 3D culture of corneal epithelial cells in the laboratory.
- It took a long time for EMT to be recognized as a potential mechanism for carcinoma progression because of the following reasons:
 - ♦ EMT cannot be followed in time and space in human tumors.
 - ♦ Great diversity of cellular organization in human tumors.
 - ♦ Recognition of carcinomas (epithelial origin) and sarcomas (mesenchymal origin) as two separate entities, not thought to interconvert (except a rare tumor known as sarcomatoid carcinoma).
- The mechanisms that govern EMT are now being unraveled and many parallels are being found between EMT in embryonic development and EMT in tumor development.

Role of EMT

- Under physiological conditions, EMT plays an important role in embryonic development.
- EMT also plays a crucial role in pathological conditions like tissue reconstruction after injury (wound healing), chronic inflammation, tissue fibrosis in response to injury (lung, kidney, liver), carcinogenesis, tumor metastasis and invasion.
- However, the difference between normal development and pathological processes is at cellular and molecular level.
 - ♦ Events follow highly regulated spatial and temporal plans during physiological development.
 - ♦ During pathologic transformation, the order of events may be stochastic and time-independent or particular events may be bypassed.
- During tumorigenesis EMT may increase motility and invasiveness of cancer cells and malignant transformation may be associated with signaling pathways promoting EMT.

Epithelial Plasticity is Bidirectional

- The reverse phenomenon, back to epithelial phenotype is called, mesenchymal-epithelial transition (MET).
- MET occurs at various stages of morphogenesis, alternating with EMT during the formation of heart, somites, and kidney, and in the formation of coelomic cavities.
- *Metastable phenotype* refers to the ability of cells to express attributes of both epithelial and mesenchymal phenotypes.

E-CADHERIN AND EMT

- E-cadherin is a calcium-dependent transmembrane glycoprotein which functions as an adhesion molecule. It is present in most epithelial cells in embryonic and adult tissues.
- Both EMT and MET are dependent on E-cadherin.
- The cells undergoing EMT downregulate E-cadherin.
- In various human carcinomas, functional loss of E-cadherin may result from
 - ♦ Production of a defective protein
 - ♦ Promoter hypermethylation
 - ♦ Gene mutation
 - ♦ Transcriptional repression may result from the activation of repressors, such as Snail, Slug, Sip1, Ets, Twist.
- **Under physiologic conditions**, the signaling pathways that lead to cell proliferation, differentiation and migration begin with growth factor binding to a specific receptor.
- This is followed by activation of signal-transducing proteins which transmit the signal across cytosol to nucleus.
- This leads to induction and activation of nuclear regulatory factors which initiate DNA transcription.

Signaling Pathways in EMT

- Various classes of molecules that change in expression, distribution, and/or function during the EMT, and that are involved, include (Table 1.1):
 - ♦ Growth factors [e.g. transforming growth factor (TGF)- β , Wnt]
 - ♦ Transcription factors (Snails, SMAD, LEF, and nuclear β -catenin)

Table 1.1: Pathways involved in the regulation of EMT

<i>Receptor</i>	<i>Ligand</i>	<i>Signaling molecule</i>	<i>Intermediate signaling endpoint</i>	<i>Effect</i>
TGF- β receptor	TGF- β	RhoA	MRTF-A	Stress fibers migration
FGF receptor	FGF	Smad	SNAIL2	Cytoskeleton activation migration, focal adhesion, rearrangement
Tyrosine kinase	HGF	Sarc	SNAIL2	Increased migration
Integrins	EGF	Ras, MAPK	SNAIL1	E-cadherin downregulated, reduced cell adhesion
Frizzled	Collagen, fibronectin	Paxillin, Rac	GIT1	Stress fibers migration

- ◆ Molecules of the cell-to-cell adhesion axis (cadherins, catenins)
- ◆ Cell-to-ECM adhesion axis (integrins, focal contact proteins, ECM proteins)
- ◆ Cytoskeletal modulators (Rho family)
- ◆ Extracellular proteases (matrix metalloproteinases, plasminogen activators).
- ◆ Other molecular changes seem to occur after the initial behavioral change; for example, there is often a trend to replace cyokeratin intermediate filaments with other types, typically vimentin.

INDUCTION OF EMT

- The first event in EMT is the proteolytic digestion of the basement membrane by metalloproteinases.
- Local expression of TGF- β , EGF, IGF-II and FGF-2 facilitates EMT by binding to receptor with ligand-inducible intrinsic kinase activity.
- Overexpression of master regulators of EMT, such as the transcription factors like Twist, Snail, and SIP1 repress the expression of E-cadherin.

TGF- β Pathway or Smad Signaling Pathway

- TGF- β interacts sequentially with two membrane receptors.
- TGF binds first to the type II receptor and then the ligand-receptor complex associates with type I TGF receptor.
- T β IR phosphorylates T β IR.
- Activated TGF-RI propagates the signal downstream by phosphorylating Smad2 and Smad3 which form complexes with Smad4 and translocate into the nucleus.
- In combination with T cell factor (TCF) family transcription factors, they down-regulate E-cadherin genes and initiate EMT.

Wnt/ β -catenin Signaling Pathway

- Wnt signals through a family of cell surface receptors called frizzled (Frz).
- It stimulates several pathways, the central one involves **β -catenin** and APC.
- It regulates the amounts of β -catenin protein available within the cell for binding cadherins.
- It mediates
 - ◆ cell-cell adhesion
 - ◆ adhesion to cytoskeletal (F actin) elements
- In resting cells, β catenin forms complex containing glycogen synthase kinase3 (GSK3 β), axin and APC protein.
- Phosphorylated β -catenin is degraded by ubiquitination.
- Hence, intracellular level of β -catenin is kept low.
- Wnt-Frz leads to dissociation and inactivation of GSK3 β , which can no longer phosphorylate β -catenin.
- Free β -catenin translocates to the nucleus, thereby inducing gene expression in a complex with T cell factor (TCF) down-regulating E-cadherin and initiating EMT.
- For example, in APC-mutated in colon cancer, cells behave as if they are under constant stimulation by Wnt pathway.
- This leads to EMT induction as β -catenin translocates to nucleus.

Tyrosine Kinases Pathway

- Several growth factors can induce EMT by binding to receptor tyrosine kinases.
- FGF, EGF, TGF- α and IGF-2 can induce EMT.

TRANSCRIPTION FACTORS ASSOCIATED WITH EMT

Twist

- Twist transcription factors cooperate with mitogenic oncoproteins in cancer cells.
- Twist contributes to the malignant transformation via the following:
 - ♦ Overrides premature senescence (inhibits apoptosis).
 - ♦ Downregulates E-cadherin.
 - ♦ Upregulates mesenchymal markers like Vimentin, SMA.
 - ♦ Induces EMT allowing tumor progression and dissemination.

Snail

- Snail is a strong direct repressor of E-cadherin.
- It confers tumorigenic, invasive and migratory properties.
- It inhibits apoptosis.
- It inversely correlates with the degree of differentiation and is associated with lymph node metastasis.

Steroid Receptor Coactivators Interacting Protein (SIP)

- SIP, a novel ankyrin repeat containing protein, sequesters steroid receptor coactivators in the cytoplasm.
- It contains a Smad-binding domain and may therefore modulate TGF signaling pathway, which is known to induce EMT.

EMT MARKERS

- Table 1.2 gives a list of markers that can be used for diagnosing EMT.

Table 1.2: Markers for EMT	
Proteins that increase in abundance	
N-cadherin	MMP-2
Vimentin	MMP-3
Fibronectin	MMP-9
Snail1 (Snail)	Integrin $\alpha\beta$ 6
Snail2 (Slug)	N-cadherin
Twist	Vimentin
FOXC2	Fibronectin
Sox10	Snail1 (Snail)
Proteins that decrease in abundance	
E-cadherin	Cytokeratin
Desmoplakin	Occludin

Contd.

Table 1.2: Markers for EMT

Proteins that decrease in abundance	
β -catenin	Snail1 (Snail)
Smad-2/3	Snail2 (Slug)
NF- κ B	Twist
	β -catenin
Proteins whose activity increases	
ILK	Rho
GSK-3 β	
<i>In-vitro</i> functional markers	
Increased migration	Increased scattering
Increased invasion	Altered cell shape

EMT in Cancer

- Turning an epithelial cell into a mesenchymal cell requires alterations in morphology, cellular architecture, adhesion and migration capacity.
- Thus there is a derangement of apicobasal polarity and cell-to-cell adhesive architecture and function, lack of basal lamina integrity.
- The occurrence of EMT during tumor progression allows tumor cells (i.e., ones that are noninvasive and nonmetastatic) to acquire the capacity to infiltrate surrounding tissue and ultimately metastasize to distant sites.
- The most compelling evidence for the involvement of EMT in oncogenesis is the ability of multiple EMT regulators to enhance tumor formation and/or metastasis.

Studies to Prove Role of EMT in Cancer

- For example, expression of Snail1 increases the aggressiveness of experimentally induced breast tumors, and high Snail1 expression correlates with an increased risk of tumor relapse and poor survival rates in human breast cancer.
- Loss of E-cadherin is a hallmark of metastatic carcinoma, and proteomic analysis of breast cancer reveals that circulating mammary tumor cells, or those found as micrometastases, show evidence of mesenchymal conversion.
- Nuclear localization of β -catenin is frequently used as an EMT marker, and nuclear β -catenin is a marker for a poor prognosis in colorectal cancer.

THERAPEUTIC IMPLICATIONS

- Strategies to block any steps during EMT would have a major impact on EMT and, thereby, on fibrosis, and cancer metastasis.
- Blockade of growth factor, cytokine and integrin pathways that lead to activation of signaling elements such as β -catenin and transcription factors Slug, Snail, Twist which assume critical roles in EMT may constitute potent strategies to counteract the progression from localized cancers to disseminated disease.

- EMT-associated molecules can be used as markers for the prediction of prognosis and response to targeted therapy.
- Commonly used molecular markers for EMT include increased expression of N-cadherin, vimentin, and integrins.
- IHC may be used to detect the expression of these molecular markers. IHC of EMT involved molecules may also be used to predict metastatic behavior.
- Nuclear localization of β -catenin is frequently used as an EMT marker, and nuclear β -catenin is a marker for a poor prognosis in colorectal cancer.
- Loss of E-cadherin is a hallmark of metastatic carcinoma.
- Blockade of EGFR signaling pathway by using anti-EGFR antibody or EGFR tyrosine kinase inhibitors such as gefitinib or erlotinib inhibit invasion.
- BMP-7 antagonizes TGF- β driven EMT in fibrotic kidney and heart inhibit disease development.

POINTS TO REMEMBER

- Accumulating evidence in recent years indicates that EMT is a critical process not only in development but also in tumorigenesis.
- Acquisition of EMT properties during tumor progression is associated with dissolution of epithelial integrity, increased migration, local invasion and, ultimately, metastasis.
- For its association with invasion and early steps of metastasis, inhibition of EMT appears a viable strategy for novel approaches to cancer therapy.
- However, given the complex, intertwined circuitry regulating EMT, effective deployment of anti-EMT therapeutics cannot be done without understanding the molecular alterations of the specific tumor being targeted.

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