Neu Differentiation Factors: A Family of Alternatively Spliced Neuronal and Mesenchymal Factors (43746)

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Abstract. **The Neu proto-oncogene (also called ErbB-2 and HER-2) encodes a tyrosine kinase transmembrane receptor homologous to the epidermal growth factor receptor (EGF-R). Overexpression, a point-mutation, and co-expression with EGF-R activate the oncogenic potential of the Neu protein by permanent coupling to signal transducing pathways. The search for ligands that elevate tyrosine phosphorylation of Neu led to the discovery of a 44-kDa glycoprotein that acts either as a differentiation factor or as a mitogen for mammary tumor cells. This protein, termed Neu differentiation factor (NDF), is derived from a transmembrane precursor that contains an EGF-like motif and an immunoglobulin-like domain. Alternative splicing generates a dozen NDF-related proteins that are expressed in a variety of mesenchymal and neuronal tissues. This unprecedented multiplicity raises the possibility that different isoforms fulfill distinct biological roles.** [P.S.E.B.M. 1994, Vol 206]

Normal cell growth and differentiation depend on the ability of each cell to receive, interpret and respond to a plethora of extracellular signals. The primary processing of such signals is performed at the cytoplasmic membrane by a multitude of specific receptors. Binding of a ligand to its receptor triggers a cascade of events that modulate various cellular functions, including gene expression, and may eventually result in cell proliferation, differentiation, and morphogenic changes. Aberrations in this signal transduction cascade can lead to deregulated cellular proliferation and differentiation, and thus, ultimately to tumorigenesis (1). Cytoplasmic receptors that carry intrinsic tyrosine kinase (TK) activity comprise the largest family of receptors for growth factors. There are at least seven distinct TK receptor subfamilies containing structurally related members (2). Many of the TK receptors have no known ligands (orphan receptors), and have been discovered through their homology to members of their subfamily or by their oncogenic potential (3). The most frequently implicated receptors and growth factors in human cancer are members of the epidermal growth factor receptor (EGF-R) subfamily and their ligands (1). In addition to EGF-R, this subfamily includes Neu/HER-2/ErbB-2 (4), HER-3/ErbB-3 (5), and the recently discovered HER-4/ErbB-4 (6). Despite the structural and functional homologies among members of the EGF-R family, only ligands for the EGF-R were identified and none of them activates the other receptors. Recently a ligand that elevates tyrosine phosphorylation of Neu/HER-2 was identified and cloned (7, 8), and, consequently, a whole family of structurally related ligands was cloned and studied (9–11). This review will analyze the structure, function, and possible biological role of these factors.

Neu and Oncogenesis

Neu was originally described as a transforming gene in pregnant rats treated with the carcinogen ethylnitrosourea. Upon treatment at a specific time of gestation (Day 15), the offspring developed neuroblastomas and glioblastomas (12). The transforming allele contained a single-point mutation changing a valine residue to a glutamic acid at Position 664 of the pre-

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dicted transmembrane domain (13). This single point mutation causes constitutive activation of the TK (14) by stabilizing receptor dimers (15, 16). In the human gene (Neu/HER-2), no transforming mutation was found, but site-directed mutagenesis revealed that a similar point mutation can render Neu/Her-2 oncogenic. Wild-type Neu/HER-2 is overexpressed in approximately 20% of breast, ovarian, stomach, pancreatic, and bladder carcinomas. In breast and ovarian cancer, overexpression of Neu is associated with poorer prognosis . It seems that the major pathway of oncogenic activation of Neu/HER-2 in human malignancies is by amplification and overexpression of the wild-type gene. Overexpression probably results in the stabilization of receptor dimers, which are essential for maintaining the TK in its active state.

Signaling by Neu

To circumvent the absence of a known Neu ligand, a chimeric receptor in which the extracellular domain was derived from the EGF-R and the cytoplasmic domain from Neu was constructed. Upon activation of the receptor with the heterologous ligand, a strong mitogenic effect was observed in murine fibroblasts (17, 18). Additional events that followed Neu activation included increased turnover of inositol lipids, accelerated transport of glucose across the plasma membrane and rapid induction of *fos* and *jun* expression. Ligand binding, oncogenic mutation, or overexpression of Neu all lead to the stabilization of receptor dimers. This is an essential initial step in the signal transduction cascade. Following dimer formation, the intracellular TK is activated and Neu undergoes autophosphorylation on five tyrosine residues located on the non-catalytic C-terminus of the protein. These autophosphorylated tyrosine residues function as docking sites for proteins that contain the *src* homology-2 domain (SH-2). Specific SH-2 containing proteins become associated with Neu through binding with phosphorylated tyrosine residues of the activated receptor. These include phospholipase Cγ (PLCγ), the p85 subunit of phosphatidylinositol 3′ kinase, and the GTPase activating protein of RAS. Recently, it was shown that the most distal autophosphorylation site of Neu confers oncogenicity to this receptor through coupling of the TK to a biochemical pathway that includes Rasγ, MAP-kinase and c-*jun* (Ben-Levy R *et al.*, submitted for publication).

Ligands That Activate Neu

An activity that stimulates tyrosine phosphorylation of Neu was detected in various biological sources, including the growth medium of Ras-transformed fibroblasts (Rat1-EJ), breast cancer cell lines, hematopoietic cells such as transformed T cells and activated macrophages, newborn calf serum, and extracts of calf kidney (reviewed in 19). However, the active molecule was isolated and cloned from only two such sources. Neu differentiation factor (NDF) (7, 8) was isolated from the growth medium of Rat1-EJ cells, and its human homologue, termed heregulin (HRG) (9), from the growth medium of MDA-MB-231 breast cancer cells. NDF, a 44-kDa heat-stable glycoprotein, stimulates tyrosine phosphorylation of Neu in human tumor cells of breast, colon, and neuronal origin. When NDF is applied to certain breast cancer tumor cells (AU-565 and MDA-MB453) it induces phenotypic differentiation that includes morphological changes and synthesis of milk components (casein and lipids), as well as growth-arrest in the G2/M phase (7). However, NDF/heregulin is mitogenic to other breast cancer cell lines (SKBR-3 and MCF7) (9). *In situ* hybridization analysis indicated that NDF expression is confined predominantly to the central and peripheral nervous systems (20) and implied a neural function. This was confirmed by the finding that two recently cloned neural factors, namely the glial growth factor (GGF) (10), which is mitogenic to Schwann cells, and an avian acetylcholine receptor inducing factor (ARIA) (11) show very high homology to NDF/HRG. The primary structure of NDF, HRG, GGF, and ARIA indicated that these molecules comprise a new family of polypeptide factors. These mosaic proteins are encoded by a single gene that was mapped to the short arm of human Chromosome 8 (20). The basic structure of NDF includes a N-terminal region, an immunoglobulin (Ig) motif, a glycosylation-rich spacer domain, an EGF-like domain, a hydrophobic transmembrane domain, and a cytoplasmic tail. However, many variants of this structure exist. All of the isoforms of NDF are probably generated through alternative splicing at three domains (Fig. 1), the EGF-like domain, the juxtamembrane region and the cytoplasmic tail. Two major subtypes of NDF, denoted α and β, are classified according to the terminal 18–21 amino acids of their EGF-like domain. This region includes the third disulfide loop of the EGF-like domain, so that the α/β variation alters the sequence between the fifth and sixth cysteines of this domain (Fig. 1 and 2). Subtypes 1 through 5 are classified according to the juxtamembrane sequence distal to the EGF-like motif. Three additional subtypes (a–c) are classified according to the C-terminal sequence of the cytoplasmic tail following a common stretch of 157 amino acids (Fig. 2), which includes an invariant transmembrane domain. Importantly, members of the NDF family fall into two groups, either mesenchymal or neuronal (Fig. 2), on the basis of their tissue of origin. Presumably these

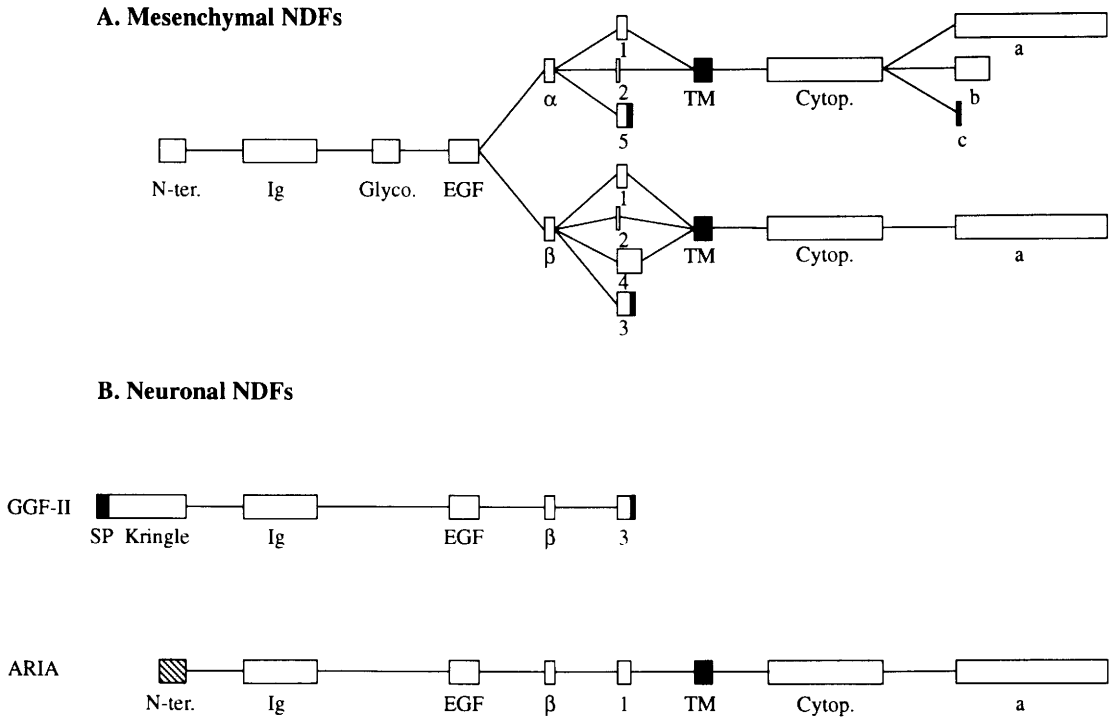

Figure 1. Schematic representation of the mosaic structure of NDF isoforms and their structural variation. The structures of the two groups of NDFs are represented by boxes that correspond to the major structural motifs. The boxes are drawn to scale and correspond to blocks of coding regions. The recognizable domains include an amino terminal (N-*ter*) region, an immunoglobulin-like motif (*Ig*), a glycosylated spacer sequence (*Glyco*), an EGF-like motif (*EGF*), which is subdivided into α and β isoforms, a juxtatransmembrane region subdivided into isoforms 1–5, a hydrophobic transmembrane domain (*TM*), a common cytoplasmic tail (*Cytop*), and a variable-length cytoplasmic tail (*a–c*). Note that the neuronal isoforms possess no glycosylated spacer domain and have a variable N-terminal region. GGFII contains a putative kringle motif (*Kringle*) and a hydrophobic signal peptide (*SP*). The hatched box represents the unique N-terminal sequence of ARIA.

groups are generated by tissue-specific splicing events.

NDF Isoforms

Table I summarizes the information available on the molecularly cloned members of the NDF family. There are 15 fully cloned variants from rodent, avian, bovine, and human sources. Because some variants were cloned from more than one source, the overall number of distinct isoforms is 10. These are α2a, α2b, α2c, β1a, β2a, β3, β4a, β1, β1-kringle and β1a-ARIA. The α2 isoforms are the most prevalent in various epithelial human tumors and in non-neuronal tissues; the β1 isoforms are enriched in neural tissues. The existence of multiple isoforms of NDF has no precedence in other families of growth-regulatory secreted proteins and raises questions regarding the structural basis and the functional role of the many related factors, as well as the relationships between the domain structure and specific functions. These aspects are briefly discussed below.

The N-Terminus. All of the isoforms of NDF, except for GGFII and ARIA, share a 50 amino acid-long hydrophilic N-terminal sequence. This domain undergoes proteolytic cleavage upon maturation of the molecule. Of note is the absence of a hydrophobic signal peptide in this region. By contrast, GGFII contains a putative kringle domain and a hydrophobic 22 amino acid-long signal peptide in its N-terminal region and lacks the N-terminal sequence shared by most other NDFs. ARIA displays a unique N-terminal region that contains no kringle domain or a signal peptide.

Immunoglobulin Domain. All of the isoforms contain an Ig-like domain that is transcribed from two separate exons (10). The amino acid sequence suggests that it belongs to the C2-set of Ig homology units. The ubiquitous presence of this domain in all isoforms suggests that it has an essential function. Nevertheless deletion of the Ig-like domain or of the whole N-terminal region does not affect the binding of NDF to cells (9, 21), indicating that this domain is not involved in receptor recognition.

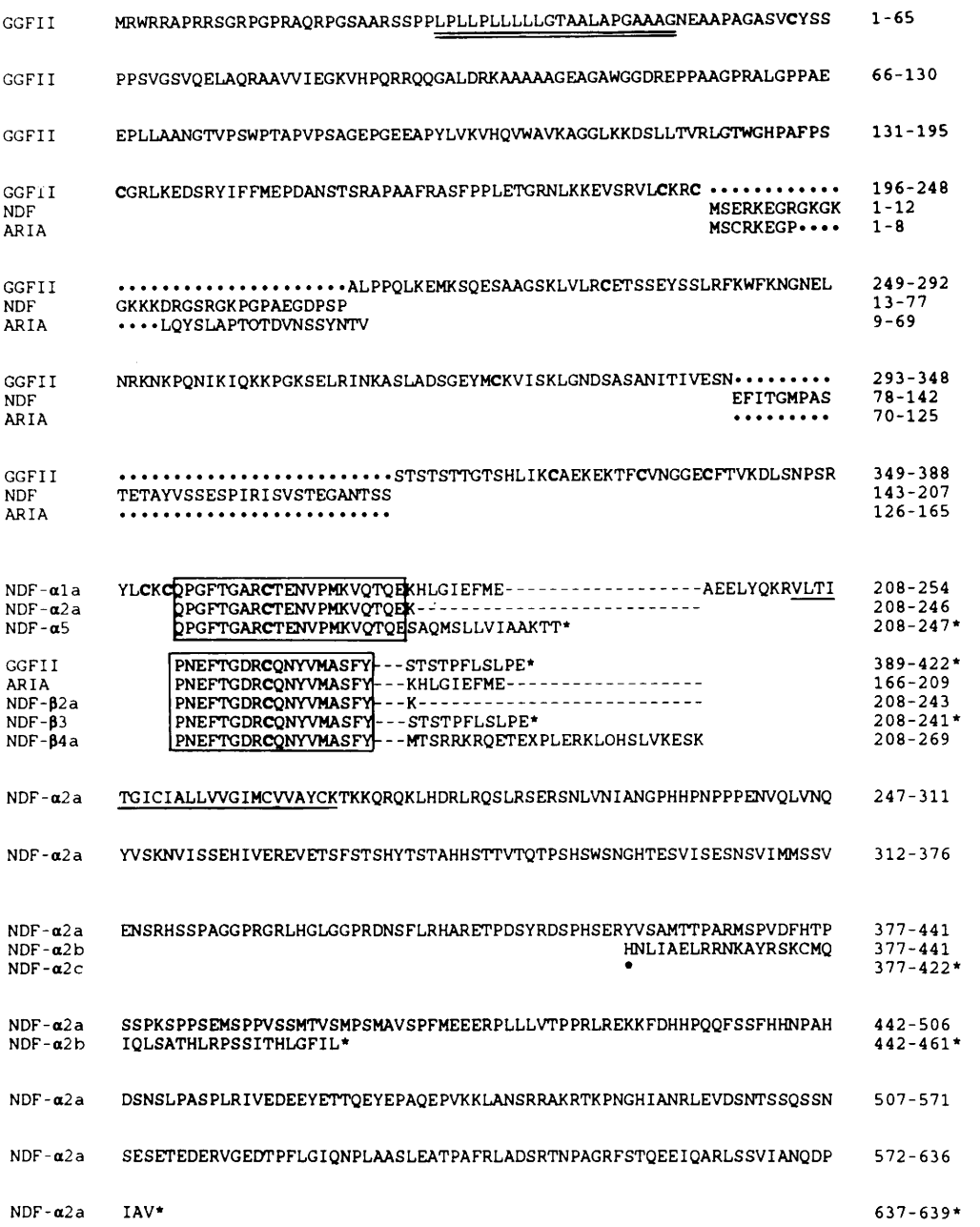

Figure 2. Amino acid sequences of the NDF family. Refer to Table I for the corresponding accession numbers in the GeneBank Database. Note that GGFII has the largest N-terminal sequence. Asterisks, COOH-terminal amino acids; dashes, gaps introduced to facilitate sequence alignment; dots, actual gaps in the amino acid sequence; single underline, putative transmembrane domain; double underline, putative signal peptide. The boxed regions correspond to the α (upper box) and β (lower box) groups of isoforms. Amino acid numbers are indicated on the right column.

The Spacer Domain. This 34 amino acid-long region connects the Ig domain to the EGF motif and contains many sites of N- and O-linked glycosylation. This region is absent in two of the neural forms of NDF, namely GGFII and ARIA, suggesting that it is transcribed from a separate exon. All other isoforms of NDF (except β3) are heavily glycosylated (7). The NDF-β3 subtype, which contains no transmembrane domain and undergoes no secretion, is unglycosylated. The function of the spacer domain is unknown. However, one possible role is to act as a stiff separator of the Ig and EGF domains, thus keeping them exposed for molecular interaction. Nevertheless, bacterially expressed NDFs, which are not glycosylated, possess full capacity to induce tyrosine phosphorylation of Neu (9, 22).

EGF-Like Domain. This motif is defined by six cysteine residues that are characteristically spaced over a sequence of 40 amino acids and are predicted to fold into a typical structure with three disulfide-linked loops. The EGF-like domain functions as the receptor binding site of NDF. Deletion mutants that contain

Table I. Biochemical Characteristics of Ligands That Interact with Neu/ErbB-2

Name	Isoform	Source	Protein	Receptor binding/ activation	Unique activities/ properties	Reference/ GeneBank accession #
NDF	α2c	Rat1-EJ (Ras-transformed Rat1 cells).	44 kDa glycoprotein, secreted into the medium. 60–75 kDa proNDF present in cell lysates.	Phosphorylation of Neu in breast, colon and neuroblastoma cells, and cross-linking to Neu in phosphorylation-responsive cells. No activity on Neu-expressing ovarian cells and fibroblasts.	Induces cell growth arrest at G_2/M and differentiation of AU-565 breast cancer cells. Slow processing, (proteolytic-cleavage)	8, 21 S35165; UO2324
	α2a	Rat1-EJ.	40–44 kDa secreted glycoprotein. 95 kDa proNDF.	Phosphorylation of Neu in MDA-MB 453 cells.	Rapid processing, (proteolytic-cleavage).	21 UO2323; UO2321
	α2b	Rat1-EJ.	40–44 kDa secreted glycoprotein. 60–75 kDa proNDF.	Phosphorylation of Neu in MDA-MB 453 cells.		21 UO2316; UO2317
	β1	PCR amplified cDNA of rat neural tissue.	40–44 kDa secreted glycoprotein. 60–75 kDa proNDF.	Not tested.	Present mainly in neural tissues.	21 Not Submitted
	β2a	Rat1-EJ.	40–44 kDa secreted glycoprotein. 95 kDa proNDF.	Phosphorylation of Neu in MDA-MB 453 cells.		21 UO2318
	β3	Rat1-EJ.	30 kDa nonglycosylated protein.	No secreted form. The intracellular form not tested.	Not secreted.	21 UO2315
	β4a	Rat1-EJ.	40–44 kDa secreted glycoprotein. 95 kDa proNDF.	Phosphorylation of Neu in MDA-MB 453 cells.		21 UO2322
Heregulin	α2a	MDA-MB-231 (human breast cancer cell line).	45 kDa secreted glycoprotein.	Phosphorylation of Neu in breast cancer cell lines and cross-linking to Neu in the same cells.	Mitogenic in SKBR-3 and MCF-7 breast cancer cell lines.	9 M94165
	β1a	MDA-MB-231.	45 kDa secreted glycoprotein.	Phosphorylation of Neu in breast cancer cell lines and cross-linking to Neu in the same cells.	Mitogenic in SKBR-3 breast cancer cell line.	9 M94166
	β2a	MDA-MB-231.	Not studied.	Phosphorylation of Neu in breast cancer cell lines.	Not studied.	9 M94167
	β3	MDA-MB-231.	Protein is not secreted.	The intracellular form was not tested.		9 M94168
ARIA	β1a	Chick brain.	33–42 kDa secreted protein.	Phosphorylation of Neu in MDA-MB 453 cells.	Stimulates the synthesis of muscle acetylcholine receptors. No spacer domain.	11 L11264
GGFII-GGFHBS5	β3-kringle	Human brain and spinal cord cDNA.	45 kDa secreted protein.	Phosphorylation of a 185 kDa Schwann cell protein.	Mitogenic to Schwann cells. Contains a putative kringle domain and a signal peptide in the N-terminal. No spacer domain.	10 L12259
GGFHFB1	β3	Human brain/spinal cord cDNA.	Protein not secreted.	Not studied.	Mitogenic to Schwann cells. Not secreted.	10 L12260
GGFBPP5	β3	Bovine pituitary cDNA.	Protein not secreted.	Not studied.	Mitogenic to Schwann cells. Not secreted.	10 L12261

only this domain fully retain their affinity to the receptor. Conversely, a mutant that contained only the first two disulfide loops of the EGF-like domain displayed no specific cellular binding and could not displace cell-bound NDF (21). Two types of the EGF-like domain are found in the NDF family: α and β. The β1 isoform binds to mammary cells with an affinity that is approximately 10-fold better than the α2 isoform, and this was attributed to the α/β variation rather than to the 1/2 differences (21).

Juxtamembrane Domain. The stalk that connects the EGF domain to the transmembrane domain

undergoes proteolysis upon release of soluble NDF from the cell. This domain displays the most extensive variation, as five different variants of it exist. The subtypes range in size from a single amino acid (Isoform 2) to 27 amino acids (Isoform 4, see Fig. 2). Isoforms 3 and 5 terminate in a stop codon and thus code for proteins that lack a transmembrane region. A putative proteolysis site (Lys-Arg) is shared by all of the transmembrane forms of NDF, but the various juxtamembrane domains may contain additional proteolysis sites.

Transmembrane Domain. The transmembrane domain is composed of 23 amino acids that are identical in all forms of the transmembrane NDFs. Isoform β3 has essentially the entire extracellular portion of NDF but lacks the transmembrane domain because of a stop-codon in the juxtatransmembrane domain. This isoform is not released from transfected COS-7 cells. Its function as Neu activator is intact in cell lysates (9).

Cytoplasmic Domain. All of the transmembrane isoforms contain a common sequence of 157 amino acids, followed by two variable regions (a or b), or by a stop codon (Isoform c). The a isoform extends for an additional 217 amino acids, and Isoform b extends for an additional 39 different amino acids. The longest isoform, namely α2a, is processed most rapidly, while the shortest isoform, α2c, undergoes slow processing and release (21).

Perspectives

Although different NDF isoforms activate tyrosine phosphorylation of Neu, not all cell lines that express Neu bind to NDF. Specifically, ovarian cancer cell lines (SKOV-3) and transfected fibroblasts that express Neu do not bind NDF (22), suggesting that a still undefined molecule acts as a receptor for NDF. The expected pattern of expression of this putative receptor corresponds to the tissue distribution of the recently identified homolog of Neu, namely HER-4/ErbB-4. The multiplicity of NDFs raises the question of the function and biological role of specific isoforms. NDFs are mitogenic to epithelial and neuronal cells, promote differentiation in certain breast cancer cells, and increase the synthesis of acetylcholine receptors. Since these effects were observed in different cell lines, as well as with different ligands, it is unclear whether the multiple responses are due to different isoforms, different receptor subtypes or different downstream effectors in specific cells. Similarly, the biological role of the transmembrane topology of the NDFs and the function of the relatively long cytoplasmic domains are unknown. Although our knowledge regarding NDFs has grown significantly, it is clear that we have only scratched the surface in understanding the true role these factors play in development, differentiation, and oncogenesis.

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