

The nucleocrine pathway comes of age

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Abstract

More than 20 years ago, it was initially predicted that hormones and growth factors might promote cell growth by binding and thereby inactivating tumor suppressors, as exemplified by the proposed complex formation between insulin and retinoblastoma protein (RB). This mainly intracellular/nuclear growth-regulatory circuit was termed “the nucleocrine pathway” and the physical interaction between insulin and RB was subsequently proven through several methods, primarily by immunofluorescence and co-immunoprecipitation. Meanwhile, additional nucleocrine pairs have emerged through further experimental studies, specifically the FGF1-p53 and angiogenin-p53 heterodimers. Moreover, first experimental clues have been obtained as to the intranuclear presence of the previously surmised heterodimer between the EGF precursor and the p130 tumor suppressor. In addition, RB-binding motifs have recently been discovered in interleukin-6 (IL-6) and cellular apoptosis susceptibility (CAS) protein. These findings point to a more general significance of the nucleocrine pathway in cell growth regulation and as a particularly useful target in cancer therapy.

Keywords: nucleocrine pathway, hormone, growth factor, tumor suppressor, insulin, retinoblastoma protein (RB).

More than a century ago, Ernest Starling introduced the term “hormone” into medical science [1] and almost 50 years ago, Charles Huggins delivered his Nobel Lecture on the “endocrine-induced regression of cancers” [2], in which he outlined that “hormones are of crucial significance for survival of several kinds of hormone-responsive cancers”. Meanwhile, evidence has accumulated to a major extent as to the central role of host-derived hormones and growth factors in cancer pathophysiology [3]. Such conceptual overlap between endocrinology and oncology – that was initially based on the studies regarding “endocrine” and “paracrine” growth-regulatory pathways – received an additional dimension when Michael Sporn and Anita Roberts recognized the principle of “autocrine” cell growth [4]. Accordingly, some tumor cells can produce and respond to their own hormones and growth factors, thus potentially rendering them independent from their host environment. The latter was postulated to apply particularly to host-derived hormones as well as growth factors and thus to contribute to the so-called growth autonomy of some types of malignancies.

As a variant on the autocrine theme, Bert O’Malley suggested a few years later that there might also exist an “intracrine” pathway of (malignant) cell growth whereby the hormone/growth factor, after its cellular synthesis, is not being secreted (and, as a result, may not interact with its cell surface receptor), but instead remains intracellularly where it can, for instance, bind its equally intracellularly retained and/or internalized receptor [5]. This “intracrine” concept – that was proposed to include also functional intracellular hormone and growth factor subunits [6] – has been validated experimentally not only for IL-3 [7] and FGF-3 [8], but also for many other growth factors over the past two decades [9–11]. In this context, it is notable that some growth factors such as basic FGF (*i.e.*, FGF-2) may be retained intracellularly and promote cell growth in an intracrine manner even though they carry a signal peptide for secretion [6, 12].

More than 20 years ago, I then expanded the endocrine, paracrine, autocrine and intracrine principles by proposing a novel concept according to which (host- and/or tumor-derived) hormones and growth factors might promote cell growth by binding and thereby inactivating tumor suppressors [6, 13]. Since these latter proteins (that are different or distinct from classical hormone receptors) are mainly located inside the cell, particularly in the cell nucleus, I subsequently termed this signal transduction mechanism the “nucleocrine” pathway [14]. Its paradigm has been the complex formation between insulin and the key tumor suppressor retinoblastoma protein (RB) and this interaction was predicted based on an analysis of the amino acid sequences of these two molecules [6, 13, 14]. A summary of the nucleocrine and endocrine principles can be gathered from Table 1.

Initial experimental clues for the existence of the anticipated insulin-RB heterodimer were obtained by means of the ELISA method [15]. Subsequently, the intracellular complex formation between insulin and RB was proven by immunofluorescence studies that involved epitope masking and unmasking aspects [16] as well as by co-immunoprecipitation of endogenous insulin and RB [17, 18] and, moreover, through precipitation of RB with agarose-bound insulin [17]. Importantly, it was also shown that the binding of insulin to RB coincided with increased cell proliferation [16], in keeping with the previously advanced hypothesis [6, 13, 14].

Meanwhile and intriguingly, further nucleocrine pairs have emerged. Specifically, it was shown that acidic fibroblast growth factor (FGF) or, respectively, FGF1 – which is a potent growth factor – physically interacts with the p53 tumor suppressor in the cell nucleus in order to prevent p53-mediated cellular apoptosis [19]. Moreover, the pro-angiogenic growth factor angiogenin (AGN) equally forms a nuclear complex with p53 and, as a result, promotes cell survival by inhibiting various aspects of the pro-apoptotic function of p53 [20].

Table 1 – Overview of endocrine and nucleocrine principles. The examples (a), (b), and (c) represent endocrine or, respectively, nucleocrine pairs of ligands and their corresponding interaction partners. The paracrine, autocrine and, respectively, intracrine principles can be regarded as variations on the endocrine theme in that they equally rely on physical interactions between growth factors and their corresponding receptors, albeit the range of their actions is spatially more limited, specifically at the tissue, cellular and, respectively, intracellular levels (vs. the systemic, organism-wide range of endocrine actions)

Mode of action	Ligand	Interaction partner for ligand	Preferential location of interaction
Endocrine	<i>Hormone/Growth factor (GF)</i> Examples: (a) insulin; (b) FGF-1; (c) angiogenin.	<i>GF receptor</i> Examples: (a) insulin receptor; (b) FGF-1 receptor; (c) angiogenin receptor.	Extracellular/Cell surface
Nucleocrine	<i>Hormone/Growth factor (GF)</i> Examples: (a) insulin; (b) FGF-1; (c) angiogenin.	<i>Tumor suppressor</i> Examples: (a) RB; (b) p53; (c) p53;	Intracellular/Cell nucleus

Both of these experimentally proven interactions are entirely consistent with the theoretical considerations and predictions advanced in the initial 1995 paper on the nucleocrine pathway [14], which specifically stated: “... I am now proposing the adoption of the term ‘nucleocrine’ to specifically denominate those subsets of endocrine, paracrine, autocrine and intracrine effects that, subsequent to or instead of an initial interaction of insulin or any other nuclear growth factor with its receptor on the cell surface or intracellularly, may involve an association of the respective growth factor (and its receptor) with a distinct tumour suppressor protein in the cell nucleus... An important corollary of this prediction is, for instance, the existence of a nuclear tumour suppressor which is targeted by fibroblast growth factor (FGF) as part of the latter’s nuclear actions given the evidence for a direct involvement of FGF in gene regulation (Mason, 1994). Conversely, it is conceivable that there are growth factors and/or their receptors which influence gene expression through binding to the central tumour suppressor p53.”

In addition to these insulin-RB, FGF1-p53 and angiogenin-p53 nucleocrine complexes, it was also demonstrated by immunofluorescence (Figure 1) that the EGF precursor protein [21] co-localizes in the cell nuclei of HepG2 human hepatoma cells with the p130 tumor suppressor protein, a member of the RB family of proteins [22], specifically only in proliferating cells, but not in quiescent cells.

On the one hand, this result partly confirmed a previous structure-based prediction on the physical interaction between these two proteins [23]. On the other hand, it is reminiscent of the differential nuclear localization of IGFBP-3 that equally occurs only in proliferating cells, not in resting cells [24]. In this context, it is interesting to note that, by contrast to human IGFBP-3, which contains a nuclear localization sequence (NLS) that is bipartite [25], the human EGF precursor harbors a putative NLS similar to the NLS of HIV-1 Tat protein (R. T. Radulescu, unpublished observation).

Moreover, this nuclear localization of the EGF precursor parallels the presence and growth-regulatory actions of other growth factor *precursors* in the cell nucleus, such as the EGF-like precursor [26] and the IL-1 α precursor [27].

Furthermore, the recent demonstration [28] of the equally intracellular, albeit cytoplasmic complex formation between the growth factor alpha-fetoprotein (AFP) and the tumor suppressor PTEN should be considered as an additional example for a relevant interaction between a growth factor and a tumor suppressor and as a (cytoplasmic) variant on the nucleocrine theme.

In this context, it might be worthwhile to explore whether the “gating” of nucleocrine interactions is controlled by specific (cytoplasmic) growth factor proteases, thus extending the paradigm of insulin-degrading enzyme (IDE) that degrades insulin and thereby prevents it from translocating to the cell nucleus and, as a result, from subsequently binding and inactivating (nuclear) RB [29, 30]. Such proteases specifically degrading FGF1, AGN and, respectively, AFP, if identified, may represent, similar to IDE [29, 30], further candidate tumor suppressors that preserve or protect the functions of the tumor suppressors p53 and PTEN by degrading their growth-promoting ligands. Consistent with this concept, it has been demonstrated that a FGF1-specific single-chain antibody blocks the nuclear translocation of FGF1 and, consequently, inhibits tumor growth and metastasis [31]. Along similar lines, the ribonuclease inhibitor protein has been shown to display anti-tumor effects *in vivo* [32] and, more recently, to bind AGN in the cytosol which may also interfere with the latter’s nuclear translocation [33].

Most recently, I discovered RB-binding motifs in the growth factors interleukin-6, briefly: IL-6 (Figure 2a) and cellular apoptosis susceptibility (CAS) protein (Figure 2b), suggesting that each of them may bind and thereby inactivate the RB tumor suppressor, thus promoting cell growth through this nucleocrine mechanism.

More specifically speaking, I have detected, on the one hand, the LXSXE RB-binding motif, which is similar to the classical LXCXE RB-binding motif in human IL-6 (Figure 2a). This LXSXE motif equally occurs in the protein phosphatase type 1 catalytic subunit, briefly PP-1 α (Figure 2a) and has previously been surmised to mediate this protein’s binding to RB [34].

This proposed *direct* RB inactivation by IL-6 is in line with reports on the intracrine role of IL-6 [35, 36] and, moreover, complements the previously unraveled *indirect* RB inactivation ensuing from increased phosphorylation of RB caused by IL-6 [37].

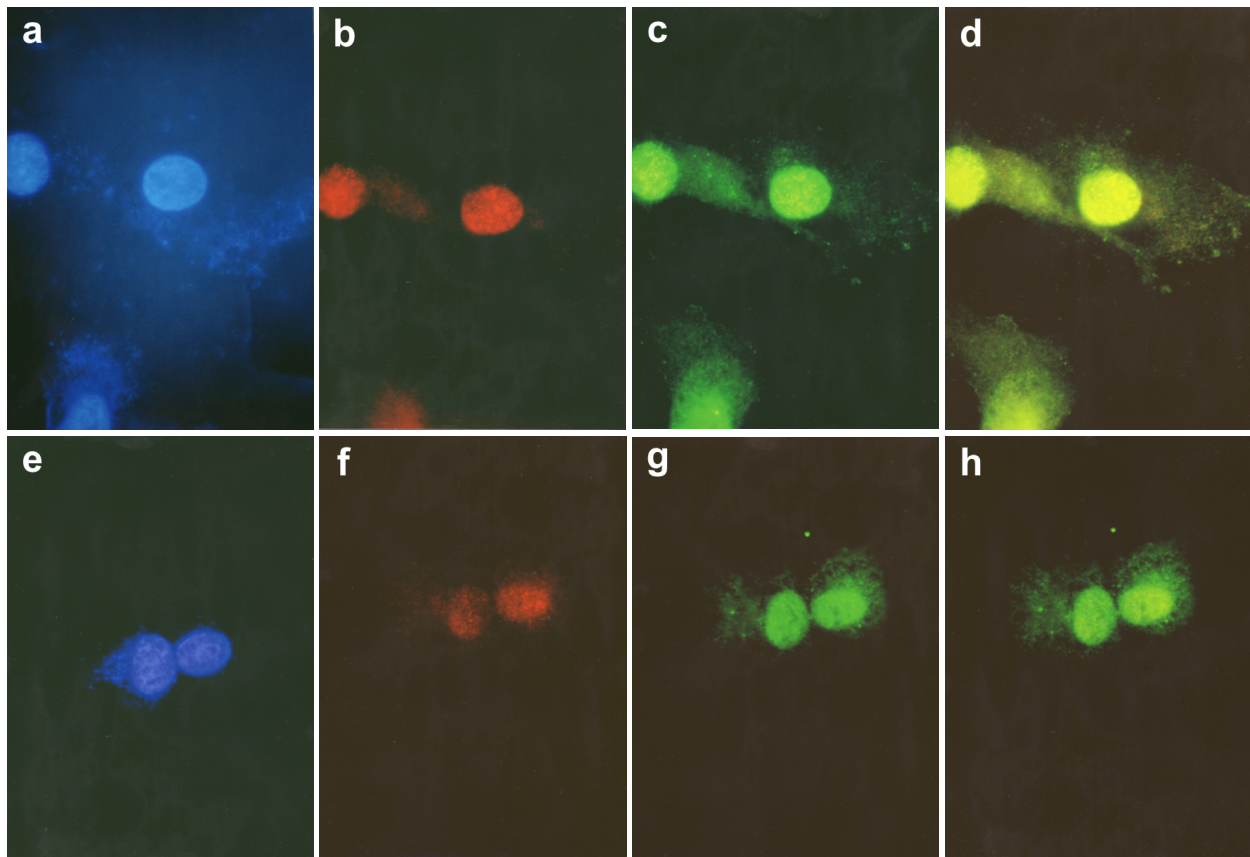


Figure 1 – Nuclear co-localization of EGF precursor protein with 130 protein occurs to a greater extent in HepG2 human hepatoma cells stimulated by insulin (a–d) than in starved (growth-arrested) cells (e–h), i.e., cells that have not been stimulated by a growth factor such as insulin. Insulin-stimulated, proliferating cells (upper row): nuclei (a), nuclei with antibody to nuclear EGF precursor (b), nuclei with antibody to nuclear p130 (c) and overlay or, respectively, merge of the preceding two pictures (d). Quiescent cells (lower row): nuclei (e), nuclei with antibody to nuclear EGF precursor (f), nuclei with antibody to nuclear p130 (g) and overlay of the preceding two pictures (h). The experimental protocol was briefly as follows: HepG2 cells were seeded on cover slips, allowed to adhere and subsequently incubated with RPMI/5% FCS for 24 hours. The experiment proceeded with a 24 hour-period of serum starvation after which some of these cells were further serum starved for another 24 hours, whereas others were growth stimulated with bovine insulin (Sigma) at 100 µg/mL RPMI/5% FCS equally for 24 hours. Following this treatment, all cells were processed according to a previously described protocol [16]. Briefly, cells were fixed with 2% paraformaldehyde for 30 minutes at 4°C and permeabilized with 0.2% Triton X-100 for 5 minutes at room temperature. Cells were then incubated with primary antibodies for 90 minutes and subsequently with fluorescently labeled secondary antibodies for 60 minutes. Finally, nuclei were counterstained with Hoechst 33342 dye (Serva Feinbiochemica) and mounted with ProLong antifade (Molecular Probes) for immunofluorescence microscopy. Primary antibodies and dilutions were as follows: mouse anti-human EGF precursor antibody M5 (a gift from Johan Stenflo, University of Lund, Malmö, Sweden, cf. Ref. [21]) diluted 1:20 and rabbit anti-human p130 antibody C-20 (Santa Cruz Biotechnology) equally diluted 1:20. Secondary antibodies and dilutions were as follows: Cy3-labeled goat anti-mouse antibody (Amersham Pharmacia Biotech) diluted 1:50 and Alexa 488-labeled goat anti-rabbit antibody (MoBiTec) equally diluted 1:50. Stained cells were examined using an Axioskop microscope (Zeiss) with a ×100 objective. Images were captured using 400 ASA slide films (Kodak).

a) **Leu Lys Ser Arg Glu** PP-1alpha₅₁₋₅₅

b) **Leu Thr Ser Ser Glu** human interleukin-6₇₇₋₈₁

Figure 2a – Alignment of LXSXE RB-binding motifs in PP-1alpha (a) and human interleukin-6 (b). Amino acids are displayed in three-letter code. Crucial residues are highlighted in bold letters.

Furthermore, I have identified the LXFEXE RB-binding motif, i.e., another LXCXE-like motif, in CAS (Figure 2b). This LXFEXE motif is present in the viral oncoprotein Tax (Figure 2b), and has been implicated as an RB-binding motif [38].

This proposed *direct* RB inactivation by CAS is in

a) **Leu Leu Phe Glu Glu** HTLV-1 Tax oncoprotein₃₀₆₋₃₁₀

b) **Leu Val Phe Thr Glu** human CSE1L/CAS₆₄₆₋₆₅₀

Figure 2b – Alignment of LXFEXE RB-binding motifs in viral Tax (a) and human CAS (b). Amino acids are displayed in three-letter code. Crucial residues are highlighted in bold letters.

line with reports on the pro-metastatic role of CAS [39] and its intranuclear presence [40].

The anticipated heterodimers between IL-6 and RB and, respectively, CAS and RB are remarkable also in the light of the fact that IL-6 and CAS are candidate markers for oncoprotein metastasis, briefly: OPM [41]. Hence,

these presumed heterodimers considerably strengthen the potential importance of the nucleocrine pathway in OPM that has initially been proposed to apply to the (RB-binding) insulin molecule [42], but may also include the (p53-binding) FGF1 protein considering its presence in the serum of patients with (advanced) cancer disease [43, 44]. The latter is also interesting given the fact that FGF1 lacks a signal peptide, thus suggesting the involvement of an alternative, signal peptide-independent secretory pathway in its release [45].

In conclusion, the data presented here suggest that the nucleocrine pathway is employed by various hormones and growth factors in order to decisively influence cell fate. By contrast to signal transduction pathways from the

cell membrane to the nucleus [46–48] and cytoplasmic/nuclear growth-regulatory processes [49–53] that involve **many relay stations** (Figure 3) to convey a cell proliferation signal and are thus characterized by a high degree of redundancy that translates into a marked tendency to develop resistance to therapy and/or post-treatment disease recurrence, the nucleocrine pathway [14, 16] is rather simple in that it relies on **a single** protein-protein interaction (*i.e.*, between a growth factor and a tumor suppressor) in order to directly (or yet indirectly through just a few mediators, cf. Figure 3) and specifically promote (accelerated) cell growth and survival, hence displaying a low level of redundancy or, respectively, minimal capacity to resist therapy [30, 54, 55].

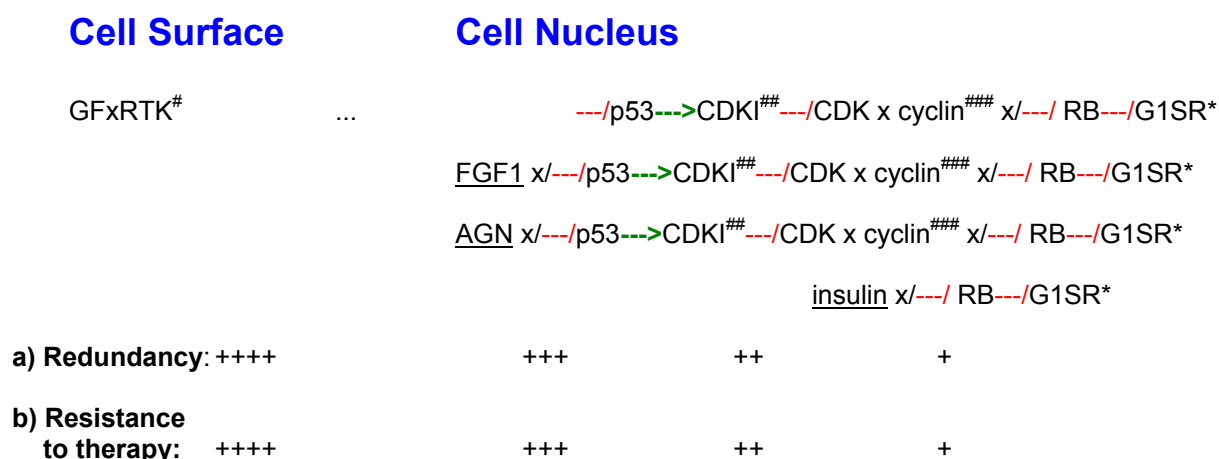


Figure 3 – Overlap of nucleocrine [14] and RB [50] pathways and their relationships to a) redundancy of biological information and b) resistance to therapy. Nucleocrine growth factors are underlined. [#]Mitogenic signal transduction cascades (...) initiated by the binding of growth factors (GFs) to their cell membrane-bound receptors, primarily receptor tyrosine kinases (RTK); ^{##}CDKI: Cyclin-dependent kinase inhibitor, *e.g.*, p21 (N.B. p21 inhibits CDKs and cell growth only at relatively high concentrations, whereas at low concentrations it has the opposite effect); ^{###}*e.g.*, cyclin D1; *G1SR: G1/S restriction point; x: Binding; ---: Inhibition; -->: Activation; x/---: Combined binding and inhibition; p53: p53 tumor suppressor protein; CDK: Cyclin-dependent kinase; RB: Retinoblastoma tumor suppressor protein; AGN: Angiogenin; FGF1: Fibroblast growth factor 1.

Therefore, antiproliferative substances which target the nucleocrine pathway, briefly: anti-nucleocrine pharmaceuticals such as MCR peptides, *i.e.*, RB-derived compounds that interfere with insulin-RB complex formation in cancer cells [16, 18, 56–66] and likely also in morphologically normal, yet intrinsically premalignant cells [42, 54, 64] should be effective anti-cancer therapeutics in the clinical setting.

After decades of drug development in endocrine pharmacology, during which the focus has been on interfering with the action of hormones at the level of their cell membrane receptors (*e.g.*, through beta-adrenergic blockers and angiotensin antagonists for the treatment of hypertension and through the Herceptin antibody for the therapy of a subset of breast cancer), the knowledge base has meanwhile reached a stage that enables to go beyond these receptor-directed approaches and also to conceptually bypass so-called second messengers. Accordingly, a clinical paradigm shift is within reach that consists of drugs that directly block the action of hormones (as *primary* messengers) inside the cell, hence contributing to establishing a fundamentally novel intracrine and nucleocrine pharmacology. This would entail

that these drugs prevent intracellular hormones/growth factors from inactivating tumor suppressors and, as a result, maintain or restore the beneficial and protective functions of tumor suppressors for the health of the entire organism.

In this context, the discovery and validation of individual tumor suppressor peptides of a potential therapeutic interest could be implemented by initially determining their recognition and binding of a specific hormone/growth factor *in vitro* [15], in keeping with the nucleocrine principle [14], and by subsequently measuring their activity in a semi-quantitative and rapid manner by a sort of plaque-forming assay (Figure 4), in analogy to those plaque-forming assays previously reported by Fleming *et al.* in bacteriology in 1929, Delbrück *et al.* in bacteriophage virology in 1939 and by Jerne *et al.* in immunology in 1963. Such proposed novel bioassay – a proposed “suppressogram”, in analogy to the antibiogram – should considerably advance and accelerate the development of many new tumor suppressor-based drugs – for which the RB-derived MCR peptides [66] might serve as an important paradigm – for the effective treatment of cancer and infectious diseases.

Untreated half of plate

Tumor suppressor peptide-treated
half of plate

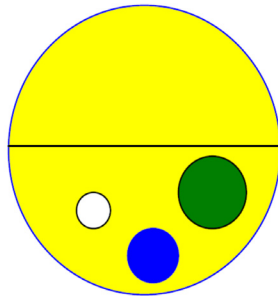


Figure 4 – Proposed plaque-forming assay for the biological evaluation of distinct hormone/growth factor-binding tumor suppressor peptides, e.g., against cancer cells or bacteria. The antineoplastic/antibacterial activity of such peptides could be quantified by the diameters of visible plaques which reflect the efficiently treated areas of cancer cell or, respectively, bacterial cultures (symbolized by yellow color). Depending on their specific growth characteristics, cancer cells could be cultured in soft agar (if they are reproducing in suspension) or, respectively, might be attached to the plate (if they grow by adherence). Similar to the principles of the antibiogram or, respectively, plaque assay in bacteriology, the diameters of the above-depicted “suppressogram” plaques would correlate with the potency of the respective tumor suppressor peptides or, respectively, their minimal inhibitory concentration (MIC). Accordingly, in the above-specified diagram, the three plaques reflect the distinct effects (i.e., the white, blue and green plaques symbolize low, moderate and high activities, respectively) of three different tumor suppressor peptides (e.g., derived from p16, p53 and, respectively, RB) depending on the cells’ resistance to the treatment with each of these peptides.

Conflict of interests

The author declares that he has no conflict of interests.

Acknowledgments

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