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Differentiation and Malignant Transformation: Two Roads Diverged in a Wood

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Abstract Growth factors and their receptors are known to send at times contradictory signals, such as proliferation or differentiation. Recent developments in our knowledge of growth factor receptors and their signaling pathways have clarified the basis for this puzzling behavior. Separate domains of a receptor and/or the availability of specific substrates determine the fate of a cell stimulated by the same growth factor. In some models, the difference between malignant transformation and differentiation (leading to cell death) may depend on the presence or absence of a single agonist or antagonist molecule. The type 1 insulin-like growth factor receptor will serve as the paradigm for this review. J. Cell. Biochem. Suppls. 32/33:68–75, 1999.

Growth of any population of cells, whether normal or abnormal, depends on the environmental signals, among which growth factors (either stimulatory or inhibitory) are perhaps the most important. Until recently, stimulatory growth factors were considered merely as mitogenic agents. In recent years, a new aspect of growth factor's action has emerged, that is, the ability of growth factors and/or their receptors to protect cells from cell death in general and apoptosis in particular. The anti-apoptotic effect of growth factors is perfectly compatible with their mitogenicity, as both actions favor the growth of cell populations, normal or abnormal. But there is another aspect of growth factor action that is more difficult to explain, and that is their ability to send contradictory signals. We are not referring to growth stimulatory versus growth inhibitory growth factors. These are different signals originating from different growth factors. We are referring to the same growth factor or growth factor receptor sending opposite signals. We will give three of the best known examples, but other examples can be easily found: (1) transforming growth factor-β (TGF-β) has long been known to be mitogenic for certain cell types (usually epithelial cells) and to cause inhibition of growth in other cell types [Moses et al., 1985]; (2) epidermal growth factor (EGF) is generally considered a stimulatory growth factor, but in certain cells that over-express the EGF receptor, EGF can actually induce growth arrest [Carpenter et al., 1982]; and (3) insulin-like growth factor I receptor (IGF-IR), activated by its ligands, is known to be mitogenic both in vivo and in vitro; it also promotes growth in size of the cell, is one of the most powerful anti-apoptotic receptors, and is quasi-obligatory for the transformation of cells [Baserga et al., 1999]. Yet, the IGF-IR can also induce cell differentiation, an effect that is in clear contradiction to its other functions.

When confronted with these contradictory signals originating from the same receptor, we often take refuge in the explanation that the outcome depends on "the cell context." Such an explanation is obviously not an explanation. Another alternative is simply to ignore the contradictions. Many of us like to play the tribal game of my growth factor is better than yours, my receptor is better than yours, my cell line is better than yours, and so forth. As a consequence, as long as the cells do what we want them to do, we do not care what else they could be doing. Neither of these attitudes is satisfactory. In this review, we will try to convince the

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reader to accept the contradictions and to see whether we can find some explanations for the "cell context." We will take as the main example the IGF-IR, not because of the tribal reasons given above, but because it is the one we know best. We are sure the lesson we can draw from the IGF-IR is valid (with modifications) also for other receptors.

IGF-I-MEDIATED PROLIFERATION AND DIFFERENTIATION

It is known that, in certain hematopoietic cells, the granulocyte colony-stimulating factor (G-CSF) sends at the same time a proliferative and a differentiating signal [Ward et al., 1999]. The cells grow in number, but they also differentiate and, apparently, one depends on the other. Differentiation occurs only if the cells can undergo one or two rounds of replication [Valtieri et al., 1987]. In the case of the IGF-IR, we know that, in most cell types (e.g., mouse embryo fibroblasts like 3T3 cells, human diploid fibroblasts, some epithelial cells), the IGF-IR sends an unambiguous mitogenic signal. Indeed, IGF-I was originally classified as a stimulatory growth factor necessary for the transition of cells from G/1 to S phase [Scher et al., 1989].

But in other cell types, IGF-I and IGF-II can stimulate either proliferation or differentiation, or both. For instance, under certain conditions, myoblasts, osteoblasts, adipocytes, oligodendrocytes, neurons, and hematopoietic cells can be induced to differentiate by IGF-I [reviewed by Petley et al., 1999]. The role of the IGF system in differentiation has been studied in greater detail in myoblasts. Myoblasts in cultures are undifferentiated cells, which can grow indefinitely in serum, but differentiate into myocytes, if the serum is removed or decreased. If, after serum removal, the cells are incubated with either IGF-I or IGF-II, they are stimulated to proliferate, but the stimulation is short-lived and is followed by differentiation [Navarro et al., 1997]. Similarly, if myoblasts are stably transfected to express IGF-II constitutively, they proliferate normally in serum, but undergo enhanced differentiation when they are placed in decreased serum conditions [Stewart et al., 1996]. Differentiation, especially in neurons and hematopoietic cell lines, is usually followed by cell death. We will now examine in more detail the response of hematopoietic cells to IGF-I, taking 32D cells as a model.

THE CASE OF 32D CELLS

32D cells are murine hematopoietic cells, which undergo apoptosis within 24 h after withdrawal of interleukin-3 (IL-3) [Zhou-Li et al., 1997]. An important characteristic of 32D cells is that they have low levels of IGF-I and insulin receptors, and no IRS-1 (or IRS-2), one of the major substrates for both receptors [Wang et al., 1993; Zhou-Li et al., 1997]. When 32D cells over-express the IGF-IR, they survive in the absence of IL-3 and, with the addition of IGF-I, they actually grow for about 48 h [Valentinis et al., 1999; Soon et al., 1999]. The cells then begin to differentiate along the granulocytic pathway, eventually decreasing in number [Valentinis et al., 1999], as one would expect from terminally differentiated cells [Maruoka et al., 1997]. The fact that 32D cells do not express IRS-1 gave an important clue to the question: why does the IGF-IR, usually so mitogenic, induce differentiation of 32D cells? Indeed, if the cells overexpressing the IGF-IR are stably transfected with the IRS-1 cDNA, the cells no longer differentiate [Valentinis et al., 1999], grow indefinitely in the absence of IL-3, and actually form tumors in animals. Conversely, if 32D cells are transfected with a plasmid expressing Shc proteins (another major substrate of the insulin and IGF-I receptors), they rapidly differentiate. To complete the story, a dominant negative of Shc will partially reduce differentiation in 32D cells that express the IGF-IR [Valentinis et al., 1999]. Thus, at least in the case of 32D cells and the IGF-IR, the "cell context" is simple: when IRS-1 is the predominant substrate, the cells are programmed for proliferation. If Shc proteins predominate, the cells have a tendency to differentiate. In other words, the cell context is the availability of individual substrates for the IGF-IR.

There are some interesting corollaries to these findings. In the first place, 32D cells that overexpress both the IGF-IR and IRS-1 not only do not differentiate, but they actually undergo malignant transformation. As mentioned above, they can be passaged indefinitely in the absence of IL-3 and form tumors both in syngeneic and in nude mice. 32D cells expressing only the IGF-IR cannot be passaged without IL-3, nor do they form tumors in animals, as one would expect from cells undergoing differentiation. If 32D cells over-express only IRS-1, they do not even survive in the absence of IL-3

[Zamorano et al., 1996; Zhou-Li et al., 1997; Valentinis et al., 1999]. Thus, IRS-1 or the IGF-IR, singly, cannot transform 32D cells, or even induce their prolonged survival. In combination, they cause malignant transformation of 32D cells. If the IGF-IR is over-expressed in cells that already have IRS-1, as for instance in myoblasts, it inhibits differentiation and causes transformation [Navarro et al., 1997]. In the case of 32D cells, the presence of a single transducing molecule makes the difference between malignant transformation and terminal differentiation.

Another intriguing aspect of this system (32D/IGF-IR cells vs 32D/IGF-IR/IRS-1 cells) is that 32D IGF-IR cells are stimulated to proliferate for the first 48 h, before they differentiate. Therefore, the difference between these two cell lines, in terms of their response to IGF-I, has to do with transformation, not with mitogenesis. This is very important, because it is customary to determine the mitogenicity of the IGF-IR (or the insulin receptor, or IRS-1) by measuring thymidine incorporation during the first 24 h after stimulation with the appropriate ligand. Clearly, with this assay, we would have not been able to detect much difference between 32D IGF-IR and 32D IGF-IR/IRS-1 cells, as they are both stimulated to proliferate by IGF-I, at least for the first 2 days. We also know that certain markers of differentiation in hematopoietic cells can be detected very early, while the cells are still proliferating [Borregard and Cowland, 1998]. It seems that a differentiating agent, such as IGF-I or G-CSF, can induce simultaneously the proliferative and the differentiating programs, with the latter eventually prevailing. One could speculate that the main function of IRS-1 in this model is to inhibit the activation of the differentiation program, while leaving intact the proliferation program, resulting in continuous cell proliferation.

STRUCTURAL BASIS FOR RECEPTOR DIVERSITY

The next step is even more fascinating. Recently, in an elegant experiment, Fambrough et al. [1999] showed that various mutations of the PDGF-β receptor, which inactivate certain specific signaling pathways, end up activating the same immediate early genes (IEG), that set in motion the cell cycle machinery. Their finding indicates that there is a network of signaling pathways, all leading with some redundancy to the same genes [Pawson and Saxton, 1999]. In other words, no particular receptor domain is absolutely required for mitogenesis (or, at any rate, induction of IEG), as different domains can substitute for others in delivering the same mitogenic signal. This lovely experiment formally provided us with the explanation for an observation we had been making on the IGF-IR for several years. Our observation was that the only mutants of the IGF-IR that did not deliver a mitogenic signal were disabled receptors, that is, receptors that had completely lost their functions. For instance, receptors with a mutation at the ATP-binding site or the tyrosine kinase domain have lost all their functions, including mitogenicity [Baserga et al., 1997, 1999]. But there are several mutants of the IGF-IR that are no longer transforming, or are incapable of sending a differentiation signal, that are still mitogenic [Romano et al., 1999; Valentinis et al., 1999]. Table I summarizes the properties and functions of several mutants of the IGF-IR (I have omitted differentiation, because the studies in this area have not been yet completed). A mutation in the ATP-binding site (lysine 1003) results in a receptor that does not autophosphorylate and fails to transmit a mitogenic signal; it is, in fact, for all practical purposes, a nonfunctional receptor. Similarly, a triple mutation in the tyrosine kinase domain (Y1131,Y1136, and Y1136), results in a receptor that is seriously defective and that fails to transmit either a mitogenic or a transforming signal [Gronborg et al., 1993; Li et al., 1994]. A double mutation at Y950 and the kinase domain also results in an inactive receptor. But a mutation at Y950 only impairs transformation and has no effect on mitogenesis [Romano et al., 1999]. There are, in fact, several mutants of the IGF-IR that are fully mitogenic but no longer transforming; the C-terminus of the receptor is clearly dispensable for mitogenesis but is necessary for transforming activity [Hongo et al., 1996]. The transforming domain can be tentatively located between residues 1245 and 1310, where at least three domains seem to be involved, the tyrosine residue at 1251, the serine residues at 1280–1283 and, more weakly, the residues at 1293–1294. More details and the appropriate references on the domains of the IGF-IR can be found in the review by Baserga et al. [1999].

There is a dissociation not only between mitogenicity and transforming ability (colony formation in soft agar), but also between transform-

TABLE I. Mutational Analysis of the IGF-I Receptor

Type of receptor	No. of recep-tors	Mito-genicity	Trans-form.	Protection from apoptosis
No receptors	zero	−	−	−
Wild-type	50	+	+	+
Wild-type	0.7	−	−	−
Wild-type	1.5	−	−	−
Wild-type	2.2	+	−	+
Wild-type	3.0	+	+	+
KA 1003	>50	−	−	−
Y950	>100	+	−	+
3YF	16	−	−	−
Y950/3YF	20	−	−	−
1280–1283	50	+	−	+
Y1250	>50	+	+	+
Y1251	>100	+	−	+
Y1316	>100	+	+	+
del.1245	>100	+	−	+
Y950/del.1245	20	+	−	−

The type of receptor either is wild-type or has the indicated mutation. Y indicates a tyrosine residue. 3YF is a triple mutation in the tyrosine kinase domain: tyrosines 1131, 1135, 1136. del. means truncation at the indicated residue. Mitogenicity means ability to induce IGF-I mediated cellualr proliferation in stably transfected R-cells. Transformation means ability to make R-cells capable of forming colonies in soft agar. The protection from apoptosis was determined in two different cell types: murine hemopoietic cells stably transfected with the indicated receptors [O'Connor et al., 1997], and in mouse embryo fibroblasts, where apoptosis was induced by OKA [D'Ambrosio et al., 1997] or by anoikis [Romano et al., 1999]. The receptor numbers were reported in the papers mentioned above, were determined by Scatchard analysis, and are expressed as $\times 10^4$ per cell. The R-cells with different numbers of IGF-IR/cell were described by Rubini et al. [1987].

ing ability and ability to protect cells from apoptosis as well. The ability of the IGF-IR to protect cells from apoptosis was tested in murine hematopoietic FL5.12 cells [O'Connor et al., 1997] after interleukin-3 (IL-3) withdrawal, and in murine embryo fibroblasts in anoikis [Romano et al., 1999] or in those treated with OKA [D'Ambrosio et al., 1997]. In all systems, for instance, the IGF-IR truncated at residue 1245 (non-transforming) protects cells from apoptosis. Clearly, transformation and protection from apoptosis can also be separated at the level of the receptor. It will be noted that mitogenicity and protection from apoptosis go together [Peruzzi et al., 1999]: the only exception (the Y950/δ1245 mutant) may be more apparent than real. This mutant, thus far, has been tested for survival only in 32D cells, which do not have IRS-1. We predict that it will protect mouse embryo fibroblasts from anoikis.

When the IGF-IR mutants are tested for their ability to induce differentiation, a similar situation emerges. Both Y950 and the C-terminus, dispensable for mitogenesis, are required for differentiation [Valentinis et al., 1999]. This is true of hematopoietic cells and of neuronal cells in culture [Morrione A., Romano G., Navarro M., Reiss K., Valentinis B., Dews M., Eves E., Rosner M.R., and Baserga R., submitted]. It seems that there is an essential receptor, necessary and sufficient for mitogenesis and survival, and a deluxe receptor, required for transformation and differentiation. In the case of the IGF-IR, the essential receptor comprises the ATP-binding site and the tyrosine kinase domain. The C-terminus and other residues (especially Y950) confer to the receptor the possibility of modulating other functions, such as transformation and differentiation. This model is obviously related to the findings of Fambrough et al. [1999]: several mutations that can affect IGF-IR signaling will not impair the mitogenicity of the receptor, as other redundant pathways come into action.

THE INSULIN RECEPTOR

An easy way to test this hypothesis is to look at the insulin receptor (IR), that shares 77% homology with the IGF-IR [Ullrich et al., 1986]. An over-expressed IR is mitogenic and protects cells from apoptosis, if the cells have IRS-1, as in mouse embryo fibroblasts [Prisco et al., 1999]. In 32D cells, the IR has zero survival value, the cells over-expressing the IR dying as fast as the parental cells [Peruzzi et al., 1999]. In addition, the IR cannot transform cells that have no endogenous IGF-IR, like R- cells [Miura et al., 1995]. Finally, the IR cannot induce differentiation of hematopoietic cells [Valentinis et al., 1999] or neuronal cells. An example is shown in Figure 1, where we used H19–7 cells, a rat hippocampal neuronal cell line. H19–7 cells grow normally at 34°C but are induced to differentiate at 39°C [Eves et al., 1994]. Figure 1A shows that H19–7 cells over-expressing the IGF-IR differentiate at 39°C when induced by IGF-II or insulin at high concentrations. By contrast, cells that over-express the IR cannot differentiate with either IGF-II or insulin and differentiate only when induced with basic fibroblast growth factor, which induces differentiation even in parental H19–7 cells [Eves et al.,

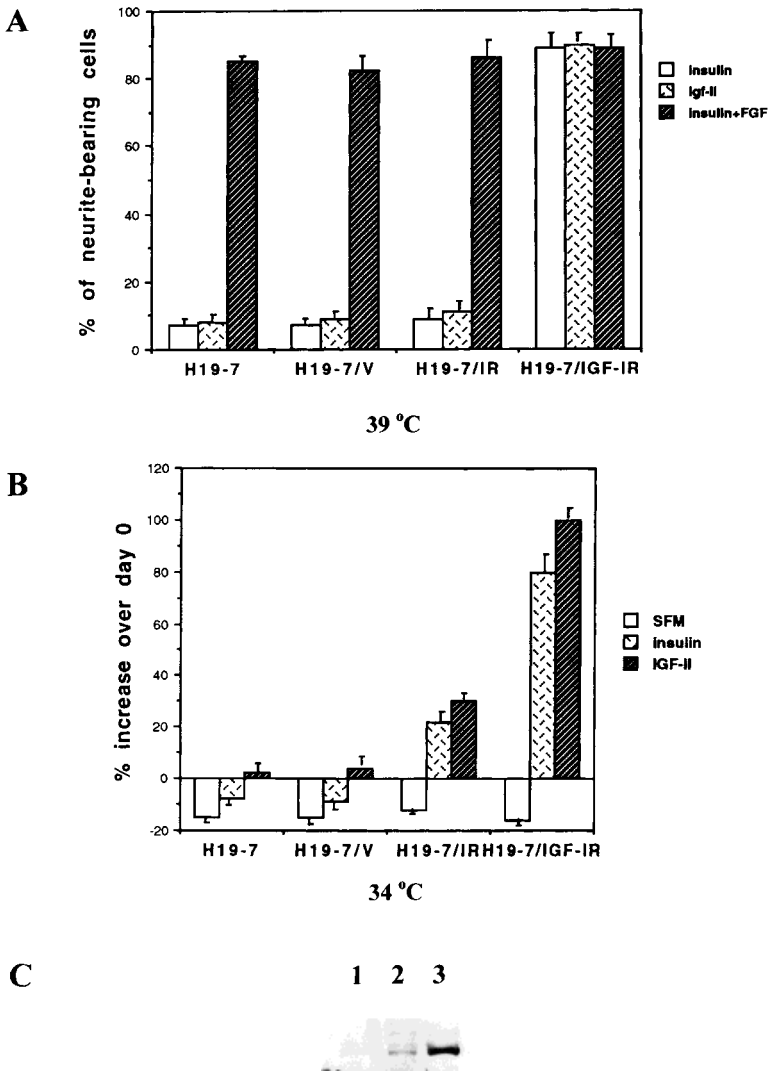

Fig. 1. The insulin receptor cannot differentiate neuronal cells. The cells used were H19–7 cells, a rat hippocampal neuronal cell line, which grows normally in serum at 34°C, but which undergoes differentiation at 39°C, when induced by basic fibroblast growth factor (FGF). Several cell lines are derived from the parental cell lin: a cell line over-expressing the insulin receptor (H19–7/IR), a cell line over-expressing the IGF-I receptor (H19–7/IGF-IR), and a cell line transfected with the empty plasmid vector, used for the other two cell lines (H19–7/V). Only the H19–7/IGF-IR cells can be induced to differentiate by IGF-II or insulin at high concentrations (**A**). All cell lines differentiate with bFGF. At 34°C, the IR is mitogenic in response to both IGF-II and insulin, although not as efficiently as the IGF-IR (**B**). **C:** The IR is over-expressed in H19–7/IR cells (**lane 3**), as compared with parental cell line (**lane 1**), or cells transfected with the empty vector (**lane 2**).

1994]. At 34°C, the IR is mitogenic, albeit not as strongly as the IGF-IR (Fig. 1B). The homologies between the IGF-IR and the IR are very low in the C-terminus, where the deluxe functions of the IGF-IR often localize. As mentioned above, an IGF-IR truncated at residues 1245 (last 92 amino acids) is mitogenic [Hongo et al., 1996], protects cells from apoptosis [O'Connor et al., 1997], but is nontransforming [Hongo et al., 1996] and is defective in inducing differentiation of 32D cells [Valentinis et al., 1999]. Clearly, certain signals originate from the C-terminus of the IGF-IR that are not shared with the IR.

IGF-IR SIGNALING

This review is not meant to discuss the signaling pathways of the IGF-IR, which are very complex and redundant, and which overlap with signaling pathways of other receptors. However, on the basis of our studies with IGF-IR mutants, we offer a schematic diagram of how

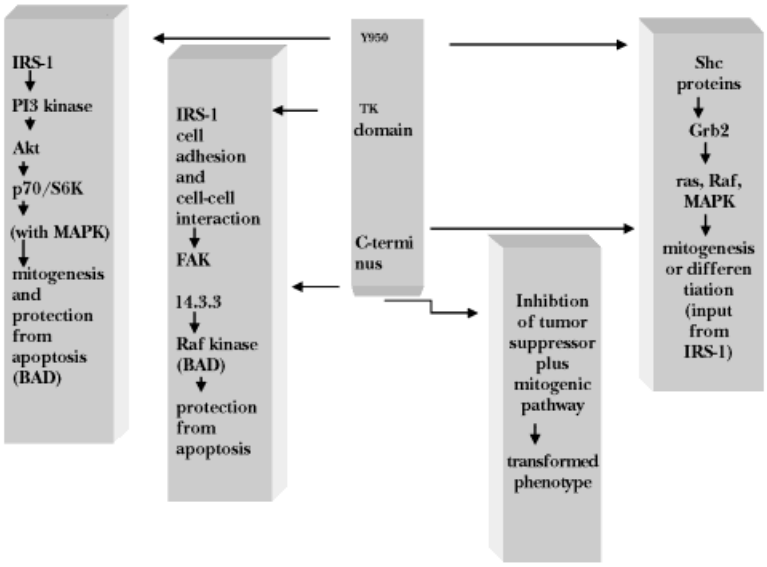

Fig. 2. Schematic diagram of the signaling pathways of the IGF-I receptor. Mitogenicity and protection from apoptosis generally go hand-in-hand and largely depend on signaling through IRS-1, although Y950 and the C-terminus may contribute to it as well, especially in cells that do not have IRS-1. Residues in the C-terminus and Y950 are involved in malignant transformation, but, obviously, malignant transformation also requires mitogenicity. Thus, the IGF-IR must have the mitogenic domains in addition to the transforming domains to induce malignant transformation. Differentiation may use more than one residue or substrate, or both but the most important requirement is weak signaling from IRS-1.

certain domains of the receptor, and certain substrates, contribute to the four major functions of the IGF-IR: mitogenicity, survival, malignant transformation, and differentiation (Fig. 2). Briefly, signaling through IRS-1 promotes cell adhesion, mitogenesis, and protection from apoptosis, while signaling through Shc proteins favors differentiation (if IRS-1 is absent or very low). Malignant transformation requires all the mitogenic signals, plus something else originating from the C-terminus of the receptor. Finally, in the absence of IRS-1, the IGF-IR has alternative pathways for survival, that center on Raf-1 and its translocation to mitochondria [Peruzzi et al., 1999].

TRANSFORMATION VERSUS GROWTH ARREST

We have mentioned that IRS-1 can change the fate of 32D/IGF-IR cells from differentiation to transformation. We hypothesized that, in this system, IRS-1 plays a role similar to that played by the Rho family of proteins in Ras transformation. Recent reports in the literature have indicated that certain cellular oncogenes also send contradictory signals, that is,. transformation in some instances and growth arrest in others. As an example, we shall use Ras, for many years considered as the prototype of a transforming oncogene. It is now accepted that Ras also sends a growth arrest signal, by inducing $p21^{waf1}$ [Olson et al., 1998], and that, to achieve transformation, Ras requires the co-operation of the Rho family of proteins [Khosravi-Far et al., 1995; Qiu et al., 1995]. These proteins seemingly repress the transcription of $p21^{waf1}$ activated by Ras. These results make sense, as $p21^{waf1}$ has always been considered an inhibitor of cell proliferation and an inducer of differentiation [see review by Gartel and Tyner 1999]. Unfortunately, we tested our hypothesis in 32D cells, which are of hematopoietic origin. It turns out that p21WAF1 is not always an inhibitor of cell proliferation. Broxmeyer and coworkers [Mantel et al., 1996] have provided strong evidence that, in hematopoietic cells, $p21^{waf1}$ expression actually stimulates cell proliferation. The most convincing evidence was that marrow cells of p21 −/− knockout mice proliferate poorly, and the mice themselves show impaired hematopoiesis. Thus, when we tested 32D/IGF-IR/IRS-1 cells for inhibition of $p21^{waf1}$ expression, we found none [Valentinis B., Navarro M., Zanocco-Marani T., Edmonds P., Morrione A., Sacchi A., Reiss K., and Baserga R., in preparation].. Despite the fact that our hypothesis was incorrect (a frequent occurrence in research), the contradictory effects of the ras

oncogene remain a good example of how signal transduction can send opposite signals, even halfway through transduction.

CONCLUSIONS

The main conclusion is that contradictory signals from a growth factor receptor are not due to those mysterious causes embodied in the notion of "cell context." At least in the case of the IGF-IR, differentiation or proliferation depends on the domains of the receptor and the availability of its substrates. "Cell context" is therefore reduced to the simple fact that different types of cells express different genes, a concept we have known for decades (all cells of an individual animal have the same genes, but different types of cells express different genes).

The Ras example extends this concept beyond the receptors to their signal transduction pathways. Even more intriguing is the observation that the difference between differentiation (with inevitable cell death) and malignant transformation hangs by a thread. The thread can be a single molecule: IRS-1 in the case of the IGF-IR, or Rho protein(s) in the case of Ras. This should give occasion for some thought, both to a basic scientist as well as to clinicians.

Finally, a secondary conclusion I would like to offer for consideration is the idea of an essential receptor and a deluxe receptor. The essential receptor (whether it is the IGF-IR or other growth factor receptors) provides the essential functions (mitogenesis and survival), while the nonessential domains of the receptor provide modulatory functions, like differentiation or malignant transformation.

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