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Communication of Signals and Responses Leading To Cell Death Using Engineered Regulatory Networks

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ABSTRACT

This paper demonstrates the cell death/ apoptosis pathways considering three inputs Tumor Necrosis Factor (TNF), Epidermal Growth factor (EGF) and Insulin. Basically death pathway is a two process pathway: extrinsic pathway and intrinsic pathway. Later on pathways are converted into truth tables considering '1' as cell survival and '0' as cell death. The truth tables are implemented in VHDL using XILINX ISE, CMOS using SPICE and Fuzzy Logic using MATLAB. Out of three processes Fuzzy Logic is the best implementation in terms of reproducibly, computation time and ease in computation.

Keywords: VHDL, CMOS, Fuzzy

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INTRODUCTION

The signaling system underlying cell death allows the cell to process input signals capturing information coming from the environment of the cell to lead to one of two possible outputs: cell survival or cell death [1, 2, 3]. The system output is typically a phenotypic readout (death or survival); however, it can also be determined by measuring “early” signals that perfectly correlate with the death/ survival output. Examples of such early signals include phosphatidylserine exposure, membrane permeability, nuclear fragmentation and caspase substrate cleavage. The Fig.1 illustrates the system under study linking the three input signaling such as TNF [4], EGF [5, 6, 7] and Insulin [8, 9, 10] and four output signals phosphatidylserine exposure, membrane permeability, nuclear fragmentation and caspase substrate cleavage leading to cell death/ survival.

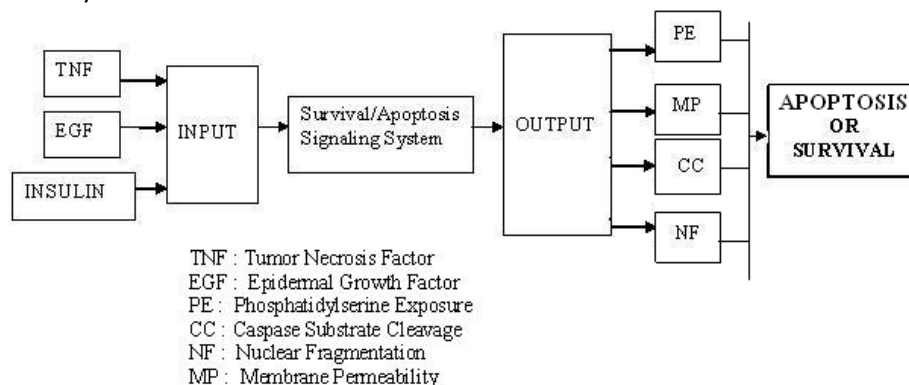


Fig 1 Illustration of the system model of biological cue-signal-response system. In presence of three input signals (in single or in combination) the system model make logical decisions based on the prevailing intracellular and extra cellular conditions and produce a reliable behavior. In general the apoptotic cell death is described based on chromatin condensation, fragmentation of DNA, blebbing of the plasma membrane, and formation of "apoptotic bodies", which are phagocytes by neighboring cells.

Biological systems have the immense capability to generate complex structures from very simple systems. With simple rules and few inputs, a biological system can grow from a single cell to a multi cellular organism in a relatively short time. Biological systems, however, have been accurately synthesizing nano-scale machines for millions of years. Logic gates are the basic building blocks in electronic circuits that perform logical operations. These have input and output signals in the form of 0's and 1's; '0' signifies the absence of signal while '1' signifies its presence. Similar to the electronic logic gates, cellular components can serve as logic gates. In this paper, I make the following contributions:

SPICE

SPICE is a program that simulates electronic circuits in our PC. We can view any voltage or current waveform in our circuit [11, 12]. SPICE calculates these voltages and currents versus time (Transient Analysis) or versus frequency (AC Analysis). Most SPICE programs also perform other analysis like DC, Sensitivity, Noise and Distortion. Today, SPICE is available from many vendors who have added schematic drawing tools to the front end and graphics post processors



to plot the results. SPICE simulators and applications have been expanded to analog and digital circuits, microwave devices, and electromechanical systems [13].

VHDL coding

VHDL is a programming language that has been designed and optimized for describing the behavior of digital systems [14, 15]. It is a hardware description language that can be used to model a digital system at many levels of abstraction, ranging from the algorithmic level to the gate level. The complexity of the digital system being modeled could vary from that of simple gate to the complete digital electronic system or anything in between. The digital system can also be described hierarchically. Timing can also be explicitly modeled in the same description. The resulting VHDL simulation models can then be used as building blocks in larger circuits (using schematics, block diagrams or system-level VHDL descriptions) for the purpose of simulation [16, 17].

FUZZY LOGIC

Fuzzy Logic is a departure from classical two-valued sets and logic that uses "soft" linguistic (e.g. large, hot, tall) system variables and a continuous range of truth values in the interval [0,1], rather than strict binary (True or False) decisions and assignments [18, 19]. Formally, fuzzy logic is a structured, model-free estimator that approximates a function through linguistic input/output associations. Fuzzy rule-based systems apply these methods to solve many types of "real-world" problems, especially where a system is difficult to model, is controlled by a human operator or expert, or where ambiguity or vagueness is common. A typical fuzzy system consists of a rule base, membership functions, and an inference procedure. Fuzzy logic is the part of artificial intelligence or machine learning which interprets a human's actions [20]. Computers can interpret only true or false values but a human being can reason the degree of truth or degree of falsehood. Fuzzy models interpret the human actions and are also called intelligent systems.

BIOLOGY OF APOPTOSIS

Almost all cells, regardless of their phylogenetic origin or physiological specialization, ultimately senesce and die. There are several ways of dying, depending on the nature and severity of the death stimulus, type of the cell affected, and homeostatic conditions of the cell and its surroundings. The two major forms of cell death recognized today are apoptosis and necrosis [10, 21]. The main difference is thought to be the requirement for energy: apoptosis is an active process consuming energy and requiring macromolecular synthesis, while necrosis occurs passively. In spite the fact that there are several features that can distinguish these processes from each other, also common and overlapping characteristics appear, and there are cases where no clear cut distinction between apoptosis and necrosis can be found [22]. While the characterization of differences between apoptotic and necrotic cell death remains incomplete, recent findings suggest that apoptotic cell death differs even from cell to cell, and each induction strategy is likely to involve a unique set of genes shown in Table 1.

Table 1 : Programmed Cell death pathways

APOPTOSIS	NECROSIS
Caspase activation inhibition of mRNA translation	Pro-inflammatory signaling and cytokine production
Condensation of cell and organelles	Swelling of the cell and organelles
Chromatin condensation DNA fragmentation	Mottled chromatin condensation
Loss of membrane asymmetry	Loss of membrane asymmetry
Membrane remains impermeable	Rapid loss of membrane permeability
Cell falls apart into apoptotic bodies	Cell membrane explodes. Remains stay together

Apoptosis is a highly regulated process, being controlled by various ligands and signaling pathways [23]. However, some pathways and events of the apoptotic program have been conserved among species and are considered as mediators of fundamental events in apoptotic signaling and cell death process itself, and therefore, they appear to be of particular significance. These include for example, the action and regulation of the bcl-family of proteins, cytochrome c release from mitochondria which is accompanied with mitochondrial dysfunction, and activation of caspases. The molecular genetic studies in the nematode *Caenorhabditis elegans* (*C. elegans*) have revealed the existence of conserved genes and gene families, such as ced-3 (homologs of mammalian caspases), ced-4 (Apaf-1), and ced-9 (the bcl-family of proteins), which are involved in apoptosis.

SYSTEM APPROACH

During apoptosis, a complex death program is initiated that ultimately leads to the fragmentation of the cell. The death program can be either initiated by the cell itself or by certain external stimuli. These external stimuli may induce apoptosis by targeting one of two pathways [24, 25, 26]. The ‘extrinsic’ pathway is initiated by triggering cell death receptors on the cell surface, leading to activation of the intracellular apoptotic machinery (*death signal-induced, death receptor-mediated pathway*). The ‘intrinsic’ pathway of apoptosis is initiated via the mitochondria by cellular stress, such as chemotherapeutic drugs and radiation (*the stress-induced, mitochondrion-mediated pathway*) (i.e. a caspase-9-dependent pathway) shown in Fig. 2. The elucidation of the molecular mechanisms regulating these processes is of primary interest.

Caspases constitute one of most specific cysteine proteases with an absolute requirement for cleavage site after aspartic acid (hence the name *caspase*). At least four amino acids to the NH₂ terminal side of aspartate serve as the recognition site for cleavage and functional catalysis. The tetra peptide recognition motif differs significantly among caspases, explaining the diversity of their biological functions. The cleavage of protein by caspases is very specific and efficient. Caspases are specific cysteine-rich proteases [24]. The involvement of caspases has been proved by a discovery of CED-3, a cell death gene product of a nematode (*Caenorhabditis elegans*). Caspases are requested for terminal differentiation of specific cell types, whether this differentiation process leads to enucleation or not. These enzymes also play

a role in T and B lymphocyte proliferation and, in some circumstances, appear to be cytoprotective rather than cytotoxic. These pleiotropic functions implicate caspases in the control of life and death but the fine regulation of their dual effect remains poorly understood. Deregulation of the pathways in which caspases exert these non-apoptotic functions is suspected to play a role in the pathophysiology of several human diseases. A total of 12 cysteine proteases known as caspases have been identified in mammals: caspase-1 to -10, caspase-12 and caspase-14. The protein initially named caspase-13 was later found to represent a bovine homolog of caspase-4, and caspase-11 is most likely the murine homolog of human caspase-4 and -5. They are divided into three groups: apoptosis activators like caspase 2, 8, 9, 10 that contain a long prodomain at the N-terminus; apoptosis executioners such as caspase 3, 6, 7 that have a short prodomain and inflammatory mediator with caspase 1, 4, 5, 11, 12, 13 and 14.

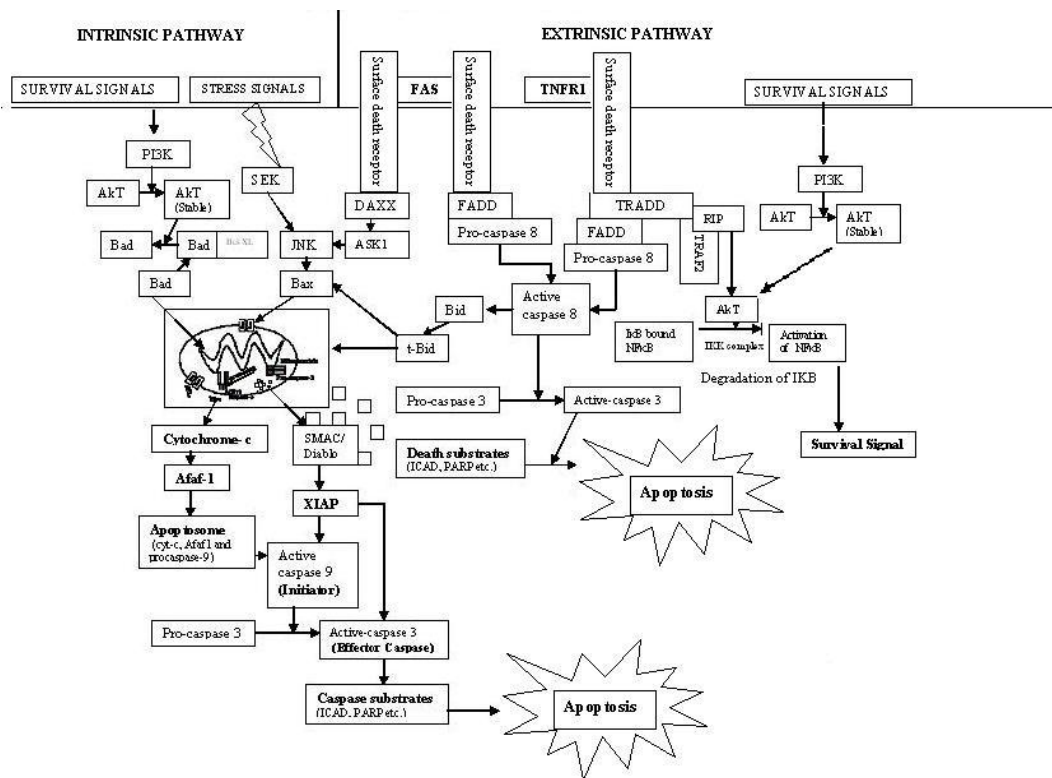


Fig 2. Schematic representing the core components of apoptosis pathways. In the extrinsic pathway, TNF super family members including Fas Ligands binding to a death receptor and forming a death inducing signalling complex (DISC), which activate caspase-8. In the intrinsic pathway, cytochrome c released from mitochondria causes apoptosome formation and caspase-9 activation. Both caspase-8 and caspase-9 activate downstream caspases like caspase-3 and leading to apoptosis.

Death receptor-mediated procaspase-activation pathway

Death receptor-dependent procaspase-activation pathway of caspase-8/caspase-10:

Cell death signals, such as Fas ligand (FasL) and tumor necrosis factor (TNF)-2, can be specifically recognized by their corresponding death receptors, such as Fas or TNF receptor (TNFR)-1, in the plasma membrane. Their binding will in turn activate the death receptors. Fas can bind to the Fas-associated death domain (FADD) (or TNFR-associated death domain, TRADD) and cause FADD aggregation and the emergence of DEDs [24, 27]. These exposed DEDs interact with the DEDs in the prodomain of procaspase-8, which will induce the oligomerization of procaspase-8 localized on the cytosolic side of the plasma membrane. Then a massive molecule complex known as the death-inducing signal complex (DISC) is formed. In DISC, two linear subunits of procaspase-8 compact to each other followed by procaspase-8 autoactivation to caspase-8. The activation of the downstream pathways of caspase-8 varies with different cell types (Figure 1). In Type I cells (cells of some lymphoid cell lines), caspase-8 is vigorously activated and can directly activate the downstream procaspases (e.g. procaspase-3). In Type II cells (other than Type I cells), caspase-8 is only mildly activated and unable to activate procaspase-3 directly. However, it can activate the mitochondrion-mediated pathway by truncating Bid (a pro-apoptotic Bcl-2 family member), a kind of proapoptotic protein in the cytosol, into its active form, tBid. tBid will trigger the activation of the mitochondrion pathway: cytochrome c, apoptosis-inducing factor (AIF) and other molecules are released from mitochondria, and apoptosis will be induced.

The activation pathway mediated by procaspase-10, with a DED-containing prodomain, is similar to that mediated by procaspase-8. Caspase-10 functions mainly in the apoptosis of lymphoid cells. It can function independently of caspase-8 in initiating Fas- and TNF-related apoptosis. Moreover, Fas crosslinking in primary human T cells leads to the recruitment and activation of procaspase-10 [28]. Although caspase-8 and caspase-10 both interact with the DED of FADD in death receptor signaling, they may have different apoptosis substrates and therefore potentially function distinctly in death receptor signaling or other cellular processes.

Death receptor-dependent pro caspase-activation pathway of caspase-2

Once death signals bind to their corresponding death receptors on the plasma membrane, death receptors will be activated. The activated receptors recruit procaspase-2 by adaptors, such as receptor-interacting protein (RIP), RIP associated ICH-1/CED-3 homologous protein with a death domain and TRADD, by means of the prodomain of procaspase-2. Procaspase-2 is activated after the recruitment. Very little has been understood so far concerning the downstream substrates of caspase-2.

Mitochondrion-mediated procaspase-activation pathway

Mitochondrion-mediated procaspase-activation pathway of caspase-8

Apart from being recruited to form a DISC complex after autoactivation, procaspase-8 could also be activated through a cytochrome c-dependent pathway [29, 30]. After cytochrome c is released from mitochondria to the cytosol, caspase-6 is the only cytosolic caspase with the ability to activate procaspase-8, which depends solely on procaspase-6 activation by prodomain cleaving. It means that, in the cytochrome c-dependent pathway, the activation of procaspase-8 requires neither the interaction with FADD nor the formation of a DISC complex.

Mitochondrion-mediated procaspase-activation pathway of caspase-9

When cellular stress (e.g. DNA damage) occurs, proapoptotic proteins in the cytosol will be activated, which will in turn induce the opening of mitochondrion permeability transition pores (MPTPs). As a result, cytochrome c localized in mitochondria will be released to the cytosol. With the presence of cytosolic dATP (deoxyadenosine triphosphate) or ATP, apoptotic protease activation factor-1 (Apaf-1) oligomerizes. Together with cytosolic procaspase-9 [31], dATP and cytochrome c, oligomerized Apaf-1 can result in the formation of a massive complex known as apoptosome. The N-terminal of Apaf-1 and the prodomain of procaspase-9 both have CARDs, with complementary shapes and opposite charges. They interact with each other by CARDs and form a complex in the proportion of 1:1. Activated caspase-9 can in turn activate procaspase-3 and procaspase-7. The activated caspase-3 will then activate procaspase-9 and form a positive feedback activation pathway.

In the mitochondrion-mediated activation pathway, Apaf-1 is a central component of the apoptosome [30]. Apaf-1 has three distinct domains: an N-terminal CARD, a nucleotide-binding domain and 12–13 repeats of WD40 near its C-terminal. At least four different isoforms of Apaf-1 have been found, all of which contain the three domains resulted from the alternative splicing of Apaf-1 pre-mRNA. CARD is responsible for binding the prodomain of procaspase-9, thus it is important in procaspase-9 recruitment and activation. The sequence of the nucleotide-binding domain is very similar to CED-4 in *C. elegans*. For this reason, the domain is also referred to as the CED-4-homologous domain. This domain is responsible for Apaf-1 oligomerization in the presence of cytochrome c and dATP [32, 33]. The dATP-binding ability of Apaf-1 alone is poor, but with cytochrome c it can be greatly enhanced. Procaspase-9 also has a synergic promotion to the binding. WD40 repeats are involved in the interaction of Apaf-1 and cytochrome c. Recently, there have been many reports concerning the activation of caspase-9, which have challenged traditional ideas. Under normal physiological conditions, inactive caspase-9 exists in the form of a monomer. When caspase-9 is artificially crystallized or is recruited by Apaf-1 *in vivo*, the formation of a caspase-9 dimer results in the activation of caspase-9. According to these new results, alternative ideas have been brought forward about how procaspase-9 is activated and what molecules are required during the activation. One view generally held is that, although the prodomain of procaspase-9 is cleaved, the formation of the caspase-9 (or procaspase-9) dimer, rather than the cleavage, is essential to the activation of

caspase-9 [31]. However, under some circumstances, the activation of procaspase-9 may be independent of mitochondrial factors, such as cytochrome c.3

Many proteins involved in this process that interact systematically regulating a specific pathway or cross talk with other proteins of different pathways [34, 35]. As a result many pathways activated simultaneously leading to many biochemical and physiological changes inside the cell. The final outcome of whether a cell dies or survives depends in the concentrations of key players among the pathways. In this work we have engineered regulatory network for cell death (Fig. 3),

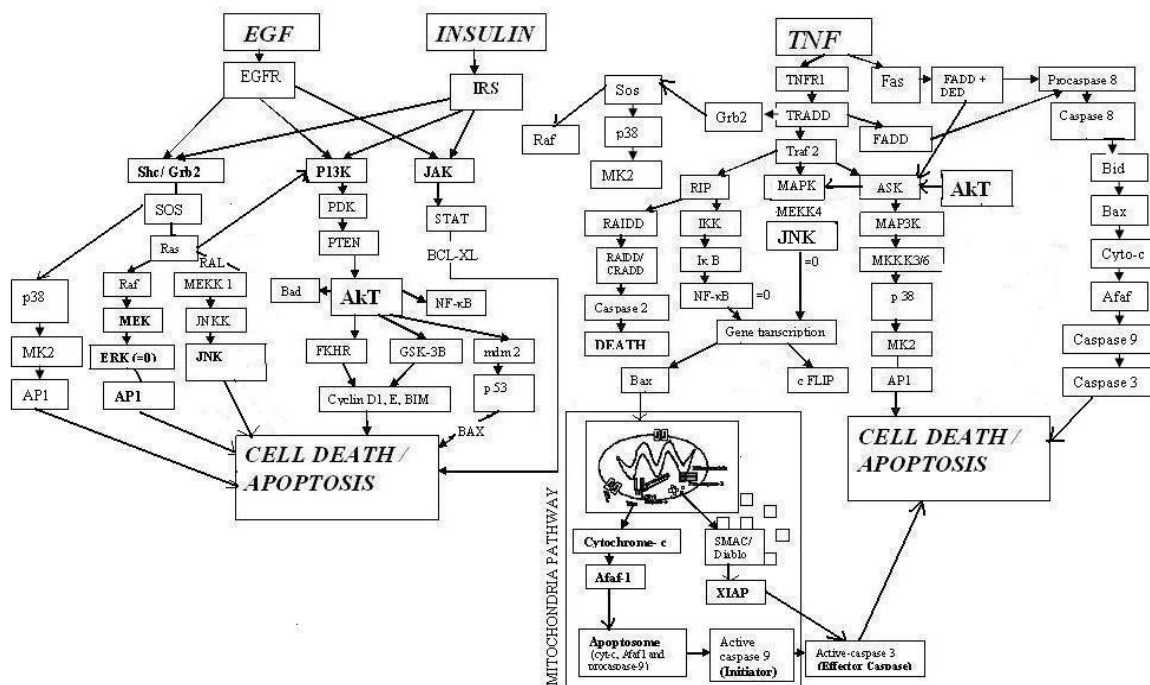


Fig 3. Illustration of cellular communication induced by combination of TNF, EGF and insulin leading to Cell death

TRUTH TABLES

On the basis of above models we have made the *truth tables* for every possible pathways encountered for cell death/ survival for pro death cytokine *TNF*, and pro survival cytokine *EGF* and *Insulin* and combining the three taking '1' as *cell survival* and '0' as *cell death*. For cell survival the ten different proteins i.e. P13K, TNFR1, EGFR, IRS, IKK, Grb2, SOS, Ras, TRADD, Traf2 should be present. If any one of them is absent than there is a cell death. This is the necessary condition.

Table 2: Truth Table for TNF, EGF and Insulin combined

<u>Raf</u>	MEK	ERK	Output
0	0	0	0
0	0	1	0
0	1	0	0
0	1	1	0
1	0	0	0
1	0	1	0
1	1	0	0
1	1	1	1

RAL	MEKK	JNK	Output
0	0	0	0
0	0	1	0
0	1	0	0
0	1	1	0
1	0	0	0
1	0	1	0
1	1	0	0
1	1	1	1

<u>mTOR</u>	PTEN	<u>Akt</u>	Output
0	0	0	0
0	0	1	0
0	1	0	0
0	1	1	0
1	0	0	0
1	0	1	1
1	1	0	0
1	1	1	0

RIP	<u>IκB</u>	<u>NF-κB</u>	Output
0	0	0	0
0	0	1	0
0	1	0	0
0	1	1	0
1	0	0	0
1	0	1	0
1	1	0	0
1	1	1	1

p 38	MK2	Output
0	0	0
0	1	0
1	0	0
1	1	1

Above are all truth tables made from the computational model of Cell Survival and Cell death. In the later chapter we will implement all these truth tables using different electronic implementation.

RESULTS AND DISCUSSIONS

SYSTEM IMPLEMENTATION OF CMOS USING SPICE

SPICE is a powerful general purpose analog and mixed-mode circuit simulator that is used to verify circuit designs and to predict the circuit behavior. This is of particular importance for integrated circuits. It was for this reason that SPICE was originally developed at the Electronics Research Laboratory of the University of California, Berkeley (1975), as its name implies: **S**imulation **P**rogram for **I**ntegrated **C**ircuits **E**mphasis.

The response of input signal (in the combination of TNF, EGF and Insulin) in presence of key players regulating different pathways by using CMOS is shown in Fig 4. As the ten marker proteins are present ('1') then, it activates five different pathways. V(31) is represented as output for CMOS Details of input notation for CMOS are shown in Table 3.

Table 3 : Different key proteins involved in communicating signal through different pathways for CMOS implementation

	CMOS	Combination of marker proteins
Pathway 1	V(5)	RAF, MEK, ERK
Pathway 2	V(11)	RAL, MEKK, JNK
Pathway 3	V(22)	p38, MK2
Pathway 4	V(27)	mTOR, PTEN, AkT
Pathway 5	V(17)	RIP, IκB, NF-κB

Now to activate first pathway all the three marker proteins should be present ('1') leading to cell survival. Similarly for pathways 2, 4 and 5. But to activate pathway 3, marker protein PTEN should be absent ('0') than it will lead to cell survival.

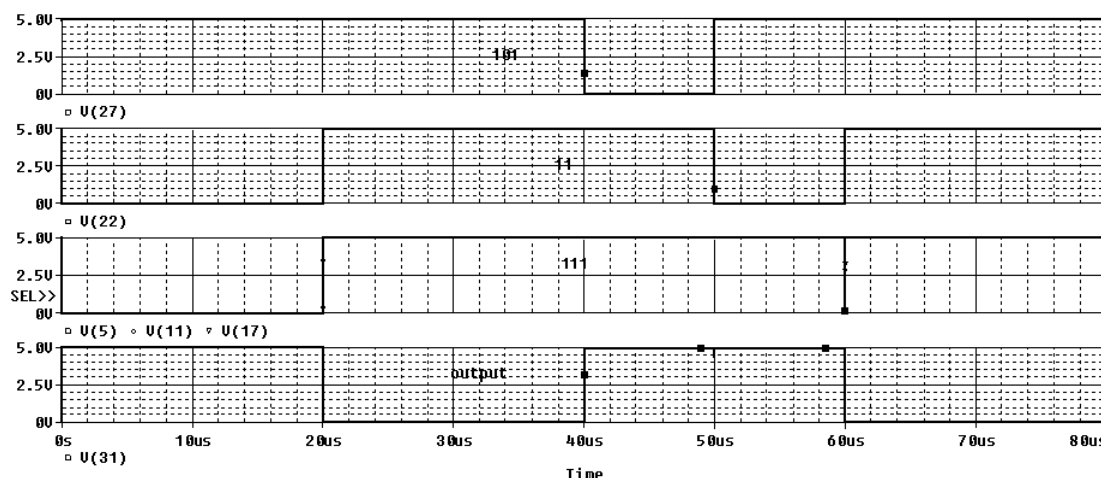


Fig 4 System Implementation of TNF, EGF and Insulin combined signal cascade using CMOS

SYSTEM IMPLEMENTATION USING HDL

VLSI stands for "Very Large Scale Integration". This is the field which involves packing more and more logic devices into smaller and smaller areas.

Without going into details, we can say that the VHDL, can be called as the "C" of the VLSI industry. VHDL stands for "VHSIC Hardware Definition Language", where VHSIC stands for "Very High Speed Integrated Circuit". This language is used to design the circuits at a high-level, in two ways. It can either be a behavioral description, which describes what the circuit is supposed to do, or a structural description, which describes what the circuit is made of. VHDL is also a

general-purpose programming language; just as high-level programming languages allow complex design concepts to be expressed as computer programs.

The response of input signal (in the combination of TNF, EGF and Insulin) in presence of key players regulating different pathways. Table 4 shows the five different pathways, its input notation and output notation used to implement VHDL coding which is shown in Fig 5. As the ten marker proteins are present ('1') then, it activates five different pathways.

Table 4 Different key proteins involved in communicating signal through different pathways for VHDL implementation of TNF, EGF and Insulin combined

	Input	Combination of marker proteins	Output
Pathway 1	'b'	RAF, MEK, ERK	'k'
Pathway 2	'c'	RAL, MEKK, JNK	'l'
Pathway 3	'd'	p38, MK2	'm'
Pathway 4	'e'	mTOR, PTEN, AkT	'n'
Pathway 5	'f'	RIP, IκB, NF-κB	'o'

Now to activate first pathway all the three marker proteins should be present ('1') than only it will lead to cell survival. Similarly for pathways 2, 4 and 5. But to activate pathway 3, marker protein PTEN should be absent ('0') than it will lead to cell survival.

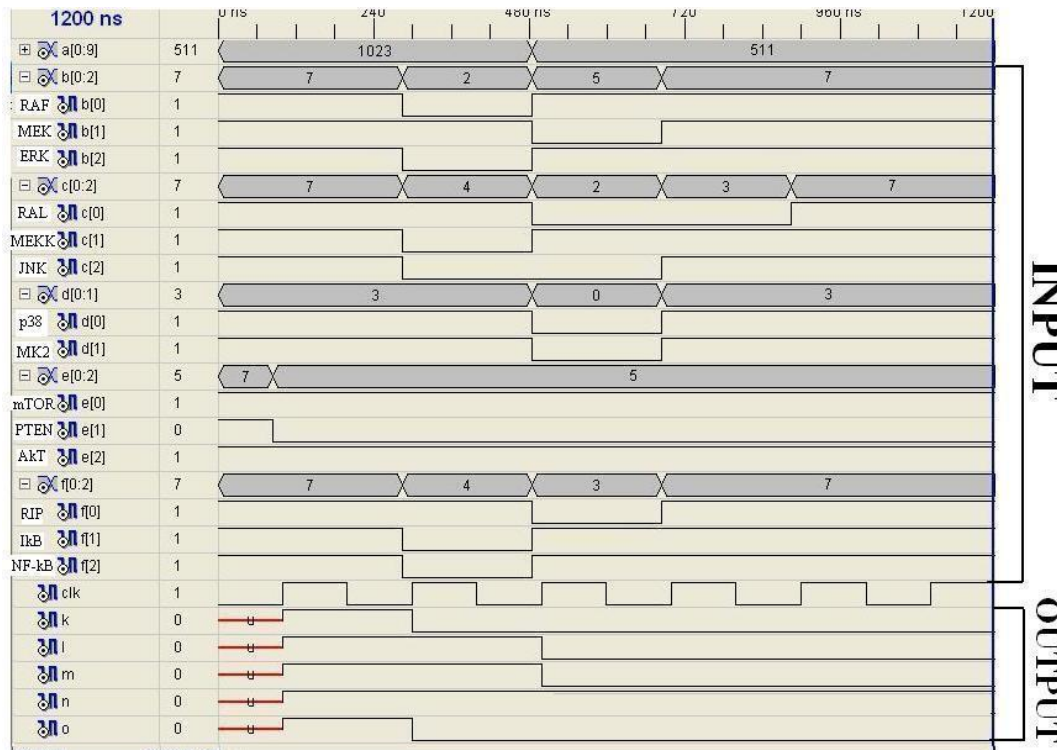


Fig 5. System Implementation of TNF, EGF and Insulin combined signal cascade using VHDL

SYSTEM IMPLEMENTATION USING FUZZY SYSTEM

In the Fuzzy Logic , there are five parts of the fuzzy inference process shown in Fig 6. Fuzzification of the input variables, application of the fuzzy operator (AND or OR) in the antecedent, implication from the antecedent to the consequent, aggregation of the consequents across the rules, and defuzzification.

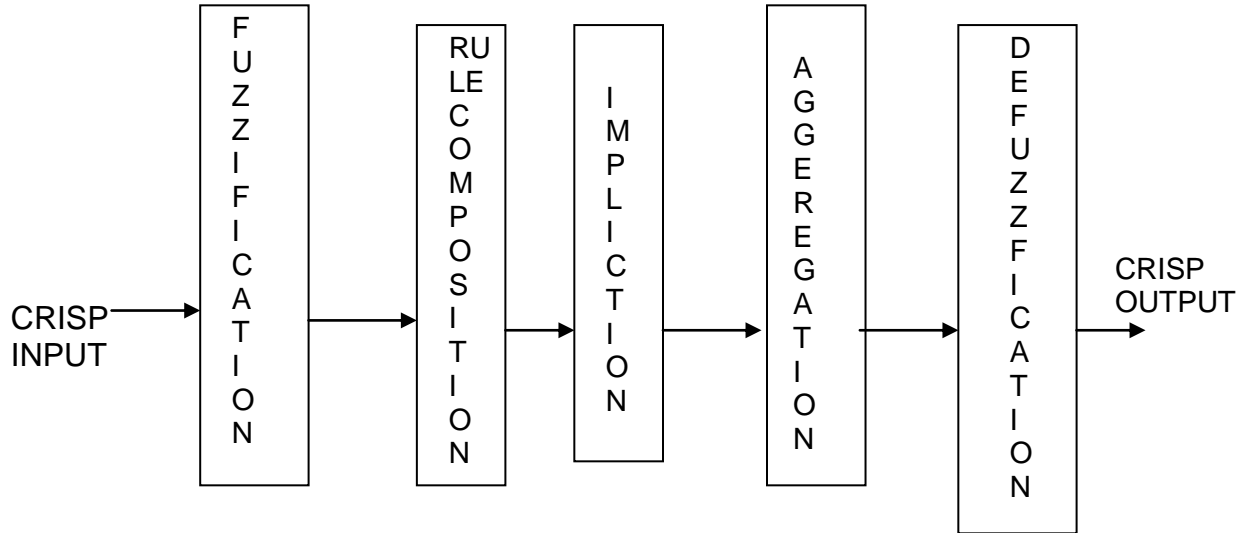


Fig 6. Fuzzy System Model

The pathways involving TNF that regulates cell survival and cell death are as follows

- 1) $TNF / TRAF2 / MAPK \rightarrow JNK$ (= 1 Cell Survival, = 0 Apoptosis); (Result Shown in Fig 7(a))
- 2) $TNF / MAPK / p38 \rightarrow MK2$ (= 1 Cell Survival, = 0 Apoptosis); (Result Shown in Fig 7(b))
- 3) $TNF / RIP1 / IKK \rightarrow NF\kappa B$ (= 1 Cell Survival, = 0 Apoptosis); (Result Shown in Fig 7(c))
- 4) $TNF / RAS / MEK \rightarrow ERK$ (= 1 Cell Survival, = 0 Apoptosis); (Result Shown in Fig 7(d))
- 5) $TNF / FAS \rightarrow CASPASE 8$ (Apoptosis). (Result Shown in Fig 7(e))

To get the output of all the above pathways of TNF, we have used Fuzzy Tool box of MATLAB by taking data as: Type = 'Mamdani', And Method = 'Min', Or Method = 'Max', Implication method = 'Min', Aggregation Method = 'Max', Defuzzification Method = 'Centroid' shown in Fig 7 (a-e). Yellow filled boxes are treated as '1' i.e. high, while blank ones are '0' i.e. low. In last column, blue filled part in Fig. 7 (a, b, c, d) represents Cell Survival while Fig 7(e) represents Cell death.



Fig 7. System Implementation of TNF signal cascade using Fuzzy (a) Output for TNF/ TRAF2/ MAPK = JNK pathway. (b) Output for TNF/ MAPK/ p38 = MK2 pathway. (c) Output for TNF/ RIP1/ IKK = NF-κB pathway. (d) Output for TNF/ RAS/ MEK = ERK pathway. (e) Output for TNF/ FAS = Caspase 8 pathway.

CONCLUSION

It has been revealed that cell death signals induced by TNF, EGF and insulin are temporarily separated and this is reflected in our model by the differences between the values of the parameters used. Simulations based on electronic implementation (VHDL, SPICE, Fuzzy) recapitulate most features of the data and generate several predictions involving pathway crosstalk and regulation. We uncover a relationship between the key proteins involved in TNF, EGF and insulin cellular signalling pathways that might account for the cell death decision of the cells. Out of the three processes Fuzzy Logic is the best, because it takes intermediate values also instead of using '0' and '1' only as in VHDL and SPICE. More generally, these models are flexible, able to incorporate qualitative and noisy data, and powerful enough to produce quantitative predictions and new biological insights about the operation of signalling networks.

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