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SCREENING STUDIES TO DESIGN NOVEL AND POTENT TGF-B SUPER FAMILY SIGNALLING

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ABSTRACT

The transforming growth factor beta (TGF-β) superfamily is a large family of structurally related cell regulatory proteins that was named after its first member, TGF-β1. Many proteins have since been described as members of the TGF-β superfamily in a variety of species, including invertebrates as well as vertebrates and categorized into 23 distinct gene types that fall into four major subfamilies: the Decapentaplegic-Vg-Related (DVR) related subfamily (including the bone morphogenetic proteins and the growth differentiation factors), the activin/inhibin subfamily, the TGF-β subfamily, a group encompassing various divergent members.

INTRODUCTION

Transforming growth factor-beta (TGF-beta) is a multifunctional peptide that controls proliferation, differentiation and other functions in many cell types. TGF-beta-1 is a peptide of 112 amino acid residues derived by proteolytic cleavage from the C-terminal of a precursor protein [(1-2)]. These proteins interact with a conserved family of cell surface serine/threonine-specific protein kinase receptors, and generate intracellular signals using a conserved family of proteins called SMADs [(3-4)]. They play fundamental roles in the regulation of basic biological processes such as growth [(5)], development, tissue homeostasis and regulation of the immune system. TGF-beta system is now known to be highly conserved throughout the animal kingdom, and its basic pathway provides a simple route for signals to pass from the extracellular environment to the nucleus. This process involves TGF-β1, TGF-β2 and TGF-β3, which are three different molecules that all activate the same intracellular receptor [(6)]. A family of secreted growth regulatory proteins called Transforming Growth Factor-beta receptor type-1 (TGFBR1), are one of the few known classes of proteins that can inhibit cell growth. TGF-beta is involved in an extraordinary range of biological processes, including embryonic development, wound healing and angiogenesis. In its normal state, the TGF-beta pathway restricts cell growth, differentiation and cell death [(7)].

Transforming growth factor β (TGF-β) is a multifunctional cell regulatory peptide that variably affects proliferation, differentiation, tissue repair, and extracellular matrix formation. To date, at least three TGF-β isoforms (TGF-β1, -β2, and -β3) have been identified in mammalian tissues and are known to biologically interact with their two different receptors (TGF-βR1 and TGF-βR2)[(8)]. TGF-β usually mediates cell growth suppression but may paradoxically play an important role in promoting the development of several malignant diseases. Several investigators have demonstrated the TGF-β over expression in cultured cancer cell lines and their localization in tumor tissues and have suggested that TGF-β has a potent stimulatory effect on the growth of malignant epithelial neoplasms[(9)]. As for mesenchymal neoplasms, TGF-β expression has been investigated in some osteocartilaginous-matrix-producing tumors, including osteosarcomas and chondrosarcomas. TGF-β regulates bone and cartilage formation by inducing osteoblast and osteoclast proliferation and by inducing extracellular matrix formation in normal and neoplastic tissues[(10)]. Transforming growth factor (TGF-*beta*) is a pluripotent cytokine involved in Cell growth, Differentiation, Development, Cell adhesion, Extracellular matrix (ECM) deposition, Migration, Immune response regulation[(11)].

As an important and pervasive signaling pathway, transforming growth factor β (TGF-β) super family signalling pathways regulate a wide range of biological processes at cellular and systematical levels [(12)]. It dictates not only the single cell's expansion, determination, movement and apoptosis, but also contextual interactions among different cells, tissues and organs, which guide development, immune regulation, tumorigenesis, and wound recovery[(13)]. Malfunction in these

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pathways often leads to many kinds of diseases in vertebrates. Detailed studies of these pathways at different levels shed light on the relevant biomarker screens and therapeutic application[14]. The TGF−β superfamily ligands consists of more than 30 polypeptide growth factors including TGF−βs(1-3), activins (A,B), inhibins (A,B), bone morphogenetic proteins(BMPs 1-9), growth differentiation factors including myostatin, nodal, leftys (1,2), and Mullerian inhibiting substance (MIS)[15]. These members show a similar cysteinek not structure, and are universally expressed[16]. DNA mutations or protein expression abnormality can cause many malfunctions resulting in developmental, metabolic and physiological disorders[17].

MATERIALS AND METHODS

Rational Drug Design (RDD)

The concept of RDD could be traced to the findings of Paul Ehrlich (*chemoreceptor)* and Emil Fischer (*lock and key model)* [18]. Contemporarily, HTS makes it possible to screen huge libraries of molecules within a short time span Nevertheless, initial euphoria that designated these techniques as universal lead generators subsided as a result of the considerable costs involved and disappointingly low hit rates. Lessons learnt from these strategies seek a complete shift of drug research paradigms from an empirical science to structure based analysis of macromolecule-ligand interactions [19]. Figure 1 shows a flow chart that describes different approaches that enable RDD to evolve new NCE's with greater biological activity[20].

Role of Computer Aided Molecular Design in Drug Discovery

Generation of chemical diversity *in silico*, is easily achieved using existing computational resources and algorithms: putative ligands can either be extracted from large databases of compounds, or they can be "grown" computationally by joining molecular fragments [21].

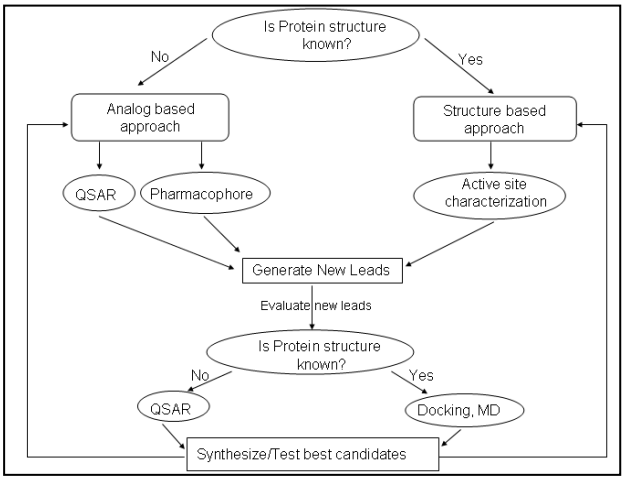

Figure 1 Different Approaches of Rational Drug Design

Approaches to RDD

On the other hand, accurate prediction of binding affinities has been a more difficult task [22]. Because of the multitude of energetic and entropic factors involved, the thermodynamics of binding cannot be analytically modelled without first simplifying the problem. Computational methods that attempt to design leads vary in the nature and in the degree of the simplifying assumptions they use [23].

The state of the art in RDD or Computer Aided Molecular Design (CAMD), can be divided into two broad categories: analog based study and structure based study based on the availability of three dimensional structure of the target [24].

Analog Based Studies

In a broad sense, analog based studies gather information from already existing drugs/ligands that are active against target biological molecule (protein or DNA/RNA) of interest. Based on this information a set of rules are framed to either design a new ligand or modify an existing ligand in order to enhance its biological activity[25].

Structure Based Studies

Structure based approaches, based on the three-dimensional structure of the target overcome many of the limitations of analog based studies. These methods help to develop a general theoretical description of the protein–ligand interactions that would enable an a priori design of new leads for a particular biological target. The first success story in structure based design is the antihypertensive drug Captopril, an inhibitor of Angiotensin Converting Enzyme (ACE)[26].

Docking

Docking in a true sense is the formation of non-covalent protein–ligand complexes *in silico*. Given the structure of a protein and a ligand, the task is to predict the structure of the complex[27]. Conceptually, docking is an energy optimization process concerned with the search of the lowest free energy binding mode of a ligand within a protein binding site. Docking constitutes two components: pose searching and scoring. Inclusion of protein flexibility is computationally expensive[28]; therefore much of the existing docking programs treat the protein either as rigid or allow flexibility only to the side chain functional groups. A good docking method estimates the forces involved in the protein–ligand recognition viz. electrostatic, van der Waals and hydrogen bonding and places the ligand appropriately in the active site [29].

Scoring

In principle, a scoring function used in docking is a mathematical function whose values are proportional to the binding affinities of the leads. A good scoring function should be able to give reliable estimates of binding affinities of structurally diverse leads for different protein targets while considering the thermodynamic aspects of binding [30]. These functions reflect a best fit with respect to the training set used but rarely achieve generality. *Force field based methods* are first principle methods that use force field parameters to score the vdW and electrostatic interactions between protein and ligand [31]. The score includes receptor-ligand interaction energy and internal ligand energy (such as steric strain induced by binding). These methods do not require calibration or training with experimental binding data. *Knowledge based methods* evaluate the frequencies of particular type of interaction-the mutual distance between particular type of atoms across the interface, in databases of protein-ligand complexes [32]. The sample distribution describes the probability of occurrence of an interaction and is compared with the reference mean. Any deviation from the mean is translated into statistical preferences using mathematical equations and related to energies in a Boltzmann-like fashion [33].

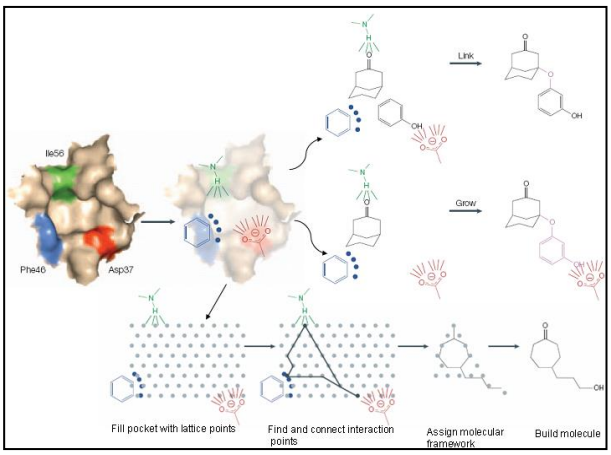

Figure 2 Strategies of de novo ligand design

De novo Ligand Design

De novo design uses structural information to "grow" a molecule into the active site by sequentially adding or joining molecular fragments instead of using libraries of existing compounds 107. Structure sampling is carried out by different methods like linking, growing, lattice-based sampling, random structure mutation, transitions driven by molecular dynamics simulations, and graph-based sampling [(34)]. Figure 1 gives a schematic description of few of these strategies. Apart from these, the ligand can also be built from recombination of bioactive conformations of known ligands for a particular target. Recombination is carried out by overlaying the known ligands and swapping the fragments of different ligands [(35)]. This procedure is carried out recursively, so that the compounds that emerge from recombination are added to the pool of known actives and participate in subsequent cycles of recombination [(36)]. The largest advantage of *de novo* design is its ability to develop novel scaffolds utilizing the whole chemical space [(37)].

Virtual Screening (VS)

VSare a knowledge driven process that uses computational chemistry techniques to analyze large chemical databases in order to identify possible new leads. VS is used as an initial screen for large databases to prune the number of compounds that are to be screened experimentally (37). This process of finding 'needles in a haystack' produces leads that may otherwise not have surfaced and therefore adds immense value to the early drug discovery stages. VS protocols include ligand based screens like 1D filters (e.g. molecular weight), 2D filters (similarity, substructure fingerprints), and 3D filters (3D-pharmacophore, 3D shape matching) and structure based screens like docking (38). The potential sources of error contributing to the identification of false positives and false negatives in VS include: 1) approximations in the scoring functions employed; 2) improper solvation terms; 3) neglect of protein flexibility and; 4) poor assessment of the protonation states of active site residues or ligands [111]. Significant improvements in VS have been made by consensus scoring of multiple scoring functions and by clustering docking poses, from multiple docking tools before scoring (39).

Molecular Dynamics

Molecular Dynamic (MD) simulations are widely used to obtain information on the time evolution of conformations of biological molecules with the associated kinetic and thermodynamic properties. The basic feature of molecular dynamics is the calculation of a trajectory of the molecule, i.e. a series of structures at regular time steps in which the system is moving under the influence of the forces acting on the atoms.[113] These are calculated from the first derivative of the potential function with respect to the atom positions. By applying Newton's equations of motion, these forces can then be used to calculate how the atomic positions change with time resulting in a dynamic trajectory. MD can be utilized to quantify the properties of a system and is therefore a valuable tool in understanding the complete profile of a model system. Breakthroughs in MD lead to its first application on the protein bovine pancreatic trypsin inhibitor for 9.2ps *in-vacuo* in 1977.[114] In 1988, cumulative advances in the MD simulations made it possible to carry out a microsecond MD of a much larger protein in solution.[115] Table 2.4 lists different MD methods where the utility of each varies with the aspects of desire.

RESULTS AND DISCUSSION

Quantitative Structure Activity Relationship (QSAR)

QSAR is one of the most widely used analog based methods. It aims at correlating structural features of a series of known compounds with their biological activities. From these correlations empirical equations are derived and subsequently used to guide the design of new leads. Early QSAR methods related biological activity to the presence (or absence) of functional groups in a series of structurally related compounds (Free-Wilson model), or to the physicochemical properties (lipophilicity, electronic properties) of the compounds in the training set (Hansch analysis). HipHop: Common feature based alignments. Pharmacophore model, or Hypothesis, consists of a three dimensional configuration of chemical functions surrounded by tolerance spheres. A tolerance sphere defines that area in space that should be occupied by a specific type of chemical functionality. Each chemical function is assigned a weight, which describes its relative importance within the hypothesis.

HypoGen: Quantitative Pharmacophores Models

It creates SAR hypothesis models from a set of molecules for which activity values are known. HypoGen selects pharmacophores that are common among the active compounds but not among the inactive compounds and then optimizes the pharmacophores using simulated annealing. The top pharmacophores can be used to predict the activity of unknown compounds or to search for new possible leads contained in 3D chemical databases. HypoGen generates hypotheses that are set of features in 3D space, each containing a certain tolerance and weight that fit to the features of the training set, and that correlate to the activity data. The hypotheses are created in three phases Constructive, subtractive and optimization phase. The constructive phase identifies hypotheses that are common among active compounds, the subtractive phase removes hypotheses that are common among the inactive compounds, and the optimization phase attempts to improve the initial hypotheses. The resulting hypotheses models consist of set of generalized chemical features in three-dimensional space as well as regression information. Therefore, the hypotheses models can be used as search queries to mine for potential leads (Figure 2.2) from a three-dimensional database or in the form of an equation to predict the activity of a potential lead.

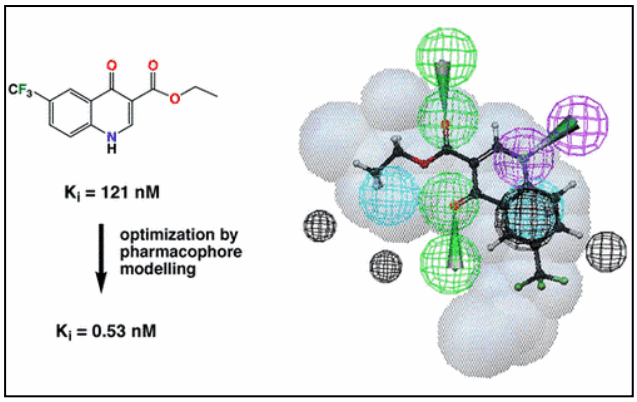

Figure 2 Lead optimization using Pharmacophores

HypoGen calculates the cost of two theoretical hypotheses, one in which the cost is minimal (Fixed cost), and one where the cost is high (Null cost). Each optimized hypothesis cost should have a value between these two values and should be closer to the Fixed than the Null cost. Randomized studies have found that if a returned hypothesis has a cost that differs from the Null hypothesis by 40-60 bits, it has 75-90% chance of representing a true correlation in the data. Another useful number is the Entropy of hypothesis space. If this is less than 17, a thorough analysis of all the models will be carried out.

Constructive phase

Constructive phase is very similar to HipHop algorithm. This is done in several steps:

1. All active compounds are identified
2. All hypotheses (maximum 5 features) among the two most active compounds are identified and stored
3. Those that fit the remaining active compounds are kept

Subtractive phase

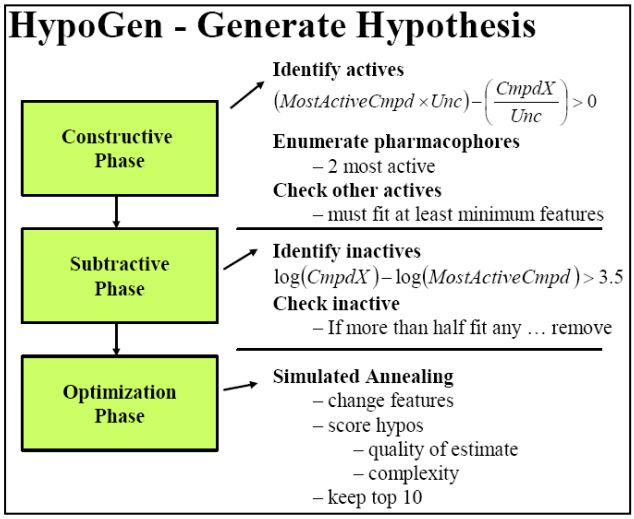

Figure 3 Hypotheses generation in Catalyst

In this phase, the program removes hypotheses from the data structure that are not likely to be useful. The hypotheses that were created in the constructive phase are inspected and if they are common to most of the inactive compounds then they are removed from consideration.

Optimization phase

The optimization is done using the well-known simulated annealing algorithm. The algorithm applies small perturbations to the hypotheses created in the constructive and subtractive phases in an attempt to improve the score.

Hypo Refine

The HypoRefine algorithm is an extension of the Catalyst HypoGen algorithm for generating SAR-based pharmacophore models which can be used to estimate activities of new compounds. HypoRefine helps to improve the predictive models generated from a dataset by a better correlating hypothesis with the steric properties that contribute to biological activity. In addition, HypoRefine can help overcome over-prediction of inactive compounds with pharmacophore features in common with other active compounds in the dataset, where inactivity is due to steric clashes with the target.

Interpreting the cost parameters in the output files

During an automated hypothesis generation run, Catalyst considers and discards many thousands of models. It distinguishes between alternatives by applying a cost analysis. The overall assumption is based on Occam's razor; that is between equivalent alternatives, the simplest model is best.

In general, if this difference is greater than 60 bits, there is an excellent chance of the model to represent a true correlation. Since most returned hypotheses are higher in cost than the fixed cost model, a difference between fixed cost and null cost of 70 or more is necessary to achieve the 60 bits difference [(38)].

Fixed cost: Cost of the simplest possible hypothesis (initial)

Null cost: Costs when each molecule estimated as mean activity acts like a hypothesis with no features

Weight cost: A value that increases in a Gaussian form as the feature weight in a model deviates from an idealized value of 2.0. This cost factor favors hypotheses in which the feature weights are close to 2. The standard deviation of this parameter is given by the weight variation parameter.

Error cost: A value that increases as the rms difference between estimated and measured activities for the training set molecules increases. This cost factor is designed to favor models for which the correlation between estimated and measured activities is better. The standard deviation of this parameter is given by the uncertainty parameter (39).

Configuration cost

A fixed cost depends on the complexity of the hypothesis space being optimized. It is equal to the entropy of the hypothesis space. This parameter is constant among all the hypotheses (40). The main assumption made by HypoGen is that an active molecule should map more features than an inactive molecule.

In other words, the molecule is inactive because a) it misses important feature or b) the feature is present but cannot be oriented in correct space. Based on this assumption, the most active molecule in the dataset should map to all features of the generated hypotheses (40).

Metric for Analyzing Hit Lists and Pharmacophores

Validity of the pharmacophore model is determined by its ability to retrieve known active molecules from the various known databases (Figure 2).

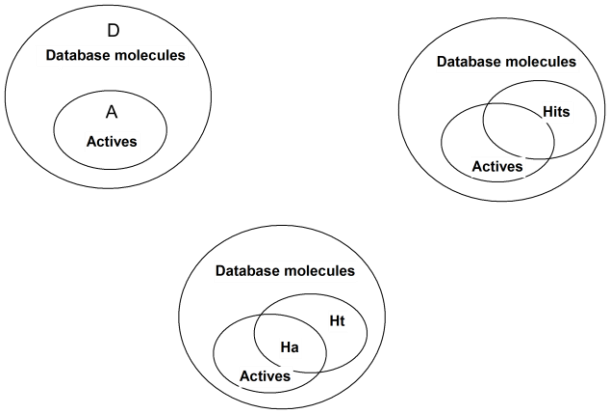

Figure 2 Database searching using pharmacophore models

D = Total number of compounds in database, A = Number of active compounds in database, H_t = Number of compounds in search hit list and H_a = Number of active compounds in hit list

Pharmacophore Validation

Percent yield of actives:
% Y = H_a / H_t x100
Percent ratio of the activities in the hit list:
% A = H_a / Ax100
Enrichment (enhancement)
E = H_a / H_t= H_ax D
A/ DH_tx A
False negatives: A - H_a
False positives:H_t- H_a

$$\text{Goodness of fit} = \left(\frac{H_a\ (3A + H_T)}{4\ H_t * A}\right) \times \left(\frac{1 - H_t - H_a}{D - A}\right)$$

The best hit list is obtained when there is perfect overlap of the hit list to the known active compounds in the database. This occurs when both conditions Ha = Ht and Ha = A, hence Ha = Ht= A, are satisfied, which is a nearly impossible case to achieve in a real-life situation. In reality, there may be many compounds in the database that may be active but either have not been listed as active, or have not been tested for specific activity. In either case, these compounds end up in the "False positives" list. Hence we consider the list of false positives as opportunities for potential leads. The objective is to improve the hit list in such a manner that the false positives can contain a large number of potential leads (41). "False negatives" list is nothing but missing the retrieval of active molecules from database. The best hit list is the one that retrieves all the actives and nothing else (i.e., Ht = Ha= A); False negatives = 0, false positives = 0. The worst list is the one that retrieves everything else but the known actives in the database (i.e., Ha = 0, Ht = D-A) False negatives = A, false positives = D-A (42). The GH score gives a good indication of how good the hit list is with respect to a compromise between maximum yield and maximum percent of activities retrieved. The

Table 1 provides an acceptable sorting of the hit lists, from best to worst, via the GH score. The Goodness of Hit formula is a convenient way to quantify hit lists obtained from searches with various queries.

ADMET Prediction

Absorption, Distribution, Metabolism, Elimination, and Toxicity (ADMET) profiles of chemical compounds have become the bottleneck and a major challenge in drug research. *In silico* prediction of physicochemical parameters of compound's ionizability (*pKa*), and lipophilicity (*log P* or *log D*), provide an indication of its likely absorption in the gut. Various computational techniques using *in silico* models are emerging that attempts to give the ADMET profile of a given compound (43).

Case	% Y	% A	Enrichment	False negatives	False positives	GH score
Best	100	100	500	0	0	1
Typical Good	40	80	200	20	120	0.60
Extreme Y	100	1	500	99	0	0.50
Extreme A	0.2	100	1	0	49,900	0.50
Typical Bad	5	50	25	50	950	0.26
Worst	0	0	0	100	49,900	0

Simulations One Step Ahead–Future Directions

Advances in computations in structural biology have made it possible to carry out virtual cell simulations that mimic the cell environment and the cellular events therein.[120] A number of programs have been developed in this area. For example *NEURON* and *GENESIS* simulate the electrophysiological behavior of single neurons and neuronal networks. One step ahead of these, *E-CELL* (44) constructs a model of a hypothetical self-sustaining whole-cell with 127 genes sufficient for transcription, translation and energy production. Alternately, *M Cell* provides a modeling tool for realistic simulation of cellular signalling in the complex 3-D cellular microphysiology-subcellular microenvironment in and around living cells, using Monte Carlo algorithms to track the stochastic behaviour of discrete molecules in space and time.[12]*Virtual Cell* is another program that models cell biological processes. Future prospects of such virtual cell simulations would hopefully reduce the limitations of simulations using isolated proteins which will never exist in real situations and interrelated ADMET properties.

CONCLUSION

After its first member, TGF-1, the transforming growth factor beta (TGF-) superfamily is a vast family of structurally related cell regulating proteins. Since then, numerous proteins have been identified in a wide range of species, including invertebrates and vertebrates, as belonging to the TGF- super family. These proteins have been divided into 23 different gene types that fall into four major subfamilies: the Decapentaplegic-Vg-Related (DVR) related subfamily, which includes the bone morphogenetic proteins and the growth differentiation factors, the activin/inhibin subfamily, and the TGF- subfamily.

References

1. Leveen P, Carlsen M, Makowska A, *et al*. TGF-beta type II receptor-deficient thymocytes develop normally but demonstrate increased CD8 proliferation in vivo. Blood. 2005; 106:4234-4240.
2. de Boer J, Williams A, Skavdis G, *et al*. Trans-genic mice with hematopoietic and lymphoid specific expression of Cre. Eur J Immunol. 2003; 33: 314-325.
3. Ghilardi N, Li J, Hongo JA, Yi S, Gurney A, de Sauvage FJ. A novel type I cytokine receptor is expressed on monocytes, signals proliferation, and activates STAT-3 and STAT-5. J Biol Chem. 2002; 277:16831-16836.

4. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C (T)) Method. Meth-ods. 2001; 25:402-408.
5. Kralovics R, Teo SS, Buser AS, *et al*. Altered gene expression in myeloproliferative disorders correlates with activation of signaling by the V617F mutation of Jak2. Blood. 2005; 106:3374-3376.
6. Dupic F, Fruchon S, Bensaid M, *et al*. Duodenal mRNA expression of iron related genes in re-sponse to iron loading and iron deficiency in four strains of mice. Gut. 2002; 51:648-653.
7. Nemeth E, Rivera S, Gabayan V, *et al*. IL-6 medi-ates hypoferremia of inflammation by inducing the synthesis of the iron regulatory hormone hepcidin. J Clin Invest. 2004; 113:1271-1276.
8. Yamamoto K, Yoshida K, Miyagoe Y, *et al*. Quan-titative evaluation of expression of iron-metabo-lism genes in ceruloplasmin-deficient mice. Bio-chimBiophys Acta. 2002; 1588:195-202.
9. Murthy L, Menden EE, Eller PM, Petering HG. Atomic absorption determination of zinc, copper, cadmium, and lead in tissues solubilized by aqueous tetramethylammonium hydroxide. Anal Bio-chem. 1973; 53:365-372.
10. He W, Dorn DC, Erdjument-Bromage H, Tempst P, Moore MA, Massague J. Hematopoiesis con-trolled by distinct TIF1gamma and Smad4 branches of the TGFbeta pathway. Cell. 2006; 125:929-941.
11. Carlino JA, Higley HR, Creson JR, Avis PD, Ogawa Y, Ellingsworth LR. Transforming growth factor beta 1 systemically modulates granuloid, erythroid, lymphoid, and thrombocytic cells in mice. Exp Hematol. 1992; 20:943-950.
12. Thomas DA, Massague J. TGF-beta directly tar-gets cytotoxic T cell functions during tumor evasion of immune surveillance. Cancer Cell. 2005;8: 369-380.
13. Gorelik L, Flavell RA. Abrogation of TGFbeta signaling in T cells leads to spontaneous T cell differ-entiation and autoimmune disease. Immunity. 2000; 12:171-181.
14. Larsson J, Blank U, Helgadottir H, *et al*. TGF-beta signaling-deficient hematopoietic stem cells have normal self-renewal and regenerative ability in vivo despite increased proliferative capacity in vitro. Blood. 2003; 102:3129-3135.
15. Hahn SA, Schutte M, Hoque AT, *et al*. DPC4, a candidate tumor suppressor gene at human chro-mosome 18q21.1. Science. 1996; 271:350-353.
16. Nagatake M, Takagi Y, Osada H, *et al*. Somatic in vivo alterations of the DPC4 gene at 18q21 in hu-man lung cancers. Cancer Res. 1996; 56:2718-2720.
17. Schutte M, Hruban RH, Hedrick L, *et al*. DPC4 gene in various tumor types. Cancer Res. 1996; 56:2527-2530.
18. Maesawa C, Tamura G, Nishizuka S, *et al*. MAD-related genes on 18q21.1, Smad2 and Smad4, are altered infrequently in esophageal squamous cell carcinoma. Jpn J Cancer Res. 1997; 88:340-343.
19. Friedl W, Kruse R, Uhlhaas S, *et al*. Frequent 4-bp deletion in exon 9 of the SMAD4/MADH4 gene in familial juvenile polyposis patients. Genes Chromosomes Cancer. 1999; 25:403-406.
20. Howe JR, Roth S, Ringold JC, *et al*. Mutations in the SMAD4/DPC4 gene in juvenile polyposis. Sci-ence. 1998; 280:1086-1088.
21. Takaku K, Miyoshi H, Matsunaga A, Oshima M, Sasaki N, Taketo MM. Gastric and duodenal polyps in Smad4 (Dpc4) knockout mice. Cancer Res. 1999; 59:6113-6117.
22. Xu X, Brodie SG, Yang X, *et al*. Haploid loss of the tumor suppressor Smad4/Dpc4 initiates gastric polyposis and cancer in mice. Oncogene. 2000; 19:1868-1874.
23. Jenkins BJ, Grail D, Nheu T, *et al*. Hyperactiva-tion of Stat3 in gp130 mutant mice promotes gas-tric hyperproliferation and desensitizes TGF-beta signaling. Nat Med. 2005; 11:845-852.
24. Kim BG, Li C, Qiao W, *et al*. Smad4 signalling in T cells is required for suppression of gastrointesti-nal cancer. Nature. 2006; 441:1015-1019.
25. Liu D, Wang J, Kinzel B, *et al*. Dosage-dependent requirement of BMP type II receptor for mainte-nance of vascular integrity. Blood. Prepublished on May 11, 2007, as DOI 10.1182/blood-2006-11-058594.
26. Handra-Luca A, Condroyer C, de Moncuit C, *et al*. Vessels' morphology in SMAD4 and BMPR1A-related juvenile polyposis. Am J Med Genet A. 2005; 138:113-117.
27. Wang RH, Li C, Xu X, *et al*. A role of SMAD4 in iron metabolism through the positive regulation of hepcidin expression. Cell Metab. 2005; 2:399-409.
28. Pak M, Lopez MA, Gabayan V, Ganz T, Rivera S. Suppression of hepcidin during anemia requires erythropoietic activity. Blood. 2006; 108:3730-3735.
29. Karlsson G, Blank U, Moody JL, *et al*. Smad4 is critical for self-renewal of hematopoietic stem cells. J Exp Med. 2007; 204:467-474.
30. Roberts AB, Sporn MB. Physiological actions and clinical applications of transforming growth factor-beta (TGF-beta). Growth Factors 1993; 8(1):1-9.
31. Wakefield LM, Smith DM, Flanders KC, Sporn MB. Latent transforming growth factor-beta from human platelets. A high molecular weight complex containing precursor sequences. J Biol Chem 1988; 263:7646-7654.
32. Wakefield LM, Smith DM, Masui T, Harris CC, Sporn MB. Distribution and modulation of the cellular receptor for transforming growth factor-beta. J Cell Biol 1987; 105(2):965-975.
33. Annes JP, Munger JS, Rifkin DB. Making sense of latent TGFbeta activation. J Cell Sci 2003; 116(Pt 2):217-224.
34. Massague J, Chen YG. Controlling TGF-beta signaling. Genes Dev 2000; 14(6):627-644.
35. Bharathy S, Xie W, Yingling JM, Reiss M. Cancer-associated transforming growth factor beta type II

receptor gene mutant causes activation of bone morphogenic protein-Smads and invasive phenotype. Cancer Res 2008; 68(6):1656-1666.

36. Konig HG, Kogel D, Rami A, Prehn JH. TGF-{beta} 1 activates two distinct type I receptors in neurons: implications for neuronal NF-{kappa} B signaling. J Cell Biol 2005; 168(7):1077-1086.
37. Lebrin F, Deckers M, Bertolino P, Ten Dijke P. TGF-beta receptor function in the endothelium. Cardiovasc Res 2005; 65(3):599-608.
38. Daly AC, Randall RA, Hill CS. TGF-β-induced Smad1/5 phosphorylation in epithelial cells is mediated by novel receptor complexes and is essential for anchorage-independent growth. Mol Cell Biol. 2008
39. Massague J, Chen Y-G. Controlling TGF-β signaling. Genes Dev 2000; 14:627-644.
40. Cui W, Fowlis DJ, Cousins FM, Duffie E, Bryson S, Balmain A, *et al*. Concerted action of TGF-beta 1 and its type II receptor in control of epidermal homeostasis in transgenic mice. Genes Dev 1995;9 (8):945-955.
41. Glick AB, Kulkarni AB, Tennenbaum T, Hennings H, Flanders KC, O'Reilly M, *et al*. Loss of expression of transforming growth factor beta in skin and skin tumors is associated with hyperproliferation and a high risk for malignant conversion. Proc Natl AcadSci USA 1993; 90(13): 6076-6080.
42. Glick AB, Weinberg WC, Wu IH, Quan W, Yuspa SH. Transforming growth factor beta 1 suppresses genomic instability independent of a G1 arrest, p53, and Rb. Cancer Res 1996;56(16):3645-3650.
43. Ge R, Rajeev V, Subramanian G, Reiss KA, Liu D, Higgins L, *et al*. Selective inhibitors of type I receptor kinase block cellular transforming growth factor-beta signaling. BiochemPharmacol 2004; 68(1):41-50.
44. Xie W, Mertens JC, Reiss DJ, Rimm DL, Camp RL, Haffty BG, *et al*. Alterations of Smad signaling in human breast carcinomas are associated with poor outcome: a tissue microarray study. Cancer Res 2002;62(2):497-505.

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