Instituto Juan March
de Estudios e Investigaciones

63

CENTRO DE REUNIONES
INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

TGF-β Signalling in Development and Cell Cycle Control

Organized by

J. Massagué and C. Bernabéu

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INTRODUCTION

Joan Massagué and Carmelo Bernabéu

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The proliferation and differentiation of cells in higher organisms is controlled by extracellular polypeptides known as "growth factors" or "cytokines". These factors are produced by many cell types and act on neighboring cells, forming an intercellular signaling network. Growth factors exert their effects by contacting receptor proteins on the membrane of target cells. Based on the enzymatic activity on their cytoplasmic half, these receptors can be grouped in three major classes: the G protein-coupled receptors, the tyrosine kinase-coupled receptors and the receptor serine/threonine kinases. Much work over the past two decades has been directed towards identifying these receptors and understanding the biochemical mechanisms that carry and translate their signals into specific effects on cell division, differentiation and death processes.

TGF-β and related factors constitute one of the most diverse and fascinating families of growth factors. Factors of the TGF-β family are present in organisms ranging from the fruitfly to man, and are involved in controlling crucial steps during embryogenesis as well as maintaining and repairing adult tissues and functions. Three years ago, the receptor mechanism employed by TGF-β was elucidated with the finding that this factor binds to a receptor serine/threonine kinase known as the "type II" receptor. Upon binding, the type II receptor recruits, phosphorylates and activates a related kinase, known as the "type I" receptor, which then propagates the signal by phosphorylating in turn other proteins. Other members of the TGF-β family such as the activins and the bone morphogenetic proteins (BMPs) seem to employ a similar receptor mechanism. This process of receptor activation is highly regulated. In fact, in addition to these receptor kinases, TGF-β interacts with another class of membrane receptors known as the "type III" receptors. This class includes two important molecules, betaglycan and endoglin, which are specialized in controlling the access of TGF-β to the signaling receptors.

During the past year, a series of milestone findings have allowed for the first time to piece together a pathway linking these receptors to their target genes in the nucleus. The first breakthrough came through studies on the Dpp pathway in the fruitfly *Drosophila*. Dpp is the fly homologue of the vertebrate proteins BMP-2 and BMP-4, and plays key roles during development of the embryo and the larva. Recently, genetic screens for mutations that would exacerbate the phenotype of

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defective Dpp receptors led to the identification of a gene called Mad. A family of Mad homologues, known as Smad proteins, were quickly identified in human, mouse, frog and nematode. More importantly, the Smads were rapidly shown to act downstream of the receptors for TGF-β, activin and BMP. Furthermore, biochemical analysis of the Smad proteins has demonstrated that they are the direct substrates of the type I receptors. Upon phosphorylation by the receptor, the Smad proteins move from the cytoplasm into the nucleus. Once in the nucleus, Smads are thought to associate with subunits with specific DNA binding activity generating complexes that directly control gene expression. Smads associate with themselves in trimers of diverse composition, generating a combinatorial systems that may underlay the functional complexity of the TGF-β family. This year has therefore witnessed the dawn of the TGF-β/Smad pathway.

Although the TGF-β family has many effects on virtually every cell type, certain cellular responses to these factors have attracted special attention because of their biological significance. Examples include the ability of TGF-β to inhibit cell proliferation, to stimulate cell adhesion and migration, and to suppress immune cell function, as well as the ability of activin and BMP to induce the formation of mesoderm in the frog, and the ability of Dpp to control wing and eye formation in *Drosophila*. Some of the genes mediating these responses have been identified. For example, TGF-β activates the expression of p15, a protein that inhibits cyclin-dependent kinases (CDKs). CDKs are essential for cell commitment to DNA replication and mitosis, and constitute a major nexus of positive and negative growth stimuli. CDK inhibitors such as p15, p16, p21 and p27 represent one of the principal controls over the activity of these kinases. Work is in progress to determine whether p15 and other gene responses to TGF-β and related factors are directly mediated by the Smads.

Progress in understanding the mechanisms of TGF-β signaling is beginning to shed light into the molecular basis of several important disease conditions. Disruption of the TGF-β growth inhibitory mechanism is often observed in human cancer. Almost all cases of a certain form of human colon cancer have lost the TGF-β type II receptor whereas a majority of human pancreatic carcinomas have

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lost Smad4/DPC4, a Smad family member required for signaling by all TGF-β factors. When the growth inhibitory response to TGF-β is lost, cells may be left with the ability to migrate and invade tissue in response to this factor. Indeed, studies on TGF-β transgenic animals have shown that skin and mammary carcinoma cells become much more invasive when they are stimulated by TGF-β. A completely different type of pathology originates when deleterious mutations appear in the gen encoding endoglin, a component of the membrane TGF-β receptor system. Endoglin in chromosome 9q34, is the target gene for Hereditary Hemorrhagic Telangiectasia type 1 (HHT1), also kown as Rendue-Osler-Weber syndrome. HHT is an autosomal dominant vascular disorder associated with frequent nose bleeds, telangiectases, and lung and brain arteriovenous malformations. These abnormalities are probably due to a defect in the TGF-β responses of the endothelial cells.

In experiments with animal models, the generation of TGF-β knockout mice have unveiled non-overlapping phenotypes among the β1, β2, or β3 isoform null animals. These knockout mice indicate that during embryogenesis TGF-β1 functions in preimplantation development and implantation, and in the adult it prevents autoimmunity. On the other hand, TGF-β2 plays an important role in epithelial-mesenchymal interactions in the development of the cranial, axial and appendicular skeleton, eyes and ears, heart, and urogenital tract. TGF-β3 plays an essential role in secondary palate fusion. An important finding is that the viability of the TGF-β1 knock out mice highly depends on the genetic background, suggesting the existence of genetic modifiers and opening new avenues in the characterization of the corresponding genes.

In summary, it appears that in spite of the high complexity of the TGF-β system, the TGF-β field has undergone important advances which are helping to integrate and understand its genetic, molecular, cellular and developmental implications as a whole.

Joan Massagué and Carmelo Bernabéu

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SESSION 1. RECEPTORS AND SIGNAL TRANSDUCTION (I)

Chair: Anita B. Roberts

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SIGNAL TRANSDUCTION BY OLIGOMERIC TGF-β RECEPTORS

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The TGF-β receptor is composed of two subunits: type I (Tβ RI) and type II (Tβ RII). Binding of TGF-β 1 to Tβ RII causes Tβ RII to associate with and phosphorylates Tβ RI, interactions essential for TGF-β 1- activated signal transduction. An early report (Cheifez S. *et al. JBC* 265: 20533, 1990) showed that pretreatment of Mink lung epithelial cells with dithiothreitol (DTT) suppresses the binding and cross-linking of [^{125}I]TGF-β to the Tβ RI but not to the type II receptor, Tβ RII. Since DTT has metal chelator activity, we decided to study a possible metal requirement for binding of TGF-β to the TGF-β receptors. We showed that the cytosolic domain of Tβ RI but not Tβ RII binds Zn^{++} with an affinity of 1 μM. Zn^{++} is essential for the interaction of Tβ RII with Tβ RI, as shown by TGF-β 1 binding and cross-linking assays in which membrane permeable thiols able to chelate Zn^{++} abolish the TGF-β 1- induced association of Tβ RI with Tβ RII. Further, we have developed a novel *cell-free* TGF-β binding and cross-linking assay, and showed that Zn^{++}- specific chelators block Tβ RI/Tβ RII interactions as well as the TGF-β 1- induced phosphorylation of Tβ RI, presumably by Tβ RII. Our current model predicts that the TGF-β type I receptor requires Zn^{++} binding to a specific region in order to fold correctly and be fully functional. These findings represent the first example of a Zn^{++} binding cytokine receptor and provide a novel mechanism for receptor oligomerization.

Using TGF-β -responsive mink lung epithelial cells (Mv1Lu), we have determined the half lives of the endoplasmic reticulum (ER) and mature forms of these receptors. In metabolically-labeled cells, approximately 90% of newly-synthesized type II receptor undergoes modification of N-linked sugars in the Golgi, with a half life of 30-35 minutes; the Golgi-processed form of the receptor has the relatively short metabolic half life of 2.5 hours. In contrast, only 50% of pulse-labeled type I receptor is converted to the Golgi-processed and therefore endoglycosidase H (Endo H)-resistant form, and the Endo H-sensitive ER form has a half life of 2.8 to 3 hours. Addition of 100 pM TGF-β 1 causes the Golgi-processed type II receptor to become less stable, with a half life of 1.7 hours, and also destabilizes the Golgi-processed type I receptor. TGF-β 1 binding and cross-linking experiments on cells treated with tunicamycin for various times confirm different ER to cell surface processing times for Tβ RI and Tβ RII. Our results, which suggest that stable

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complexes between type I and II TGF-β receptors do not form until the proteins reach a post-ER compartment (presumably the cell surface), have important implications for our understanding of complex formation and receptor regulation.

Analysis of TGF-β signaling pathways has been hampered by the lack of cell lines in which both Tβ RI and Tβ RII are deleted, and by the inability to study signal transduction by Tβ RI independent of Tβ RII since Tβ RI does not bind TGF-β directly. To overcome these problems, we constructed and expressed chimeric receptors with the extracellular domain of the erythropoietin receptor (EpoR) and the cytoplasmic domains of Tβ RI or Tβ RII. When expressed in Ba/F3 cells, which do not express EpoR, Epo induces the formation of a heteromeric complex between cell surface EpoR/Tβ RI and EpoR/Tβ RII chimeras. Neither the EpoR/Tβ RI nor EpoR/Tβ RII chimera interacts with endogenous TGF-β receptors. Ba/F3 cells expressing both EpoR/Tβ RI and EpoR/Tβ RII chimeras, but not EpoR/Tβ RI or EpoR/Tβ RII alone, undergo Epo-induced growth arrest. When expressed in Ba/F3 cells in the absence of the EpoR/Tβ RII chimera, EpoR/Tβ RI ($T^{204}D$), a chimeric receptor with a point mutation in the GS domain of Tβ RI that is autophosphorylated constitutively, triggered growth inhibition in response to Epo. Thus, both homo dimerization of the cytoplasmic domain of the type I TGF-β receptor and heterodimerization with Tβ RII is required for intracellular signal transduction leading to inhibition of cell proliferation.

The Tβ RII kinase is intricately regulated by autophosphorylation on at least three serine residues. Ser213, in the membrane-proximal segment outside the kinase domain, undergoes intra-molecular autophosphorylation which is essential for the activation of Tβ RII kinase activity, activation of Tβ RI, and TGF-β -induced growth inhibition. In contrast, phosphorylation of Ser409 and Ser416, located in a segment corresponding to the substrate recognition T-loop region in a three-dimensional structural model of protein kinases, is enhanced by receptor dimerization and can occur via an inter-molecular mechanism. Phosphorylation of Ser409 is essential for Tβ RII kinase signaling, while phosphorylation of Ser416 inhibits receptor function. Mutation of Ser416 to alanine results in a hyperactive receptor that is more able than wild-type to induce Tβ RI activation and subsequent cell cycle arrest. Since on a single receptor either Ser409 or Ser416, but not both simultaneously, can become autophosphorylated, our results show that Tβ RII phosphorylation is intricately regulated and affects TGFb receptor signal transduction both positively and negatively.

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TGF-β signaling via serine/threonine kinase type I and type II receptors

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Transforming growth factor -β (TGF-β) superfamily members exert their cellular signals by inducing distinct heteromeric complexes of two different serine/threonine kinase receptors, denoted type I and type II receptors (1). In mammals five type II receptors and seven type I receptors have thus far been identified (2-4). The ligands for ALK-1 and ALK-7, predominantly expressed in endothelial cells and nervous system, respectively, remain to be identified.

We investigated the in vivo phosphorylation sites in TGF-β type I receptor (TβR-I) and TGF-β type II receptor (TβR-II) after complex formation (5). Phosphorylation of TβR-II was observed at serine residues in the C-terminal tail and in the juxtamembrane region. TβR-II was constitutively phosphorylated and TGF-β addition did not induce further phosphorylation in TβR-II. TGF-β induced phosphorylation of serine and threonine residues in the GS-domain of TβR-I and more N-terminal of this region (Ser165). Mutations of Ser165 in TβR-I resulted in an increase in TGF-β-mediated growth inhibiton and extracellular matrix formation, whereas TGF-β-induced apoptosis was decreased. Thus TβR-I/Ser165 phosphorylation appears important for signal regulation of the activated heteromeric receptor complex.

Smads play a key role in the intracellular signaling pathways of TGF-β superfamily members downstream of serine/threonine kinase receptors. In transfected COS cells, we observed that TGF-β and activin, but not BMP receptor activation induced phosphorylation of Smad2, and that activation of BMP receptors and to a lesser extend TGF-β receptors, but not activin receptors, induced phosphorylation of Smad1 (6). More importantly, in nontransfected Mv1Lu cells TGF-β stimulated the phosphorylation and nuclear translocation of Smad2, but not Smad1. Significant serine (and little threonine, but no tyrosine) phosphorylation of Smad2 was observed 15-30 min after addition of TGF-β, and plateaued after about 2 h incubation at 37 °C (6). The characterization of Smad2 phosphorylation sites and their functional significance, as well as the identification of downstream nuclear effectors that result in the transcriptional modulation of specific genes, will be the subject of future studies.

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ENDOGLIN MODULATES CELLULAR RESPONSES TO TGF-β

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Endoglin is an homodimeric membrane glycoprotein which can bind the -β1 and -β3 isoforms of transforming growth factor-β (TGF-β). The gene encoding endoglin has been localized to human chromosome 9q34qter and identified as the target gene for the autosomal dominant vascular disorder known as hereditary haemorrhagic telangiectasia type 1, a disease characterised by repeated and abundant nose bleeds, telangiectases, pulmonary and cerebral arteriovenous malformations and gastrointestinal bleeding. To understand the biology of endoglin, regulation and functional studies were conducted in monocytic cells. Endoglin is up-regulated during monocyte differentiation and TGF-β itself can stimulate the expression of endoglin in cultured human monocytes and in the U-937 monocytic line, suggesting the involvement of autocrine TGF-β1 in the up-regulation of its own receptor. To study the functional role of endoglin, stable transfectants of U-937 cells were generated which overexpress L- or S- endoglin isoforms, differing in their cytoplasmic domain. Inhibition of cellular proliferation and down-regulation of c-*myc* mRNA which are normally induced by TGF-β1 in U-937 cells were totally abrogated in L-endoglin transfectants and much reduced in the S-endoglin transfectants. Inhibition of proliferation by TGF-β2 was not altered in the transfectants, in agreement with the isoform specificity of endoglin. Additional responses of U-937 cells to TGF-β1, including stimulation of fibronectin synthesis, cellular adhesion, platelet/endothelial cell adhesion molecule 1 (PECAM-1) phosphorylation and homotypic aggregation were also inhibited in the endoglin transfectants. However, modulation of integrin and PECAM-1 levels and stimulation of mRNA levels for TGF-β1 and its receptors R-I, R-II and betaglycan ocurred normally in the endoglin transfectants. No changes in ligand binding were observed in L-endoglin transfectants relative to mock, while a 1.5 fold increase was seen in S-endoglin transfectants. The degradation rate of the ligand was the same in all transfectants. Elucidating the mechanism by which endoglin modulates several cellular responses to TGF-β1 without interfering with ligand binding or degradation should increase our understanding of the complex pathways which mediate the effects of this factor.

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TGF-β receptors and Smads

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TGF-β signals through two types of serine/threonine kinase receptors, the type II and type I receptors, which form a heteromeric complex presumably consisting of two type II and two type I receptors. Whereas several type I receptors can physically interact with the type II TGF-β receptor (TβRII), only one of them, TβRI, has been shown to mediate the gene induction and growth inhibitory responses in Mv1Lu epithelial cells. This specificity is remarkably considering the high degree of sequence conservation among the cytoplasmic domains of the different type I receptors. To explore the structural basis for this signaling specificity, we have analyzed the cytoplasmic domains of TβRI and the related Tsk7L type I receptors and of various chimeric derivatives. Based on our analyses of the chimeric and mutant receptors, we conclude that the differential abilities of the cytoplasmic domains of the TβRI and Tsk7L receptors to mediate TGF-β signaling is associated with a short segment in the kinase domain that shows a striking sequence difference and is predicted to be exposed at the protein surface of the receptor.

We have identified the Mad-related proteins, Smad-3 and Smad-4/DPC4, as downstream mediators of TGF-β signaling, i.e. induction of both gene expression and growth inhibition. Smad-3 is associated with the receptor complex, whereas Smad-4 is not. In addition, Smad-4 cooperates with Smad-1 and Smad-2 in mediating BMP-2/4 and activin-like responses. Furthermore, Smad-4 synergizes with Smad-1, -2 and -3 in different responses. Based on these observations, Smad-4 is presumably a common mediator of responsiveness to different TGF-β family members, which is required in cooperation with different Smads. The physiological cooperativity of Smad-4 and -3 is based on their physical association. Thus, Smad-4 directly interacts with Smad-3 in a heteromeric complex, whereas Smad-3 and -4 also homodimerize. Homomeric and heteromeric associations of Smad-3 and -4 are presumably both required for their biological activity, since Smad mutations that decrease or abolish these interactions correlate with decreased activity or inactivity of these Smads.

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MADR Proteins in TGFβ Signalling

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Until recently, very little was known about the molecular nature of components required for ser/thr kinase receptor signalling. A novel class of molecules was recently identified in a genetic screen in Drosophila for enhancers of weak *dpp* alleles and was termed *mother's against dpp* (*mad*)[1]. There are no known structural motifs in MAD that suggest the function of this protein. However, we have shown that *mad* is necessary for signalling by the DPP receptor, TKV, placing this protein downstream of the receptor. Further, we have shown that Dros. MAD is also rapidly phosphorylated in response to BMP2 and in vivo, the protein translocates into the nucleus in response to DPP signalling. Identification of mammalian homologues of *mad* led to the isolation of the MAD-related (MADR) proteins, MADR1 and MADR2. Sequence comparison of these MADR proteins reveals that the amino and carboxy-terminal regions of the molecules, termed MH1 and MH2 domains, respectively, are highly conserved, while the middle portion is divergent[2]. MADR1, which is more closely related to Drosophila MAD, is rapidly and specifically phosphorylated in response to BMP, but not TGFβ or activin. This response depends on both the type II and type I receptors for BMP2 and can be induced by constitutively active versions of the BMP type I receptors ALK3 and ALK6[3]. BMP signalling also induces the rapid nuclear accumulation of MADR1 suggesting that this protein functions in transmitting signals into the nucleus[3,4]. MADR2 on the other hand is specifically regulated by TGFβ and activin signalling, but not BMPs and like MADR1, it accumulates in the nucleus upon activation of TGFβ signalling[5]. MADR2 interacts transiently with both TGFβ and activin receptors and is a direct substrate of the receptor[5]. Interaction and phosphorylation of MADR2 by the TGFβ receptor occurs via the type I kinase and requires transactivation of receptor I by receptor II. The sites of phosphorylation targeted by the TGFβ receptor have been identified and mutation of these sites generates dominant negative MADR2 which blocks TGFβ-dependent signals. Moreover, mutant MADR2 fails to accumulate in the nucleus in response to TGFβ signalling. Thus, MADR proteins function in TGFβ signalling to transmit signals directly from the receptor into the nucleus.

Given the central role that MADR proteins play in TGFβ signalling, we have sought to investigate their function in developmental and disease processes. Chromosomal mapping of *MADR2* reveals that the gene is at 18q21, a region that displays loss of heterozygosity in many human tumours and we have identified several missense mutations in sporadic colorectal carcinomas[6]. These mutations are not active in regulating early Xenopus development and act dominantly to block endogenous MADR2 function. These mutants block TGFβ signalling and in Xenopus, they block head formation, suggesting that MADR2 function is required in early development for formation of the head organizer. These studies demonstrate that mutations in components of the TGFβ signalling pathway can lead to the development of cancer and highlight the importance of this pathway not only in control of early development but also in human disease.

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SESSION 2. RECEPTORS AND SIGNAL TRANSDUCTION (II)

Chair: Harvey F. Lodish

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Decapentaplegic signalling and its roles in Drosophila development.
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Among the several TGF-beta signals known to be present in Drosophila, the DPP (decapentaplegic) signalling system has received the most attention. DPP is the Drosophila homolog to the vertebrate BMP2 and BMP4 ligands, and work elaborating this pathway in Drosophila has delineated numerous components with clear vertebrate cognates.

We have used both molecular and genetic approaches to identify and characterize components of this signalling pathway. Among the most productive of our genetic approaches are screens for maternally acting mutations that enhance (i.e., make more severe) the phenotypes of weak dpp mutations on dorsal-ventral patterning of the embryo. Specifically, these maternal effect mutations cause genotypes that make just enough DPP protein for normal dorsal-ventral pattern formation to be inviable. Out of these screens have emerged several candidate genes contributing to DPP signalling including Mad (Mothers against dpp) and Med (Medea). Progress in the genetic identification of other components of the DPP signalling pathway will be discussed.

We now know that Mad is the prototype of a family of molecules contributing to DPP signal transduction (the Smad family). We have explored the contributions of Mad to DPP signalling using morphogenesis of the embryonic gut as a model system. DPP has several roles in gut morphogenesis, but of special interest is the signalling of DPP from cells of a portion of the midgut mesoderm (parasegment 7) to the overlying gut endoderm to induce a cell fate change marked by expression of the homeotic gene labial in the endoderm cells. Using the labial induction assay, we have shown that MAD is specifically required in cells receiving (not sending) the DPP signal and is downstream of the thick veins (TKV) type I DPP receptor but upstream of labial expression. We have also demonstrated that, in vivo, a subset of the cytoplasmic localization of MAD shifts to the nucleus in response to a DPP signal, and that after that DPP signal disappears, the nuclear component is extinguished as well. In collaboration with A. Mehra, J. Wrana and L. Attisano, we have found that nuclear localization in cultured Drosophila cell lines correlates with phosphorylation of MAD in response to a DPP-like signal.

In a separate line of experiments, we have used development of pattern elements in the Drosophila wing to study the relative

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contributions of two type I receptors (encoded by the saxophone and thick veins genes) to DPP patterning. In collaboration with A. Penton and F.M. Hoffmann, we have studied the effects on wing blade patterning of homozygous mutant clones for null alleles of saxophone and partial loss-of-function alleles of thick veins. We find that these clones cause shifts in the anterior-posterior identity of their cells, consistent with the idea that these cells behave as if they are further from the source of a secreted DPP signal than they actually are. Effects of these clones on the DPP-responsive genes spalt and optomotor-blind are consistent with the effects on adult wing pattern elements. Our results are consistent with the model that a gradient of DPP with a high point midway along the anterior-posterior axis is interpreted through DPP receptors to allocate anterior-posterior fates. Taken together with our results on MAD signalling, we predict that the level of MAD translocation to the nucleus will be a readout of the response to graded DPP signals.

□

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Analysis of Downstream Components of TGFβ Signaling in *C. elegans* and *Drosophila*

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My lab is interested in identifying and characterizing signaling components of the TGFβ-like family. We use genetic and molecular techniques to study these pathways primarily in *C. elegans* and *Drosophila*. Currently, we are characterizing Smad genes from flies and worms, using genetic screens in both organisms to find new components, and are analyzing some new suppressors and enhancers from our genetic screens.

C. elegans studies

sma-2, sma-3 and sma-4 define a set of genes required for downstream signaling events.

SMA-2, SMA-3, and SMA-4 are members of a family of cytoplasmic signaling molecules, the Smads, which act downstream of TGFβ superfamily receptors. Other members of this family include the *Drosophila Mad* gene (see abstract by W. Gelbart), and the human tumor suppressor genes DPC4 and Smad2. We have previously described that *sma-2*, *sma-3*, and *sma-4* have very similar mutant phenotypes yet encode highly homologous gene products, and do not compensate for each other. These results have led to a model in which at least three Smads are required together for signal transduction in *C. elegans*. We are left with the questions: why are multiple Smads required? More specifically, are there functional differences between these genes? Are multiple Smads required in other pathways in other organisms?

sma-6 encodes a new type I receptor in *C. elegans*.

In *C. elegans*, two TGFβ-like signaling systems have been identified which utilize a common type II receptor, *daf-4*. The phenotypes of *daf-4* include constitutive dauer formation, small body size, and mail tail abnormalities. *daf-1* has previously been shown to encode a TGFβ superfamily type I serine-threonine receptor kinase, and mutations in this gene effect constitutive dauering. Based on our work in characterizing the downstream SMAD components *sma*-[2,3,4], we had postulated the existence of another type I receptor which was involved in those phenotypes shared by these SMADs and *daf-4*, namely body size and tail development.

We have cloned a novel type I receptor, and asked if it participated in the small pathway. Interestingly, it is encoded by the gene, *sma-6*. We are now interested in analyzing the developmental role of *sma-6* in more detail. We have obtained new alleles of *sma-6*, *wk7*, *wk8*, and *wk9* in screens for small mutants, and we also have performed a non-complementation screen using the canonical *e1482* allele.

Because the phenotypes of the *daf-4* and the *sma*-[2,3,4] genes, when mutant, also result in characteristic

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defects in tail development, we have analyzed the male tails of the *sma-6* alleles. It can be hypothesized that another type I receptor is involved in this process, but our results argue against this. Animals homozygous for the *e1482* allele do not exhibit the abnormalities, specifically crumpled spicules and ray fusions, present in other genes in the small pathway. However, the finding that these deformities are present in *wk7*,*wk8*, and *wk9* males confirms the notion of *daf-4*, *sma-2*, *sma-3*, *sma-4*, and *sma-6* functioning in a common pathway. These findings genetically demonstrate the potential for disparate TGFβ signaling pathways to intersect at a common type II receptor, and to elicit different biological outcomes.

lon-1 may be a negative regulator of the signaling pathway.

If a reduction-of-function results in small (i.e. short) animals, then mutations in a negative regulator might result in long animals. We have examined the three existing long loci in *C. elegans* and have shown that *lon-1* is epistatic to sma-6 and *sma-*[*2,3,4*]. The question still remains whether or not lon-1 is part of the downstream signaling, but further experiments add evidence to this hypothesis. Transformants of *sma-6* (which probably overexpress the gene 2-5 times normal levels) show a long body phenotype. Further, in cosmid rescue experiments for *lon-1*, we find that many transformed lines generate small animals (i.e. short). These experiments suggest a link between being short and being long and reflect positive and negative aspects of the same pathway. Efforts are underway to examine *lon-1* molecularly and determine if it is a negative regulator of the small pathway.

Genetic screens for new signaling components in *C. elegans*.

Our finding that the small phenotype and male tail ray transformations are mutant phenotypes indicative of the TGFβ pathway, has suggested that other genes in the pathway may exhibit similar mutant phenotypes. Screens for small animals have not been done systematically in *C. elegans*, and therefore previous screens have not saturated for this phenotype. We have screened 17,000 genomes for small and long animals and have recovered 45 mutations, which fall into about 5-7 complementation groups. Attempts are being made to clone some of these loci to determine what they encode and eventually how they intersect with the TGFβ signaling pathway.

Drosophila studies

Medea and *Mad* cooperate to effect the *decapentaplegic* signal.

In *C. elegans*, we have shown that multiple Smad genes are required for transducing a TGFβ-like signal. In an effort to identify novel *Drosophila* Smads, other than *Mad*, that may be required for DPP signalling, we have isolated a new Smad. Further studies show it is encoded by *Medea*. *Medea* and *Mad* are dominant maternal enhancers of *dpp* that, interestingly, derive from different classes of the Smad family. To assay their roles in DPP signalling, we have generated somatic clones of *Medea* and compared them with clones of *Mad* in the eye. We find that posterior clones of both genes result in a transformation to head cuticle, suggesting that both are necessary for furrow initiation. *Medea* clones in the anterior part of the eye show minor defects, like the defects of *Mad* clones reported by the Mlodzik and colleagues. This data, plus other data by Raftery and Gelbart, show that *Mad* and *Medea* mutant phenotypes are similar and that they are part of the *dpp* pathway. Using tissue culture, we have examined the subcellular localization of the two Smads and have observed biochemical differences between them. In an attempt to further dissect the functioning of these two genes, we have generated transgenic flies containing various deletion constructs of *Medea* and *Mad*. Such constructs may also allow us to

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determine the epistatic relationship between these two genes. In summary, we show that a conserved feature of TGFβ signalling is that at least two Smad members, one from each of the two divergent classes, are required for signalling.

A New Smad in *Drosophila*, the *deranged* gene

We have been using a combination of PCR and genome gazing to identify other Smads in *Drosophila*. Our studies from worms and flies suggested that there should be at least two more Smads in the fly genome. These efforts have identified *deranged*, a new member of the Smad family. We are attempting to identify mutations in this gene, in order to characterize it further.

Genetic screens

We are using modifier screens to identify new components of the TGFβ like signaling pathways in *Drosophila*. This has been done by examining a set of genomic deficiencies for interactions with activated type I receptors and by doing EMS screens for suppressors or enhancers of activated type I receptors. Interacting genes have been identified and are being further characterized.

One important issue in signaling is how specificity is achieved. We have noted differences in the phenotypes produced by activated *saxophone* and *thick veins* receptors and are exploiting these differences to address this question. Since both *saxophone* and *thick veins* are part of the *dpp* pathway, one expects that many of the loci picked up in the screen will be similar, but some will be unique. In fact, *Mad* and *schnurri* mutations suppress both activated receptors, so the genes that give specificity to the signal remain to be identified.

Apoptosis and Cell Cycle

In vertebrate systems, the TGFβ subfamily negatively regulates cell growth. This is accomplished, in part, by the regulation of inhibitors of the cell cycle. The *dpp* pathway in *Drosophila* also regulates the growth of various tissues, such as the imaginal disks, although it causes a positive growth. We are interested in exploring the relationship between *dpp* signaling and the control of cell cycle. This is being carried out by PCR screens for cell cycle regulators and by the examination of suppressor mutations for their effects on cell cycle markers.

Misregulation of the cell cycle can lead to apoptosis, providing a connection between the cell cycle and cell death. One recent report (see Niswander abstract) shows that BMP signaling in the interdigits of the developing chick and duck is responsible for inducing cell death. We find that over expression of activated receptors in the wing disk can lead to increased cell death, and we are examining the role of the *dpp* pathway in apoptosis.

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Developmental Control of Cell Death and Cartilage Differentiation by BMP signaling

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We are exploring the role of the bone morphogenetic proteins (BMPs), members of the TGFβ superfamily, during vertebrate limb development using mutant type I BMP receptors. One role we have identified is in mediating programmed cell death (apoptosis) in the interdigital region where BMPs are expressed. During embryonic development, cells between the digits normally die which leads to tissue regression and separation of the digits. Retroviral-mediated expression of a dominant-negative BMPR-IB leads to a suppression of interdigital apoptosis and causes the webbing to remain between the digits (Science 272, 738-741, 1996). The opposite mutation, an activated BMPR-IB results in increased apoptosis. Thus, BMP signaling can provide a developmental trigger for programmed cell death.

In contrast, our studies indicate that an activated BMPR-IA does not alter cell death and instead affects cartilage differentiation. Infection of the embryonic limb bud with activated BMPR-IA induces ectopic and extensive mesenchyme condensation and cartilage formation. In addition, cartilage differentiation is delayed at a stage prior to formation of hypertrophic cartilage. Joint formation is disrupted and soft-tissue and muscle are not observed. Preliminary experiments in which muscle precursors cells are labeled indicate these cells are recruited into the cartilage. Studies to determine whether these cells express cartilage-specific markers are underway. Analysis of genes involved in cartilage/joint formation and differentiation will be presented, as well as a model to place BMP signaling in the context of other signaling pathways necessary for these processes.

Recently we have started to explore whether BMP signaling in the limb is antagonized by the proteins, noggin and chordin, that bind to BMPs and inhibit their action. Results will be presented on the RNA localization of noggin and the effects of overexpressing noggin in the developing vertebrate limb. (Funded by NIH-HD-32427, Human Frontier in Science, and Memorial Sloan-Kettering Cancer Center Support Grant)

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Smad Structure and Function

Joan Massague. Memorial Sloan-Kettering Cancer Center and Howard Hughes Medical Institute.

TGFβ and the related cytokines BMP and activin signal by contacting two related transmembrane serine/threonine kinases that act in sequence, with the second kinase phosphorylating Smad proteins. Different Smads are phosphorylated in response to TGFβ, activin or BMP. Smad1 responds in this fashion to BMP2/4 and Smad2 to TGFβ and activin. Another member of this family is the tumor suppressor gene DPC4/Smad4 (deleted in pancreatic carcinoma). By analyzing activin, BMP and TGFβ signaling in *Xenopus* embryo and mammalian cells, we have shown that, in all these pathways, the responses tested are dependent on a functional interaction between DPC4 and one of the other Smads. DPC4 is essential for the function of Smad1 and Smad2 in pathways that signal mesoderm induction and patterning in Xenopus embryos as well as antimitogenic and transcriptional responses to TGFβ in breast epithelial cells. DPC4 associates with Smad1 in response to BMP and with Smad2 in response to activin or TGFβ. Smad phosphorylation and association with DPC4 correlate with accumulation of Smads in the nucleus, association with a DNA binding partner (Fast1) and induction of Smad transcriptional activity. Therefore, DPC4 is a shared partner of other Smads in pathways that can be traced all the way from the ligand to the target gene. The entire Smad signaling network may be disabled in cancer cells that have lost DPC4 function. To investigate the events underlying Smad activation and association, in collaboration with Nikola Pavletich we have resolved the crystal structure of the effector domain of DPC4. The structural information sheds light on the mechanism of Smad oligomerization and its disruption in cancer.

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Does the molecular heterogeneity of Hereditary Hemorrhagic Telangiectasia (HHT) help us to understand the role of TGF-ß and its receptors in vascular disorders?
Michelle Letarte, Nadia Pece, Sonia Vera, Annie Bourdeau, Marie-Eve Paquet, Urszula Cymerman, and Robert A. White*. Division of Immunology and Cancer, Hospital for Sick Children and Department of Immunology, University of Toronto, Toronto, Canada M5G 1X8 and *Department of Diagnostics Radiology, Yale University School of Medicine, New Haven, USA.

HHT is an autosomal dominant vascular disorder, also known as Rendu-Osler-Weber syndrome, which occurs at a frequency estimated at > 1 in 10,000 individuals. It is associated with frequent nose bleeds and a variable number of mucocutaneous telangiectases. Although 20-50% of patients have life-threatening vascular malformations in lung (PAVM), brain (CAVM) or liver, the molecular mechanisms underlying the generation of these AVM are largely unknown. An AVM is defined as a direct communication between arterie(s) and vein(s) bypassing a capillary bed. Direct shunting of blood in the veins at arterial pressures causes great distension and tortuosity in the walls of the involved veins with focal deposition of layers of elastic tissue and fibroblast proliferation. CAVM which often occur in newborn and young children, are thought to arise from a disordered mesodermal differentiation between weeks 3 and 8 of human gestation. HHT is a heterogeneous disease with great variations in clinical manifestations between families as well as within a given family. Genetic linkage analysis first revealed molecular heterogeneity in HHT with some families clearly mapping to chromosome 9q34 while others do not. Endoglin, which had been mapped to that same region (Fernandez-Ruiz et al., 1993) was then identified as the target gene for HHT 1; each family so far has a different mutation, most of which lead to premature stop codons (McAllister et al, 1994, 1995). Endoglin was shown previously to be a component of the TGF-ß1 receptor complex, expressed predominantly in endothelial cells. The gene for HHT 2 was subsequently mapped to chromosome 12q (Vincent et al, 1995; Johnson et al, 1995) and identified as ALK-1 (Johnson et al, 1996). This receptor type I is abundant in endothelial cells and when transfected into COS cells can associate with TGF-ß or activin receptor type II (Attisano et al, 1993). The three ALK-1 mutations reported to date are in the conserved kinase subdomains and would likely impair enzymatic activity. Analysis of a limited number of families suggests that PAVM and CAVM are associated with HHT 1 families while a later onset of disease and a reduced incidence of AVM are generally observed in HHT 2 families. A third locus, as yet unidentified, is associated with families with liver AVM (Piantanida et al, 1996). These findings raise the interesting possibility that endoglin, ALK-1 and the third candidate gene are involved in common pathways implicated in the regulation of blood vessel formation. The observation that endoglin mutations lead to a more severe phenotype than ALK-1 suggests that endoglin acts upstream from ALK-1 and possibly interacts with more than one TGF-ß family receptor complex. To understand the relative contribution of endoglin and ALK-1 to the initiation of HHT, we have derived human endothelial cells from umbilical vein (HUVEC) from clinically defined HHT families. Note that mutations are uncharacterised in these families, genotype could be HHT1, 2, or 3 and each child has a 50% chance of carrying a mutation. To date we have processed 9 HUVEC samples from families with a history of PAVM or CAVM; four showed 50% endoglin expression by flow cytometry and S35-methionine labeling corresponding to the product of the normal allele. The mutant protein appears unstable and is degraded intracellularly suggesting that the disease is due to reduced endoglin levels rather than to interference by the mutated protein. The other five cases of HUVEC expressed normal levels of endoglin suggesting that the babies were unaffected. This will be confirmed by assessing expression of endoglin in activated monocytes of the affected parents and by mutation analysis. We also processed seven HUVEC from families with no history of PAVM and CAVM, which all showed normal endoglin level. We must now demonstrate that the affected parents do not carry endoglin mutations but do indeed express ALK-1 (and/or eventually the type 3) mutations. Having HHT endothelial cells in culture bearing defined mutations of endoglin and/or ALK-1 should allow us to determine which pathways are affected in these cells, in response to TGF-ß or related ligands implicated in the regulation of blood vessel formation.

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Relationship Between the Jun-N-Terminal Kinase Pathway and TGF-ß Signalling in *Drosophila* During Dorsal Closure

Juan R. Riesgo-Escovar and Ernst Hafen

We are interested in the control of dorsal closure during embryogenesis in *Drosophila*, a process that involves coordinated epithelial cell shape changes. For this reason, we have cloned and characterized mutations in *basket* (*bsk*), a 'dorsal open' gene where the process of dorsal closure is defective. *bsk* codes for the *Drosophila* homolog of mammalian Jun-N-Terminal kinases. Mutations in the *Drosophila decapentaplegic* (*dpp*) receptors *thick veins* and *punt* also have dorsal closure phenotypes, and *dpp* itself is expressed in the leading edge cells during dorsal closure. The leading edge cells, the dorsalmost row of cells of the lateral epithelia, have been found to initiate the process of dorsal closure. These cells also express *bsk* . We are currently investigating the relationship between *bsk* signalling and *dpp* signalling in this process. Data will be presented that favours a model where *bsk* signalling controls *dpp* expression.

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SESSION 3. DEVELOPMENT

Chair: William M. Gelbart

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Dpp signalling in Drosophila limb development

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Dpp is expressed in a subset of Drosophila imaginal disc cells and has the capacity to organize patterning over large regions of surrounding tissue. We have examined whether Dpp exerts its organizing activity directly, by moving to distant cells, or indirectly, via secondary signals. Our results show that two putative target genes of Dpp, namely *omb* and *spalt*, can be induced at a considerable distance from a Dpp source in the wing imaginal disc, but only autonomously in cells expressing constitutively activated Dpp receptors. Thus Dpp acts directly to control gene expression in remote cells. Furthermore the range of *spalt* expression depends on the levels of Dpp produced and differs from that of *omb*. We infer that Dpp controls spatial pattern by establishing, over many cell diameters, a concentration gradient that dictates distinct cellular responses.

We also have analyzed the requirement for the Dpp signal transduction components Thick veins, Punt and Schnurri and for two novel orphan receptor serine/threonine kinases. In the developing wing blade, somatic clones lacking any of the Dpp signal transduction components fail to grow when induced early in larval development. The spatial requirement for these signaling components are consistent with a long range activity of Dpp and indicate that Dpp input controls cell proliferation during limb development.

In order to understand how wing imaginal disc cells interpret and respond to the Dpp signal, we are studying the Dpp target genes *omb* and *spalt*. In a first step we identify the regulatory elements that are responsible for the transcriptional activation of these genes in response to Dpp signalling.

Our aim is to understand how the Dpp morphogen gradient arises and how it controls growth and pattern.

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Genetic Approaches to Signalling by Drosophila Decapentaplegic- F. Michael Hoffmann

The Drosophila decapentaplegic protein is important in establishing the pattern of the embryonic ectoderm, mesoderm and midgut and in establishing the pattern of the adult appendages of the fly. Dpp expression is restricted to specific groups of cells by a large array of regulatory elements that lie both 5' and 3' to the coding region of the gene. The Dpp protein secreted from these cells acts over some distance, the extent of which is still being determined by several labs, and activates responding cells by binding to Type II (punt) and Type I (thickveins and saxophone) receptors. The activated receptors act through the Drosophila MAD protein to regulate the expression of specific genes, which differ in the different tissues that respond to Dpp. Some candidate target genes include zen in the dorsal ectoderm, bagpipe in the embryonic mesoderm, Ultrabithorax, wingless and labial in the embryonic midgut and optomotor blind, spalt, vestigial and wingless in the imaginal discs.

We have produced clones of cells that are mutant for dpp receptors to determine what happens when cells cannot respond to dpp. Analysis of clones of cells unable to respond to dpp in the leg and wing imaginal discs revealed that one function of dpp in these tissues is to negatively regulate the activation of the wingless gene by hedgehog (Penton and Hoffmann, 1996, Morimura et al., 1996). This role of dpp has important consequences on patterning in the leg and wing imaginal discs. In the leg disc, hedgehog induces dpp in the dorsal part of the disc and wingless in the ventral part of the disc. At the intersection of dpp and wg expression in the center of the disc, a specialized region is induced, the end knob, that is competent to undergo distal outgrowth to produce the distal regions of the leg. Negative regulation of wingless by dpp is necessary to ensure a single site of distal outgrowth. In the third instar wing disc, hedgehog induces dpp along the anterior-posterior boundary whereas wingless is induced along the dorsal-ventral boundary, the presumptive wing blade margin, by the actions of a different regulatory pathway including apterous, Notch and Serrate. dpp signaling is required to prevent wingless expression along the anterior-posterior boundary. Cells that cannot respond to dpp express wingless along the anterior-posterior boundary leading to the generation of an ectopic wing margin down the center of the wing blade.

We also have been interested in the function of the stripe of dpp expression in the morphogenetic furrow of the eye. The furrow sweeps across the eye disk in the late third instar. Cells ahead of the furrow are in a proliferating undifferentiated sheet of epithelial cells. The furrow initiates a coordination of the cell cycle and the differentiation and patterning of the cells into the clusters of retinal and accessory cells that become the adult eye. Our analysis of dpp receptor mutant clones in the eye imaginal disc has revealed a role for dpp in facilitating the synchronization of the cell cycle associated with the morphogenetic furrow. Our current interpretation is that dpp accelerates the progression of the cells through G2-M so that they enter G1 arrest in the furrow at the same time (Penton et al., 1997).

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Another major interest of the laboratory is in using genetic screens to discover other genes involved in dpp signaling. We have carried out a genetic screen for modifiers of a sensitized genetic background produced by mutation of the dpp receptor thickveins. This screen recovered new alleles in known dpp signaling genes punt, Mad and medea. We have sequenced the new alleles of Mad and punt. Most importantly, we identified mutations in three additional genes. Potentially, these will identify novel components of the dpp signaling pathway. The genetic screen did not recover any new alleles of the gene schnurri. Our lab and two other labs have previously reported that the Drosophila gene schnurri functions in the dpp signaling pathway. schnuuri encodes a large nuclear protein with seven C2H2 zinc finger motifs and was hypothesized to be a DNA binding protein. However, we have not been able to demonstrate specific DNA binding. Our genetic analysis indicates that interference with dpp signaling may require a specific shn allele, shnIB, that is predicted to produce a truncated protein. Our current working hypothesis is that the shnIB interferes with dpp signaling but that shn protein may not itself normally be required for dpp signaling.

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The role of Bmps in mouse embryonic development.

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The Bmp superfamily consists of more than 15 members in the mouse. We have taken a variety of approaches to studying the role of these Bmp genes in embryonic development. Null mutations have now been generated in several genes by homologous recombination in embryonic stem cells and the phenotypes of some of the homozygous mutants will be described briefly. In some cases, embryonic phenotypes have not been observed in homozygous mutants, presumably because the absence of one gene is compensated for by one or more family member(s) co-expressed in the same tissue. Some insight into the role of Bmp genes that are early homozygous lethals can come from analysis of heterozygous phenotypes on different genetic backgrounds. Thus, $Bmp4^{tm1}$ heterozygous mice show right hind limb preaxial polydactyly with low penetrance on the C57BL/6 background, but this is increased to 100% penetrance on all four limbs in the double heterozygotes $Bmp4^{tm1}/+$; $Gli3^{Xtj}/+$ and $Bmp4^{tm1}/+$; $Alx4^{tm1}/+$. Models for these genetic interactions will be presented.

In order to study the role of Bmps in early mouse forebrain development we have compared the expression of five different Bmp genes - Bmp 2, 4, 5, 6, and 7 by in situ hybridization. Striking co-expression of these Bmps is observed within the dorsomedial telencephalon, coincident with a future site of choroid plexus development. Bmp co-expression overlaps that of the homeobox gene, Msx1, and the winged helix gene, Hfh4, and is complementary to that of winged helix gene, Bf1. The domain of Bmp co-expression is also associated with limited growth of the neuroectoderm, as revealed by morphological observation, reduced cell proliferation, and increased local programmed cell death. In vitro experiments using explants from the embryonic lateral telencephalic neuroectoderm reveal that exogenous BMP4 protein induces expression of Msx1 and inhibits Bf1 expression, which is consistent with their specific expression patterns in vivo. Moreover, BMP4 locally inhibits cell proliferation and increases apoptosis in the explants. These results provide evidence that BMPs function during the regional morphogenesis of the dorsal telencephalon by regulating specific gene expression, cell proliferation and local cell death.

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New Insights into the roles of TGF-β1 from study of TGF-β1 knockout mice.

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The observations that TGF-βs1, 2, and 3 are often secreted by the same cells and that their mature, bioactive forms are often interchangeable *in vitro*, suggested that they might be functionally redundant. However, each isoform is encoded by a distinct gene and each is expressed in a unique pattern *in vivo*. Study of the promoters of these genes has shown that TGF-β1 is induced selectively by a variety of signals and is the isoform most acutely regulated in the pathogenesis of fibrotic diseases, in autoimmune disease, and especially in carcinogenesis. TGF-β1 is also the only isoform present in serum and plasma.

Generation of mice with a targeted deletion of the TGF-β1 gene has provided a unique perspective on the question of redundancy of the TGF-β isoforms *in vivo*, since expression of the TGF-β2 and 3 genes is unimpaired. Study of these mice has led to has led to new concepts of endocrine and autocrine modes of TGF-β1 action and to unambiguous assignments of *in vivo* roles of TGF-β1 in autoimmune disease and suppression of expression of class I and II major histocompatibility antigens (MHC); in thymic maturation of T-cells; in regulation of nitric oxide synthase; and in regulation of hematopoiesis, especially of myeloid lineages. Surprisingly, we also showed that the absence of TGF-β1 results in improved healing of simple incisional wounds and that release of TGF-β1 from platelets is *not* required to initiate repair. Perhaps the most unexpected finding is the identification of a role for TGF-β1 in regulation of mitchondrial gene expression and of mitochondriogenesis; these studies are still in the early stages but could link dysregulation of TGF-β1 expression to various degenerative processes associated with defects in oxidative phosphorylation.

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Non-Overlapping Phenotypes of the TGFβ Knockout Mice

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To determine the *in vivo* function of TGFβ1-3 we have generated mouse strains deficient in each. Consistent with the sequence conservation of each TGFβ across mammalian species, and with considerable non-overlapping expression patterns for each gene, the knockout phenotypes are all distinct, indicating considerable lack of redundancy between the TGFβs. TGFβ1 has both developmental and immunoregulatory functions (1,2). TGFβ2 and TGFβ3 have developmental defects that lead to perinatal deaths (3). These mice indicate that during embryogenesis TGFβ1 functions in preimplantation development and implantation, and in the adult it functions to prevent autoimmunity. TGFβ2 plays essential roles in epithelial-mesenchymal interactions in the development of the cranial, axial and appendicular skeleton, eyes and ears, heart, and urogenital tract. TGFβ3 plays an essential role in secondary palate fusion. A cautionary note will be struck with respect to making distinctions between primary and secondary effects of gene ablation phenotypes.

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REQUIREMENTS FOR TGFB/DPP SIGNAL TRANSDUCTION PATHWAY IN DROSOPHILA LIMB DEVELOPMENT

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decapentaplegic (*dpp*), has been implicated in many aspects of Drosophila development, controling proliferation and patterning at different stages. For instance, the localised expression of*dpp* is associated with organising activity in the developing limbs of Drosophila. Until recently, beside the identification of its typeII (*punt*) and typeI receptors (*thick-veins* and *saxophone*), few components of the signal transduction pathway were known and the mechanisms of *dpp/TGFb* long-range activity were still unclear. By generating a constitutively activate, ligand independent form of the type I receptor *thick-veins* , *tkv**, we showed *mother-against-dpp* (*mad*) is a positive, rate-limiting component of the *dpp* pathway, downstream of *dpp* receptors[1]. We identified two*dpp* dependent target genes involved in the wing development and showed that *dpp* exerts its non-autonomous effect by direct, long range action in neighbouring cells. We also found the local concentration of the *dpp* protein, defines at least one threshold for target activation[2]. There is still some debate concerning the possible existence of an other lower threshold for target activation. We will present data adressing wether the sensitivity thresholds in the pathway activity lies in the induction step or in the the subsequent differential resistence to declining *dpp* activity as the cells move away from the *dpp* source.
dpp and*wingless* are both required for Drosophila leg development. They are involved in the early specification of the leg primordium, for distalisation and later for patterning along the dorsal/ventral axis. They show interesting interactions in that they can both repress each other for dorsal/ventral patterning and synergize for distalisation. We are currently adressing and will present data concerning the temporal and spatial requirements for these signal transduction pathways in specification of distinct cell fates along the proximal/distal axis of the leg.

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SESSION 4. CELL CYCLE AND CANCER (I)

Chair: Brigid L. M. Hogan

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Transgenic and Knockout Mice to Study TGFβ1 Functions *in vivo*: Cancer, Development and Genetic Modifiers

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In vitro and *in vivo* studies have implicated TGFβ1 in playing a role in a number of processes of relevance to both embryogenesis and neoplasia. We have focused on the use of transgenic mice, both conventional over-expressers (1,2) and the TGFβ1 knock out mice (3,4), to investigate its biological function in a whole animal system.

Tumorigenesis: TGFβ1 has been implicated in cell cycle control and carcinogenesis. To address the exact function of TGFβ1 in skin carcinogenesis *in vivo*, mice with TGFβ1 expression targeted to keratinocytes were subjected to long term chemical carcinogenesis treatment (5). TGFβ1 showed biphasic action during multistage skin carcinogenesis, acting early as a tumour suppressor, but later enhancing the malignant phenotype. The transgenics were more resistant to induction of benign skin tumours than controls, but the malignant conversion rate was vastly increased, such that the number of carcinomas was greater in transgenic than control mice. There was also a higher incidence of highly invasive, fibroblastoid spindle cell carcinomas, suggesting that TGFβ1 can enhance malignant progression by eliciting an epithelial-mesenchymal transition *in vivo* (5). This latter activity of TGFβ1 is supported by the observations of other labs that TGFβ1 can induce epithelial-mesenchymal transitions in a number of epithelial cell types *in vitro*. Furthermore, we have recently isolated and characterised clonal cells lines from a lymph node metastasis of a chemically induced mouse skin carcinoma which can undergo a reversible epithelial (squamous) to mesenchymal (spindle) transition either *in vitro* or *in vivo*. When injected subcutaneously into nude mice *in vivo*, both squamous and spindle clones give rise to spindle tumours, but the squamous clones subsequently revert to their original epithelial phenotype when tumour explants are re-cultured *in vitro*. We have shown that TGFβ is indeed necessary for the spindle cell conversion which occurs *in vivo*, and that this ability of TGFβ to enhance malignant progression results from its direct action on the carcinoma cell *per se* rather than secondary to other mechanisms, such as immunomodulation (6). The action of TGFβ1 in enhancing malignant progression may mimic its proposed function in modulating epithelial cell plasticity during embryonic development.

Development: Homozygous TGFβ1 knock out mice are non-viable, but the cause of death is variable. We previously reported that, on a mixed genetic background, ~50% of homozygous null animals die mid-gestation due to defects of the yolk sac, primarily affecting yolk sac vasculogenesis and haematopoiesis (4). The other half die post-natally (3). We have now bred the null allele through four generations onto three genetic backgrounds, C57Bl6J/Ola, NIH/Ola and 129/Sv and have examined percentage prenatal survival and embryonic phenotype on all three backgrounds, and on embryos which are F1 (NIH × C57). C57Bl6 TGFβ1$^{-/-}$ animals *all* die prenatally, mostly pre-organaogenesis (< 8.0 dpc). 80% NIH/Ola; 25% 129/Sv and 30% F1 TGFβ1$^{-/-}$ animals survive to birth, prenatal loss being due to yolk sac insufficiency in all cases. In F1 intercrosses there were no maternal effects between C57 and NIH, suggesting that different maternal TGFβ1 levels do not play a role in the differential

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prenatal loss. Utilising the two strains, NIH and C57Bl6J, which showed the largest differential in terms of prenatal survival, we performed a genome wide screen to search for genetic modifiers which determine the phenotypic outcome of the TGFβ1$^{-/-}$ genotype. A large F1 intercross, using 80 TGFβ1$^{-/-}$ neonatal animals and ~50-60 polymorphic DNA markers ~90% of the genome was screened. A major codominant modifier locus was identified linked to D5Mit268 on chromosome 5. This single modifier accounts for 80-90% of the genetic variation between NIH and C57 strains. The simplified prediction is that, at this locus, TGFβ1$^{-/-}$ mice which are homozygous NIH will survive to birth, TGFβ1$^{-/-}$ mice homozygous for C57 will die pre-organogenesis, and mice heterozygous C57/NIH will die mid-gestation from yolk sac insufficiency (7). This locus presumably encodes a gene of importance in determining TGFβ1 responses *in vivo*.

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The retinoblastoma protein pathway in restriction point control and oncogenesis.

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To make the fundamental decision whether or not to commit themselves to replication of the genome and a round of the cell division cycle, eukaryotic cells evolved a carefully controlled checkpoint mechanism strategically placed in advanced G1 phase. Defects in this checkpoint, called the 'restriction point' (or 'R' point) in mammalian cells, may lead to deregulated growth with devastating consequences for the organism. Recent evidence from several laboratories including ours strongly implicates the biochemical pathway comprising the D-type cyclins, their partner kinases CDK4 and CDK6, multiple CDK inhibitors (CDIs) from the INK4 or Kip/Cip families, and the retinoblastoma protein (pRb), as a molecular mechanism potentially governing progression through this checkpoint. The model best supported by current evidence is that pRb exerts negative control on the G1>S-phase transition by sequestering positive regulators of G1 progression, such as transcription factors of the E2F family, and that this growth-restraining ability is eliminated via phosphorylation of pRb (executed by G1 cyclin/CDK complexes) in late G1, thereby allowing the liberated E2F and other transcription factors to activate the S-phase genes. The D-type cyclins and their associated kinase activities represent a sensor and a convergent step regulated, in opposite ways, by both mitogenic and growth inhibitory signals, including TGF-β. The order of events along the G1-controlling pathway, the underlying biochemical mechanisms, and possible existence of parallel pathway(s) regulating G1>S transition will be considered.

The key components of the 'restriction point pathway' have properties of either tumour suppressors (pRB, $p16^{INK4/CDKN2}$ inhibitor of CDK4/6) or proto-oncogenes (cyclins D1 and D2, CDK4), and deregulation of at least one component of the pathway appears to be an obligatory step in the multistep development of the majority, if not all, human malignancies. On the other hand, our results indicate that biological consequences of deregulating individual components along the 'cyclin D-CDK-CDI-pRb pathway' are unequal, most likely reflecting their hierarchical roles in the G1 control. Thus, reflecting its key position in the pathway, loss of pRb totally eliminates the checkpoint, whereas aberrations of the upstream components, such as p16 and cyclin D1, can cooperate in multistep tumorigenesis. Both of these emerging concepts, namely the candidacy of the 'cyclin D-CDK-CDI-pRb pathway' as an obligatory target in multistep tumorigenesis, and the hierarchy of the individual components within the G1-controlling mechanism, will be documented by data from our laboratory. Finally, the emerging significance of these concepts for better understanding the molecular basis of the normal cell-cycle control, and for tumour diagnostics and management, will be discussed. These recent findings and particularly the unravelling of the 'pRb pathway', strengthen the homeostatic role of the G1 control mechanism(s), and the need to focus future research activities on multicomponent pathways as functional units and complex oncogenic targets.

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INTERPLAY BETWEEN CYTOKINE SIGNALLING AND ONCOGENE EXPRESSION IN THE CONTROL OF CELL VIABILITY

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Many diverse factors modulate mammalian cell apoptosis. These include oncoproteins, tumour suppressor proteins, cell cycle regulators, cytokines, toxins and physical trauma. However, how each of these impacts upon what is believed to be a basal apoptotic machinery is obscure. We have studied the induction of apoptosis in fibroblasts by the oncoproteins c-Myc and E1A, both of which promote cell death most effectively in the absence of the survival factor IGF-I. Suppression of oncoprotein-induced apoptosis by IGF-I is independent of the cell cycle, requires no *de novo* protein synthesis and its effect is exerted almost instantaneously. To investigate further the mechanism by which IGF-I suppresses apoptosis, we have taken two strategies. One is to dissect the IGF-I receptor to identify regions of the molecule that propagate survival signals. The second is to identify where within the "hierarchy" of apoptosis control IGF-I exerts its effects. We can show that IGF-I exerts an anti-apoptotic effect downstream of oncoproteins, p53, the Bcl-2 family, and the pro-enzyme form of the cysteine protease ICE. However, IGF-I does not suppress apoptosis induced by active ICE. However, comparison of the anti-apoptotic effects of IGF-I with a *bona fide* ICE protease inhibitor ZVAD.fmk indicates that IGF-I acts upstream of activation of ICE proteases. Whereas IGF-I prevents the onset of apoptosis, as judged by the start of membrane blebbing, ZVAD.fmk has no effect on membrane blebbing but acts to prevent completion of the apoptotic programme itself. Thus IGF-I acts prior to the "apoptotic trigger" whereas active ICE proteases comprise part of the actual apoptotic process itself.

3 key publications for Gerard Evan

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About the role of TGFβ in tumor cell invasiveness

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The Ha-Ras gene is mutated and/or overexpressed in 30 to 50 % of solid tumors in man. Human breast carcinomas display generally overexpression of receptor tyrosine kinases (RTKs) or non mutated Ras. Moreover, mutated Ras contributes to the formation of breast carcinomas in various rodent models.

We studied the effect of a mutated Ha-Ras oncoprotein on non tumorigenic murine mammary epithelial cells (MMECs). These epithelial cells are highly polarized in cell culture, cause however rapid tumor growth after subcutaneous injection into nude mice and undergo epithelial-mesenchymal transition during tumor outgrowth. Together with other fibroblastoid properties, the tumor cells acquire a drastically increased invasive capacity.

To investigate this extreme phenotypic flexibility of Ras-transformed mouse mammary epithelial cells in the absence of host factors we cultured epithelial cells in reconstituted collagen matrices under defined serum free conditions. Ras-transformed cells acquired a highly polarized epithelial organization similar to untransformed cells. Addition of TGFβ1 to Ras-transformed MMECs induced an epithelial-fibroblastoid cell conversion indistinguishable from the one observed during tumor outgrowth.

In vivo the initial TGFβ dose inducing the epithelial fibroblastoid conversion of tumor cells in vivo is produced by stromal fibroblasts as a paracrine host derived factor. Moreover, the same Ras transformed MMECs are growth inhibited by TGFβ only in the epithelial phenotype, become unresponsive to growth inhibition, when gaining fibroblastoid properties. Once converted fibroblastoid Ras-cells become highly invasive and produce high amounts of TGFβ themselves, stabilising their fibroblastoid phenotype. Interrupting this autocrine loop results in reversion to the epithelial polarized phenotype.

To evaluate a more general implication of TGFβ in the control of epithelial tumor cell invasiveness we investigated various human tumor cell lines for invasiveness in vitro in the presence or absence of TGFβ. All lines tested showed a strong invasive phenotype in the presence of TGFβ, but rather marginal invasiveness in its absence.

To proof this finding in vivo and to correlate it with invasiveness during tumor outgrowth in vivo, we stably expressed a dominant negative form of TGFβ receptor II in murine epithelial tumor cell lines. Tumor cell lines expressing the dominant negativ TGFβ receptor II showed drastically reduced tumor outgrowth, indicating disturbed local invasiveness of the tumor cells as well as reduced formation of lethal lung metastases. These study, carried out with cell lines from two species and different tissue origin demonstrate a clear dependence of epithelial tumor cell invasiveness on TGFβ.

Taken together these data suggest that TGFβ induces its own production in an autocrine loop and synergistically co-operates with dominant oncogenic transformation. This mechanism is markedly facilitating tumor cell invasiveness.

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TGF-β REGULATES PROLIFERATION, DIFFERENTIATION AND DEATH OF FETAL HEPATOCYTES IN PRIMARY CULTURE

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Fetal hepatocytes in primary culture are cells capable to carry out both proliferation and differentiation processes simultaneously. Previous studies have shown that these cells respond to mitogens, such as hepatocyte growth factor (HGF) or epidermal growth factor (EGF), inducing the expression of early genes, such as fos and myc.

The transforming growth factor-β (TGF-β) family is one of the most influential groups of growth and differentiation factors. We have shown that TGF-β1 inhibits fetal hepatocyte proliferation arresting these cells at G1 phase of the cell cycle and down-regulating the mitogen-induced myc early expression. However, TGF-β has no effect on the expression of other protooncogenes, such as fos and H-ras. In addition to its inhibitory role on fetal hepatocyte growth, TGF-β increases the mRNA levels of fibronectin, an extracellular matrix protein, and maintains the expression of some liver specific genes, such as albumin and alfafetoprotein, above control values. The analysis of the expression of some hepatocyte transcriptional factors has shown that TGF-β increases HNF1α, HNF1β and HNF4 mRNA levels, indicating that TGF-β may modulate liver growth and differentiation throughout fetal development.

Furthermore, TGF-β also regulates death of these cells. Dose-response analysis shows that the TGF-β concentration needed to induce hepatocyte death (2.5 ng/ml) is 5 times that needed to inhibit growth in these cells (0.5 ng/ml). In response to TGF-β, hepatocytes induce DNA fragmentation and the appearance of nuclei with a DNA content lower than 2C, typical of a programmed cell death model. TGF-β-induced apoptosis in fetal hepatocytes is preceded by an induction of reactive oxygen species production and a decrease in the glutathione intracellular content, indicating that this factor induces oxidative stress in fetal hepatocytes. Studies performed to analyse levels of c-fos mRNA - a gene whose expression is modulated by redox state - have demonstrated that only high, apoptotic concentrations of TGF-β (2.5 ng/ml) produce an increase in the mRNA levels of this gene. Gel mobility shift assays shows that the c-fos induced expression is coincident with an increase in AP-1 activity. Finally, cell death induced by TGF-β in fetal hepatocytes is partially blocked by radical scavengers, which decreased the percentage of apoptotic cells , whereas these agents did not modify the growth inhibitory effect elicited by TGF-β in these cells. In summary, these results provide evidence for the induction of an oxidative process in response to TGF-β in fetal hepatocytes, which would regulate apoptosis but not growth of these cells.

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Altered Regulation of the Cdk Inhibitors Contributes to TGF–β Resistance and Cancer Progression. **J.M. Slingerland**[1], M. Stampfer[2], and C. Catzavelos[1] University of Toronto and Sunnybrook Health Sciences Centre, Toronto, CANADA[1] and Lawrence Berkeley Laboratory, University of California, Berkley, CA, USA[2]

Loss of sensitivity to TGF–β–mediated growth arrest is common in human adenocarcinomas and may confer a growth advantage during tumor progression. To explore how sensitivity to arrest by TGF–β may be lost in cancers, we have studied the effects of TGF–β in closely related human mammary epithelial cells (HMEC) that differ in their cell cycle response to TGF–β. These cells express type I and type II TGF–β receptors and retain other TGF–β responses. The arrest resistant phenotype was not due to loss of cdk inhibitors $p15^{INK4B}$, $p21^{CIP1}$ or $p27^{Kip1}$. Cellular $p27^{Kip1}$ was not increase in TGF–β arrested cells, rather TGF–β caused shifts in the association of $p27^{Kip1}$ with target cdks. The comparison of TGF–β sensitive and resistant HMEC demonstrates that TGF–β regulates $p15^{INK4B}$ expression at at least two levels: mRNA accumulation and protein stability. In sensitive HMEC, TGF–β caused a marked increase in $p15^{INK4B}$ protein, due not only to increased p15 mRNA, but also resulting from a major increase in $p15^{INK4B}$ protein stability. As cdk4 and cdk6-associated $p15^{INK4B}$ increased during TGF–β arrest of sensitive cells, there was a loss of cyclin D1, $p21^{Cip1}$, and $p27^{Kip1}$ from these kinase complexes, and cyclin E/cdk2 associated $p27^{Kip1}$ increased. We also demonstrate that $p15^{INK4B}$ can displace cyclin D1 and KIP proteins $p21^{Cip1}$ and $p27^{Kip1}$ from cdk4 complexes in vitro. These data support the hypothesis previously raised by others, that $p15^{INK4B}$ acts to displace already associated cyclin D1 from target cdks, prevents new cyclin D1/cdk complexes from forming and TGF–β causes a shift in the equilibrium of binding of $p27^{Kip1}$ from cdk4 to cyclin E/cdk2. Comparison of TGF–β sensitive and resistant cell lines highlights the importance of post-translational regulation of $p15^{INK4B}$ by TGF–β. In the TGF–β resistant HMEC line, $184A1L5^{R}$, although the p15 transcript increased, the $p15^{INK4B}$ protein did not accumulate. $p15^{INK4B}$ half-life was not increased, and cyclin D1/cdk association and kinase activation were not inhibited.

We have found evidence for altered post-translational regulation of $p27^{Kip1}$ in primary human breast carcinomas. Immunohistochemical analysis of 168 primary breast cancers showed consistent loss of $p27^{Kip1}$ protein in both *in situ* and invasive tumors. The decrease in $p27^{Kip1}$ levels in primary tumors was confirmed by Western analysis and was not due to loss of gene expression. Reduced $p27^{Kip1}$ was a strong independent predictor of disease relapse. Loss of $p27^{Kip1}$ in human breast cancers may be part of the TGF–β resistant phenotype and represent an important step in tumor progression.

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SESSION 5. CELL CYCLE AND CANCER (II)

Chair: Rik Derynck

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c-Myc suppresses the growth-inhibitory function of $p27^{Kip1}$ and $p16^{INK4a}$ by distinct molecular mechanisms

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In normal cells, expression of the c-Myc oncoprotein (Myc) is strictly dependent on mitogenic stimuli and, conversely, is suppressed by growth-inhibitory signals. Myc has itself a strong mitogenic activity: induction of Myc in quiescent cells promotes cell cycle entry, and constitutive Myc expression prevents cell cycle exit in response to antiproliferative signals. However, the mechanisms linking Myc function to cell-cycle control remain unknown.

Progression through the cell cycle requires the enzymatic activity of cyclin-dependent kinases (CDKs). Cyclin/CDK complexes are controlled at various levels, one of which is the association with several inhibitory proteins collectively termed CKIs. CKIs mediate cell cycle arrest in response to a variety of growth-inhibitory stimuli such as TGFβ, mitogen withdrawal, contact-inhibition, and others. Strikingly, Myc overexpression is dominant to most of these stimuli. For example, Myc overcomes TGFβ-induced arrest in keratinocytes, which involves both $p27^{Kip1}$ and $p15^{INK4b}$. These observations suggested that Myc may interfere with the growth-inhibitory function of CKIs.

To test this hypothesis, we developed a double retroviral infection protocol allowing to sequentially express Myc and any CKI in cultured fibroblasts, and to biochemically analyse CKI-expressing cells shortly after infection. The results of two parallel studies will be presented, and are summarized below:

i. **Myc and p27**

In Rat1 cells, retrovirally-expressed p27 associates with cyclin E/CDK2 complexes and represses their catalytic activity. This leads to accumulation of Rb-family proteins in their hypo-phosphorylated, active forms and to G1 arrest. Prior expression of Myc, however, prevents cyclin E/CDK2 inactivation and dephosphorylation of Rb, and allows continuous cell proliferation in the presence of p27. These effects of Myc are mediated by a non-covalent sequestration of p27 in a form unable to bind cyclin E/CDK2. Neither Myc itself, nor other G1-cyclins are directly responsible for p27 sequestration (Vlach *et al.* 1996, *EMBO J.* **15**, 6595-6604). Consistent with these results, we also observed that activation of Myc in quiescent cells induces rapid sequestration of endogenous p27 leading to activation of cyclin E/CDK2 .

ii. **Myc and INK4 proteins**

As for p27, Myc prevents growth arrest by the CKIs $p16^{INK4a}$, $p15^{INK4b}$ and $p18^{INK4c}$. However, unlike for p27, Myc does not impair the molecular function of p16. In Myc-expressing cells, p16 still associates with CDK6 and prevents hyperphosphorylation of Rb-family proteins. Thus, Myc cells

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proliferate with elevated p16 levels and hypophosphorylated Rb, suggesting that Myc bypasses the function of Rb, rather than that of p16 itself.

To gain further insight into the molecular pathways involved in Myc function, we asked whether G1 cyclins (A, E, D1-3) can substitute for Myc in preventing growth arrest by p27 or p16. None of these proteins prevents cell cycle arrest by p27. For example, retrovirally expressed cyclin E associates with cellular CDK2, but these complexes are still repressed by p27, and require Myc for derepression. On the other hand, cyclin E prevents p16-induced arrest. This is surprising at first sight, since cyclin E and its partner CDK2 are not targets of p16. However, as for Myc, cells expressing cyclin E and p16 proliferate with hypophosphorylated Rb-family proteins, suggesting that cyclin E also bypasses Rb function.

Since cyclin E levels are up-regulated by Myc, we suggest that bypass of p16-induced arrest is essentially a cyclin E-driven phenomenon. However, how Myc leads to deregulated cyclin E expression remains to be elucidated. The mechanisms by which cyclin E bypasses p16/Rb-induced arrest also remain unclear. For example, we do not know whether Rb-mediated repression of E2F function is relieved by Myc or cyclin E. This is a possibility, since we and others have found that E2F proteins also prevent growth arrest by p16. However, none of the E2F proteins relieve p27-induced arrest, suggesting that cyclin E/CDK2 activity (suppressed by p27 but not p16) is essential for cell growth downstream of E2F. Indeed, the cyclin E gene is a known transcriptional target of E2F.

Together with other reports, our findings suggests that cyclin E/CDK2 complexes perform a function distinct from Rb phosphorylation or E2F activation that promotes G1-S progression and cell proliferation. Our data further suggest that this function alone can bypass the accumulation of active pRb induced by p16 or other INK4 proteins.

In summary, Myc prevents p27- and p16-induced cell cycle arrest by distinct molecular mechanisms. Both activities of Myc require the formation of transcriptionally active Myc/Max dimers, suggesting that they are mediated by the products of yet unknown Myc/Max-target genes.

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Control of gene expression by promoter proximal pausing of RNA polymerase II

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The c-*myc* gene offered the first example of an eukaryotic cellular gene controlled by regulation of RNA elongation (1, 2). RNA polymerase II (pol II) pauses immediately downstream of the RNA cap site of the major c-*myc* P2 promoter. Pausing of pol II occurs irrespective of whether c-*myc* is repressed or transcribed (3, 4). The rate of c-*myc* transcription is regulated by a controlled release of pol II from its pause site. Inhibition of phosphorylation of the carboxy-terminal domain (CTD) of the large subunit of pol II does not inhibit initiation and transcription up to the pause site. However, inhibition of CTD phosphorylation inhibits the transition from a paused to a processive transcription mode, indicating that kinase activities are required in the regulation of pol II release from its pause site (5).

In Burkitt's lymphoma (BL), a human B cell tumour, the c-*myc* gene is chromosomally translocated to the constant region of one of the three immunoglobulin (Ig) gene loci. Chromosomal breakpoints have been detected >100 kb upstream and downstream of c-*myc*. While the normal c-*myc* allele in BL cells is transcriptionally silent, the translocated allele is expressed at high levels. Pausing of pol II on the translocation chromosome is abolished suggesting that element(s) in the immunoglobulin gene loci can provide signals for activation of paused pol II (6).

Promoter proximal pausing of pol II can be reconstituted on stably transfected episomal Epstein-Barr virus-derived DNA constructs in BL cells. These constructs establish an authentic c-*myc* chromatin with paused pol II at a position identical to chromosomal c-*myc* (7, 8). The c-*myc* gene on these constructs is repressed and not inducible by PMA, Ca-ionophore and other inducers which activate c-*myc* in quiescent cells. However, sodium butyrate, an inhibitor of histone deacetylases, strongly induces the episomal c-*myc*. This activation is inhibited by TGF-β1. In contrast, repression of the translocated c-*myc* by sodium butyrate is not affected by TGF-β1 in BL cells (8).

Since regulatory elements of the Ig gene loci are suggested to influence promoter proximal pausing of pol II we became interested in the regulation of Ig gene promoters. The mouse pre-B cell line 70Z/3 carries a functionally rearranged Ig-κ gene. Nuclear transcription experiments and genomic footprint analysis revealed a paused transcription complex proximal to the Ig-κ gene promoter. The Ig-κ gene is not

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expressed in 70Z/3 cells but can be induced by bacterial lipopolysaccharide (LPS). This induction occurs without a significant decrease in the pause rate of pol II. Induction of the Ig-κ gene by LPS requires activation and binding of NF-κB to the Ig-κ intron enhancer. TGF-β1 inhibits induction of the Ig-κ gene by LPS but not binding of NF-κB to DNA (9), suggesting that TGF-β1 may specifically interfere with a signal pathway/interaction of the Ig-κ intron enhancer to the paused pol II. Since Ig-κ gene transcription induced by interferon-γ is not sensitive to TGF-β1 in 70Z/3 cells (9) different signal cascades with different sensitivity to TGF-β1 appear to converge at paused pol II complexes proximal to the Ig-κ gene promoter.

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REGULATION AND FUNCTION OF THE p16^{CDKN2a} TUMOUR SUPPRESSOR. Gordon Peters, Eiji Hara, Ignacio Palmero, Beth McConnell and David Parry. Imperial Cancer Research Fund, 44 Lincoln's Inn Fields, London WC2A 3PX.

The product of the *CDKN2a* tumour suppressor gene, p16, binds to and inhibits the cyclin-dependent kinases Cdk4 and Cdk6, which function as regulators of G1 progression by initiating the phosphorylation of the retinoblastoma gene product, pRb. Ectopic expression of p16 can induce a G1 arrest but only in cells that retain functional pRb. Paradoxically, human cell lines lacking functional pRb accumulate very high levels of p16, enough to sequester all the available Cdk4 and Cdk6 and prevent them from associating with the D cyclins. However, this is not the case in mouse cells; primary fibroblasts from *Rb1*-nullizygous mouse embryos and mouse cells transformed by SV40 T-antigen contain moderate levels of p16 and retain the cyclin D-Cdk complexes.

In seeking an explanation for this surprising species difference, we have shown that the negative feedback loop through which pRb regulates the transcription of the human p16 gene does not appear to operate in mouse cells. Other facets of the gene are conserved in both species. For example, p16 RNA and protein are relatively stable, their levels do not fluctuate appreciably during the cell cycle, and they accumulate as fibroblasts approach their maximum number of population doublings in culture. These data suggest that p16 may be important in preventing cell immortalization rather than in regulating the cell cycle.

A role for p16 in establishing replicative senescence would also account for the higher frequency of mutations, deletions and methylation observed in tumour cell lines as opposed to primary tumours. However, p16 mutations do occur at a significant frequency in both sporadic and familial tumours. We have analysed a selection of premature termination and missense mutants for their ability to bind to Cdk4 and Cdk6, to inhibit cyclin D-Cdk4 kinase activity and to block cell cycle progression. Curiously, not all of the reported mutations affect known functions of p16, although two that are associated with familial melanoma represent temperature sensitive alleles.

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CYCLIN A IS DOWNREGULATED BY cAMP AND TGF-β

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Cyclin A whose function seems to be absolutely required for S phase traverse, is likely to be involved in oncogenic processes as its gene is a site of integration for hepatitis B virus in some hepatocarcinomas. It was therefore interesting to look for a deregulation of its expression in CCL39 Chinese hamster lung fibroblasts and in various transformed variants. In all cell lines tested, cyclin A expression was correlated with establishment of S phase, with a progressive deregulation of its G1 controls. We show here that TGF-β, which inhibits α-thrombin or fibroblast growth factor-induced mitogenicity in Go arrested CCL39 cells, down regulates cyclin A expression with a potency inversely correlated with the transformation status of the variants analyzed. We have cloned both human and murine cyclin A promoters into luciferase vectors. Transient transfections of these constructs into CCL39 or NIH3T3, cells, has allowed us to delineate sequences and characterize factors susceptible to mediate serum and TGF-β effects.

ι) A short 50 bp sequence which is highly conserved between mouse and man contains a cyclic AMP responsive element (CRE), a NF-1/NF-Y site and a GC-rich box. Band shift carried out with antibodies suggest for the first two sites the presence of multiple complexes containing ATF-1 and NF-Y respectively. The nature of the protein(s) binding to the third element is still unknown.

ii) The CRE element mediates both TGF-β1 and cAMP agonists down-regulation in CCL39 cells. In the latter case a systematic survey of genes modulated by TGF-β1 in variants of CCL39 points to JUNB as a potential partner of ATF.

iii) The GC-rich site (Cell Cycle Responsive Element, CCRE) is instrumental in mediating the response of cyclin A promoter to serum or MAP kinases agonists: its mutation abolishes the transcriptional repression in quiescent cells, resulting in a promoter with constitutive high activity.

iv) Interestingly, whereas the CRE and the NF-Y sites are always occupied in vivo, the CCRE occupancy varies according to the proliferation status of the cells. This observation has been confirmed in normal T lymphocytes whose proliferation can be induced by the stimulation of cell surface receptors with monoclonal antibodies.

Finally, cyclin A expression has been linked to cell adhesion. We show here that the CCRE is required to down-regulate cyclin A promoter in the absence of cell-matrix contacts.

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POSTERS

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A calcium-dependent signal transduction pathway is implicated in the induction of transcription factor CTF-1 by TGF-β

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Transforming Growth Factor beta (TGF-β) is a pluripotent peptide hormone that regulates various activities including cell growth, differentiation and extracellular matrix protein gene expression. We have previously shown that TGF-β induces the activity of CTF-1, the prototypic member of the CTF/NF-I family of transcription factors. This induction correlates with the stimulation of collagen gene expression by TGF-β, which was previously proposed to involve CTF/NF-I binding sites. However, the mechanisms of TGF-β signal transduction remain poorly understood. In this report, we have analyzed the role of free calcium signaling in the induction of CTF-1 activity by TGF-β. We found that expression of a constitutive form of the heterodimeric calcium/calmodulin-dependent phosphatase calcineurin induces the activity of the CTF-1 transcriptional activation domain (TAD) in mouse NIH3T3 cells. In contrast, a mutated calcineurin catalytic subunit which cannot heterodimerize with the regulatory subunit does not mediate CTF-1 activation, thus arguing for a direct involvement of free calcium signaling in TGF-β action. The immunosuppressants cyclosporin A and FK506 abolish calcineurin-mediated induction of the CTF-1 TAD. Suprisingly, however, these compounds have little effect on TGF-β induction of the CTF-1 TAD, suggesting that other calcium-sensitive enzymes might mediate TGF-β action. Indeed, expression of a truncated catalytic subunit of the calcium/calmodulin-dependent kinase IV (ΔCaMKIV), but not of other related kinases like CaMKI and CaMKII, potently activates the CTF-1 TAD and mimicks TGF-β induction. Consistent with the relevance of CaMKIV in the TGF-β signal transduction pathway, the previously identified TGF-β-responsive domain of the CTF-1 TAD mediates both TGF-β as well as ΔCaMKIV responsiveness. TGF-β induction is inhibited in cells pretreated with thapsigargin, which depletes the endoplasmic reticulum calcium stores, further arguing for the proposed role of calcium mobilization in TGF-β action. Finally, TGF-β stimulates calcium influx and mediates an increase of the cytoplasmic calcium concentration in NIH3T3 cells. Thus, a calcium-dependent signaling pathway involving CaMKIV or a closely related kinase is implicated in the transcriptional induction of CTF-1 by TGF-β, providing a molecular mechanism for the effects of TGF-β on gene expression.

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Nitric Oxide Antagonizes Transforming Growth Factor-β1-induced Apoptosis in Primary Cultures of Hepatocytes

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Regulation of organ size and liver function is the result of a dynamic balance between a complex network of signals (1). Under physiological conditions senescent or damaged cells are removed by apoptosis, whereas the response to acute cell injury mostly involves a necrotic process (2). Several growth factors and cytokines have been identified as inducers of hepatocyte apoptosis both in vivo and in vitro and among them, a prominent role has been recognized for TGF-β and for the engagement of the Fas/Apo-1 receptor (3). In addition to receptor evoked apoptosis, exposure of several cell lines to high levels of NO is a sufficient condition for triggering apoptosis. In contrast to this, low concentrations of NO protect against apoptosis and cellular stress in neurons and in various cell types of lymphoid origin (4). iNOS, the enzyme which synthesizes the high-output NO pathway, is expressed in liver in response to LPS and to combinations of pro-inflammatory cytokines as well as under pathological conditions such as cirrhosis or liver regeneration after partial hepatectomy. The physiological role of the transient expression of iNOS under non-physiological circumstances has been related to an improvement of the dynamic circulation through the liver as well as an additional modulator of the regenerating process. In this way, we have investigated the role of NO on the apoptosis triggerd by TGF-β in primary cultures of hepatocytes. Our results show that nitric oxide has a dual effect in the control of apoptosis in cultured fetal hepatocytes, i.e. that whereas a low synthesis of nitric oxide counteracts the apoptotic death induced by TGF-β, large amounts of nitric oxide have deleterious effects by itself, promoting both an apoptotic and necrotic response in these cells. These results suggest that nitric oxide synthesis by hepatocytes might be involved in the protection against apoptotic death.

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Expression and function of *decapentaplegic* and *thick veins* during the differentiation of the veins in the *Drosophila* wing

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The differentiation of the veins in the *Drosophila* wing involves the coordinate activities of several signal transduction pathways, including those mediated by the transmembrane proteins Veinlet and Notch and the Transforming growth factor ß superfamily member Decapentaplegic. We find that *decapentaplegic* is expressed in the pupal veins under the control of genes that establish vein territories in the imaginal disc. The expression of *decapentaplegic* in the veins is regulated by cis-regulatory sequences located in the *short vein* region of the gene. Decapentaplegic, acting through its receptor Thick veins, activates vein differentiation and restricts expression of both *veinlet* and the Notch-ligand *Delta* to the developing veins. Genetic combinations between mutations that increase or reduce *Notch*, *veinlet* and *decapentaplegic* activities suggest that the maintenance of the vein differentiation state involves cross-regulatory interactions between these pathways.

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TGF-β Type I Receptor Kinase Subdomain Determines TGF-β Signaling Specificity

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TGF-β transduces signals through a heteromeric complex consisting of two related serine/threonine kinase receptors, the type I and type II receptors. In the heteromeric complex, the type II receptor binds ligand and phosphorylates the cytoplasmic domain of the type I receptor. The type I receptor determines the intracellular signaling specificity, presumably by phosphorylating and activating specific downstream target(s). In mink epithelial (Mv1Lu) cells, TβR-I mediates TGF-β-induced growth inhibition and gene transcriptional activation responses, while other type I receptors for TGF-β superfamily including Tsk7L and TSR-I do not respond to TGF-β signals to elicit these responses.

In this study, we have constructed a series of chimeras between TβR-I and Tsk7L, and analyzed the functions of these swap mutants in TGF-β-induced growth inhibition and gene expression in Mv1Lu cells. We found that neither the entire extracellular and transmembrane nor cytoplasmic domain of the two type I receptors was interchangeable. Further sequential replacement in the cytoplasmic domain of TβR-I with the corresponding portion of Tsk7L revealed that the juxtamembrane domain (including GS motif) and most regions in the kinase domain of both type I receptors are functionally similar, with the exception that the the loop (L45; amino acid 235-284) between β4 and β5 in the kinase domain displayed functional distinctness for the two type I receptors. Replacement or mutation in the loop of TβR-I was found to dramatically decrease or abolish the signaling capacity of TβR-I, suggesting that the loop was essential in mediating TGF-β-induced intracellular responses. Furthermore, L45 of TβR-I, when introduced in a heterologous type I receptor, e.g. Tsk7L and TSR-I, was sufficient to confer the heterologous cytoplasmic domain to transduce TGF-β signals. These results suggest that the loop region may determine the specificity for TGF-β responses by serving as a docking site to recruit specific intracellular effectors or substrates within the close proximity of the oligomeric TGF-β receptor complex.

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ARAUCAN AND *CAUPOLICAN* PROVIDE A LINK BETWEEN COMPARTMENT SUBDIVISIONS AND PATTERNING OF SENSORY ORGANS AND VEINS IN THE *DROSOPHILA* WING

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The homeobox prepattern genes *araucan* (*ara*) and *caupolican* (*caup*) are coexpressed near the anterior/posterior (A/P) compartment border of the developing *Drosophila* wing in two symmetrical patches located one at each side of the dorso/ventral (D/V) compartment border. *ara-caup* expression at these patches is necessary for the specification of the prospective vein L3 and associated sensory organs through the transcriptional activation, in smaller overlaping domains, of *rhomboid/veinlet* and the proneural genes *achaete* and *scute*. We show that *ara-caup* expression at those patches is mediated by the Hedgehog signal through its induction of high levels of Cubitus interruptus (Ci) protein in anterior cells near to the A/P compartment border. The high levels of Ci activate *decapentaplegic* (*dpp*) expression and, together, Ci and Dpp positively control *ara-caup*. The posterior border of the patches is apparently defined by repression by *engrailed*. Wingless accumulation at the D/V border sets, also by repression, the gap between the two patches. Thus, *ara* and *caup* integrate the inputs of genes effecting the primary subdivisions of the wing disc into compartments to define two smaller territories. These in turn help create the even smaller domains of *rhomboid/veinlet* and *achaete-scute* expression.

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INTERACTION OF FKBP12 WITH THE TYPE I TGF-ß RECEPTOR

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TGF-ß1 is a potent inhibitor of cell growth. Accumulating evidences suggest that disruption of growth regulation by TGF-ß1 is one of the mechanisms of oncogenesis. However, the signal transduction pathway of TGF-ß1 within the cell remains elusive. We conducted a two-hybrid screening with the cytoplasmic region of the type I TGF-ß receptor (TßR-I) as the bait and have identified FKBP12 and farnesyl transferase-α (FTα) as interacting proteins. Both constitutively active and kinase-negative TßR-I bind FKBP12 *in vivo* and ligand addition does not affect the interaction. The juxtamembrane region of TßR-I, a putative regulatory domain of the TßR-I kinase, is required for the interaction. One of the deletion mutants in this region, which mediates transcriptional response to TGF-ß1, does not bind FKBP12. Futhermore, FKBP12 is not phosphorylated by TßR-I *in vitro* whereas FTα is. Taken together, we propose that FKBP12 is not a direct substrate of the TßR-I kinase but may modulates the TßR-I function through binding to its regulatory domain.

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Petra I. Knaus

A Dominant Inhibitory Mutant of Type II Transforming Growth Factor β Receptor in the Malignant Progression of a Cutaneous T- Cell Lymphoma

In many cancers, inactivating mutations in both alleles of the transforming growth factor β (TGF-β) type II receptor (TβRII) gene occur and correlate with loss of sensitivity to growth inhibition by TGF-β. We describe a novel mechanism for loss of sensitivity to growth inhibition by TGF-β in tumor development. Mac-1 cells, isolated from the blood of a patient with an indolent form of cutaneous T-cell lymphoma, express wild-type TβRII and are sensitive to TGF-β. Mac-2A cells, clonally related to Mac-1 and isolated from skin nodule of the same patient at a later, clinically aggressive stage of lymphoma, are resistant to TGF-β. They express both the wild-type TβRII and a receptor with a single point-mutation (Asp-404 to Gly: D404G) in the kinase domain (D404G-TβRII); no TβRI or TβRII is found on the plasma membrane, suggesting that D404G-TβRII dominantly inhibits the function of the wild-type receptor by inhibiting its appearance on the plasma membrane. Indeed, inducible expression, under control of a tetracycline-regulated promoter, of D404G-TβRII in TGF-β-sensitive Mac-1 cells as well as in Hep3B hepatoma cells results in resistance to TGF-β and dissappearance of cell surface TβRI and TβRII. Overexpresion of wild-type TβRII in Mac-2A cells restores cell surface TβRI and TβRII and sensitivity to TGF-β. The ability of the D404G-TβRII to dominantly inhibit function of wild-type TGF-β receptors represents a new mechanism for loss of sensitivity to the growth-inhibitory functions of TGF-β in tumor development.

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Identification of novel interactors of Smad1

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Smad1 (Madr1, Bsp-1, DwfA) is an intermediate in the BMP signal transduction pathway. Following ligand activation, Smad1 is phosphorylated and translocated to the nucleus where it acts as a co-factor in transcriptional activation. We have used a yeast two-hybrid system to identify intracellular interactors of Smad1. Initial screens identified 4 novel clones with no sequence similarity to known genes. Two of these clones interact with full-length Smad1 but not a C-terminal mutant form. Northern blot analysis demonstrates expression patterns similar to *smad1*. Current studies are aimed at determining the functional role of these novel proteins in mediating BMP signaling through Smad1.

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TRANSFORMING GROWTH FACTOR β–1 INDUCES MITOGENESIS AND DIFFERENTIATION-SPECIFIC GENE EXPRESSION IN FETAL RAT BROWN ADIPOCYTES

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Fetal brown adipocytes show a low number of transforming growth factor β1 (TGF-β1) binding sites of high affinity, revealing the presence of type I, II and III TGF-β1 receptors and a minor-labeled species of approximately 140 KDa. The culture of cells in the presence of TGF-β1 induced the expression of the tissue-specific gene uncoupling protein in a dose- and time-dependent manner. In addition, TGF-β1 up-regulates the expression of genes involved in adipogenesis, as well as induces the expression of fibronectin, specific target gene for TGF-β1 (1). These results suggest that TGF-β1 is a major signal involved in initiating and/or maintaining the thermogenic and adipogenic differentiation of fetal brown adipocytes before birth.

The presence of TGF-β1 for 24 or 48h stimulates DNA synthesis, the percentage of cells in the S+G2/M phases of the cell cycle, and cell number, as compared to quiescent cells. The mitogenic capacity of TGF-β1 (1 pM) is similar to that shown by 10% fetal calf serum. TGF-β1 for 48 h increases by 5-fold the percentage of cells containing (^{3}H)thymidine-labeled nuclei as compared to quiescent cells. In addition, single fetal brown adipocytes, showing their typical multilocular fat droplests phenotype, become positive for (^{3}H)thymidine-labeled nuclei in response to TGF-β1. Moreover, TGF-β1 induces the mRNA expression of a complete set of proliferation-related genes, such as c-fos (30 min), c-myc and β-actin (2 h), and H-ras, cdc2 kinase, and glucose 6-phosphate dehydrogenase at 4 and 8 h, as compared to quiescent cells. Concurrently, TGF-β1 for 12 h increased the protein content of proliferating cellular nuclear antigen by 6-fold and p21-ras by 2-fold (2). Although our results demonstrate that TGF-β1 induces the expression of very early genes related to cell proliferation, TGF-β1 could be acting either as a mitogen or as a survival factor to induce proliferation in fetal brown adipocytes.This possibility merits further investigation. In conclusion, we propose TGF-β1 as a developmental factor involved in brown adipose tissue growth and differentiation.

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Evidence that BMP-2 and Activin-A Respectively Regulate Specification and Subsequent Endoderm-Mediated Support of Embryonic Cardiac Myocyte Differentiation. John Lough, Matthew Barron, Michele Brogley, Kathleen M. McCormick and Xiaolei Zhu, Medical College of Wisconsin, Milwaukee, WI. 53226 USA.

This laboratory has demonstrated that endoderm cells in the heart forming region (HFR endoderm) of the stage 5-6 chicken embryo support terminal cardiac differentiation in explanted precardiac mesoderm cells, and that FGF or activin-A can mimic endoderm's cardiogenic activity (Dev. Dyn. 200:155, 1994; Dev. Biol. 168:567,1995). More recent findings (Dev. Biol. 178:198;1996) indicating that endoderm cells secrete decapentaplegic (DPP)-like antigens prompted a degenerate polymerase chain reaction (PCR) screen to identify cDNAs in the TGF-β DPP subgroup; the only DPP subgroup member detected was BMP-2. Although BMP-2 protein, unlike FGF or activin-A, could not support survival of precardiac mesoderm, addition of BMP-2 to FGF subtly increased the extent of myocardial differentiation. To ascertain whether combined BMP-2 and FGF could induce cardiogenesis in *non*-precardiac mesoderm, explants were cultured with individual or combined growth factors. Whereas FGF alone maintained explant viability without differentiation, BMP alone could not support cell survival. In contrast to these growth factors' isolated effects, treatment with BMP-2 plus FGF induced cardiogenesis, suggesting that this combination specified cells to the cardiac lineage. Somewhat surprisingly, neither growth factor alone could up-regulate serum response factor or tinman; expression of both transcription factors was dependent upon the combined presence of BMP-2 and FGF. Although activin-A, which as revealed by follistatin inhibition participates in endoderm-mediated cardiogenesis, can support precardiac mesoderm differentiation when present alone, this growth factor cannot replace BMP-2's specification activity on non-precardiac mesoderm. These results indicate that endoderm-secreted BMP-2 specifies gastrulating mesoderm cells to the cardiac lineage, while FGF and activin-A, which are also endoderm products, are required to complete the cardiac differentiation program.

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Transforming Growth Factor -ß mediates differential inhibition of cytokine activity through interference of mitogenic signal transduction, receptor expression and induction of apoptosis.

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Transforming Growth Factor-ß (TGF-ß) is a complex glycoprotein produced by a variety of haematopoietic cell types and possesses both growth enhancing and growth inhibiting properties dependent on target cell type, their concentration in culture and the presence of other growth factors. It has been shown that TGF-ß plays a role in regulating haematopoietic growth and differentiation and we have taken advantage of the use of several multi-responsive cytokine dependent haematopoietic cell lines to investigate the interactions of TGF-ß with a wide range of other cytokines. We show that TGF-ß exhibits a differential inhibition of cytokine induced mitogenesis, the level of which is dependent on the cytokine but not the cell line. One example of this is where GM-CSF or IL-3 induced proliferation is inhibited by 10-15% whereas IL-4 and IL-5 induced activity is inhibited by 80-90% when tested on TF-1, MO7-e and AML-193 cell lines. The anti-cytokine activity of TGF-ß is strictly time dependent and does not affect the movement of cells through the cell cycle. We show that the inhibition of cytokine induced cell proliferation by TGF-ß is based on its ability to rapidly induce apoptosis, as measured by flow cytometry, ELISA, DNA breakdown, chromium release and microscopy. In order to investigate the mechanisms underlying the TGF-ß differential inhibition of cytokine induced proliferation, we investigated the cytokine receptor expression and signal transduction pathways of GM-CSF, IL-3, IL-4 and IL-5 of the multi responsive human erythroleukaemia cell line TF-1. We illustrate that TGF-ß inhibits the expression of cytokine receptors on the TF-1 cell surface by flow cytometry and that the level of this inhibition is directly correlated with its ability to inhibit the proliferation mediated by each cytokine. Therefore, IL-4 and IL-5 receptor expression is drastically reduced compared to that of GM-CSF and IL-3. We also show that in TF-1 cells, GM-CSF and IL-3 induce tyrosine phosphorylation events that are required to induce mitogenisis. However, IL-4 and IL-5 induce tyrosine dephosphorylation events that are required to induce mitogenisis. Inhibitors of tyrosine kinases and phosphatases in cell culture underline the biological differences between the two pathways induced by these cytokines whereby genistein enhances IL-4 and IL-5 induced proliferation and inhibits GM-CSF and IL-3 and orthovanadate does the reverse. TGF-ß does not affect the ability of GM-CSF or IL-3 to induce tyrosine phosphorylation but is a potent inhibitor of the IL-4 and IL-5 induced tyrosine phosphatase. This data therefore suggests that the differential ability of TGF-ß to inhibit cytokine induced proliferation is due to its ability to induce apoptosis whilst interfering with receptor expression and cytokine signal transduction

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THE ROLE OF BONE MORPHOGENETIC PROTEINS IN VERTEBRAL DEVELOPMENT

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This study first shows a striking parallel between the expression patterns of the BMP4, *Msx1* and *Msx2* genes in the lateral ridges of the neural plate before neural tube closure and later on, in the dorsal neural tube and superficial midline ectoderm. We have previously shown that the spinous process of the vertebra is formed from *Msx1*- and 2-expressing mesenchyme and that the dorsal neural tube can induce the differentiation of subcutaneous cartilage from the somitic mesenchyme (Takahashi, Y. et al. (1992) *Proc. Natl. Acad. Sci. USA* 89, 10237-10241; Monsoro-Burq, A.-H. et al. (1994) *Proc. Natl. Acad. Sci. USA* 91, 10435-10439). Moreover, implantation of mouse (m) BMP4-producing cells in the somitic mesenchyme induces *Msx1* and *Msx2* gene expression subcutaneously followed by cartilage differentiation (Watanabe, Y. and Le Douarin, N.M. (1996) *Mech. Dev.* in press), as does the dorsal neural tube (Takahashi et al., 1992). We show here that mBMP4- or human (h) BMP2-producing cells grafted dorsally to the neural tube at E2 or E3 increase considerably the amount of *Msx*-expressing mesenchymal cells which are normally recruited from the somite to form the spinous process of the vertebra. Later on, the dorsal part of the vertebra is enlarged, resulting in vertebral fusion and, in some cases (e.g. grafts made at E3), in the formation of a "giant" spinous process-like structure dorsally. In strong contrast, BMP-producing cells grafted laterally to the neural tube at E2 exerted a negative effect on the expression of *Pax1* and *Pax3* genes in the somitic mesenchyme, which then turned on *Msx* genes. Moreover, sclerotomal cell growth and differentiation into cartilage were then inhibited. Dorsalization of the neural tube, manifested by expression of *Msx* and *Pax3* genes in the basal plate contacting the BMP-producing cells, was also observed.

In conclusion, this study further demonstrates that differentiation of the ventrolateral and dorsal parts of the vertebral cartilage is controlled by different molecular mechanisms. The former develops under the influence of signals arising from the floor plate-notochord complex (Pourquié, O. et al. (1993) *Proc. Natl. Acad. Sci. USA* 90, 5242-5246). These signals inhibit the development of dorsal subcutaneous cartilage forming the spinous process, which requires the influence of BMP4 to differentiate.

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by Aristidis Moustakas.

TGF-β signalling and the regulation of cytoskeletal organization.

Transforming growth factors β (TGF-β) are multipotent proteins that regulate cell growth, differentiation and extracellular matrix deposition. TGF-β arrests cell cycle progression and loss of responsiveness to this factor has been proposed as an important mechanism for tumorigenesis and progression. TGF-β signals through cell membrane hetero-oligomeric receptors that belong to the receptor serine-threonine kinase family. Among the few downstream signalling effectors described yet are the new family of MAD signal transducers which constitute a novel class of tumor supressors and the regulatory α-subunit of the isoprenyl-transferases. In the present work, the involvement of MAD and the isoprenyl-transferases is examined in the regulation of cell morphology of malignantly transformed fibroblasts by TGF-β.

Cell shape regulation by TGF-β involves the dynamic control of cell adhesion to its substratum and to its neighbouring cells. TGF-β upregulates both the synthesis of extracellular matrix and of integrin receptors on the cell membrane. TGF-β also upregulates actin biosynthesis at both the transcriptional and translational level, albeit to a low degree. The actin-based cytoskeleton (e.g. stress fibers) organization induced by TGF-β has also been reported in a variety of cell types but at low levels.

Malignantly transformed and tumorigenic Rat-1 and NIH-3T3 fibroblasts expressing the activated H-Ras (Val-12) oncogene stably (Rat-1/EJ-1) or under inducible conditions by dexamethazone (NIH-3T3/MMTV-H-Ras) and the corresponding normal fibroblasts have been exploited. TGF-β was found to completely revert the phenotype of the transformed fibroblast from spindle-form cells with intense cortical (peripheral ring-like) actin networks to flat, adherent cells full of dense stress fiber networks and multiple focal adhesion plaques. The effect was time and dose dependent. As low as 2 pM TGF-β1 could produce the reversion effect and at 100 pM TGF-β1, reverted cells were clearly observed after 10 min treatment, the phenomenon peaked at 2 hr and started regressing after 4 hr.

This reversion phenomenon is complex since it involves both rapidly acting pathways which are presumably directed towards the cytoskeleton and immediate-early gene responses which accumulate their effects within the 2 hr interval post TGF-β1 treatment. Results will be presented focusing on the following questions: 1) The mechanism of direct signal trasduction toward Ras and actin-based cytoskeleton regulation. 2) The involvement of members of the Rho/Rac family of G-proteins in the described pathways. 3) The role of MAD proteins in mediating the above TGF-β responses.

This research is addressing a) the important and complex issue of TGF-β signal transduction toward the regulation of cell morphology and adhesion and b) the mechanistic aspects of anti-tumorigenic action that TGF-β can exhibit under a variety of conditions.

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DISTINCT ORGANIZING ACTIVITIES OF HEDGEHOG AND DECAPENTAPLEGIC SIGNALS PATTERN THE *DROSOPHILA* WING DISC

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In *Drosophila*, Decapentalegic, a member of the TGF-β family, is considered to be an important signal for patterning of structures. The study of the functions of this molecule in this organism sheds light on how the members of the TGF-β family act in other organisms, including vertebrates. In the *Drosophila* wing imaginal disc, the Hedgehog (Hh) signal molecule induces the expression of *decapentaplegic* (*dpp*) in a band of cells abutting the antero-posterior (A/P) compartment border. It has been proposed that this Dpp signal organizes the patterning of the entire wing disc. We have tested this proposal by studying the local response to distinct levels of ectopic expression of Hh and Dpp. To this aim, the sensory organ precursors (SOPs) of the wing and notum and the presumptive wing veins were used as positional markers relative to the A/P border. Here, we show that the Dpp signal specifies the position of most SOPs in the notum and some of those in the wing in a concentration dependent manner. Close to the A/P compartment border, however, SOPs are specified by Hh rather than Dpp. We also show that late signaling by Hh, after setting up *dpp* expression, is responsible for the localization of veins 3 and 4 and the formation of the scutellar region. One of the effector target genes of Hh that mediates these actions is the zinc-finger protein Cubitus interruptus (Ci). These results indicate that Hh and not Dpp alone organizes the patterning in the region of the wing disc near the A/P border

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DIFFERENTIAL EFFECT OF TGF-β_1 IN NORMAL AND TRANSFORMED MOUSE EPIDERMAL KERATINOCYTES.

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We have studied the long-term effects of TGF-β_1 in a panel of mouse epidermal cell lines that includes nontumorigenic immortalized keratinocytes and cell lines derived from benign papillomas and squamous cell carcinomas. TGF-β_1 affects differentially the growth and differentiation properties of those cell lines. Nontumorigenic keratinocytes from immortalized or papilloma-derived cell lines responded to TGF-β_1 by a block of cell proliferation and by the induction of a process of terminal differentiation leading to cell death. However, carcinoma cell lines that escaped the arrest of proliferation exerted by the growth factor, when cultured in the presence of TGF-β_1, elicited an epithelial-mesenchymal transdifferentiation (EMT) that correlated with increased expression, secretion and activity of extracellular matrix proteases (uPA, MMP-9) and enhanced invasive and metastatic properties. Interestingly, immortalized keratinocytes transformed by transfection of a Ha-ras oncogene were sensitive to EMT, whereas keratinocytes transfected with a normal Ha-ras gene behaved as the parental cell line. These results suggest that TGF-β_1 acts as a stimulator of malignant progression in later stages of mouse epidermal carcinogenesis. On the other hand, transformation of epidermal keratinocytes by a Ha-ras oncogene confers a different cell sensitivity to TGF-β_1.

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Complementary activities of two TGFß isoforms (ß2 and ß3) in mediating epithelial-mesenchymal cell transformation in the embryonic heart.

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Epithelial-mesenchymal cell transformations are essential for a variety of embryological events. During early development both gastrulation and neural crest cell formation require epithelial-mesenchymal cell transformation. Later, organogenic events, including in the proper formation of the valves of the heart depend on controlled epithelial to mesenchymal cell transformation. In the heart, valve formation begins by the localized formation of mesenchymal cells derived from endothelium in the atrioventricular canal and the outflow tract. This laboratory previously showed that TGFßs 2 and 3 are present in the chick heart at the time of cell transformation and that TGFß3 was critical to the transformation process (J.D. Potts et al. 1991: 1992). Due to the prominent distribution of TGFß2 in the mouse heart shown by Akhurst and colleagues and the recent demonstration of heart defects in the TGFß2 knockout mouse, we reinvestigated the role of the TGFß isoforms in cell transformation in the chick heart. In situ hybridization and immunolocalizations were performed to show that TGFß2 was present in avian endothelial and myocardial tissues prior to the time of cell transformation. TGFß3 was present in the myocardium but does not appear to be expressed in the transforming endothelial tissue until transformation has begun. Cardiac explant cultures were prepared and exposed to either exogenous TGFß Isoforms or to blocking antibodies specific for each of the isoforms. These experiments revealed that anti-TGFß2 blocked the initial "activation" or cell-cell separation that begins epithelial-mesenchymal cell transformation. Blocking monoclonal antibodies against TGFß3 prevented cell transformation but did not block the initial cell-cell separation process. Addition of exogenous TGFß isoforms produced a differential expression of several markers of the cell transformation process including cell surface adhesion molecules, extracellular matrix proteins, and cytoskeletal components. These data have lead to a model where epithelial-mesenchymal cell transformation in the chick heart appears to utilize the sequential activity of TGFß2 for initial activation, TGFß3 for cell shape change and invasion and then one or both of these molecules for enhanced cell migration after cell transformation. As addition of either TGFß Type II (C. B. Brown et al., 1996) or TGFß Type III (C.B. Brown et al, unpublished) receptor antibodies can block cell transformation in these assays, we are attempting to resolve the relationship between specific TGFß isoforms, receptors and transformation markers in this model system.

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List of Invited Speakers

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TGF-β SIGNALLING IN DEVELOPMENT AND CELL CYCLE CONTROL

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