

1st International p53 Isoforms Meeting

SEPTEMBER 13-15 2010
LYON, FRANCE

www.iarc.fr/p53isoforms/



An international meeting jointly organised by the International Agency for Research on Cancer (IARC/WHO) and the University of Dundee

**“p53 isoforms through evolution:
from identification to biological function”**



Meeting Website:

www.iarc.fr/p53isoforms/

Contact:

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Meeting location:

International Agency for Research on Cancer
150 Cours Albert Thomas
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France

MEETING SCOPE

It is estimated that up to 90% of human protein-coding genes express multiple protein isoforms due to alternative promoters, splicing and initiation of translation thus increasing the coding capacity of the human genome.

Like most genes, several p53 protein isoforms are produced by the tumor suppressor TP53 gene. p53 isoforms have been described since the very early days of p53 research; however, it is only during the past eight years that they have become the focus of systematic research. Undoubtedly, one of the main triggers for this expanding interest is the fact that p63 and p73, the two homologues of p53 discovered in the late 1990s, are expressed as multiple protein isoforms with specific expression patterns and distinct biological functions.

In recent years, about ten p53 protein isoforms have been identified (Figure 1), and studies have accumulated demonstrating that p53 isoforms are involved in a wide range of biological functions and pathologies. In addition, p53 protein isoforms also include polymorphic variants due to single nucleotide polymorphisms (SNPs) in the TP53 gene, such as the codon 72 (R72P). The description of the p53 protein isoforms opens up a broad new area for understanding the diversity of p53 functions.

The First International p53 Isoforms Meeting will provide a multi-disciplinary forum for researchers involved in p53 isoforms and for all those interested in their biological and pathological significance. The main topics to be addressed include:

- Lessons from animal models
- Genetic and epigenetic control of p53 isoform expression
- Biological functions in cell model systems
- Identification and characterization of p53 isoforms
- Involvement of p53 isoforms in human diseases.

The meeting will also offer opportunities for specialist debates on essential technical issues:

- Panels of available animal models for p53 isoforms expression studies
- Methods for detection of p53 isoforms
- Cell standards for detection and measuring variations in p53 isoform expression
- Immunological definition of p53 isoforms
- Approaches for analysing p53 isoform dysregulation in human diseases, including human cancers, and for compiling this information into public databases.

With this meeting, we hope to stimulate research on p53 isoforms and encourage collaboration.

Fig.1 p53 protein isoforms in animal models.

(A) Schematic representation of the human TP53 gene (upper panel) and of the human p53 protein isoforms (lower panel).

The human TP53 gene contains 11 exons (boxes) encoding several p53 products. The usage of the distal promoter (P1) leads to the production of p53 and Δ40p53 isoforms, while the internal promoter (P2) regulates the expression of Δ133p53 and Δ160p53 isoforms.

The classical p53 protein contains a transactivation domain (TAD - blue), a proline-rich domain (PXXP - purple), a DNA-binding domain (DBD - orange) and a C-terminal domain (green) with a nuclear localisation signal (NLS) and an oligomerisation domain (OD). It has been shown that p53 protein conserved 5 domains through evolution (from I to V - grey boxes). The theoretical molecular weight of p53 is indicated in kD on the left (53kD).

Three N-terminal p53 isoforms have been identified: **Δ40p53**, which is produced either by alternative splicing of the intron 2 or by internal initiation of translation using ATG40 of p53 transcript that results in the lack of the first TAD; **Δ133p53**, which is encoded by a transcript initiated in intron 4 where an internal P2 promoter has been identified thus resulting in the lack of the TAD, PXXP and part of the DBD; and **Δ160p53**, which is produced by internal initiation of translation using ATG160 of Δ133p53 transcript that results in the loss of the TAD, PXXP and a larger part of the DBD.

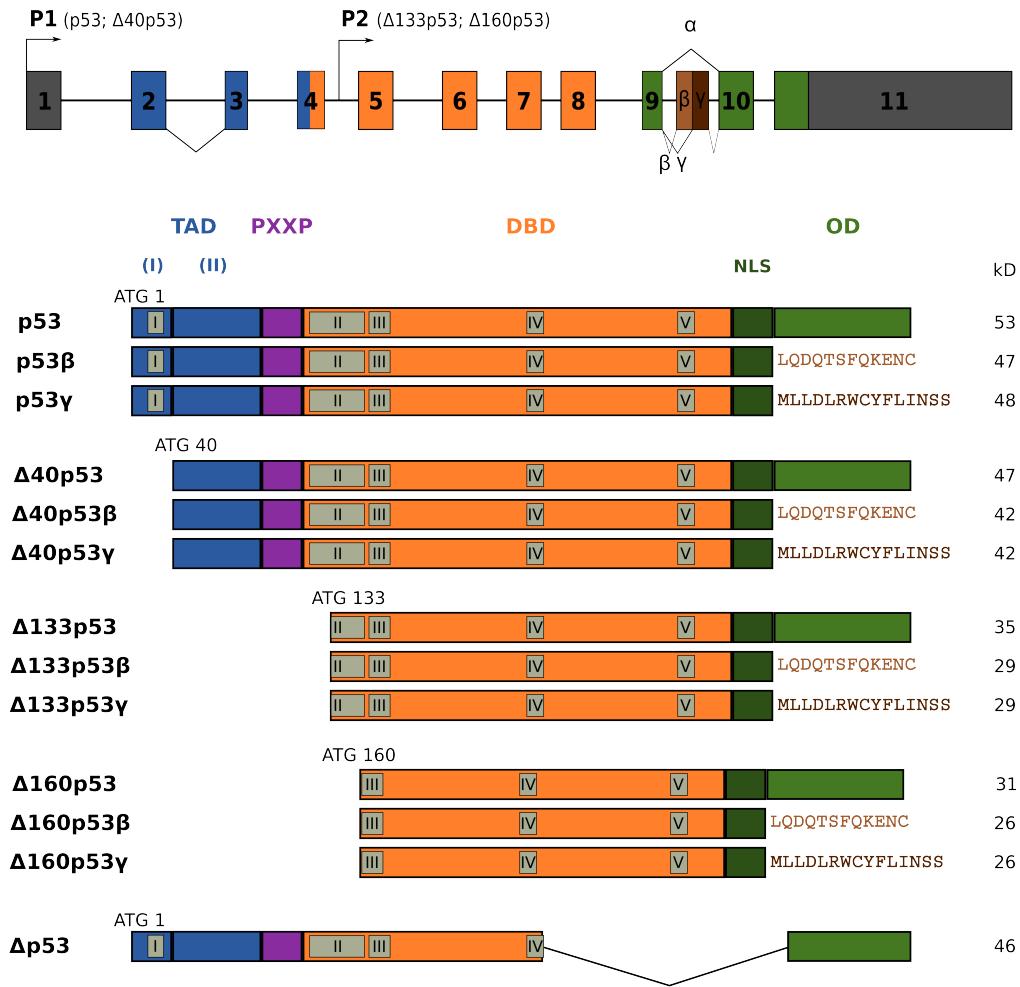
Three C-terminal p53 isoforms have been detected in humans: **p53α** (or p53), which corresponds to the classical p53 protein with the NLS and the OD; **p53β**, which is produced by an alternative splicing in intron 9, leading to the replacement of the OD by 10 new residues; and **p53γ**, also produced by an alternative splicing in intron 9, leading to the replacement of the OD by 15 new residues.

An additional p53 protein isoform has been described, **Δp53**, which lacks part of the DBD and the NLS.

(B) Nomenclature of the p53 protein isoforms through animal models.

The table aims to recapitulate the different names used to designate the p53 protein isoforms in human, mouse, drosophila and zebrafish models. The usual name (i.e. the most cited in the literature) and the other names used in the literature are presented. For some isoforms ("others"), no parallels have been done between animal models. Purple: novel identified isoforms described for the first time during the meeting.

A



B

HUMAN		MOUSE		DROSOPHILA		ZEBRAFISH	
<i>Usual name</i>	<i>Other name</i>	<i>Usual name</i>	<i>Other name</i>	<i>Usual name</i>	<i>Other name</i>	<i>Usual name</i>	<i>Other name</i>
p53	p53α FLp53 TAp53(α)	p53		Dmp53L	Dp53 p53B	Zp53	p53
p53β	TAp53β						
p53γ	TAp53γ	p53AS					
Δ40p53	Δ40p53α ΔNp53(α) p53/p47 p47	Δ40p53	p53:p44 p44			ZΔNp53	ΔNp53
Δ40p53β		Δ40p53AS					
Δ40p53γ							
Δ133p53	Δ133p53α ΔN133p53					ZΔ113p53	Δ113p53
Δ133p53β							
Δ133p53γ							
Δ160p53	Δ160p53α	Δ157p53					
Δ160p53β		Δ157p53AS					
Δ160p53γ							
Others	Δp53		Δ122p53				
					DmNp53		

ORGANIZERS

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FUNDING BODIES

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SPONSORS



MEETING PROGRAMME

Sunday, 12 September

16:00 - 18:00 Registration
Dinner & Overnight Leisure time

Monday, 13 September

7:30 - 9:30 Registration
9:15 Meeting Introduction
All day 1st p53 Isoforms Meeting
All day Poster exhibition
End of day Cocktail in the main entrance at IARC

Tuesday, 14 September

All day 1st p53 Isoforms Meeting
All day Poster exhibition
13:45 - 16:00 Poster session
Evening Dinner at Brasserie Georges

Wednesday, 15 September

All day 1st p53 Isoforms Meeting
All day Poster exhibition
15:15 Meeting Conclusion
Afternoon Departure of participants

SCIENTIFIC PROGRAMME

Monday 13 September 2010

Session 1 – Meeting Introduction and Keynote Addresses

- 09:15 - 09:30 Jean-Christophe BOURDON, *UK* and Pierre HAINAUT, *France*
09:30 - 10:30 Varda ROTTER, *Israel*

10:30 - 11:00 *Coffee break*

Session 2 – p53 isoforms in Animal Models

p53 family in Animal Models

- 11:00 - 11:30 Guillermo LOZANO, *USA*
11:30 - 11:45 Adam ODELL, *UK*
11:45 - 12:15 Alea MILLS, *USA*
12:15 - 12:45 Gerry MELINO, *Italy*

12:45 - 13:30 *Lunch*

p53 isoforms in Animal Models: Zebrafish (I)

- 13:30 - 14:00 Jin Rong PENG, *China*
14:00 - 14:30 Ulrich RODECK, *USA*
14:30 - 15:00 Elizabeth PATTON, *UK*
15:00 - 15:15 Jun CHEN, *China*

15:15 - 15:45 *Coffee break*

p53 isoforms in Animal Models: Drosophila (II)

- 15:45 - 16:15 Andreas BERGMANN, *USA*
16:15 - 16:45 John TOWER, *USA*
16:45 - 17:15 Bertrand MOLLEREAU, *France*

p53 isoforms in Animal Models: Mouse (III)

- 17:15 - 17:30 Marie KHOURY, *UK*
17:30 - 18:00 Anthony BRAITHWAITE, *New Zealand*
18:00 - 18:30 Frank TOLEDO, *France*

18:30 *Cocktail in the main entrance*

Tuesday 14 September 2010

Session 3 – p53 isoforms in cellular models

p53 family in cellular models

09:15 - 09:45 Daniel ABERDAM, *France*

09:45 - 10:00 Arnaud VIGNERON, *UK*

p53 isoforms in cellular models (I)

10:00 - 10:30 Group photo in the main entrance

10:30 - 11:00 *Coffee break*

11:00 - 11:30 Jean-Christophe BOURDON, *UK*

11:30 - 12:00 Pierre ROUX, *France*

12:00 - 12:30 Anne-Catherine PRATS, *France*

12:30 - 12:45 Virginie MARCEL, *UK*

12:45 - 13:45 *Lunch*

13:45 - 16:00 Poster Session and Coffee

p53 isoforms in cellular models (II)

16:00 - 16:30 Bennett VAN HOUTEN, *USA*

16:30 - 17:00 Greg MATLASHEWSKI, *Switzerland*

17:00 - 17:15 Kanaga SABAPATHY, *Singapore*

17:15 - 17:45 Reiner JANICKE, *Germany*

Dinner

Wednesday 15 September 2010

Session 4 – Regulation of p53 isoforms expression and activities

p53 family in cellular models

- 09:15 - 09:45 Jamal TAZI, *France*
09:45 - 10:00 Charlotte SAGNE, *France*
10:00 - 10:15 Sandra GHAYAD, *France*
~~10:15 - 10:30 Arandkar SHARATH CHANDRA, *India* (cancelled)~~
10:30 - 11:00 Frances FULLER-PACE, *UK*
- 11:00 - 11:30 *Coffee break*

Session 5 – Deregulation of p53 isoforms expression in human cancers

- 11:30 - 12:00 Pierre HAINAUT, *France*
12:00 - 12:30 Alastair THOMPSON, *UK*
12:30 - 13:00 Neda SLADE, *Croatia*
- 13:00 - 14:00 *Lunch*
- 14:00 - 14:30 Bjorn GJERTSEN, *Norway*
14:30 - 14:45 Gerda HOFSTETTER, *Austria*
14:45 - 15:00 Sofia KOIDOU, *Greece*
15:00 – 15:15 Stefano LANDI, *Italy*

Session 6 – Meeting conclusion

- 15:15 -15:30 Jean-Christophe BOURDON, *UK* and Pierre HAINAUT, *France*

PRESENTATIONS

**ORAL
COMMUNICATIONS**

LECTURE 1

p53 ISOFORMS AND MUTANTS

Rotter V

Department of Molecular Cell Biology, The Weizmann Institute of Science, Rehovot, Israel

p53 plays a central role in guarding genomic fidelity. It is therefore expected that in order to secure efficient genomic stability such a gene will consist of a family of genes that are structured of multiple alternative spliced forms. As of today two important remotely related p53 relative genes were discovered, p63 and p73. In spite of their homology to p53 structure, each of these genes exhibits a rather distal biological activity.

Even though it was expected that such an important gene would at least generate a back up system by generating genetic expression of alternative spliced forms, only one mouse interesting alternative spliced p53 protein was initially discovered. By screening cDNA mouse libraries we identified at the early 80s the p53AS that was generated as a result of a 96bp insertion that was obtained because of an alternative acceptor-splicing site in intron 10. This generated a longer cDNA molecule yet because of a new stop codon, encoded for a shorter protein that also lost the PAb421 epitope. The p53AS was found to consist 30% of the mRNA population. Availability of such a C-terminal altered protein permitted the discovery that this domain, that contains a negative control element for DNA binding, that is important for p53 to execute its apoptotic activity.

Using the same approach we were not able at that time to identify similar alternatively spliced p53 proteins from human cDNA libraries. Instead we found some genomic interesting polymorphisms in the human p53 gene, an interesting one is the 72 Arg/Pro that its biologically significance is ambiguous. Never the less, the existence of alternative spliced human p53 proteins, was later discovered by other laboratories using more advanced technologies.

In addition to the heterogeneity of wild type p53 proteins there is emerging interest in mutant p53 molecules which seem to present a large population of various types which probably contribute to malignant transformation by various mechanisms.

LECTURE 2

CONFLICTING ROLES OF MDM2 SPLICED VARIANTS

Lozano G

U.T. MD Anderson Cancer Center

pending

SHORT COMMUNICATION 1

TAKING A FRESH LOOK AT THE HUPKI MOUSE AS A MODEL SYSTEM FOR CANCER RESEARCH

Odell AF¹, Wei Q², Whibley C¹, Bourdon JC³ and Hollstein M¹

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²Department of Genetic Alterations in Carcinogenesis, German Cancer Research Center, Heidelberg, Germany

³University of Dundee, CR-UK Cell Transformation Research Group, Dundee, United Kingdom

In the realm of p53 research, the human p53 knock-in mouse (Hupki) has become a useful model system for examining both the mechanisms leading to p53 mutations and their cellular consequences. Whilst our group has focused on the methods of senescence by-pass utilised by murine embryonic fibroblasts (MEFs), where both p53-dependent and independent routes exist, the role of p53 isoforms and other p53-family members in immortalisation and tumourigenesis has been neglected. Recently, we have begun to address whether any isoforms of p53 are involved, or indeed present, in the Hupki model. In addition, we have successfully developed a novel method of rapidly generating human p53 knock-in MEFs utilising an attP/attB-PhiC31 integrase platform mouse system and have begun addressing the impact of 'suppressor-site' mutations on p53 gain-of-function mutants. Furthermore, we hope to extend the Hupki mouse system to address the contribution of p53 and its isoforms to tumour-induced angiogenesis.

LECTURE 3

THE MANY FACES OF P63: UNVEILING THE ROLE OF P63 ISOFORMS IN DEVELOPMENT, AGING, AND CANCER

Alea A. Mills

Cold Spring Harbor Laboratory

The realization that the *p53* homologue *p63* encodes multiple proteins has revolutionized the *p53* field. Indeed, dual promoters and extensive alternative splicing leads to the generation of at least six different *p63* proteins; these can be categorized into the TAp63- and the DNp63 isoform classes, which contain and lack a *p53*-like transactivation domain, respectively. Having generated mice lacking *p63* and discovering that *p63* was essential for development, we more recently discovered an unexpected link between *p63* deficiency, cellular senescence, and aging *in vivo*. Despite these advances, the question remained as to which of the *p63* isoforms were regulating the tumor suppressive mechanism of cellular senescence. Our current work demonstrates that Δ Np63 α is an oncogene that induces expression of the chromatin remodeler Lsh, thus promoting stem-like proliferation. We also discovered that TAp63 induces senescence and suppresses tumorigenesis *in vivo*. Thus, these findings demonstrate that *p63* encodes isoforms that promote, as well as isoforms that prevent, tumorigenesis. Work of others has revealed that *p53* also encodes multiple isoforms, some of which have oncogenic- and others that have tumor suppressive potential. Thus, the multi-faceted capability of *p63* appears to be an emerging theme for members of the *p53/p63/p73* family.

LECTURE 4

TAp73, AN ANCESTRAL MEMBER OF THE p53 FAMILY, IS INVOLVED IN NEURONAL DEVELOPMENT VIA miR-34a. DIFFERENTIAL ROLE OF ISOFORMS.

Melino G

University Tor Vergata, Rome, Italy; Medical Research Council, Toxicology Unit, Leicester, UK

In the last ten years, p63 and p73 have been identified as the ancestral members of the p53 family. Despite the high sequence and structural similarity, the mouse knockouts revealed a crucial role in neural development for p73 and in epidermal formation for p63. We identified several transcriptional targets, the mechanisms of regulation of cell death, and the p63 isoform involved in epithelial development. Both genes are involved in female infertility as well as in cancer formation, although with distinct mechanisms.

p73 steady state protein levels are kept low under normal physiological conditions through degradation by the 26S proteasome, mediated by the HECT-containing E3 ubiquitin ligase ITCH. We developed an ELISA high throughput screening for ITCH auto-ubiquitylation, resulting in several positive compounds that are able to modulate chemosensitivity at 10 mM concentration. These compounds could be effective in cancer treatment. In addition to this major degradation pathway, we have also described additional novel mechanisms of degradation. (1) the orphan F-box protein FBXO45 targets p73 for degradation. (2) a novel transcriptional target of TAp73, the ring finger domain ubiquitin ligase PIR2 (p73-induced Ring Finger 2) regulates the proteasomal degradation of the ΔNp73 isoforms. (3) the antizyme ubiquitin-independent, proteasome-dependent pathway targets ΔNp73 for degradation.

Here, we describe the involvement of p73 in neuronal development. TAp73 knockout mice (TW Mak G&D 2008) show hippocampal dysgenesis. Conversely, ΔNp73 knockout mice (TW Mak G&D 2010) show signs of moderate neurodegeneration with a significant loss of cellularity in the cortex. TAp73 is able to drive the expression of miR-34a, acting on specific binding sites present on the miR-34a promoter. In agreement with these *in vitro* data, miR-34a transcript expression is significantly reduced *in vivo* both in the cortex and hippocampus of p73-/ mice. In keeping, we show a role for miR-34a, in parallel to TAp73 expression, during *in vitro* differentiation of ES cells. Expression of miR-34a increases during *in vitro* neuronal terminal differentiation, of ex vivo primary cortical neuronal cultures, in parallel with the expression of TAp73. Moreover, we also detect an increase *ex vivo* of miR-34a steady state expression during postnatal development of the brain and cerebellum, when synaptogenesis occurs. We further confirm a role for miR-34a in synaptogenesis, as overexpression or silencing of miR-34a results in an inverse expression of a number of synaptic genes, via their 3'-UTR. In particular, miR-34a overexpression decreases synaptotagmin I and syntaxin-1A expression, and the endogenous levels of miR-34a are able to regulate only synaptotagmin I expression. Our findings show that p73 drives the expression of miR-34a during terminal synaptic differentiation.

LECTURE 5

DEF: A MODULATOR OF THE p53-Δ113p53 PATHWAY

Tao T¹, Shi H¹, Chen J², and Peng J¹

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Def (Digestive organ expansion factor) is a novel nuclear localized protein. Loss-of-function of Def (*def*-/- mutant) leads to underdevelopment of the liver, exocrine pancreas and intestine in zebrafish due to cell cycle arrest rather than increased cell apoptosis. During the course of studying the molecular mechanism responsible for the *def*-/- phenotype we identified the *p53* isoform Δ113p53 whose expression is aberrantly elevated in the *def*-/- mutant. Detailed molecular characterization revealed that Δ113p53 expression is directly regulated by p53 and Δ113p53 specifically antagonizes the p53 apoptotic activity. Δ113p53 is a counterpart of human p53 isoform Δ133p53, suggesting that Δ133p53 likely plays a fundamental role in the p53 pathway in human. In view of the facts that Δ113p53 transcript level rather *p53* transcript level is highly elevated in the *def*-/- mutant and Δ113p53 expression is totally p53-dependent, two key questions need to be addressed: 1) what is the biochemical relationship between Def and p53? And 2) does Def function alone or by forming a complex with other proteins during digestive system development in zebrafish? Thus far, we have performed yeast two-hybrid screen and identified 16 putative Def-interacting proteins. Whole-mount *in situ* hybridization showed that expression of 15 of these genes is, as of the *def* gene, enriched in one or more digestive organs. Functional analysis of five genes via morpholino-mediated gene expression knockdown approach showed that morphants in all cases were defective in the development of the digestive system and exhibited a phenotype similar to the *def*-/- mutant. Some of these Def-interacting factors (e.g RYBP) are known to be involved in the p53 pathway, therefore, we are in the process to link Def, p53 and Δ113p53 for their function during digestive organ development in zebrafish.

1. Chen, J., Ng, S.M., Chang, C.Q., Zhang, Z.H., Bourdon, J.C., Lane, D.P., and Peng, J.R. (2009) p53 isoform Δ113p53 is a p53 target gene that antagonizes p53 apoptotic activity via BclxL activation in zebrafish. **Genes & Development** 23:278-290.
2. Chen, J., Ruan, H., Ng, S.M., Gao, C., Soo, H.M., Wu, W., Zhang, Z.H., Wen, Z.L., Lane, D.P., Peng J.R. (2005) Loss-of-function of def selectively up-regulates Δ113p53 expression to arrest expansion growth of digestive oegans in zebrafish. **Genes & Development** 19: 2900-2911.

LECTURE 6

DIFFERENTIAL REGULATION OF p53 FUNCTION BY THE N-TERMINAL ΔNp53 AND Δ113p53 ISOFORMS IN ZEBRAFISH EMBRYOS

Davidson WR², Kari C³, Ren Q¹, Daroczi B¹, Dicker AP¹ and Rodeck U^{3, §}

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³Department of Dermatology, Thomas Jefferson University, Philadelphia, PA

The p53 protein family coordinates stress responses of cells and organisms. Alternative promoter usage and/or splicing of p53 mRNA gives rise to at least nine mammalian p53 proteins with distinct N- and C-termini which are differentially expressed in normal and malignant cells. The three known N-terminal p53 variants contain either the full-length (FL), or a truncated (\square N/ \square 47) or no transactivation domain (Δ 113/ Δ 133) altogether. The functional consequences of coexpression of the different p53 isoforms in whole organisms are poorly defined. Here we identified and investigated the role of the zebrafish Δ Np53 ortholog in the context of FLp53 and Δ 113p53 coexpressed in the developing zebrafish embryo.

We cloned the zebrafish Δ Np53 ortholog and determined that ionizing radiation increased expression of steady-state Δ Np53 and Δ 113p53 mRNA levels in zebrafish embryos. Ectopic Δ Np53 expression caused hypoplasia and malformation of the head, eyes and somites, but partially counteracted lethal effects caused by concomitant expression of FLp53. FLp53 expression was required for developmental aberrations caused by Δ Np53 and for Δ Np53-dependent expression of the cyclin-dependent kinase inhibitor 1A (CDKN1A,p21,Cip1,Waf1). Knockdown of p21 expression markedly reduced the severity of developmental malformations associated with Δ Np53 overexpression. By contrast, forced Δ 113/133p53 expression had little effect on Δ Np53-dependent embryonal phenotypes. Despite marked sequence differences these functional attributes were shared between zebrafish and human Δ Np53 orthologs ectopically expressed in zebrafish embryos. All isoforms could be coimmunoprecipitated with each other after transfection into Saos2 cells.

Both alternative N-terminal p53 isoforms are expressed in response to cell stress and antagonize lethal effects of FLp53 expression in developing zebrafish to different degrees. However, in contrast to Δ 113/133p53, forced Δ Np53 expression itself can lead to developmental defects which depend, in part, on p21 transactivation. In contrast to FLp53, the developmental abnormalities caused by Δ Np53 are not counteracted by concomitant expression of Δ 113p53.

LECTURE 7

**BRAF^{V600E} CO-OPERATING MUTATIONS DIRECT MELANOMA
PATHOLOGY**

Richardson J¹, Mathers ME², den Hertog J³, Lister JA^{4*} and **Patton EE^{1*}**

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The BRAF^{V600E} kinase active mutation is the most frequent mutation in melanoma. BRAF^{V600E} is also frequently found in benign nevi, suggesting that additional genetic mutations co-operate with BRAF^{V600E} to promote melanoma development and progression. Using the zebrafish as a model system, we have previously shown that human BRAF^{V600E} expression in melanocytes is sufficient to promote nevi formation, and that an additional co-operating mutation in p53 is required for melanoma progression. BRAF^{V600E} p53 melanoma in zebrafish is highly invasive, often unpigmented, and shares many of the pathological characteristics with human melanoma.

To identify additional BRAF^{V600E} co-operating mutations, we have tested the function of BRAF^{V600E} in zebrafish deficient for Pten or Mitf activity. Zebrafish have two *pten* genes (*a* and *b*) that are prone to blood, ocular and intestinal tumors. BRAF^{V600E} expression in *pten* deficient zebrafish generates large ectopic nevi that appear to slowly develop into a heavily pigmented, endophytic melanoma. In contrast, BRAF^{V600E} expression in a *mitf* hypomorphic background develop nodular and spreading melanoma in the epidermis.

Comparative histopathology revealed consistent differences in tumour histology between the BRAF^{V600E} zebrafish models. Significant variations were seen in growth pattern, cytological characteristics and degree of melanin pigmentation. The *p53* melanomas showed the most marked cytological atypia, and tended to an expansile growth pattern. The *pten* tumours were heavily melanotic and highly infiltrative. In the *mitf* model, the tumours had a predominantly superficial growth pattern. Our BRAF^{V600E} genetic melanoma models reveal that BRAF^{V600E} co-operating mutations exert significant influence on melanoma pathology, and these observations may allow us to make some early comparisons with the differing growth patterns seen in the various sub-types of human melanoma.

SHORT COMMUNICATION 2

PROTEIN INTERACTION BETWEEN p53 AND Δ113p53 IS ESSENTIAL FOR Δ113p53 ANTI-APOPTOTIC FUNCTIONS

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²College of Life Sciences, Zhejiang University, Zijingang Campus, Hangzhou, Zhejiang, China, 310058.

Δ113p53 is an N-terminal truncated p53 isoform and functions to antagonize p53-mediated apoptotic activity. Δ113p53 does not work simply as dominant-negative towards p53 but rather modulates differential gene expression to protect cells from apoptosis. Δ113p53 retains the oligomerisation domain of p53. Our preliminary data showed that Δ113p53 and p53 can form a complex. However, it is not known whether protein interaction between p53 and Δ113p53 is required for Δ113p53 to inhibit the apoptotic activity of full-length p53. To address this question, we created a series of point mutations in the oligomerisation domain of Δ113p53. Among these 10 mutants, two of them lost the ability to interact with p53. These two Δ113p53 mutants also lost the abilities to modulate p53 target gene expression and to inhibit p53 induced cell apoptosis. On the other hand, those Δ113p53 mutants, which can interact with p53 retain the abilities to antagonize p53's apoptotic activity. Our data demonstrated that protein-protein interaction between Δ113p53 and p53 is essential for Δ113p53 anti-apoptotic function.

LECTURE 8

GENETIC CHARACTERIZATION OF THE *DROSOPHILA* p53 GENE

Bergmann A, Fan Y, Herz HM, Jain A and Barton MC

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The mammalian p53-family consists of p53, p63 and p73. While p53 accounts for tumor suppression through cell cycle arrest and apoptosis, the functions of p63 and p73 are more diverse and also include control of cell differentiation. The *Drosophila* genome contains only one *p53* homolog, *Dp53*. Previous work has established that Dp53 induces apoptosis, but not cell cycle arrest. Here, by using the developing eye as a model, we show that *Dp53*-induced apoptosis is primarily dependent on the pro-apoptotic gene *hid*, but not *reaper*, and occurs through the canonical apoptosis pathway. Importantly, similar to mammalian p63 and p73, expression of Dp53 also inhibits cellular differentiation of photoreceptor neurons and cone cells in the eye independently of its apoptotic function. Intriguingly, expression of the human cell cycle inhibitor p21 or its *Drosophila* homolog *dacapo* can suppress both Dp53-induced cell death and differentiation defects in *Drosophila* eyes. These findings provide new insights into the pathways activated by Dp53 and reveal that Dp53 incorporates functions of multiple p53-family members.

An open question in the *Drosophila* p53 field is how Dp53 may be regulated in the absence of an identifiable Mdm2 gene in the genome. We have identified a different E3 ubiquitin ligase, called Bonus, which may serve as a putative Dp53 regulator. Mutations in *bonus* cause apoptosis, which can be rescued by Dp53 depletion. We found that the mammalian ortholog of Bonus, termed Trim24, ubiquitylates and negatively regulates p53 levels, suggesting that the function of Bonus is evolutionary conserved.

LECTURE 9

REGULATION OF DROSOPHILA LIFE SPAN BY p53

Tower J

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Drosophila melanogaster contains a single *p53* gene with two promoters, predicted to produce two *p53* protein isoforms, *p53A* and *p53B*. The *p53A* isoform is typically referred to as wild-type *p53*, and is the most studied. We have found that *p53A* has sexually-dimorphic effects on life span. Over-expression of *p53A* in the adult fly nervous system caused decreased life span in males and increased life span in females. In contrast, tissue-general over-expression produced the opposite pattern: increased life span in males and decreased life span in females. In a *foxo* null background, *p53A* life span effects in males were reversed, becoming similar to the effects in females. These data demonstrate that wild-type *p53A* over-expression can regulate life span independent of *foxo*, and suggest that *foxo* acts in males to produce sexually antagonistic life span effects of *p53A*. Mutations of the endogenous *p53* gene also had sex-specific effects on fly life span. Currently we have generated transgenic fly strains to allow for conditional over-expression of both *p53A* and *p53B* isoforms in tissue-specific patterns, and the characterization of these strains is underway.

LECTURE 10

DIFFERENTIAL FUNCTIONS OF DROSOPHILA P53 ISOFORMS

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Drosophila melanogaster is well suited to study the functions of p53 isoforms. *Drosophila melanogaster* p53 (Dm-p53) is the only member of the p53/p63/p73 family of genes found in *Drosophila*. Only two Dmp53 isoforms, Dm-p53 long (Dm-p53L) and short (Dm-p53S), have been identified. In addition, Dmp53L is similar to the human P53 full-length isoform, while Dmp53S has a smaller transactivation domain and resembles to the human P53 Delta 40 isoform.

Our goal is to study the individual function of each of the Dmp53 isoforms and their specific roles in the regulation of apoptosis, autophagy and tissue regeneration during *Drosophila* development. First, we have found that both Dmp53L and Dmp53S are able to induce apoptosis and autophagy in developing tissues. Second, we have observed that Dmp53S, but not Dmp53L, induces strong Wingless expression, a homologue to the mammalian Wnt and tissue regeneration. These data indicate a distinct ability of each isoform in mediating tissue regeneration. Considering the implication of P53 and the Wnt pathway in mammalian tissue regeneration, *Drosophila* offers a unique possibility to decipher the role of P53 isoforms in this process.

SHORT COMMUNICATION 3

THE EXPANDING UNIVERSE OF p53: IDENTIFICATION OF NEW ACTIVE MOUSE p53 ISOFORMS

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Our laboratory has previously identified p53 isoforms in human and drosophila cells and highlighted an association of some p53 isoforms with survival in cancer patients. We have also shown that human p53 isoforms modulate p53 transcriptional and tumour suppressor activities. In order to determine the physiological relevance of the p53 isoforms during embryonic development, ageing and carcinogenesis, it is required to develop a mouse genetic model. As the mouse p53 isoforms were not completely explored, we set out to identify and characterise them.

Here we report that the mouse *p53* gene expresses 6 different p53 isoforms (p53, p53AS, Δ40p53, Δ40p53AS, Δ157p53 and Δ157p53AS), confirming the previously described alternative splicing of intron 10 leading to p53AS expression (1). Interestingly, we determine that Δ40p53 isoform can also be obtained in mouse by alternative splicing of intron 2. Moreover, we demonstrate that intron 4 of the mouse *p53* gene contains a promoter region that leads to the expression of Δ157p53 isoforms. Additionally, we show that p53 isoforms are differentially expressed in normal mouse tissues.

With the aim to investigate the biological activities of the mouse p53 isoforms, scientific tools were developed. We show that some p53 isoforms have p53-dependent and -independent transcriptional activities and can modulate p53 transcriptional activity in a promoter-dependent manner.

- (1) Arai N, Nomura D, Yokota K, Wolf D, Brill E, Shohat O, Rotter V. Immunologically distinct p53 molecules generated by alternative splicing. Mol Cell Biol. 1986 Sep;6(9):3232-9.

LECTURE 11

WIDESPREAD INFLAMMATION AND CANCER IN MICE EXPRESSING A Δ133p53-LIKE ISOFORMS

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Up to nine isoforms of human p53 have now been reported. A number of these isoforms have been found to be over expressed in a range of human tumours, and three of them (Δ 40p53, p53 β , Δ 133p53) have been reported to moderate/antagonize normal p53 activities. These data suggest that one or more of the p53 isoforms may play a role in tumorigenesis. Here we test *in vivo* whether p53 isoforms increase tumour susceptibility. We created a knock-in p53 mouse mutant expressing an N-terminally deleted p53, Δ 122p53, which is essentially equivalent to the human isoform Δ 133p53 α . This mutant lacks the Mdm2 binding site, the transactivation domain and the proline rich domain and is thus incompetent for normal activities of p53. Further results show that the Δ 122p53 is a dominant oncogene. Δ 122p53 mice have decreased survival and develop a complex tumour spectrum distinct from p53 null (p53 $^{-/-}$) mice. In addition, mice heterozygous for Δ 122p53 and wild-type p53 have decreased survival compared to heterozygous p53 null (p53 $^{+/-}$) mice. As well as being highly tumour prone, the mice also exhibit widespread inflammation and show elevated levels of pro-inflammatory cytokines in the serum. Our investigations of Δ 122p53 suggest that human Δ 133p53 α plays a role in inflammatory pathways that when deregulated, can cause chronic inflammation resulting in cancer. .

LECTURE 12

p53 REGULATION: LESSONS FROM MOUSE MODELS EXPRESSING ONLY A SUBSET OF p53 ISOFORMS

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TP53, the gene encoding p53, is mutated in more than half of human cancers, and many other tumors present alterations of proteins interacting with p53. Understanding the regulation of p53 is therefore of major clinical importance. It was recently found that p53 isoforms may participate in the regulation of the p53 full-length protein (FL-p53). In humans, *TP53* encodes 9 isoforms, from the use of 2 promoters, 3 translation start sites and 3 alternate splicing events. At least 3 of these isoforms appear to have a significant biological role : FL-p53, Δ133p53 (expressed from an internal promoter in intron 4), and p53β (with a distinct C-terminal domain resulting from alternative splicing). Overexpression of Δ133p53 or p53β in cultured human cells was found to affect the p53 transcriptional response, and both isoforms are misregulated in some cancers.

The mouse *Trp53* gene also comprises an internal promoter in intron 4 and an alternative splicing (AS) exon encoding a distinct C-terminal domain. To analyze the role of p53 isoforms *in vivo*, we decided to target at the *Trp53* locus the specific deletion of either the internal promoter, or of the alternative splicing exon. We thus generated two mouse models with conditional mutations: one may express all p53 isoforms but those from the internal promoter, and the other may express all p53 isoforms but those with the AS C-terminal domain. A preliminary analysis of these mutants is presented.

LECTURE 13

PLURIPOTENT STEM CELLS AS CELLULAR MODELS FOR P63-RELATED PHYSIOPATHOLOGY

Aberdam D

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Pluripotent stem cells are able to differentiate into many cell types *in vitro*, thus providing a potential unlimited supply of cells for cell-based therapy. As they recapitulate the main steps of embryogenesis, they represent as well a powerful cellular model for cognitive *in vitro* studies on normal development and congenital diseases. We reported their efficient ability to recapitulate the reciprocal instructive ectodermal-mesodermal commitments, for the formation of an embryonic skin and that the transcription factor p63, a member of the *p53* family, is mandatory for epidermal commitment. The production of pluripotent cell (iPS) lines derived from patient affected by ectodermal dysplasia (ED) fibroblasts further allowed us to decipherate the congenital p63-linked pathways defective in ED skin formation.

p63 gene encodes two main isoforms, TAp63 and ΔNp63, with opposing functions. Recently, we report an unexpected role of p63 in heart development. TAp63 deficiency prevents expression of pivotal cardiac genes and in turn cardiogenesis, resulting in the absence of beating cardiomyocytes. Our observations indicate that TAp63, expressed by sox-17 endodermal cells, acts in a non-cell-autonomous manner by modulating expression of cardiogenic factors. Remarkably, we found that p63-null mouse embryos exhibit severe defects in embryonic cardiac development, including pronounced dilation of both ventricles, a defect in trabeculation and abnormal septation. This was accompanied by myofibrillar disarray, mitochondrial disorganization and reduction in spontaneous calcium spikes. This unexpected discovery was made on knock-out mice that have been produced a decade ago and thus confirms the powerful of pluripotent stem cells for cognitive studies linked to physiopathology.

In summary, our findings uncover a critical role for p63 in both epidermal and cardiovascular fate and suggest that p63 could be a candidate gene for orphan congenital heart diseases.

SHORT COMMUNICATION 4

REGULATION OF THE HIPPO PATHWAY BY ASPP1

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ASPP1, a p53 binding protein is a coactivator of p53, specifically functioning to activate the expression the apoptotic p53 target genes. However, immunofluorescence experiments and cell fractionation have revealed a cytoplasmic localisation of ASPP1 indicating some other functions for this protein. Our experiments have shown an unexpected role of ASPP1 in the control of the Hippo pathway via the binding and the inhibition of the Lats1 Kinases. ASPP1 expression leads to an increase of YAP translocation to the nucleus and transcription of its target genes, resulting in a reduced sensitivity to different stress like dNTP depletion, low serum or anoikis. The regulation of YAP by ASPP1 also lowers the expression of LATS2, another important regulator of YAP and p53, and prevents the induction of p21 by the LATS2/p53 pathway. This activity of YAP reduces the induction of senescence and increase the clonogenic potential of cells treated by different inducers of p53 like DNA damaging treatment or ROS induction. These functions of cytoplasmic ASPP1 confer a potential oncogenic role to this protein, balancing previous results showing a tumour suppressive role for nuclear ASPP1.

LECTURE 14

REGULATION AND BIOLOGICAL ACTIVITIES OF HUMAN P53 ISOFORMS.

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p53 protein regulates multiple biological activities including cell cycle progression, cell death, angiogenesis, cell motility. p53 is a transcription factor tightly regulated at the transcriptional, translational and post-translational levels. One the most burning questions in the field is how p53 contributes to the decision making upon cellular stress?

We investigated the activities of human p53 isoforms on cell cycle and apoptosis as well as the regulation of p53 isoforms expression at the mRNA and protein levels.

We established that the internal p53 promoter is directly transactivated by p53 in response to cellular stress inducing the expression of $\Delta 133p53\alpha$ isoform at the mRNA and protein levels. Using a low dose of doxorubicin, U2OS cells trigger p53-mediated G2 cell cycle arret but not apoptosis. After depletion of $\Delta 133p53$ isoform expression using specific siRNA and in response to a low dose of doxorubicin, U2OS cells change their decision in cell fate outcome triggering G1 cell cycle arrest and apoptosis. By rescue experiments, we established that $\Delta 133p53\alpha$ prevents p53-mediated apoptosis and G1 arrest without inhibiting p53-mediated G2 cell cycle arrest in response to low dose of doxorubicin. It indicates that $\Delta 133p53\alpha$ does not exclusively act by inactivating p53 but rather by regulating gene expression.

Results regarding the transcriptional activity of other p53 isoforms and the degradation of p53 isoforms by the proteasome will be presented. We will discuss our molecular model.

LECTURE 15

N-TERMINAL DELETED p53 ISOFORMS CONTROL MIGRATION AND INVASION

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The *p53* tumour suppressor is the most frequently mutated gene in human cancers. Our latest contribution to this field has been to highlight that the role of *p53* during tumour progression is not restricted to the control of cell proliferation, but is extended also to the regulation of cell invasion. Specifically, we have shown that *p53* modulates cell migration, one of the first steps in metastasis formation.

Loss of *p53* activity promotes formation of filopodia, the actin-containing membrane extensions implicated in cell locomotion. *p53* implements its effects on cell migration by regulating cancer cell invasion, since *p53* loss in fibroblasts cultured in a three-dimensional (3D) matrix induces a morphological switch (from an elongated to a markedly spherical and flexible shape) associated with significantly increased invasive properties. Our work shows that this transition requires the activation of RhoA GTPase and of ROCK kinase, its main effector. This suggests that genetic alterations of *p53* in tumours are sufficient to promote cell motility and invasion, thereby contributing to metastasis formation. In addition, by regulating E-Cadherin expression, *p53* inhibits Epithelial-Mesenchymal Transition (EMT), which constitutes a novel facet of its tumour suppressor function.

Because of alternative splicing, initiation of translation and use of internal promoter nine different *p53* isoforms that can regulate its native activity are expressed. These isoforms have different effects on cell fate outcome, in regulating cell cycle arrest, apoptosis and replicative senescence. However, their role in the process of cancer cell migration and invasion is not yet documented. We found that expression of N-terminally-deleted splice variants of *p53* is associated with poor prognostic features in breast cancer patients. Mechanistic analysis of the role of $\Delta 133p53\beta$ isoform has shown that it promotes migration and invasion of breast and colon carcinoma cells that still express wild type *p53*. Over-expression of $\Delta 133p53\beta$ induces disruption of E-Cadherin-dependent adherent junctions allowing cells to detach from the epithelium and migrate by using amoeboid-like movements. This phenotype requires the activity of ROCK and is associated with activation of RhoA. Our data demonstrate that deregulated expression of $\Delta 133p53\beta$ confers increased motility and invasiveness to cancer cells.

LECTURE 16

THE p53 ISOFORM, Δ133p53, STIMULATES ANGIOGENESIS AND TUMOR PROGRESSION

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#Equal contribution

Tumor suppressor p53, involved in DNA repair, cell cycle arrest and apoptosis, also blocks new blood vessel formation, i.e. angiogenesis, a process strongly contributing in tumor development. P53 exists as 9 proteins, including Δ133p53 isoforms that lack the N-terminal transactivation domain. Δ133p53 is overexpressed in various human tumors however its role in tumor progression has remained unelucidated. In the present study, we have examined the involvement of Δ133p53 in tumoral angiogenesis and tumor growth in the highly angiogenic human glioblastoma U87, by a knockdown approach. Data show that Δ133p53 knockdown, in contrast to p53 knockdown, blocks endothelial cell migration and tubulogenesis without affecting cell proliferation in vitro. In vivo, siRNAs against Δ133p53 strongly block angiogenesis and growth of glioblastoma tumors, both in the chicken chorio-allantoïc membrane and in mice xenografts, indicating that Δ133p53 exhibits pro-angiogenic and pro-tumoral features. We also show, by Taqman Low Density Array, that Δ133p53 specifically modulates the angiogenic balance without affecting vascular endothelial growth factor A or fibroblast growth factor 1 and 2 expression. These data reveal Δ133p53 as an activator of angiogenesis and tumor progression, acting by a mechanism that does not involve the major angiogenesis signalling pathways, therefore providing a new potential therapeutic target in cancer treatment.

SHORT COMMUNICATION 5

EXPRESSION OF Δ133p53 TRANSCRIPT REGULATED BY p53 FAMILY MEMBERS ENCODES TWO P53 ISOFORMS: Δ133p53 AND Δ160p53

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The *TP53* gene expresses several p53 proteins isoforms. Among them, Δ133p53α is a N-terminal truncated p53 isoform that lacks the whole transactivation domain and part of the DNA-binding domain. It has been reported that Δ133p53α inhibits p53-mediated replicative senescence, apoptosis and G1 arrest through modulation of gene expression. Δ133p53α protein is encoded by a specific transcript driven by an internal promoter P2 located between intron 1 and exon 5 of *TP53* gene. The transcription factors regulating P2 promoter activity remain unknown.

We demonstrated that the P2 promoter activity is regulated by the p53 tumour suppressor protein. In response to doxorubicin treatment, Δ133p53α expression is increased at both mRNA and protein levels in wild-type. In addition, chromatin immunoprecipitation and luciferase assays showed that p53 binds p53 response elements located within the P2 promoter and transactivates P2 promoter. In addition, we observed that p63β, ΔNp63α, ΔNp63β and ΔNp73γ transactivated the P2 promoter..

By siRNA transfection and site-directed mutagenesis, we identified a fourth N-terminal p53 isoform, Δ160p53α. This novel p53 isoform is produced by internal initiation of translation at ATG160 using Δ133p53α transcript. We detected endogenous Δ160p53α protein in three different cell lines: U2OS, T47D and K562. In K562 cells, the *TP53* gene presents an insertion at codon 136 leading to a premature stop at codon 148. Thus, K562 cells do not express p53 or Δ133p53α but retain the ability to express Δ160p53α protein.

Therefore, we showed that Δ133p53 is a novel target of p53 that encodes two proteins by alternative initiation of translation at ATG133 and ATG160.

LECTURE 17

CANCER CELL BIOENERGETICS AND p53 ISOFORMS

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Over 70 years ago, Otto Warburg proposed that tumor cells have altered bioenergetics, displaying a decreased dependence on oxidative phosphorylation (OXPHOS) with a concomitant increase in glycolysis. This hypothesis has been confirmed in some tumor types and not others. We have examined the bioenergetics of several breast cancer cell lines using a Seahorse XF24 extracellular flux analyzer. This instrument measures oxygen consumption (a measure of OXPHOS) and pH (a measure of lactate production) changes in real time. We found a four-fold variation in both OXPHOS and glycolysis among a panel of 12 different breast cancer cell lines. Those cell lines with the lowest OXPHOS also had the lowest steady state levels of ATP. Levels of glycolysis were independent of OXPHOS levels. Furthermore no correlation was observed between p53 status and levels of OXPHOS. This lead us to investigate the effects of p53 levels on cellular bioenergetics using HCT116 isogenic cell lines that are either +/+, +/- or -/- for wt p53. We found that loss of one p53 allele was sufficient to decrease OXPHOS by about two-fold. However, there was no compensatory increase in glycolysis. Further loss of the second allele did not decrease OXPHOS. Surprisingly, steady-state levels of ATP were the same in all three cell lines. We have also examined the effects of overexpression of p53 using a tet-off system in SaOS2 cells. Unexpectedly, high levels of WTp53 were also associated with a decrease in OXPHOS, perhaps due to cell-cycle arrest. We have initiated a series of experiments with several cell lines expressing both WT and different p53 isoforms including: p53beta, d133p53, d133p53beta. Results will be presented.

LECTURE 18

EXPRESSION OF N-TERMINAL TRUNCATED p53, p47 THROUGH ALTERNATIVE SPLICING

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The p53 gene is part of a larger gene family that includes the p63 and p73 genes. The p63 and p73 genes express different isoforms that either contain the full length protein or are truncated in the N-terminal trans-activation domain due to alternative splicing. In general, the N-terminal truncated isoforms of p63 and p73 are able to impair the activity of the full length proteins. We have therefore investigated whether alternative splicing of the p53 likewise gives rise to an N-terminal deleted isoform of p53. In this presentation, evidence will be provided that alternative splicing involving exon 4 results in a splice variant expresses an N-terminal truncated isoform of p53 termed p47. Previous work by other groups has shown that p47 can also arise through alternative initiation of translation from the same mRNA through an IRES. Therefore, p47 can arise by at least 2 distinct mechanisms. We are currently evaluating whether the p47 arising through alternative splicing is able to regulate p53 activity.

SHORT COMMUNICATION 6

p47: ALTERNATE MECHANISM OF GENERATION AND FUNCTIONAL CHARACTERIZATION

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p53, as we know, exists as various N- and C-terminally truncated forms, due to alternate translation initiation at the N-terminus and alternate splicing at the C-terminus. Of them, the major form besides the full-length (FL) p53 is p47, which is initiated from the ATG in exon 4, due to alternate IRES entry sites, and also due to alternate splicing of a novel p53 RNA form, which contains intron 2 of p53 (known as the p53[EII] form). P47 lacks the transactivation domain, and hence is also known as DeltaN (DN)p53 or Delta40p53. Functionally, p47 is thought to be both able to induce cell death, depending on the context, and also inhibit it. However, the physiological relevance of this form of p53 is not well understood yet.

We report here the identification of an intronic promoter that leads to p47 expression, as an alternate mechanism for its production. Expression from this intron alone is sufficient for p47 expression. 5' race experiments indicate that the p47 initiated from the intronic activity starts from the codon 44 of p53. Functional analysis indicates that p47 is capable of inhibiting cell growth, by inducing apoptosis, as efficient as p53 alone. Interestingly, p47 co-expressed with p53 reduces the latter's ability to induce cell growth, highlighting that these 2 forms of p53 need to be coordinately expressed to induce the desired outcome. Mechanistically, p47 was found to selectively induce p53-target genes such as AIP-1 and PIG3, but not p21 or MDM2. Moreover, similar to the effect on cell death, co-expression of p47 with p53 led to reduced p53-dependent target gene activation. Hence, it appears that p47-mediated cell death works through an alternate pathway to induce apoptosis.

We have also analyzed the role of endogenous p47, by utilizing cells that lack p53 but still express p47. Endogenous p47, which is highly abundant in the absence of stress, is further induced by a variety of stresses, but to a much reduced extent compared to p53, concomitantly selectively inducing its target genes. Silencing p47 expression leads to reduction of cell death. Importantly, p47 is not easily detected in parental p53 positive cells, even after stress stimulation, indicating that p53 exerts some form of inhibitory effect on p47 expression. Detailed mechanistic insights on role and regulation of p47 will be presented.

LECTURE 19

p53 β AND p53 γ : MODULATORS OF p53 FUNCTION?

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Upon DNA damage and other stresses, the transcription factor p53 elicits numerous responses including DNA repair, cell cycle arrest and apoptosis. Although these properties make p53 surely a prototype tumor suppressor, p53 exhibits also tumorigenic functions. Thus, p53's diverse activities require tight control mechanisms that, however, are only insufficiently understood. Recently, it was found that the *p53* gene allows expression of at least nine different isoforms that arise from multiple splicing events and the usage of alternative promoters. Several of these isoforms interfere with the function of the full-length p53 mainly by acting in a dominant-negative manner. In addition, an isoform-dependent selective activation of p53 target genes was also observed. For example, the C-terminally truncated p53beta was shown to increase expression of Bax and p21 thereby contributing to p53-dependent apoptosis and senescence, respectively. However, as p53beta almost completely lacks the C-terminal located oligomerization domain, it is unknown how this isoform interacts with and modulates transcriptional stress responses of full-length p53. Therefore, we studied the impact of p53beta and also p53gamma, a similarly spliced p53 isoform, on the function of the full-length p53 protein. The results will be discussed.

LECTURE 20

THERAPEUTIC MODULATION OF ALTERNATIVE SPLICING BY SMALL MOLECULES

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Almost all protein-coding genes are spliced and their majority is alternatively spliced. Alternative splicing is a key element in eukaryotic gene expression that increases the coding capacity of the human genome and now an increasing number of examples illustrates that the selection of wrong splice sites causes human disease. A fine-tuned balance of factors regulates splice site selection. In addition to conserved sequences at the splice junctions, splice site selection also depends upon different sets of auxiliary cis regulatory elements known as exonic and intronic splicing enhancers (ESEs and ISEs) or exonic and intronic silencers (ESSs and ISSs). Specific binding of SR proteins to their cognate splicing enhancers as well as binding of splicing repressor to silencer sequences serve to enhance or inhibit recognition of weak splice sites by the splicing machinery.

Given that the vast majority of human genes contain introns and that most pre-mRNAs containing multiple exons undergo alternative splicing, mutations disrupting or creating such auxiliary elements can result in aberrant splicing events at the origin of various human diseases. The rapidly emerging knowledge of splicing regulation now allows the development of treatment options. In the past few years, numerous studies have reported several approaches allowing correction of aberrant splicing events by targeting either the mutated sequences or the splicing regulators whose binding is affected by the mutation.

My talk will be focused on small molecules that modulate the activity of SR splicing factors to bring out those holding the greatest promises for the development of therapeutic treatments either alone or in combination with antisense oligonucleotides.

SHORT COMMUNICATION 7

G-QUADRUPLEX STRUCTURE IN TP53 GENE ARE INVOLVED IN THE SPLICING OF INTRON 2

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The tumor suppressor gene *TP53*, encoding p53, is expressed as several transcripts. The fully spliced (FSp53) transcript encodes the canonical p53 protein. The alternatively spliced p53I2 transcript retains intron 2 and encodes Δ Np53 (or Δ 40p53), an isoform lacking the first 39 N-terminal residues corresponding to most of the main transactivation domain.

We demonstrate the formation of *G-quadruplex* structures (G4) in a GC-rich region of intron 3 that modulates the splicing of intron 2. First, we show the formation of G4 in synthetic RNAs encompassing intron 3 sequences by UV melting, thermal difference spectra and circular dichroism spectroscopy. In this region, p53 pre-mRNA contains a succession of very short exons (exons 2 and 3) and introns (intron 2 and 4) covering a total of 333 bp. Site-directed mutagenesis of G-tracts putatively involved in G4 formation decreased by about 30% the excision of intron 2 in a GFP-reporter splicing assay. Moreover, treatment of lymphoblastoid cells with 360A, a synthetic ligand that binds to single-strand G4 structures, increases the formation of FSp53 and decreases p53I2.

These results indicate that G4 structures in intron 3 regulate the splicing of intron 2, leading to differential expression of transcripts encoding distinct p53 isoforms.

SHORT COMMUNICATION 8

POTENTIAL INVOLVEMENT OF p53 IN TRANSLATIONAL FIDELITY AND IRES-DEPENDENT TRANSLATIONAL INITIATION CONTROL

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Protein synthesis is a fundamental cell process and ribosomes are the main effectors of this process, particularly through the ribosomal RNA (rRNA) that displays ribozyme activity. Ribosome biogenesis is a very complex process involving transcriptional as well as many post-transcriptional steps to produce quality-controlled functional cytoplasmic ribosomes. It is now well demonstrated that ribosome production is enhanced in cancer cells and that ribosome biogenesis plays a crucial role in tumor progression. However, at present, there is an important lack of data to determine whether the entire process of ribosome biogenesis and ribosome assembly is modified during tumor progression and its potential impact on the dysregulation of translational control of cancer cells.

In this study, we have analyzed the major steps of ribosome biogenesis, the structure of ribosome and the translational activity in a model of human breast cancer progression in a well characterized cellular model (Elenbaas *et al.*, Genes Dev, 2001). Our results show an unanticipated p53-dependent modification of rRNA methylation pattern that is responsible for the impairment of translational fidelity and for the increase of Internal Ribosome Entry Site (IRES)-dependent translational initiation of genes playing key roles in oncogenesis. Therefore, by demonstrating that p53 is not only involved in the control of the rate of production of ribosomes but also in their structure and function, our study point out a novel role for p53 that, when altered, could be responsible for, on one hand, a “translational instability” of cancer cells since the proteome would not reflect the expected correctly translated transcriptome and on the other hand, an uncontrolled expression of the growing class of genes that are now recognized as key players of oncogenesis: those containing IRES elements in their 5' UTR.

SHORT COMMUNICATION 9

TRANS ACTING FACTORS REGULATE DIFFERENTIAL SYNTHESIS OF p53 ISOFORMS

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p53 mRNA has been shown to be translated into two isoforms, the full-length p53 (fl-p53) and a truncated isoform ΔNp53, which acts as a dominant-negative inhibitor of fl-p53. Previously, we have shown that translation of p53 and its N-terminally truncated isoform ΔN-p53 can be initiated at the Internal Ribosome Entry Sites (IRES). The two IRESs regulate the translation of p53 and ΔN-p53 in a distinct cell-cycle phase-dependent manner. We have also demonstrated that polypyrimidine tract binding protein (PTB) positively regulates the IRES activities of both the p53 isoforms by shuttling from nucleus to the cytoplasm during stress conditions. Our recent results suggest that the structural integrity of the p53 RNA is critical for the IRES function. We have compared the secondary structure of the wild-type RNA with cancer-derived silent mutant p53 RNAs having mutations in the IRES elements. These mutations result in the conformational alterations of p53 IRES RNA that affects the IRES function. Interestingly, these mutant RNAs failed to bind to some *trans*-acting factors (p37/38, p44 etc) which could be critical for the IRES function. By Immunoprecipitation of RNP complexes and super shift assay using anti hnRNPC1/C2 (p44) antibody, we have demonstrated that the mutant RNA showed reduced binding to this protein factor. Also, partial silencing of hnRNPC1/C2 inhibited the IRES function considerably. Taken together, our observations suggest pivotal role of several *trans* acting factors in regulating the p53-IRES function, that in turn influences the synthesis of different p53 isoforms.

Cancelled

LECTURE 21

THE RNA HELICASE p68 MODULATES EXPRESSION AND FUNCTION OF THE Δ133 ISOFORM(S) OF p53, AND IS INVERSELY ASSOCIATED WITH Δ133p53 EXPRESSION IN BREAST CANCER

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We have previously shown that the 'DEAD box' RNA helicase p68 is a potent co-activator of p53-dependent transcription and is important for the p53 response to DNA damage. We, and others, have demonstrated that p68 and the Δ133p53 isoforms, which modulate the function of full-length p53, are aberrantly expressed in breast cancer. In a study of 200 primary breast cancers we identified a striking inverse association between p68 and Δ133p53 expression. Consistent with these observations, we found that siRNA depletion of p68 in cell lines results in a p53-dependent increase of Δ133p53 in response to DNA damage, suggesting that increased Δ133p53 expression could result from down-regulation of p68 and providing a potential mechanistic explanation for our observations in breast cancer. Δ133p53α, which has been shown to negatively regulate the function of full-length p53, reciprocally inhibits the ability of p68 to stimulate p53-dependent transcription from the *p21* promoter suggesting that Δ133p53α may be competing with p68 to regulate p53 function. This hypothesis is underscored by our observations that p68 interacts with the C-terminal domain of p53, co-immunoprecipitates Δ133p53α from cell extracts and interacts only with p53 molecules that are able to form tetramers. These data suggest that p68, p53 and Δ133p53α may form part of a complex feedback mechanism to regulate the expression of Δ133p53, with consequent modification of p53-mediated transcription, and may modulate the function of p53 in breast and other cancers that harbour wild type p53.

LECTURE 22

DNP53 (D40P53) ISOFORM: REGULATION OF EXPRESSION AND POSSIBLE ROLES IN REGULATING BASAL LEVELS OF p53 ACTIVITY

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Δ Np53 (also termed D40p53) lacks the N-terminal transactivation domain of p53 and a functional counterpart of the major Δ N isoforms of p63 and p73. It also lacks the Mdm2 binding domain and is thus activated by stress signals in the same way as full-length p53. It is expressed by two mechanisms: internal initiation of translation at codon 40 in fully-spliced p53 mRNA (FSp53) or alternatively spliced p53 mRNA retaining Intron 2 (p53I2) and using codon 40 as main initiation codon.

We have recently shown that alternative splicing of p53 has influenced by the presence of a G-quadruplex structure encompassing a G-rich sequence in intron 3. Mutation of G bases or the use of a pharmacological ligand that stabilize G4 alter the normal pattern of expression of FSp53 vs. p53I2.

The G4 sequence in intron 3 partially overlaps with a common polymorphism consisting of a 16bp repeat (frequency of duplicated allele in Caucasians: 0.2). Studies in Li-Fraumeni families from Brazil have shown that this polymorphism is a very strong modifier of the penetrance of germline TP53 mutation. Whereas childhood cancer is one of the hallmarks of LFS, subjects with a wild-type allele carrying a duplicated 16-bp motif in intron 3 show a 20 to 30 years delay in the age at first cancer diagnosis. These results suggest that a structural motif regulating p53 isoform expression has a strong impact of the basal activity of wild-type p53, thereby affecting susceptibility to cancer.

Reference: Marcel et al. J Med Genet 2010.

LECTURE 23

CLINICAL IMPLICATIONS OF THE P53 ISOFORMS IN BREAST CANCER

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The p53 gene in breast cancer appears to be particularly complex. P53 mutations can be detected in some 25% of cancers and have prognostic significance, particularly in key patient subgroups. Indeed, the p53 functional status of breast cancer has been used to seek a predictive marker for taxane rather than anthracycline therapy (the EORTC 10994 trial) in the neoadjuvant setting.

Immunohistochemistry of the p53 network in breast cancer, as in other solid tumour types, has also proved complex. Recently, panels of p53 network proteins have been identified which might correlate with patient outcome and reflect the mutational status of the p53 gene in breast cancer.

The spectrum of p53 isoforms in breast cancer, originally identified by Bourdon et al in 2005, may provide explanations as to the difficulties scientists and clinicians have had in rationalising the role of p53 in clinical breast cancer.

Using RNA extracted from primary, previously untreated breast cancers, RT-PCR was used to examine the expression of the β and γ isoforms (each identified in a third of cancers) and the N terminal truncated $\Delta 133p53\beta$ isoform (identified in 11% of cancers) in a series of over 100 patients. For comparison, the Roche p53 Amplichip was used to detect p53 mutation in DNA from the same cancers. Expression of the isoforms and p53 mutation status was compared with the clinical and pathological data including follow up for all patients.

Expression of the γ isoform in cancers with p53 mutation was associated with a better than expected prognosis, appearing to abrogate the effects of p53 mutation on prognosis, even though the isoform contained the same p53 mutation identified in the cancer. Conversely, the $\Delta 133p53\beta$ isoform appeared to confer a more aggressive tumour behaviour and poorer outcome.

While these data require confirmation in further series including clinical trials settings, key p53 isoforms may moderate the effects of p53 mutation in breast cancer and provide an explanation for the inconsistent literature on the roles and clinical importance of p53 in breast cancer.

LECTURE 24

THE EFFECT OF p53 ISOFORMS ON p73 ACTIVITY IN TUMOR CELLS

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TP53 tumor suppressor protein is crucial in the cell growth control and the maintenance of genomic stability. These activities are due, at least in part, to its ability to form tetramers that bind to specific DNA sequences and activate transcription. The homologues of p53, proteins p63 and p73, can transcriptionally activate p53 target genes *in vivo*. Both p63 and p73 generate transactivating forms (TAp73/TAp63) as well as a number of N-terminally truncated transactivation-deficient transdominant isoforms (called ΔTAp73/ΔTAp63). It was recently discovered that *p53*, like *p73*, has a second promoter P2 and undergoes alternative splicing to generate multiple isoforms that might play important roles in carcinogenesis. Since some mutant p53 proteins form complexes with TAp73α or TAp73β it was important to find out whether p53 isoforms can do the same and potentially act as dominant-negative inhibitors of TAp73 and TAp63. All six p53 isoforms can form complex with TAp73β, while only isoforms Δ133p53, Δ133p53β and Δ133p53γ can form complex with TAp73α. Inhibitory interactions of two proteins in complex often lead to their stabilization. Our results have shown that only three isoforms (Δ133p53, Δ133p53β and Δ40p53) stabilize TAp73β. Furthermore, we have shown that all isoforms of p53 inhibit transcriptional activity but with different efficiency. The apoptotic acitivity of TAp73β was augmented by coexpression of p53β, but Δ133p53 and Δ133p53β inhibit its apoptotic activity most efficiently. The half-lives of different p53 isoforms were determined - p53γ isoform has the shortest, while Δ133p53γ has the longest half-life. Defining the interactions between p53 family members would gain insight into how the p53 isoforms modulate the functions of p73. The discovery of p53/p73 network could have a major clinical impact in prognostic use and p53 targeted drug design.

LECTURE 25

**THE ROLE OF p53 FULL LENGTH, BETA AND GAMMA ISOFORMS IN
MYELOID DEVELOPMENT AND ACUTE MYELOID LEUKEMIA**

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Tumor suppressor p53 play a role in differentiation of myeloid progenitor cells and their aggressive malignant counterparts acute myeloid leukemia (AML), but limited is known about the role of p53 isoforms in the myeloid cell compartment of the bone marrow. In various malignancies, e.g. breast cancer and leukemia, mutated TP53 is strongly associated with resistance to conventional anti-cancer therapeutics. In leukemic patients, the TP53 gene is non-mutated in approximately 90%, and successful and persistent remission seems to depend on wild type TP53 in the leukemic cells.

We have described different expression of p53 and beta/gamma isoforms in normal leukocytes, monocytes and neutrophil granulocytes. The p53 protein isoforms are modulated during chemotherapy. In acute myeloid leukemia, p53 protein is modulated within few hours after start of chemotherapy, skewing the isoform ratio towards full length forms relative to beta-gamma. Our studies indicate that the isoform expression correlate with molecular features of mutational status of NPM1/B23 and the receptor tyrosine kinase Flt3 as well as therapy response and overall survival. Short survival and the relapse marker mutated Flt3 correlates with enhanced full length p53 protein expression, while longer survival and the good prognostic marker mutated NPM1 correlate with relative beta/gamma expression. Our current efforts examine the modulation of p53 in AML patients undergoing non-genotoxic differentiation therapy. Understanding of p53 isoform function in normal bone marrow and myeloid malignancies may have impact in future therapeutic strategies in leukemia.

SHORT COMMUNICATION 10

THE CLINICAL RELEVANCE OF p53 ISOFORMS IN OVARIAN CANCER

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C-terminally truncated p53 isoforms were present in 18 of 34 ovarian cancer cell lines (52.9%) and 134 of 245 primary ovarian cancers (54.7%). Besides p53ΔE6 and p53β, we identified p53ζ, p53δ, and p53ε, arising from alternative splicing of intron 6 and 9, respectively. p53δ expression constituted an independent prognostic marker for recurrence-free and overall survival (hazard ratio 1.854, 95% confidence interval 1.121 - 3.065, $P = 0.016$, and hazard ratio 1.937, 95% confidence interval 1.177 - 3.186, $P = 0.009$, respectively). p53β expression was associated with adverse clinicopathologic markers, i.e. serous and poorly differentiated cancers ($P = 0.002$ and $P = 0.008$, respectively) and correlated with worse recurrence-free survival in patients exhibiting functionally active p53 ($P = 0.049$).

Δ40p53 but not Δ133p53 expression was up-regulated in ovarian cancers in comparison to normal ovarian tissue. High Δ40p53 expression was associated with improved recurrence-free survival compared to low expression in ovarian cancers exhibiting functionally active p53 ($P = 0.015$). The expression N-terminally truncated p53 isoforms did not correlate with the functional p53 status and clinicopathologic parameters.

SHORT COMMUNICATION 11

CONSERVATION OF EXONIC SPLICING REGULATORY ELEMENTS AND EPIGENETIC LANDMARKS IN HUMAN AND MOUSE p53

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The impact of point mutations and SNPs on splicing regulation has been, until presently, addressed in few studies. Nevertheless, small genetic changes can lead to significant modifications of splicing enhancer recognition and epigenetic alterations appear to exert a major role on splicing regulation, probably through RNA polII stalling. The *p53* homology between mouse and human is not very extensive, even in the genetic region corresponding to the DNA binding domain of TP53 (<85%). Moreover, mouse DNA lacks the *p53* ATG 133 codon and, as a result, the corresponding Δ133 human TP53 isoforms. In addition, considerable genetic differences are observed in *p53* between different species including mice, regarding the apoptosis-related response elements. We presently investigated the distribution of splicing-regulatory elements in human and mouse *p53* sequences including epigenetic landmarks (CpGs) which, in human *p53* exons 5-8, co-localize with multiple exonic splicing enhancer (ESE) elements, or neighbor splice site sequences.

Although there is considerable homology close to splice sites in the human and mouse *p53* genes, there is limited overall homology even in the conserved exons 5-10 (81.3-89.7%). Computational analysis of the distribution of ESEs [1] in this region also reveals considerable differences, particularly in exons 6 and 9 which are alternatively spliced, as well as in exon 4 and close to the 133 codon in exon 5. The human *p53* exon 3 contains no ESE contrary to the mouse exon 3 sequence (one SF2/ASF, SF2/ASF(IgM-BRCA1) binding element), but includes a CpG close to the 3' end splice site. Conservation in constitutively spliced exons is more prominent for the ESEs recognizing SF2/ASF and SF2/ASF(IgM-BRCA1) which are primarily responsible for splicing. On the contrary, ESEs recognizing SRp40 and SRp55, involved in the regulation of cellular response to intra- and extracellular conditions [2,3] are less conserved (this is also observed between the mouse and rat *p53* sequences). Smaller differences in the ESE distribution are observed in exons 7 and 8. In exon 10, where considerable genetic homology is observed between human and mouse *p53* (83.2%) and which is responsible for the TP53 cellular distribution, there are extensive differences in the ESE distribution. Limited homology is also observed in the CpG distribution in the conserved exons (65.85%) except in exon 10, but the total number of CpGs in these exons shows small variation (32 and 28). In several exons (4, 5, 6 and 8) CpGs are observed close to the 3' splice sites in both organisms. In conclusion, the distribution of ESEs in genetic regions which are alternatively expressed shows considerable variation between the human and mouse *p53*, mainly with respect to specific ESEs, but there is a significant balance of epigenetic landmarks, particularly in the homologous exons 5-10.

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SHORT COMMUNICATION 12

ΔN133p53 EXPRESSION LEVELS IN RELATION TO HAPLOTYPES OF THE TP53 INTERNAL PROMOTER REGION

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The transcription of the ΔN133p53 isoform is controlled by an internal promoter region (IPR) that, following re-sequencing of 47 Caucasians, showed eight polymorphisms in eleven haplotypes. We assayed the functional effects of the commonest six haplotypes on the promoter activity with a luciferase reporter system, in HeLa and 293T cells. Our study showed that different IPR haplotypes are associated with differences in the promoter activity. These results imply that A1 and A6 haplotypes exhibit the highest baseline levels of ΔN133p53, whereas the A5 and A8 the lowest. In vivo quantitative-PCR on human tissues confirmed that ΔN133p53 have different baseline levels, in relation to the individual IPR haplotypes. Such differences followed the same trend observed in the in vitro experiments. Interestingly, we observed the same trend also when cell lines were treated with camptothecin, that induces a raise of promoter activity. Following in silico analysis, we assayed with the electrophoretic mobility shift assay the rs179287 polymorphism and found changes in the pattern of protein bindings, partially explaining our findings. Thus, we showed that the expression of ΔN133p53 is under genetic control, and suggested the presence of interindividual differences underlying this mechanism.

POSTERS

POSTERS

POSTER 1

CHROMATIN DOMAIN ORGANIZATION OF THE *TP53* LOCUS IN NORMAL MAMMARY EPITHELIAL AND BREAST CANCER CELL LINES CORRELATES WITH THE TRANSCRIPTIONAL STATUS OF p53

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p53 is a tumor suppressor protein critical for genome integrity. Although its control at the protein level is well known, the transcriptional regulation is still unclear. We have analyzed the organization of the *TP53* gene domain using DNA arrays in several breast cancer and control cell lines. We have found that in the control breast epithelial cell line, HB2, the *TP53* gene is positioned within a relatively small DNA domain, encompassing 50 kb, delimited by two nuclear matrix attachment sites. Interestingly, this domain structure was found to be radically different in the studied breast cancer cell lines, MCF7, T47D, MBA-MD-231 and BT474, in which the domain size was increased and *TP53* transcription was decreased. We propose a model in which the organization of the *TP53* gene domain correlates with the transcriptional status of p53 and neighboring genes.

POSTER 2

OCCURRENCE OF GERMLINE TP53 MUTATIONS AMONG CHILDREN WITH ADRENOCORTICAL TUMORS, CHOROID PLEXUS TUMORS AND RHABDOMYOSARCOMAS, AND IN FAMILIES WITH MULTIPLE CHILDHOOD TUMORS.

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Aim: The purpose of our study was to evaluate the contribution of *TP53* germline mutations for development of childhood adrenocortical tumors (ACT), choroid plexus tumors (CPT) and rhabdomyosarcomas (RMS), and further evaluate the *TP53* mutational status in families with more than one childhood cancer patient.

Material: Children diagnosed with ACT (≤ 18 yrs; n=3), CPT (≤ 18 yrs; n=7) and RMS (≤ 5 yrs; n=30) during the time period 1958–2008 were identified through the population based Cancer Registry in the South Health Care Region in Sweden. Patients who were still alive were invited to participate in the study, and blood samples were collected from those who accepted. From a cohort of 196 childhood cancer patients diagnosed between 1962 and 2009, where both a blood sample and family history of cancer were available families with multiple childhood tumors (n=18) were identified. Mutational screening of *TP53* was performed using direct sequencing and multiplex ligation-dependent probe amplification (MLPA).

Results: Screening for *TP53* mutations was performed in 3 patients diagnosed with ACT (1 adenoma, 2 carcinoma), 5 patients with CPT (4 papilloma, 1 carcinoma), 18 patients with RMS (15 embryonal RMS, 2 alveolar RMS, 1 unspecified RMS) and in 18 childhood cancer patients with a family history of childhood cancer. Germline *TP53* mutations were found in 1/3 patients with ACT (1/2 carcinomas) and in 1/18 patients with RMS (1/15 embryonal RMS). No mutations were identified in children with CPT. In childhood cancer patients, with a family history of at least one other childhood cancer case, no mutations were detected. None of the children with an identified germline mutation had a family history of cancer compatible with the criteria's for classical Li-Fraumeni syndrome.

Conclusion: In a population based material we confirm that a fraction of children with ACT and RMS have a germline *TP53* mutation irrespectively of a family history of classical Li-Fraumeni syndrome. Screening for *TP53* mutations in cases of ACT could be considered as a clinical option for better diagnosis and management. *TP53* mutations do not seem to be of importance in families with multiple cases of childhood tumors and should not be an indication for mutation screening in absence of adult Li-Fraumeni associated tumors.

POSTER 3

ORAL SQUAMOUS CELL CARCINOMA (OSCC) AND EXPRESSION OF p53 GENE POLYMORPHYISM/S IN PAKISTAN

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Oral squamous cell carcinoma (OSCC) is the leading cause of death in the developing countries like Pakistan. The major risk factor for developing OSCC is excessive chewing habits of tobacco, *niswar* (a type of raw chewing tobacco) *gutka* (the preparation of crushed betel nut, tobacco and sweet or savory flavors) and *manpuri* (the powder of betel nut, tobacco and slaked lime). The p53 gene is the extensively studied tumor suppressor gene involved in the suppression of tumor. The germ line mutation/ polymorphism of p53 gene involved in the multiple steps of carcinogenesis. This study aimed to find out the loss of p53 gene functions due to mutation/ polymorphism caused by genomic alteration and interaction with tobacco and its related ingredients in Pakistan. The total of 250 OSCC patient's tissue and blood specimens was collected with informed consent from local hospitals of Karachi. p53 mutation analyses of exons 2, 3, 4, 5, 6, 7, 8, 9, 10 and 11 of p53 genes were examined by polymerase chain reaction-single-strand conformation polymorphism (PCR-SSCP) and direct DNA sequencing. The PCR-SSCP analysis showing mobility shift bands in tumor samples were purified and directly sequenced. In exon 4 of the p53 gene, a C to G missense mutation at nucleotide position 215 of the coding sequence was identified. This change substitutes amino acid proline with arginine at position 72 of p53 protein. The change was significantly observed in OSCC tumor sample that may be responsible for causing OSCC in Pakistan.

Key words:

OSCC, p53 polymorphism, PCR-SSCP, missense mutation, direct DNA sequencing

POSTER 4

INTRIGUING DISCREPANCIES IN SEARCH FOR HETEROZYGOUS TP53 MUTATIONS *IN VITRO* AND *IN VIVO*

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Heterozygous mutations of *TP53* were frequently described. Presence of wild type allele in cancer cells is used to support hypothesis of dominant negative effect of missense *TP53* mutants. Moreover, frequency of heterozygous *TP53* mutations could help in determining how rational would it be to search for a therapy based on recovering *TP53* actions. To this end, we have determined frequency of heterozygous *TP53* mutations *in vivo*. Our experimental data and IARC database analysis showed high frequency of these mutations *in vivo*. Moreover, *in vivo* data analysis suggested the existence of an unknown mechanism eliminating wild type *TP53* mRNA and/or favoring mutated allele in cells presenting heterozygous mutation of *TP53* (*Szybka et al Br J Cancer 2008*). To this end, we decided to define this mechanism by the means of functional assays performed using: cancer cell lines established in our laboratory, and commercially available cell lines – defined in the databases as presenting heterozygous mutations of *TP53*. Several cell lines were analyzed: ST-486, PF-382, RPMI-8402, MOLT-13, (MOLT-13 boost), LS-123, BFTC-909, H-318. Nevertheless we met strong obstacles trying to find cell lines showing stably and/or in reality, single heterozygous mutation of *TP53*. The reasons for the discrepancies between frequency of *in vitro* and *in vivo* *TP53* mutations and reliability (credibility) of current *in vitro* cell culture models will be the subject of further analysis.

POSTER 5

DETECTION OF MUTATIONS IN THE EXONS 4 TO 8 OF THE p53 TUMOR SUPPRESSOR GENE IN CANINE MAMMARY GLANDS

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Fifteen female canines with mammary tumors and 6 normal females were used for the study of mutations in exons 4 to 8 of the p53 gene. The size of mammary tumors was 7.4 ± 5.9 cm in diameter, and they were characterized as carcinoma and mixed malignant tumors. The adjacent mammas tissue did not present histological alterations as well as the normal animals. DNA samples from the tumors, respective adjacent normal mammary tissue and mammary glands from the healthy animals were sequenced and analyzed for the presence of mutations. Mutations were found in 71.8% of the tumors samples and the most frequent were missense mutations. The most attacked exons in the mammary tumor were 5, 7 and 8, with 23.4, 31.6 and 23.4% mutations, respectively. In the adjacent mammary tissue, exons 4, 5 and 8 were the most frequently altered (30.6, 25.6 and 35.3%, respectively). Among the more frequent mutations reported here were G (17.5%) and C (13.3%) deletions and A (15.4%) and T (14.7%) insertions. However, it was not possible to identify a single nucleotide polymorphism that repeated in all tumors and could be considered a diagnostic factor, and there were no relationships between mutations and tumor type. Although few samples exhibited mutations in the codons related to the binding of p53 protein to the DNA in canine species, the majority of the alterations were located near them. These codons were responsible for the structural conformation of the protein, which were all in the central domain of DNA binding. The results of the present study indicate that abnormalities in the Tp53 gene are involved in the genesis of canine mammary tumors. Moreover, these abnormalities may be present early in normal tissue, as the mutations were detected in the macroscopically and histologically normal mammary tissue adjacent to the tumors. These mammary tissues can lead to recurrence if not removed together with the tumor.

POSTER 6

EXPRESSION OF p53 PROTEIN AND POLIMORPHISMS IN EXON 8 OF Tp53 GENE IN CANINE MAMMARY CARCINOMAS

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This study was undertaken with the aim of evaluating p53 expression, applying the immunohistochemical technique to malignant primary mammary neoplasms histopathologically diagnosed in female dogs and to investigate exon 8 of the Tp53 suppressor gene for mutation types by means of PCR-RFLP pattern of bands. Nineteen healthy glands were used as a control group (group 1). Samples from 18 cases diagnosed with malignant tumors (group 2), and with contralateral mammary glands (group 3) were collected during the UFRPE Veterinary Hospital routine. The histological tumors were identified and classified into grades. The streptavidin-biotin peroxidase method was used for analyzing the immunohistochemical expression of p53, evaluated according to the location and intensity of stain. Expression of p53 protein was not observed in the samples of group 1. On the contrary, it was observed in all malignant tumors located either alone in the nucleus or in both the nucleus and cytoplasm in the samples of group 2. In group 3, expression of the p53 protein was observed both in the tumor (2 samples) and normal mammary tissues (4 samples). For the molecular analyses, genomic DNA was extracted and submitted to PCR-RFLP with the following endonuclease enzymes: AluI, BsoBI, Ddel and SmaI. The band pattern showed polymorphism between groups, but not between histological variants of tumors. This polymorphism detected mutations in the fragment studied - exon 8 of Tp53 - which could account for changes in nucleotides, located in the restriction sites of the endonuclease enzymes. These findings lead to the conclusion that immunoexpression had no correlation with histological subtype or malignity grade, but might be related to the expansive process of mammary tumors in female dogs. Thus, PCR-RFLP could be used for the early diagnosis of mammary cancer in tissues where histopathological alterations are absent. Accordingly, the adoption of these parameters in association with other prognosis markers used for humans could possibly be useful in the study of canine malignant mammary tumors.

POSTER 7

p53 REGULATES THE Otx1 EXPRESSION IN BREAST CANCER

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Otx1, a homeobox containing gene of the Otx family, regulates nervous system development during embryogenesis. Postnatally Otx1 is transcribed in the anterior pituitary gland, where activates transcription of the pituitary hormones (1), and plays a role in hematopoiesis, enhancing pluripotent cells and erytroid differentiation. Otx1 can still be detected in mature cells of the erythroid and megakaryocytic lineage (2). Recently it has been reported that Otx1 is overexpressed in non-Hodgkin Lymphomas (3) and in neural tumors (4). In our study we demonstrate that the Otx1 gene is expressed in human breast cancer, in rodent mammary gland cancer stem cells (LA7) and during mouse mammary gland development.

The mammary gland is the unique organ that undergoes extensive remodeling and differentiation in adults. Recently it has been demonstrated the presence of mammary stem cells (MaSCs) in the ducts. Breast cancer is due to transforming events in a cancer stem cell that accumulates additional genetic changes and drives tumor progression with symmetrical divisions. The tumor suppressor p53 regulates polarity of divisions in MaSCs, in fact, while wild-type p53 suppresses self-renewal, inducing cellular differentiation, the loss of p53 promotes symmetric divisions of cancer stem cells, contributing to tumor growth (5).

The aim of the study was to verify the relation between Otx1 and p53, in order to demonstrate the possible coregulation of the Otx1 and theTp53 genes during mammary and breast cancer stem cells differentiation.

We analyzed the Tp53 and Otx1 gene expression levels in human ductal and lobular invasive breast cancer by quantitative real time RT-PCR, using normal breast tissue as a control. We obtained a correlation coefficient of $r=0.864$ ($p<0.001^{**}$), demonstrating the coexpression of Otx1 and p53.

Chromatin immunoprecipitation and luciferase assay showed the direct transcriptional regulation of Otx1 promoter by p53 protein.

In order to verify the possible role of this pathway in tumor cell differentiation we analyzed the Otx1 and p53 expression levels in LA7 undifferentiated and differentiated (LA7D). The expression of both Otx1 and p53 genes was increased in LA7D.

In addition, we studied the Otx1 expression during the mouse mammary gland development, and we demonstrated the Otx1 overexpression essentially during the lactation, confirming the role of Otx1 in the cell differentiation.

In conclusion, these data demonstrate that p53 protein activates the transcription of Otx1 gene and that this pathway could be involved in both mammary and breast cancer stem cells differentiation.

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POSTER 8

TAp63 AND Δ Np63 DEFICIENT MOUSE MODELS REVEAL ISOFORMS AFFECTING ECTRODACTYLY-ECTODERMAL DYSPLASIA-CLEFTING (EEC) SYNDROME

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The *p63* gene has a complex structure with two promoters giving rise to the TAp63 and Δ Np63 transcripts. Alternative splicing further diversifies the TAp63 and Δ Np63 isoform classes into α , β and γ isoforms. The prominent phenotypes observed in the *p63* deficient mouse models have clearly established *p63* as a master regulator of epidermal development. Further, *p63* deficient mice provided clues that led to the discovery that *p63* mutations are the cause of several different human developmental syndromes. We have established a mouse model for one of these human syndromes—Ectrodactyly-Ectodermal dysplasia-Clefting (EEC) syndrome—by knocking in the R279H mutation that causes EEC in humans.

This “EEC mouse model” has phenotypes similar to those found in human EEC patients, including craniofacial clefting and defects within keratinocytes of the skin. R279H is located within the DNA binding domain of *p63*; therefore, this mutation is present in each of the currently known *p63* isoforms. It is currently unknown which *p63* isoform contribute to the pathology of human EEC. To address this issue, we have used chromosome engineering technology to generate a mouse model in which TAp63 isoforms are absent, yet Δ Np63 isoforms are still expressed. Using the same strategy, we are generating mouse models that allow specific ablation of Δ Np63 isoforms in either the germline or within somatic cells. The phenotypes of EEC mice in TAp63- and Δ Np63 deficient backgrounds are currently being assessed in order to determine which isoforms affect the EEC-like phenotypes. Our current findings on determining the mechanism whereby *p63*-modulated pathways contribute to the pathological features of EEC will be discussed.

POSTER 9

EXPRESSION OF p53 FAMILY ISOFORMS IN MELANOMA AND MYELOID LEUKEMIA

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The *TP63* gene encodes six isoforms. While three C-terminal ends are generated by alternative splicing, a two-promoters usage leads to the production of full-length (TA) or truncated (DeltaN) N-terminal isoforms. DeltaNp63 plays a crucial role in the maintenance of the self-renewal potential, on the proliferation of epithelial progenitors, on the acquisition of the epithelial phenotype and on adhesion. It has been recently found to be expressed in normal human mammary stem cells.

The deregulation of the *TP63* gene, resulting in an increased DeltaN/TA ratio, is a common feature in epithelial tumors, in particular in breast carcinoma. Thus, DeltaNp63 could have a main role in the maintenance of both normal and tumor mammary progenitors.

We characterized luminal- and bipotent-restricted progenitors from human normal mammoplasty samples by cell sorting with EpCAM and CD10 markers, respectively (Bachelard-Cascales *et al.*, Stem Cells, 2010). We demonstrated that the CD10⁺ population contains very immature cells. In parallel, we characterized an immortalized mammary cell line (MCF10A) that is considered as a bipotent cell line, able to generate both luminal and myoepithelial cells. These two models were used to study DeltaNp63 expression and function in mammary progenitor cells.

The enforced expression of DeltaNp63 in MCF10A cells by the use of a Human Embryonic Fibroblasts Conditioned Medium (HEF.CM) resulted in an enrichment of immature cells with an increased potential to form spheres and to generate myoepithelial colonies. The HEF.CM also induces a switch toward cadherins that are specific of epithelial cells.

Together, these results confirm that DeltaNp63 favors the engagement of immature mammary cells towards the myoepithelial lineage. They also suggest that microenvironment is able to control stem cell differentiation, by acting on DeltaNp63 expression. Thus, we hypothesize that aberrant secretion of soluble factors by microenvironment, by overexpressing DeltaNp63, could lead to tumorigenesis. Understanding these mechanisms could allow the identification of new targets for breast cancer therapy.

POSTER 10

EXPRESSION OF p53 FAMILY ISOFORMS IN MELANOMA AND MYELOID LEUKEMIA

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The p53 family consists in numerous proteins encoded by the three genes *TP53*, *TP63* and *TP73*. Alternative promoters' usage and alternative splicing lead to the expression of at least 9 isoforms for p53, to potentially more than 35 for p73. Some of these isoforms (TA) contain a transactivation domain and act as tumor suppressors, while other ones are deleted of this domain (ΔN or ΔTA) and act as dominant negative of TA isoforms and then as potential oncogenes.

Unlike *TP53*, mutations of *TP63* and *TP73* are not frequently observed in human tumors. Nevertheless, overexpression of N-terminally truncated isoforms of p63 and p73 have been described in several tumor types. Furthermore this up-regulation is frequently associated with resistance to chemotherapy and poor prognosis.

Melanoma, Chronic Myeloid Leukemia (CML) and Acute Myeloid Leukemia (AML) are tumors that share some common properties, like resistance to conventional therapies and rare *TP53* mutations.

In order to understand how p53 family members could be involved in the outcome of these tumor types, we started in collaboration with clinicians analyses of the expression of p53, p63 and p73 N-terminal variants in Melanoma, AML and CML samples, both at mRNA and protein levels.

Preliminary results confirm the overexpression of N-terminally truncated isoforms of p73 in advanced melanomas, AML and CML and indicate that the expression of one of the p53 truncated isoforms is also deregulated in some melanomas.

Using well documented collections of tumors available, further investigations will allow characterizing the relationship between p53 and p73 isoforms expression and resistance to treatment and clinical outcome.

POSTER 11

THE ROLE OF p53 β AND p53 γ ISOFORMS IN ACUTE MYELOID LEUKAEMIA

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Acute myeloid leukaemia (AML) is a heterogeneous disease characterized by the accumulation of myeloid progenitors in the bone marrow and peripheral blood. In contrast to solid cancers, 90% of AML patients exhibit wild-type *TP53*. Although *TP53* is rarely mutated in AML, its function is frequently abrogated by several negative regulators. The discovery of multiple protein isoforms originating from the *TP53* gene has further complicated the understanding of p53's role in *TP53* wild-type cancers. Investigation of their expression, function and role in AML is critical to understand and subsequently exploit their diagnostic and therapeutic potential.

Significantly, we have found that the expression of p53, p53 β , and p53 γ protein isoforms correlate to response to therapy and survival in a group of AML patients ($n = 68$) through a novel protein mapping and bioinformatic technique. Based on these remarkable findings we have focused our efforts on further investigation of the function of these p53 isoforms.

cDNA constructs containing isoform sequences p53/p53 β /p53 γ , were separately transduced into the p53 $^{-/-}$ cell lines HL60 (AML), H1299 (non-small cell lung carcinoma) and SAOS-2 (osteosarcoma), in addition to bone marrow cells from C57BL/6 p53 $^{-/-}$ mice. This provides us with a model for characterization of the specific isoform phenotypes and mapping of their responses to different AML therapies. The function of these isoforms were examined through a variety of techniques such as flow cytometry, colony assay, immunofluorescence staining, immunoblotting and treatment response assays.

Preliminary results show dissimilar response to different p53 isoform incorporation, which indicate an individual functionality among the isoforms studied. p53 β and p53 γ expression attenuated clonogenicity in HL60 cells and altered morphology of cytoplasm and nucleus consistent with distinct effects on differentiation. Colony assay analysis of the HL60 cells showed distinct reduction of growth in HL60 cells expressing p53 γ . Also, co-transfection of p53 with p53 β or p53 γ in SAOS-2 cells altered p53s effect on specific GFP-coupled p53 responsive elements. We anticipate that future findings will contribute to the understanding of the clinical importance of p53 protein isoform expression. The deciphering of the role of p53 protein isoforms in AML may provide a sorely needed diagnostic tool and a predictor of treatment response.

POSTER 12

DOWN-REGULATION OF p53 AND p53 ISOFORMS EXPRESSION DURING HEPATOCYTIC DIFFERENTIATION OF HEPARG LIVER PROGENITOR CELLS

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Background: The HepaRG cell line is a naturally immortalized human liver cell line with progenitor properties and bipotent differentiation-inducible capabilities, established from the non-tumoral region of a resected HepatoCellularCarcinoma (HCC) (1). Induced differentiation leads HepaRG cells to evolve from a homogeneous, dedifferentiated and depolarized phenotype into a structurally defined monolayer displaying the morphology of either biliary epithelial cells or primary hepatocytes. Furthermore, differentiated HepaRG cells are susceptible to HBV infection.

Objectives: We have assessed the patterns of expression of the tumour suppressor protein p53 and of its isoforms DeltaNp53 and Delta133p53 during differentiation and infection by HBV to identify whether changes in p53 expression may be involved in the differentiation process.

Methods: HepaRG cells were cultured for 14 days in proliferation conditions and differentiation was induced by DMSO and EGF for 14 days. Cells were harvested, mRNA and proteins extracted and analyzed by RTqPCR or Western Blot, respectively.

Results: Analysis of genomic DNA revealed that HepaRG contain wild-type *TP53* sequences. In differentiated cells p53 protein was detectable by Western blot and underwent accumulation upon DNA damage, similar to normal p53 in hepatocytes. During differentiation, we observed a consistent down-regulation of the expression of the fully spliced form of the p53 mRNA (FSp53), encoding the full-length p53 protein. In Western blot, differentiation was accompanied by a 2 to 5 fold reduction in p53 protein levels. Similarly, levels of both DeltaNp53 and Delta133p53 were decreased (the latter detected only at mRNA level). The drop in p53 expression was accompanied by decreased levels of the products of p53-target genes *P21WAF1* and *MDM2*. Upon infection of differentiated cells with HBV, levels of full-length p53 were further decreased at mRNA and protein levels, whereas the expression of isoforms was not affected.

Conclusions: These results demonstrate that p53 exists in a wild-type form in HepaRG and is strongly regulated at the transcriptional level during differentiation and infection. This decrease may correspond to a survival mechanism by which differentiated cells may get rid of a pro-apoptotic factor, making cells permissive to HBV processing and replication after infection.

(1): Gripon et al. Proc Natl Acad Sci USA, 99:15655-15660, 2002

POSTER 13

DIFFERENTIAL FUNCTIONS OF DROSOPHILA p53 ISOFORMS IN TISSUE REGENERATION

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Drosophila melanogaster is an animal model well suited to study the functions of p53 isoforms. Dp53 is the only member of the p53/p63/p73 family of genes found in *Drosophila*. Only two *Drosophila* P53 (Dp53) isoforms, Dp53 long (Dp53L) and short (Dp53S), have been identified despite the complexity of p53 activities and the cellular processes it regulates. In addition, Dp53L is similar to the human P53 full length isoform, while Dp53S has a smaller transactivation domain and resembles to the human P53 Delta 40 isoform.

Our goal is to study the individual functions of each of the Dp53 isoforms and their specific roles in the regulation of apoptosis and tissue regeneration during *Drosophila* development. First, we have observed that Dp53L and Dp53S have distinct transcriptional expression profile during development and adulthood. Second, we have found both isoforms are able to induce apoptosis in developing tissues, but each activates specific apoptotic genes. Third, we have observed that Dp53S, but not Dp53L, induces strong Wingless expression, a homologue to the mammalian Wnt and tissue regeneration. These data indicate a distinct ability of each isoform in mediating tissue regeneration. Considering the implication of P53 and the Wnt pathway in mammalian tissue regeneration, *Drosophila* offers a unique possibility to decipher the role of P53 isoforms in this process.

POSTER 14

**A MOUSE MODEL TO EVALUATE THE ROLE OF p53 ISOFORMS
EXPRESSED FROM THE INTERNAL *TRP53* PROMOTER**

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Trp53, the gene encoding the p53 protein is mutated in more than half of tumors, and many other cancers present the alterations of proteins interacting with p53. Understanding the regulation of p53 is therefore of major clinical importance.

It was recently suggested that p53 isoforms may participate in the regulation of the p53 full-length protein (FLp53). The internal promoter of *TrP53*, conserved from drosophila to man, drives the expression of proteins lacking the transactivation domain of p53. In humans, one isoform expressed from this internal promoter, D133p53, is absent in normal breast tissue but present in breast cancers, moreover, its expression is altered in colon adenoma and carcinoma. In addition, transfection experiments suggested that overexpression of this isoform reduces the pro-apoptotic capacity of FL-p53. Furthermore, in zebrafish, the homologous D113p53 isoform was found to be essential to antagonize FLp53-mediated apoptosis by activating *bcl-2l*.

To analyze the role of isoforms expressed from the *TrP53* internal promoter in a mammalian system *in vivo*, we generated a conditional mouse model expressing all p53 isoforms but those transcribed from the internal promoter. A preliminary analysis of this mouse mutant is presented.

POSTER 15

A MOUSE MODEL TO EVALUATE THE ROLE OF C-TERMINAL ALTERNATIVE SPLICING AT THE *Trp53* LOCUS

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The p53 tumor suppressor protein is a transcription factor that is stabilized and activated in response to stress, to induce the transcription of genes involved in various responses including cell cycle arrest and apoptosis.

p53 is a modular protein and the complexity of its regulation is partially based on its unique structure, harboring two distinct DNA binding domains with different properties. The evolutionary conserved core DNA binding domain is essential for p53 to function as a transcription factor, as it recognizes specific DNA sequence elements in the promoters of target genes. The p53 C-terminal domain binds to DNA structures rather than specific DNA sequences.

A new layer of complexity has been added by the recent discovery of multiple p53 isoforms. The murine isoform p53AS, so called for Alternatively Spliced, has a distinct C-terminal domain that may alter its DNA binding properties. Transfection experiments suggest that p53AS may regulate the apoptotic activity of the p53 full-length protein, and alter the transcriptional activation of some of the p53 target genes.

To better understand the importance of the p53AS isoform, we decided to target in ES cells a specific deletion of the AS exon at the Trp53 locus. Mice were obtained from the recombinant ES cells. A preliminary analysis of these mice is presented.

POSTER 16

ROLE AND REGULATION OF p47 – THE ALTERNATE FORM OF p53

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p53 has been known to exist as several isoforms mainly as a result of alternate splicing at the C-terminus or alternate translation initiation at the N-terminus. Recently, one of the isoforms that has been gaining immense attention is the p47 isoform in which the first 40 amino acids of p53 are absent and is produced via an internal ribosome entry site (IRES)-mediated translation. It has also been reported to be generated from a novel *p53* RNA form that retains the intron 2 (In2), known as p53E(II), that was identified in a cDNA library constructed from primary human foreskin fibroblast. Controversial observations of p47 functions as a cell death inducer have been reported. However, the physiological relevance of p47 has not been very well understood.

Here in this study, we report the importance of In2, which acts as a promoter that leads to p47 expression. 5'RACE on promoterless constructs which consist only In2 and the rest of the exons in *p53* showed a novel transcript which starts at codon 44 of p53. Such constructs showed low expression of p47. Nevertheless, these constructs exhibited cell death in colony formation assays (CFA). Functional analysis using p47 expressing constructs driven by pCMV showed remarkable cell death and selective induction of p53 target genes - AIP1 and Pig3 - whilst no induction of p21 or MDM2. However, co-expression of p47 and p53 had no additive effect on cell death and enhancement of target gene activation. Instead inhibition of p53-dependent death was observed. This suggested the existence of an alternate p47-mediated apoptotic pathway. In addition, the physiological presence of In2 in the novel *p53* RNA transcript, p53E(II), was confirmed in different cell lines and primary normal skin fibroblasts. p47 expressed from p53E(II) that was mutated not to express p53 was as capable of inducing cell death as p47 expressed from *p53* RNA confirming the above observation.

Lastly, cells lacking p53 expression but expressing p47 endogenously and its isogenic controls were used to verify the data above. Detailed results will be presented.

POSTER 17

MODULATION OF p53 TRANSCRIPTIONAL ACTIVITY BY Δ40p53, A p53 ISOFORM LACKING THE TRANSACTIVATION DOMAIN

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The tumor suppressor protein p53 is a transcription factor ubiquitously expressed as a major isoform of 53 kDa (also termed TAp53). Recently, several forms of lower molecular weight have been identified. Δ40p53 is a p53 isoform lacking the first forty residues in the N-terminal domain, which contains the transcriptional activation domain. Δ40p53 isoform is often detected at low levels in p53 expressing cells, as well as in normal tissues. While there is evidence that this protein isoform has low, if any, transcriptional activity on p53-target genes, its effects on p53 transcriptional activity are not well described.

By co-transfected Δ40p53, TAp53 and a beta-galactosidase reporter construct (p53 consensus response elements upstream the *LacZ* gene), we showed that Δ40p53 isoform exhibits reduced transactivation capacity compared to TAp53. In addition, Δ40p53 counteracts transcriptional activity of TAp53. Co-immunoprecipitation experiments indicated that both p53 isoforms can form hetero-oligomers, suggesting that Δ40p53 could modulate TAp53 transcriptional activity through protein interaction. We also demonstrated that Δ40p53 is able to bind specifically to p53 consensus DNA sequence *in vitro* and competes with wild-type TAp53 in specific DNA-binding assays.

Overall, these results suggest that Δ40p53 has the potential to work as a regulator of TAp53 activity, in particular when present at low levels as compared to TAp53, compatible with the expression patterns observed in many cells and tissues.

POSTER 18

REGULATION OF BCL-2 EXPRESSION BY Δ133p53α ISOFORM

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The *TP53* gene expresses several isoforms due to the usage of alternative promoters, splicing and translational initiation sites. The internal promoter P2 located within *TP53* gene regulates the expression of Δ133p53α isoform, which lacks the whole transactivation domain and part of the DNA-binding domain.

Several studies have shown that Δ133p53α can modulate p53 suppressive functions through regulation of gene expression. Indeed in human fibroblasts, knock-down of Δ133p53α expression promotes p53-dependent replicative senescence by modulating *p21* and *mir-34* expression. Recently, our group also reported that Δ133p53α inhibits p53-dependent apoptosis and G1 arrest without inhibiting p53-dependent G2 arrest. These observations were attributed to the differential regulation of *p21*, *Bcl-2* and *Hdm2* gene expression. However, the molecular mechanism by which Δ133p53α regulates gene expression remains unknown.

In this study, we investigated whether Δ133p53α regulates the transcriptional expression of Bcl-2, an anti-apoptotic protein. First, we observed that knock-down of Δ133p53α expression was associated with a decrease of Bcl-2 expression at both mRNA and protein levels. Second, transient transfection leading to Δ133p53α over-expression resulted in an increase of Bcl-2 mRNA and protein levels. In addition, Bcl-2 expression by Δ133p53α was regulated in a dose-dependent manner, low doses of Δ133p53α being sufficient to modulate Bcl-2 expression. Finally, we investigate the regulation of Bcl-2 promoter activity in presence of Δ133p53α.

Therefore, our results support the hypothesis that Δ133p53α has an intrinsic activity, involved in differential modulation of gene expression.

POSTER 19

TRANSCRIPTIONAL REGULATION OF Δ133p53 ISOFORM BY p53

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The *TP53* gene expresses several p53 proteins isoforms. Among them, Δ133p53α is a N-terminal truncated p53 isoform that lacks the whole transactivation domain and part of the DNA-binding domain. It has been reported that Δ133p53α isoform modulates p53-mediated senescence, apoptosis and cell cycle arrest in response to stress. Δ133p53α protein is encoded by a specific transcript, p53I4, driven by an internal promoter P2 located between intron 1 and exon 5 of *TP53* gene. The transcription factors regulating P2 promoter activity remain unknown.

Here, we demonstrated that the P2 promoter activity is regulated by the p53 tumour suppressor protein. In response to doxorubicin treatment, Δ133p53α expression is increased at both mRNA and protein levels in wild-type but not in mutant p53 cells. In addition, chromatin immunoprecipitation and luciferase assays showed that p53 binds p53 response elements located within the P2 promoter and transactivates P2 promoter. However, mutant p53 had no effect on P2 promoter activity. We also observed that Δ133p53α protein does not bind specifically p53 consensus DNA sequence *in vitro*, but competes with wild-type p53 in specific DNA-binding assays.

Therefore, we showed that Δ133p53 is a novel target of p53 that may participate in a negative feedback loop modulating p53 tumour suppressive functions.

POSTER 20

Δ133p53 TRANSCRIPT ENCODES TWO p53 ISOFORMS: Δ133p53 AND Δ160p53

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The *TP53* gene contains an internal promoter P2, which regulates the expression of Δ133p53α mRNA. This transcript encodes Δ133p53α isoform, which lacks the whole transactivation domain and part of the DNA-binding domain. It has been reported that Δ133p53α inhibits p53-mediated replicative senescence, apoptosis and G1 arrest through modulation of gene expression.

By siRNA transfection and site-directed mutagenesis, we identified a fourth N-terminal p53 isoform, Δ160p53α. This novel p53 isoform is produced by internal initiation of translation at ATG160 using Δ133p53α transcript. We detected endogenous Δ160p53α protein in three different cell lines: U2OS, T47D and K562.

In K562 cells, the *TP53* gene presents an insertion at codon 136 leading to a premature stop at codon 148. Thus, K562 cells do not express p53 or Δ133p53α but retain the ability to express Δ160p53α protein. Two C-terminal splicing variants of Δ160p53 were detected in K562, Δ160p53α and Δ160p53β. In addition, we observed that Δ160p53β protein expression is regulated by hemin treatment, which induces erythrocyte differentiation.

Therefore, we described for the first time that the human Δ133p53 transcript encodes two proteins by alternative initiation of translation at ATG133 and ATG160.

POSTER 21

ENDOGENOUS p53 β PROTEIN IS REGULATED BY THE PROTEASOME INDEPENDENTLY OF HDM2

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The *TP53* gene encodes a C-terminal p53 isoform, p53 β produced by alternative splicing in intron 9. It has been shown that p53 β modulates p53 suppressive functions. In particular, p53 β promotes p53-mediated apoptosis and replicative senescence. In addition, p53 β has intrinsic pro-apoptotic activities that may be correlated to its capacity to bind DNA and to regulate *Bax* promoter activity. However, the regulation of p53 β expression is unknown.

To investigate the regulation of p53 β stability, we took advantage of the neuroblastoma SK-N-AS cells, which express, at endogenous level, a truncated p53 protein (R342X) and wild-type p53 β . We show that endogenous p53 β is accumulated in response to MG132, indicating that p53 β protein is degraded by the proteasome

To determine whether p53 β degradation is promoted by MDM2, we studied its degradation in cells devoid of p53 expression after transient transfection thus avoiding the interplay with other p53 isoforms. We observed that p53 β protein has the same half-life than p53; (2) is accumulated in response to MG132 treatment; (3) is ubiquitinated by the E3-ligase, Hdm2; and (4) is co-immunoprecipitated with Hdm2. Although p53 β is ubiquitinated by HDM2, p53 β degradation is not promoted by HDM2.

Therefore, our data support the hypothesis that p53 β expression can be regulated at the protein level through the proteasome.

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