697 Bcl-2 functionally compensates for down-regulation of CHIP and protects cancer cells from cell death

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Background: Carboxy terminus of Hsp70p-Interacting Protein (CHIP) is a ubiquitin E3 ligase. CHIP adds ubiquitin to misfolded and excess proteins to induce protein degradation by proteasome pathway. Namely, CHIP controls protein quality and maintains cellular homeostasis. In our previous study, we discovered that CHIP suppresses breast cancer cell growth and metastasis. We clarified the mechanism underlying metastasis pathway. In contrast, the suppression of tumour growth by CHIP is little understood. In our experiments, we generated CHIP knocked-down MCF-7 cells by RNA interference (RNAi) (CHIP KD cells). In CHIP KD cells, we found that the levels of B-cell lymphoma 2 (Bcl-2) expression was up-regulated. Bcl-2 is known as an anti-apoptotic protein. Then we hypothesized that up-regulation of Bcl-2 promotes anchorage independent cell growth, and it may cause the enhancement of tumour growth in CHIP KD cells. Therefore, we focused on Bcl-2 and tried to elucidate the mechanism how to regulate Bcl-2 expression by CHIP.

Materials and Method:

1. Bcl-2 and CHIP were double knocked down in MCF-7 cells by RNAi. Using this cell lines, soft-agar colony formation assay was performed.
2. Bcl-2 transcriptional activity was examined in CHIP KD cells by nuclear Run-on assay.
3. CHIP expression was recovered in CHIP KD cells by adenovirus infection. Then the protein level of Bcl-2 was examined.
4. CHIP was knocked down by RNAi in MCF-7 cells. Then, the number of colonies was counted. Besides, the same examination was performed in Bcl-2 KD or over-expressed MCF-7 cells.
5. Bcl-2 expression level in MCF-7 cells was measured by flow cytometry.

Results:

1. Bcl-2 was a major factor to enhance anchorage independent cell growth by CHIP KD.
2. CHIP KD cells, Bcl-2 transcription was up-regulated.
3. Bcl-2 expression was not changed, when CHIP expression was recovered in CHIP KD cells.
4. CHIP KD induced cell death and it was suppressed by Bcl-2.
5. Bcl-2 expression level was heterogeneous in MCF-7 cells.

Conclusion: From our data, it is suggested that cellular protein homeostasis is disrupted and cell death is induced when CHIP expression is suppressed. However, portion of cancer cells that highly expresses Bcl-2 may be able to survive, because high expressing Bcl-2 functionally compensates for down-regulation of CHIP. Finally, we hypothesized that high Bcl-2 expressing cells become a majority of cellular population and they exhibit enhanced anchorage independent cell growth.

698 Nucleolar protein MYBBP1A is required for nucleolar stress-induced p53 acetylation

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The tumour suppressor protein p53 responds to a diversity of stresses and regulates many target genes whose products induce cell cycle arrest, apoptosis, senescence, and DNA repair. DNA damage is widely believed to activate p53 as a transcription factor through post-translational modifications such as phosphorylation, ubiquitination, and acetylation. Phosphorylation of p53 in response to DNA damage is mediated by various kinases. phosphorylation of Ser15 in p53 greatly increases its ability to interact with p300 and CBP. The phosphorylation-dependent interaction of histone acetyltransferases p300 with p53 drives p53 acetylation. Previous reports show that p53 acetylation is indispensable for p53 activation. Recently, a number of external and internal insults were shown to induce nucleolar stress by disrupting nucleolar structure. The impairment of nucleolar function stabilizes and activates p53. However, the mechanisms underlying this regulation are still unclear.

Previous report showed that flux of 489 endogenous nucleolar proteins and nearly 300 proteins translocate from the nucleolus to the nucleoplasm following nucleolar disruption (Andersen et al., Nature, 2005). From these 300 proteins, we excluded 200 based on their known function and selected 100 candidates for investigation. We then generated siRNAs against mRNAs for these candidate proteins and examined the effect of each siRNA on both the acetylation status and quantity of p53 proteins in cells that had been treated with TIF-IA siRNA to increase p53 acetylation.

We show that nucleolar disruption induces acetylation and accumulation of p53 without phosphorylation. Recent evidences demonstrated that p53 acetylation is an indispensable event for p53 activation. Using siRNA library, we screened nucleolar proteins involved in acetylation of p53, and identified MYBBP1A. Nucleolar disruption led to translocation of MYBBP1A from nucleolus to nucleoplasm. MYBBP1A then binds to p53 and facilitates the complex formation among p53 and p300 to induce p53 acetylation. MYBBP1A depletion significantly abrogated p53 activation and apoptosis induced by nucleolar disruption.

MYBBP1A is a signal transducer of nucleolar stress, which can directly bind to p53, bypass phosphorylation step, and induce acetylation and accumulation of p53.

699 The effects of ER alpha ligands on breast cancer metastasis

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Background: Breast cancer is typically a hormone-dependent tumour; estrogen may increase breast cancer proliferation by binding to estrogen receptor, ER alpha. ER alpha is a valuable prognostic factor for early-stage breast cancer. ER alpha-negative cancers, however, typically exhibit higher histological grades than ER alpha-positive cancers during progressive stages. In contrast, TGF-beta acts as a tumour suppressor early in tumour development, whereas it promotes invasion and metastasis at later stages. These biphasic effects of ER alpha and TGF-beta have been well known, however the molecular mechanisms remain poorly understood.

Materials and Methods:

1. MCF-7 cells were treated with ER alpha ligands, and then examined pS2 (target gene for ER alpha) or PAI1 gene (target gene for TGF-beta signaling) expression by real-time RT-PCR.
2. The effects of ER alpha ligands on ER alpha and phosphorylated Smads Protein levels were examined by Western Blotting.
3. Invasive potentials of MCF-7 cells were tested with a Matrigel-coated transwell assays in the absence or presence ER alpha ligands.

Results and Conclusion: We found that ER alpha, which is the prognostic factor of breast cancer, inhibits TGF-beta signaling. This inhibition of TGF-beta signaling is mediated by inducing the degradation of signal transducer Smad proteins in an estrogen-dependent manner. ER alpha also suppressed the breast cancer metastasis in a mouse model which evaluates cancer metastatic behavior for 6 weeks. In addition, we showed that the inhibitory effects of ER alpha expression on TGF-beta signaling were abrogated by the treatment with pure antagonist ICI182,780, which induces rapidly ER alpha protein degradation in MCF-7 cells. The ER alpha-dependent inhibition of TGF-beta signaling was not changed by the treatment with tamoxifen, which is one of the selective estrogen receptor modulators. The migratory or invasive potential of MCF-7 cells were also enhanced by ICI182,780 treatment, but not affected by tamoxifen, suggesting that the clinically treatment with ICI182,780 may induces breast cancer metastasis.

Taken together, our study indicates that breast tumour invasiveness and metastasis are inhibited by estrogen, enhanced by ICI, and not affected by tamoxifen through the regulation of TGF-beta signaling. Now we are exploring a new compound which downregulates both of tumour growth and metastasis of breast cancer.

700 BORIS and its paralogue CTCF exhibit similar biological functions

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Background: BORIS, a paralogue of the multifunctional, multivalent 11 zinc finger protein CTCF, is distinguished by its aberrant expression in both cancer cell lines and primary tumours of multiple origins. Whilst CTCF has been characterised as a putative tumour suppressor, BORIS has previously been regarded as a potential oncogene. The aim of this study was to further define the roles of CTCF and BORIS in carcinogenesis, both *in vitro* and *in vivo*.

Material and Methods: A tet-dependent multicistronic lentiviral system was constructed, allowing the regulated expression of either BORIS or CTCF in human and mouse primary cells and cancer cell lines. Assays were performed *in vitro* to assess the influence of BORIS and CTCF on cellular processes, including colony formation, proliferation, cell cycle and apoptosis. Bioluminescence imaging of an orthotopic tumour model in NOD-SCID mice was employed to determine the roles of BORIS and CTCF in tumour growth *in vivo*.

Results: Surprisingly, both BORIS and CTCF expression mediated a significant decrease in cell proliferation and clonogenic capacity, suggesting that BORIS may be acting as a tumour supressor. In asynchronous cells over-expressing CTCF, decreased cycling led to a significant increase in the percentage of cells in G0/G1. A similar trend was observed in BORIS over-expressing cells. In a UV damage induced model of apoptosis, BORIS mediated pro-apoptotic effects in primary cells, and CTCF exhibited a similar trend consistent with our previous reports. Finally, using bioluminescence imaging we demonstrate for the first time, the tumour suppressor role of CTCF in breast cancer cells *in vivo*. The growth of BORIS over-expressing tumours

was also found to be inhibited, but not to the same extent observed in CTCF over-expressing tumours.
Conclusions: These findings indicate that, rather than acting in an oncogenic fashion, BORIS and CTCF mediate similar functions suggesting that BORIS is best characterised as a tumour supressor gene.

701 RNA content in the nucleolus alters p53 acetylation

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Background: p53 protects against cancer through its capacity to induce cell cycle arrest or apoptosis under a large variety of cellular stresses. It was reported that a common denominator in all p53-inducing stresses is nucleolar disruption. Thus, the existence of a stress sensor that monitors nucleolar structure and function and regulates p53 levels was proposed. Recently, we screened nucleolar proteins involved in the accumulation and acetylation of p53 using a siRNA library and identified three gene products, Myb-binding protein 1a (MYBBP1A), RPL5, and RPL11. We revealed that MYBBP1A translocated from the nucleolus to the nucleoplasm under nucloeolar stress conditions and enhanced acetylation of p53. RPL5 and RPL11 have been shown to be involved in the stabilization of p53. However, the relationships among MYBBP1A, RPL5, and RPL11 were not investigated.
Material and Methods: To evaluate the relationships among MYBBP1A, RPL5, and RPL11, MYBBP1A was knocked-down together with either RPL5 or RPL11. MCF-7 cells were treated with a combination of siRNAs, and the expression and acetylation levels of p53 were analyzed by immunoblot. Furthermore, the effects of RPL5 or RPL11 knockdown on MYBBP1A localization were examined by immunofluorescence.
Results: Treatment of cells with siRNAs for MYBBP1A and RPL5 or for MYBBP1A and RPL11 showed no additive effects in the protein or acetylation levels of p53, which had been reduced by RPL5 or RPL11 siRNA. This result suggested that MYBBP1A and RPLs functioned on the same pathway that regulated p53. Immunostaining showed that knockdown of RPL5 or RPL11 abrogated the nucleolar stess-induced translocation of MYBBP1A into the nucleoplasm. Interestingly, RNase treatment caused translocation of MYBBP1A into the nucleoplasm, which indicated that MYBBP1A was anchored in the nucleolus through binding to nucleolar RNA. We found that RPL5 and RPL11 were necessary for rRNA transport. Knockdown of RPL5 or RPL11 abrogated export of rRNA from the nucleolus and counteracted reduction of nucleolar RNA levels caused by inhibition of rRNA transcription. As a result, RPL5 or RPL11 knockdown inhibited MYBBP1A translocation to the nucleoplasm and p53 activation.
Conclusions: The nucleolar RNA content is maintained by a dynamic equilibrium between RNA generation and export. The loss of this balance due to stress alters the nucleolar RNA content and modulates p53 activity through translocation of MYBBP1A from the nucleolus to the nucleoplasm.

702 mRNA and miRNA expression profiles of pre- and postoperative breast cancer tumours

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Background: Several gene expression studies have been performed on breast cancer. These studies, however, are predominantly done using tumour tissue obtained during breast cancer surgery. In this study we wanted to examine how surgery and preoperative procedures influence the expression profiles of breast cancer tumours.
Material and Methods: This study includes 15 randomly selected patients for which both a preoperative and a postoperative tumour sample were available. The preoperative samples were taken as a core needle biopsy by an experienced radiologist while the postoperative samples were obtained as an additional core sample from the tumours after the patients had been operated with ablatio mamma (surgical removal of the entire breast). 12 of the patients had undergone Sentinel Node (injection with radioactive substance to examine spread of cancer cells to the lymph nodes) as part of the standard procedure before operation.
Total RNA was isolated from all the tumour samples and whole genome gene expression and miRNA expression micro-array analysis were performed using Agilent microarrays per the manufacturer's instructions.
Results: Examination of the gene expression profiles revealed that two of the postoperative samples contained almost only normal tissue and these two patients were therefore excluded from further analysis. Paired Significance Analysis of Microarrays (SAM) of the remaining 13 pairs revealed 235 differently expressed genes (FDR < 2.5). The gene list contains several early response stress-related genes as well as genes of which the expression has previously been associated with cancer.
For the miRNA expression analysis 10 pairs were used and only 2 miRNAs (miR-923 and miR-1274b) were significantly differently expressed according to paired SAM. Neither of these miRNAs are studied earlier.
Conclusions: The expression profiles of preoperative breast cancer tumours differ from that of postoperative tumours for a small fraction of genes (235 out of 44K). However the expression of these genes in concert affects specific biological pathways, which may relate to stress responses in the tissue after the injection with radioactive substance or during operation. Since most expression profiles are obtained from post-operative specimen it is important to take in to consideration that findings in these pathways may reflect the timing of when samples are taken.

703 KinaseSwitch: a technology platform for the inducible and reversible inhibition of kinases using chemical genetics in vivo

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Background: KinaseSwitch uses chemical genetics *in vivo* – a complementary approach switching any selected kinase ON/OFF in a pharmacological manner. A 'silent' point mutation is introduced in the ATP-binding pocket at a defined position (gatekeeper), leaving the kinase protein fully functional. Thus, novel functional alleles of kinases are created which can be inhibited by proprietary, well characterized ATP analogues (PP1-based derivatives). These analogues are highly selective for one mutated kinase and show at least 100 times higher affinity compared to all other WT kinases *in vivo*. This technology has been developed by Prof. Kevan Shokat (UCSF) and is widely published as ASKA (A chemical switch for inhibitor-sensitive alleles of any protein kinase; Bishop et al., *Nature*, Vol 407, 21 September 2000) technology.
Materials and Methods: Any S/T or Tyr kinase can be analysed using this technology. Bioinformatic analysis is used to identify potential residues for mutagenesis. Mutant kinase alleles are tested *in vitro* to select the most potent mutations that do not interfere with kinase activity, but allow specific inhibition of the kinase using a selected ASKA inhibitor. IC50 values of WT and mutant kinase are established to validate specificity. Once the *in vitro* analysis is completed, mutations are inserted into the mouse genome, allowing inhibition of the target kinase *in vivo* after administering ASKA compound to animals.
Results: Examples for kinases Akt1, Btk, c-met, EphB4 and the TrkA/B/C family are presented *in vitro*/*in vivo*. These examples show the broad utility of this kinase technology platform. Inducible and complete inhibition of kinases *in vivo* can be achieved using the selected ASKA compound.
Conclusion: KinaseSwitch mice can serve various important purposes along the discovery workflow in target validation and compound testing. The function of the kinase is inhibited *in vivo* in a pharmacological way – similar to a drug. KinaseSwitch is dose dependant, inducible and reversible. The mutated protein is expressed, unlike in other KO technologies, and can serve its tasks in its spatial context. KinaseSwitch phenotypes provide a baseline phenotype of inhibiting only the target kinase, allowing direct comparison with candidate kinase inhibitors and characterization of ON and OFF target effects of a drug development candidate.

704 Lysine monomethylation of HMGA2 by the monomethyltransferase Set7/9

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Background: The architechtural transcription factor High Mobility Group AT-hook 2 (HMGA2) is frequently rearranged and amplified in tumours of mesenchymal origin, and several studies have suggested an important role in adipocytic cell growth and development. As being an oncofetal gene, HMGA2 is expressed in undifferentiated cells, and becomes re-expressed during cancer development. It is known as a chromosomal protein, and as the histones, this protein is post-translationally modified. We aim to further understand the role of HMGA2 in cancer and normal biology with respect to post-translational modifications of the protein.
Material and Methods: Bioinformatic rediction of lysine methylation of HMGA2 followed by *in vitro* methyltransferase assays with recombinant proteins, and thereafter verified in cell lines by immunodetection.
Results: We report a novel mechanism of HMGA2 regulation through lysine monomethylation by the Set7/9 methyltransferase. We have shown for the first time that HMGA2 is lysine methylated on one specific residue located in between the second and third AT-hook, and that the protein is methylated in mesenchymal tumours. The AT hooks are responsible for the binding to the minor groove of DNA, whilst the linker regions are more flexible in nature. We suggest, through our findings, that this region is comparable in function to the protruding tails of the histones, and involves cross-talk between different modifications.
Conclusion: We have revealed a new way of regulating HMGA2 through lysine monomethylation.