UHNMAC News

The University Health Network Microarray Centre Newsletter – Winter 2007

UHN Microarray Centre

Welcome to the Winter 2007 edition of the UHNMAC News. This newsletter will be published quarterly and will provide general UHNMAC news, a review of a recent journal article that uses microarrays manufactured at the UHNMAC, and a feature report pertaining to microarray technology.

ANNOUNCEMENTS

New lower prices for all services!

In November 2006, the UHNMAC lowered all service prices. Please visit our website, http://www.microarrays.ca/services/services.html, for details.

UHNMAC aCGH Survey

The UHNMAC Survey about Array Comparative Genomic Hybridisation is still open. If you can spare 10 minutes, please complete the survey. There is also a link from the home page.

SEMINARS

- 1st Annual Toronto Functional Genomics Symposium scheduled for June 18-19, 2007 Check the website for updates soon!
- Please check our website for upcoming seminars hosted by the UHNMAC

FEATURE ARTICLE & REVIEW

Vigano MA, Lamartine J, Testoni B, Merico D, Alotto D, Castagnoli C, Robert A, Candi E, Melino G, Gidrol X, Mantovani R. New p63 targets in keratinocytes identified by a genome-wide approach. EMBO J, 2006, 25(21):5105-16

Getting under your skin - using keratinocytes to identify p63 gene targets

Now that the human genome has been sequenced, the task of identifying the function of each gene has begun. For transcription factors, the identification of specific targets will allow us to better understand complex transcriptional networks and to treat diseases associated with mutations in transcription factor genes.

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FEATURE REPORT : microRNA

MicroRNA (miRNA) are single-stranded regulatory RNA molecules found in nematodes, plants, and animals. miRNA has been implicated as regulators of developmental timing, neuronal differentiation, cell proliferation, programmed cell death, and fat metabolism[1,2]. Not much is known about miRNA in mammals although it is thought to act as post-transcriptional modulator of gene expression[1].

miRNA represent about 0.01% of the mass of total RNA[3]. miRNA occur in three different forms: long pri-miRNA, hairpin pre-miRNA, and short mature miRNA. In the nucleus, pri-miRNA is cleaved by the nuclease Drosha to form pre-miRNA[4]. Pre-miRNA is 70-125 nucleotides long and form

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As always, general questions about microarrays can be addressed to help@microarrays.ca, orders for UHNMAC array products can be placed at orders@microarrays.ca, and questions about any of our services can be addressed to geneservice@microarrays.ca. If you have any suggestions for newsletter articles or questions you'd like addressed, please contact general@microarrays.ca.

p63 Gene Targets

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p63 is a master regulatory gene of multilayered epithelia such as keratinocytes[1] and there is evidence that p63 is involved in developmental processes[2,3]. In particular, the ΔNp63α isoform is thought to play a key role in the asymmetric division of epithelial cells and is involved in ankyloblepheron-ectodermal defects (AEC), ectrodactily, ectodermal dysplasia (EEC), and the split hand/foot malformation (SHFM) syndromes[4]. One of the difficulties in finding targets specific to p63 lies in its homology with other transcription factors, like p53.

Conventional strategies for finding genes targeted *in vivo* by p63 include profiling analysis of cells in which p63 and p63α are overexpressed, silencing p63 with siRNA, and by studying p53-controlled genes as p63 is homologous to p53. More recently, however, the ChIP-on-chip technique, which combines chromatin immunoprecipitation (ChIP) and the use of DNA microarrays (chips) containing CpG islands and promoter regions, has been used to identify genome-wide TF targets.

Vigano *et al.* used the ChIP-on-chip technique to screen for p63 targets using both promoter arrays and human CpG island 12k arrays. HaCaT keratinocytes were used as p63 is involved in the development of ectodermal tissues and HaCaT cells partially mimic basal keratinocytes. By identifying novel targets of p63, scientists could better understand the molecular mechanisms of this complex transcription factor.

This study found that many of the p63 targets are themselves transcription factors or co-regulators. As expected, p63 regulated classes of genes important for developmental and differentiation processes like cell adhesion, cytoskeleton, cell proliferation, and cell cycle. Unexpectedly, p63 was found to target genes involved with immunity and vascular processes. Other novel targets included 8 genes carrying the WD40 domain, which is involved in signal transduction, pre-mRNA processing, and cytoskeleton assembly[5], genes acting as MAPK modulators and genes belonging to the ubiquitin-dependent protein processing pathways. Vigano *et al.* speculate that ubiquitin-dependent regulation targets may themselves be regulators of p63 activity in a way similar to the p53/MDM2 feedback regulatory loop.

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The targets were validated by promoter transactivation studies, immunostaining normal tissue to confirm regulation by p63, and by expression analysis in differentiated HaCaT cells and in cells overexpressing ΔNp63α, which verified ΔNp63α targets were regulated during keratinocyte differentiation. *In vivo* p63 binding experiments in primary keratinocytes were also performed and results show that half of the loci were also bound by p53, which indicates that both proteins regulate common sets of genes. One of the limitations of the ChIP-on-chip technique identified in this study was the varying degree of gene enrichment during the generation of ChIP probe amplicons. The authors suggest that variations could have been a result of sub-optimal selection of the amplified region or due to binding of p63 mediated by other DNA-binding proteins. In addition, ChIP experiments represent average binding within the entire cell population that is heterogeneous with respect to cell-cycle phases and thus the *in vivo* affinity of p63 for specific targets may have varied. Another source of variation that is not discussed is the way in which immunoprecipitated DNA was amplified (by LM-PCR). Such issues should be kept in mind when performing ChIP-on-chip studies.

This study illustrates the robustness of the ChIP-on-chip approach. Vigano *et al*. were able to identify 183 new ΔNp63α target genes, 88% of which were effectively bound *in vivo*, thus enabling us to better understand its molecular mechanisms. This study has also demonstrated that ChIP-on-chip is a relatively accurate high-throughput method for identifying transcription factor targets.

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microRNA

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hairpin structures with an overhang of 2 nucleotides at the 3` end. Pre-miRNA is exported into the cytoplasm by RanGTP and exportin5 proteins[5]. In the cytoplasm, pre-miRNA is processed by the Dicer ribonuclease to form the mature miRNA[6]. Mature miRNA are 19-23 nucleotides long.

In plants, miRNA target specific mRNA with high complementarity and are likely to trigger the degradation of the mRNA similar to the action of small interfering RNA (siRNA)[7]. Eukaryotic miRNA appear to act predominantly as translational repressors but can guide RNA degradation in some cases[8].

Historically, miRNA were first found in *C. elegans*[1,7]. Two miRNAs, lin-4 and let-7, were found to regulate developmental timing by pairing with the 3` untranslated region of target mRNAs and attenuating their translation[7]. miRNA can be identified by forward genetics (mutation analysis), as in the case of lin-4 and let-7, by sequencing of size-fractionated cDNA libraries, and more recently by computational prediction.

It is difficult to define a "true" miRNA. The generally accepted definition of miRNA is that 1) the mature miRNA is expressed as a distinct transcript (about 22 nucleotides) and is detectable by Northern blot or other experimental means, 2) the mature miRNA originates from a precursor with a characteristic secondary structure (hairpin), and 3) the mature miRNA should be processed by the Dicer ribonuclease[9]. Phylogenetic conservation is another criterion often considered.

In 2003, Lim *et al*. estimated that there are about 250 miRNA in vertebrates[10], however, recent studies (2005) suggest that there are at least 800 human miRNAs[11]. A public miRNA database, miRBase (http://microrna.sanger.ac.uk/) provides access to a searchable database of published miRNA sequences and annotation, predicted miRNA targets, and the miRBase Registry that provides researchers with unique names for novel miRNA genes prior to publication of results. As of October 2006, the number of entries on miRBase for potential miRNAs was 4361, although there may be some overlap, discrepancy on what defines a "true" miRNA, and unverified sequences.

Microarrays to profile miRNA expression

The goal of profiling miRNA expression is to discover the specific mRNA molecules regulated by each miRNA molecule. Bioinformatic analyses of developmentally regulated miRNAs have suggested potential mRNA targets[11]. Studies have found that miRNA are differentially associated with polyribosomes. The significance of this is the suggestion that a complex hierarchical regulation of miRNAs exists. It is possible that miRNAs may compete for binding with the mRNA target and that the dynamic association of miRNAs with different RNP complexes may affect their interaction with different mRNA targets[11].

Microarrays are a good method for high-throughput expression profiling, however, one of the challenges of profiling miRNA expression is that the small size of the miRNA entities leaves little room for labelling or designing specific probes[2]. Another challenge is the fact that miRNA exists in three forms (pri-miRNA, hairpin pre-miRNA and short mature miRNA). Expression profiling of short mature miRNA will require that signal from hairpin pre-miRNA and pri-miRNA can be eliminated[2]. In addition, microarrays made with standard DNA probes are often unable to distinguish single nucleotides. Despite these challenges, researchers have successfully used microarrays to profile miRNA expression.

Krichevsky and colleagues were the first to use an array-based approach to profile miRNA expression in 2003[7]. miRNA arrays were prepared by spotting oligonucleotides (54-72-mers), anti-sense to the miRNA sequence, onto NEN membranes. Total RNA was filtered by size to exclude molecules larger than approximately 60 nucleotides and these small RNA molecules were end-labelled with ^{33}P dATP by T4 polynucleotide kinase[7]. Following hybridisation of the labelled samples to the array overnight, the membranes were washed and exposed to phosphorimager screen. The screens were scanned using a Phosphor Imager and hybridisation signals were quantified.

Other protocols for array-based miRNA profiling have been developed. For example, Sun *et al.* used CodeLink-activated slides (GE Healthcare) and pin-based dispensing system to spot 5` amino-modified (C6 linker) oligonucleotides of sense miRNA sequences[12]. These oligonucleotides were designed to hybridise to biotin end-labelled antisense miRNA targets[12]. Total RNA was primed with random hexamer and SuperScript II reverse transcriptase was used to generate first strand cDNA. The 3` end of each cDNA was labelled with biotin-ddUTP using a Terminal Labelling kit. After hybridisation and washing, the slides were double stained with R-Phycoerythrin Streptavidin to increase overall signal levels.

More recently, problems with microarray cross-hybridisation and specificity have been overcome with the use of LNA-modified array elements[13]. Locked Nucleic Acids (LNA) are synthetic analogues of RNA and DNA bases in which the ribose ring is locked with a methylene bridge connecting the 2`-O atom and the 4`-C atom. These analogues are characterised by increased thermostability of nucleic acid duplexes when LNA bases are incorporated into oligonucleotides. The properties of LNA are exploited to design probe sets for uniform, high-affinity hybridisations that are able to discriminate single nucleotide differences[13]. Castoldi *et al.* found that LNA-modified capture probes yield a several-fold-increase in hybridisation signal in comparison with unmodified DNA probes when the same amount of total RNA (as little as 2.5 µg) is used[13]. The increased sensitivity can eliminate the need for RNA size selection and/or amplification.

miRNA research using microarray platforms

In the past couple of years, researchers have found miRNA expression profiles can be used as markers of lung cancer and to identify miRNA involved in the regulation of tumour growth. Yanaihara *et al*. found that miRNA expression profiles are diagnostic and prognostic markers of lung cancer[14]. For example, they found that high has-miR-155 and low has-let-7a-2 expression correlated with poor survival. Meng *et al*. used miRNA microarrays to identify miRNA involved in the regulation of cholangiocarcinoma growth and response to chemotherapy[15]. By comparing the miRNA expression of malignant and non-malignant cholangiocytes using microarrays, they found that miR-21, miR-141 and miR-200b were highly over-expressed in malignant cholangiocytes compared to non-malignant, and that inhibition of miR-21 and miR-200b increased the cells sensitivity to gemcitabine (modified nucleoside used in chemotherapy) and inhibition of miR-144 decreased cell growth. Meng *et al*. concludes that aberrantly expressed miRNA or their targets will provide mechanistic insight and therapeutic targets for cholangiocarcinoma[15].

Other studies involving viral infections and antisense-based strategies to block miRNA function indicate that miRNA may represent novel therapeutic targets. It appears that cellular miRNA may play a role in the establishment of latent viral infections and in evading the host's immune system[16]. The advantages of miRNA for viruses are that miRNA provides a specific way to downregulate host gene products that may interfere with viral replication, miRNA are not antigenic, and that mature miRNA occupy less than 200 nucleotides of the viral genome. However, one major disadvantage for most RNA viruses, as well as one group of DNA viruses, is that they replicate and express their genome in the cytoplasm, not in the nucleus (cellular location of Drosha)[16]. Despite being a RNA virus that does not seem to encode any miRNA itself, Jopling *et al*. have recently reported that human hepatitis C needs cellular miR-122a, after infection, to allow for replication[17]. This finding suggests that miRNA (in this case, miR-122a) may represent novel targets for anti-viral intervention. Meister *et al*. adapted an antisense-based strategy to block miRNA function in cell extracts and cultured cells[8]. This study used 2`-o-methyl oligoribonucleotides for the sequence-specific inactivation of miRNPs, showing that such antisense reagents could be valuable for therapeutic intervention to control miRNA activity in disease states[8].

Future microarray-based studies will provide more insight into the way in which miRNA modulates gene expression. By further investigating the involvement of miRNA in specific disease states, novel diagnostic markers and therapeutic targets will be found.

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