DNA Methylation Analysis – Reliable Cell Characterization in Regenerative Medicine

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Abstract

Persistent gene regulation is a primary role of epigenetics. Methylation of DNA is frequently associated with long term down regulation of gene expression, and thus is well suited to ensuring stable commitment along a cell lineage. We demonstrate that DNA methylation patterns can serve as characteristic markers to distinguish different cell types. We have identified panels of methylation markers that are specific to mesenchymal stem cells or various differentiated cell types in the mesenchymal lineage (adipocytes, chondrocytes, etc.). This method of cell type identification has a number of advantages over conventional markers in that it is robust, is both qualitative and quantitative, and can be extended to a high level of informational complexity.
Applications of DNA methylation analysis in regenerative medicine will be in two areas: (1) test kits for quality control of therapeutic cell products, which characterize cell identity, purity and potency and (2) screening assays for the identification of novel growth and differentiation factors.

Benefits of DNA Methylation Analysis

Properties	DNA Methylation	mRNA based	Protein based
technical	+ stable molecule + high sensitivity + high reproducibility	- very instable + high sensitivity - low reproducibility	- medium stable - low sensitivity + high reproducibility
biological	+ determination of cell type / long term specialization of cell	- Determination of short term cell status	- Determination of combination of cell type and cell status

The Phenomenon of DNA Methylation

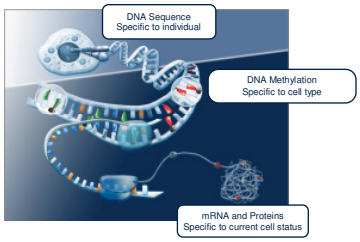
DNA Methylation
- Epigenetic regulation in higher organisms
- 5-Methyl-Cytosine
- Methylation Target: CpG dinucleotides

CpGs non-random distribution:
- Rare in most of genome (every 100 dinucleotides) and methylated
- Frequent in 'CpG Islands' (every 10 to 20 dinucl.), mostly unmethylated

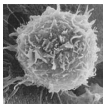
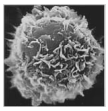
CpG islands:
- ~1 Kb long
- Usually associated with promoter and first exon
- >50% of genes have promoter proximal CpG Island

Methylation = Gene repression

The Hierarchy of Regulation



Concept: DNA Methylation as Barcode

Cell type A (e.g. stem cell) — Methylation Pattern: 011010101

Cell type B (e.g. T-cell): 110001101

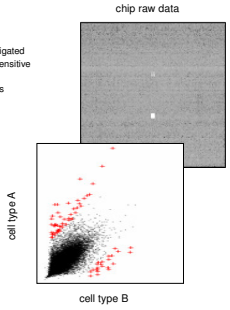
DNA-Methylation pattern is a fingerprint of each cell type

Genome-wide Chip Based Marker Discovery (DMH)

Process
- Genomic DNA is digested and linkers are ligated
- Fragments are digested with methylation sensitive restrictases
- PCR amplification of unrestricted fragments
- Unspecific fragmentation, labelling
- Detection on custom Affymetrix chip

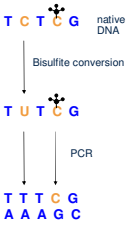
Chip characteristics
- Nearly all human CpG islands
- 50,000 fragments
- Multiple features per fragment

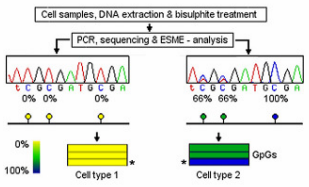
Alternative: candidate gene approach



Bisulfite Conversion / Sequencing

Methylation specific conversion of DNA

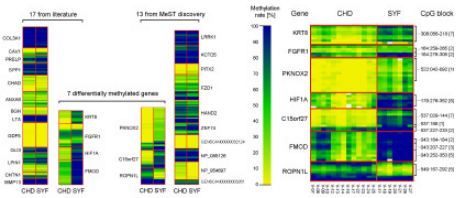




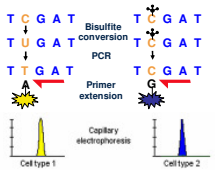
Candidate Markers, Marker Validation

Candidate Marker Discovery
- 17 candidates from literature approach
- 13 candidates from chip discovery (DMH)
- 7 differentially methylated genes are identified

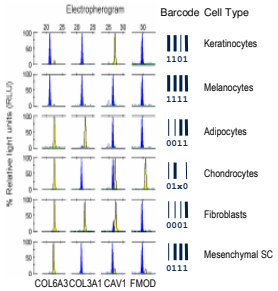
Marker Validation
- Training set comprising 17 chondrocytes and 9 synoviocytes (open label)
- Support vector machine was trained to calculate combined marker panel (CMP) score for sample classification



Methylation-Specific Primer Extension (MS-SNuPE)



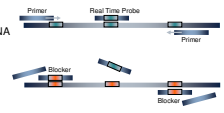
- Easy fingerprinting
- Quantitative
- Can be multiplexed



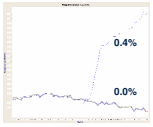
Barcode	Cell Type
1101	Keratinocytes
1111	Melanocytes
0011	Adipocytes
01x0	Chondrocytes
0001	Fibroblasts
0111	Mesenchymal SC

Methylation Specific Real Time PCR

General Method
- Bisulfite conversion of genomic DNA
- Specific PCR amplification of individual methylation markers
- High sensitivity
- Detection limit: 10 cells, 0.025% contamination



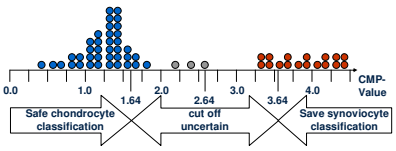
Detection of a minute melanocyte contamination in an adipocyte sample
- Detection limit: 400pg methylated DNA in presence of 100ng background DNA
- ≡ 57 cells within 14,286 cells (0.4%)



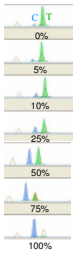
Classification of Blinded Samples

Blinded Study of 59 samples
- 39 samples safely classified as chondrocytes
- 11 samples safely classified as synoviocytes (more than 1.0 from cut off)
- 6 samples classified as synoviocytes with relative certainty (0.6 from cut off)
- 3 samples could not be classified with certainty

Subsequent Unblinding
- **Chondrocytes, correctly classified**
- **Mixed origin, not classified**
- **Synoviocytes, correctly classified**

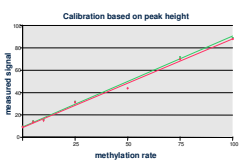


Quantitation of Mixed Cell Populations



Test system: Defined mixtures of unmethylated and methylated DNA

Determination of ratio: cg/(cg+tg) based on peak height

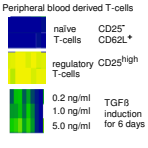


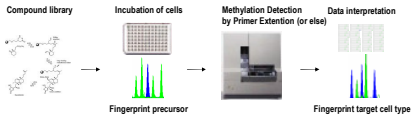
Current quantification sensitivity is at ca. 3% methylation difference

Concept – Growth Factor Screening

Provide an integrated HT- screen for growth factor identification:
- In vitro differentiation is monitored by changes in methylation patterns
- Stimulatory and inhibitory effect of compounds is detectable
- Applicable for dose response tests
- Adaptable for high throughput screening

Peripheral blood derived T-cells





Product vision: A general cell-based drug screen for all applications

Conclusion

It was shown that the analyzed cell types from the mesenchymal lineage and other lineages possess distinct DNA methylation patterns. DNA methylation markers can be assayed by SNuPE and methylation specific real time PCR to determine the cell type and the purity of a cell sample as well as to detect minute contaminations. This technology can be used for quality control of cultured cells. Furthermore, the concept of using DNA methylation analysis to detect differentiation processes was presented and is applicable for screening assays allowing the identification of novel growth and differentiation factors.