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Gene Expression Signatures in Stem Cells - Lessons for Therapy

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ABSTRACT Stem cells that reside in adult tissues are principally quiescent, yet harbor enormous capacity for self renewal and differentiation. The "stemness" of these cells is dependant on the cues from the microenvironment or the niche, as well as from the genetic program of these cells, which was poorly understood till recent times. It has now been shown in model organisms that gene expression profiles comprise a phenotype that differs between individuals of different genetic backgrounds. Recent knowledge has also helped in identifying the molecular signature of the stem cells, that included several potentially relevant members of secretory pathways which would play a crucial role in identification of a core set of molecular components that define pluripotent cells. With the recently emerging models of cell- based therapies for different clinical situations, this information would be very useful in understanding the pathobiology of stem cells and also in evaluating the long-term success. It will also help in understanding the processes by which stem cells adopt specific cell fates, specifically with reference to pleuripotent stem cells that have autologous as well as potential allogenic clinical applications.

INRODUCTION

The potential of stem cells in regenerative medicine is now one of the most sought after field in clinical and translational research. Stem cells are defined as cells with self renewal and capacity to differentiate into other tissues and could be either embryonic or adult stem cells (Hall et al. 1989). The common pathways for all stem cells include asymmetric division, and regenerative capacity, governed by various intrinsic and extrinsic factors (Schoffield et al. 1983). The autonomous regulators could be in the form of external signals, proteins that regulate the asymmetric cell division, nuclear factors that control gene expression, chromosomal modifications in daughter cells, biologic clock of transient amplifying cells and many other known and unknown factors (Watt et al. 2000; Guo et al. 1996; Jan et al.1998; Lui et al.2000). Till recently, scientists identified stem cells by the way they behaved and by chemical markers on the cell surface. One of the recently emerging information is on molecular signatures or gene expression signatures of stem cells. These refer to genes that are coordinately expressed in samples related to certain defined criteria eg., cell type, differentiation state, or signaling response. The large body of available gene expression data allow us to define the coordinately expressed genes, termed gene expression signatures, which characterize the states of cellular physiology that reflect cellular differentiation, activation of signaling pathways, and the action of transcription factors.

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WHAT ARE THE METHODS TO DETERMINE GENE EXPRESSION SIGNATURES?

The methods for detection of differential gene expression in tissues consist of a range of techniques that estimate qualitative and quantitative differences in mRNA with the more recently developed methods, microarray and serial analysis of gene expression enabling automated, high throughput analysis while other older techniques have the power to detect multiple differences in gene expression but involve identification of altered genes individually.

Microarray

A microarray (also known as a 'chip') is a miniature ordered array of nucleic acid, protein, or tissue fixed on a flat surface thereby allowing interactions with genes or gene products. The principle on which microarray-based interactions

occur may be nucleic acid (DNA-DNA or DNA-RNA) hybridization or protein-antibody/protein-protein interaction. Nucleic acid arrays usually have oligonucleotides or cDNA sequences. These are used for a whole range of applications involving the study of gene expression, gene function and sequence variations. An extensively used application relevant to this article is the identification of profiles of expressed genes in a specific tissue in response to specific stimuli or at any particular stage of disease or development. The major advantage of microarrays is that they enable a simultaneous analysis of thousands of different genes. More than 30,000 cDNA/ oligonucleotide sequences can be spotted onto a chip, which is a glass slide or a membrane such as nitrocellulose or nylon using robotic applicators that create individual spots of 300-400 micrometers spaced uniformly on the surface.

The immobilized nucleic acids are the probes and the mRNA or cDNA preparation from tissues of interest is the target. The mRNA is reverse-transcribed into cDNA using a fluorescent probe to obtain labeled cDNAs. Hybridization and washing of the target cDNA from the chip is followed by detection of the extent of interaction of the cDNA assayed with probe sequences on the chip. This is accomplished by the use of a scanner or imager. Analysis and interpretation of microarray results require the computer-managed processes of quantitation and normalization of data, including corrections for background, difference in intensity due to artifacts, and estimation of 'interesting variation'. The most crucial and challenging task is to derive meaningful biological correlations and conclusions from the data. The main issues here are the interpretation of the magnitude of gene expression changes and the clustering of genes based on similarities in properties (Chaudhuri 2005).

A major drawback with microarrays is the likelihood of getting artefactual results due to their high sensitivity and the extent of variability in results from one experiment to another. This has necessitated the validation of microarray-generated results by other independent laboratory-based methods of RNA or protein analysis.

Serial Analysis of Gene Expression

Serial analysis of gene expression (SAGE) is a technique that is useful for detecting and quanitifying transcripts of thousands of genes. It is widely used for large scale profiling of gene expression similar to microarrays, but has the advantage that it does not require any prior knowledge of gene sequences thus making it possible to use on uncharacterized organisms and genomes as well. It is also a more suitable technique than microarray when amounts of starting material are small since it involves an amplification step. SAGE is based on the principle that a short sequence of transcript (typically about 10 base pairs, known as a SAGE tag) close to the polyA tail is sufficient to uniquely identify an mRNA species. Second, joining SAGE tags end to end to create a concatenated multitag molecule allows sequencing of several tags in the same reaction.

In SAGE, mRNA is purified by being bound on oligo dT-coated magnetic beads. Double-stranded cDNA is synthesized on the beads, and then digested with a restriction enzyme *NlaIII*, a 4-base cutter that is expected to have cut-sites every 256 bases. Thus it produces short fragments of approximately 256 bp long adjoining the polyA-tail. The *Nla III* is known as the *anchoring enzyme*. The cut cDNAs are divided into two tubes, ligated with two different linkers containing a recognition site for *BsmF*1 enzyme known as the *tagging enzyme*. The *BsmF1* enzyme cuts 10 bases 3' from the anchoring enzyme cut site, thereby generating SAGE tags. The 2 different tags generated from the 2 reactions are joined together and then released from linkers, and series of tags are ligated to each other and cloned. Clones from test and control samples are sequenced to identify the profile and quantity of transcripts. A modification of the SAGE method is the use of a different tagging enzyme so as to generate longer tags of 17 bp (LongSAGE). This method has the power to identify all sequences in an unannotated genome (Patino et al. 2002).

Massively Parallel Signature Sequencing (MPSS)

This technique, developed by S. Brenner and colleagues (Brenner et al. 2000) has the capacity to simultaneously screen millions of cDNA templates without separation of individual cDNAs from the mixture, and has been used for identification of gene expression profiles (Jogeneel et al. 2003). The cDNA library is cloned

into a vector containing any one of a set of 16 nucleotide-long 'tag' sequences to generate combinations of different tags with different cDNA sequences. The tag-cDNA fragment is fluorescently labeled and attached to microbeads, in such a way that each microbead has ~10^5 identical cDNAs attached. The cDNA-loaded microbeads are arrayed in a planar array into a flowcell that is connected to a detector that reads fluorescent signals on the whole array at once. Sequencing by a process of successive cycles of adaptor ligation and restriction enzyme cleavage generates short 20 nucleotide 'signature sequences' for each cDNA, sufficient to identify it (Brenner et al. 2000).

Differential Display

The technique of differential display was first described by Liang and Pardee (1992) and has been used extensively for comparisons of gene expression profiles of different tissues. It involves the reverse transcription of mRNAs of test and control samples separately, and then amplification of the cDNA pools, by using oligo dT in combination with short primers of arbitrary sequence. This is followed by resolution and display of both test and control preparations on denaturing polyacrylamide gels. Bands are visualised by silver staining or by radioactive labeling during cDNA synthesis followed by autoradiography. Bands that are differentially present in control vs test are excised out of the gel, re-amplified and sequenced or subcloned. Though technically very simple, this method is labor-intensive and is limited by the resolving power of the acrylamide gel.

Subtractive Hybridisation

Differences in test and control mRNA populations are identified by creation of a subtracted cDNA library (Vitek et al. 1981). The test cDNA is hybridized with either the control mRNA or cDNA preparation. The cDNAs that are not represented in control samples will not have corresponding complementary transcripts to hybridise to, and will thereby remain single-stranded. The sscDNA species are separated by chromatography on hydroxyapatite and then cloned into a cDNA library for sequencing and identification. A newer method of producing subtracted cDNA libraries, known as suppression subtractive hybridization (SSH) was developed (Diatchenko et al. 1996). This method is also used for detection and enrichment of differentially expressed transcripts between two populations.

GENE EXPRESSION SIGNATURES IN STEM CELLS

The idea that stem cells possess unique characteristics responsible for their ability to self-renew as well as differentiate into different lineages led to the search for gene expression 'signatures' or sets of genes that are co-ordinately expressed in stem cells as distinct from other differentiated cell types. Numerous studies on embryonic stem cells have attempted to obtain a global profile of gene expression in these cells in an effort to define the molecular basis of "stemness".

Studies on profiling of stem cells have used methods such as subtractive hybridization (Phillips et al. 2000) or microarrays containing a defined set of a few hundred cDNAs (for example, Kelly et al. 2000). The scope of such studies has been greatly enhanced by the use of high-density oligonucleotide arrays containing over ten thousand genes. The first studies that used such an approach attempted to obtain gene expression profiles that govern stemness in embryonic, hematopoietic and neural stem cells (Ivanova et al. 2002; Ramalho-Santos et al. 2002). The gene expression profiles described by both groups of investigators for each of the stem cell types consisted of ~2000 genes that were over-expressed relative to control cells. While both of these studies showed a variable degree of overlap in profile between different stem cell types, both identified ~200 genes common to all 3 stem cell profiles, belonging to a variety of functional categories including transcriptional regulation, cell cycle, signal transduction and DNA repair. A feature that validated the methodology used was that known stem cell markers for each type of stem cell were indeed found to be overexpressed in that cell type. Thus, expression of *Oct4, Zfp42/ Rex I, Brachury, Smad4, p53, Fgf4,* and several others known to be ES-cell markers were found in ES cells, genes such as *nestin, EgfR, FgfR1 and Notch 1* in neuronal stem cells and *Kit 1, Notch1, Abcg2, Tie1, Tie2, Flk 2* and others in hematopoeitic stem cells (Ramalho-Santos et al. 2002).

However, a comparison of the data obtained by these two groups, which used the same types

of stem cells and the same approach, showed that their results were largely different from each other with only about 15 genes being common to the entire list of genes identified as stem-cell specific by each (Rao and Stice 2004). This raised doubts regarding whether it is in fact possible to obtain a definitive stem cell signature. It has been proposed that stem cells reflect a transient state that any cell can potentially acquire rather than an entity (Zipori 2004). Apart from the idea that there is an inherent variability in the cells themselves, these differences between studies may reflect differences in the analytical methods, experimental methods such as the culture conditions, number of passages, and methods of isolation of stem cells that could lead to heterogeneity in cell populations used. Heterogeneity in transcription profiles due to differences in extent of culturing and between separate isolations has been demonstrated with neurospheres, thus questioning the value of characterizing them using gene expression profiles (Sievertzon et al. 2005). An additional source of variation could be that the reference or control cells used in each study against which stem cell-specific of gene expression is measured, are somewhat different though they are generally differentiated cells.

Since human embryonic cells are available as different cell lines, studies using these cells have attempted to generate comparative data on expression profiles between different HES cell lines. Gene expression profiles of human embryonic cell lines show a common group of expressed genes shared by different lines as well as differences in gene expression patterns between individual lines (Abeyta et al. 2004; Bhattacharya et al. 2004; Skottman et al. 2005). A set of 92 genes was found to be common to six different ES cell lines (Bhattacharya et al. 2004) and all 6 ES lines formed a cluster in gene expression profiles. Broadly, the study by Bhattacharya et al., found the 'stem cell signature' comprising 92 genes having the following features: DNA repair enzymes were highly expressed while genes of the p53 and Rb pathways are low to absent, members of the *wnt* signaling pathway, activin and retinoid signaling were overexpressed, cyclins were overexpressed and most markers of differentiation were absent except a few such as *keratin 8, keratin 18, beta tubulin 5, cardiac actin and troponin T1*. A similar clustering of gene expression was noted in five different human ES cell lines as compared to a range of other tumor and somatic cell lines in another study (Sperger et al. 2003). A comparison of ES cells with embryonal carcinoma (EC) cells by Sperger et al showed that 895 genes were commonly expressed in both groups suggesting that these genes may be related to pluripotency. Among the genes that were highly expressed in ES and EC cells were *Pou5FI (Oct4), FOXD3 and SOX2*, coding for transcription factors that are considered to be specific to pluripotent cells, DNA synthesis enzymes, receptors of the *wnt-beta catenin* signaling pathway, fibroblast growth factor receptors (*fgfR*) and bone morphogenetic protein (BMP) receptor. Profiling of ES cells by a different method, MPSS, using pooled RNA from three different human ES cell lines also revealed SOX2, Oct4 and DNMT3â as among the most highly expressed gene signatures (Brandenberger et al. 2004).

Interestingly, independent evidence points to a critical role for transcription factors *Oct4*, *Nanog* and *SOX2* in maintaining pluripotency (Niwa et al. 2000; Avilion et al. 2003; Mitsui et al. 2003). Further efforts have been made to define the downstream targets for these transcription factors in ES cells. Loh et al (2006) mapped binding sites for these factors in the mouse ES cell genome. They identified a core set of 345 genes, of which 30 encode DNA binding regulatory proteins that are downstream targets for these factors. Similar studies have mapped binding sites for Oct 4 and Nanog in human cells (Boyer et al.2005). These studies may reveal the proteins that function in regulatory cascades determining cell fate.

Attempts have been made not only at defining stemness in hematopoetic stem cells (HSCs), which are fairly well characterized with respect to phenotypes and life cycle, but also to generate temporal gene expression profiles in relation to self renewal and differentiation (Bruno et al. 2004; Venezia et al. 2004).

Ivanova et al. (2002) using murine HSCs obtained a gene expression profile in which 45% of genes included transcription factors, intracellular signaling proteins, cell-surface receptors, and ligands. The gene expression profile in this study showed a 50% overlap with that obtained in a similar study of HSCs based on subtractive hybridization by Phillips & co-workers (Phillips et al. 2000). Possible support of this data is also provided by the observation of a

40% overlap of the HSC-specific gene expression profile between mouse and human HSCs (Ivanova et al. 2002). Temporal mapping of gene expression during lineage-specific differentiation of hematopoeitic precursor cells by examining levels of various regulatory factors (Cheng et al. 1996) revealed differental changes in levels of transcription factors such as GATA-1, GATA-2, C/EBP, PU.1, and NF-E2 in differentiation to erythrocytes, monocytes and granulocytes. The levels of GATA-1 and GATA-2 increased during erythrocyte differentiation, whereas PU.1 increased during granulocyte/monocyte differentiation. A recent study by Venezia et al (2004) reported 'proliferation-specific' as well as 'quiescence-specific' gene expression signatures on HSCs. This was done by treatment of HSCs with 5-fluorouracil and determination of gene expression profile over a time course using microarray analysis. The 'P-signature' identified was a group of 338 genes specific to proliferating cells containing cell cycle genes, DNA synthesis, ATP synthesis and energy metabolism genes. The 'Q-signature' consisted of 298 genes expressed in quiescent cells, belonging to the categories of signaling molecules and cell-cell adhesion. Encouragingly, a comparison of the P and Q-signatures obtained by Venezia et al., with the gene expression profiles for long term (LT) HSCs and short term (ST) HSCs isolated by different methods in an earlier study (Akashi et al. 2003) showed that most genes in the LT HSC profile were also part of the Q-signature while most genes in the ST HSC expression profile were part of the P-signature, supporting the functional equivalence of LT HSCs with quiescence and ST HSCs with proliferation.

The data available so far suggests that stem cells overexpress a large variety of genes belonging to different functional categories. It appears that many of the genes identified would be relevant to all cell types rather than a particular type of cell. It has been observed that ribosomal proteins and housekeeping genes make up a large proportion of the signal in stem cell profiles (Ramalho-Santos et al. 2002). These are obviously ubiquitous in function and expression and may be expressed at a higher level in stem cells. Common structural genes may also be present for similar reasons. To arrive at a unique signature, it is desirable to identify genes that specifically define the distinguishing properties of stem cells. To enable this would also require the characterization of all genes in the genome. The presence of a large number of uncharacterized ESTs and novel genes in stem cell expression profiles identified in different studies (Ramalho-Santos et al. 2000; Brandenberger et al. 2004) renders a large part of the data uninterpretable until knowledge about all these genes is available. It has been suggested that the defining stem cell signature may entail identifying a pattern(s) of gene expression rather than looking at individual genes or collections thereof and looking at protein as well as transcription profiles (Zipori 2004). Clearly, independent functional studies on genes relevant to different stem cell types are also required to provide clues to stemness. For example, studies on animal models have shown that PU.1 transcription factor which is important for differentiation of myeloid lineages, is also a self renewal factor for erythroid progenitor cells and is required to maintain hematopoietic stem cell populations (Back et al. 2004; Kim et al. 2004). It would eventually require a combination of different approaches to determine the basis of stemness.

Another interesting hypothesis postulated by Moore's research team (Charbord P and Moore K 2005) was that the microenvironmental or stromal cells provide a complex molecular milieu which helps mediate and balance the self-renewal and commitment potentials of stem cells and therefore they aimed at defining these molecules. They compared the genes active in the supportive cells (HSC-supportive stromal cell lines.) to those in cells that did not support stem cells (fetal liver stromal cell lines). Their data suggested that HSC-supportive cells are immature, sessile, and highly reactive after binding to integrin ligands and cytokines and thus provide a dynamic space poised to respond to molecular cues elaborated within the stem cell niche, thus highlighting the complex network of intercellular signaling and communication involved in the organization of the niche space. Mohamed et al. (2006) have shown that different combinations of growth factors and cytokines and optimize the ex-vivo expansion of cord blood stem cells.

Future Directions of Gene Expression Signatures- for Stem Cell Therapy

Modern therapeutics is a rapidly adapting field that takes inputs from the latest technology

that helps in understanding the basic biology at cellular, molecular or biochemical level. Molecular signatures are one such application, being explored for development of therapeutic intervention, drug development, measure of cellular response/benefit. So the question now arises- how it is going to affect the stem cell research and its clinical application? Whether it is with respect to embryonic stem cells or adult stem cells, the expectations from this include:

- will it provide consistent and reliable information on how the ES or undifferentiated MSCS take the path of differentiation or maintain stemness and queiscence?
- Can we identify the specific cues from niche which help in differentiation of stem cells and which would be involved in homing of the transplanted stem cells?
- Can the gene expression signatures be controlled/modified by external factors- eg addition of growth factors, cytokine?
- Can we arrest the desired cells at a particular stage of differentiation?
- Can we control the differentiation/ growth/ death of the cells after transplantation?
- Can we observe and monitor the survival of transplanted cells in the new niche?
- Can we evaluate the functional integrity of the transplanted cells and the talk between the new niche and the transplanted cell?
- Can they predict the life span of the stem cells and their mechanism of cell death and degeneration?
- Will the gene expression signatures be altered if and when the stem cells undergo any malignant transformation or any other abnormal differentiation and will it be possible to document and predict these changes?
- Can these signatures help or improve the cryopreservation and banking facilities?

The emerging information on gene signatures has as many questions as methods, especially with respect to clinical application, which probably are already being is explored and addressed by various groups, but as of today, the published literature is scant. It is also pertinent to mention here that this article aims to highlight the concepts on this aspect and due to space constraints, it may not be possible to review all the papers on this subject. This is probably similar to the knowledge gain in the field of tumor biology, which has many similarities as well as dissimilarities to stem cells. Both have unique properties of cell growth and differentiation and are governed by both intrinsic and extrinsic factors. The differences are that tumor biology is probably better understood and the focus there is on new therapeutic drugs, whereas in stem cell biology the main thrust is in its potential clinical application of the stem cells that would repair and replace the damaged tissues and are expected to be functionally competent cells.

CONCLUSIONS

In summary, there is tremendous potential in the information provided by gene expression signatures of stem cells. However, the methods of establishing the signatures, and the conditions that affect the results are so variable, that caution is warranted before this information is directly extrapolated to viable human stem cells in their natural niche or in ex-vivo conditions. As rightly said, the information is more like library facilities wherein the specific information on a particular aspect has to be searched in detail and applied in the right context.

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