

Expression Profiling of Genes involved in the Control of Pluripotency Using cDNA Microarray

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Abstract

To identify genes implicated in the control of pluripotency as well as characteristics of stem cells, we analyzed expression profiles of genes derived from mouse morulas, blastocysts, embryonic stem cells, mesenchymal stem cells, and uterus tissue using cDNA microarray. Comparative analyses of their expression profiles identified putative clones that expressed specifically in specific samples or not in a specific sample. The expression pattern of these candidate clones was analyzed using RT-PCR and non-radioactive *in situ* hybridization. Functional annotation of these clones on pluripotency and stem cell plasticity is in ongoing. These studies may further our understanding on the nature of the stem cells and molecular mechanisms underlying many facets of mammalian development and differentiation.

Introduction

Stem cells are unique cell populations with the ability to undergo both self-renewal and differentiation simultaneously (Blau *et al.*, 2001; Hall and Watt, 1989; Weissman, 2000). A wide variety of adult mammalian tissues also harbors stem cells, yet "adult" stem cells may be capable of reconstituting only a limited number of cell types. In contrast, embryonic stem cells (ESCs), derived from inner cell mass (ICM) of blastocyst-stage of early mammalian embryos, are capable of differentiating into any fully differentiated somatic as well as germ cell lineages (namely pluripotency) when conjoined with blastomeres of early mouse

embryos. To our surprise, adult stem cells have been recently reported to have potential to trans-differentiate into other tissue stem cells under specific internal environments (namely stem cell plasticity).

In the unmanipulated blastocyst-stage embryo, stem cells of ICM promptly differentiate to generate primitive ectoderm, which ultimately differentiates during gastrulation into the three embryonic germ layers, whereas the trophectoderm differentiates to generate extra-embryonic tissues. When removed from their normal embryonic environment and cultured under appropriate conditions, ICM cells give rise to cells that proliferate and replace themselves indefinitely. While in this undifferentiated state in culture, they maintain the developmental potential to form advanced derivatives of all three embryonic germ layers (Bradley *et al.*, 1984; Nagy *et al.*, 1990). On the other hand, many adult tissues contain populations of stem cells that have the capability for renewal after trauma, disease, or aging. The cells may be found within the tissue or in other tissues that serve as stem cell reservoirs. For example, bone marrow is the major source of adult hematopoietic stem cells (HSCs) that renew circulating blood elements (Till and McCulloch, 1961; Civin *et al.*, 1984). The adult bone marrow also contains mesenchymal stem cells (MSCs), which contribute to the regeneration of mesenchymal tissues such as bone, cartilage, muscle, ligament, tendon, adipose, and stroma (Friedenstein *et al.*, 1987; Haynesworth *et al.*, 1992; Kuznetsov *et al.*, 1997). Among adult stem cells, MSCs and HSCs are known to be sole stem cells that could replicate as undifferentiated cells *in vitro* and also be induced to differentiate into appropriate cells (Pittenger *et al.*, 1999; Akashi *et al.*, 1999).

Yet, neither the detailed molecular mechanisms underlying nor fate signaling genes involved in stem cell-ness and plasticity are known, although the fate decision choices in stem cells should be highly regulated by intrinsic signals and the external micro-environments (Watt and Hogan, 2000). Several molecules have been shown to play roles in early aspects of stem cell-ness, but it has not been possible to elucidate regulatory pathways that function at the level of self-renewing stem cells. The elucidation of regulatory mechanisms characterizing for stem cells requires an extensive description of the molecular components available to the stem cell, that is, its genetic program. Here, to understand establishment, maintenance, and differentiation of embryonic stem cells at the

molecular level, and also to identify genes implicated in the control of pluripotency as well as characteristics of stem cells, we analyzed expression profiles of genes in mouse morulas, blastocysts, ESCs, MSCs, and uterus tissue using cDNA microarray. We confirmed expression profiles of several candidate genes during *in vitro* differentiation of ESCs or MSCs and in developing mouse embryos by RT-PCR and *in situ* hybridization analyses, respectively.

Materials and Methods

1. RNA isolation from mouse embryos and stem cells

Superovulation was induced by injection of PMSG and hCG. Morulas and blastocysts were obtained from 80 and 96 hrs of postcoitus ICR mice. Total RNA was prepared from 300 embryos per group using RNeasy Kit (Qiagen). Total RNAs was also prepared from embryonic stem cell line, CCE, and mesenchymal stem cell line, C3H10T1/2 (ATCC Cat No CCL-226). ESCs and MSCs were maintained in an undifferentiated state by culturing cells in the Dulbecco's MEM supplemented with 10% FBS in the presence or absence of 1000 u/ml recombinant LIF (ESGRO, Chemicon), respectively.

2. cDNA microarray analysis

The [α]³³P-dCTP labeled 1st strand cDNA probes were synthesized from the purified RNAs by a reverse transcription reaction. Genefilter GF400 mouse cDNA microarray membrane (Research Genetics) containing 5355 mouse cDNA were hybridized with probes and washed according to the protocol provided by the manufacturer. Expression profiles of cDNA clones obtained by a phospho-imaging were analyzed using a Pathways 3 program (Research Genetics).

3. Functional annotation of the candidate clones and confirmation of their expression in stem cells

Candidate clones to be more than two fold differences in their expression in comparing groups were identified. Candidates were further characterized their functional aspects using conventional databases. Candidates that identified already or corresponding to ESTs were further analyzed for their expression profiling

during early developmental stages, from unfertilized egg through blastocyst stage, and during *in vitro* differentiation of ESCs and MSCs with a RT-PCR experiment. Differentiation of ESCs was induced by culturing cells in the bacterial petri-dish in the absence of LIF (Kim, 1996). Differentiation of MSCs was induced by supplementation of 5-azacytidine to the culture medium (Lassar *et al.*, 1986) or supplementation of retinoic acid (Piitenger *et al.*, 1999). Tissue specific expression of candidate clones was monitored by nonisotope *in situ* hybridization. cDNAs corresponding each clone were obtained by RT-PCR for the ESC RNA and then subcloned into pGEM-T Easy vector (Promega). Digoxigenin (Boehringer Mannheim) labeled RNA probes were prepared by *in vitro* transcription reaction.

Results and Discussion

1. Quality control of the cDNA microarray data

The quality of data obtained by the cDNA microarray was tested by plotting of two independent RNA samples from same group. The data was processed further only when expression profiles of each clones in two samples were within less than two-fold differences. Examples are shown in Fig. 1.

2. Comparison of expression profiles among samples

Expression profiling of genes among mouse morulas, blastocysts, ESCs, MSCs, and uterus tissue were compared sequentially as shown some examples in Fig. 2. Most clones showed no significant difference in their expression between morula and blastocyst, between ESCs and blastocyst, and between ESCs and MSCs, such that only 2.8% and 2.6% of clones were differentially expressed between morula and blastocyst and between ESCs and MSCs, respectively.

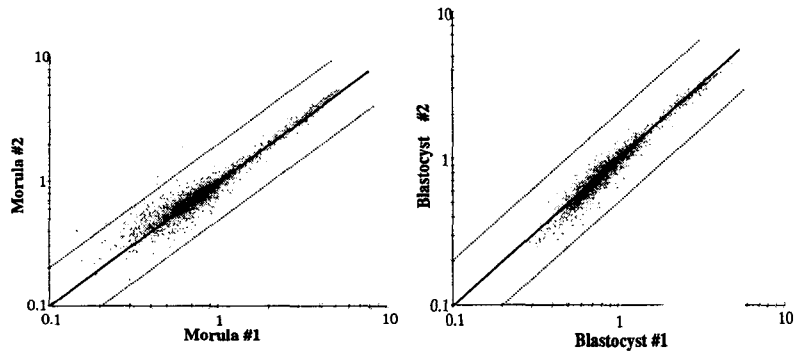


Fig. 1. Scatter plot analyses of log-transformed expression data for two independent mRNA populations isolated from mouse morulas and blastocysts.

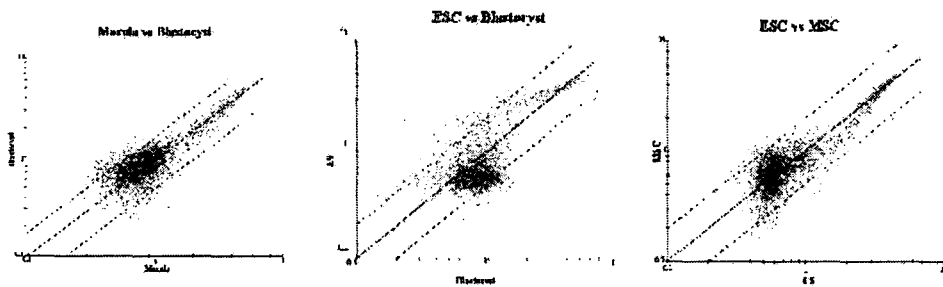


Fig. 2. Scatter plot analyses of log-transformed expression data among samples isolated from mouse morulas, blastocysts, ESCs, and MSCs.

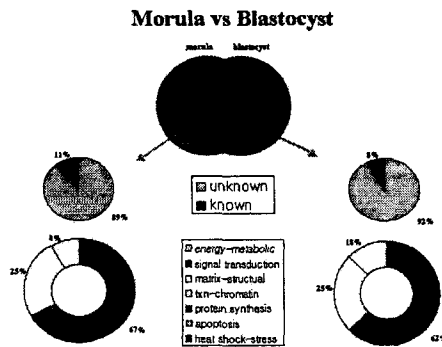


Fig. 3. Analyses of clones preferentially expressed in morulas or blastocysts.

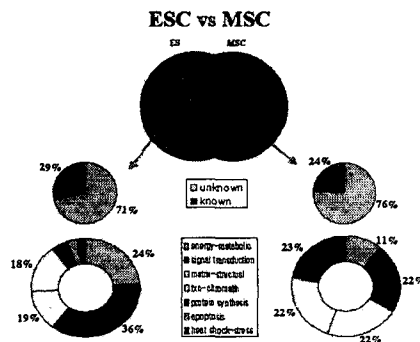


Fig. 4. Analyses of clones preferentially expressed in ESCs or MSCs.

Clones that preferentially expressed in morula and blastocyst were 2.03% and 1.89%, respectively, when compared between morula and blastocyst. Most of these developmental stage-preferential clones were ESTs (Fig. 3). Among the identified clones that preferentially expressed in morulas or blastocysts, those involved in the signal transduction and cell structural integrity were prevailing, showing more than 60% and 25%, respectively. Similarly, ESCs- and MSCs -preferentially expressed clones were 2.48% and 0.46%, respectively, and their functions were not known (Fig. 4). Clones involved in signal transduction (36%) and energy and metabolism (24%) were prevailing among the identified clones in ESCs, whereas most of them were involved in signal transduction (23%), protein synthesis (23%), cell

structural integrity (22%), and transcription (22%) in MSCs.

3. Classification of candidate clones according to their expression pattern

We classified preferentially expressed clones into 13 types according to the clustering of their expression pattern (Fig. 5). Only 3% of clones were categorized into these 13 types.

Type B clones are those expressed preferentially in MSCs, but not in ESCs or early embryos, and thus they may be involved in the maintenance of MSCs-specific characteristics or the differentiation potential of MSCs. On the other hand, type G clones are those expressed preferentially in early embryos and ESCs, but not in MSCs, suggesting that they are involved in the maintaining of pluripotency or the differentiation of ESCs.

4. RT-PCR analyses of candidate clones

Clones showing preferential expression in early embryos, ESC, or MSCs, were further characterized for their expression during *in vitro* differentiation of ESCs and MSCs by RT-PCR method. Proper differentiation of ESCs was confirmed by the monitoring of the expression of known marker genes involved in each three germ layer, whereas differentiation of MSCs was by alkaline phosphatase staining and oil red O staining. As shown in Fig. 6, clones 1, 2, 4 increase their expression, while clone 3 decreases its expression during *in vitro* differentiation of ESCs.

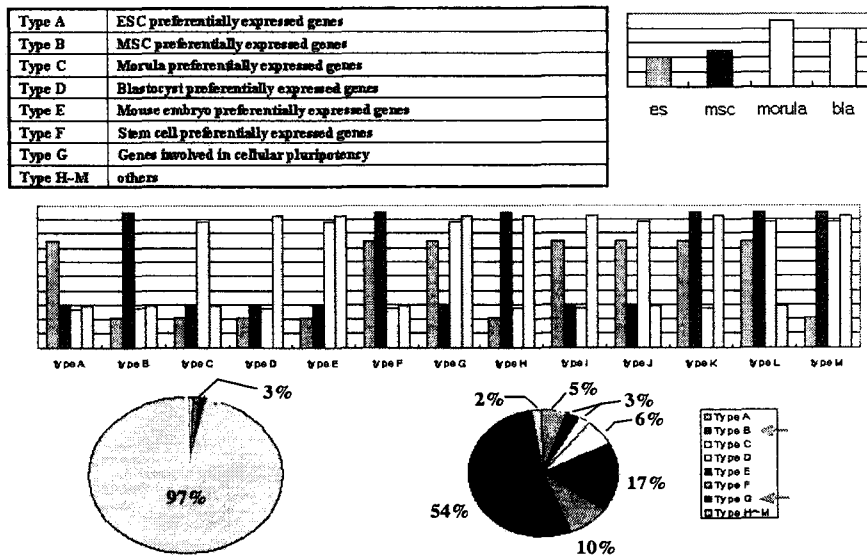


Fig. 5. Grouping of clones into 13 types according to the clustering of their expression pattern among morula, blastocyst, ESC, and MSC.

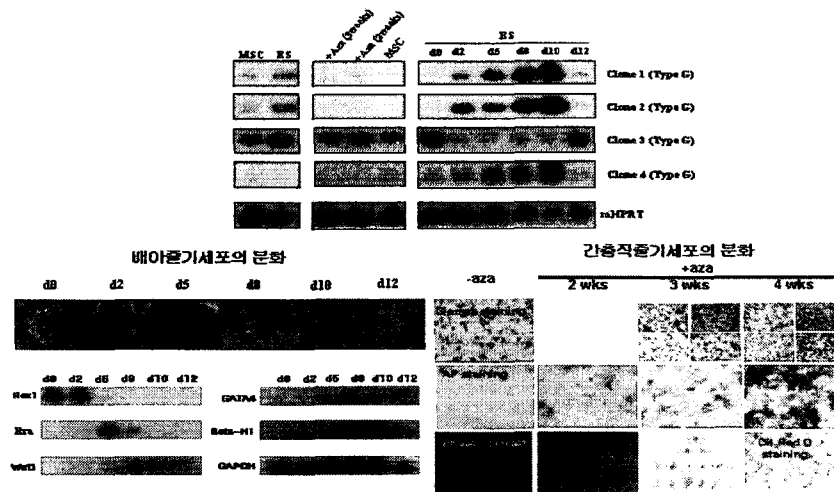


Fig. 6. RT-PCR analysis of candidate clones during *in vitro* differentiation of ESCs and MSCs.

5. Analysis of expression pattern by *in situ* hybridization

Clone 2 that increases its expression during *in vitro* differentiation of ESCs was further characterized by *in situ* hybridization on the cryosectioned 13 dpc mouse embryo tissues. The expression of clone 2 was only detected in the brain region, and specifically in the subependymal layer of forebrain (Fig. 7). Astrocytes in subventricular zone of ependymal layer

were known to be neural stem cells in the adult mammalian brain (Doetwch *et al.*, 1999). Thus, it is suggested that clone 2 involves in the maintenance or differentiation of neural stem cell although further characterization is required.

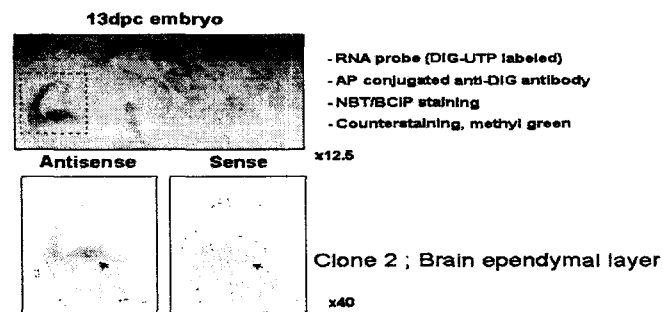


Fig. 7. *in situ* hybridization of clone 2 in the brain of 13 dpc mouse embryos.

In this study, we demonstrated massive search of clones preferentially expressed in early embryos, ESCs, and MSCs with cDNA microarray system and identified several candidate clones that might be involved in pluripotency of ESCs or stem genes leading specific fate decision during early development. Although further functional characterization of candidates is required, our data obtained in this study may be fundamental for further massive analysis of clones involved in stem cell-ness and stem cell plasticity. The panel of developmental marker genes can also be used in a genetic screen for stage- or cell lineage-specific genes.

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