Stem cell gene expression in MRPS18-2-immortalized rat embryonic fibroblasts

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We have recently found that primary rat embryonic fibroblasts (REFs) could be immortalized by overexpression of the human mitochondrial ribosomal protein MRPS18-2 (S18-2). A derived cell line, designated 18IM, expressed the embryonic stem cell markers SSEA-1 and Sox2. Upon inoculation into severe combined immunodeficiency mice, 18IM cells differentiated to express pan-keratin. They were not tumorigenic. Here we report the gene profiling of 18IM, compared with REF cells. Pathways involved in oxidative phosphorylation, ubiquinone (Coenzyme Q 10) biosynthesis, fatty acid elongation in mitochondria, PI3K/AKT signaling, a characteristic of rapidly proliferating cells, were upregulated in 18IM. Genes involved in the transcription/translation machinery and redox reactions, like elongation factors, ATP synthases, NADH dehydrogenases, mitogen activated kinases were upregulated as well. 18IM cells produced more pyruvate, indicating enhanced ATP synthesis. The expression of Oct4, Sox2, and Nanog that can contribute to the experimental induction of pluripotency in primary fibroblasts was also elevated, in contrast to Klf4 and C-myc that were downregulated. Subsequently, three new immortalized cell lines were produced by S18-2 overexpression in order to check the representativeness of 18IM. All of them showed anchorage-independent growth pattern. Two of three clones lost vimentin and smooth muscle actin, and expressed Sox2 and Oct4. We suggest that S18-2 is involved in the developmental regulation.

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Mouse embryonic fibroblasts have been converted into pluripotent stem cells (iPSC) in vitro, by expressing four genes, SOX2 (NM_003106), OCT4 (NM_002701), KLF4 (NM_004235), and C-MYC (NM_002467)1-3 or, alternatively, N-MYC (NM_005378).4 Rat fibroblasts could also be turned into iPSC by the same four genes.5 Human primary fibroblasts could be converted into stem cells by the same or a slightly different set of genes, such as OCT4, SOX2, NANOG (XM_002344645), and LIN28 (NM_024674).6 Also, OCT4 and SOX2 could induce pluripotency in human fibroblasts by themselves, in conjunction with valproic acid, a histone deacetylase inhibitor.7 SOX2 and OCT4 could generate iPSCs from cord blood cells without valproic acid.8,9

We have accidentally discovered that overexpression of the human mitochondrial ribosomal protein MRPS18-2 (S18-2, NP_054765) immortalized primary rat embryonic fibroblasts (REFs).10 The derived cell line, designated 18IM, lost its sensitivity to contact inhibition and acquired the ability for anchorage-independent growth in soft agar with a cloning efficiency of 94%. It expressed the embryonic stem cell markers SSEA-1 and Sox2 that were not detected in the original REFs, nor in C-MYC- and HA-RAS-transformed REFs. Moreover, the 18IM cells lost the expression of mesodermal markers like vimentin and smooth muscle actin (SMA). In contrast, they turned on the ectoderm- and endoderm-specific pan-keratin, ectoderm-specific beta-III-tubulin, and mesoderm-specific MHC class II markers in confluent culture. Part of the cells differentiated into Oil red O stainable fat cells.

Upon subcutaneous inoculation into severe combined immunodeficiency (SCID) mice, 18IM cells differentiated to express pan-keratin. Unlike C-MYC and HA-RAS-transformed fibroblasts, they were not tumorigenic.10 Here we report the gene expression pattern of 18IM cells and three new S18-2-immortalized cell lines, in comparison with control REFs.

Results

Stem cell marker expression in 18IM cells. The gene expression profile of 18IM cells was compared with REFs by microarray techniques. Genes that showed a twofold and higher difference with FDR-corrected P-value <0.05 were selected for further studies.

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Abbreviations: REF, rat embryonic fibroblasts; S18-2-MRPS18-2, mitochondrial ribosomal protein S18-2; SCID, severe combined immunodeficiency; 18IM–REFs, immortalized by overexpression of MRPS18-2 (S18-2) protein; iPSC, induced pluripotent stem cells; IMDM, Iscove’s Modified Dulbecco’s Medium; Q-PCR, quantitative PCR

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Supplementary Table S1 shows the differential gene expression. More than 4000 differently expressed genes are shown in an overview (Figure 1).

Ingenuity Pathway Analysis (IPA, Ingenuity Systems Inc., Redwood City, CA, USA) was performed for Gene ontology studies on differential expression set (4209 genes). Several canonical pathways, including 'human embryonic stem cell pluripotency', the 'NANOG pathway', and others were activated in 18IM cells (Table 1).

At least 19 cellular pathways were altered in the S18-2-immortalized cells (Table 1). Uregulated genes include COX and NDUF family genes, involved in proliferation, oxidative phosphorylation, cellular respiration, and other redox reactions, suggesting that 18IM cells are metabolically more active. Their increased pyruvate release suggested an elevated level of ATP production (Supplementary Figure S1), in accordance with higher proliferation rate, as reported earlier.10

Genes involved in the WNT, BMP, and other self-renewal pathways, like β-catenin (Ctnn-β), Bmp3 (BMP receptor), Smad1/5/8, Stat3, Spp1, Sox11, and Sh3glb1 were upregulated. In contrast, genes involved in mesenchymal differentiation, such as Runx2, Hoxc8, Creb, and Tlx2, or endoderm development, as Foxd3, were downregulated. Essential factors in the TGF-β and BMP pathways, like TGFB3, BMP1, BMP7, SMAD1, and CREB1 were also upregulated with pathway expectation (−log (P-value)) of 1.64 and 1.19, respectively. Many genes involved in the regulation of stem cell development and proliferation were differentially expressed in 18IM and REF cells. Genes involved in the proliferation of embryonic cells (30 genes), proliferation of neural stem cells (9 genes), and survival of embryonic cells (11 genes) were also differentially expressed.

Quantitative PCR validation of microarray data. Fifteen genes that have an important role in stem cell phenotype maintenance were chosen to validate the microarray data by quantitative PCR (Q-PCR). The elongation factors Eef1A2 (NM_012660) and Tceas3 (NM_001015008) were strongly upregulated in 18IM cells, in agreement with the microarray data (Figure 2). The expression of Eef1A2 increased 10,000-fold (8933 according to microarray) and of Tceas3 increased by 393-fold (500 by microarray), suggesting enhanced RNA synthesis. Gata3 (NM_133293) that may be involved in histone acetylation11 was overexpressed (Figure 2, middle panel) while Gata4 (NM_144730) and Gata6 (NM_019185), which are usually expressed in differentiated tissues, were downregulated (Figure 2, lowest panel). Genes influencing cell proliferation like Tiam1 (NM_001105784), Epas1 (NM_023090), and Jmjd4 (NM_001105784) were expressed at a higher level in 18IM cells. Similar changes were recorded by Q-PCR and microarray for Tiam1 (566/106) and Jmjd4 (98/145).

18IM cells also showed a high expression of the testis-specific Tssk1 gene (NM_001011900).12 EST data showed that this gene is expressed in the brain and in the germ cell tumors. The placenta-specific Cdx2 gene (NM_023963) was 100 times more highly expressed in 18IM, compared with REF cells. Cdx2 expression was also reported in gastrointestinal and colorectal tumors, gliomas, and embryonic tissue. Genes that participated in the experimental induction of stem cell markers in normal fibroblasts, such as Oct4, Nanog, and Sox2 were also more highly expressed in 18IM cells. However, C-myc and Klf4 were downregulated in the immortalized cells.

There was no significant change in the methylation status of the promoter region of Oct-4, Sox2, Nanog, Lin28, and C-myc in 18IM cells compared with REFs (see Supplementary Table S3), nor was there any difference in total genome methylation (Supplementary Figure S2).

Protein expression. Protein expression was assayed by western blotting and immunostaining. The immortalized 18IM cells have lost vimentin and SMA expression, concomitantly with the rise of Sox2, HIF1A, p53, RB, and p14ARF protein levels, confirming our previous data (Figure 3). Despite lower levels of C-myc mRNA, C-myc protein was expressed at the higher level in 18IM cells, compared with REFs, probably, due to protein stabilization.

It is noteworthy that the embryonic stem cell marker SSEA-1 was expressed on 30% of 18IM cells that have grown in culture for >2 years (Supplementary Figure S2A). They were non tumorigenic in SCID mice, after the inoculation of 0.5–2 × 10⁵ cells.

Experiments on newly generated S18-2-immortalized REF lines. Three new S18-2-immortalized cell lines were generated by transfecting REFs with GFP-S18-2 and pBabe-S18-2 plasmids, followed by selection on G418. They were followed in culture for 12 months. Clones 10 and 12 that expressed GFP-S18-2 at a high level grew similarly to 18IM cells, forming foci with a dense center, whereas clone 6 that expressed a much lower level of S18-2 protein from a pBabe construct did not form compact foci, but grew as a monolayer, showing a high degree of cellular heterogeneity. Clones 10 and 12 but not clone 6 showed anchorage-independent growth (Figure 4). Cells (0.5 × 10⁶) were inoculated subcutaneously in SCID mice. After 2 months no tumor formation was detected.

The protein expression pattern of the three new clones was compared with 18IM and REF cells by western blotting. Clone 6 showed no loss of vimentin and SMA, in contrast to 18IM
Table 1 Pathways that are regulated differently in 18IM cells compared with REFs

| Pathway Description | Log2 Ratio | Ratio | Downregulated Genes | Upregulated Genes |
|---------------------|------------|-------|---------------------|-------------------|
| Oxidative phosphorylation | 5.15 | 1.33 e+05 | 1/66 (5%) | 0/66 (0%) |
| Mitochondrial dysfunction | 4.89 | 1.16 e+05 | 8/172 (5%) | 0/172 (0%) |
| Ubiquinone biosynthesis | 3.83 | 1.01 e+05 | 3/119 (3%) | 0/119 (0%) |
| Butanolate metabolism | 2.58 | 7.52 e+05 | 7/133 (5%) | 0/133 (0%) |
| Lysine degradation | 2.49 | 7.69 e+05 | 6/234 (3%) | 0/234 (0%) |
| Valine, leucine and isoleucine degradation | 2.31 | 9.01 e+05 | 8/111 (8%) | 0/111 (0%) |
| Fatty acid elongation in mitochondria | 1.84 | 8.89 e+04 | 2/45 (4%) | 0/45 (0%) |
| Agrin interactions at neuromuscular junction | 1.00 | 8.36 e+04 | 4/132 (3%) | 0/132 (0%) |
| TGFβ signaling | 1.84 | 1.16 e+04 | 12/86 (14%) | 0/86 (0%) |
| Citrate cycle | 1.52 | 8.47 e+04 | 1/59 (2%) | 0/59 (0%) |
| Synthesis and degradation of ketone bodies | 1.47 | 1.58 e+05 | 8/19 (5%) | 0/19 (0%) |
| Choline | 1.46 | 1.17 e+04 | 9/87 (16%) | 0/87 (0%) |
| Role of NANOG in mammalian embryonic stem cell pluripotency | 1.45 | 9.66 e+04 | 11/114 (10%) | 0/114 (0%) |
| BMP signaling pathway | 1.19 | 1.0 e+04 | 3/80 (10%) | 0/80 (0%) |
| Human embryonic stem cell pluripotency | 0.91 | 9.64 e+04 | 8/144 (6%) | 0/144 (0%) |
| NIFα1 signaling | 0.49 | 5.57 e+04 | 11/105 (10%) | 0/105 (0%) |
| PI3Kα/PTEN signaling | 3.19 | 5.15 e+04 | 17/136 (13%) | 0/136 (0%) |
| C/EBP signaling | 0.13 | 5.36 e+04 | 23/168 (14%) | 0/168 (0%) |
| Sonic hedgehog signaling | 3 e+03 | 6.45 e+04 | 4/31 (13%) | 0/31 (0%) |

*Upregulated genes are indicated in bold. Magenta: ATP synthases, Blue: cytochrome C oxidases (or COX assembly proteins), Green: NADH dehydrogenases, Pink: succinate–ubiquinone oxidoreductase, Red: mitogen-activated kinases (MAPK), Violet: bone morphogenetic proteins and BMP receptors.*
and clones 10 and 12 that expressed S18-2 at a high level (Figure 5). Clones 10 and 12 expressed high levels of Oct4 and Sox2, and about 5% of them expressed SSEA-1. Only the 18IM cells (that were cultured for 12 months) expressed E-cadherin.

**Discussion**

The MRPS18-2 (MRSPS18B, S18-2) protein is encoded by a gene on human chromosome 6p21.3, adjacent to the MHC class II gene cluster. It was first cloned from CD34+ hematopoietic stem/progenitor cells by Zhang et al.\(^{13}\) Similarly to the other two members of the S18 family, S18-2 is localized on the surface of the small subunit (28S) of the mammalian mitochondrial ribosome.\(^{14,15}\) We have previously found that S18-2 is involved in the regulation of the RB pathway. It binds to both hypo- and hyper-phosphorylated retinoblastoma protein (RB) but not to other pocket proteins, such as p107 and p130.\(^{16}\) In EBV-transformed lymphoblastoid cells a great portion of the normally cytoplasmic S18-2 is targeted to the nucleus by one of the virally encoded transforming proteins, EBNA-6.\(^{17}\) Its binding to RB displaces E2F1, leading to an increase of free E2F1. This may presumably lift the RB-dependent block of S-phase entry;
We now report that numerous pathways, characteristic for rapidly proliferating cells, are upregulated in 18IM. They include genes involved in the transcription/translation machinery, such as the elongation factors Eef1A2 and Tcea3, and enzymes that participate in metabolic and redox reactions, like the cytochrome C oxidases Cox4, Cox6A2, Cox4I1-2, Cox8, and the NADH dehydrogenases NdufA3, 6, 11, 13, NdufB2 and 8 (Table 1). SOD2 (superoxide dismutase), GPX4, and GPX7 (glutathione peroxidases) genes were also upregulated in 18IM cells. Moreover, ATP synthases (ATP5-E, F1, H, I, J2, and ATP50, ATP6-V0A1, and ATP6-V1E1), usually located in the inner membrane of the mitochondria, were also elevated in 18IM cells. They also showed increased release of pyruvate (Supplementary Figure S1) that may be associated with enhanced ATP production.

The expression of Oct4, Sox2, and Nanog that have been used for the experimental induction of PSC differentiation in primary fibroblasts was elevated in 18IM (Figure 2). Other genes in the same experimental category, such as Klf4 and C-myc, were downregulated in 18IM cells. However, C-myc protein was expressed at the higher level in immortalized cells. It is noteworthy that 18IM cells was unable to produce tumors in SCID mice, they transdifferentiated. We may speculate that C-myc protein was stabilized in immortalized cells but its transactivating ability was inhibited.

As a step towards exploring the developmental role of the S18-2 protein, we inhibited its expression in the Danio rerio (Zebrafish) model. Introduction of a S18-2-specific morpholino into the fish eggs led to the block of S18-2 translation. This resulted in embryonic lethality (24–72 h). Forty-eight hours after the introduction of the morpholino into 308 eggs left no living fish, in contrast to >88% of normally looking fish in the control experiments. Simultaneous injection of morpholino and in-vitro-transcribed S18-2 mRNA permitted the development of normal fish. Further studies are in progress to explore the developmental role of S18-2 in development in zebrafish, xenopus, and mouse models.
Materials and Methods

Cell cultures. Primary REFs and 18IM cells were cultured at conditions similar to those of 37 °C, using Iscove's Modified Dulbecco's Medium (IMDM) medium supplemented by 10% of fetal bovine serum and appropriate antibiotics. 18IM cells were cultured without feeder layer in IMDM that contained 0.5 mg/ml of G418.

Microarray analysis. Total RNA was extracted from samples using the RNeasy Mini kit (Qiagen Inc., Hilden, Germany), according to the manufacturer’s recommendations. For each sample, 200 ng total RNA was reverse transcribed, linear amplified, and labeled with Cy3 using the one color Low RNA Input Linear Amplification Kit PLUS (Agilent Technologies, Santa Clara, CA, USA), according to the manufacturer's instructions and purified using the RNeasy Mini kit (Agilent Technologies). After labeling, samples were measured on a Nanoporn microarray module for labeling efficiency and quantification. Samples were then hybridized on Agilent 4 × 44K whole rat genome GE arrays (Agilent Design #014850) at 65 °C for 17 h. After washing and GE washing buffer, the slide was scanned with Agilent Microarray Scanner G2565BA (Agilent Technologies) at 5 μm resolution. Feature extraction software (Version 9.5.3.1) was used to convert the image into gene expression data. Data were normalized by the quantile method, using Partek. Only genes with a false discovery rate < 0.05 were selected as significant with fold change cutoff of 2. Molecular functions of genes were classified according to Gene Ontology function using Ingenuity Pathway Analysis (IPA).

Conflict of Interest

The authors declare no conflict of interest.

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