Aberrant Genomic Imprinting in Rhesus Monkey Embryonic Stem Cells

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Key Words. Imprinting • Embryonic stem cells • Rhesus monkey • Embryos

ABSTRACT
Genomic imprinting involves modification of a gene or a chromosomal region that results in the differential expression of parental alleles. Disruption or inappropriate expression of imprinted genes is associated with several clinically significant syndromes and tumorigenesis in humans. Additionally, abnormal imprinting occurs in mouse embryonic stem cells (ESCs) and in clonally derived animals. Imprinted gene expression patterns in primate ESCs are largely unknown, despite the clinical potential of the latter in the cell-based treatment of human disease. Because of the possible implications of abnormal gene expression to cell or tissue replacement therapies involving ESCs, we examined allele specific expression of four imprinted genes in the rhesus macaque. Genomic and complementary DNA from embryos and ESC lines containing useful single nucleotide polymorphisms were subjected to polymerase chain reaction–based amplification and sequence analysis. In blastocysts, NDN expression was variable indicating abnormal or incomplete imprinting whereas IGF2 and SNRPN were expressed exclusively from the paternal allele and H19 from the maternal allele as expected. In ESCs, both NDN and SNRPN were expressed from the paternal allele while IGF2 and H19 showed loss of imprinting and biallelic expression. In differentiated ESC progeny, these expression patterns were maintained. The implications of aberrant imprinted gene expression to ESC differentiation in vitro and on ESC-derived cell function in vivo after transplantation are unknown. STEM CELLS 2006;24:595–603

INTRODUCTION
As many as 200 of the 30,000 genes in the eutherian mammalian genome may be imprinted. In some cases, imprinting is absolute with only one parental allele expressed, whereas for others it is relative depending on developmental time and cell or tissue type [1, 2]. The best candidate for the imprinting mark is methylation of the cytosine residue in CpG dinucleotides because propagation of cytosine methylation is readily achieved with DNA methyltransferase and because methylation has been associated with the repression of genes on the inactive X chromosome in female mammals as well as the inactive state of tissue-specific genes and silenced transgenes [3]. Studies on mice deficient in methyltransferase also support the conclusion that methylation is involved in at least the somatic maintenance of allele-specific gene expression [4]. Disruption of imprinted gene expression results in postimplantation lethality as demonstrated in uniparental embryos, gynogenotes, or androgenotes [5] and has been associated with disease and carcinogenesis in humans [6–8]. Abnormal expression of NDN and SNRPN, located on chromosome 15q11-q13 in the human, results in the neurogenetic disorder known as Prader-Willi Syndrome [9, 10], whereas loss of IGF2 and H19 imprinting is associated with the etiology of Beckwith-Wiedemann Syndrome and Wilms’ tumors [11]. H19 and IGF2 are located near each other and are coordinately controlled by a common differentially methylated region or imprinting center. Some imprints are altered in response to environmental insults (e.g., H19 in the mouse [7, 12]) or to in vitro manipulations such as intracytoplasmic sperm injection (ICSI) [13]. Embryonic stem cells (ESCs) carry potential for therapies based on cell or tissue replacement. First derived in mice, ESCs are now routinely available in primates, including rhesus macaques and humans [14, 15]. Because of marked differences between mouse and primate ESCs and the practical, ethical, and legal limitations of human ESC research, we conducted studies in the rhesus macaque, from which
preimplantation stage embryos and ESC lines are available. We recently confirmed the imprinted status of NDN, SNRPN, IGF2 (paternal), and H19 (maternal) in skeletal muscle of the rhesus monkey with the description of single nucleotide polymorphism (SNPs) useful for allele specific expression analysis [16]. Here, we established the expression patterns of these genes in preimplantation stage embryos generated by ICSI. We then addressed the stability of these imprints in both undifferentiated and in vitro differentiated ESCs. The safety and efficacy of ESC-based treatments should ideally be extensively examined in the nonhuman primate before clinical applications in patients are initiated.

**Materials and Methods**

**Embryo Production and Nucleic Acid Extraction**

Controlled ovarian stimulation and oocyte recovery have been described previously [17]. Briefly, cycling females were subjected to follicular stimulation using twice-daily intramuscular injections of recombinant human follicle-stimulating hormone as well as concurrent treatment with Antide, a GnRH antagonist, for 8–9 days. Unless indicated otherwise, all reagents were obtained from Sigma-Aldrich (St. Louis, http://www.sigma-aldrich.com) and all hormones and Antide were obtained from Ares Advanced Technologies, Inc. (Norwell, MA, http://www.serono.com/index.jsp). Responding females also received recombinant human luteinizing hormone on days 7–9 and recombinant human chorionic gonadotropin (hCG) on day 10. Cumulus-oocyte complexes were collected from anesthetized animals by laparoscopic follicular aspiration (28–29 hours after hCG) and placed in HEPES-buffered TALP (modified Tyrode solution with albumin, lactate, and pyruvate) medium containing 0.3% bovine serum albumin (TH3) at 37°C. Hyaluronidase (0.5 mg/ml in TH3) was added directly to aspirate-containing tubes at 37°C for 30 seconds before the contents were poured onto a cell strainer (Falcon, 70-μm mesh size; Becton, Dickinson and Company, Franklin Lakes, NJ, http://www.bd.com). The strainer was immediately backwashed with TH3, and the oocytes retained on the mesh were collected by medium flush-and sonication (1:4; Irvine Scientific, Santa Ana, CA, http://www.irvinescientific.com), and a 5-

**Monkey ESC Culture and DNA/RNA Extraction**

Rhesus monkey ESC culture has been described previously [21]. Briefly, ESCs were grown on feeder layers of mitotically inactivated mouse embryonic fibroblasts (MEFs) in medium consisting of 85% Dulbecco’s modified Eagle’s medium (DMEM)/F-12 (Invitrogen, Carlsbad, CA, http://www.invitrogen.com) with 15% defined FBS (HyClone), 1 mM L-glutamine (Invitrogen), 0.1 mM β-mercaptoethanol, and 1% nonessential amino acids (Invitrogen). Medium was changed daily, and ESC colonies were split every 5–7 days by manual disaggregation with collected cells replated on fresh MEF layers.

For the production of differentiated cells, entire ESC colonies were loosely detached from feeder plates and transferred with ESC medium into ultra-low attachment six-well plates (Corning Inc., Corning, NY, http://www.corning.com). Aggregates were cultured in suspension for 5 days to form embryoid bodies (EBs). To induce further differentiation, EBs were transferred into gelatin-coated culture dishes to allow attachment of cells. After EBs attached and spread out, the medium was replaced with serum-free DMEM/F12 containing 1% ITS supplement (insulin, transferrin, and sodium selenite; Invitrogen) and fibronectin (5 μg/ml; Invitrogen). After 8 further days of culture, medium was replaced with N2+FGF2 medium (DMEM/F12 containing 1% N2 supplement [Invitrogen]) and FGF2 (10 ng/ml; R&D Systems Inc., Minneapolis, http://www.rndsystems.com) to induce progenitor cell production. For neuronal phenotypes (serotonin positive, ectodermal lineage), a previously described protocol was employed [22]. Briefly, after 10–14 days of culture, FGF2 was removed and progenitor cells were cultured for an additional 10 days. Cell clumps with elongated fiber bundles were collected and subjected to total RNA extraction. For pancreatic phenotypes (C-peptide positive, endodermal lineage), progenitor cells were cultured in medium supplemented with exendin-4 and nicotinamide as described previously [23]. The presence of the desired phenotypes was quantitated by counting individual cells after immunocytochemical staining with the following antibodies: anti-serotonin (1:1,000; ImmunoStar, Hudson, WI, http://www.immunostar.com), anti-tubulin-β-III (1:500; Chemicon, Temecula, CA, http://www.chemicon.com), or anti-C-peptide (1:500; Linco Research, Inc., St. Charles, MO, http://www.lincoresearch.com).

Expression of OCT4 in both undifferentiated and differentiated...
ESC populations used the primers and polymerase chain reaction (PCR) protocols described previously [24]. gDNA was extracted from ESC colonies using QiAamp DNA Micro Kit (Qiagen) according to the manufacturer’s instructions, and PCR reactions involving gDNA were performed as described for peripheral blood leukocytes [16]. Total RNA was extracted from ESCs using RNeasy Mini Kit (Qiagen) according to the manufacturer’s instructions.

PCR-Based Amplification and Allele-Specific Expression Analysis

Total RNA from embryos or ESCs was treated with DNAase I before cDNA preparation using SuperScript™III First-Strand Synthesis System for reverse transcription (RT)-PCR (Invitrogen) according to the manufacturer’s instructions. Characteristics of the SNPs employed for allele-specific expression analysis are summarized in Table 1. For RT-PCR, the following primers were designed to augment those described by us previously for gPCR (Fig. 1 in [16]). Nec-3F; 5'-CCGGAAATCATCAAGGTGACAAACATC-3' Nec-3R; 5'-AGCCGAAGCACCACCCCATGTC-3' SNRPN-5F; 5'-CTCTTGGTCAAGGTTAAGCTTG-3' SNRPN-5R; 5'-AGCTCAGAACACTCTCACA-3' SNRPN-6R; 5'-CTCTTCAAGCGTGAAGCCATATA-3' H19-2F; 5'-TGGAGAGTTCAAGGCTCCAGAC-3' H19-2R; 5'-CTCAGGAGCCAAAGCAGAGC-3' GAPD-2F; 5'-CTACACTGGCCACCCAGAAC-3' GAPD-2R; 5'-CACCCTGTTGTGGTTAGCCAAATTC-3' Nec-3F/R were the internal primers based on the rhesus monkey sequence of Nec-1F/R. SNRPN primers were based on monkey sequence of Nec-1F/R. SNRPN primers were based on initial screening using Avai restriction enzyme at 37°C for 16 hours, electrophoresed on a UV transilluminator. Restriction digestion of SNRPN amplicons with XbaI resulted in 308- and 90-bp fragments from the A-containing allele, whereas the G-containing allele remained uncleaved. Restriction enzyme digestion of H19 amplicons with Avai resulted in 204- and 46-bp fragments for the C-containing allele, whereas the T-containing allele remained intact. For NDN, IGF2, H19, and GAPD, homozygous parental combinations were used. For SNRPN, only heterozygous parents were available. To examine allele-specific expression of SNRPN in embryos, heterozygosity in gDNA samples was confirmed before RT-PCR analysis. For genomic PCR of SNRPN exon 8 in embryos, a primer located on intron 7 for hemi-nested PCR was transferred to a 20-μl volume containing the same concentration of MgCl₂, dNTP mix, DNA polymerase, and internal primers (Nec-3F/R, SNRPN-5F/6R, and H19-2F/R, respectively). PCR conditions were the same as the first amplification. For IGF2, PCR primers and conditions were the same as those used with genomic PCR, except that the number of amplification cycles was increased to 45 to ensure adequate amplification from small amounts of transcript. For GAPD, PCR conditions were 45 cycles at 94°C, 60°C, and 68°C for 30, 60, and 45 seconds, respectively. Expressed alleles were determined by direct sequence analysis of the RT-PCR amplicons or by restriction fragment length polymorphism (RFLP). In all RT-PCR reactions, samples were analyzed without RT in order to exclude the possibility of gDNA contamination. For direct sequence analysis, the same primers used in PCR amplification were employed. RFLP analysis using XbaI and Avai was available for NDN SNP-1 and H19 SNP-3, respectively [16]. For RFLP, 5 μl of PCR products were digested in a 20-μl volume with 10 IU of restriction enzyme at 37°C for 16 hours, electrophoresed through agarose gels, stained with ethidium bromide, and visualized on a UV transilluminator. Restriction digestion of NDN amplicons with XbaI resulted in 308- and 90-bp fragments from the A-containing allele, whereas the G-containing allele remained uncleaved. Restriction enzyme digestion of H19 amplicons with Avai resulted in 204- and 46-bp fragments for the C-containing allele, whereas the T-containing allele remained intact.

Table 1. Characteristics of the single nucleotide polymorphisms (SNP) employed for allele-specific expression analysis in rhesus monkey embryos and embryonic stem cells

<table>
<thead>
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<th>Accesion no.</th>
<th>Position</th>
<th>Polymorphism</th>
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</thead>
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<tr>
<td></td>
<td>2</td>
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<td>795</td>
<td>T &gt; C</td>
</tr>
<tr>
<td>SNRPN</td>
<td>9</td>
<td>AY 624142</td>
<td>743</td>
<td>G &gt; A</td>
</tr>
<tr>
<td></td>
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<tr>
<td></td>
<td>11</td>
<td>AY 624142</td>
<td>810</td>
<td>G &gt; A</td>
</tr>
<tr>
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<td>AY 624138</td>
<td>169</td>
<td>C &gt; T</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>AY 624138</td>
<td>286</td>
<td>C &gt; T</td>
</tr>
<tr>
<td></td>
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<td></td>
<td>3</td>
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<td>457</td>
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</tr>
<tr>
<td>GAPD</td>
<td>1</td>
<td>AY 624140</td>
<td>358</td>
<td>G &gt; A</td>
</tr>
</tbody>
</table>

The nucleotide position is based on polymerase chain reaction of genomic DNA and direct sequence analysis of the amplicon produced. The primers employed can be found in [16].

Quantitative RT-PCR

Real-time RT-PCR was used for the quantitiation of allele-specific expression of IGF2 as described by Suda and coworkers [25] using a custom TaqMan fluorogenic detection system (Custom TaqMan SNP Genotyping Assays; Applied BioSystems, Foster City, CA, http://www.appliedbiosystems.com). Oregon rhesus monkey embryonic stem cells (ORMES)-1, -6 and -7 were C/T heterozygous for IGF2 SNP-4 and amenable to analysis. RNA extraction and RT was as described above. PCR reaction conditions were 50°C for 2 minutes, 95°C for 10 minutes, 92°C for 15 seconds, and 60°C for 1.25 units of AccuSure DNA polymerase. PCR conditions were as follows: NDN, 35 cycles at 94°C, 60°C, and 68°C for 30, 60, and 45 seconds, respectively; SNRPN, 35 cycles at 94°C, 60°C, and 68°C for 30, 60, and 45 seconds, respectively; and H19, 35 cycles at 95°C, 62°C, and 68°C for 90, 60, and 45 seconds, respectively. After amplification, 1 μl of the first-round product was transferred to a 19-μl volume containing the same concentration of MgCl₂, dNTP mix, DNA polymerase, and internal primers (Nec-3F/R, SNRPN-5F/6R, and H19-2F/R, respectively). PCR conditions were the same as the first amplification. For IGF2, PCR primers and conditions were the same as those used with genomic PCR, except that the number of amplification cycles was increased to 45 to ensure adequate amplification from small amounts of transcript. For GAPD, PCR conditions were 45 cycles at 94°C, 60°C, and 68°C for 30, 60, and 45 seconds, respectively. Expressed alleles were determined by direct sequence analysis of the RT-PCR amplicons or by restriction fragment length polymorphism (RFLP). In all RT-PCR reactions, samples were analyzed without RT in order to exclude the possibility of gDNA contamination. For direct sequence analysis, the same primers used in PCR amplification were employed. RFLP analysis using XbaI and Avai was available for NDN SNP-1 and H19 SNP-3, respectively [16]. For RFLP, 5 μl of PCR products were digested in a 20-μl volume with 10 IU of restriction enzyme at 37°C for 16 hours, electrophoresed through agarose gels, stained with ethidium bromide, and visualized on a UV transilluminator. Restriction digestion of NDN amplicons with XbaI resulted in 308- and 90-bp fragments from the A-containing allele, whereas the G-containing allele remained uncleaved. Restriction enzyme digestion of H19 amplicons with Avai resulted in 204- and 46-bp fragments for the C-containing allele, whereas the T-containing allele remained intact.

For NDN, IGF2, H19, and GAPD, homozygous parental combinations were used. For SNRPN, only heterozygous parents were available. To examine allele-specific expression of SNRPN in embryos, heterozygosity in gDNA samples was confirmed before RT-PCR analysis. For genomic PCR of SNRPN exon 8 in embryos, a primer located on intron 7 for hemi-nested PCR amplification of exon 8 was designed: SNRPN-5F. For genomic PCR of SNRPN from embryos, first-round amplification using SNRPN-5F/2R was followed by a second amplification with SNRPN-2F/2R. gDNA from ESCs and the parents of the embryos from which the ESC lines were derived were screened for polymorphisms in the transcribed regions of NDN, SNRPN, IGF2, H19, and GAPD as described by us previously [16]. RT-PCR of total RNA extracts of ESC lines that contained informative SNPs, based on parental analysis, generated amplicons that were subjected to sequence analysis.
GAPD gene, in 3% Agarose gels. This blastocyst (B1) expressed predominately the maternal allele. For gDNA, only amplification with H19-2F/R was performed. Amplicons were digested with and without RT-PCR (cDNA), the first round of nested PCR used H19-1F/R primers, whereas the second round of amplification was performed with H19-2F/R primers.

For cDNA, first-round amplification involved Nec-1F/1R followed by second-round amplification with Nec-3F/3R. For gDNA, amplification with only Nec-3F/3R was performed. Amplicons were digested with and without XbaI at 37°C for 16 hours and analyzed by electrophoresis in 1.6% Agarose gels. Bl-1 expressed both parental alleles after XbaI digestion, whereas Bl-2 expressed predominately the maternal allele. (B): Chromatogram showing paternal expression of SNRPN in an informative blastocyst. gDNA from the father was homozygous C/C, whereas maternal gDNA was heterozygous C/T. Blastocyst gDNA was heterozygous with paternal origin of the C allele and maternal origin for the T allele. An expressed C (or paternal) allele was detected. The polymorphic nucleotide is identified by bold red font.

Figure 1. Expression analysis of four imprinted and one nonimprinted genes in individual rhesus monkey blastocysts. (A): Restriction fragment length polymorphism (RFLP) analysis with XbaI digestion of NDN amplicons. cDNA from each blastocyst and gDNA from the parents were used as templates. For cDNA, first-round amplification involved Nec-1F/1R followed by second-round amplification with Nec-3F/3R. For gDNA, amplification with only Nec-3F/3R was performed. Amplicons were digested with and without XbaI at 37°C for 16 hours and analyzed by electrophoresis in 1.6% Agarose gels. Bl-1 expressed both parental alleles after XbaI digestion, whereas Bl-2 expressed predominately the maternal allele.

We first studied blastocysts, because this is the embryonic stage at which implantation occurs and from which ESCs are routinely derived. Animals, heterozygous or homozygous for specific SNPs identified previously [16], were selected based on their ability to create embryos (upon oocyte collection and fertilization by ICSI) that could be analyzed for allele-specific expression. After culture for 7–9 days, expanded blastocysts containing approximately 250 cells were subjected to RNA isolation, and subsequent RT-PCR amplification of the polymorphic regions and sequence analysis of the resultant amplicons were then used to determine the expressed allele(s). We analyzed NDN and SNRPN, imprinted genes that are expressed predominantly in the developing and adult brain [26] and that therefore might be relatively stable during early development. H19 and IGF2 were selected because they have been implicated in growth regulation during early development and might be especially susceptible to changes during embryo culture and ESC derivation [27]. GAD served as a nonimprinted gene.

Allele-specific expression in NDN was examined using a G/A polymorphism (SNP-1) for which three animal pairs were homozygous, G for the male and A for the female, and 27 expanded blastocysts were examined individually. Of the 24 expressing embryos, 22 were biallelic and two showed maternal expression (Fig. 1A). Blastocyst ages varied between 7 and 9 days, but there was no apparent correlation between NDN ex-
pression and age. With SNRPN, nine embryos were analyzed from an animal pair in which the male was C homozygous and the female C/T heterozygous (SNP-11 and primers SNPRT5F/6R). Genomic PCR revealed that three embryos were C/T heterozygous, and subsequent sequence analysis from their cDNAs indicated expression from the C, or paternal, allele (Fig. 1B). IGF2 expression was examined in five informative blastocysts from two animal pairs carrying T/C polymorphisms (SNPs-1 and 4). Only the paternal allele was expressed (Fig. 1C). For H19, six heterozygous blastocysts were examined from two animal pairs that were homozygous for a T/C polymorphism (SNP-3). Expression from the maternal allele was observed (Fig. 1D). Finally, using an A/G polymorphism in one parental combination (SNP-1), we recovered two embryos and confirmed biallelic expression of GAPD (Fig. 1E).

Imprinting in Undifferentiated ESCs
Next, we defined imprinting in the undifferentiated ESC phenotype. Several ESC lines derived from ICSI-produced embryos were available in the ORMES series [28] with normal karyotypes: ORMES-3 and -7 were 42 XY, and ORMES-6, -8, and -10 were 42 XX. Additionally, two lines with stable translocation or inversion were studied: ORMES-1 (42, XY, t[11:16]) and -5 (42 XY, inv [1]). Colonies of undifferentiated ESCs consisted of morphologically unique cells expressing the pluripotent cell markers, OCT4, SSEA-4, TRA-1-60, and TRA-1-81 [28]. ESC line selection was based on the presence of the appropriate SNPs in the transcribed region of the genes of interest. Four ESC lines were heterozygous for a polymorphism in NDN (SNP 1 or 2). With ORMES-6, gDNA from the male and female and the ESC line were analyzed for a C/T polymorphism: genotypes of the paternal, maternal, and ESCs were C/C, C/T, and C/T, respectively, indicating that the C in the ESC genome originated from the paternal and the T from the maternal genome. The cDNA from undifferentiated ORMES-6 contained the C (or paternal) allele, indicating normal imprinting (Fig. 2A). Similarly, RT-PCR and direct sequence analysis of amplicons from ORMES-7, -8, and -10 indicated that expression was from the paternal allele (Table 2). For SNRPN, analysis of both parental and ESC gDNA for a G/A polymorphism (exon KB-1 SNP-10 or -11) revealed that ORMES-6, -7, and -8 cells expressed paternal alleles (ORMES-6 results in Fig. 2B) consistent with normal imprinting of SNRPN. Similar results were obtained for ORMES-1 using SNP-9 (Table 2). Four polymorphisms in IGF2 were useful in allele-specific expression analysis. As an example in ORMES-6, a C/T polymorphism (SNP-2) was examined. The maternal genome contained only the T allele, whereas the paternal genome was heterozygous at this position. Both the gDNA and cDNA from undifferentiated ORMES-6 were heterozygous, C/T, indicating biallelic expression (Fig. 2C). A total of seven ESC lines were examined, and all expressed both parental IGF2 alleles (Table 2). Two C/T polymorphisms in H19 allowed expression analysis in ORMES-5, -6, -8, and -10. For ORMES-6 (SNP-3), the paternal genome was C/T heterozygous whereas the maternal genome contained only the C allele. Both gDNAs and cDNAs from ORMES-6 were C/T heterozygous, indicating biallelic expression (Fig. 2E). ORMES-8 and -10 also expressed both parental alleles (Table 2), indicating that H19 imprinting was lost. However, ORMES-5 showed predominantly maternal expression,

Figure 2. Allele-specific expression analysis in undifferentiated and differentiated rhesus monkey ESC (ORMES-6) populations. The polymorphic nucleotide is identified in chromatograms by bold red font. (A): Chromatograms showing paternal expression of NDN in ORMES-6. The paternal gDNA was homozygous, C/C, whereas the maternal and ESC gDNAs were heterozygous C/T. The C allele in the ESC line must have a paternal origin, whereas the T allele was derived from the mother. cDNA from undifferentiated and neuronally differentiated ESC populations contained only the C (or paternal) allele. (B): Chromatograms showing paternal expression of SNRPN in ORMES-6 based on a G/A polymorphism. (C): Chromatograms showing biallelic expression of IGF2. (D): Chromatograms showing biallelic expression of GAPD. (E): Restriction fragment length polymorphism (RFLP) analysis of H19 showing biallelic expression in undifferentiated and in one neurally differentiated (OR6N1, passage 24) cell population, and maternal in another (OR6N3, passage 28). Abbreviations: ESC, embryonic stem cell; gDNA, genomic DNA; ORMES, Oregon rhesus monkey embryonic stem cells.
the expected outcome for this imprinted gene. Finally, a G/A polymorphism in \textit{GAPD} in ORMES-6, -7, -8, and -10 was exploited. As an example, the paternal and maternal genomes of ORMES-6 were homozygous, G/G and A/A, respectively, whereas cDNA from ORMES-6 was heterozygous G/A, indicating biallelic expression (Fig. 2D). Although we observed no differences in expression between undifferentiated ESC lines (with the exception of \textit{H19} in ORMES-5), imprinting could still vary as a function of passage number. Therefore, we examined allele-specific expression of \textit{IGF2} and \textit{H19} from very early passage numbers [8] to modest passage numbers [20–30] in ORMES-1, -6, and -8 but observed no differences.

We also applied quantitative RT-PCR to confirm the ratios of biallelic expression of \textit{IGF2} and \textit{H19} from very early passage numbers [8] to modest passage numbers [20–30] in ORMES-1, -6, and -8 but observed no differences.

### Imprinting in Differentiated ESCs

Finally, anticipating that therapies involving ESCs will use progenitor or terminally differentiated progeny, allele-specific expression in neural or pancreatic phenotypes was examined. ESCs were differentiated into neural progenitor populations that were more than 70% nestin and Musashi 1–positive [21]. After exposure to N3 medium as described by Salli and coworkers [22], up to 70% of the treated cells were serotonin-positive. Similarly, between 67% and 78% of exendin-4- and nicotinamide-treated cells were C-peptide–positive (results not shown). OCT4 expression was negative, confirming the absence of undifferentiated cells. \textit{NDN} transcripts were examined in ORMES-6–derived neuronal populations at passages 24 and 26. Complimentary DNA showed the presence of the C (or paternal) allele, indicating normal paternal expression (Fig. 2A; Table 3). For \textit{SNRPN}, ORMES-6–derived differentiated cells at passages 24 and 26 expressed the paternal allele (Fig. 2B; Table 3). These results show that monoallelic expression of \textit{NDN} and \textit{SNRPN} was maintained after differentiation. \textit{IGF2}, which was not imprinted in undifferentiated ORMES lines, was also expressed from both parental alleles in differentiated cell populations (Fig. 2C; Table 3). Expression of the silent maternal allele in \textit{IGF2} was confirmed by real-time PCR analysis for both neuronal and pancreatic phenotypes in ORMES-1, -6, and -7 (Fig. 3). Paternal- to maternal-allele ratios varied from 1.11 to 1.47 for neuronal populations and from 1.16 to 1.35 for pancreatic populations. Significant differences ($<.05$; Student’s $t$ test) were noted between the averaged ratios for ORMES-1, -6, and -7 when undifferentiated and differentiated phenotypes were compared, although in all cases biallelic expression was observed.

### Table 2. Summary of allele-specific expression studies in undifferentiated populations from ORMES cell lines

<table>
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<tr>
<th></th>
<th>NDN SNP no.</th>
<th>H19 SNP no.</th>
<th>\textit{IGF2} SNP no.</th>
<th>SNRPN SNP no.</th>
<th>\textit{GAPD} SNP no.</th>
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</table>

SNPs are defined in [16]. The absence of a result indicates that no informative SNP was available.

Abbreviations: B, biallelic expression; M, maternal expression; ORMES, Oregon monkey embryonic stem cells; P, paternal expression; SNP, single nucleotide polymorphism.

### Figure 3.

Quantitative polymerase chain reaction analysis of \textit{IGF2} allele-specific expression in undifferentiated and differentiated cell populations for ORMES-1, -6, and -7 cell lines. Each sample was run at least in triplicate. Undifferentiated $\bullet$; neuronal $\square$; pancreatic $\circ$.
Abbreviations: ESC, embryonic stem cell; ORMES, Oregon monkey embryonic stem cells.

cell lines examined. The number of unique parental combinations producing blastocysts is indicated in brackets. The numbers in parenthesis refer to the number of blastocysts or ORMES cell lines expressing per number of blastocysts or ORMES.

Discussion

The assisted reproductive technologies have facilitated the development of invasive procedures from ICSI to preimplantation genetic diagnosis, somatic cell nuclear transfer, and ESC derivation. Studies in mice suggest that preimplantation embryos taken from the natural environment and exposed to the stress induced by in vitro culture or manipulation can lead to disruption of epigenetic marks at imprinted loci, resulting in aberrant growth and morphology at fetal and perinatal stages of development [8, 12, 13, 27]. In cloned animals, a major cause of fetal wastage is aberrant imprinted gene expression secondary to incomplete nuclear reprogramming [29]. Additionally, abnormal imprinting occurs in mouse ESCs derived from natural and cloned blastocysts [30, 31]. Here, we present an analysis of allele-specific gene expression in ICSI-produced embryos and their ESCs in rhesus macaques (Table 3). The use of ICSI as opposed to conventional in vitro fertilization or the recovery of in vivo fertilized embryos was based on both convenience and our focus on allele-specific expression analysis in ESCs. Three of the studied genes, H19, SNRPN, and IGF2, including those expected to be sensitive to environmental stress, were normally imprinted in monkey blastocysts derived by ICSI. This observation was particularly interesting in view of concerns over the possibility of increased risks for imprinting diseases in assisted reproduction technology (ART)–produced children [32] and is consistent with the first demonstration of imprinting in a human gene, IGF2, in the preimplantation embryo [33]. Moreover, despite evidence that culture conditions can dramatically impact the uniparental expression of H19 in the mouse [12], this conclusion could not be extrapolated to primates based on the analysis of six individual blastocysts in the present study. In human embryos, detection of the paternal imprint on H19 in pooled 8– to 32–cell stage embryos was noted using restriction enzyme digestion [34], and uniparental H19 expression was observed in a single blastocyst analyzed directly by RT-PCR [35].

In monkey blastocysts, NDN was expressed either from the maternal allele or from both alleles. This result could arise from expression of residual maternal mRNA, an unlikely possibility given the expectation that most maternal transcripts are absent by the time of embryonic gene activation [36]. Alternatively, it could reflect the erasure of imprinting in the embryonic genome or simply that the imprint mark had not yet been established.

The need for overlapping epigenetic signals has been recognized, including alterations in chromatin configuration [37], and precludes a definitive decision in this matter without additional experimental evidence. Our observation that NDN in ESCs was exclusively expressed from the paternal allele suggests that it was simply a matter of timing and that imprinting had not yet been established in the day-7 to -9 blastocyst. The outcome was unexpected, however, because SNRPN was imprinted in the monkey blastocyst and in four–cell stage human embryos [38] and both SNRPN and NDN expression is regulated by a common imprinting center [9]. In previous studies, NDN expression was rarely detected in amplified cDNA derived from single human embryos up to and including the blastocyst stage [35]. The role of NDN, which encodes the neuronal protein, necdin, has not been elucidated during preimplantation development, although it is implicated in the neonatal period [39].

In monkey ESC lines, NDN and SNRPN showed paternal expression similar to the adult; however, we observed loss of monoallelic expression of H19 and IGF2 with paternal–maternal-allele ratios for IGF2 in the range of 0.84 to 1.64. Interestingly, human embryonic germ [40] cells showed monoallelic expression of three imprinted genes, whereas in two of the studied lines partially relaxed imprinting of IGF2 was seen with allele ratios of 4:1 or 5:1. With human ESCs, normal imprinting was reported for the five genes studied, with the single exception of one line that displayed biallelic expression of H19. The extent of expression of the silent allele in this case was passage number–dependent, increasing from 25% to 58% p66-p76 versus p91-p101, respectively, leading the authors to suggest that mechanisms controlling H19 expression are subject to perturbation [41]. Biallelic expression of both H19 and IGF2 was seen at a very early passage number in the present study and even in karyotypically abnormal ESC lines. The latter may represent the expected outcome on occasion of a balanced translocation or when the chromosome carrying the gene under study is not implicated. Based on the coordinated expression of the H19/IGF2 gene pair, this suggests that alterations have occurred at the common imprinting center located upstream of H19 [42]. Cui and coworkers [42] have suggested that loss of IGF2 imprinting commonly involves altered methylation at the imprinting center but not mutations in the CTCF (CCCTC-binding factor; zinc finger protein) or its binding site. The role

### Table 3. Summary of expression analysis of four imprinted genes and one nonimprinted gene in rhesus monkey blastocysts, ESCs, and ESC-derived neuronal cell populations

<table>
<thead>
<tr>
<th>Gene</th>
<th>Blastocysts</th>
<th>ESCs</th>
<th>ESC-derived neuronal cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>NDN</td>
<td>No expression (3/27)</td>
<td>Paternal (9/9)</td>
<td>Paternal (1/1)</td>
</tr>
<tr>
<td>SNRPN</td>
<td>Paternal (9/9)</td>
<td>[1 pair]</td>
<td>[1 pair]</td>
</tr>
<tr>
<td>IGF2</td>
<td>Paternal (5/5)</td>
<td>[2 pairs]</td>
<td>[2 pairs]</td>
</tr>
<tr>
<td>H19</td>
<td>Maternal (6/6)</td>
<td>[2 pairs]</td>
<td>[2 pairs]</td>
</tr>
<tr>
<td>GAPD</td>
<td>Biallelic (2/2)</td>
<td>[1 pair]</td>
<td>[1 pair]</td>
</tr>
</tbody>
</table>

The numbers in parenthesis refer to the number of blastocysts or ORMES cell lines expressing per number of blastocysts or ORMES cell lines examined. The number of unique parental combinations producing blastocysts is indicated in brackets. Abbreviations: ESC, embryonic stem cell; ORMES, Oregon monkey embryonic stem cells.
of imprinting center methylation in the monkey is currently under active investigation by us. Abnormal expression of DNA methyltransferase I (DNMT1) could account for imprint loss, a process that has resulted in embryonic lethality in DNMT1-deficient mice [43]. Differentiated ESCs retained the expression pattern seen in undifferentiated cells with the exception of H19, in which expression varied between maternal and biallelic as a function of passage number. This variability could represent the sampling of different cell populations in a given culture or in the cell phenotypes present at different passage numbers.

The significance of imprint loss in monkey ESCs to cell function and fate after transplantation of progeny into a host animal remains unknown. Epigenetic instability in murine ESCs is not incompatible with normal development, because murine ESCs can support the routine generation of normal chimeric phenotypes present at different passage numbers. This variability could represent the sampling of expression varied between maternal and biallelic as a function of passage number. This variability could represent the sampling of different cell populations in a given culture or in the cell phenotypes present at different passage numbers.

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