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Specific Impairment of Cardiogenesis in Mouse ES Cells Containing a Human Chromosome 21

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Down syndrome (DS) leads to cardiac defects which are common and significant in babies with DS. We recently generated chimeric mice carrying a human chromosome (hChr) 21. The contribution ratio of embryonic stem (ES) cells containing a hChr 21 was specifically low in the heart, compared to other organs, and cardiovascular malformations were observed, suggesting that an additional copy of hChr 21 also disrupts the normal development of heart in mice. Here we describe that the presence of hChr 21 in ES cells delays the appearance of beating cardiomyocyte during differentiation, whereas differentiation into other cell types is not disrupted. Furthermore, the defect in cardiogenesis was restored following the deletion of a specific region of hChr 21. Therefore, we conclude that the imbalance of specific gene(s) on hChr 21 may lead to the disturbance of cardiogenesis and that this may be a useful system to model and investigate the cardiac defects of human DS. © 2000 Academic Press

Key Words: **human chromosome 21; Down syndrome; ES cells; *in vitro* differentiation; cardiomyocyte; heart malformation.**

Down syndrome (DS) is a congenital disease caused by the trisomy of human chromosome (hChr) 21 and it is characterized by distinct facial and physical features, and mental retardation. Some patients have congenital heart defects (1, 2). An animal model that can provide access to tissues is required to examine the developmental consequences of DS. Previously, mice have been generated with segmental trisomy for mouse chromosome 16, which shares a large region of genetic homology with hChr 21 (3). However, no animal model has been generated so far that accurately models any of the disorders associated with DS (4, 5). Recently, to address these problems, we generated chimeric mice carrying an intact hChr 21 as a freely segregating extra chromosome retained at a high frequency through microcell-mediated chromosome transfer of hChr 21 into embryonic stem (ES) cells (6). Analysis of these chimeric mice revealed cardiovascular malformations (reported elsewhere). These findings suggest that these mice may serve as a model of the heart disease exhibited in DS. Furthermore, we found that the contribution ratio of embryonic stem (ES) cells containing a hChr 21 in chimeras was about 50% in adult heart, while it was more than 90% in other organs such as brain, liver, and lung (reported elsewhere). These findings prompted us to investigate the possibility that the extra hChr 21 interfered with cardiogenesis during development. In this study, we examined the effect of the additional hChr 21 in ES cells on differentiation and cardiogenesis *in vitro.*

Abbreviations used: DS, Down syndrome; ES, embryonic stem; hChr, human chromosome.

MATERIALS AND METHODS

ES cell culture and construction of ES cells containing a hChr 21. The TT2F mouse ES line was maintained in an undifferentiated state by culture on mitomycin C-treated mouse embryonic fibroblasts (MEF) feeder layers according to standard protocols. The ES culture medium used was high glucose Dulbecco's modified Eagle's medium (DMEM) containing 15% fetal bovine serum, 0.1 mM β-mercaptoethanol, 1 mM sodium pyruvate, 1× nonessential amino acids, 2 mM glutamine, and 1000 U/ml recombinant human leukemia inhibitory factor (LIF; Gibco-BRL).

The introduction of hChr 21 into ES cells was performed as we reported previously (7). Briefly, mouse A9 cells containing a hChr 21 tagged with pST*neo* were used as a source of microcell donors for microcell-mediated chromosome transfer into mouse ES cells. The hybrid cells were maintained in nonselective culture medium for 24 h and then selected in culture medium containing 300 μg/ml of G418 (Funakoshi, Tokyo, Japan). We isolated G418-resistant clones and screened genomic DNA from these clones by PCR using hChr 21 specific markers analysis using PCR. The following markers and primer pairs were used to amplify fragments from 50 ng of genomic DNA: pAW32a (5′-TCATACAGAGTATAACACCAGGAC-3′ and 5′-

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GTCTTATTGTGATAGGCTTGC-3′, amplifies a 270-bp fragment); D21S411 (5′-GAACCATTATAAGTTGACCATC-3′ and 5′-ACATGTTAACATGCTATATCTGT-3′, amplifies a 154-bp fragment); D21S120 (5′-TGTGTCTGCCATTTCTGGGTGTAG-3′ and 5′-GATCCTGGGACAAAGTAGTCTCTAA-3′, amplifies a 318–330-bp fragment); APP (5′-CTGGGCAATAGAGCAAGACC-3′ and 5′-ACCCATATTATCTATGGACAATTGA-3′, amplifies a 115-bp fragment); SOD (5′-ATTCTGTGATCTCACTCTCAGG-3′ and 5′-TCGCGACTAACAATCAAAGT-3′, amplifies a 133-bp fragment); SIM2 (5′-GGGCCTCATGGTAAGAGTCA-3′ and 5′-GAAAAATGTCGGTGGTATCTCC-3′, amplifies a 250-bp fragment); ETS2 (5′-TACCATGCCAATGGTTTATAAGG-3′ and 5′-ATGTGACTGGGAACATCTTGC-3′, amplifies a 177-bp fragment), and D21S334 (5′-CCTAAATAAATGATGGTCCCTG-3′ and 5′-AGTGATACCGTGCCTGGTC-3′, amplifies a 278-bp fragment). Amplifications were performed with an annealing temperature of 62°C for 30 cycles and the products were analyzed on a 3% agarose gel.

Fluorescence in situ hybridization (FISH) analysis. Two-color FISH analysis was performed on fixed metaphase spreads of ES cell clones using digoxigenin (Boehringer) labeled human COT-1 DNA (Gibco-BRL) and biotin labeled pST*neo* plasmid DNA, as we reported previously (7, 8). The hybridized probes were detected with anti-digoxigenin-rhodamine (Boehringer) and FITC-avidin (Vector), respectively. Chromosomes were counterstained with DAPI (Sigma).

Embryoid body (EB) formation and in vitro differentiation. Induction of differentiation into a variety of cell types including cardiac myocytes was performed according to the method of Oyamada *et al.* using the hanging drop culture method (9). Briefly, approximately 400 ES cells in 30 μl aliquots of differentiation medium, which consisted of ES culture medium without LIF (see above), were placed on the undersurface of a petri dish lid, inverted and cultured for 3 days. The resulting EBs were transferred individually to separate wells of 24-well tissue culture plates (Costar). For the coculture of two EBs that were derived from different cell lines, one EB was allowed to attach to the bottom of 12-well plate (Costar) for 3 h, followed by the transfer of the second EB and attachment to a different position. Culture medium was changed every 2 days. For the quantitative estimation of differentiation, cultures were monitored every day under a microscope to detect the presence of rhythmically beating cardiac myocytes in the dense center of EBs. For every value, three independent experiments were performed in which more than 50 EBs were examined. The approximate number of cardiomyocyte aggregates per EB was 1–4. The day hanging drop culture was initiated was defined as day 0 of culture in all experiments. In addition to TT2F EBs differentiating into cardiac myocytes, we could observe differentiation into cartilage, contracting skeletal muscle cells, smooth muscle cells, and cells forming tubular structures reported previously for other ES cells such as the J1 and D3 lines (10, 11). However, unlike other ES cell lines, we could not detect differentiation into neuronal cells, even when EBs were treated with retinoic acid, which is known to promote the differentiation to neuronal cells (12).

RESULTS

Differentiation of ES Cells into Beating Cardiac Myocytes Is Impaired

Mouse ES cells differentiate spontaneously into a variety of cell types including beating cardiac myocytes *in vitro.* To investigate whether the presence of hChr 21 in mouse ES cells has any effects on formation of beating cardiac myocytes, we generated 4 ES cell lines containing a hChr 21 by microcell-mediated chromosome transfer. These cell lines have 40 mouse chromosomes and one hChr 21 which is intact [TT2F(#21-7) and TT2F(#21-11)] or partially deleted [TT2F(#21-5) and TT2F(#21-10)] (Fig. 1). Comparisons were made among three independent ES cell lines containing a hChr 21, TT2F(#21-7), TT2F(#21-10) and TT2F(#21-11), and wild type TT2F by *in vitro* differentiation analysis. The region containing the ETS2 locus on hChr 21 was deleted spontaneously during microcell-mediated chromosome transfer to the TT2F(#21-10) hybrid, while the hChr 21 in TT2F(#21-7) and TT2F(#21-11) hybrids was intact for all the regions that were tested. The introduction of up to four TT2F(#21-10) cells into 8-cell-stage embryos led to the development of chimeric adult animals which carry a hChr 21 in approximately 90% of their cells in many organs except the heart. Some of these mice exhibited cardiovascular malformations. Analysis of chimeric mice derived from TT2F(#21-7) and TT2F(#21-11) hybrids has not been performed. All cell lines had a similar doubling time (approximately 12 h) in the undifferentiated state, and gave rise to EBs of similar size and gross morphology in hanging drop cultures in the absence of LIF (data not shown). After 7 days in culture, rhythmically beating cardiac myocytes appeared at the dense center of EBs derived from all cell lines, the ratio of cardiogenic differentiation in EBs derived from the TT2F(#21) clones was lower than that of EBs derived from wild type ES cells (Fig. 2A). Approximately 80% of EBs derived from TT2F cells were beating by day 8. The ratio of EBs that resumed beating reached to approximately 80% by day 8 in EBs derived from wild type. The behavior of EBs derived from TT2F cells closely resembled a previous report using the J1 ES cell line (9). In EBs derived from TT2F(#21) clones, the ratio of beating EBs remained significantly lower than this from days 7 to 12. From days 12 to 17, the ratio of beating EBs derived from TT2F(#21) cells reached approximately 80%. To investigate whether a specific region of hChr 21 is responsible for the retardation of maximum cardiomyocyte differentiation in TT2F(#21) cell lines, we investigated the differentiation of TT2F(#21-5) cells, which contain a hChr 21 with a partial deletion, established spontaneously in the process of microcell-mediated chromosome transfer. Interestingly, the EBs derived from this cell line could differentiate into cardiomyocytes in a similar manner to EBs derived from wild type ES cells. This result suggests that the region containing SOD1 or D21S120 may be responsible for the delay of maximum differentiation to cardiomyocytes and that this phenotype is not due to clonal variation.

To test whether the retardation of differentiation in ES cells containing a hChr 21 is specific to cardiac myocytes, we analyzed EBs derived from each ES cell line for the differentiation into cartilage, using the same system as before. As shown in Fig. 2C, differentiation into cartilage occurred with a similar timing and essentially to the same extent in all the cell lines.

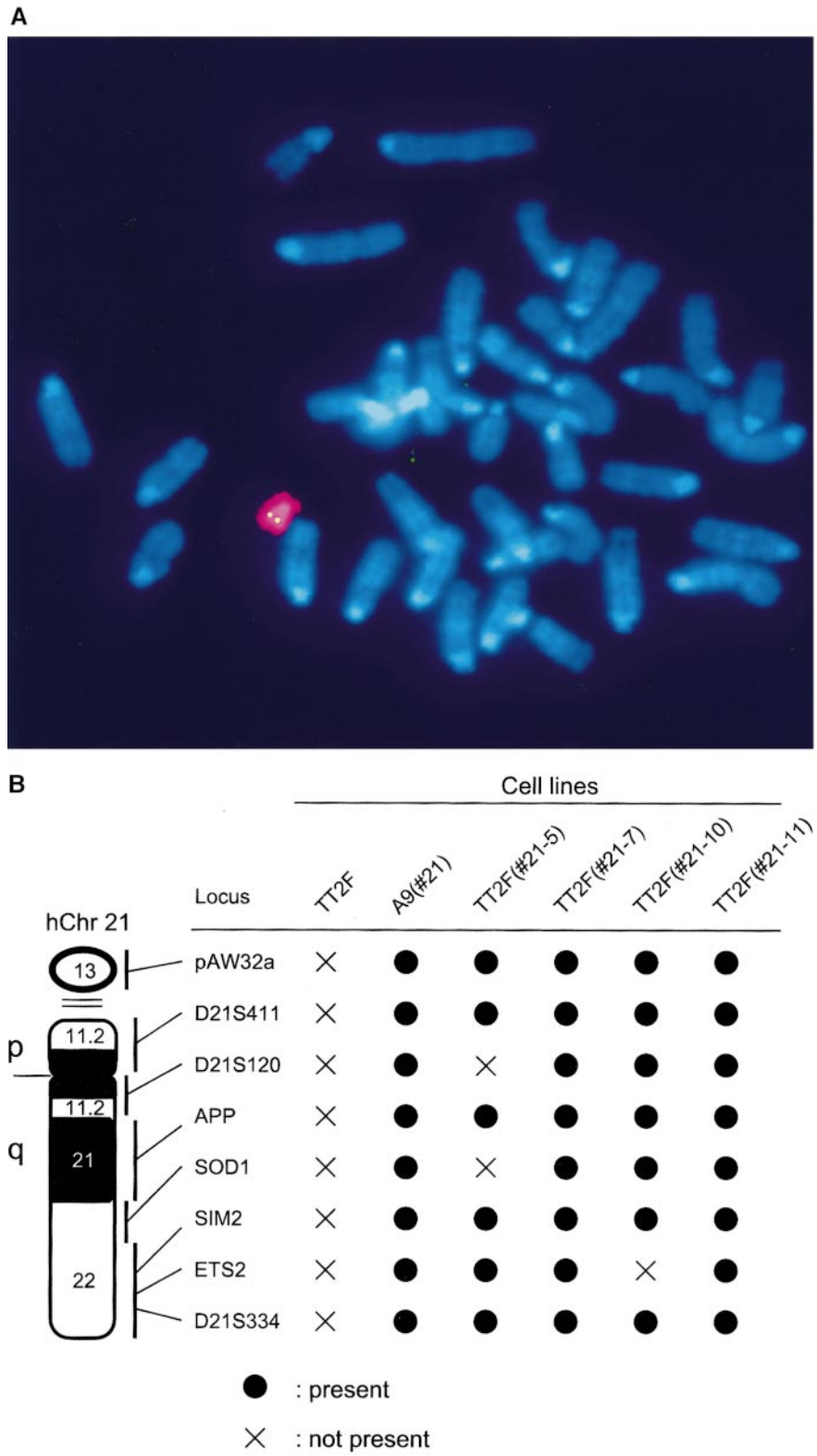

FIG. 1. FISH analysis of TT2F(#21-10) cells with a human specific probe and a summary of the regions of hChr 21 retained by various cell lines. (A) A photograph of metaphase spreads of TT2F(#21-10) cell line, hybridized with human COT-1 (red) and pST*neo* probes (yellow). The integrated pST*neo* plasmid on hChr 21 in TT2F(#21) was mapped to 21q22.2. Mouse chromosomes were counterstained with DAPI (blue). (B) Summary of the region of hChr 21 retained by various cell lines. Dots denote the retention of the corresponding hChr 21 loci indicated on the left side of the figure, based upon the detection of appropriately sized bands by PCR with human specific primers. Absence of human loci is shown with an X, which indicates a deletion in hChr 21.

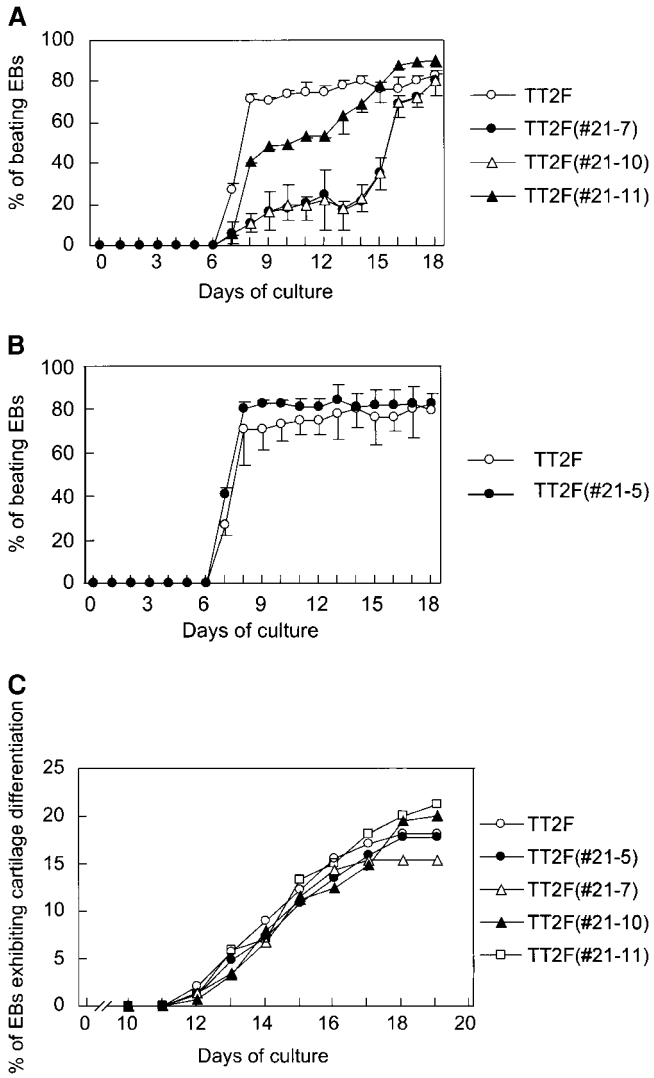

FIG. 2. Percentages of EBs differentiating to beating cardiomyocytes or cartilage *in vitro*. (A) Percentages of EBs exhibiting cardiomyocyte differentiation. The EBs derived from wild type ES cells (TT2F) and ES cells containing a hChr 21 [TT2F(#21-7), TT2F(#21-10), and TT2F(#21-11)] were used to monitor differentiation into cardiomyocytes. TT2F(#21-10) was used previously to generate chimeric mice carrying a hChr 21. The data points are shown as means (bars, standard deviation) ($n = 3$). (B) Percentages of EBs exhibiting cardiomyocyte differentiation. EBs derived from wild type ES cells (TT2F) and ES cells containing a hChr 21 [TT2F(#21-5)] were used to monitor differentiation into cardiomyocytes. The data points are shown as means (bars, standard deviation) ($n = 3$). (C) Percentages of EBs exhibiting cartilage differentiation. EBs derived from ES cells used in A and B were used to monitor the differentiation into cartilage. The data points are shown as means ($n = 3$).

Differentiation into skeletal muscle also occurred in a similar manner in all these cell lines, although the frequency of forming the skeletal muscle was much lower than that of cardiac myocyte and cartilage in this system (data not shown). These results are consistent with the fact that TT2F(#21-10) cells could contribute with a high ratio to the organs *in vivo*.

Taken together, we concluded that the hChr 21 specifically delays the maximum differentiation of beating cardiac myocytes in this *in vitro* system.

Rescue of Impairment of in Vitro Differentiation into Cardiac Myocytes in TT2F(#21) Cells by the Coculture with Wild-Type ES Cells during EB Formation

Next, we examined how an additional hChr 21 in ES cells delays the maximum differentiation into cardiomyocytes and we investigated why ES cells containing a hChr 21 can develop into adult heart *in vivo*, although TT2F(#21) cells have a defect in cardiogenesis *in vitro*. To mimic the chimerism level in adult hearts, 1:1 or 1:9 mixtures of wild type ES and either TT2F(#21-10) or TT2F(#21-11) were cocultivated during the formation of EBs. As shown in Fig. 3A, EBs derived from a 1:1 mixture differentiated into beating cardiac myocytes in essentially the same fashion as EBs derived from wild type ES cells, while the maximum levels of differentiation were delayed in EBs derived from the 1:9 mixture. This result suggests that, if the distribution ratio of wild type ES cells in EBs is 50%, the EBs carrying a hChr 21 could normally differentiate into cardiac myocytes. Although it is not clear whether ES cells containing a hChr 21 differentiate to cardiac myocytes with the same timing as wild type ES cells in the same EBs, this result is consistent with the findings that ES cells containing a hChr 21 could contribute to the adult chimeric hearts at the ratio of approximately 50%.

To investigate which factor(s) cause the difference in cardiomyocyte differentiation between ES cells with or without a hChr 21, we cocultured two EBs in a single well, one derived from wild type ES cells and the other from either TT2F(#21-10) or TT2F(#21-11) cells. The time course of differentiation in EBs derived from each clone were much the same to those when EBs derived from each clone were cultured separately (Fig. 3B). This result suggests that the hChr 21 does not delay the maximum differentiation of cardiomyocytes in EBs by modulating the expression of soluble factors.

DISCUSSION

In this study we examined the role of an extra hChr 21 in ES cells during cardiogenesis using genetically altered ES cells. We found that the presence of a hChr 21 in ES cells results in a specific retardation of *in vitro* differentiation into cardiac myocytes, which was consistent with our predictions, which were based on the low contribution of ES cells containing a hChr 21 to heart tissue in chimeric mice. Based on the results of *in vitro* and *in vivo* differentiation of ES cells, we concluded that mice carrying a hChr 21 could be models for the heart defects seen in DS. Furthermore, we

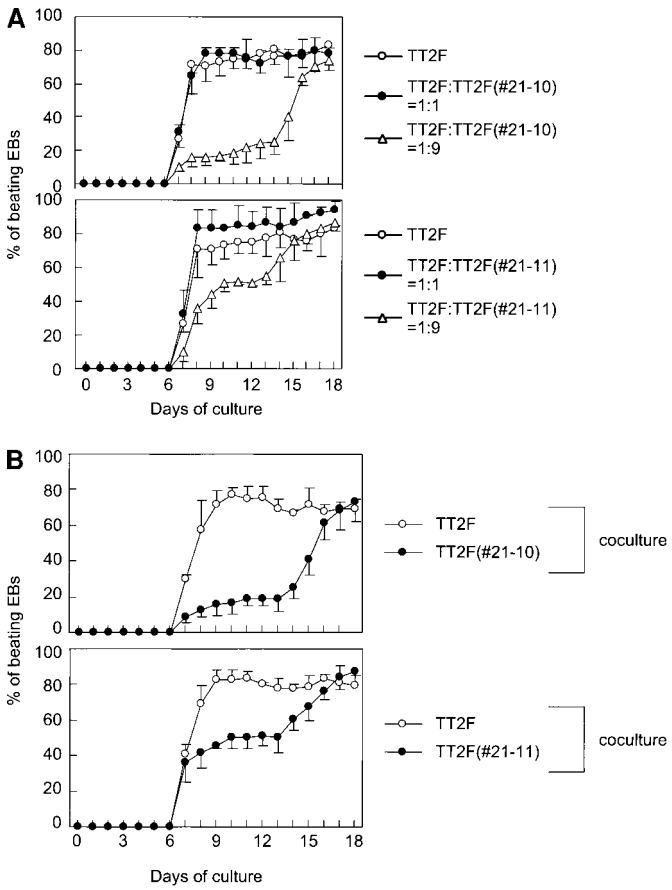

FIG. 3. Rescue of mutant phenotype by the coculture of wild type ES cells during the EB formation but not after the EB formation. (A) Percentages of EBs exhibiting cardiomyocyte differentiation. 1:1 or 1:9 mixtures of wild type ES and either TT2F(#21-10) or TT2F(#21-11) cells were cocultivated during the formation of EBs. The data points are shown as means (bars, standard deviation) (n = 3). (B) Percentages of EBs exhibiting cardiomyocyte differentiation. After the formation of EBs derived from TT2F, TT2F(#21-10) or TT2F(#21-11) separately, an EB derived from wild type ES and an EB derived from either TT2F(#21-10) or TT2F(#21-11) were cocultured in the same dish. The data points are shown as means (bars, standard deviation) (n = 3).

speculated that a delay in cardiogenesis during development caused by trisomy of hChr 21 could be one of the causes that leads to cardiovascular malformations and heart disease in babies with DS, and death of embryos with DS during pregnancy. It should be noted, however, that further study of *in vivo* differentiation in chimeric mice is required to demonstrate how a delay in the differentiation into cardiomyocytes leads to these phenotypes.

The next question to be addressed is the mechanism of how the hChr 21 in ES cells retards the maximum differentiation into cardiac myocytes. Although we found that the difference in the differentiation between wild type ES cells and ES cells containing a hChr 21 is not likely caused by soluble factors, the mechanism is largely unknown. Because EBs derived from equal amounts of wild type ES cells and TT2F(#21) cells could normally differentiate into cardiac myocytes, we speculate that the factors that are different between TT2F and TT2F(#21) cells may be signalling molecules that control neighboring cells in the development of cardiac myocytes, such as paracrine signaling factors or adhesion molecules. These genes could be on hChr 21 or could be downstream genes located on mouse chromosomes that are up- or down-regulated because of the presence of hChr 21. In this context, the gap junction protein connexin43 is an candidate because this molecule is known to be essential for normal heart formation and function (13, 14). Several transcription factor genes expressed in precardiac mesoderm, such as Cript-1, Nkx2.5, Mef2C, eHAND, and dHAND were predicted be responsible for the commitment of mesodermal cells to the cardiac lineage (10, 15). Thus, to approach the precise mechanism of how an extra hChr 21 impairs differentiation of mouse ES cells into cardiac myocytes, it is useful to compare the expression of these classes of genes in EBs derived from TT2F or TT2F(#21) cells.

Animal models that reflect human DS and systems to investigate DS *in vitro* have not yet been established. Although it remains to be determined whether the fragments of hChr 21 that delay the cardiogenesis *in vitro* correspond to those that induce cardiovascular malformations and other phenotypes of DS *in vivo,* the system described in this study has the potential to be a novel system to investigate DS *in vitro.* Furthermore, the chimeric mice from ES cells in this study should provide a novel and valuable model to examine DS *in vivo.* Thus, it would be possible to identify the critical genes for DS using these systems and extend this approach using gene targeting techniques in ES cells.

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