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Trisomy 12 in HESC Leads to No Selective In Vivo Growth Advantage in Teratomas, but Induces an Increased Abundance of Renal Development

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Abstract The aim of this investigation was to examine the impact of chromosome 12 amplification (tri-12 cells) in human embryonic stem cells (HESC), following in vivo engraftment to an immunodeficient xeno-model. For this we used sublines from the HESC line HS181, spontaneously exhibiting either low or high frequencies of tri-12 cells. Fluorescent in situ hybridization (FISH) analysis revealed a random distribution of tri-12 cells in the HS181 colonies in vitro. Similarly, the contribution of tri-12 cells to the development of various tissues in teratomas in vivo seemed to be fully random with no particular preference regarding in vivo differentiation pathway of tri-12 HS181 cells compared to HS181 cells with disomy 12 (di-12 cells). On the other hand, following in vivo transplantation the ratio of tri-12/di-12 cells was significantly reduced ($P < 0.001$), indicating a negative selection for this trisomy in vivo. Moreover, injection of HS181 cultures containing tri-12 cells resulted in a significantly increased abundance of areas compatible with renal formation ($P < 0.001$), relative teratomas derived from injection of di-12 HS181 cells. However, such areas included no increased relative frequency of tri-12 cells, suggesting indirect mechanism(s) for the increased abundance of renal development. The reasons for such developmental bias are unknown and warrant further investigation. J. Cell. Biochem. 100: 1518–1525, 2007.

Key words: HESC; trisomy; chromosome 12; teratoma; renal development

Though optimal in vitro culture conditions of human embryonic stem cells (HESC) are yet to be fully developed, it is today well established that HESC cultured under stringent conditions can exhibit a surprisingly stable chromosomal constitution also during extended in vitro culture [e.g., Brimble et al., 2004; Carpenter et al., 2004; Rosler et al., 2004], contrary to the experience from all other mammalian cell culture systems. However, it is also well established from several laboratories, including our own, that inadvertent genetic HESC variants can be found, concerning changes in several separate chromosomes, for example, 1, 5, 6, 8, 12, 13, 17, 18, 20, or X [Buzzard et al., 2004; Draper et al., 2004a; Inzunza et al., 2004; Pera et al., 2004; Hoffman and Carpenter, 2005; Mitalipova et al., 2005; Plaia et al., 2006; Imreh et al., 2006]. Long-term culture per se seems to enhance accumulation of mutations in the dividing cell population [Maitra et al., 2005] and the possibility of mutations is thus a concern particularly during expansion of HESC.

As previously reported, the HESC line HS181 has been repeatedly shown to exhibit the karyotype 46,XX, by Q-banding at passage 22 [Hovatta et al., 2003], with CGH at passage 39 [Inzunza et al., 2004], and by G-banding at passages 45 and 54 [Imreh et al., 2004]. However, following bulk expansions up to $>10^7$ cells a selective amplification of the whole chromosome 12 could be detected [Imreh et al., 2006], suggesting a growth advantage of tri-12

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cells. A selective advantage of amplified chromosome 12 for the propagation of undifferentiated HESC has been reported previously [Draper et al., 2004b]. Since the conditions in vitro differ substantially from the far more complex environment in vivo, results from phenotypic studies in vitro may differ compared to studies in vivo, and we wanted to evaluate the consequences of this specific genetic abnormality for HESC development in vivo. HESC can spontaneously differentiate into derivatives of all three germ layers in vivo [Thomson et al., 1998; Reubinoff et al., 2000]. We have previously described the ability of HESC cells to form organoid structures composed of tissue elements reminiscent of human organs, showing organization and orientation as indicated by morphology and tissue-specific markers [Gertow et al., 2004]. The study gives further support to the use of HESC teratoma formation as a relevant model, providing new insights of cellular interactions in early developmental stages.

MATERIALS AND METHODS

Ethical Permissions

This study was performed with the permission of the Local Ethics Committee at Karolinska Institute (114/00) and the Regional Committee of South Stockholm for Animal Experimentation (S 172-03).

Cells

The HESC line HS181 [Hovatta et al., 2003] and spontaneously arising sublines with chromosomal abnormalities were cultured in vitro, at indicated passage numbers, as previously described [Imreh et al., 2004]. In brief, cells were maintained in HESC medium consisting of 80% KnockOut-Dulbecco's Modified Eagle Medium (KO-DMEM), 20% KnockOut-Serum Replacement (KO-SR), 2 mM L-glutamine, 1% nonessential amino acids, 0.1 mM β-mercapoethanol, 4 ng/ml basic fibroblast growth factor (bFGF) (all from Invitrogen AB, Stockholm, Sweden) at 37°C, 6.8% CO_2 and high humidity. Commercially available human foreskin fibroblasts (CRL-2429; ATCC, Manassas) were used as feeder cells. The fibroblasts were cultured in IMDM, supplemented with 10% fetal bovine serum (FBS) (Invitrogen). The cells were mitotically inactivated by irradiation at 35Gy before seeding on a 6-well plate (Corning, NY) at a density of 2.1×10^4 cells/cm^2. The HESC culture medium was changed daily and the cells were passaged every 4–6 days by incubating in dispase enzyme (10 mg/ml) (Invitrogen) for 5–7 min at 37°C and mild mechanical splitting. The degree of spontaneous differentiation of HS181 cells was 10–15% in the in vitro cultures used for the experiments reported here.

Immunohistochemistry

Immunohistochemistry using antibodies for WT-1, CD34, and CD31 was performed on 5 μm thick sections from the paraffin-embedded teratomas. The sections were deparaffinized in xylene and rehydrated through a graded series of ethanol. Stainings were performed on a TechMate TM 500 Plus (DAKO Cytomation, Glostrup) using the ChemMate Detection Kit and Peroxidase/DAB (DAKO Cytomation) followed by hematoxylin counterstaining.

Cytogenetic Studies

Samples of cells were treated with colcemid KaryoMAX, 0.07 μg/ml (GIBCO, Paisley, Scotland) overnight. After washing, the cells were incubated in 0.4% trypsin solution (GIBCO) for 2–3 min. Cells were treated with 1,400 IU/ml collagenase (Worthington, Lakewood, NJ) at 37°C for 20 min and harvested using standard procedures. The metaphases were analyzed after Q-banding.

Fluorescent In Situ Hybridization (FISH)

The commercially available probe Vysis CEP 12 was used to verify the copy number of chromosome 12. The probe was treated according to the manufacturer's instructions, added to the chromosome preparations or deparaffinized (see IHC) tissue sections and covered by a 22×22 mm coverslip. The probe and target DNA were denatured for 8 min at 74°C. Hybridization was performed in a moist chamber at 42°C overnight. After hybridization, the slides were washed for 1 min in 1× SSC at 72°C and for 1 min in 2× SSC at room temperature and air-dried. The slides were mounted with Vectashield containing DAPI (4,6-diamino-2-phenyl-indole) (VectorLabs, Burlingame, CA).

Mice

Immunodeficient male, C.B.-17/GbmsTac-scid-bgDF N7 mice (6 weeks old), allowing the

xeno-grafting of HESC, were obtained from M&B (Denmark) and kept under isolator conditions in M2 cages on aspen wood chips (Beekay bedding, Scanbur B&K AB, Sweden) with free access to water and rodent diet (RM3(P)irr, Special Diet Services, UK), using artificial light from 6.a.m. to 6 p.m., room temperature $24 \pm 2^{\circ}C$, and humidity of $55 \pm 10\%$.

Xeno-Graft Model in Immunodeficient Mice

This was performed as described previously [Gertow et al., 2004]. Briefly, HS181 cells were harvested mechanically immediately prior to implantation and approximately 10^4 HESC were implanted beneath the testicular capsule of a young (6 weeks) SCID/Beige male mice (C.B.-17/GbmsTac-scid-bgDF N7, M&B) anesthetized using Isoflurane (Baxter). Teratoma growth was determined by palpation and the mice were sacrificed (cervical dislocation) 6.5–8.5 weeks post implantation. The tissue was fixed in 4% neutral buffered formaldehyde overnight, dehydrated through a graded series of alcohols to xylene, and embedded in paraffin.

RESULTS

Random Distribution In Vitro of Tri-12 Cells in Spontaneously Arising Mutant Sublines of HS181

Using FISH analysis with a chromosome 12 specific probe, it was possible to positively identify chromosome 12 disomic (di-12) or trisomic (tri-12) HS181 cells in the cultures. No differences in morphology between di-12 and tri-12 cells could be observed by light microscopy (data not shown), and the analysis of spontaneous mutant sublines of HS181 revealed a heterogeneous distribution of cells with positive signals. While some colonies were entirely trisomic (Fig. 1G,H), some were mixed disomic/trisomic (Fig. 1B, D, and F), or completely devoid of trisomic cells (Fig. 1A, C, and E). Merged colonies may arise from several smaller parts of disrupted colonies after passage, and the cells of the resulting new colonies may intermix (Fig. 1B,D), thereby supporting a random distribution of the tri-12 cells.

Increased Abundance of Areas Compatible With Renal Formation in Teratomas Generated From HS181 Cultures With Tri-12 Cells

HS181 subcultures with defined frequencies of tri-12 cells were injected under the testis capsule of immunodeficient mice and the resulting teratomas analyzed (Table I). Based on the present findings as well as previously found dominant tissue types in HS181 teratomas [Gertow et al., 2004], six major groups of tissues were identified and their respective prevalence evaluated: (I) bone/cartilage, (II) loose mesenchyme; (III) euronal, (IV) epithelial/gut, (V) kidney, and (VI) muscle. The relative proportions of various tissues were noted by visual observations on Hematoxylin and Eosin (HE) stained sections using a scoring system (see footnote of Table I), and a relative index for each tissue type was estimated for each teratoma. Next, the abundance of the tissues in the teratomas derived from tri-12 cells (#1–10 in Table I) were compared to teratomas from karyotypically normal, diploid

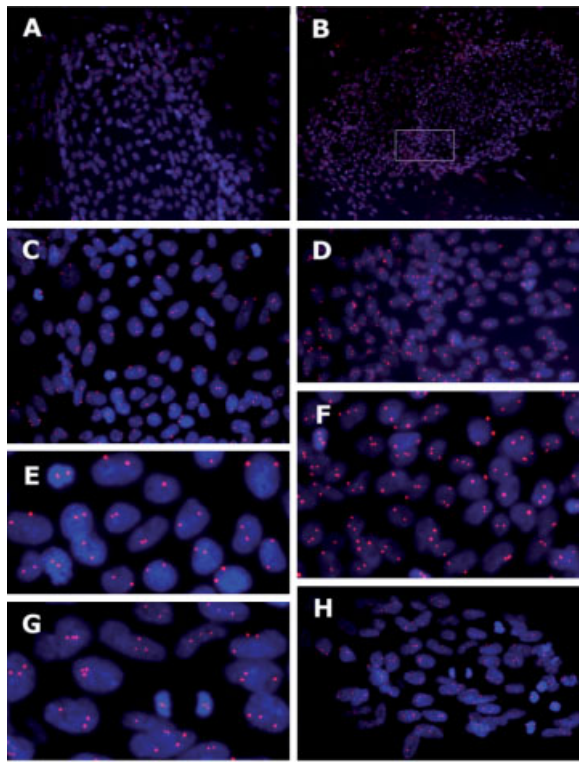

Fig. 1. Distribution of tri-12 cell in vitro, illustrated by FISH. HESC culture with a colony exhibiting a dominance of tri-12 cells (**A**), shown with increasing magnifications in (**C**) and (**E**). **B**: A colony in which the left side is trisomic and the right side disomic. This polarized appearance could possibly be due to merging of fragments from predominantly di- and trisomic colonies transferred from the previous passage. Boxed area in (B) is shown in a higher magnification in (**D**). A mixed contribution is seen in (**F**) and an example of a dominantly trisomic colony is shown in (**G**) and (**H**), where (G) is a magnification of (H). Original magnification of (A) and (D) = 20×; (B) = 10×; and (C) and (E–H) = 40×.

TABLE I. Relative Abundance of Tissues in HS181 Teratomas

No.	% Tri-12 (passage)	Teratoma harvested at day	(I) Bone/ cartilage[a]	(II) Loose mesenchyme	(III) Neuro	(IV) Epithelium and gut	(V) Renal	(VI) Muscle
#1	68 (p72)	D60	+++	+++++	++	+	+	+
#2	68 (p72)	D60	++	+++	++	++	+	−
#3	68 (p72)	D60	+++	+++	+++	+	++	−
#4	30 (p32)	D60	++	++++++	++	++	+	+
#5	30 (p32)	D60	++	++++	++++	+	+	+
#6	30 (p32)	D60	+	+++++	++	+	+	−
#7	14 (p36)	D45	−	+	++++++	+	+	−
#8	14 (p36)	D45	−	+	++++++	+	+	−
#9	14 (p36)	D45	−	+++	+++++	++	−	−
#10	0 (p18)	D42	++	++	+++++	++	−	−
#11	0 (p18)	D42	−	++	++++++	++	−	−
#12	0 (p18)	D52	+++	+++++	++	+	−	−
#13	0 (p49)	D65	−	++++++	+++	−	−	−
#14	0 (p49)	D65	++	++++	++++	+	+	+
#15	0 (p49)	D65	++	++++++	+	+	−	−
#16	0 (p49)	D75	+++	+++++	+	++	−	+
#17	0 (p49)	D66	+	++++++	+	++	−	+
#18	0 (p15)	D140	−	+++++	−	++++	−	+

−, 0%; +, 1–10%; ++, 11–20%; +++, 21–30%; ++++, 31–40%; +++++, 41–50%; ++++++, >50%.
[a]Visual evaluation of the relative area (% of total) for each type of tissue in teratoma sections.

HS181 cells (#10–18 in Table I). An overview of one teratoma is presented in Figure 2A. All teratomas were well encapsulated without any sign of invasiveness. The overall abundance of tissues and structures were strikingly similar between the di-12 and tri-12 groups. In line with previous findings by us [Gertow et al., 2004] and others [Thomson et al., 1998; Odorico et al., 2001; Heins et al., 2004; Przyborski, 2005], areas compatible with renal development were a rare finding from diploid HESC. Furthermore, in a separate ongoing study including nine separate HESC lines derived by our network, we found only 2/9 teratomas positive for renal development [Gertow et al., unpublished]. It was thus unexpected that the tri-12 containing HS181 teratomas showed a significant ($P < 0.001$, χ^2) higher abundance of areas compatible with renal development (8/9 teratomas #1–9 vs. 1/9 teratomas #10–18, in Table I). In addition, in each renal positive teratoma, there was a higher abundance of renal foci in the analyzed sections (Fig. 2A). Occurrence of renal development, including primitive glomeruli and ducts, was indicated with a morphology resembling a nephrogenic mesenchyme (Fig. 2B–L). Immunohistochemistry staining for protein of the Wilm's Tumor-associated gene (WT1) using monoclonal Mouse anti-human WT1 (DAKO, clone 6F-H2) supported the morphological appearance of renal development (Fig. 2G–I). WT-1-positive staining was found in the more immature regions such as metanephrogenic mesenchyme, while excluded from mature glomeruli and tubulus. Positive staining with CD34 and negative with CD31 within the glomerular areas indicated immature vascularization of the nephronic structures (Fig. 2E,F). As expected from renal development [Jin et al., 1991; Lahr et al., 1993; Nouwen et al., 1993], CD56 was intensely expressed in the mesenchyme (Fig. 2J). Furthermore, since renal differentiation involves a mesenchymal to epithelial conversion, the finding of both E-cadherin (Fig. 2K) and CK18 (Fig. 2L) in the tubular parts associated with the glomeruli was expected.

The findings in teratomas #7–9 with a high abundance of areas compatible with neural development and a low frequency of areas with development of loose mesenchymal tissue and muscle were most likely the result of the time point of harvest. These teratomas were harvested at day 45 after injection, at which stage neural development is prevailing and dominant, while loose mesenchyme and muscle development is sparse [Gertow et al. unpublished].

Tri-12 Cells Contribute Arbitrarily to Tissue Formation in the Teratomas, and the Ratio Tri-12/Di-12 Cells Is Reduced Following In Vivo Transplantation

Four teratomas (#1–3 and #6 in Table I) generated from HS181 cultures with tri-12 cells were subjected to a detailed analysis regarding

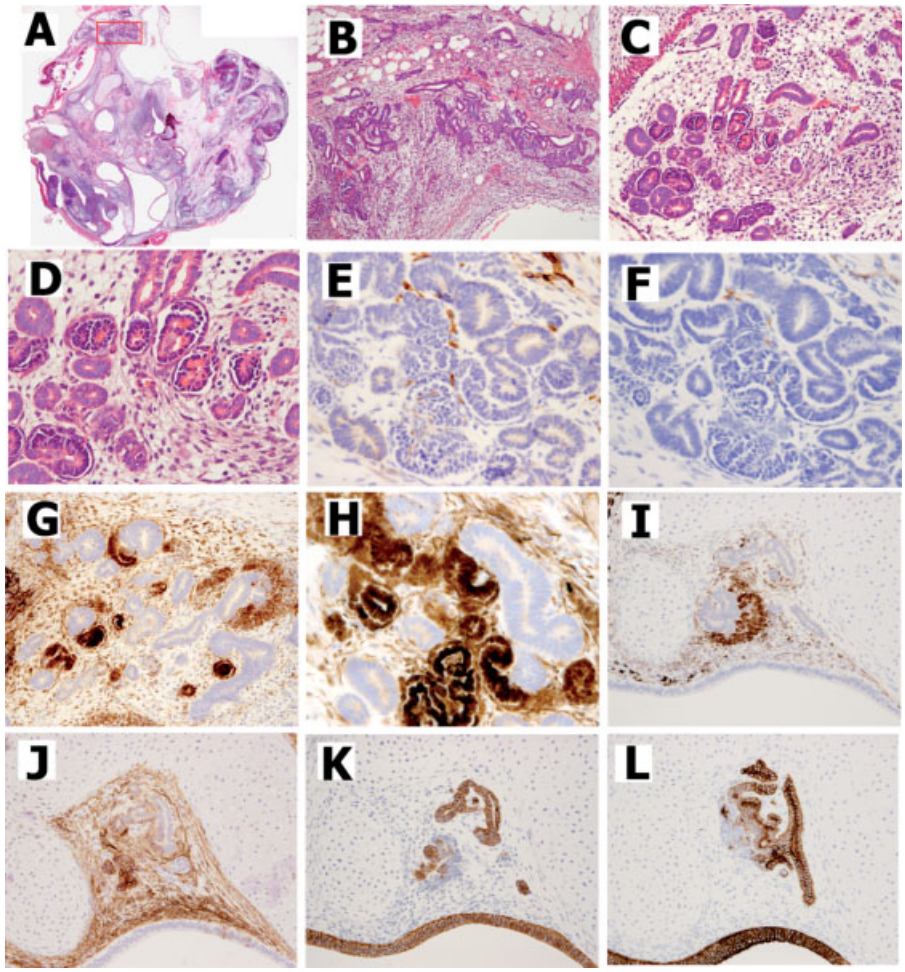

Fig. 2. Renal development. Hematoxylin and Eosin (HE) staining of a representative tri-12 containing HESC teratoma (**A**), from which one of the areas with development compatible with renal development is highlighted (box) and shown in higher magnification in (**B**). Similar development is further illustrated with increasing magnifications in (**C**) and (**D**). As seen in (**E**) immature human vascularization is indicated by positive CD34 (DAKO) staining showing structures with high resemblance of vascularized glomerulus and in (**F**) negative CD31 (DAKO) staining of the adjacent section. WT-1 staining (DAKO) supports the appearance of renal development showing a varying degree of maturity (**G** and **H**). **I–L**: Areas compatible with glomerulus development stained with WT1 (I), CD56 (J), E-Cadherin (K), or CK18 (L). Original magnification of (A) = 2×; (B) = 4×; (C) = 20×; (D–H) = 40×; (I–L) = 2×.

the proportional contribution of trisomic cells to the formation of various tissues. The frequency of tri-12 cells was evaluated for each of the six major groups of tissues identified (see above). Figure 3 shows representative data from FISH analysis of areas with cartilage development and the corresponding HE view. A centrally located cartilage of human origin (red) is seen in close apposition to the neighboring mouse tissue above (Fig. 3A,B). Individual cells of the cartilage are visualized in higher magnification in Figure 3C,D in which both disomic and trisomic cells can be seen. The neighboring mouse tissue is demonstrated in Figure 3E and magnified in Figure 3F. Only clearly separable cells with a distinct double or triple signal were included in the analysis and 4,000 cells were counted for each teratoma. A marked reduction of the total proportion of tri-12 cells (in all groups of tissues), relative to the frequency of tri-12 cells in the HESC population used for injection, was observed for all four teratomas analyzed. Teratomas #1–3 were derived from a HS181 population with 68% tri-12 cells and at day 60 after transplantation they exhibited a total frequency of tri-12 cells of 23.6%, 12.8%, and 26.8%, respectively ($P < 0.001$, χ^2). Similarly, teratoma #6 showed a significant reduction from 30% tri-12 cells in the injected HS181 population to a frequency of

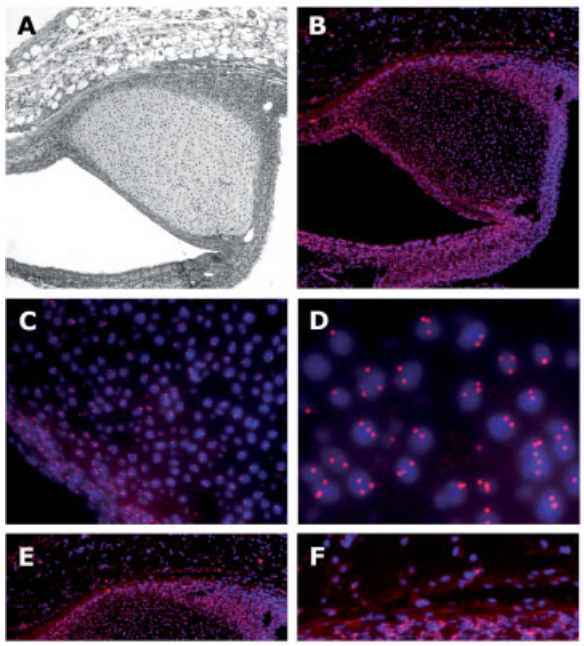

Fig. 3. Tri-12 contribution in vivo. Hematoxylin and Eosin (HE) staining of HESC teratomas showing a cartilage in close approximation to the mouse capsule (**A**) with FISH analysis of the corresponding area (**B**), and with increasing magnification shown in (**C**) and (**D**). The mouse tissue is shown to be negative in (**E**), with a higher magnification in (**F**). (Original magnification of (A–B) and (E–F) = ×10; (C–D) = ×20).

11.0% tri-12 cells in the teratoma tissue at day 60 ($P < 0.001$, χ^2). When the above-described six groups of identifiable tissues were evaluated separately in the analysis, no significant differences between the groups regarding frequencies of tri-12 cells were found (data not shown). Specifically, renal areas showed a range of 11.0–26.8% tri-12 cells (mean $18.3 \pm 11.7\%$), compared to a range of 10.9–31.8% (mean $18.8 \pm 7.3\%$) for all tissues analyzed (NS, χ^2). Thus, in spite of the clearly demonstrated increased abundance of renal formation in teratomas with tri-12 cells, no significant difference in the frequency of tri-12 cells was found for renal forming areas, compared to other tissues.

DISCUSSION

Similar to the findings in vitro, random variations were observed in vivo regarding occurrence of tri-12 cells in separate areas within the HESC teratomas. For instance, areas with cartilage formation could be completely devoid of, be more or less dominated by, or be composed of mixed populations of diploid and tri-12-cells. However, the range and means of tri-12 frequencies did not differ between different tissue types. We cannot rule out that there could be selective advantages for tri-12 cells in tissues other than the six types analyzed, or in subpopulations of cells, but our findings argue against a general growth advantage in vivo for cells trisomic for chromosome 12. On the contrary, although tri-12 cells clearly contribute to all studied tissue formation in the teratomas, there appears to be a significant selection against the persistence of tri-12 cells and their participation in the development following transplantation in vivo.

Another unexpected finding was the enhanced abundance of areas compatible with renal formation found in teratomas derived from HESC cultures with tri-12 cells. This result raised the unanticipated possibility that tri-12 cells could have a preference for renal development. If so, the tri-12 cells would be expected to dominate in the renal forming areas, and it was thus of particular interest to evaluate the contribution of tri-12 cells in such areas. However, this was clearly not the case. Similar to the observations in other tissues, also in the renal looking tissue the tri-12 cells were randomly distributed and their frequency was not elevated beyond the average of all tissues, that is, in fact it was identical. An alternative interpretation could instead be that tri-12 cells are able to support, or enhance, the induction of other cells toward renal differentiation. It must be noted though that although trisomy of chromosome 12 was validated as the only detected abnormality using Q-banding, we have not excluded involvement of other more minor genetic, or epigenetic, changes in the tri-12 cells.

A possible causal link between increased abundance of renal development and tri-12 cells awaits explanation. Amplification of chromosome 12 is a consistent clinical finding in germline tumors [Looijenga et al., 2003], thus providing an indirect link of the amplification of chromosome 12 with diseased urogenital development. There are, however, to our knowledge no data published suggesting a link between the early normal urogenital development and genes on chromosome 12. As an alternative, we hypothesize an indirect mechanism of gene dosage from genes on chromosome 12 to promote the development of intermediate mesoderm, favoring urogenital development and for

some reason renal development in particular. Recombining metanephric mesenchyme with dorsal neural tubes in culture is well known to induce development of renal tubules. Since neural development and tissues are among the dominating components of HS181-derived teratomas [Gertow et al., 2004], we would currently favor a model where reciprocal interactions between the metanephric mesenchyme and neural cells potentially result in renal differentiation and development. This line of thinking finds additional support from recent findings of Darr et al. [2006] who found increased expression of NANOG (chromosome 12) upon the early primitive ectodermal differentiation from HESC, possibly leading to further enhancement of the neural compartment and the proposed inductive effect by neural cells on metanephric mesenchyme.

In summary, our study underlines differences between in vitro and in vivo effects on phenotype, resulting from karyotype variations. Importantly, the findings reported here highlight the importance of HESC mutant sublines for studies on human development. An advantage of amplification of chromosome 12 has previously been indicated for the propagation of HESC in vitro [Draper et al., 2004a; Imreh et al., 2006], but surprisingly tri-12 cells were here shown to be selected against following transplantation in vivo. Furthermore, the findings of increased abundance of areas compatible with renal development from cultures containing tri-12 cells warrant a deeper analysis on a causal link between renal development and genes on chromosome 12. Like previously suggested [Imreh et al., 2006], exploring genetic integrity and phenotype analysis of HESC mutant variants could reveal important knowledge also on the process of tumor transformation and progression.

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