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FOCAL ADHESION KINASE IS NOT ESSENTIAL FOR *in vitro* AND *in vivo* DIFFERENTIATION OF ES CELLS

Dusko Ilic[1,2,3], Yasuhide Furuta[1,3], Toshio Suda[4], Tadao Atsumi[1], Jiro Fujimoto[2], Yoji Ikawa[1], Tadashi Yamamoto[2] and Shinichi Aizawa[1,3,*]

[1]Laboratory of Molecular Oncology, Tsukuba Life Science Center, The Institute of Physical and Chemical Research (RIKEN), 3-3-1 Koyadai, Tsukuba, Ibaraki 305, Japan

[2]Department of Oncology, The Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minatoku, Tokyo 108, Japan

[3]Department of Morphogenesis and [4]Department of Cell Differentiation, Institute of Embryology and Genetics, Kumamoto University School of Medicine, 2-2-1 Honjo, Kumamoto 860, Japan

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Focal adhesion kinase, FAK, is a unique protein tyrosine kinase found in cellular focal adhesions. It is widely expressed and highly phosphorylated during embryogenesis. To examine the function of FAK in cell differentiation, we made FAK-deficient embryonic stem (ES) cells by homologous recombination. However, FAK-deficiency did not interfere with differentiation of the ES cells into cells of three germ layers when implanted subcutaneously into nude mice or when treated with retinoic acid *in vitro*, nor was there any evidence of defects in hematopoiesis *in vitro*.

Protein phosphorylation on tyrosine is one of the major regulatory mechanisms of cellular activities. Studies of the tyrosine phosphorylation during development revealed a large number of proteins temporally tyrosine-phosphorylated (1). The tyrosine phosphorylation was detected in all embryonic tissues at high levels during the early stages of development. Two of the most prominent such proteins had molecular weight of 70 and 120 kD. They have not yet been identified, but recent studies have suggested that both of them are focal adhesion proteins (2,3). Paxillin may represent a major phosphotyrosine-containing protein of 70 kD and FAK protein of 120 kD.

* To whom correspondence should be addressed.

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Reorganization of cytoskeleton is essential in cell growth, division and differentiation during embryogenesis. It generates and maintains morphology and movements of cells that underlie morphogenesis. Cell architecture is regulated by cell-cell and cell-extracellular matrix (ECM) interactions. Focal adhesions are areas of the plasma membrane by which a cell attaches to ECM. Integrins mediate cell-ECM interactions to cytoskeletal structures (4). The question of how integrins regulate cytoskeletal organization, and the interaction of focal adhesion proteins with cytoplasmic domains of integrin molecules is currently of intense interest. Tyrosine phosphorylation of proteins may be one of the main regulatory mechanisms in the interactions. For example, treatment with the protein tyrosine kinase inhibitor, herbimicyn A, inhibited the formation of focal adhesions (5).

FAK itself is a non-receptor protein tyrosine kinase that is unique in having no SH2 or SH3 domains, myristylation site or homology with any other known non-receptor protein tyrosine kinase (6). It is widely expressed in embryonic tissues and localized in cellular focal adhesions (6-9). An increasing number of *in vitro* observations have suggested that FAK mediates signaling from ECM through integrins and plays an important role in the organization of cytoskeleton (5,7,8,10), but no direct functional analyses have been reported. As an initial step in clarifying the role of FAK in cell-ECM interactions during embryogenesis, we examined here the effects of FAK-deficiency in the processes of cell differentiation from embryonic stem (ES) cells that are considered equivalent to inner cell mass (ICM). Unexpectedly, however, we observed no evidence of defects in cell differentiation with FAK-deficiency.

Materials and Methods:

Isolation of FAK genomic DNA: A DNA fragment spanning the 987^{th} to 1846^{th} bp positions of murine FAK cDNA (7) was used to screen a genomic library of TT2 ES cells (11) in which DNAs were cloned into the SalI site of λ cloning vector λFixII (Stratagene). Hybridization to nitrocellulose filters (Schleicher & Schuell) containing replicas of bacteriophage plaques was performed under highly stringent conditions (50% formamide; 4 x SSC; 50 mM HEPES, pH7.0; 10 x Denhardt's solution; 100 μg/ml salmon sperm DNA) as described (12). From 6 x 10^5 plaques screened, three independent clones were identified. The phages were purified, and SalI insert was recloned into pBluescript II SK vector (Stratagene) and mapped for restriction enzyme sites.

Vector construction: The *pgk-1 neo*^{r} cassette lacking a polyadenylation signal was inserted into the AfI III site after conversion into the BgI II site, located in the exon spanning the 1442^{th} to 1525^{th} bp position of the murine FAK cDNA (7). Negative selection for homologous recombinant was made with HSV-TK gene at the 5' end and DT-A gene at the 3' end of the targeting vector (13-15). For electroporation, the targeting vector was linearized with XhoI.

Cell culture and selection: Primary cells were cultured from 14-16 day old embryos of the neomycine resistant YF4 strain of mice, and feeder cells were prepared as described (16). ES cells used were TT2 established from an F1 embryo between a C57BL/6 female and a CBA male (11), and cultured on the feeder cells with ES medium [DMEM, 20% FCS; 10^4 units/ml leukemia inhibitory factor (LIF) (AMRAD, ESGRO); 10^{-4} M β-mercaptoethanol; nonessential amino acid (Flow Lab.); sodium pyruvate (Flow Lab.)]. 1 x 10^7 cells singly suspended in 0.5 ml HBS (25 mM HEPES, pH 7.05; 137 mM NaCl; 5mM KCl; 0.7 mM KCl; 0.7 mM Na_2HPO_4; 6 mM dextrose) were electroporated with 12 nM linearized DNA using a gene pulser (250 V, 960 μF) (Bio-Rad). The cells were selected against 150 μg/ml G418 (GIBCO) and 0.2 μM FIAU (Bristol Meyers), and resistant colonies were screened for homologous recombinants by Southern blot analysis. ES cells homozygous for FAK mutation were obtained by selecting FAK heterozygous ES cells against 5 mg/ml G418 for 10-14 days (17).

Southern blot analysis: DNAs were extracted and Southern blot analyses were performed as described (12).

RT-PCR: Poly(A)$^+$ RNAs were isolated using magnetic beads with a covalently bound oligo(dT) sequence (Dynal) and transcribed by reverse trancriptase ("SuperScriptII", GIBCO BRL) with the oligo dT primer. The products were serially diluted and subjected to PCR in 50 μl reaction mixtures [0.2 mM each dNTP (Pharmacia); 1.5 mM $MgCl_2$; 50 pM each primer; 10 mM TRIS/HCl, pH 9.0; 50 mM KCl; 0.1% Triton X-100; 2.5 U Taq DNA polymerase (Promega)] at 30 cycles (45 s at 94°C, 25 s at 55°C and 3 min at 72°C). Sequences of primers used were: A, 5'-TCAACAGGTGAAGAGTGACTA-3'; B, 5'-GCAGCCTTTGCTTGTCAGGTA-3'; C, 5'-CCAGGGATTATGAGATTCAGA-3'; D, 5'-GACACCAGAACATTCCGAGCA-3'; E, 5'-CCTGCAGACAGCTACAATGAG-3'; F, 5'-TGGCCGTGTCTGCCCTAGCAT-3'; N, 5'-CAGGATGATCTGGACGAAGA-3'. The PCR products were analyzed by 8% polyacrylamide gel electrophoresis (PAGE). The RT-PCR assays for *Brachyury*, activin β_B, collagen α IV, GATA-1 and HPRT expressions were performed semiquantitatively as described (18).

Western blot: Cells were washed with 1mM Na_3VO_4 in PBS and lysed in RIPA buffer (1% NP-40; 150 mM NaCl; 10 mM TRIS/HCl, pH 7.4; 1mM EDTA; 0.1% SDS; 0.1% DOC; 1mM Na_3VO_4; 50 mM Na_2MoO_4; 1 mM NaF; 1mM PMSF; 1 μg/ml aprotinin; 1 μg/ml leupeptine). 10 μg of whole lysates per lane was run on 8% SDS-PAGE and transferred to nitrocellulose membrane (Schleicher & Schuell). The membrane was blocked with 5% skim milk in Tween/TBS and treated with mouse monoclonal 2A7 anti-FAK antibody (UBI) (19) and AP-conjugated goat anti-mouse IgG. Blots were stained with 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium (GIBCO).

In vivo and *in vitro* differentiation: To generate teratoma, 2 x 10^6 cells in 200 μl PBS were injected subcutaneously into 5 week old male CD-1 nude mice (Charles River, Japan). Tumors were dissected after 3 to 4 weeks, fixed in formalin and stained with hematoxylin-eosin.

To induce differentiation by retinoic acid (RA), ES cells were cultured with LIF-free ES cell medium supplemented with 0.3 μM RA. To induce embryoid body formation, the cells were cultured in dishes coated with poly(2-hydroxyethyl methacrylate) (Sigma) to prevent cell attachment to substrate. Hematopoiesis was examined by culturing the ES cells on OP9 feeder cells as described (20).

Results and Discussion:

FAK gene disruption

The FAK gene was disrupted in TT2 ES cells by homologous recombination (11,15,21) (Figure 1). The targeting vector used contained 10 kb of FAK genomic DNA. A sequence encoding the

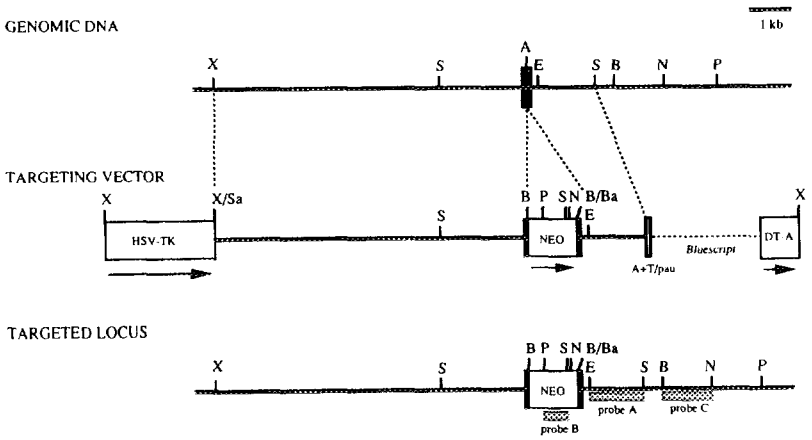


Figure 1. Targeting of FAK allele. Top: restriction map of the FAK locus. Only relevant sites are indicated. Black box indicates targeted exon spanning 1442th to 1525th bp position. X, XhoI; S, SphI; A, AflIII; E, EcoRI; B, BglII; N, NcoI; P, PstI. Middle: map of targeting vector. AflII site was converted into BglII site and *neo*^{r} gene with *pgk-1* promoter lacking poly(A)$^{+}$ signal was inserted into this site. HSV-TK, thymidine kinase cassette; A + T/pau, mRNA destabilizing sequence and pausing signal; DT-A, diphtheria toxin cassette. Arrows indicate orientation of inserted cassettes. Bottom: predicted map of the mutated allele following homologous recombination with targeting vector. Shaded boxes indicate location of fragments used as probes in Southern blot analyses (probes A, B and C).

beginning of the kinase domain (7) was disrupted by insertion of a 3-phosphoglycerate kinase gene promoter (22) and a neomycine phosphotransferase (*pgk*-1 *neo*^{r}) expression cassette without poly(A)$^{+}$ signal positioned in the same orientation. Herpes simplex virus thymidine kinase (HSV-TK) and diphtheria toxin A (DT-A) cassettes were placed on the 5' and 3' ends, respectively, to increase the frequency of homologous recombinants by negative selection (13-15). After selection with G418 and 1-(2-deoxy-2-fluoro-β-D-arabino-furanosyl)-5-iodouracil (FIAU), 340 double resistant colonies were picked up and screened by Southern blot analysis using a probe inside of the targeting vector (probe A) after digestion with SphI (Figure 2a). This should detect 4.1 kb product in the wild type allele and 2.0 kb in the targeted allele. The analysis suggested that ten clones were homologous recombinants, and these were further analyzed by digesting with Bgl II, Nco I and Pst I. They should yield 3.5, 3.7 and 5.5 kb bands, respectively, from the targeted allele. Including the analyses with probe B, inside the *neo*^{r} gene (Figure 2b), and probe C, outside of the targeting vector (Figure 2c), the results were consistent with the homologous nature of recombinations without any random integration in all ten clones.

Two heterozygous recombinant clones (+/-) (#103 and #257) were randomly chosen to isolate homozygous mutant clones (-/-) by culturing them at high concentrations of G418 (17). Homozygous mutants were obtained at the frequency of 17/19 in clone #103 and 1/38 in clone #257 after selection at 5.0 mg/ml G418 (Figure 2a). The remainder retained the normal allele intact with rearrangement of the mutated allele (Figure 2d). No difference was found in the following observations among homozygous mutant clones thus obtained, and no specification was made about which clone was used in each experiment.

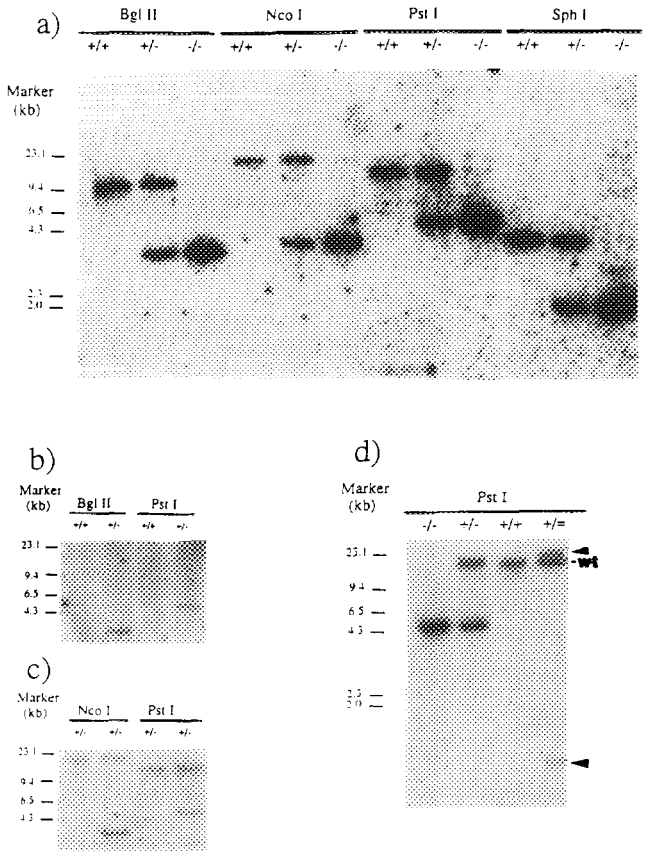


Figure 2. Southern blot analyses of targeted ES clones. (**a**) Southern blot analysis using a probe outside of the targeting vector (probe A) Genomic DNAs from TT2 parental ES cells (+/+), targeted clones (+/-) and double targeted clones (-/-) were digested with BglII, NcoI, PstI or SphI. SphI digestion generates 4.1 kb product in the wild type allele, but the precise locations of BglII, NcoI and PstI sites in the 5' upstream of wild type genomic DNA are unknown; they were out of the genomic clone used. SphI digestion generated 4.1 kb product in the wild type. Recombinant alleles yielded 3.5, 3.7, 5.5 and 2.0 kb hybridizing bands after BglII, NcoI, PstI and SphI digestion, respectively. (**b**) Southern blot analysis using a probe in the *neo*^{r} cassette (probe B). DNAs from wild type ES cells (+/+) and recombinant clones (+/-) were digested with BglII and PstI. Recombinant allele generated bands of expected sizes, 3.5 and 5.5 kb, respectively. (**c**) Southern blot analysis using a probe outside of targeting vector (probe C). Digests of DNAs from wild type ES cells (+/+) and recombinant clones (+/-) with NcoI and PstI yielded 3.7 and 5.5 kb bands, respectively. (**d**) Southern blot analysis with probe A for homozygous mutant clones after PstI digestion. Heterozygous #257 (+/-) clone yielded two types of recombinants after culture at a high concentration of G418. Disruption of both FAK alleles occurred in only one clone (D257-8), but others preserved wild type allele with the identical pattern of change (arrows) in recombinant allele; an example is shown on D257-10 (+/=). +/+ indicate parent TT2 cells.

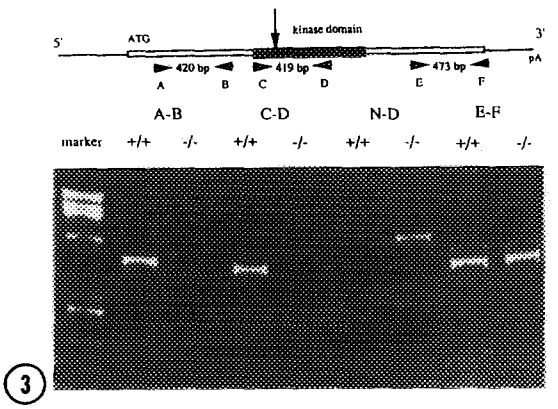
Null nature of disruption

FAK-homozygous mutant cells did not show any change in cell growth or cell attachment to feeder cells or gelatin-coated dishes (data not shown), suggesting a non-essential role of FAK in undifferentiated state. To confirm the inactivation of the FAK gene, Northern blot analysis was

first made on RNAs from wild type, heterozygous and homozygous mutant ES cells. Not only was no 4.5 kb normal transcript detected but neither were any truncated products in homozygous mutants (data not shown). To verify this, RT-PCR analyses were performed with several primers on homozygous mutants (Figure 3). No amplification encompassing the site of *neo*^{r} gene integration was observed with primers C and D as expected, nor was any evidence of transcription found on the 5' upstream region of the integration site using primers A and B. The integration might disrupt the transcriptional machinery, or such product might be unstable and short-lived. The amplification with primers E and F on the 3' region of the *neo*^{r} integration site yielded products of two different sizes even in homozygous mutants. However, these were viewed as the transcripts in fusion with the *neo*^{r} gene directed by *pgk-1* promoter of this gene, since the gene did not have a polyadenylation signal. Indeed, the PCR encompassing *neo*^{r} gene and the 3' side with primers N and D did yield amplifications. The nature of the two different transcripts is unknown, but their difference could be due to alternative splicing since there are several exons in this region. In any event, such transcripts would not be translated because of the presence of the stop codon at the 3' end of the *neo*^{r} gene. Western blot analysis on cell lysates of each genotype of ES cells using monoclonal anti-FAK antibody (19) also did not reveal any product in the homozygous mutant ES cells (Figure 4).

Retinoic acid - induced differentiation

RA induces differentiation of ES cells that may be equivalent to relatively earlier steps of ICM-differentiation *in vivo* (23). The possible effect of FAK-deficiency on cell differentiation was first



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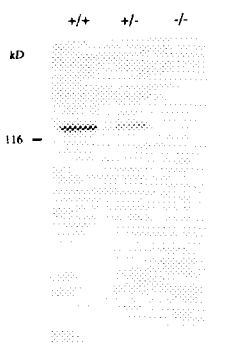

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Figure 3. RT-PCR analysis for null nature of FAK gene disruption. Top: map of FAK cDNA. ATG, start of translation; A, B, C, D, E, F, and N indicate locations of primers used; arrowheads, orientation of primers; an arrow, place of the *neo*^{r} cassette insertion. Bottom: RT-PCR analysis of cDNAs from wild type (+/+) and FAK-deficient ES cells (-/-) with indicated primers. DNA size marker is ϕx174-HaeIII digest.

Figure 4. Western blot analysis. 10 μg of cell lysates from wild type (+/+), heterozygous (+/-) and homozygous mutant (-/-) ES cells were run in each lane, and FAK protein (125 kD) was detected with monoclonal anti-FAK antibody 2A7 (19).

tested with this system. The molecular markers of cell differentiation used (18) were T gene (*Brachyury*) for primitive mesodermal cells (24), activin β_B for primitive ectodermal cells (25), collagen α IV for primitive endoderm (26) and GATA-1 for the primitive hematopoiesis (27). The analysis was performed by semi-quantitative PCR using hypoxanthine phosphoribosyl transferase (HPRT) expression as standard (18). No apparent change in the expression of either of these markers was found with FAK-deficiency (Figure 5).

Differentiation of hematopoietic cells

Efforts have been made to set up a differentiation system in a specific lineage of cells *in vitro* from ES cells, and this was successful for hematopoietic cells (20). Effects of FAK-deficiency on hematopoiesis were then examined with this system. FAK-deficient ES cells differentiated into erythroid cells, neutrophiles, megakaryocytes and mastocytes, showing no evidence of defects in hematopoiesis (Figure 6).

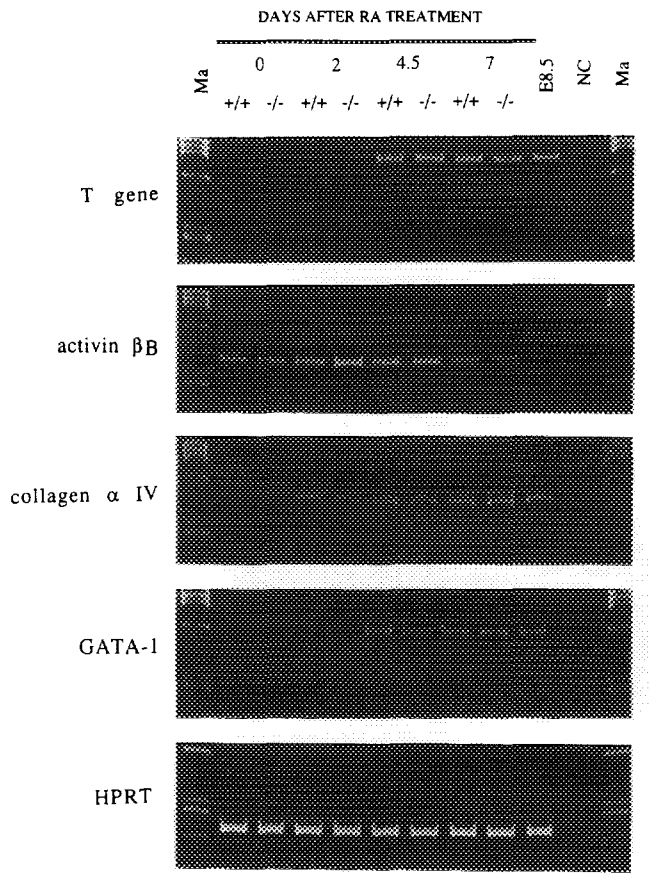

Figure 5. Expression of marker genes by RA-induced differentiation. DNA size marker (Ma) is ϕx174-HaeIII digest; +/+, normal ES cells; -/-, FAK-deficient ES cells; E8.5, 8.5-day normal B6 mouse embryos used as positive control; NC, negative control (no cDNAs).

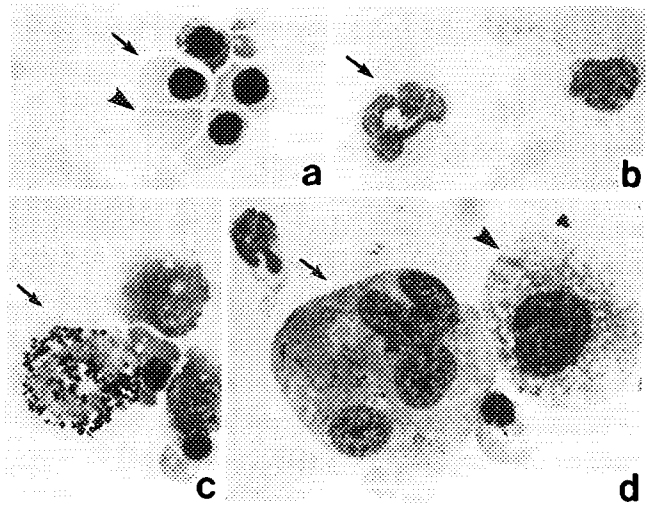

Figure 6. Apparently normal hematopoiesis from FAK-deficient ES cells. FAK-deficient ES cells differentiated into (**a**) megakaryocyte, (**b**) mast cell, (**c**) neutrophil and (**d**) erythroblast (all are indicated by arrows) as normal ES cells did in the presence of the stromal cells OP-9 (20). Arrowheads indicate a stromal cell in (a) and enucleated erythroid cell in (d).

Differentiation through embryoid body formation

An alternative method of ES cell differentiation *in vitro* is embryoid body formation after aggregation of the cells by suspension culture. Cystic embryoid bodies thus formed are considered to represent peri-primitive streak stage embryos (28). FAK-deficient ES cells yielded

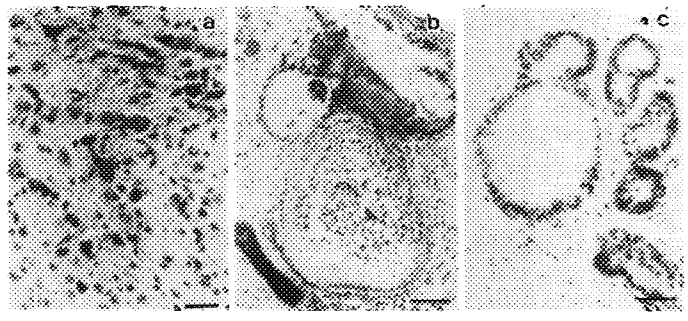

Figure 7. Examples of tissue differentiation in subcutaneous teratomas derived from FAK -/- ES cells. (**a**) Mature neuronal tissue as an example of ectodermal derivative. (**b**) Examples of mesodermal tissues. A cartilaginous nodule with ossificated area (indicated by arrows) is shown in the lower center and a longitudinal section of muscle fibers is seen at upper left. (**c**) An example of endodermal component; secretory tubules composed of columnar epithelial cells containing numerous goblet cells. No differences in variation or amount of these tissues were found among teratomas originated from FAK -/- , +/- and wild type ES cells. Bars indicate 25μm in (a), and 50μm in (b) and (c), respectively.

embryoid bodies normally. No difference was observed in their size during 7 day culture in suspension, and the embryoid bodies formed were histologically typical cystic ones (data not shown).

In vivo differentiation of FAK-deficient ES cells

TT2 ES cells could differentiate into a variety of cells of the three germ layers yielding teratomas when transplanted subcutaneously into nude mice. Effects of FAK-deficiency on cell differentiation were next tested by this system which may represent relatively terminal differentiation (16). No difference was found by FAK-genotype in outgrowth of teratomas when $2x10^6$ cells were transplanted. Furthermore, FAK-deficient ES cells differentiated into derivatives of each germ-layer, such as neuronal, cartilaginous and columnar epithelial tissues, without any apparent difference from heterozygous or wild type ES cells (Figure 7).

Prospect:

The significance of the present observations should be confirmed by generating FAK-deficient mice. Analyses of the mice will be reported elsewhere.

Acknowledgments:

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