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Short Contribution

The generation and *in vivo* differentiation of murine embryonal stem cells genetically null for either N-cadherin or N- and P-cadherin

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ABSTRACT Many mutations of the murine genome are recessive embryonic lethals precluding phenotype analysis at subsequent stages of development. This is true for embryos genetically lacking either N-cadherin or N- and P-cadherin. To circumvent this, we have generated pluripotent embryonal stem (ES) cells of the same genotype *in vitro* and differentiated them *in vivo* in the form of teratomas. All of the ES cells isolated in this study had a normal ES cell morphology *in vitro* and were able to generate teratomas. Histological analysis revealed that some differentiation and histogenesis had occurred within the teratomas. Epithelial formation was, for example, unaffected in all cadherin null cells. Surprisingly, however, the differentiation of cells lacking both N- and P-cadherin was, in general, even more pronounced both quantitatively and qualitatively. Tumours lacking either N- cadherin or N- and P-cadherin contained more striated muscle (apparently cardiac muscle) than heterozygote controls, and this was most strikingly conspicuous in teratomas from N- and P-cadherin null cells. This more pronounced differentiation was not seen for all tissues, however, as structures with a simple neural tube-like morphology were never found in teratomas lacking both N- and P-cadherin and organoid-like structures were rare in Ncad-/-tissue.

KEY WORDS: *teratoma, cadherin, ES cells*

Embryonal stem (ES) cells are pluripotent stem cells derived from the inner cell mass of the preimplantation blastocyst (Evans and Kaufman, 1981). The cells can be propagated *in vitro* in an apparently undifferentiated euploid state upon a heterotypic feeder layer of fibroblasts. This pluripotent state is maintained by several cytokines released by the feeder layer (e.g. Piquet-Pellorce *et al.,* 1994). Upon cytokine withdrawal ES cells spontaneously differentiate, initially recapitulating the first stages of normal embryonic differentiation but eventually forming derivatives of all the three primary germinal layers of the embryo. ES cells can also be inoculated into a histocompatible host, where they form a complex and polymorphic tumour termed teratoma (Evans and Kaufman, 1981). Histologically, the differentiated cells within a teratoma are those which ultimately originate from all three embryonic germ layers of a normal embryo and although differentiation appears to be chaotic, it tends to occur in a clonal fashion so that identical cell types are clustered. In fact, a unique characteristic of teratomas is that the differentiated cells are often found in primitive organoid-like structures (Martin, 1980).

Cadherin cell adhesion molecules (CAMs) promote inter-cell adhesion in a strictly Ca^{2+} dependent and homophilic manner (Takeichi, 1995). The 'classical' cadherins include epithelial (E-), neural (N-) and placental (P-) cadherins and, as their names suggest, they tend to be tissue specifically expressed. N-cadherin, for example, is found in mesodermal and neuroectodermal-derived tissue (Hatta and Takeichi, 1986) and P-cadherin in extraembryonic tissues, uterine decidua and the proliferative basal layers of some stratified epithelia (Nose and Takeichi, 1986). Using ES cell technology, the mutation of most of the cadherin genes has been performed. Embryos homozygous null for N-cadherin develop to embryonic day 9.5 but then die because of a cell adhesion defect in the developing myocardium (Radice *et al.,* 1997b). Mice homozygous null for P-cadherin are viable and can be bred (Radice *et al.*, 1997a), but show a mild hyperplasia of the mammary epithelium. Furthermore, embryos homozy-

Abbreviations used in this paper: ES, embryonal stem; MEFs, murine embryonic fibroblasts.

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gous null for both N- and P-cadherin have more severe morphological perturbations when compared to that seen for the N-cadherin null embryo alone (G.L. Radice, unpublished observations).

After mating mice inheriting recessive and null mutations of N- or P-cadherin and culturing their blastocysts *in vitro,* we have generated pluripotent ES cells genetically null for either N-cadherin (i.e., Ncad -/-) or both N- and P-cadherin (i.e., NPcad -/-). Contrary to the P-cadherin null condition, where embryos are capable of differentiating into all lineages, either Ncad -/- or NPcad -/- embryos are non-viable. Hence, to avoid the lethal phenotypes and analyse differentiation in the absence of cadherin CAMs, teratomas were derived from Ncad -/-, NPcad -/- and heterozygous control ES cells and histologically examined.

The candidate ES cells isolated were genotyped by Southern blot or PCR (data not shown). ES cells with all of the expected genotypes were generated (Table 1), but because of the relatively small numbers of clones involved we cannot yet confidently say that all genotypes were recovered at a Mendelian frequency. Phenotypically, all of the ES cells generated in this study were morphologically indistinguishable from wild-type ES cells (data not shown) suggesting that E-cadherin, the predominant cadherin synthesised by ES cells, is exclusively responsible for the uniquely adherent phenotype of ES cells. Furthermore, all ES cell genotypes inoculated were able to proliferate and form teratomas over a roughly similar time span (Table 2). Surprisingly, none of the teratomas ever caused a mortality or metastasised within the hosts (despite inoculation near lymph nodes), perhaps due to the fact that the ES cells still expressed E-cadherin.

Histological analysis clearly indicated that some differentiation and histogenesis had occurred within the teratomas. In these respects, the mutant ES cells in this study are different from E-cadherin -/- ES cells, which are unable to form organised structures within teratomas (Larue *et al.*, 1996). Furthermore, as illustrated in Figure 1, many of the structures formed within the teratomas appeared to be well adherent. The most predominant constituents of Ncad -/- tumours were cells which, for simplicity, we have termed 'light' and 'dark' cells. Light cells (Fig. 1A) were diffusely spread throughout the tumour and formed no characteristic structures, but had a strong histological resemblance to CNS white matter. For example, the nuclei of light cells showed anisonucleosis and poorly stained chromatin, and the nucleoli were not visible. Cytoplasms were not well defined but appeared to be composed of a palely stained fibrillar material (Fig. 1A, insert). Light cells were seen in equal quantity in both Ncad -/- and in +/- tumours. In contrast, dark cells were smaller, their nuclei were darkly stained and the cytoplasms were even less well defined (Fig. 1B). The cells were mostly found in either a rosette like pattern (arrowheads in 1B) or formed structures resembling primitive neural tubes (arrow in 1B). Dark cells forming rosette like structures had uniformly elongated, darkly stained nuclei and the cells were arranged in a circular fashion. The central part of the rosette was pinkish and slightly fibrillar and the lumen was either very small or not visible. Primitive neural tubes were larger than rosettes, typically oval in shape (Fig. 1B), and were composed of two different parts; inner cells which surrounded a well defined lumen composed of several rows of dark cells and an outer part composed of more cuboidal cells. Dark cells were also seen in clusters where they formed no obviously recognisable structures. Both rosettes and primitive neural tubes were found in Ncad +/- and -/- teratomas but both were less numerous in Ncad +/- than in -/- tumours. These findings are notable because even in the absence of N-cadherin some differentiation towards simple neural structures was possible, a phenomenon also seen in vertebrate and invertebrate N-cadherin mutants where early events of neural patterning occur (Iwai *et al.,* 1997; Radice *et al.,* 1997b).

Both Ncad +/- and -/- tumours contained very well differentiated and polarised epithelial cells, typically lining a cyst in an organoid-like structure (Fig. 1C). For example, we observed structures aligned by either squamous epithelium or cylindrically ciliated epithelium containing some goblet cells. This is perhaps not too surprising as E-cadherin, still present within the cells used in this study, is known to be essential for epitheliogenesis (Marrs *et al.,* 1995). Islands of cartilage were also found and, especially in Ncad -/- tumours, bundles of muscles were seen (Fig. 1D). The muscles had cross striations and relatively large centrally located nuclei resembling cardiac muscle (Fig. 1D, insert). More organoid structures were found in Ncad +/- tumours compared to Ncad -/- tumours. In tumours from both lines, one could detect small nests of completely undifferentiated cells which were located in close proximity to organoid structures.

Dark and light cells were also observed in tumours originating from NPcad -/- ES cells. Dark cells had the same morphology as described above (Fig. 1E), but they generated mostly rosette like structures and never primitive neural tubes. Additionally, there were also small clusters of dark cells with no tendency to form any of the above mentioned structures although these were relatively rare in NPcad -/- in comparison to Ncad -/- tumours. The islands of light cells had a different morphology from those described above from Ncad -/- teratomas. The vast majority of light cells were more differentiated and had a clear neuronal like morphology. In some parts of the tumour they were arranged in tandem rows (Fig. 1F). These light cells were larger in size than their counterparts in Ncad -/- tissue and had

TABLE 1

FREQUENCY OF ES CELL GENOTYPES ISOLATED

(i) After crossing N-cadherin +/- mice *inter se*

ES cell genotype	N-cadherin +/+ (Ncad +/+)	(Ncad +/-) N-cadherin +/-	N-cadherin -/- (Ncad -/-)
Frequency	1	7	4

(ii) After crossing P-cadherin -/-; N-cadherin +/- mice *inter se*

ES Cell Genotype	P-cadherin -/-; N-cadherin +/+	P-cadherin -/-; N-cadherin +/-	P-cadherin -/-; N-cadherin -/- (NPcad -/-)
Frequency	2	5	2

TABLE 2

SUMMARY OF TERATOMA INDUCTION

Genotype of ES cell line	Number of independent ES cell lines inoculated	Number of teratomas/ Number of of hosts	Average time taken to form a teratoma of 1.5-2 cm diameter (days)
Ncad +/-	3	8/9	21
Ncad -/-	4	15/15	23
NPcad +/-	1	2/3	28
NPcad -/-	2	7/9	36
All		32/36 (88.8%)	

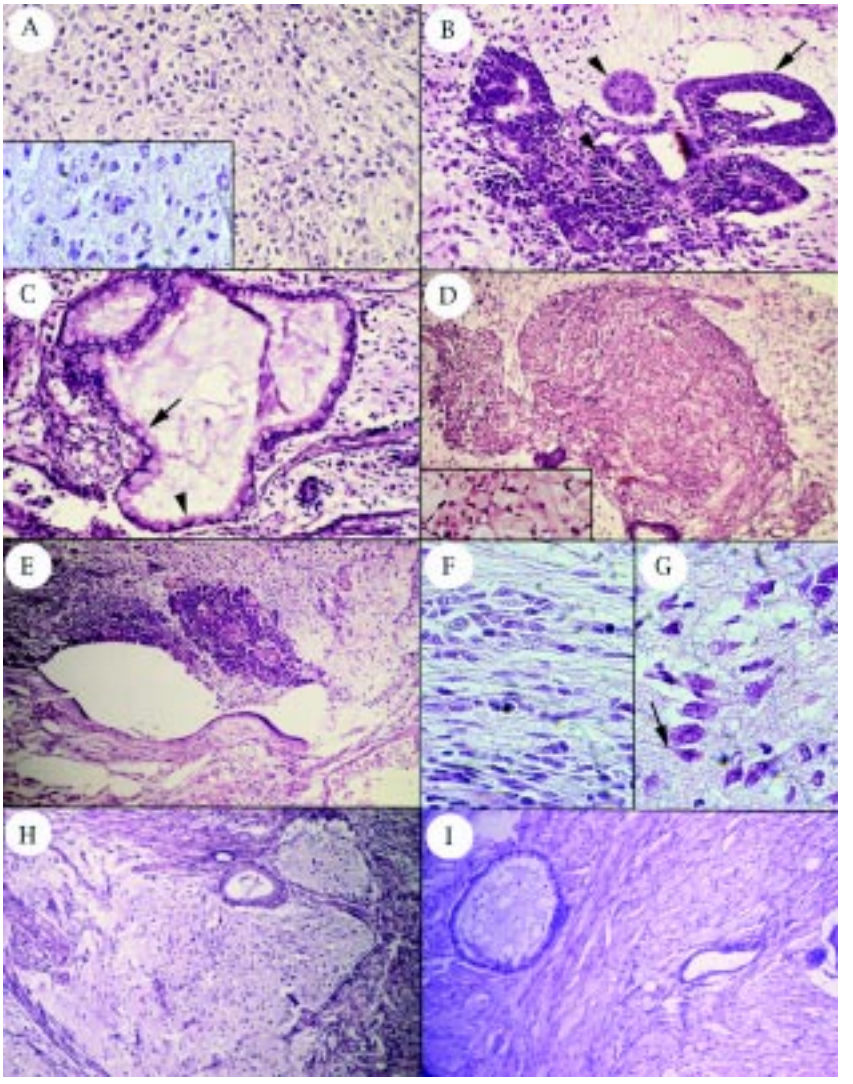

Fig. 1. Histological characterisation of teratoma tissue. *The micrographs are representative examples of morphogenesis occurring in Ncad -/- (A to D) and NPcad -/- (E to I) teratomas.* **(A)** *The light cells predominate in a Ncad -/- tumour. They have pale nuclei and not well defined cytoplasm (which looks like pale fibrillar material between nuclei). Insert shows light cells at a higher magnification. x250 (insert x1000).* **(B)** *The second predominant component of Ncad -/- tumours are the dark cells, which were found in either a rosette like pattern (arrowheads) or forming structures like primitive neural tubes (arrow). x250.* **(C)** *A cystic organoid structure found in a Ncad -/- tumour. This particular organoid appeared to be a gland-like structure which, as it surrounded a cavity, appeared to be specialised for secretion. It was composed of secretory cells with diffusely stained cytoplasm located towards the lumen (arrowhead) and goblet cells. Both cell types had nuclei which were, characteristic of epithelial, basally located. Cilia (arrow) were also located on the luminal surface. x250.* **(D)** *Bundles of striated muscle were seen in Ncad -/- tumours. Note that the cells are quite compact and not diffusely arranged. Insert shows a higher magnification of the striated muscles which resembled cardiac muscles. x160 (insert x450).* **(E)** *A very small cluster of dark cells which also formed rosette like structures was found in NPcad -/- tumours. x160.* **(F)** *Primitive neuronal differentiation of light cells in NPcad -/- tumours. Cells are arranged tandemly, typical of a CNS lamina. x1000.* **(G)** *Light cells were larger than in Ncad -/- tumours, with well defined cytoplasm and a fine process (arrow). x1000.* **(H)** *Small parts of the NPcad -/- tumours were composed of light cells which were intermingled between muscle bundles. x160.* **(I)** *An organoid structure and bundles of muscles found in a NPcad -/- tumour. x250.*

a well defined cytoplasm from which a fine process emanated (arrow in Fig. 1G). Additionally, the nuclei were smaller and the chromatin was more condensed and therefore darker stained. This enhanced differentiation is a very surprising finding, as one would have expected removal of cadherin CAMs to perturb, not enhance morphogenesis. This observation is, however, not without precedent as β1 integrin -/- ES cells show an accelerated neuronal differentiation (Rohwedel *et al.,* 1998). The light cells were intermingled between muscle bundles, which were the most predominant constituent of tumours originating from NPcad -/- ES cells (Fig. 1H). Although we cannot conclusively identify the precise type of muscle formed within these tumours, it had the typical histological characteristics of cardiac muscle. Beneath the muscle bundles within NPcad -/- teratomas there were a lot of organoid structures (Fig. 1I) with gland differentiation, squamous differentiation, salivary gland differentiation, cartilage and bone marrow structures. Also between bone trabeculi one could detect bone marrow cells. Collectively, the extent of differentiation of these organoid structures was much greater than that seen in Ncad -/- tumours. In NPcad -/- tumours one also found small clusters of undifferentiated cells in close proximity to epithelial structures.

In both Ncad -/- and NPcad -/- tumours we observed an excess of striated muscle in comparison to heterozygote controls. Notably, a mild hyperplasia of the virgin mammary epithelium was observed in P-cadherin null mice (Radice *et al.,* 1997a). The fact that P-cadherin is typically expressed in the undifferentiated proliferative layers (Nose and Takeichi, 1986) suggests that it may prevent cells from prematurely differentiating. Furthermore, two reports have described that introduction of N-cadherin into either CHO (Levenberg *et al.,* 1999) or BHK (Redfield *et al.,* 1997) cells resulted in cell cycle withdrawal. Levenberg *et al.* (1999) demonstrated that the cyclin/cyclin-dependent kinase inhibitor p27 was elevated in N-cadherin transfected CHO cells. Hence, in the absence of either N- or N- and P-cadherin, the activity of cyclin-dependent kinases would not be repressed, allowing cell cycle progression.

ES cells can now be recovered from murine blastocysts at a 100% success rate (Brook and Gardner, 1997). This implies that the generation of murine ES cells which are compound mutants for many genes will become a reality. The ability of such cells to uniformly differentiate along a developmental pathway, autonomously in the absence of any exogenous influence, is desired particularly as cells with apparent ES cell like characteristics have now been isolated

from human embryos (Thomson *et al.,* 1998). Our observations of the differentiation of cadherin deficient ES cells may be useful in this respect, and may eventually be applied to somatic cell therapy within a mouse model system.

Experimental Procedures

ES cell isolation

In order to generate N-cadherin -/- ES cells, mice heterozygous for the N-cadherin mutation (Radice *et al.,* 1997b) were mated *inter se*. To generate N-cadherin -/-; P-cadherin -/- ES cells, a line of mice homozygous mutant for P-cadherin (Radice *et al.,* 1997a) but heterozygous for the N-cadherin mutation was established and crossed *inter se*. Heterozygote control ES cells (N-cadherin +/-; P-cadherin +/-) were made by crossing P-cadherin -/- male with N-cadherin +/- female mice followed by the genotyping of individual cell lines. N-cadherin heterozygote and homozygote null ES cells were given the prefix Ncad +/- and Ncad -/-, respectively. ES cells containing mutations of both N- and P-cadherin genes were given the prefix NPcad. For example, cells lacking both N- and P-cadherin were termed NPcad -/-, whereas cells heterozygote for both genes were termed NPcad +/-.

ES cells were isolated from embryonic day 3.5 blastocysts as described by Nagy *et al.* (1993) (day of vaginal plug was taken as 0.5). Briefly, flushed blastocysts were added to a single 1 cm diameter well containing X-ray irradiated MEFs and 0.5 ml R1 medium (Nagy *et al.,* 1993). After 4 days the wells were trypsinized and re-plated (passage number 0). ES cell colonies were typically visible after 6 days and clones were expanded by repeated passage every 2 days. All embryos in this study were the result of natural matings and all of the mice used to generate them had a 129/Sv genetic background. DNA for genotyping was extracted from ES cells growing upon gelatinised plates (without MEFs). The N-cadherin genotype was assigned by Southern blot analysis (using probe N1) (Radice *et al.,* 1997b) and the P-cadherin genotype by PCR (using primers P11F and P11R) (Radice *et al.,* 1997a).

Teratoma induction and histological analysis

To induce teratomas, ES cells ($3x10^7$) were trypsinized and preaggregated in a bacteriological dish *in vitro* in R1 medium for 2 days. The ES cells were then washed with PBS and resuspended in 1.5 ml of PBS. Three virgin 129/Sv male mice served as hosts and each received a single unilateral subcutaneous injection at the flank with 0.5 ml of this suspension (approximately 10^7 cells). Hosts were sacrificed and teratomous growths were retrieved when they had reached a diameter of 1.5-2 cm. The time between inoculation and tumour retrieval was noted. DNA was extracted from random biopsies of the teratomas in order to confirm the genotype. Teratomas were fixed by immersion in 4% paraformaldehyde overnight at 4°C. A complete gross examination of the host was performed. The tumours were then embedded in paraffin and sectioned at 3 μm, stained with Mayer´s hematoxylin and eosin (H&E) and examined by conventional light microscopy. All magnifications stated on the figure legend are original magnifications.

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References

BROOK, F.A. and GARDNER, R.L. (1997). The origin and efficient derivation of embryonic stem cells in the mouse. *Proc. Natl. Acad. Sci. USA 94:* 5709-5712.

EVANS, M.J. and KAUFMAN, M. (1981). Establishment in culture of pluripotential cells from mouse embryos. *Nature 292:* 154-156.

HATTA, K. and TAKEICHI, M. (1986). Expression of N-cadherin adhesion molecules associated with early morphogenetic events in chick development. *Nature 320:* 447-449.

IWAI, Y., USUI, T., HIRANO, S., STEWARD, R., TAKEICHI, M. and UEMURA, T. (1997). Axon patterning requires *D*N-cadherin, a novel neuronal adhesion receptor, in the *Drosophila* embryonic CNS. *Neuron 19:* 77-89.

LARUE, L., ANTOS, C., BUTZ, S., HUBER, O., DELMAS, V., DOMINIS, M. and KEMLER, R. (1996). A role for cadherins in tissue formation. *Development 122:* 3185-3194.

LEVENBERG, S., YARDEN, A., KAM, Z. and GEIGER, B. (1999). p27 is involved in N-cadherin-mediated contact inhibition of cell growth and S-phase entry. *Oncogene 18:* 869-876.

MARRS, J.A., ANDERSSON-FISONE, C., JEONG, M.C., COHEN-GOULD, L., ZURZOLO, C., NABI, I.R., RODRIGUEZ-BOULAN, E. and NELSON, W.J. (1995). Plasticity in epithelial cell phenotype : modulation by expression of different cadherin cell adhesion molecules. *J. Cell Biol. 129:* 507-519.

MARTIN, G.R. (1980). Teratocarcinomas and mammalian embryogenesis. *Science 209:* 768-776.

NAGY, A., ROSSANT, J., NAGY, R., ABRAMOW-NEWERLY, W. and RODER, J.C. (1993). Derivation of completely cell culture-derived mice from early passage embryonic stem cells. *Proc. Natl. Acad. Sci. USA 90:* 8424-8428.

NOSE, A. and TAKEICHI, M. (1986). A novel cadherin cell adhesion molecule: its expression patterns associated with implantation and organogenesis of mouse embryos. *J. Cell Biol. 103:* 2649-2658.

PIQUET-PELLORCE, C., GREY, L., MEREAU, A. and HEATH, J.K. (1994). Are LIF and related cytokines functionally equivalent? *Exp. Cell Res. 213:* 340-347.

RADICE, G.L., CELESTE FERREIRA-CORNWELL, M., ROBINSON, S.D., RAYBURN, H., CHODISH, L.A., TAKEICHI, M. and HYNES, R.O. (1997a). Precocious mammary gland development in P-cadherin-deficient mice. *J. Cell Biol. 139:* 1025-1032.

RADICE, G.L., RAYBURN, H., MATSUNAMI, H., KNUDSEN, K.A., TAKEICHI, M. and HYNES, R.O. (1997b). Developmental defects in mouse embryos lacking N-cadherin. *Dev. Biol. 181:* 64-78.

REDFIELD, A., NIEMAN, M.T. and KNUDSEN, K.A. (1997). Cadherins promote skeletal muscle differentiation in three-dimensional cultures. *J. Cell Biol. 138:* 1323-1331.

ROHWEDEL, J., GUAN, K., ZUSCHRATTER, W., JIN, S., AHNERT-HILGER, G., FÜRST, D., FÄSSLER, R. and WOBUS, A.M. (1998). Loss of β1 intgerin function results in a retardation of myogenic, but an acceleration of neuronal, differentiation of embryonic stem cells *in vitro. Dev. Biol. 201:* 167-184.

TAKEICHI, M. (1995). Morphogenetic roles of classic cadherins. *Curr. Opin. Cell Biol. 7:* 619-627.

THOMSON J.A., ITSKOVITZ-ELDOR, J., SHAPIRO, S.S., WAKNITZ, M.A., SWIERGIEL, J.J., MARSHALL, V.S. and JONES, J.M. (1998). Embryonic stem cell lines derived from human blastocysts. *Science 282:* 1145-1147.

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