CHANGES IN THE EARLY DIFFERENTIATION PATHWAY OF MONOPARENTAL EMBRYONIC STEM CELLS IS NOT AFFECTED BY THE EXPRESSION OF LIF RECEPTORS

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Parthenogenetic and androgenetic embryos do not reach term due to the process of genomic imprinting, an epigenetic modification of the DNA that results in parental-specific gene expression (Fundele and Surani, 1994). This phenomenon is being widely analysed by using embryonic stem cells (ES cells) derived from the epiblast of blastocysts at the time when such genome modification is supposed to occur. The restricted distribution of parthenogenetic and androgenetic ES cells in tissues has been studied in chimeras with normal host cells and in teratomas formed by ectopic transfer (Mann *et al.*, 1990; Allen *et al.*, 1994). However, considering the dynamic nature of epigenetic changes during early development and the instability of imprinting in ES cells, it is particularly interesting to examine their earlier differentiation pathways.

We analysed parthenogenetic (PK1), androgenetic (AK1) and biparental (AB1) cells with a series of differentiation markers before and after induction of differentiation *in vitro* by culturing them in a medium without Leukemia Inhibitory Factor (LIF). Markers used were: alkaline phosphatase (AP) and anti-SSEA-1 for undifferentiated cells (Johnson *et al.*, 1977; Fox *et al.*, 1981), anti-SSEA-1 and TROMA antibody (anti-cytokeratin 8; Kemler *et al.*, 1981) for differentiation to visceral endoderm, TROMA and anti-vimentin for parietal endodermal (Lehtonen *et ál* .,1983) and anti-vimentin for mesodermal cells (Franke *et al.*, 1982).

Results and Discussion

Biparental ES cells were positive for alkaline phophatase activity and anti-SSEA-1 staining (Fig. 1c and 1d). The same markers of undifferentiation were observed in parthenogenetic ES cells. However, some PK1 cells show cytokeratin staining, which is in agreement with our previous results with parthenogenetic embryos. On the other hand, androgenetic ES cells were highly heterogeneous in showing their AP activity, with colonies of cells completely negative. Similarly, AK cells did not react with anti-SSEA-1 antibodies (Fig. 1a and 1c). The absence of a common antigen of ES cells, such as SSEA-1, and the heterogeneity in the AP activity suggest that AK1 cells suffer from an early misregulation critical for the maintenance of the undifferentiated state. This effect may be responsible for the abnormal proliferation of extraembryonic tissues and the practical absence of embryonic tissues observed in androgenetic embryos (Surani *et al.*, 1990). The meaning of the presence of cytokeratins in the undifferentiated PK1 cells is not so clear since these filaments seem not to have a function in early embryos (Uranga *et al.*, 1995).

After 6 days of culture *in vitro* without LIF, biparental (AB1) and parthenogenetic (PK1) ES cells lose their positivity for anti-SSEA-1 and, together with AK1, acquire a network of cytokeratins positive for the TROMA-1 antibody. The AB1 cells clearly differentiate into endoderm (mainly parietal) with some of them becoming mesoderm. However, PK1 and AK1 cells differentiate into parietal endoderm-like cells, with just a few expressing vimentin. In the same way, very few become mesoderm (see Table for a summary of results). This observation may indicate that there is a failure in the regulation of the endoderm differentiation *in vitro*, probably due to the absence of some factor expressed by the mother, since *in vivo* embryos develop both endodermal cell lineages at the same time. Furthermore, it seems that monoparental embryos lack the ability to express vimentin properly and even to develop into mesoderm. In this sense, Newman-Smith and Werb (1995) reported the fact that parthenogenetic embryos differentiate *in vitro* almost exclusively into parietal endoderm, and Sturm *et al.* (1994) found that embryonic lineages, such as mesoderm, are especially affected in parthenogenotes *in vivo*. Here we show that the very early differentiation pathway of androgenetic cells is quite similar to that of parthenogenetic cells, although clear differences can be observed in the undifferentiated cell population. These differences regarding surface markers and enzyme activity

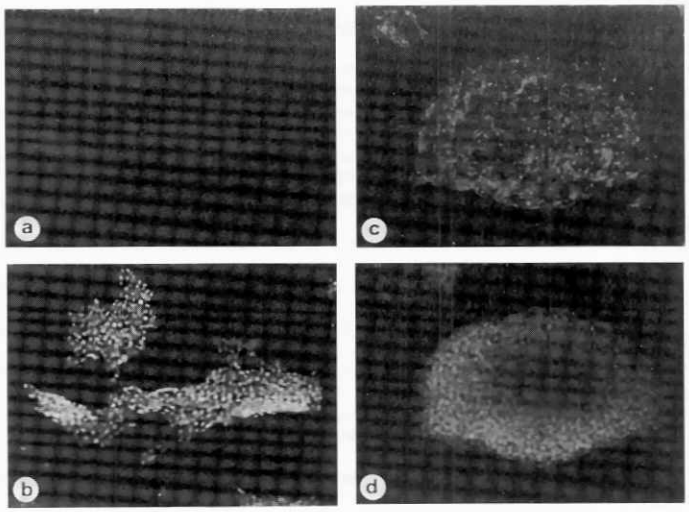

Fig. 1: AK1 and AB1 ES cells cultured with LIF. **a)** No staining for the anti-SSEA-1 antibody. **c)** Positive staining for the anti-SSEA-1 antibody. **b and d)** The same cells stained with DAPI. Magnification: 274x.

might be caused by a failure in the cascade of reactions induced by LIF since this cytokine is considered responsible for the manteinance of the undifferentiated phenotype in early embryogenesis.

		anti-SSEA-1	Alkaline Phosphat.	TROMA-1	anti-VIMENTIN
AK-1	Undiffer.	-	+/-	-	-
	Differ.	-	-	+	-
PK-1	Undiffer.	+	+	-	-
	Differ.	-	-	+	-
AB-1	Undiffer.	+	+	-	-
	Differ.	-	-	+	+

Table : Summary of results in the different cell lines assayed. Undiffer: undifferentiated cells; Differ: differentiated cells. +/-: heterogeneous activity.

Heterodimerization of low-affinity LIF receptor (LIFR) with the IL-6 receptor associated transmembrane protein gp130 is required for the biological activities of LIF since LIFR results in the formation of high-affinity binding sites. Absence of membrane-bound LIFR in mice eliminates binding of LIF with either low or high affinity (Mereau *et al.*, 1993). To investigate any misregulation in the expression of these receptors we marked undifferentiated AK1, PK1 and AB1 ES cells with anti-mouse LIF (Sigma) to detect LIF-bound LIFR, and with anti-gp130 (R&D) to localize the active complex. Surprisingly, there are no differences in the expression of both receptors in androgenetic cells as compared with parthenogenetic and biparental cells with staining restricted to cell membranes. This result is striking, it cannot be attributed to any factor different from LIF and absent from culture since biparental control cells were cultivated in the same medium and they show the normal undifferentiated phenotype. For this reason, we are working on the possibility that other maternally-expressed elements of the LIF-induced signalling pathway and different from the receptor complex must be involved in the manteinance of the stem cell state.

Materials and Methods

Cell lines were maintained in ES cell medium (Allen *et al.*, 1994; 1:1 DMEM:Ham F-12, 10% fetal calf serum supplemented with sodium piruvate, L-glutamine, nucleosides, non-essential amino acids, sodium bicarbonate and ß-2 mercaptoethanol), in culture dishes coated with 0.1% gelatin. Since ES cells cultured in such a way tend to differentiate, LIF (2000 units/ml; Sigma) was added to keep them in an undifferentiated state (Nagy *et al.*, 1993). After 6 days of culture both groups of cells (cultured with and without LIF) were fixed in methanol and in methanol/acetone 1/1 at -20°C for 10 minutes each. For immunocytochemical staining cells were blocked with fetal calf serum and marked as described elsewhere. DAPI was used for counterstaining. To detect the alkaline phosphatase activity (AP) cells fixed as previously described were incubated with the Alkaline Phosphatase Substrate Kit (VECTOR; SK-5100).

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