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Elf5 and Ets2 maintain the mouse extraembryonic ectoderm in a dosage dependent synergistic manner

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ABSTRACT

The ETS superfamily transcription factors Elf5 and Ets2 have both been implicated in the maintenance of the extraembryonic ectoderm (ExE) of the mouse embryo. While homozygous mutants of either gene result in various degrees of ExE tissue loss, heterozygotes are without phenotype. We show here that compound heterozygous mutants exhibit a phenotype intermediate to that of the more severe *Elf5*−/− and the milder *Ets2*−/− mutants. Functional redundancy is shown via commonalities in expression patterns, in target gene expression, and by partial rescue of *Elf5*−/− mutants through overexpressing Ets2 in an Elf5-like fashion. A model is presented suggesting the functional division of the ExE region into a proximal and distal domain based on gene expression patterns and the proximal to distal increasing sensitivity to threshold levels of combined Elf5 and Ets2 activity.

Introduction

The trophectoderm cells of the eutherian mammalian blastocyst give rise to the trophoblast forming the bulk of the foetal portion of the placenta (Gardner et al., 1973). In mice actively proliferating trophoblast cells are confined to the region overlying the inner cell mass. This region is termed the polar trophectoderm and expands into the blastocyst cavity after implantation at embryonic day (E) 4.5 (Copp, 1979). By E5 this proliferative region occupies the "top" (closest to implantation site) half of the egg cylinder and is termed the extraembryonic ectoderm (ExE). The bottom half of the egg cylinder ectoderm is the embryonic ectoderm that will give rise to the embryo proper. The junction between the embryonic ectoderm and ExE can be easily morphologically distinguished by virtue of a circumferential constriction. We shall here use this constriction landmark as a reference point, referring to trophoblast close to it as proximal, distant trophoblast as distal. The ExE is connected on the implantation (distal) side to a stalk of trophoblast cells termed the ectoplacental cone (EPC). The EPC attaches the conceptus to a mass of fully differentiated trophoblastic giant cells which mediate the early endometrial interactions. The proximal to distal arrangement of ExE-EPC-giant cells is maintained until midgastrulation, when the extraembryonic mesoderm and amniotic cavities push the ExE distally away from the embryo proper (Kaufman, 1995). The resultant flattened ExE lined with extraembryonic mesoderm is termed the chorion. Trophoblastic stem (TS) cells, dependent on FGF and Nodal/Activin signalling, can be isolated from polar trophectoderm and ExE but not EPC tissue during these stages (Tanaka et al., 1998; Uy et al., 2002). These TS cells, resembling from their gene repertoire ExE, can be differentiated in vitro into cells resembling chorionic derivatives such as syncytiotrophoblasts, EPC derivatives such as spongiotrophoblasts as well as into giant cells.

While numerous genes have been implicated in this sequence of trophoblast developmental events (Simmons and Cross, 2005), two are characterised by specifically being required for the early maintenance of the proliferative ExE. These are *Elf5* and *Ets2* (Yamamoto et al., 1998; Donnison et al., 2005; Georgiades and Rossant, 2006; Wen et al., 2007; Odiatis and Georgiades, 2010; Polydorou and Georgiades, 2013). Notably, both these genes code for proteins belonging to the 26 proteins making up the superfamily of ETS transcription factors (Blake et al., 2014). Loss of function of either factor results in an incompletely penetrant phenotype where, in the most severe form all (Elf5) or most (Ets2) of the ExE tissue is lost by E6.5, the start of gastrulation (Donnison et al., 2005; Georgiades and Rossant, 2006). This leads to an early arrest of trophoblast development and embryonic lethality a couple of days later. Both genes show exclusive trophoblast expression until at least late gastrulation stages and mutants can be rescued in chimeras via extraembryonic-only expression (Yamamoto et al., 1998; Donnison et al., 2005; Georgiades and Rossant, 2006). Furthermore no TS cells can be

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derived from loss of function mutants for either gene, indicating an essential requirement for both factors. In view of the similar loss of function phenotype and the observation that the consensus binding sites of Ets2 and Elf5 are nearly identical, (*a*ccGGAagt) versus (*ccc*GGAagt) (Wei et al., 2010), we hypothesised that these proteins may be able to at least partially compensate for each other.

Results

An early developmental epistatic relationship between Elf5 and Ets2

Neither Elf5 nor Ets2 mutants display an embryonic phenotype in the heterozygous condition. However, when we crossed *Elf5*+/− with *Ets2*+/− animals, we noted that 10% of deciduas were empty at E14.5 (Table 1). Similarly, at E10.5 nearly 10% of deciduas were empty or contained a severely retarded embryo. This strongly suggests that Ets2 and Elf5 are synergistically epistatic, that is, the compound phenotype is more severe than the sum of the individual phenotypes. This synergistic effect seemed to exist already at E6.5 (35% observed small embryos from compound heterozygous crosses relative to the expected 30%, as detailed in Table 1), but was not statistically significant at this stage. We estimate the penetrance of an *Elf5*+/−*Ets2*+/− phenotype to be 40% based on the E10.5 and E14.5 data (calculation in Table 2). By assuming this degree of penetrance we could explain the skewed proportions of genotyped pups obtained when crossing *Elf5*+/− with *Ets2*+/− animals (Table 2).

An Elf5 × Ets2 interaction prior to gastrulation

As the number of retarded/small embryos obtained from heterozygous crosses at the start of gastrulation (E6.5) tended to be higher than expected (Table 1), we investigated this effect in more detail by subjecting E6.5 *Elf5*+/−*Ets2*+/− embryos to whole mount in situ hybridisation. In a subset of embryos confirmed to be doubly heterozygous mutants, defective trophectoderm development was apparent. *Oct4* (*Pou5f1*) and *Cripto* (*Tdgf1*) mark the embryonic ectoderm (Rosner et al., 1990; Ding et al., 1998). In E6.5 *Elf5*+/−*Ets2*+/− embryos, *Oct4* and *Cripto* staining extended close to the ectoplacental cone (EPC), indicating a severe loss of ExE tissue as also seen in *Elf5*−/− or *Ets2*−/− mutants (Fig. 1). This effect was confirmed by additional staining for *Ascl2* (*Mash2*), which marks the EPC and ExE distal to the embryonic–extraembryonic border (DiExE; see later for more detailed descriptions). In wild type or singly heterozygous embryos the embryonic ectoderm, marked by *Cripto*, and the DiExE+EPC, labelled by *Ascl2* staining, are separated by the proximal ExE tissue (Fig. 1D, left embryo). However in the *Elf5*+/−*Ets2*+/− embryo the *Cripto* staining abuts the *Ascl2* positive domain. The *Elf5*−/− and *Elf5*−/−*Ets2*+/− phenotypes are more severe than the double heterozygotes in that *Ascl2* expression is nearly completely lost (Fig. 1D but see also Fig. 7). We conclude that while embryos heterozygous mutant for either *Elf5* or *Ets2* are normal, doubly heterozygous embryos show an incompletely penetrant phenotype whereby the proximal extraembryonic ectoderm is lost by the start of gastrulation. This indicates a positive epistatic interaction between these two transcription factors.

Table 1
Frequency of defective embryos (small or resorbed) from heterozygous crosses[a].

Stage	*n*[b]	*Observed*	*95% Conf. limits*	*Expected* [c]	*P(χ²)* [d]
E14.5 [e]	122	12 (10%)	5.2–17%	0 (0%)	**1.4E-06**
E10.5 [e]	39	3 (8%)	1.6–21%	0 (0%)	0.049
E6.5 [f]	211	73 (35%)	28–41%	63 (30%)	0.17

[a] See supplementary Table S1 for details.
[b] Number of deciduas.
[c] Expected numbers on assumption that all embryos having *Elf5*−/− and/or *Ets2*−/− genotype will be defective and single heterozygous animals will show no defects.
[d] Probability that difference between observed and expected is significant.
[e] Only double heterozygote × wild type or *Elf5*+/− × *Ets2*+/− crosses.
[f] Compound crosses (Table S1).

Table 2
Reduced frequency of double heterozygous pups (*n*=254) from (*Elf5*+/−, *Ets2*+/−) × (wild type) matings (129 strain).

Genotype	*Observed number of pups (%)*	*95% Confidence limits (%)*	*Expected if genotype were irrelevant*	*P(χ²) that diff is signif.*	*expected if 60% of Elf5+/−; Ets2+/− pups survive* [a]	*P(χ²) that diff is signif.*
Elf5+/+ *Ets2*+/+	84 (33%)	27–39%	64 (25%)		71 (28%)	
Elf5+/- *Ets2*+/+	71 (28%)	22–34%	64 (25%)		71 (28%)	
Elf5+/+ *Ets2*+/−	65 (26%)	20–31%	64 (25%)		71 (28%)	
Elf5+/− ***Ets2***+/−	35 (14%)	10–19%	64 (25%)	0.0002	42 (17%)	0.24

[a] The assumption of 40% lethality of *Elf5*+/−; *Ets2*+/− pups is derived from Table 1, where an overall 10% loss of embryos, not attributable to *Elf5*−/− or *Ets2*−/− genotypes, was observed. Thus, if original number of embryos is *x*, number of embryos conceived of each of 4 genotypes (*g*) would, according to Mendelian statistics, be (*x*/4). The observed number of embryos surviving (*n*)=0.9*x* (Table 1). If we assume that all dead (unborn) embryos are derived from one genotype, (*Elf5*+/−, *Ets2*+/−), then the surviving pups with this phenotype would be (*g*−0.1*x*)=(*x*/4)−0.1*x*=0.15*x*. This corresponds to 0.15*x*/0.9*x*=1/6th (=17%) of pups born, reflecting a 60% survivability (0.15*x*/0.25*x*).

Overlap of Elf5 and Ets2 targets in TS cells

An epistatic interaction of Ets2 and Elf5 would be expected if these transcription factors shared common target sites. This scenario is plausible as their DNA recognition consensus sequences are similar (Choi and Sinha, 2006; Wei et al., 2010). We have previously determined potential target genes of Elf5 using a siRNA knockdown approach in TS cells (Pearton et al., 2014) and now applied a similar strategy to Ets2 so as to determine commonalities. Two different *Ets2*-siRNA were able to knock down *Ets2* RNA levels by over 80% in TS cells after 48 h treatment, relative to control siRNA-treated or untreated cells (Fig. 2A). Screening of Affymetrics arrays revealed a set of 15 genes misregulated by at least 2-fold after treatment with either of the *Ets2* siRNAs (Table 3). Comparing the misregulated genes with our previously published Elf5 target gene dataset revealed four genes to be affected in a similar fashion by either Ets2 or Elf5 down regulation (Table 4). We verified the Ets2 targets by quantitative RT-PCR using numerous repeats (Fig. 2A and B). This analysis casted some doubt on the authenticity of *Ascl2*, *Emp1*, *Lgals3* and *Mbnl3* as misregulated genes (Fig. S1), but supported the microarray results of the other nine genes including three that were also Elf5 targets (Fig. 2A). Unlike what is seen after Elf5 down regulation, none of the genes downregulated after loss of Ets2 activity is known to be involved in trophoblast stem cell maintenance (Pearton et al., 2011), a result confirmed by PCR for the trophoblast stem cell markers *Cdx2*, *Eomes*, *ERRβ* and *Sox2* (Fig. 2C and D). Importantly no effect is seen on *Elf5* (Fig. 2D) nor some of the Elf5 targets (Fig. 2C), excluding the possibility that Ets2 acts simply via *Elf5* down regulation. Similarly, Elf5 knock down had no effect on *Ets2* expression (Fig. 2E), as also previously shown (Pearton et al., 2014). Since Ets2 and Elf5 do not affect each other's expression levels and as even a single shared gene, let alone three, would not be expected by chance if the target sets

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