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Imprinted expression in cystic embryoid bodies shows an embryonic and not an extra-embryonic pattern

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ABSTRACT

A large subset of mammalian imprinted genes show extra-embryonic lineage (EXEL) specific imprinted expression that is restricted to placental trophectoderm lineages and to visceral yolk sac endoderm (ysE). Isolated ysE provides a homogenous *in vivo* model of a mid-gestation extra-embryonic tissue to examine the mechanism of EXEL-specific imprinted gene silencing, but an *in vitro* model of ysE to facilitate more rapid and cost-effective experiments is not available. Reports indicate that ES cells differentiated into cystic embryoid bodies (EBs) contain ysE, so here we investigate if cystic EBs model ysE imprinted expression. The imprinted expression pattern of cystic EBs is shown to resemble fetal liver and not ysE. To investigate the reason for this we characterized the methylome and transcriptome of cystic EBs in comparison to fetal liver and ysE, by whole genome bisulphite sequencing and RNA-seq. Cystic EBs show a fetal liver pattern of global hypermethylation and low expression of repeats, while ysE shows global hypomethylation and high expression of IAPEz retroviral repeats, as reported for placenta. Transcriptome analysis confirmed that cystic EBs are more similar to fetal liver than ysE and express markers of early embryonic endoderm. Genome-wide analysis shows that ysE shares epigenetic and repeat expression features with placenta. Contrary to previous reports, we show that cystic EBs do not contain ysE, but are more similar to the embryonic endoderm of fetal liver. This explains why cystic EBs reproduce the imprinted expression seen in the embryo but not that seen in the ysE.

Introduction

Genomic imprinting is an epigenetic phenomenon that leads to parental allele-specific or imprinted expression of approximately 150 mouse genes, more than 80% of which are grouped into clusters. Gamete-specific DNA methylation of an imprint control element (ICE) controls imprinted expression of the entire cluster, as has been demonstrated by genetic deletion in seven cases (Barlow and Bartolomei, 2014). In the most common mechanism of imprinted silencing, the unmethylated ICE allele is associated with the promoter of a long non-coding (lnc) RNA that initiates silencing of imprinted genes in the cluster, as has been demonstrated for four of the seven clusters (Mancini-Dinardo et al., 2006; Meng et al., 2012; Sleutels et al., 2002; Williamson et al., 2011). While most imprinted genes show some degree of tissue-specific regulation of imprinted expression (Prickett and Oakey, 2012), two broad types have been identified: multi-lineage (ML) and extra-embryonic lineage (EXEL) specific imprinted expression. ML imprinted expression is seen in both embryonic and extra-embryonic tissues. EXEL imprinted expression is restricted to placental trophectoderm lineages and the visceral yolk sac (VYS) endoderm layer (ysE) (Hudson et al., 2011). In imprinted clusters,

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genes showing EXEL specific imprinted expression tend to be located further away from the ICE and the lncRNA that silences them, presenting a model of long-range *cis* silencing by lncRNAs (Kulinski et al., 2013).

Extra-embryonic tissues show a chromatin state distinct from embryonic lineages, for example displaying global DNA hypomethylation (Chapman et al., 1984; Popp et al., 2010; Rossant et al., 1986). EXEL imprinted expression has been suggested to arise from a more permissive chromatin organization in extra-embryonic tissues that allows the silencing mechanism to extend over greater distances (Hudson et al., 2010; Kulinski et al., 2013), or from repression of EXEL specific enhancers by imprinted lncRNAs (Pauler et al., 2012). Testing these hypotheses *in vivo* require laborious genetic experiments to manipulate the imprinted cluster in the endogenous locus in the mouse. In comparison, genetic studies in *ex vivo* model systems are faster and more cost effective. Research on the mechanism of ML imprinted expression has been facilitated by the use of an *ex vivo* model system employing ES cell differentiation (Kohama et al., 2012; Latos et al., 2009; Meng et al., 2012; Wood et al., 2010). The ysE, which arises from the primitive endoderm of the pre-implantation embryo, forms the external layer of the bilaminar VYS that encloses the embryo after the embryo turns around E8.5 and remains until birth. Although stem cells known as XEN cells that model the primitive endoderm have been derived and display some markers of visceral endoderm, they are largely unable to contribute to the visceral endoderm in chimeras (Kunath et al., 2005). In contrast, ES cells differentiated *in vitro* by aggregation into small non-attached cell clumps called embryoid bodies (EBs) eventually develop cystic structures that closely resemble the bilaminar structure of the VYS and have been considered to contain ysE (Abe et al., 1996; Doetschman et al., 1985; Kurosawa, 2007; Yasuda et al., 2009). Electron microscopy studies also showed the outer epithelial layer of cystic EBs contained microvilli and cytoplasmic vacuoles similar to the ysE (Doetschman et al., 1985). Further support that differentiating ES cells into cystic EBs provides an *ex vivo* model of ysE development comes from studies showing that the outer endodermal-like layer expresses visceral endoderm markers such as *Afp* and *Ttr*, as well as endoderm-specific transcription factors such as *Hnf1, vHnf1, Hnf3b, Hnf4* and *Gata4* (Abe et al., 1996; Doetschman et al., 1985; Koike et al., 2007; Kurosawa, 2007; Sajini et al., 2012; Sakai et al., 2011; Soudais et al., 1995). Despite this, the ability of cystic EBs to model EXEL imprinted expression of the ysE has not yet been tested.

Here we developed a robust protocol to differentiate mouse ES cells into cystic EBs and determined if this *ex vivo* system can be used as a model of ysE imprinted expression. We show that many ysE EXEL imprinted genes were either not expressed, or very lowly expressed in cystic EBs. Furthermore, while cystic EBs robustly displayed the ML type of imprinted expression, all EXEL genes expressed above baseline levels exhibited biallelic expression. Using whole genome bisulphite converted DNA sequencing (WGBS) we showed that cystic EBs exhibit a high level of DNA methylation comparable to that observed in fetal liver, and not the lower levels observed in ysE. Using RNA-seq transcriptome and WGBS analysis, we further showed that cystic EBs, rather than resembling ysE, are more closer related to the definitive endoderm derived fetal liver. Finally, we comprehensively annotated the ysE transcriptome and identified a characteristic set of genes that are absent from the cystic EB transcriptome. Together these results indicate that cystic EBs have an embryonic-like epigenetic state, contain embryonic endoderm rather than extra-embryonic endoderm, and, while a useful resource for analyzing ML imprinted expression in different tissue-types, they cannot be used as a model for EXEL imprinted expression.

Material and methods

Tissue collection and VYS layers separation

Mice were bred and housed at the IMBA/IMP facility in Vienna in strict accordance with national recommendations under Laboratory Animal Facility Permit MA58-0375/2007/4. Fetal liver, VYS and VYS endoderm (ysE) were collected from E12.5 FVB/N or CAST/EiJ × FVB/N F1 crosses. Separated ysE and mesoderm was collected from E9.25 FVB/N embryos. In each case the entire litter was pooled for each sample. The VYS endoderm and mesoderm layers were manually separated using a DispaseII pre-treatment for 1 h (E9.5) and 2 h (E12.5) as described (Hudson et al., 2011). The VYS mesoderm was collected together with the vasculature and the basement membrane, while the ysE was collected alone. The accuracy of separation was assessed using RT-qPCR for markers of ysE (*Afp*) and mesoderm (*Flk1*).

ES cell lines

Mouse ES cell lines were derived from CAST/EiJ × FVB/N crosses (described below, clones CF-C2 (XY) and FC-A2 (XY) were used), or from C57BL/6 × 129 crosses (clone A9 (XY) from Anton Wutz, clone JN (XX) from Jennifer Nichols). ES cells were maintained in DMEM based medium with 15% fetal calf serum and LIF, on irradiated E12.5 mouse embryonic fibroblast feeders according to standard protocols. ES cells were differentiated in retinoic acid (RA) as described previously (Latos et al., 2009) or into cystic EBs as described below. The A9 and JN cells showed a normal karyotype and the CF-C2 and FC-A2 clones showed normal differential methylation of tested ICE as described below. All ES cell lines also showed normal ES cell morphology and growth dynamics.

Derivation of ES cells from CAST × FVB F1 crosses

ES cells were derived from reciprocal crosses of CAST/EiJ × FVB/N using a method adapted from a published protocol (Bryja et al., 2006). In brief, harvested blastocysts were incubated in Tyrodes acid for 30–60 s till the zona pellucida dissolved. Afterwards, blastocysts were plated onto MEF feeder layers in SR-ES medium (Table S2) and left undisturbed for two days to allow attachment. Medium was changed every two days. After 6 days the blastocysts hatched and were trypsinized (0.25% trypsin–EDTA) for the first time and plated onto a new well of MEF-feeders in ES medium (Table S2). After one additional week ES cells were growing out and the best looking colonies were sub-cloned and mechanically passaged. Between 3 and 8 ES cells sub-clones were picked per blastocyst (5 CAST × FVB blastocysts, 3 FVB × CAST blastocysts, maternal allele on the left). Each clone was sex-typed, checked for differential methylation of the *Dlk*, *Igf2r* and *Kcnq1* cluster ICE, and the general morphology and growth characteristics were noted.

A robust protocol for cystic EB differentiation

To produce comparable EB populations we started differentiations from ES cells between 18 and 21 passages. Previously multiple protocols for ES cell differentiation into cystic EBs have been proposed. However, most of them depend on a random or poorly controlled aggregation of ES cells and thus are hampered by poor reproducibility of the starting aggregate size (Kurosawa, 2007). The starting cell number in the initiating clumps in suspension cultures has been shown to determine the simple EB versus cystic EB composition of the differentiated populations and substantially influence endoderm marker expression (Koike et al., 2007; Yasuda et al., 2009). To increase the reproducibility of ES cell differentiation to cystic EBs and lineage choice we used AggreWell™400

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