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„Control of *Igf2r* imprinted silencing in embryonic stem cell differentiation“

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1. Zusammenfassung

Genomische Prägung beschreibt ein epigenetisches Phänomen, welches die Expression bestimmter Gene in Säugetieren von einem der beiden elterlichen Allele darstellt. Der *Igf2r* Gen Cluster der Maus repräsentiert ein gut untersuchtes Modell für genomische Prägung. Innerhalb dieses Clusters wird die lange nicht-kodierende RNA (lnkRNA) *Airn* ausschließlich vom väterlichen Allel exprimiert und dessen entgegengesetzte Transkription über den Promoter von *Igf2r* führt zur Stilllegung des Protein-kodierenden Gens, dem eine Anreicherung von DNA Methylierung am unterdrückten *Igf2r* Promoter folgt. Der Beginn der genomischen Prägung von *Igf2r* kann während der Differenzierung von embryonalen Mausstammzellen (ESZ) nachvollzogen werden. *Airn* wird kontinuierlich während des Lebens exprimiert, wann immer *Igf2r* geprägte Expression zeigt. Es ist jedoch nicht bekannt, ob die Expression der lnkRNA ständig gebraucht wird um das Stilllegen von *Igf2r* zu erhalten oder ob *Airn* entbehrlich ist wenn DNA Methylierung am unterdrückten *Igf2r* Promoter erworben wurde. Um den Erhalt der Stilllegung von *Igf2r* besser zu verstehen wurde die *Airn* konditionelle Knock-Out (*Airn*KKO) ES Zelllinie hergestellt. Diese Zelllinie beinhaltet zwei loxP-Stellen, welche den Promoter von *Airn* flankieren. Durch Verwendung einer Tamoxifen induzierbaren CreER Rekombinase wurde die Promotersequenz entfernt, wodurch die Transkription von *Airn* zu jedem Zeitpunkt während der ESZ Differenzierung abgeschaltet werden kann. Durch das Entfernen von *Airn* konnte ich eine Re-Expression des zuvor unterdrückten *Igf2r* beobachten, jedoch war das Ausmaß der Re-Expression in später ESZ Differenzierung reduziert, wenn bereits DNA Methylierung am väterlichen *Igf2r* Promoter erworben wurde. Weiters konnte ich feststellen, dass die DNA Methylierung am *Igf2r* Promoter unabhängig von der lnkRNA *Airn* erhalten wurde. Dies zeigt, dass während ESZ Differenzierung ein Wechsel von einem *Airn*-abhängigem zu einem *Airn*-unabhängigem Stilllegen von *Igf2r* statt findet, sofern DNA Methylierung erworben wurde und dass diese *Igf2r* DNA Methylierung unabhängig von der Transkription von *Airn* erhalten wird.

1. Abstract

Genomic imprinting describes an epigenetic phenomenon whereby a small subset of mammalian genes shows parental-specific gene expression. The mouse *Igf2r* gene cluster represents a well-studied model for genomic imprinting. Within this cluster, the macro long non-coding RNA (lncRNA) *Airn* is expressed exclusively from the paternal allele and its antisense transcription over the *Igf2r* promoter causes silencing of this protein-coding gene *in cis*, followed by gain of DNA methylation at the repressed *Igf2r* promoter. Establishment of *Igf2r* silencing by *Airn* can be studied in mouse embryonic stem (ES) cells, whose differentiation recapitulates *in vivo* embryo development. *Airn* is expressed continuously throughout life whenever *Igf2r* shows imprinted expression. However, it is unknown whether the expression of the macro lncRNA is constantly needed to maintain silencing of *Igf2r* or if it is dispensable once DNA methylation is gained at the silent *Igf2r* promoter. To better understand how *Igf2r* silencing is maintained, the *Airn* conditional knock-out (*Airn*CKO) ES cell line was created. Two loxP sites were inserted around the promoter of *Airn* and conditional excision was performed with a tamoxifen-inducible Cre recombinase. This system enables the deletion of the macro lncRNA promoter at any time point during ES cell differentiation. Upon the removal of *Airn*, I observed re-expression of the previously silenced *Igf2r* allele. However, re-expression was reduced in late differentiated ES cells, when DNA methylation was gained at the paternal *Igf2r* promoter. Further, I found that this DNA methylation mark is maintained independently of the *Airn* macro lncRNA. This indicates that during ES cell differentiation a developmental switch from an *Airn*-dependent to an *Airn*-independent silencing mechanism of *Igf2r* occurs once DNA methylation is established and that maintenance of *Igf2r* DNA methylation itself is not dependent on *Airn* transcription. This study, in which maintenance of *Igf2r* silencing was investigated by conditionally deleting the macro lncRNA *Airn*, is generally relevant to improve our understanding of gene regulation by lncRNAs.

2. Introduction

2.1 Genomic imprinting

2.1.1 Definition and identification of imprinted genes

Genomic imprinting is an epigenetic mechanism leading to parent-of-origin specific expression of a subset of mammalian genes (Barlow, 2011). Mammals are diploid organisms having two copies of each chromosome inherited from their parents. In contrast to most of the genes, which are expressed from both alleles, imprinted genes are only transcribed from either the maternal or paternal chromosome (Ferguson-Smith, 2011). Genomic imprinting can also be observed in inbred mouse strains carrying genetically identical parental chromosomes, which indicates that it does not arise from differences in the DNA sequence, but rather relies on epigenetic mechanisms (Bartolomei & Ferguson-Smith, 2011). Epigenetics itself is defined as a heritable but reversible change in gene expression, which does not depend on changes of the DNA sequence. Therefore, imprinting provides an excellent model to study epigenetic gene regulation (Barlow, 2011).

Historically, parent-of-origin specific effects had been observed as early as the 1970s (Johnson, 1974), however it was only with the elegant pronuclear transplant experiments in the 1980s that the need of both maternal and paternal genomes for reproduction was formally shown (Barton et al, 1984; McGrath & Solter, 1984; Surani et al, 1984). Manipulated embryos containing either two maternal (gynogenetic) or paternal (androgenetic) pronuclei failed to develop past mid-gestation. Maternal uniparental embryos were able to develop embryonic tissues but failed to develop extra-embryonic lineages, whereas paternal uniparental conceptuses developed predominantly into extra-embryonic lineages and poorly into the embryonic one. These results suggested that developmentally important genes are not equally expressed from the two parental chromosomes. Confirming

this hypothesis, the first three mouse imprinted genes were discovered in 1991. The first one to be published was insulin-like growth factor 2 receptor (*Igf2r*) (Barlow et al, 1991), followed by insulin-like growth factor 2 (*Igf2*) (DeChiara et al, 1991; Ferguson-Smith et al, 1991) and the long non-coding RNA (lncRNA) *H19* (Bartolomei et al, 1991).

2.1.2 DNA methylation in imprinted gene clusters

So far 150 imprinted genes have been validated in the mouse genome (Williamson, 2012). The majority of these genes (>80%) are organized in clusters, whereas only a few exist as solo imprinted genes. Clusters are typically larger than 100 kilobase pairs (kbp) and contain at least two maternally or paternally expressed genes. Clustering of imprinted genes argues against a gene-specific imprinting mechanism, but rather for a long-range mechanism that acts *in cis* (i.e., on the same chromosome) to restrict expression of imprinted genes to one parental allele (Barlow, 2011).

As the two alleles of an imprinted gene reside in the same nucleus, but only one of them is expressed, a parental-specific "imprint mark" must exist to allow the cell to distinguish between them. The "imprint mark" should fulfill the following four criteria (Ferguson-Smith, 2011):

1. It has to influence the transcriptional state of the imprinted gene.
2. Establishment of the imprint mark is likely to take place when the two parental chromosomes are not in the same nucleus (for example, during gametogenesis or immediately after fertilization when the two parental pronuclei are still separated).
3. It must be heritable in somatic tissues to guarantee imprinted gene expression throughout life.
4. The mark has to be erased in the germ line to enable the establishment of a sex-specific imprint in the gametes of the next generation.

The only possible epigenetic mark, which can fulfill all the criteria mentioned above, is DNA methylation (Bartolomei & Ferguson-Smith, 2011). In mammals, DNA methylation involves transfer of a methyl group to the 5' position of cytosine within CpG dinucleotides and is associated with transcriptional repression of genes (Jones, 2012). More recent, it has been shown that methylated CpGs can be converted into hydroxymethylated CpGs, however their role as an epigenetic mark is still unclear (Wu & Zhang, 2010). CpG dinucleotides are underrepresented and not equally distributed in the mammalian genome and they often occur in CpG-rich sequences called CpG islands (CGIs). CGIs are about 1 kbp long, have increased C+G base composition compared to the genome average and are found at about 70% of mammalian gene promoters (Deaton & Bird, 2011). CGIs tend to be unmethylated and the rare methylated ones are usually associated with promoters of imprinted genes, where only one parental allele is methylated, or with promoters of genes on the inactive X chromosome in females (Abramowitz & Bartolomei, 2011; Wutz & Gribnau, 2007). Allele-specific methylated regions, which also include methylated CpG-rich sequences outside promoters, are called differentially methylated regions (DMRs) and can be distinguished into two types:

1. Gametic DMRs (gDMRs)

   These genetic elements gain parental-specific DNA methylation during gametogenesis, in haploid oocytes or sperm respectively and thus represent the candidate "imprint mark" (Barlow, 2011). So far 21 gDMRs have been identified, of which seventeen show maternal- and four paternal-specific methylation (Tomizawa et al, 2011). Interestingly, gDMRs established in oocytes are found at gene promoters, whereas paternally methylated gDMRs are located in intergenic regions (Edwards & Ferguson-Smith, 2007). Investigation of gDMR function revealed so far eight of them as imprint control elements (ICE) (also called imprint control regions (ICR) or imprinting centers (IC)), the master regulators of parental-specific gene expression (Barlow, 2011; Ferguson-Smith, 2011).

2. Somatic or secondary DMRs (sDMRs)

   Unlike gDMRs, sDMRs acquire their parental-specific DNA methylation mark in diploid cells after fertilization and therefore do not qualify as “imprint mark”. sDMRs depend on the presence of a gDMR and mark the silent allele of an imprinted gene, but only a few imprinted protein-coding genes have an sDMR (Barlow, 2011; John & Lefebvre, 2011). Further, secondary methylation marks are mostly gained after imprinted genes become repressed, indicating a role in maintaining rather than initiating the silent state of an imprinted gene (John & Lefebvre, 2011).

DNA methylation patterns are established and maintained by a family of enzymes known as DNA methyltransferases (Dnmts). DNMT3A and 3B are *de novo* DNA methyltransferases and are present in the female and male germ lines as well as in early embryonic stages. Another member of this family is DNMT3L, a DNA methyltransferase 3-like protein, which lacks enzymatic activity, however it is required as a regulatory factor (Vassena et al, 2005). Knockout experiments have shown that only DNMT3A and 3L are needed to establish the gametic imprints, whereas *Dnmt3b* deletion showed no phenotypical changes (Bourc'his et al, 2001; Kaneda et al, 2004). Still, it is not clearly understood how the DNMT3A/3L complex can distinguish between maternal- or paternal-specific regions to methylate in oocytes and sperm, respectively (Ferguson-Smith, 2011).

DNMT1 instead is required for maintaining the methylation pattern. After DNA replication hemimethylated DNA, made up of an old methylated DNA strand and a newly synthesized unmodified one, is present. DNMT1 has a high affinity for the unmethylated DNA strand and adds a methyl-group on the new CpG dinucleotides; therefore it fulfills the important function of DNA methylation heritability (Goll & Bestor, 2005). By deleting *Dnmt1* in mice, which leads to the loss of imprinted gene expression, the crucial role of DNA methylation in genomic imprinting has been demonstrated. Interestingly, in the absence of DNMT1 imprinted lncRNAs are re-

expressed from their normally silent alleles, whereas imprinted protein-coding genes are repressed (Li et al, 1993).

2.1.3 Mechanisms of imprinted gene expression

As mentioned above, the master regulator of imprinted expression is the ICE. This DNA element is a gDMR and it is inactivated on one allele by a parent-of-origin specific DNA methylation mark established in the germ line. So far eight gDMRs have been identified as ICEs (Fitzpatrick et al, 2002; Lin et al, 2003; Shiura et al, 2009; Thorvaldsen et al, 1998; Williamson et al, 2006; Wutz et al, 1997; Yang et al, 1998; Yoon et al, 2002). Deletion of the unmethylated ICE leads to re-expression of the imprinted protein-coding genes from the silent allele in the whole cluster, whereas deletion of the methylated ICE has no effect. This indicates that the unmethylated ICE acts as a repressor for imprinted protein-coding genes, which are only expressed if the ICE is methylated (Koerner et al, 2009).

Allele-specific silencing can occur by different mechanisms: methylation-sensitive insulator, silencing lncRNAs or alternative polyadenylation. In the *Igf2* cluster, the insulator-binding CTCF protein regulates imprinted gene expression by binding specifically to the unmethylated ICE (Bell & Felsenfeld, 2000). In six out of the eight ICE regulated clusters, the unmethylated ICE controls the expression of a lncRNA, which are by definition longer than 200nt and their function does not depend on processing to smaller RNAs (Gingeras, 2007). For the four lncRNAs *Airn*, *Kcnq1ot1*, *Nespas* and *Ube3a-as*, their silencing activity upon protein-coding genes has been shown in the *Igf2r*, *Kcnq1*, *Gnas* and *PWS/AS* imprinted cluster, respectively (Mancini-Dinardo et al, 2006; Meng et al, 2012; Sleutels et al, 2002; Williamson et al, 2011). Finally, the imprinted expression of the *H13* gene is established via allele-specific alternative polyadenylation, regulated by the differentially methylated promoter of the imprinted *Mcts2* gene (Wood et al, 2008). A similar mechanism operates at the *Mest/Copg2* and *Herc3/Nap1/5* imprinted clusters (Cowley et al, 2012; MacIsaac et al, 2012).

2.1.4 Life cycle of genomic imprinting

Genomic imprinting is a developmentally regulated phenomenon and regulation occurs at multiple levels. In the germ line, when the parental chromosomes are separate, sex-specific imprints are established by *de novo* DNA methylation of gDMRs, though at different time points in male and female germ cells. In males, methylation is gained in prospermatogonia between mitotic arrest and birth, whereas maternal-specific methylation takes place only after birth in the oocyte growth period (Smallwood et al, 2011). In early stages of embryonic development, the genome undergoes a massive epigenetic reprogramming including active and passive DNA demethylation, which is needed to initiate embryonic-specific gene expression. However, imprints are protected from this wave of demethylation and are maintained during mitosis in diploid cells to ensure the correct allele specific expression pattern. Finally, in primordial germ cells (PGCs) of 12.5 days post-coitum (dpc) embryos, imprints are erased to enable sex-specific *de novo* DNA methylation in oocytes and sperm, respectively (Jones, 2012).

It is important to notice that the establishment of the imprint (i.e., ICE methylation) and initiation and maintenance of imprinted expression are distinct processes. By adding a methyl group to CpG dinucleotides within the ICE in germ cells the imprint is set, however this does not automatically initiate imprinted gene expression (Santoro & Barlow, 2011). Despite the fact that the imprint itself is present in every somatic cell and at every developmental stage, the majority of imprinted genes does not show ubiquitous allele-specific expression but rather shows tissue- or cell type-specificity as well as developmental regulation. This stresses the fact that, apart from the ICE, genomic imprinting depends on additional factors, themselves differentially regulated to initiate and maintain parent-of-origin specific expression (Santoro & Barlow, 2011). For example, the macro lncRNA *Airn* in the *Igf2r* imprinted gene cluster is not always expressed during development and in every cell type and *Igf2r* imprinted expression only arises when *Airn* is expressed (Pauler et al, 2005; Yamasaki et al, 2005).

2.2 The *Igf2r* imprinted gene cluster

The *Igf2r* imprinted gene cluster is located on mouse chromosome 17 and contains the maternally expressed protein-coding genes *Igf2r* (insulin-like growth factor 2 receptor), *Slc22a2* and *Slc22a3* and the paternally expressed macro lncRNA *Airn* (antisense-*Igf2r*-RNA-non-coding) (Barlow et al, 1991; Wutz et al, 1997; Zwart et al, 2001a) (Fig.1).

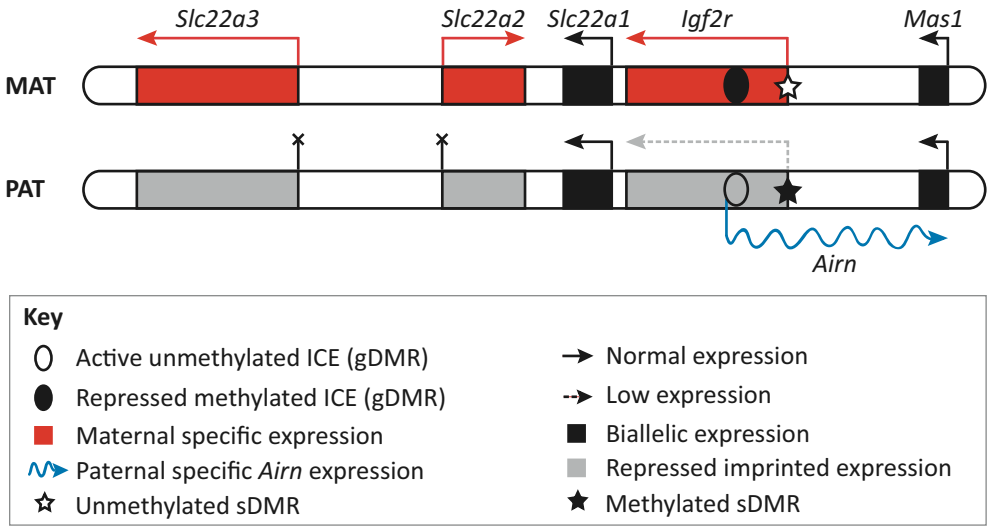

Figure 1. The *Igf2r* imprinted gene cluster.
Methylation of the ICE on the maternal chromosome in the second intron of *Igf2r* inhibits the expression of the macro lncRNA *Airn* and therefore allows the expression of the protein-coding genes *Igf2r*, *Slc22a2* and *Slc22a3*. On the paternal chromosome the methylation on the gDMR is absent, *Airn* is expressed and silences the three imprinted genes *in cis*. Additionally, DNA methylation is gained on the paternal promoter of *Igf2r* (sDMR) upon silencing.

Igf2r, also called cation-independent mannose-6-phospate receptor, codes for a scavenger transmembrane receptor, which binds *Igf2* and determines its lysosomal degradation. This interaction is necessary to regulate normal fetal growth. Loss of *Igf2r* leads to embryonic overgrowth as well as organ and skeletal abnormalities resulting in neonatal lethality (Lau et al, 1994; Wang et al, 1994). In contrast to *Igf2r*, *Slc22a2* and *Slc22a3*, which encode for membrane-spanning solute carrier proteins, are not essential for mouse development (Zwart et al, 2001b).

The ICE, which regulates the parent-of-origin expression in the imprinted cluster, is located in intron 2 of *Igf2r*. It is a 3.7kb gDMR and includes the 1.5kb CGI of the *Airn* promoter. Maternal specific methylation is established in oocytes, resulting in repression of the *Airn* macro lncRNA on the maternal chromosome. In contrast, the paternal allele carries an unmethylated ICE and *Airn* is expressed from the paternal chromosome (Lyle et al, 2000; Stoger et al, 1993).

Airn is a 118kb-long transcript overlapping the promoter of *Igf2r* in antisense orientation. Its 3'end also overlaps the *Mas1* gene, which is not affected by genomic imprinting and therefore expressed from both alleles. *Airn* is an atypical RNA polymerase II transcript, which is mostly unspliced, nuclear localized and unstable. These features indicate that the *Airn* transcript itself may not be needed to silence the protein-coding genes within the cluster, instead the act of transcription could play a crucial role (Seidl et al, 2006). Truncating *Airn* to 4% of its total length caused the same phenotype as deleting the ICE from the paternal allele, meaning biallelic expression of *Igf2r*, *Slc22a2* and *Slc22a3* (Sleutels et al, 2002; Wutz et al, 2001). This demonstrated that *Airn* expression is needed to repress *in cis* all three genes in the cluster. Since *Airn* is transcribed in antisense orientation to *Igf2r* and overlaps its promoter completely, the question arises whether the act of transcription or the *Airn* transcript itself causes *Igf2r* repression (Pauler et al, 2012). Further truncation experiments of *Airn* showed that only the transcription over the *Igf2r* promoter, but not the ncRNA product, is necessary to induce *Igf2r* silencing. This supports the hypothesis of transcriptional interference as the mechanism controlling *Igf2r* imprinted silencing (Latos et al, 2012).

As described above, imprinted genes often show developmental and tissue-specific monoallelic expression. In case of *Igf2r*, imprinted gene expression arises after embryo implantation and is maintained ubiquitously in the adult mouse, except in testis and post-mitotic neurons, correlating with loss of *Airn* transcription (Lerchner & Barlow, 1997; Yamasaki et al, 2005). *Igf2r* is therefore referred to as a multi-lineage (ML) imprinted gene. Upon repression, a secondary methylation mark is

gained on the silent paternal promoter of *Igf2r* (Stoger et al, 1993). *Dnmt1* knockout mice, which express *Airn* and silence *Igf2r* biallelically, showed that even in the absence of methylation on the *Igf2r* promoter, *Airn* is able to silence *Igf2r*, indicating no primary role for the sDMR in initiating *Igf2r* imprinted expression (Li et al, 1993).

In contrast to ML imprinted genes, some genes show parental specific expression only in extraembryonic tissues. In the *Igf2r* cluster, *Slc22a2* and *Slc22a3* show biallelic expression in most adult tissues and are only maternally expressed in placenta and visceral york sac (VYS) (Hudson et al, 2011; Zwart et al, 2001a). Therefore, they are termed as extra-embryonic lineage (EXEL) imprinted genes. The promoters of *Slc22a2* and *Slc22a3* lie 157kb and 243kb upstream of the ICE, respectively, and are not overlapped by *Airn* and neither gene gains a sDMR when silenced. Note that *Slc22a3*, which lies further away from the ICE, loses imprinted expression at 15.5-16.5 dpc, whereas *Slc22a2* retains imprinted expression throughout embryonic development (Hudson et al, 2011; Verhaagh et al, 1999; Zwart et al, 2001a). It has been shown that *Airn* maintains *Slc22a3* silencing by recruiting the repressive histone methyltransferase EHMT2 to the paternal *Slc22a3* promoter in placenta, *Igf2r* silencing, instead, is independent of EHMT2 (Latos et al, 2009; Nagano et al, 2008). This indicates that *Airn* uses a distinct mechanism to silence *Igf2r* and *Slc22a3*. Recently, an enhancer transcriptional interference model, whereby transcription of *Airn* on the paternal allele interrupts enhancer interaction with the promoters of *Slc22a2* and *Slc22a3*, has been proposed to explain how *Airn* might silence non-overlapped genes (Hudson et al, 2010; Pauler et al, 2012).

The murine *Igf2r* cluster is also conserved in humans. In both species, the order of genes is the same and furthermore the maternal specific DNA methylation within intron 2 of *Igf2r*/*IGF2R* can be found in mice and humans (Smrzka et al, 1995). However, *IGF2R* imprinted expression is absent in all tested adult tissues (Kalscheuer et al, 1993; Ogawa et al, 1993). *IGF2R*, *SLC22A2* and *SLC22A3* were only found to be imprinted in a minority of human placenta samples and are therefore referred to as polymorphic imprinted genes (Monk et al, 2006). Further, *IGF2R* shows polymorphic

imprinted expression in some early embryonic tissues and Wilms' tumors (Xu et al, 1993; Xu et al, 1997). In humans, the expression of a homologous *AIRN* ncRNA is not detected in all tested tissues, except for some samples of Wilms' tumors, although the human intronic CGI of *IGF2R* was shown to be a functional promoter in mouse transgenic experiments (Yotova et al, 2008).

2.3 ES cell models to study developmental regulation of *Igf2r* imprinted expression

2.3.1 Embryonic stem cells

Mouse embryonic stem (ES) cells derive from the inner cell mass (ICM) of blastocyst-stage embryos (Evans & Kaufman, 1981; Martin, 1981). Two hallmarks of ES cells are long-term self-renewal and pluripotency, which made them an indispensable tool in genetics. Self-renewal is the ability to perform potentially unlimited cell divisions while maintaining the undifferentiated state. Pluripotency represents the potential to develop into all embryonic cell lineages. The pluripotent capacity of ES cells was demonstrated *in vivo* by injecting them back into blastocysts and observing that they contributed to all types of cells including the germ line during embryo development. Under appropriate conditions ES cells can be cultivated *in vitro* and still keep their self-renewal and pluripotency (Wobus & Boheler, 2005).

The three key transcription factors required for pluripotency are *Oct4*, *Nanog* and *Sox2. Oct4* and *Sox2* have been identified as crucial regulators, since their expression is mainly restricted to pluripotent cells. *Nanog* represents the third important factor for acquiring pluripotency, however it seems to be dispensable once pluripotency is achieved (Yeo & Ng, 2013). Various experiments have shown that the capacity of pluripotency in ES cells depends on a precise expression balance of all three genes. To ensure self-renewal *Oct4*, *Nanog* and *Sox2* on one hand regulate their own

transcription and on the other hand they repress lineage-specific factors and activate other pluripotency genes (Li, 2010). To keep the pluripotent capacity of ES cells *in vitro*, cultivation on inactivated mouse embryonic fibroblast (MEF) feeder cells is required (Evans & Kaufman, 1981; Martin, 1981). Fibroblasts provide a crucial factor to maintain pluripotency of ES cells *in vitro*, the leukemia inhibitory factor (LIF) (Smith et al, 1988; Williams et al, 1988). LIF is a member of the interleukin (IL)-6 family and functions through signal transduction and activation of transcription (STAT) signaling, which inhibits the cellular differentiation program (Wobus & Boheler, 2005). Upon withdrawal of LIF and feeder cells, ES cells start to differentiate and differentiation can be directed to specific lineages by using appropriate differentiation factors (Williams et al, 2012). Two conventional ways of differentiating ES cells are either in monolayer with retinoic acid (RA) or as a cultured aggregate, a so-called embryoid body (EB) (Martin & Evans, 1975; Strickland & Mahdavi, 1978).

2.3.2 *Airn* and *Igf2r* expression in the ES cell differentiation system

So far, only a few examples have been published of ES cell models used to study imprinted gene expression (Kohama et al, 2012; Latos et al, 2009; Wood et al, 2010). Due to the fact that imprinted expression mainly occurs in a tissue specific way, *in vivo* studies of whole organs composed of multiple tissue types can lead to false results (Hudson et al, 2010). *In vitro* differentiated ES cells resemble early embryonic development and are therefore a good model to study various events, including the onset and maintenance of imprinting, at this developmental stage. An advantage of ES cells is that modification can be easily introduced into the genome without using time-consuming *in vivo* mouse models. Nether the less, *in vitro* systems also harbor possible pitfalls including the loss of the *in vivo* cell identity, as well as instability of DNA methylation. Thus, cultivation of ES cells requires good practice and frequent testing of their epigenetic make up (Barlow, 2011).

An ES cell system to study developmental regulation of *Airn*-mediated *Igf2r* imprinted expression has been described (Latos et al, 2009). This study showed that ES cells could mimic the *in vivo* situation, concerning the molecular mechanisms involved in the onset of imprinted expression in the *Igf2r* cluster (Fig. 2). Since imprinting is an allele-specific event, the authors had to find a suitable assay to study parent-of-origin gene expression in inbred mouse ES cells. Therefore a single nucleotide polymorphism (SNP) was inserted in the maternal copy of *Igf2r* exon 12. This SNP mutated a naturally occurring PstI restriction site, thus allowing identification of the two parental alleles (Latos et al, 2009).

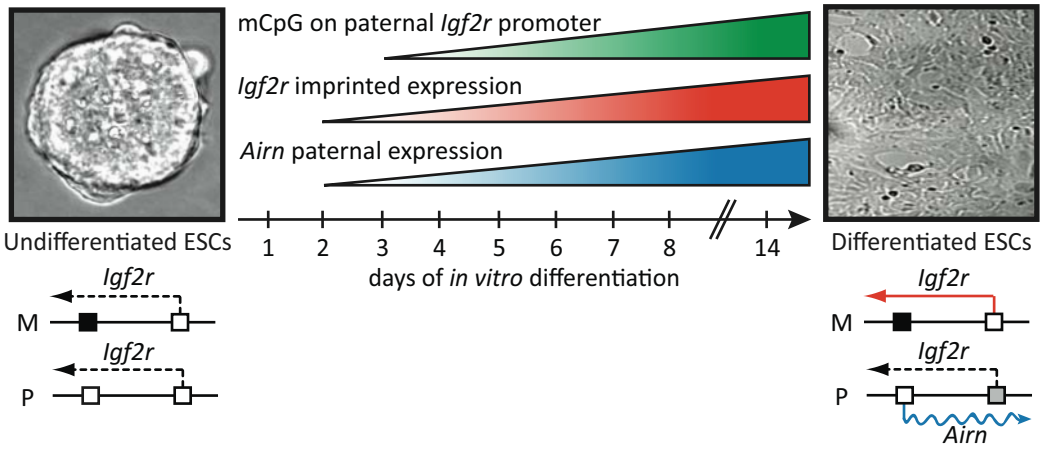

Figure 2. The developmental regulation of the *Igf2r* imprinted gene cluster in ES cells (modified from Santoro et al, in press).
Undifferentiated ES cells (top left) show low-level biallelic expression of *Igf2r* (dashed arrow) from both alleles. Upon differentiation at day 2-3 expression of maternal *Igf2r* is up-regulated (red triangle and red arrow), however low-level expression of *Igf2r* is maintained on the paternal chromosome. *Airn* expression from the paternal chromosome (blue triangle and blue wavy arrow) follows the same kinetics as *Igf2r*. Upon differentiation, DNA methylation is gained on the silent paternal *Igf2r* promoter (green triangle and grey square). Black square: Methylated ICE. White square: Unmethylated CGI. M: Maternal. P: Paternal.

In pre-implantation embryos as well as in undifferentiated ES cells *Igf2r* is biallelically expressed, whereas expression of *Airn* is absent (Lerchner & Barlow, 1997; Stricker et al, 2008; Szabo & Mann, 1995). However, *Igf2r* expression from both parental alleles is very low at this developmental stage. Upon ES cell differentiation or embryo implantation expression of maternal *Igf2r* as well as paternal *Airn* is up-regulated at the same time, indicating that the two transcripts follow the same expression kinetics (Latos et al, 2009). Co-regulation of imprinted

ncRNAs and mRNAs during development has also been shown for the *Kcnq1* imprinted cluster (Lewis et al, 2006). Interestingly, paternal *Igf2r* is not completely silenced during differentiation. It keeps its low expression level already detected in undifferentiated ES cells and the maternal to paternal *Igf2r* expression ratio increases from 1:1 in undifferentiated cells up to 10:1 in differentiated cells, suggesting that imprinted gene expression arises not from complete silencing of paternal *Igf2r*, but through the prevention of its up-regulation (Latos et al, 2009).

As mentioned above, the paternal *Igf2r* promoter represents a sDMR with partial DNA methylation gain in 13.5 dpc embryos, which is completed after birth (Stoger et al, 1993). In undifferentiated ES cells, when *Igf2r* is biallelically expressed, DNA methylation is absent at the *Igf2r* promoter. Upon ES cell differentiation, partial DNA methylation is gradually gained on the paternal allele (Latos et al, 2009). As DNA methylation of the paternal *Igf2r* promoter occurs relatively late, after the establishment of imprinted expression, this indicates that it is not involved in the initiation of allele-specific expression (Latos et al, 2009). Once switched on, *Airn* is expressed continuously during development whenever *Igf2r* shows imprinted expression (Pauler et al, 2005; Yamasaki et al, 2005). Still it is unknown whether continuous *Airn* expression is needed to maintain *Igf2r* imprinted expression once established or if it is dispensable once DNA methylation is gained.

2.3.3 The *Airn* conditional knockout (CKO) cell line

The *Airn* modified ES cell line analyzed in this thesis was generated by Federica Santoro as part of her Ph.D. project and also described in a publication that I contributed to (Santoro et al, in press).

To investigate whether continuous transcription of *Airn* is needed to maintain silencing of paternal *Igf2r*, the *Airn* conditional knockout (*Airn*CKO) cell line was created to turn *Airn* transcription off at different time points of ES cell differentiation (Fig. 3) (Santoro et al, in press). The D3 ES cell line S12/+, which

carries a SNP within maternal *Igf2r* exon 12 and therefore enables discrimination of the two parental alleles, was used (Latos et al, 2009).

The mouse ROSA26 locus provides a good target to insert genes, as it is expressed constitutively and ubiquitously during development (Zambrowicz et al, 1997). Therefore, the CreER^{T2} gene, a Cre recombinase coupled to a mutant ligand-binding domain of the human estrogen receptor (ER), was inserted via homologous recombination into the ROSA26 locus of S12/+ cells and the resulting cell line is referred to as S12^{RC}/+ (Fig. 3A) (Santoro et al, in press). The CreER gene product is located in the cytoplasm until 4-hydroxytamoxifen (TAM), an estrogen antagonist, promotes its activation and translocation into the nucleus (Feil et al, 1997).

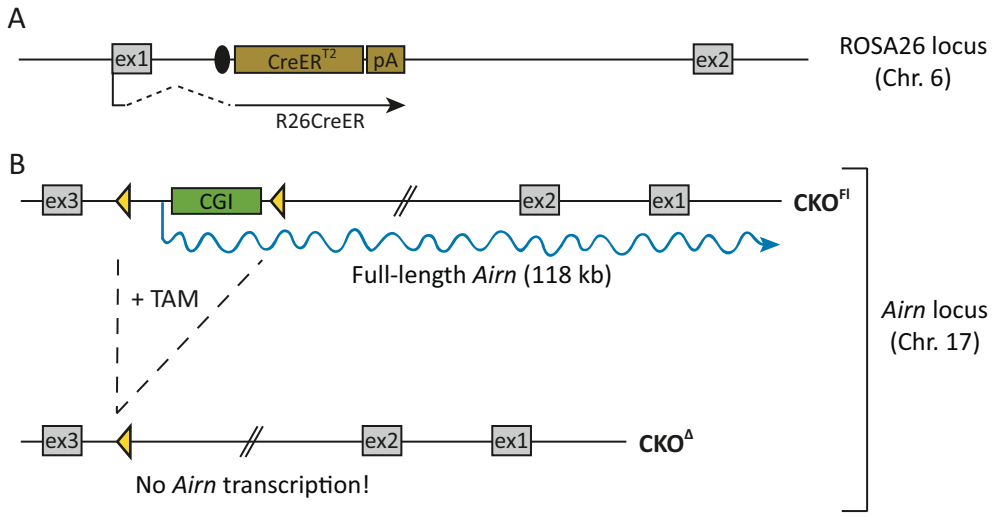

Figure 3. The *Airn* conditional knockout (CKO) ES cell line.
(A) Modified *ROSA26* (*R26*) locus on mouse chromosome 6. The R26CreER allele was generated by homologous recombination in S12/+ ES cells. Ex: *R26* exons. Black circle: splice acceptor site. pA: Polyadenylation signal. Black arrow: *R26CreER*.
(B) Modified *Airn* locus on mouse chromosome 17. The CKO^{FL} allele was generated by homologous recombination in S12^{RC}/+ ES cells (top). Two loxP sites (yellow triangles) flank a 1.9kb region containing the *Airn* promoter and CGI (green rectangle) located between exon 2 and 3 of *Igf2r* (grey rectangles). Upon TAM treatment Cre recombinase removes the DNA sequence between the two loxP sites resulting in the CKO$^{\Delta}$ allele (bottom). Ex: *Igf2r* exons. Blue wavy arrow: *Airn*.

The CKO ES cell line, CKO^{FI}, was created by inserting two loxP sites flanking a 1.9kb region, which contains the *Airn* transcriptional start site (TSS) and CGI on the paternal allele (Fig. 3B, top) (Santoro et al, in press). Upon TAM treatment, the Cre recombinase is translocated into the nucleus, where it binds specifically to the

introduced loxP sites. In case of the CKO ES cell line the two loxP sites are orientated in the same direction resulting in the deletion of the intervening sequence with one residual loxP site left (Sternberg & Hamilton, 1981). The cell line carrying the *Airn* promoter deletion is referred to as CKO^{Δ} (Fig. 3B, bottom). The inducible Cre-loxP system thus provides a powerful tool to remove the promoter of *Airn* at any time point during ES cell differentiation.

2.4 Aim of the project

I investigated the developmental control of *Igf2r* silencing by *Airn* in ES cell differentiation. Previous studies have revealed that the transcription of the macro lncRNA *Airn* is sufficient to initiate *cis*-silencing of the antisense overlapped *Igf2r* promoter via transcriptional interference (Latos et al, 2012). *Igf2r* silencing is accompanied by gain of DNA methylation at the silent paternal *Igf2r* promoter (Latos et al, 2009; Stoger et al, 1993). *Airn* is transcribed continuously throughout life whenever *Igf2r* shows imprinted expression (Pauler et al, 2005; Yamasaki et al, 2005). However, one issue that has not been addressed is whether continuous *Airn* transcription is needed to maintain *Igf2r* silencing once established or if it is dispensable once DNA methylation is gained. With the usage of the *Airn*CKO cell line (Santoro et al, in press), I was able to tackle this problem in the ES cell differentiation system. In this project I mainly worked on setting up optimal conditions for consistent ES cell differentiation either in monolayer with retinoic acid or via embryoid body formation. I induced the deletion of the *Airn* promoter by TAM treatment at various time points during ES cell differentiation followed by the analysis of allele-specific gene expression and the DNA methylation state.

3. Results

In this thesis, I used the *Airn*CKO ES cell line (described in detail in section 2.3.3, Fig. 3), which was generated by Federica Santoro as part of her Ph.D. project. Further description can be found in a publication I contributed to (Santoro et al, in press). I investigated whether continuous *Airn* expression is needed to maintain *Igf2r* silencing or DNA methylation at the paternal *Igf2r* promoter alone is sufficient to keep the repressed state. Some of the figures presented in this section are modified from the paper, whereby I performed all shown experiments by myself.

3.1 Cre-mediated excision of *Airn* promoter in undifferentiated *Airn*CKO ES cells

In the *Airn*CKO ES cell line two loxP sites flank a 1.9kb region including the TSS and the CGI of *Airn*. Further, the CreER^{T2} gene was inserted in the ROSA26 locus to ensure expression throughout ES cell differentiation. This inducible system enables the Cre-mediated recombination of the genomic sequence between the two loxP sites and therefore excision of the *Airn* promoter by administering TAM to the cells. TAM is commonly used for treating breast cancer, because of its antiestrogen and therefore antiproliferating activity (Osborne, 1998). Therefore, in experiments with TAM-inducible CreER it is important to choose the right dosage to achieve efficient recombination, however not to inhibit cell growth.

Studies investigating Cre recombination in ES cells reported already that ~ 0.2µM TAM are sufficient to induce efficient recombination with the CreER^{T2} introduced in the ROSA26 locus (Hameyer et al, 2007). Federica Santoro tested the effect of the TAM treatment on ES cell growth by using 2 to 10µM TAM and noticed that above 2µM cells grew slower (part of Ph.D. thesis of Federica Santoro, in preparation). To see whether I could optimize the assay still more, undifferentiated CKO^{Fl} ES cells, these cells have an intact *Airn* promoter flanked by two loxP sites, were treated with

different amounts of TAM ranging from 0.1 to 1µM for 6 to up to 48 hours (h). To determine the recombination efficiency of Cre, I used a Southern blot assay, whereby the genomic DNA (gDNA) was digested with EcoRI and hybridized to probe AirT (Fig. 4A). Untreated control cells show a 6.3kb fragment for the CKO^{FL} allele and a 6.2kb fragment for the wt maternal allele, which cannot be distinguished on this blot (Fig. 4B, lane 1). Upon TAM treatment the intervening DNA sequence between the two loxP sites is removed resulting in a 4.4kb fragment representing the CKO^{Δ} allele (Fig 4B, lanes 3-21).

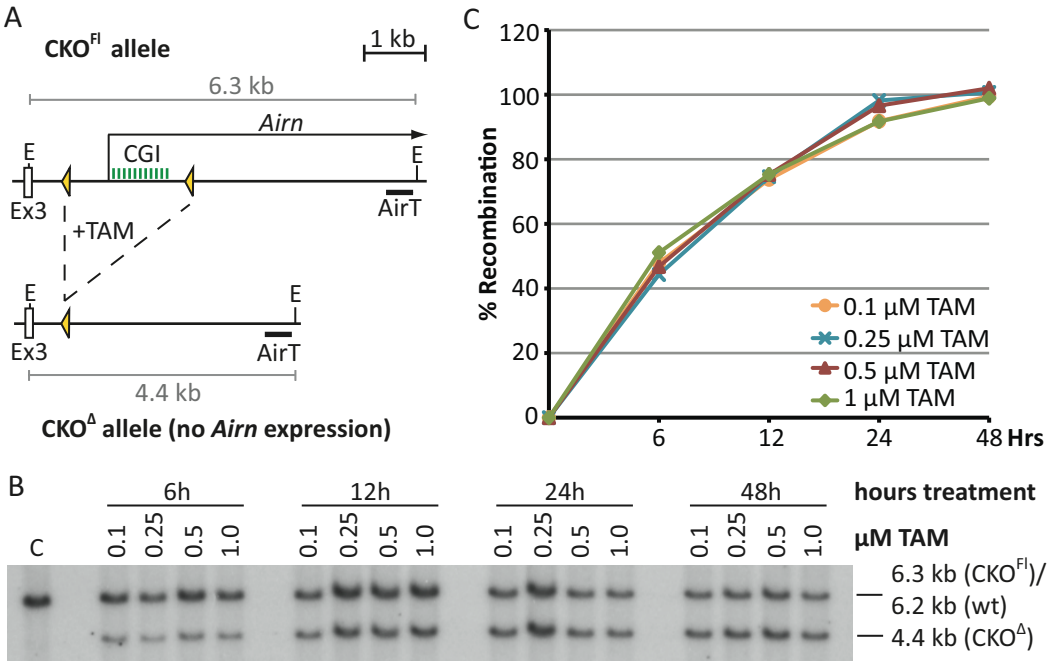

Figure 4. Cre recombination efficiency in undifferentiated *Airn*CKO ES cells (modified from Santoro et al, in press).

(A) Southern blot strategy. The *Airn*CKO allele as described in Fig. 3. The gDNA was digested with EcoRI (E) and hybridized to probe AirT (black bar). Note that the EcoRI fragment coming from the wt maternal allele is 6.2kb (not indicated). Yellow triangle: LoxP site. Green dashed line: CGI of *Airn*. Black arrow: *Airn*. Ex: *Igf2r* exon. Grey line: Expected Southern blot band size.

(B) Southern blot analysis to determine Cre-mediated recombination efficiency. Undifferentiated *Airn*CKO ES cells were treated with 0.1, 0.25, 0.5 or 1µM TAM for 6, 12, 24 or 48 hours (h). Control (C) cells were treated with vehicle only and harvested 48h later. Top band shows the wt maternal (6.2kb) and unrecombined paternal (CKO^{Fl} 6.3kb) alleles, which cannot be distinguished on this blot. Bottom band indicates the promoter deleted paternal allele (CKO^{Δ} 4.4kb).

(C) ImageQuant quantification of recombination efficiency. Equal intensity of the upper and lower bands in B indicates complete recombination. Percentage recombination is shown as % lower band/[(upper + lower band)/2]) in the graph. Independently of the amount of TAM used, complete recombination is achieved between 24 and 48h in undifferentiated CKO ES cells.

Equal intensity of the lower and upper bands indicates complete excision of the *Airn* promoter. The signal intensity was quantified and plotted in a graph (Fig. 4C). Already after 24h, deletion of the *Airn* promoter is detected in more than 80% of the cells and complete recombination can be observed after 48h of treatment. Interestingly, there is no dose-dependent difference in the efficiency of Cre recombinase, indicated by the similar excision kinetics seen with amounts of TAM ranging from 0.1 to 1µM.

3.2 *Airn*CKO ES cells differentiated with RA

Expression of *Airn* as well as up-regulation of maternal *Igf2r* arises at day (d) 2-3 of ES cell differentiation resulting in imprinted *Igf2r* expression (Fig. 2). To test whether continuous expression of the macro lncRNA is required after the establishment of *Igf2r* imprinted expression, *Airn*CKO ES cells were differentiated in monolayer with retinoic acid (RA) and excision of the *Airn* promoter was induced at day 5, 9 or 13 by adding 1µM TAM. Considering the fact that *Airn* has a half-life time of approximately two hours, only 1.6% of the transcript should be still present after 12 hours from deleting its promoter (Seidl et al, 2006). The cells were harvested four days after recombination induction, at day 9, 13 and 17, respectively.

For the experiments described below, three biological replicates were performed. Control cells, either treated with vehicle only (no TAM) or in which recombination was induced prior to differentiation, were co-differentiated in parallel. Southern blot and reverse transcription-polymerase chain reaction (RT-PCR) data are always shown for one representative replicate (two other replicates are shown in Santoro et al, in press, Fig. S3-S5). Quantitative PCR (qPCR) results are displayed as pooled data from all three biological replicates with standard deviation.

3.2.1 Southern blot quantification of Cre recombination in RA differentiated *Airn*CKO ES cells

To test whether the *Airn* promoter can be efficiently deleted during ES cell differentiation, I used the same Southern blot assay as described for undifferentiated ES cells (Fig. 4A). In untreated *Airn*CKO ES cells only the 6.3kb CKO^{Fl} and 6.2kb wt maternal allele are detected, because recombination was not induced in these cells (Fig. 5, samples 1-4). When TAM was added at day 0 and the cells were harvested after 17 days of differentiation, efficient recombination was achieved, as indicated by the equal intensity of the upper and lower bands (Fig. 5, sample 5). In contrast, excision of the *Airn* promoter was progressively less efficient when TAM was added during differentiation. Whereas Cre-mediated recombination at day 5 occurred still with 88% efficiency (Fig. 5, sample 6), the efficiency dropped to 72 and 58% when TAM was added at day 9 or 13, respectively (Fig. 5, samples 7 and 8).

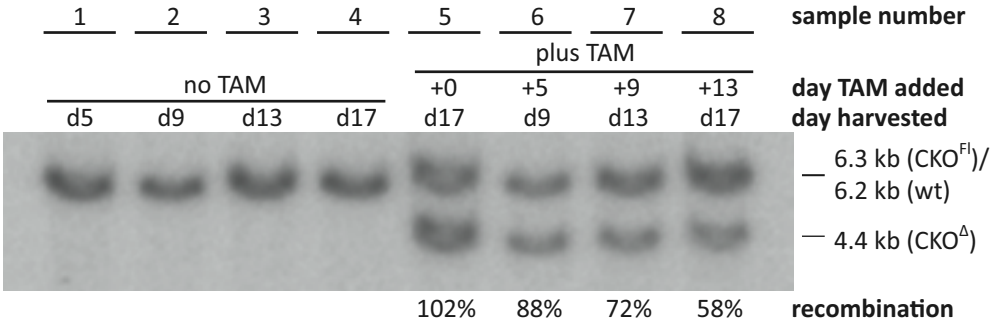

Figure 5. Inefficient Cre recombination in RA differentiated *Airn*CKO ES cells (modified from Santoro et al, in press).
One representative Southern blot of three biological replicates to detect Cre-mediated recombination in CKO ES cells differentiated with RA. Southern Blot strategy is shown in Fig. 4A. gDNA from differentiated CKO ES cells was digested with EcoRI and hybridized to probe AirT. Top band corresponds to unrecombined paternal (CKO^{Fl} 6.3kb) and wt maternal (6.2kb) alleles, which are not separated on this blot. Bottom band shows paternal allele after Cre recombination (CKO^{Δ} 4.4kb). Equal intensity of both bands indicates complete recombination shown as % lower band/[(upper + lower band)/2] underneath the blot. The *Airn* promoter is removed efficiently when cells are treated with TAM prior to differentiation, however reduced recombination is observed in ES cells treated with TAM during differentiation.

Note that the Southern blot shown in Fig. 5 represents one of three biological replicates but all of them showed comparable results for Cre-mediated

recombination (shown in Santoro et al, in press, Fig. S3): sample 5: 97-98%, sample 6: 89-91%, sample 7: 62-65% and sample 8: 51-60%.

3.2.2 Developing a qPCR assay to measure Cre recombination efficiency

As recombination in late differentiated *Airn*CKO ES cells is incomplete, as determined by quantification of the intensity of Southern blot bands, I decided to develop a qPCR assay in order to measure the extent of recombination more precisely. Federica Santoro designed the primers for this experiment. A common reverse primer, which spans part of the downstream loxP site, ensures that only the paternal but not the unmodified maternal allele is amplified. To distinguish between the two paternal alleles, the unrecombined CKO^{Fl} and the recombined CKO^{Δ} alleles, two forward primers were strategically designed to enable exclusive detection, due to the length of the amplicon, of one but not the other allele (Fig. 6A).

To verify the allele-specificity of the primers, both primer combinations, CKO-R + F1 specific for the CKO^{Fl} allele and CKO-R + F2 specific for the CKO^{Δ} allele, were tested on plasmids containing gDNA corresponding to either the unrecombined or the recombined paternal allele (Fig. 6B). Primers are specific, as the level of cross-hybridization to the wrong allele is low (0.001 and 0.0002%).

I then tested different amounts of input gDNA and whether this influences the extent of cross-hybridization (Fig. 6C). *Airn*CKO ES cells were treated either with TAM (+) or vehicle only (-) when the cells were still in the undifferentiated state and the treatment was kept throughout differentiation for 14 days. This should ensure a homogenous population carrying almost exclusively the unrecombined or the recombined paternal allele. Both primer combinations were tested on a total of 10, 50 or 100ng gDNA from TAM treated or untreated ES cells. The cross-hybridization of the primers to the "wrong" allele was again low, indicating that the primers are specific to their target allele not only on plasmid DNA but also on total gDNA. However, it seems that different amounts of gDNA used for the qPCR influences the

extent of cross-hybridization. For the CKO$^{\Delta}$ allele, the extent of cross-hybridization was low for all tested amounts of gDNA. Due to the smallest cross-hybridization reaction seen for the CKO^{Fl} allele, I decided to use a total of 50ng gDNA for this assay.

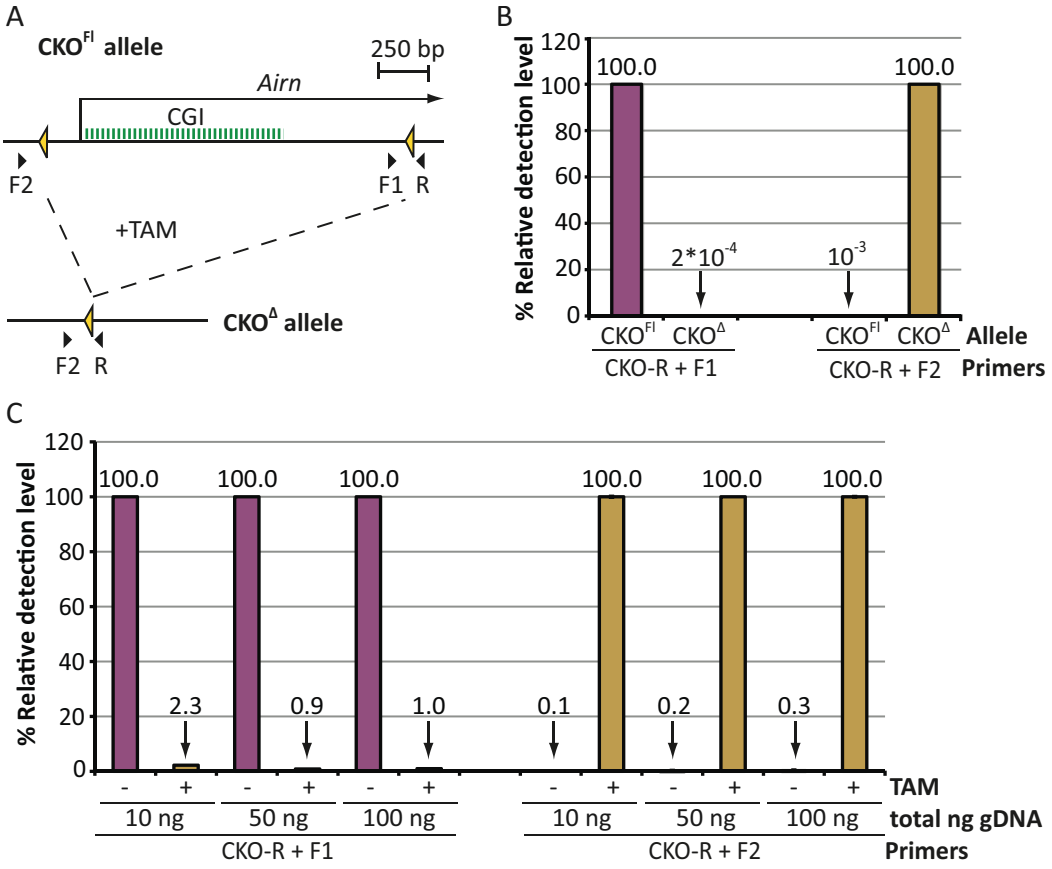

Figure 6. Optimization of Cre recombination qPCR assay.
(A) qPCR strategy to detect Cre-mediated recombination in CKO ES cells. Genomic positions of the three qPCR primers (black arrow head): one common reverse (R) primer and two different forward (F1, F2) primers to distinguish between paternal unrecombined (CKO^{Fl}, top) and recombined (CKO$^{\Delta}$, bottom) alleles. Yellow triangle: LoxP site. Green dashed line: CGI of *Airn*. Black arrow: *Airn*.
(B) Test qPCR using both possible primer combinations on plasmids containing gDNA corresponding to the unrecombined (CKO^{Fl}) or recombined (CKO$^{\Delta}$) alleles. Detection levels of the correct allele were set to 100%; detection levels of the "wrong" allele due to primer cross-hybridization are displayed relative to this value. Primers show high specificity to target sequence when tested on plasmids. Purple: CKO^{Fl} plasmid. Yellow: CKO$^{\Delta}$ plasmid.
(C) Test qPCR using both possible primer combinations on indicated amounts of gDNA of TAM treated (+) or untreated (-) CKO ES cells. Detection levels of the correct allele were set to 100%, detection levels of the "wrong" allele due to cross-hybridization are displayed relative to this value. The least cross-hybridization for the CKO^{Fl} allele is obtained when a total of 50ng gDNA is used. For the CKO$^{\Delta}$ allele, the extent of cross-hybridization is low for all tested amounts of gDNA. Purple: TAM untreated CKO ES cells (CKO^{Fl}). Yellow: TAM treated CKO ES cells (CKO$^{\Delta}$).

3.2.3 qPCR quantification of Cre recombination in RA differentiated *Airn*CKO ES cells

The newly established qPCR assay was used to quantify recombination efficiency in the RA differentiated cells shown in Fig. 5. The qPCR data show the pooled results of three biological replicates (Fig. 7). In untreated samples only the unrecombined CKO^{Fl} allele is detected (Fig. 7, samples 1-4), whereas in the sample, which was treated with TAM prior to differentiation, this same allele is virtually undetected, confirming that efficient recombination is achieved (Fig. 7, sample 5). The data for samples treated with TAM at day 5, 9 and 13 (Fig. 7, sample 6-8) confirm the results seen by Southern blot (Fig. 5). Cre-mediated excision of the *Airn* promoter at day 5 of differentiation occurs with 83% efficiency, however the excision rate drops to 59-63% when TAM was added at day 9 or 13, respectively.

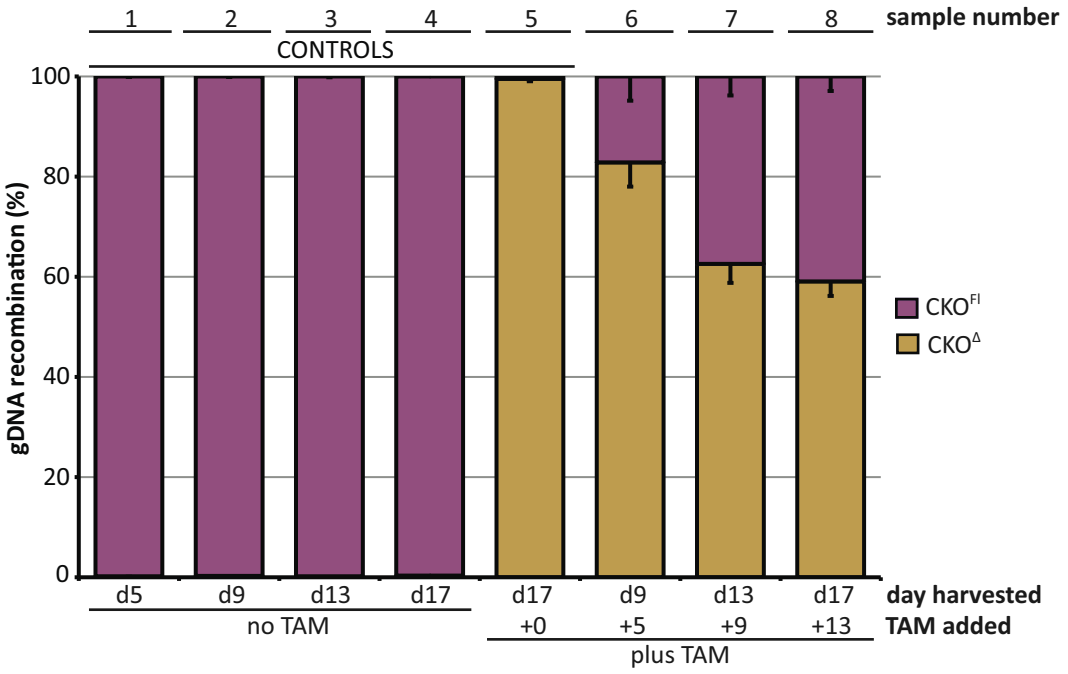

Figure 7. Quantification of Cre recombination efficiency in RA differentiated *Airn*CKO ES cells by qPCR (modified from Santoro et al, in press).
qPCR quantification of recombination efficiency of RA differentiated CKO ES cells confirms decreasing Cre efficiency upon differentiation. Samples from three biological replicates were analysed with the qPCR assay shown in Fig. 6. Combined levels of CKO^{Fl} (purple) and CKO^{Δ} (yellow) allele were set to 100 and each bar shows the contributed percentage as the mean and s.d. of three biological replicates.

3.2.4 Reduced *Airn* transcription in RA differentiated *Airn*CKO ES cells

Although with decreasing efficiency, the inducible Cre-loxP system used in *Airn*CKO ES cells works during differentiation, therefore the removal of the *Airn* promoter should lead to a reduction of transcription of the macro lncRNA. I used an RT-qPCR assay with primers specific for *Airn* to measure *Airn* steady-state levels in three biological replicates (Fig. 8).

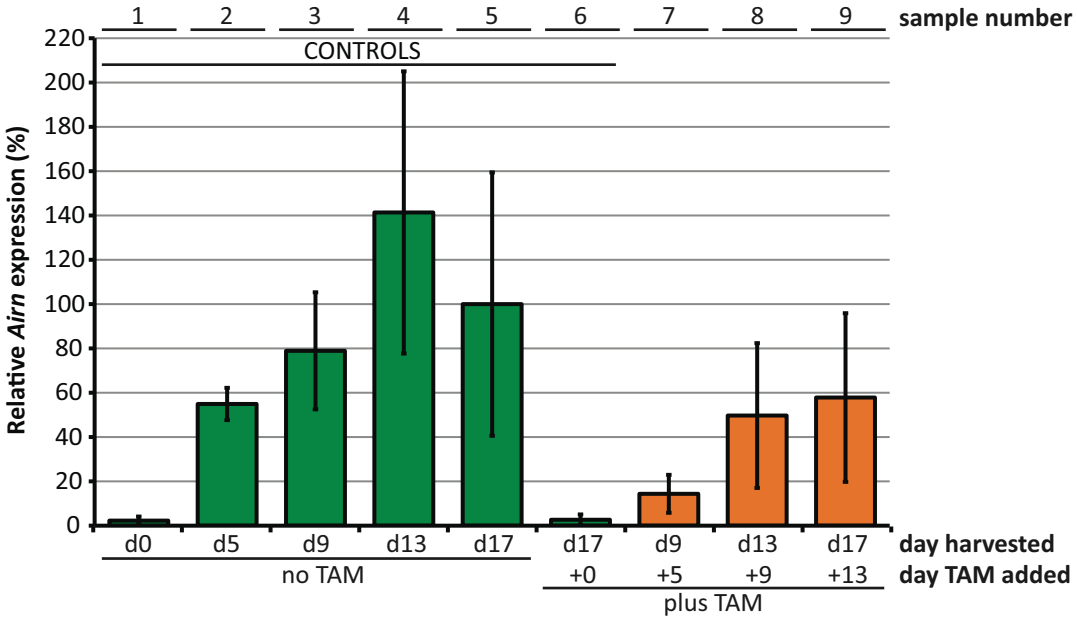

Figure 8. Deletion of the *Airn* promoter reduces *Airn* expression in RA differentiated *Airn*CKO ES cells (modified from Santoro et al, in press).
Reduced *Airn* expression levels in CKO ES cells detected by RT-qPCR with primers specific for *Airn*. Relative expression levels in untreated d17 cells were set to 100. Upon TAM treatment *Airn* levels are reduced in early (sample 7) but not in late (sample 8-9) time points. Bars and error bars: mean and s.d. of three biological replicates. Green bars: control samples. Orange bars: *Airn*CKO.

In undifferentiated cells, *Airn* is not expressed and upon differentiation up-regulation of the macro lncRNA is detected (Fig. 8, samples 1-5), as described previously (Latos et al, 2009). The observed *Airn* expression pattern in differentiated samples indicates that the introduced loxP sites in the CKO^{FL} allele do not interfere with the endogenous promoter activity. When recombination is induced at day 0 and cells are harvested at day 17, the transcription of *Airn* is completely abolished (Fig. 8, sample 6), confirming the results of efficient recombination seen in this sample (Fig. 5, sample 5; Fig. 7, sample 5). Residual *Airn* expression of 14% is

detected, when TAM is added at day 5 of differentiation (Fig. 8, sample 7). However, the removal of the *Airn* promoter in later stages during RA differentiation of CKO ES cells results in high residual expression levels of *Airn* with up to 58% (Fig. 8, samples 8-9). This can be explained by the inefficient Cre-mediated recombination seen in these samples by Southern blot (Fig. 5, samples 7-8) and qPCR (Fig. 7, samples 7-8). Together the data show that the *Airn* promoter can be deleted at any time point during ES cell differentiation resulting in a reduction of *Airn* expression.

3.2.5 *Igf2r* is re-expressed upon *Airn* removal in RA differentiated *Airn*CKO ES cells

To test the effect of the conditional *Airn* promoter deletion on imprinted expression of *Igf2r*, I used an allele-specific assay to distinguish between maternally and paternally expressed *Igf2r* (Fig. 9). *Airn*CKO ES cells were derived from a previously modified cell line in the lab carrying a SNP within exon 12 of the maternal *Igf2r* allele, which destroys an endogenous PstI restriction site (Latos et al, 2009).

Fig. 9A shows one representative non-quantitative allele-specific assay (two other replicates see Santoro et al, in press, Fig. S4), in which ES cell cDNA was amplified with primers specific for *Igf2r* exon 12 and digested with PstI. The upper band corresponds to undigested maternal *Igf2r*, since its restriction site is destroyed by the introduced SNP, whereas the two lower bands correspond to the paternal *Igf2r* RT-PCR product that is cut by PstI. In undifferentiated ES cells, *Igf2r* is expressed biallelically, indicated by the presence of maternal and paternal specific bands (Fig. 9A, sample 1). Upon differentiation of *Airn*-expressing CKO cells the paternal-specific *Igf2r* bands becomes fainter compared to undifferentiated ES cells, consistent with maternal-specific *Igf2r* up-regulation during differentiation (Fig. 9A, samples 2-5). The strong paternal-specific bands in the sample, in which the *Airn* promoter was removed at day 0, indicate that in the absence of *Airn* *Igf2r* fails to establish imprinted expression (Fig. 9A, sample 6). This result agrees with previous deletion studies of the *Airn* promoter resulting in biallelic *Igf2r* expression (Stricker et al, 2008; Wutz et al, 2001). To address the issue whether *Airn* is needed to maintain

silencing of *Igf2r* once imprinted expression has been established, expression of the macro lncRNA was turned off at day 5, 9 or 13, when *Igf2r* already shows allele-specific expression (as seen in Fig. 9A, samples 2-4). In the absence of *Airn*, paternal *Igf2r* is re-expressed at all tested time points, as indicated by stronger paternal-specific cut bands compared to control samples expressing *Airn* (Fig. 9A, compare samples 7-9 to 2-5). This shows that until day 13 *Airn* is needed to maintain silencing of *Igf2r*, however the extent of paternal *Igf2r* re-expression decreases at later time points of differentiation. Note that this RT-PCR is a non-quantitative assay. The PstI cut paternal *Igf2r* is separated into two bands, meaning that when compared to maternal *Igf2r* the intensity of both paternal-specific fragments has to be considered. Further, longer PCR fragments incorporate more ethidium bromide thus influencing the detected band intensity.

Allele-specific expression of *Igf2r* was therefore quantified with an RT-qPCR assay using a common reverse primer and a forward primer specific for the wt paternal or the SNP-carrying maternal allele (Koerner et al, 2012). The RT-qPCR was performed on three biological replicates and is shown as pooled data (Fig. 9B). Since in the absence of *Airn*, *Igf2r* is expressed biallelically, the ratio of maternal:paternal *Igf2r* expression in prior to differentiation treated samples was set to 50:50 (Fig. 9B, sample 5). In *Airn* expressing control samples (no TAM), maternal-specific *Igf2r* imprinted expression is clearly detectable with low-level (9-12% of total *Igf2r*) paternal-specific expression (Fig. 9B, samples 1-4), as previously described (Latos et al, 2009). Upon deletion of the *Airn* promoter at different time points when imprinted expression is already established, silencing of paternal *Igf2r* is relieved compared to wt control samples (Fig. 9B, samples 6-8, blue bars). Confirming previous results of the non-quantitative assay (Fig. 9A), paternal *Igf2r* is re-expressed to 38% of total *Igf2r* when TAM was added at day 5 (Fig. 9B, sample 6, blue bar), but when *Airn* is removed at day 9 or 13 the amount of paternal *Igf2r* that is re-expressed is only 30 or 27% (Fig. 9B samples 7-8, blue bars). However, incomplete promoter deletion was observed at these two time points (Fig. 5; Fig. 7). If only the population of ES cells which shows complete recombination is

considered, corrected values for paternal *Igf2r* indicate paternal *Igf2r* re-expression to ~40% of total levels at any time point of differentiation (Fig. 9B, samples 6-8, black bars). Altogether, the loss of *Airn* expression allows re-expression of paternal *Igf2r* at any time point during ES cell differentiation.

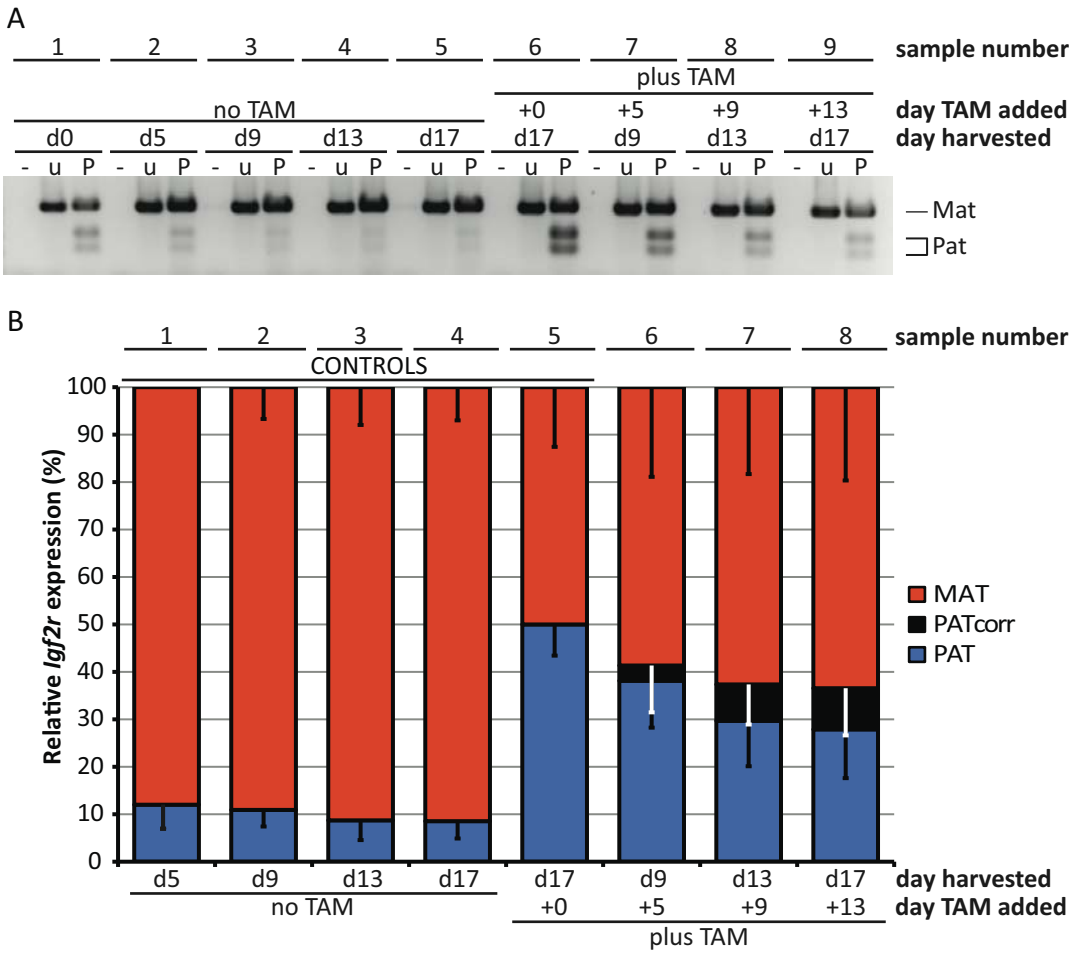

Figure 9. Continuous *Airn* transcription is needed to silence *Igf2r* in RA differentiated *Airn*CKO ES cells (modified from Santoro et al, in press).
(A) RT-PCR and paternal-specific PstI digest of one representative biological replicate out of three to examine allele-specific *Igf2r* expression. Maternally expressed *Igf2r* generates a 541bp fragment and paternal expression two 318 and 223bp fragments. Upon removal of the *Airn* promoter paternal *Igf2r* is re-expressed to different amounts. Mat: Maternal. Pat: Paternal. -: Minus RT. u: Undigested. P: PstI digested.
(B) Allele-specific *Igf2r* RT-qPCR confirms paternal *Igf2r* re-expression upon TAM treatment however with less efficiency in late time points of differentiation. Maternal (red bars) and paternal (blue bars) *Igf2r* levels are shown as a total of 100% with mean and s.d. of three biological replicates. In sample 5, maternal:paternal level is set to 50:50 since removal of the *Airn* promoter prior to differentiation leads to biallelic *Igf2r* expression. Data were corrected for recombination efficiency, quantified in Fig. 7, to show the results only for recombined cells (black bars).

3.2.6 DNA methylation of paternal *Igf2r* promoter in RA differentiated *Airn*CKO ES cells

Igf2r silencing by *Airn* is followed by moderate gain of DNA methylation at CpG dinucleotides within the paternal *Igf2r* CGI (Latos et al, 2009; Stoger et al, 1993). Previous experiments have shown that this methylation mark is not needed to establish or maintain *Igf2r* silencing until 8.5dpc of embryonic development, which is the lethal period of embryos lacking the *Dnmt1* maintenance methyltransferase gene (Li et al, 1993; Seidl et al, 2006). However, DNA methylation might still play a role in maintaining the silencing of *Igf2r* in the absence of *Airn* after this time.

To examine the methylation state of the paternal *Igf2r* promoter in *Airn*CKO ES cells, I performed a Southern blot using a NotI methyl-sensitive restriction site within the *Igf2r* CGI, which overlaps two CpG dinucleotides that can be methylated (Fig. 10A). A double digest of the gDNA with EcoRI and NotI was performed and hybridized to probe NEi. This results either in a 5kb fragment, if the CpG dinucleotides within the NotI site are methylated, or a shorter 1kb fragment if the NotI site is unmethylated.

Fig. 10B shows Southern blot analysis of one representative *Airn*CKO RA differentiation of three biological replicates (two other replicates shown in Santoro et al, in press, Fig. S5). In differentiated CKO ES cells lacking the *Airn* promoter, *Igf2r* is expressed from both parental alleles and its promoter is not methylated, indicated by the lack of the upper band (Fig. 10B, sample 5). In control cells expressing the *Airn* macro lncRNA throughout differentiation (no TAM), methylation of *Igf2r* is progressively gained resulting in up to 20% methylation by 17 days of RA differentiation (Fig. 10B, samples 1-4). Note that 50% methylation represents a fully methylated paternal allele, reflected by equal intensity of the lower and upper bands. The percentage of paternal *Igf2r* promoter methylation is calculated by dividing the intensity of the methylated band by the sum of the intensities of the methylated and unmethylated bands. In ES cells lacking *Airn* expression from the beginning of the differentiation, *Igf2r* imprinted expression is not established and no

methylation at the paternal *Igf2r* promoter is detected (Fig. 10B, sample 5). Upon the removal of *Airn* during RA differentiation, similar methylation levels of paternal *Igf2r* promoter are achieved compared to the matching untreated samples, which express *Airn* (Fig. 10B, samples 6-8). The two other biological replicates showed comparable DNA methylation results (Santoro et al, in press, Fig. S5): sample 1: 4-7%, sample 2: 10-16%, sample 3: 13-21%, sample 6: 7-14%, sample 7: 11-20% and sample 8: 15-23%.

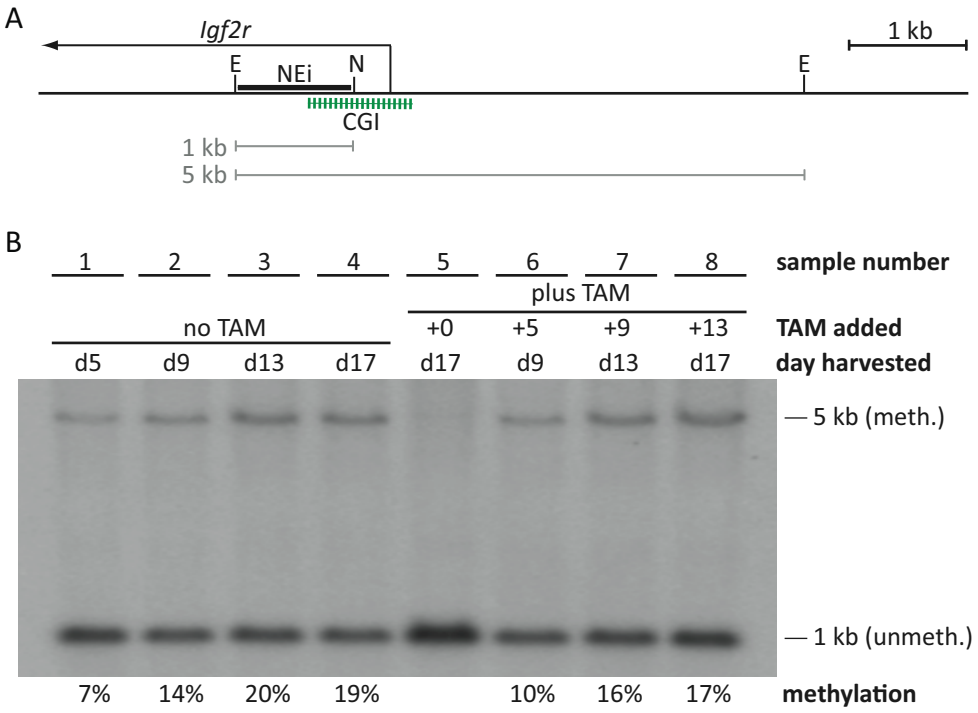

Figure 10. Methylation of the *Igf2r* promoter in RA differentiated *Airn*CKO ES cells (modified from Santoro et al, in press).
(A) Southern blot strategy to determine methylation status of the *Igf2r* promoter. gDNA was digested with EcoRI (E) and methyl-sensitive NotI (N) hybridized to probe NEi (black bar). The NotI restriction site lies within the CpG island of *Igf2r* (green dashed line) and contains two CpG dinucleotides. Black arrow: *Igf2r*. Grey line: Expected Southern blot band sizes.
(B) One representative Southern blot of three biological replicates analyzing *Igf2r* promoter methylation in RA differentiated CKO ES cells upon removal of *Airn*. Top band corresponds to a methylated *Igf2r* promoter (meth, 5 kb). Bottom band indicates unmethylated promoter (unmeth, 1 kb). In untreated cells (no TAM) methylation of the paternal *Igf2r* promoter is progressively gained during differentiation (samples 1-4) indicated by the appearance of the 5kb fragment. However the DNA methylation is maintained independently of *Airn* (samples 6-8) in CKO cells. Paternal *Igf2r* promoter methylation % meth/(meth+unmeth) is shown below the blot. Note maximum methylation is 50% as only one allele is methylated.

This shows that DNA methylation at the paternal *Igf2r* promoter is progressively gained upon ES cell differentiation, however without *Airn* expression, which results

in biallelic *Igf2r* expression, no methylation is detected. Further, the data indicate that the amount of DNA methylation, which is gained until the *Airn* promoter deletion is induced, is maintained despite the absence of *Airn*.

3.2.7 *Igf2r* promoter methylation does not interfere with Cre recombination in CKO^{Fl} ES cells

The levels of NotI methylation on the paternal *Igf2r* promoter reached a similar maximum level in cells expressing *Airn* at day 9, 13 and 17 (Fig. 10B, samples 2-4) compared to cells that had the *Airn* promoter deleted 4 days prior to analysis (Fig. 10B, samples 6-8). Since, RA differentiated *Airn*CKO ES cells only show 59-63% recombination when *Airn* promoter deletion is induced at day 9 or 13 (Fig. 7, samples 7-8), this population of cells assayed for *Igf2r* promoter methylation consist of both CKO^{Fl} cells that carry a non-deleted *Airn* promoter and CKO$^{\Delta}$ cells that have a deleted *Airn* promoter. This raises the question if Cre recombination, which deletes the *Airn* promoter, preferentially occurs in day 9 or 13 differentiated cells that still carry an unmethylated *Igf2r* promoter. Although the 28kb separating the *Airn* and *Igf2r* promoters make this unlikely, if this was correct, then DNA methylation would negatively correlate with Cre-mediated *Airn* promoter excision and I would not be able to conclude that *Igf2r* methylation is maintained independently of *Airn*. To test whether Cre recombination only occurs in ES cells, which have not gained DNA methylation, I developed an assay to distinguish between different scenarios on the paternal allele, regarding the recombination state of the *Airn* promoter as well as the methylation state of *Igf2r*. I performed a triple digest of the gDNA with PacI (a non-methyl-sensitive 8 basebair cutter that is rare in mammalian DNA) and methyl-sensitive MluI and NotI, followed by a pulsed field gel electrophoresis (PFGE) and a Southern blot using probe OT2.4 (Fig. 11A). Pulsed-field gel electrophoresis is able to separate larger DNA molecules than conventional unidirectional electrophoresis so that fragments greater than 20kb can be identified by a Southern blot assay (Herschleb et al, 2007).

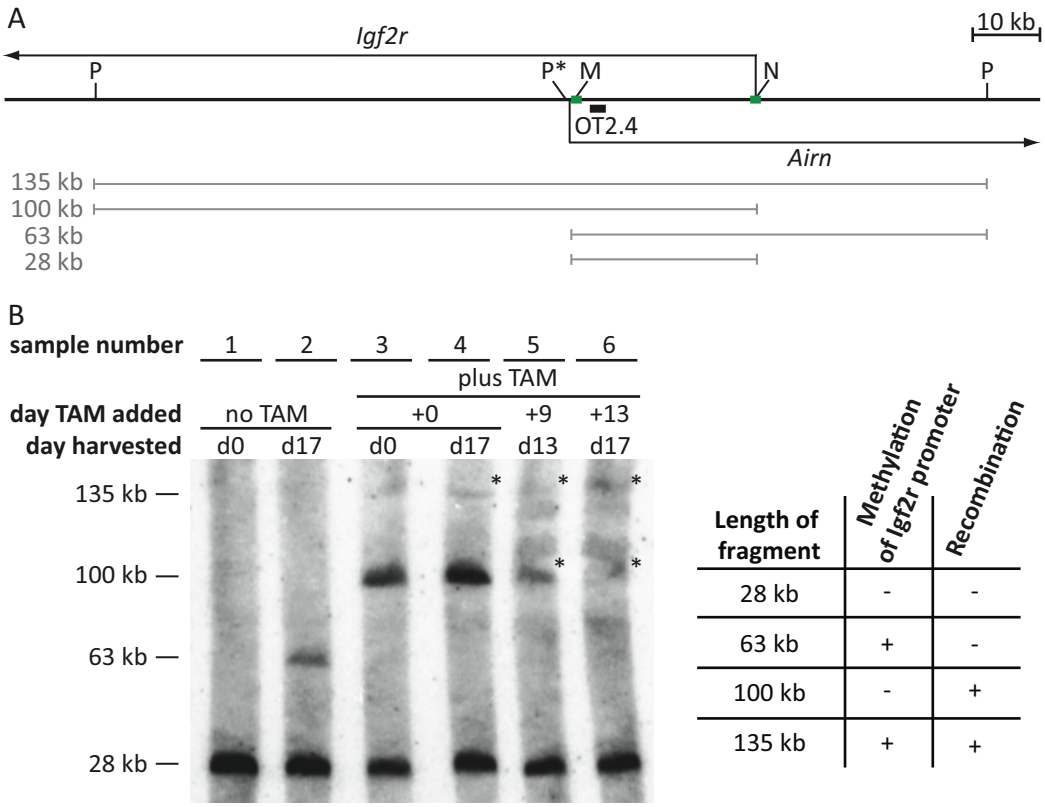

Figure 11. Cre recombination can occur in *Airn*CKO ES cells with a methylated *Igf2r* promoter.
(A) Pulse field gel electrophoresis (PFGE) strategy assaying the correlation of Cre-mediated recombination and paternal *Igf2r* promoter methylation in TAM treated CKO ES cells. gDNA was digested with PacI (P), methyl-sensitive MluI (M) and NotI (N) and hybridized to probe OT2.4 (black bar). MluI restriction site lies within the CGI (green bar) of *Airn* and NotI within the CGI of *Igf2r*. Note that the central PacI site (P*) is deleted in the CKO allele and that Cre recombination removes the MluI site. Black arrows: *Igf2r* and *Airn*. Grey line: Expected Southern blot band sizes.
(B) PFGE analysis of RA differentiated CKO ES cells reveals no influence of *Igf2r* promoter methylation on Cre recombination. Table (right) shows all different combinations of recombination and methylation and the expected length of fragments. Asterisks (*) indicate faint bands. The missing 63kb fragment in ES cells treated with TAM upon differentiation (samples 5-6) shows that Cre can remove the *Airn* promoter although the paternal *Igf2r* promoter is methylated.

Fig. 11A shows a map of the investigated region. MluI does not cut the wt maternal allele due to complete methylation of the *Airn* CGI (Stoger et al, 1993), thus a triple PacI-MluI-NotI digest always generates a maternally derived 28kb PacI*-NotI fragment in all cells. On the paternal CKO^{Fl} allele, PacI does not cut at the central PacI* site, since generation of the CKO^{Fl} allele caused deletion of this site (Santoro et al, in press). The paternal *Airn* CGI is unmethylated (Stoger et al, 1993) and MluI digests the CKO^{Fl} allele. However, upon Cre recombination and excision of the *Airn* promoter the MluI site is removed on the CKO^{Δ} allele. The methyl-sensitive NotI restriction site is located in the CGI of *Igf2r* and is used to determine paternal-

specific *Igf2r* DNA methylation (described in Fig. 10A). Thus after a triple digest, the paternal allele can generate the following bands (Fig. 11B, right):

(1) 28kb: MluI - NotI (CKO^{FI} allele with an unmethylated NotI site not distinguished from the maternal PacI*-NotI fragment that is 0.7kb smaller)

(2) 63kb: MluI - PacI (CKO^{FI} allele with a methylated NotI site)

(3) 100kb: PacI - NotI (CKO^{Δ} allele with an unmethylated NotI site)

(4) 135kb: PacI - PacI (CKO^{Δ} allele with a methylated NotI site)

Since the maternal allele always contributes to the 28kb fragment, I only focus on the bands derived from the paternal allele (Fig. 11B, left). In untreated control ES cells the paternal *Airn* promoter is present. Undifferentiated cells show no methylation at the *Igf2r* promoter, resulting in a strong 28kb band from both alleles (Fig. 11B, sample 1). Untreated day 17 samples gain some DNA methylation at the *Igf2r* promoter, which can be detected as a 63kb fragment (Fig. 11B, sample 2). If *Airn*CKO ES cells are treated with TAM prior to differentiation, Cre-mediated recombination is extremely efficient (Fig. 5; Fig. 7). Removing the macro lncRNA promoter results in loss of the MluI site within the CGI of *Airn*. In TAM treated day 0 samples, this results in a 100kb band (Fig. 11B, sample 3), whereas in later differentiation an additional faint 135kb fragment, indicating cells with a methylated *Igf2r* promoter, is detected (Fig. 11B, sample 4). Removal of the *Airn* promoter at day 9 or 13 and harvesting the cells 4 days later results in faint 100kb and 135kb bands (Fig. 11B, samples 5-6). Importantly, no 63kb fragment is visible, which would represent the unrecombined CKO^{FI} allele with a methylated *Igf2r* promoter. Together, this indicates that methylation of the *Igf2r* promoter does not negatively correlate with Cre recombinase efficiency and that recombination does not occur only in cells carrying an unmethylated *Igf2r* promoter.

3.3 *Airn*CKO ES cells differentiated via EB formation

In the first part of this thesis, *Airn*CKO ES cells were differentiated in monolayer by adding RA. Unfortunately, this revealed some problems in particular the inefficient Cre-mediated excision of *Airn* at later time points of differentiation as well as little DNA methylation (~20%) gained at the *Igf2r* promoter. The resulting non-homogenous cell population did not allow me to obtain a clear answer to the question whether in the absence of *Airn* DNA methylation is sufficient to maintain silencing of *Igf2r*. A previous study has shown that ES cells differentiated via EB formation gained near complete methylation at the paternal *Igf2r* promoter (Latos et al, 2009). Therefore, I repeated the analysis by EB formation. Altogether, three biological replicates using the same experimental setup as for RA differentiated ES cells, adding TAM at day 5, 9 or 13 and harvesting the cells 4 days later, were performed.

3.3.1 Cre recombination in EB differentiated *Airn*CKO ES cells

To test the efficiency of the TAM inducible Cre-loxP system in EB differentiated *Airn*CKO ES cells, the same Southern blot (Fig. 4A) and qPCR assay (Fig. 6A) were used as described for RA treated cells.

Southern blot analysis revealed that in untreated control ES cells (no TAM) only the 6.3kb band for the paternal unrecombined CKO^{FL} as well as the 6.2kb wt maternal fragment are detected (Fig. 12A, samples 1-4). In the prior to differentiation treated sample, recombination worked efficiently, as indicated by the equal intensity of the upper (CKO^{FL} + wt) band and the lower (4.4kb recombined CKO^{Δ}) band (Fig. 12A, sample 5). This was also seen in RA differentiated *Airn*CKO ES cells (Fig. 4; Fig. 6). However, if the excision of the macro lncRNA promoter was induced during EB differentiation at day 5, 9 or 13, recombination worked with similar efficiency throughout differentiation (Fig. 12A, samples 6-8). Similar results can be observed in

the two other biological replicates (see Santoro et al, in press, Fig. S3): sample 5: 97-99%, sample 6: 95-96%, sample 7: 96% and sample 8: 75-86%.

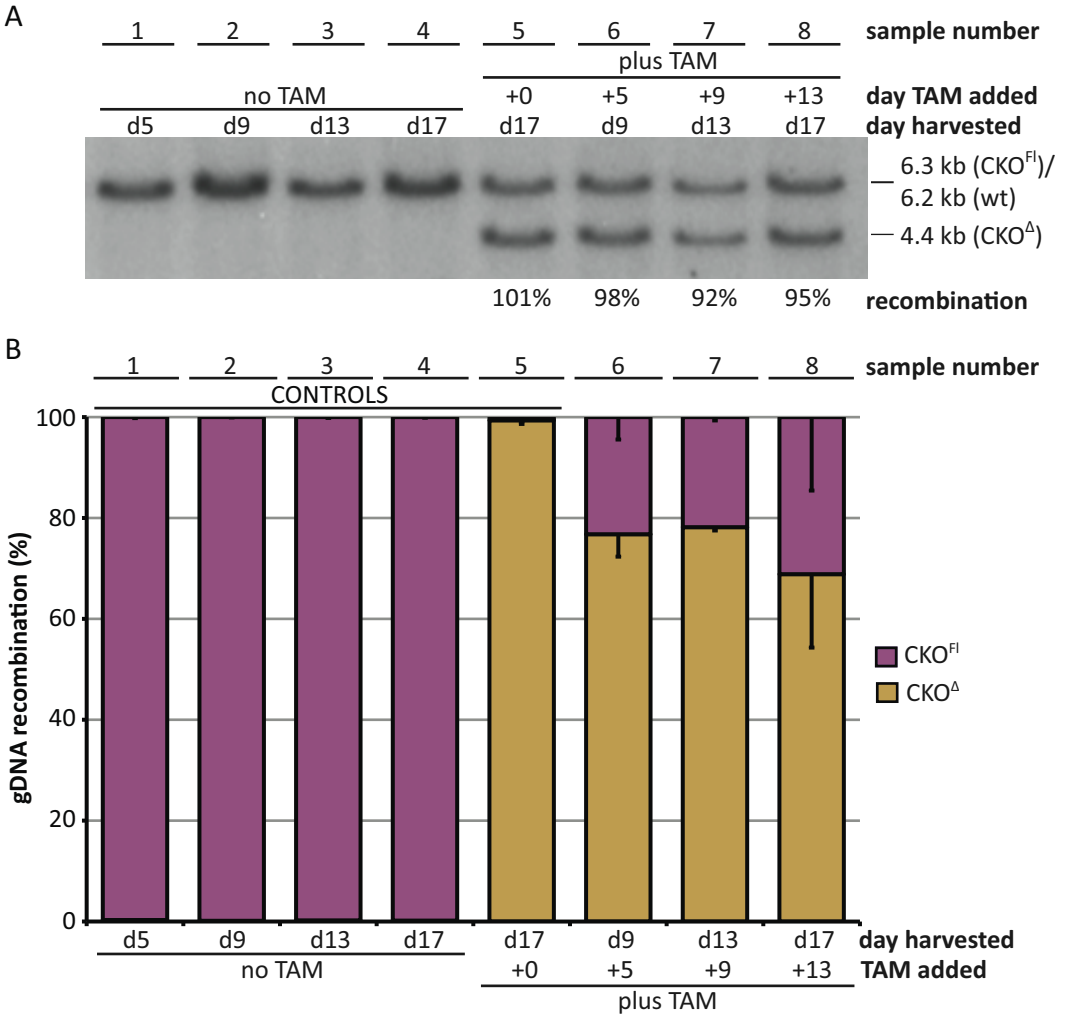

Figure 12. Cre recombination works more efficiently in EB differentiated *Airn*CKO ES cells (modified from Santoro et al, in press).
(A) One representative Southern blot of three biological replicates showing efficient Cre-mediated recombination in CKO ES cells differentiated via EB formation. Southern blot strategy is explained in Fig. 4A. gDNA was digested with EcoRI and hybridized to probe AirT. Unrecombined paternal (CKO^{Fl} 6.3kb) and wt maternal (6.2kb) alleles, which are not separated on this blot, correspond to the top band. Bottom band corresponds to paternal allele with *Airn* promoter deletion (CKO^{Δ} 4.4kb). Equal intensity of both bands indicates complete recombination shown as % lower band/[(upper + lower band)/2] underneath the blot.
(B) qPCR quantification of recombination efficiency in EB differentiated CKO ES cells confirms efficient removal of *Airn* promoter at any time point. Samples of three biological replicates were analysed with the qPCR assay shown in Fig. 5A. Combined levels of CKO^{Fl} (purple) and CKO^{Δ} (yellow) alleles were set to 100 and each bar shows the contributed percentage as mean and s.d. of three biological replicates.

To confirm the data observed by Southern blot, the gDNA was analyzed by qPCR using the previously described assay (Fig. 5A). In untreated control ES cells (no TAM)

only the unrecombined CKO^{FL} allele was detected (Fig. 12B, samples 1-4), whereas TAM treatment of cells prior to differentiation led to complete recombination (Fig. 12B, sample 5). Upon removal of the *Airn* promoter at day 5, 9 or 13 during EB differentiation, 22-32% residual CKO^{FL} allele was detected by qPCR (Fig. 12B, samples 6-8). These values are comparable to the ones observed in RA differentiated ES cells treated with TAM at day 5 and importantly do not decrease in late EB differentiation.

3.3.2 Reduced *Airn* transcription in EB differentiated *Airn*CKO ES cells

As EB formation of *Airn*CKO ES cells showed almost complete Cre-mediated recombination at any time during differentiation, I expected reduced residual *Airn* expression compared to RA differentiated ES cells. The same RT-qPCR assay described for RA differentiated cells was used to detect expression of the macro lncRNA in EB differentiated cells (Fig. 8). In untreated control cells (no TAM), *Airn* is not expressed at day 0 (Fig. 13, sample 1) and its expression is up-regulated upon differentiation (Fig. 13, samples 2-5). ES cells treated with TAM at day 0 showed complete recombination, resulting in very low residual expression of *Airn* (Fig. 13, sample 6). Inducing the excision of the promoter at day 5, 9 or 13 resulted in only 4-17% residual *Airn* expression (Fig. 13, samples 7-9). These results are in accordance with the efficient Cre-mediated recombination seen at all time points during EB differentiation (Fig. 12). Together, the data suggest that the TAM induced Cre-mediated excision of the *Airn* promoter works more efficiently in EB differentiated ES cells than in RA differentiated ones.

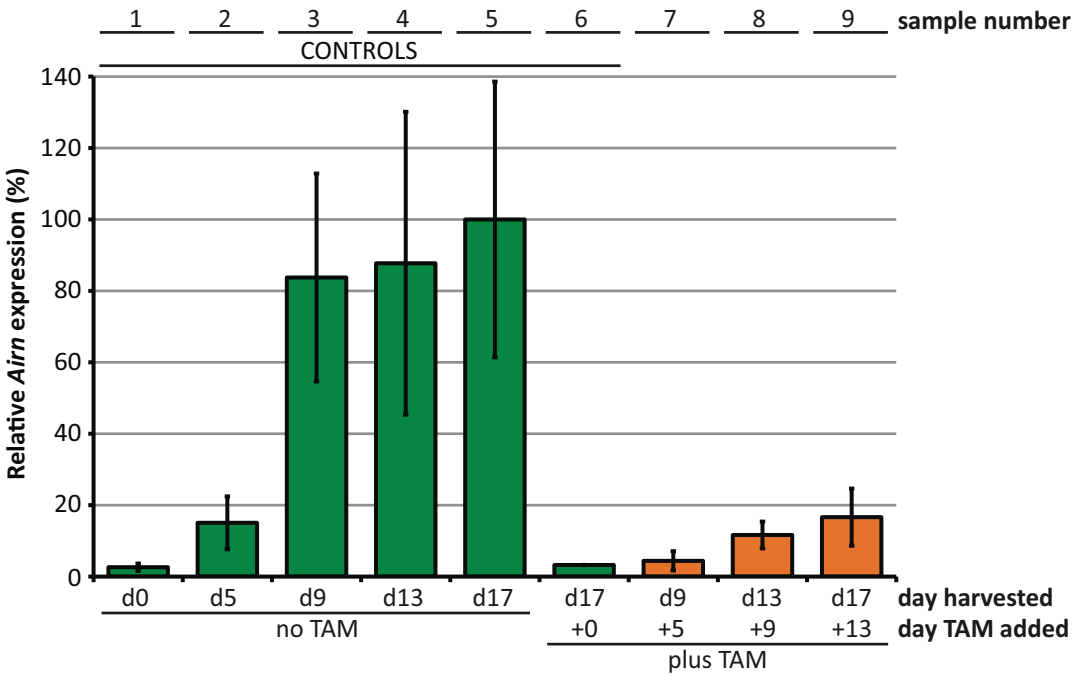

Figure 13. Deletion of the *Airn* promoter in EB differentiated *Airn*CKO ES cells removes *Airn* expression (modified from Santoro et al, in press).
RT-qPCR with primers specific for *Airn* detects only low residual expression of *Airn* in TAM treated CKO ES cells differentiated via EB formation. Relative *Airn* expression levels in untreated day 17 cells were set to 100. Upon removal of the *Airn* promoter during ES cell differentiation, expression levels are greatly reduced at all time points. Bars and error bars: mean and s.d. of three biological replicates. Green bars: control samples. Orange bars: *Airn*CKO.

3.3.3 *Igf2r* re-expression upon *Airn* removal in EB differentiated *Airn*CKO ES cells

Compared to RA differentiated *Airn*CKO ES cells EB differentiated ones showed almost perfect recombination and almost complete removal of *Airn* expression at any tested time point. Therefore they provide a more efficient system to find out whether the macro lncRNA is needed to maintain silencing of *Igf2r*. RT-PCR and RT-qPCR, as described for RA differentiated ES cells, were used to examine allele-specific *Igf2r* expression (Fig. 9).

As described previously, the non-quantitative RT-PCR assay makes use of the SNP in maternal *Igf2r* exon 12, which destroys an endogenous PstI site. Digestion of the PCR product with this restriction enzyme leads to one longer maternally derived fragment and two short paternal bands. Fig. 14A shows one representative biological replicate (two other replicates shown in Santoro et al, in press, Fig. S4). In

undifferentiated ES cells, *Igf2r* is biallelically expressed, as indicated by the presence of both maternal and paternal fragments (Fig. 14A, sample 1). Upon EB differentiation of untreated ES cells (no TAM), which express *Airn*, the signal of the paternal-specific bands gets fainter compared to the day 0 sample, consistent with maternal-specific *Igf2r* up-regulation (Fig. 14A, samples 2-5). The prior to differentiation treated sample showed strong maternal- and paternal-specific signals, indicating a failure in establishing imprinted expression in these cells (Fig. 14A, sample 6). TAM induced Cre-mediated excision of the *Airn* promoter during EB differentiation at day 5, 9 and 13 leads to re-expression of paternal *Igf2r*, indicated by a stronger signal of paternal-specific bands in treated compared to matching untreated cells (Fig. 14A, compare samples 7-9 to samples 2-5). However, if *Airn* is removed at late time points of EB differentiation, paternal *Igf2r* shows less re-expression.

To quantify *Igf2r* allele-specific expression, I used the allele-specific RT-qPCR assay described previously (Fig. 9B). In the absence of *Airn Igf2r* is expressed biallelically, therefore the ratio of maternal:paternal *Igf2r* expression in samples 5 was set to 50:50 (Fig. 14B, sample 5). *Airn*-expressing ES cells establish *Igf2r* imprinted expression upon EB differentiation, with low paternal and high maternal *Igf2r* levels (Fig. 14B, samples 1-4). Removing *Airn* at an early time point of differentiation leads to re-expression of paternal *Igf2r* to 47% of total *Igf2r* levels (Fig. 14B, sample 6). A similar result was observed in RA differentiated *Airn*CKO ES cells treated at day 5 (Fig. 9B, sample 6). However upon deletion of the *Airn* promoter at day 9 or 13 of EB differentiation, the extent of paternal *Igf2r* re-expression decreases to only 24% of total *Igf2r* levels (Fig. 14B, samples 7-8). As the excision of the *Airn* promoter worked efficiently at all time points in EB differentiated cells, the incomplete paternal *Igf2r* re-expression observed upon removal of *Airn* at day 9 or 13 can not be explained by inefficient Cre recombination as for RA differentiated cells, but must be due to other factors.

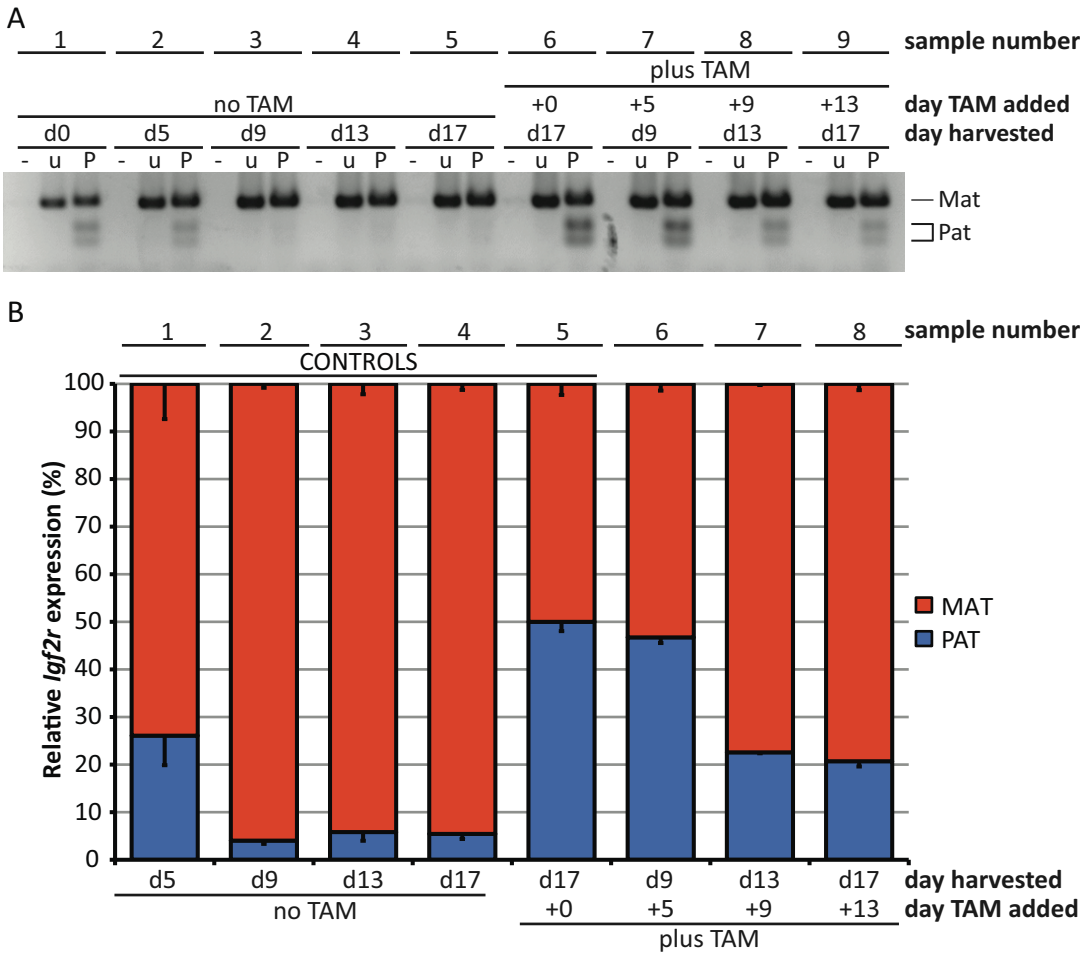

Figure 14. Continuous *Airn* transcription is needed to silence *Igf2r* in EB differentiated *Airn*CKO ES cells (modified from Santoro et al, in press).
(A) Allele-specific RT-PCR and PstI digest of one representative biological replicate out of three shows re-expression of paternal *Igf2r* in the absence of *Airn* in EB differentiated CKO ES cells. Maternally expressed *Igf2r* generates a 541bp fragment and paternal expression two 318 and 223bp fragments. Upon *Airn* removal, *Igf2r* silencing is released to different extents. Mat: Maternal. Pat: Paternal. -: Minus RT. u: Undigested. P: PstI digested.
(B) RT-qPCR analysis of *Igf2r* imprinted expression using a paternal-specific SNP confirms paternal *Igf2r* re-expression upon removal of *Airn* promoter. Maternal (red bars) and paternal (blue bars) *Igf2r* levels are shown as a total of 100%. Maternal:paternal level is set to 50:50 in sample 5 as *Igf2r* shows biallelic expression in the absence of *Airn*. Note that paternal *Igf2r* is only re-expressed to 24% in late points of differentiation despite efficient recombination. Bars and error bars indicate mean and s.d. of three biological replicates.

3.3.4 DNA methylation of paternal *Igf2r* promoter in EB differentiated *Airn*CKO ES cells

To determine whether DNA methylation is responsible for the maintenance of paternal *Igf2r* silencing in late differentiated EBs in the absence of *Airn*, the methylation state of the *Igf2r* promoter was determined by digestion of ES cell

gDNA with EcoRI and methyl-sensitive NotI restriction enzyme and hybridization with probe NEi, as described for RA differentiation (Fig. 10A). Upon EB differentiation in untreated *Airn*CKO ES cells (no TAM), DNA methylation is progressively gained at the paternal *Igf2r* promoter indicated by the gain of the 5kb methylated band, with a maximum of 42% by day 17 (Fig. 15, samples 1-4). Note that maximum methylation is 50%, as only the paternal *Igf2r* allele is methylated. In the absence of *Airn*, *Igf2r* is expressed biallelically and no DNA methylation is seen at the paternal CGI (Fig. 15, sample 5). However, after the removal of the *Airn* macro lncRNA at day 5, 9 or 13 the methylation levels are similar compared to the corresponding wt controls, suggesting that the DNA methylation, which is gained until the removal of *Airn*, is maintained independently of *Airn* (Fig. 15, samples 6-8). These data are confirmed by two biological replicates (shown in Santoro et al, in press, Fig. S5): sample 1: 4%, sample 2: 30-31%, sample 3: 37-41%, sample 4: 35-37%, sample 6: 11-13%, sample 7: 39-40% and sample 8: 37-44%.

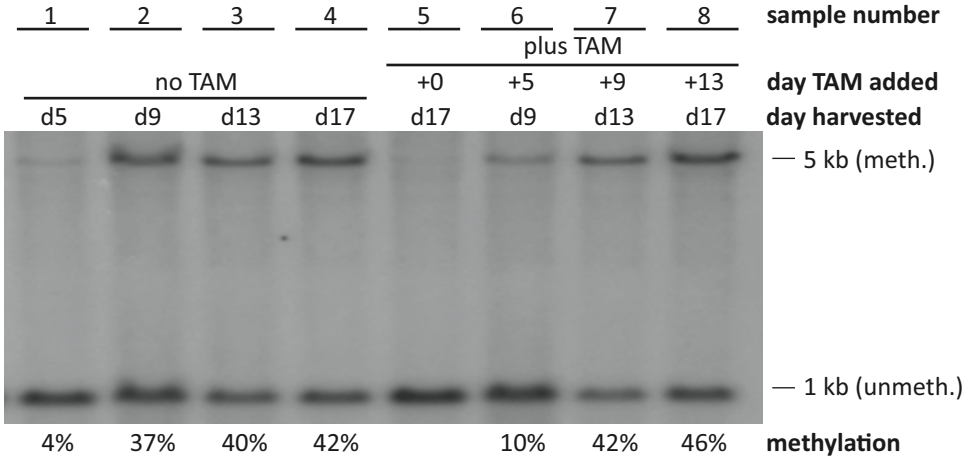

Figure 15. Methylation of the *Igf2r* promoter in EB differentiated *Airn*CKO ES cells (modified from Santoro et al, in press).
One representative Southern blot of three biological replicates assaying DNA methylation of the *Igf2r* promoter in EB differentiated CKO ES cells upon deletion of the *Airn* promoter. gDNA was digested with EcoRI and methyl-sensitive NotI and hybridized to probe NEi (for strategy see Fig. 10A). Top band shows methylated *Igf2r* promoter (meth, 5kb). Bottom band indicates unmethylated promoter (unmeth, 1kb). In untreated control cells (no TAM) DNA methylation is gained progressively during differentiation (samples 1-4). Removal of *Airn* upon differentiation does not affect the methylation state of the paternal *Igf2r* promoter (samples 6-8). Paternal *Igf2r* promoter methylation % [meth/(meth+unmeth)] is shown below the blot. Note maximum methylation is 50% as only one allele is methylated.

3.4 Proliferation marker expression in differentiated *Airn*CKO ES cells

The analysis of DNA methylation at the paternal *Igf2r* promoter showed that upon loss of *Airn* and re-expression of *Igf2r*, DNA methylation is maintained independently of the macro lncRNA (Fig. 10B; Fig. 15). Passive DNA demethylation occurs if cells divide and do not methylate the newly synthesized DNA strand (Chen & Riggs, 2011). To rule out the possibility that the observed maintenance of DNA methylation is due to cell cycle arrest in late differentiated ES cells, the expression of two proliferation marker genes was tested at different time points in RA and EB differentiated *Airn*CKO cells (Fig. 16). Further, an undifferentiated day 0 ES cell sample and five different adult mouse tissues, containing terminally differentiated cells, were used as control samples.

Ki67 is a commonly used marker for cell proliferation and cancer diagnosis. Previous studies have shown that it is exclusively expressed in proliferating cells and it is detectable in all phases of the cell cycle (Gerdes et al, 1983). If cells exit the active cell cycle, mRNA and protein levels of *Ki67* are rapidly down-regulated, pointing out the strong correlation between cell proliferation and *Ki67* expression. Therefore, *Ki67* represents a good marker to test whether cells are in an active cell cycle (Scholzen & Gerdes, 2000). Undifferentiated ES cells have the ability to self-renew and therefore a high proliferation capacity, resulting in high *Ki67* expression levels (Fig. 16A, bar 1). In adult mouse tissues that contain terminally differentiated cells, *Ki67* levels are only 1-5% compared to the day 0 sample (Fig. 16A, bars 10-14). *Airn*CKO ES cells were either RA or EB differentiated and harvested at day 5, 9, 13 or 17 (Fig. 16A, bars 2-9). The highest *Ki67* expression was detected in day 9 samples. *Ki67* expression levels decrease upon differentiation, to 42-54% at day 17 compared to day 0, however still showing higher levels than adult mouse tissues.

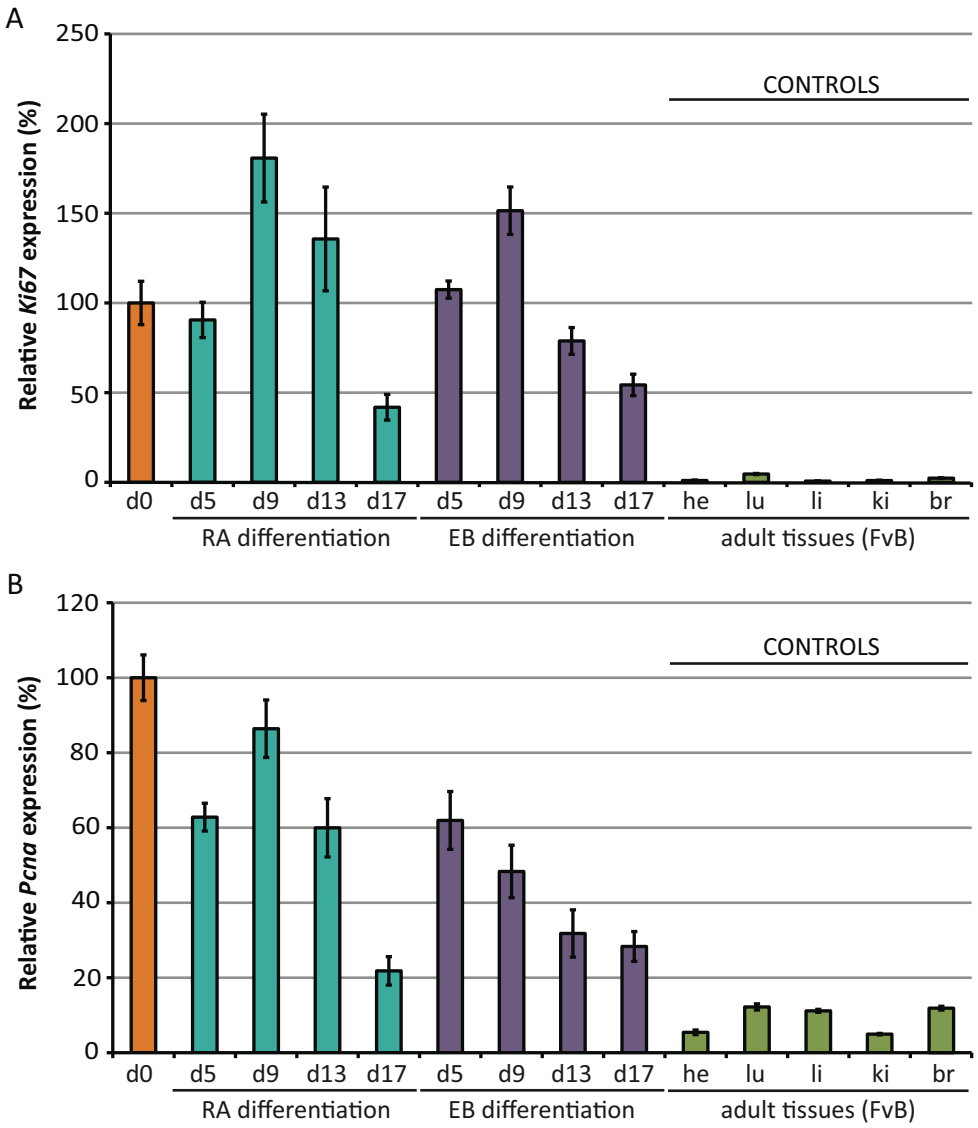

Figure 16. *Airn*CKO ES cells proliferate even in late stages of differentiation (modified from Santoro et al, in press).
RT-qPCR to measure relative expression levels of proliferation markers in RA and EB differentiated CKO ES cells using *Ki67* (A) and *Pcna* (B) as proliferation markers. Undifferentiated (orange bar), RA differentiated (turquoise bars) and EB differentiated (purple bars) CKO ES cells were compared to adult mouse tissues (green bars). Relative expressions levels were set to 100 in undifferentiated ES cells. Even in late differentiation, ES cells still show higher expression levels of both *Ki67* and *Pcna* than adult mouse tissues. Bars and error bars: mean and s.d. of three technical replicates. he: Heart. lu: Lung, li: Liver. ki: Kidney. br: Brain.

Proliferating cell nuclear antigen (*Pcna*) is another frequently used proliferation marker gene (Takasaki et al, 1981). *Pcna* has been identified as a cofactor of DNA polymerase δ that helps to hold the later on to the DNA strand, which is being replicated. Further, it unwinds the DNA during synthesis (Bowman et al, 2004). Therefore, *Pcna* is a key player for the entry into the synthesis phase of the cell cycle

and a good marker for dividing cells. In undifferentiated ES cells, *Pcna* expression levels are high due to their high proliferation capacity (Fig. 16B, bar 1). In adult mouse tissues, *Pcna* expression is very low, ranging between 5-12% compared to day 0 levels (Fig. 16B, bars 10-14). RA or EB differentiated *Airn*CKO ES cells show a reduction of expression at late differentiation stages resulting in 22-28% expression at day 17 compared to day 0 levels (Fig. 16B, bars 2-9). Never the less, relative expression of *Pcna* in late differentiation is twice as high compared to terminally differentiated tissues.

In summary, *Ki67* and *Pcna* expression analysis shows that *Airn*CKO ES cells still proliferate in late differentiation compared to adult mouse tissues containing terminally differentiated cells.

4. Discussion

4.1 Summary of the results

I used the *Airn*CKO ES cell line to investigate the maintenance of *Igf2r* silencing by the *Airn* macro lncRNA during ES cell differentiation induced either by RA addition or by EB formation. This cell line contains a TAM-inducible Cre recombinase gene and loxP sites flanking the *Airn* promoter that allowed me to remove *Airn* transcription at any time point of differentiation. Therefore, I was able to test whether *Igf2r* imprinted silencing can be maintained in the absence of *Airn*. The data suggest that in RA differentiation or early stages of EB differentiation, when only a very low-level of DNA methylation is gained at the paternal *Igf2r* promoter, removal of *Airn* causes loss of *Igf2r* silencing. However, if the macro lncRNA is removed during late EB differentiation, when the cells gain ~2 fold more *Igf2r* methylation, incomplete loss of *Igf2r* silencing can be observed. This indicates that continuous expression of *Airn* is needed to silence *Igf2r*, but only until DNA methylation is gained at the repressed *Igf2r* promoter. Interestingly, the data also show that *Igf2r* DNA methylation is maintained independently of the *Airn* macro lncRNA.

4.2 ES cells provide a good model to study the epigenetic mechanisms involved in genomic imprinting

ES cells have been intensively used as a model system to study the epigenetic mechanisms involved in X chromosome inactivation (XCI) in female eutherian mammals. XCI is the dosage compensation of X-linked genes between females and males achieved by random inactivation of one of the two female X chromosomes during embryo implantation. Initiation and maintenance of XCI during embryo development is recapitulated in ES cell differentiation. In undifferentiated ES cells, both X chromosomes are active, however upon differentiation expression of the

lncRNA *Xist* from one of the X chromosomes, followed by a complex series of epigenetic mechanisms leads to inactivation of most of the genes on that X-chromosome. Therefore, ES cells provide a good model to study the sequence of events as well as the factors involved in XCI, without using time-consuming and expensive *in vivo* mouse model systems (Navarro & Avner, 2010).

As with XCI, genomic imprinting is also a developmentally regulated epigenetic phenomenon. Since ES cell differentiation resembles early embryonic development, ES cells are becoming more appreciated as a model system to study imprinted gene expression (Kohama et al, 2012; Latos et al, 2009). A recent study of the *Kcnq1* imprinted cluster in ES cells reported that the sDMR of *Cdkn1c*, an imprinted gene within this cluster, does not gain DNA methylation upon ES cell differentiation although parental-specific silencing is established (Wood et al, 2010). As the *Cdkn1c* sDMR is established during *in vivo* mouse development, the authors concluded that ES cells might not be an appropriate model system to study imprinted expression, since the *in vivo* situation was not always reproducible. However, when I tested *Cdkn1c* DNA methylation in EB differentiated *Airn*CKO ES cells, using the same Southern blot strategy described in Wood et al, 2010(Wood et al, 2010), I could detect ~20% methylation at the CGI of *Cdkn1c* (Santoro et al, in press). Interestingly, differentiating cells in monolayer with RA as reported in (Wood et al, 2010)Wood et al, 2010(Wood et al, 2010), caused only a low gain of methylation at the *Cdkn1c* promoter (Santoro et al, in press). The data reported here for the *Igf2r* cluster confirm the benefit of using ES cells as a model system to study imprinted gene expression, as described previously (Koerner et al, 2012; Latos et al, 2012; Latos et al, 2009; Stricker et al, 2008). I could successfully reproduce the *in vivo* onset of *Igf2r* imprinted expression and study the maintenance of monoallelic *Igf2r* expression by *Airn* and DNA methylation. However, I was able to show that it is important to consider that different ES cell differentiation protocols can yield different results, thus affecting the interpretation of the experiments.

4.3 Efficient recombination using a TAM inducible Cre-loxP system

The *Airn*CKO ES cell line, used in this study, contains a TAM inducible Cre-loxP system. The two loxP sites were strategically placed around the *Airn* promoter, including the *Airn* CGI and TSS on chromosome 17. Further the $CreER^{T2}$ gene was inserted in the ROSA26 locus on chromosome 6. As a result, the activation of the Cre recombinase upon TAM treatment allows the removal of the *Airn* macro lncRNA (Santoro et al, in press). It has already been shown that 0.2µM TAM are sufficient to induce recombination of target loxP sites in $CreER^{T2}$-expressing ES cells (Hameyer et al, 2007). To optimize TAM treatment in our ES cell line, I tested 0.1 to 1µM TAM for different periods of time ranging from 6 to up to 48 hours in undifferentiated ES cells. First, I could confirm that already a low dosage of TAM (≥ 0.1µM) induces complete recombination and that recombination efficiency does not increase at higher doses, as reported previously (Hameyer et al, 2007). Additionally, I observed a time-dependent effect of TAM, with increasing recombination efficiency over time, independently of the dose used.

Upon differentiation of *Airn*CKO ES cells, the deletion of the *Airn* macro lncRNA was induced at day 5, 9 or 13 and cells were harvested four days later to allow time for *Airn* to decay. Interestingly, in RA differentiated cells Cre-mediated recombination is efficient at day 5 but decreases to ~60% at later time points of differentiation. However, when I used the EB differentiation protocol, Cre recombination was efficient at all tested time points, both early and late. The difference in recombination efficiency between the two differentiation protocols could be due to promoter activity since *Airn* is more highly expressed in late differentiated cells and also showed, in general, higher expression levels in RA compared to EB differentiated ES cells (Santoro et al, in press). This suggestion is also supported from the finding that loxP sites inserted 16kb downstream of the *Airn* promoter show complete recombination in both early and late RA differentiated ES cells (Santoro et al, in press). During transcription, the promoter serves as a platform for assembling the transcription machinery including RNA polymerase II and transcription factors and also gains active histone marks (Thomas & Chiang, 2006). If

the loxP sites are inserted close to a promoter, as in the *Airn*CKO ES cell line, assembly of transcription complexes on the promoter or active histone modifications might interfere with Cre recombinase binding, which is necessary to induce excision of the intervening DNA sequence (Abremski & Hoess, 1984). Therefore, increased *Airn* transcription might explain the reduced recombination seen in late RA differentiated ES cells.

Recombination efficiency in EB differentiated cells was nearly complete and comparable to reported Cre recombination in developing mouse embryos. For example, in a recent study, the conditional *in vivo* deletion of the lncRNA *Kcnq1ot1* was achieved by inserting loxP sites flanking the promoter sequence in mice (Mohammad et al, 2012). Similar to my data for EB differentiated *Airn*CKO ES cells, residual *Kcnq1ot*1 expression levels of 10-20% were detected in mouse embryos upon TAM treatment (Mohammad et al, 2012).

4.4 Continuous *Airn* transcription is needed to silence *Igf2r* until DNA methylation is established

The macro lncRNA *Airn* initiates *Igf2r* silencing by transcriptionally interfering with functional RNAPII recruitment to the *Igf2r* promoter (Latos et al, 2012). *Igf2r* imprinted expression occurs in all tissues that co-express *Airn* (Yamasaki et al, 2005). Further, *Igf2r* silencing is accompanied by the gain of DNA methylation at the repressed *Igf2r* promoter, however it is not complete until after birth of the mouse embryo (Stoger et al, 1993). DNA methylation at promoters is associated with transcriptional gene silencing and is generally propagated from one cell to the next via the *Dnmt1* complex during DNA replication (Jones, 2012). Does *Airn* therefore need to be expressed continuously, if DNA methylation has been set at the *Igf2r* promoter?

By deleting *Airn* conditionally, I was able to show that continuous *Airn* expression is needed to maintain *Igf2r* silencing, however the data indicate that *Airn* is only required until the *Igf2r* promoter gains DNA methylation. Deleting *Airn* at day 5 in EB differentiated ES cells, which only showed less than 10% methylation, resulted in complete loss of *Igf2r* silencing. A similar result could be observed in late RA differentiated ES cells, where ~20% methylation is gained. Interestingly, in late differentiated EBs up to ~40% DNA methylation was detected at the paternal *Igf2r* promoter. TAM induced deletion of the *Airn* promoter at day 9 or 13 of EB differentiation led to incomplete loss of *Igf2r* silencing. This suggests that continuous *Airn* expression is needed to repress *Igf2r*, but only until the *Igf2r* promoter gains methylation, indicating a developmental switch from an *Airn*-dependent to an *Airn*-independent silencing of *Igf2r*. I could also observe that DNA methylation established before the removal of *Airn* was maintained independently of the *Airn* macro lncRNA. To exclude the possibility that the observed maintenance of *Igf2r* DNA methylation was due to cell cycle arrest, I tested two proliferation marker genes in RA and EB differentiated ES cells. According to expression levels of both *Pcna* and *Ki67*, even at late stages, differentiating ES cells are highly proliferative compared to terminally differentiated adult tissues.

In a recent study, *Kcnq1ot1* lncRNA-mediated maintenance of imprinted gene expression in the *Kcnq1* cluster was investigated by creating a conditional knockout mouse for *Kcnq1ot1* (Mohammad et al, 2012). Upon deletion of the *Kcnq1ot1* promoter, loss of silencing of the ubiquitously imprinted genes in the cluster was observed in early and late embryonic development. Additionally, DNA methylation at the promoters of two silenced genes was lost when the *Kcnq1ot1* lncRNA was removed. This indicates that in the *Kcnq1* cluster the maintenance of both silencing of ubiquitously imprinted genes and, unexpectedly, of the somatic DNA methylation mark depends on the expression of *Kcnq1ot1* (Mohammad et al, 2012). Similarly, my data shows that *Airn* expression is continuously needed to silence *Igf2r* in the absence of DNA methylation. However, *Igf2r* DNA methylation itself is maintained in a lncRNA-independent fashion. In the case of *Kcnq1ot1*, interaction of the lncRNA

with the maintenance methyltransferase *Dnmt1* has been shown (Mohammad et al, 2010), indicating that *Kcnq1ot1* directs *Dnmt1* to the sDMRs in the cluster to maintain their methylation status. In contrast, in the *Igf2r* cluster the *Dnmt1* hemi-methyltransferase is most likely recruited via the canonical DNA replication machinery to copy methylation patterns onto newly replicated DNA (Bostick et al, 2007; Sharif et al, 2007).

4.5 *Airn* and DNA methylation together maintain stable *Igf2r* silencing during development

The investigation of the conditional deletion of the *Airn* promoter during ES cell differentiation, raises questions regarding the developmental regulation of *Igf2r* silencing. My data indicate that, in agreement with the transcriptional interference model (Latos et al, 2012), continuous *Airn* transcription is needed to silence *Igf2r*, but only until the repressed *Igf2r* promoter gains DNA methylation. It is therefore unclear why *Airn* transcription continues throughout embryonic development and adult life (Pauler et al, 2005). A similar situation can be observed for *Xist*, the lncRNA that initiates XCI. *Xist* is expressed exclusively from the inactive X chromosome and loss-of-function experiments showed that initiation of XCI depends on the expression of *Xist* (Brockdorff et al, 1991; Brown et al, 1991; Marahrens et al, 1997; Penny et al, 1996). Similar to *Airn*, *Xist* is expressed throughout ES cell differentiation and adult life, although it is not needed to maintain repression of the inactive X chromosome (Csankovszki et al, 1999; Wutz & Jaenisch, 2000). It is interesting to note that *Xist* does not silence the inactive X chromosome via transcriptional interference, but through an RNA-tethering mechanism. The *Xist* lncRNA recruits factors required for heterochromatin formation, which then lead to stable XCI (Brockdorff, 2011). Although *Airn* and *Xist* induce silencing via different mechanisms, continuous expression of both lncRNAs is not needed to maintain the repressed state. This indicates that independently of how lncRNA-mediated

silencing is initiated, continuous expression of the lncRNAs may serve as a safeguard mechanism to prevent accidental re-expression of epigenetically silenced genes.

On the other hand, if *Airn* is transcribed continuously, the need for DNA methylation at the *Igf2r* promoter is unclear. sDMRs are rare in imprinted gene clusters and only occur at very few ubiquitously silenced genes (Barlow, 2011). Many imprinted genes show parental-specific expression only for a limited timeframe during development and their imprinted expression is often restricted to a certain type of tissue (Santoro & Barlow, 2011). Therefore, a dynamic system could be needed to ensure a rapid switch between biallelic and monoallelic expression, which might explain the absence of DNA methylation for most imprinted genes. In contrast, the presence of DNA methylation, in addition to continuous lncRNA expression could provide a more long-term epigenetic repression, which is especially important for those imprinted genes with fundamental roles in development (John & Lefebvre, 2011).

4.6 Significance of understanding the regulatory function of lncRNAs

This study investigated the maintenance of *Igf2r* silencing by the *Airn* lncRNA during ES cell differentiation. The hereby gained knowledge is not only useful for a better understanding of the mechanism of action of lncRNAs in other imprinted clusters, but also of the regulatory function of non-imprinted lncRNAs in general. To date, only few lncRNAs have been functionally studied, although the number of newly identified lncRNAs is constantly increasing, especially those associated with diseases (Wang & Chang, 2011). Angelman syndrome, for example, is a neurodevelopmental disease caused by the mutation or deletion of the imprinted protein-coding gene *Ube3a* on the maternal allele (Kishino et al, 1997; Matsuura et al, 1997; Sutcliffe et al, 1997). In neurons, the paternal copy of this gene is intact and its silencing correlates with expression of the *Ube3a-as* lncRNA (Chamberlain & Brannan, 2001; Rougeulle et al, 1998). In a recent drug screen, topoisomerase inhibitors have been identified as capable of reactivating the silent paternal *Ube3a* gene and down-

regulating the antisense lncRNA (Huang et al, 2012). However, the mechanism involved is not known.

As lncRNAs become more and more interesting as drug targets to relieve silencing of their target genes, two key aspects have to be considered. First, it is important to know whether the act of transcription or the transcript itself is responsible for gene repression. In the case of *Airn*, which acts via transcriptional interference to induce *Igf2r* silencing, a hypothetical drug to reactivate the silent paternal *Igf2r* copy would have to block the act of *Airn* transcription. Secondly, before embarking in drug discovery, one has to consider what maintains lncRNA-mediated silencing. My data for the *Igf2r* imprinted cluster suggest that both *Airn* and DNA methylation maintain *Igf2r* silencing. In a case like this following blocking of *Airn* transcriptional elongation, an additional drug that removes DNA methylation at the repressed promoter would be needed. Several epigenetic drugs have been approved for clinical use, however they often have broad and unspecific effects (Arrowsmith et al, 2012). Altogether, this emphasizes the importance of understanding the underlying mechanism of gene regulation by lncRNAs before developing therapeutic strategies against them.

5. Materials and methods

5.1 Materials

5.1.1 Cell line

Airn CKO ($S12^{RC}/CKO^{FI}$) (described in Santoro et al, in press)

5.1.2 Chemicals and consumables

100bp+ ladder	Fermentas
1kb ladder	Fermentas
3MM Whatmann paper	Whatmann
4-hydroxytamoxifen (TAM)	Sigma-Aldrich
α^{32}P-dATP	Hartmann Analytic
β-mercaptoethanol	Invitrogen
Acetic acid	Applichem
Agarose	Biozym
Gold Agarose	Biozym
Boric acid	Applichem
Bromo chloropropane (BCP)	MRC
Bovine serum albumin (BSA)	Fermentas
Deoxycytidine triphosphate (dCTP)	Bioron
Deoxyguanosine triphosphate (dGTP)	Bioron
Deoxythymidine triphosphate (dTTP)	Bioron
Disodium hydrogen phosphate (Na_2HPO_4)	Merck
Dimethyl sulfoxide (DMSO)	Sigma
dNTP Mix 10mM	Fermentas
Dulbecco's Modified Eagle Medium 1x (DMEM)	Invitrogen
Dulbecco's Phosphate Buffered Saline 1x (DPBS)	Invitrogen
Ethylenediaminetetraacetic acid (EDTA)	Merck
Ethanol 96%	Merck

Ethidium bromide	Applichem
Fetal bovine serum (FBS)	PAA
Gelatin solution	Sigma
Gentamycin	Invitrogen
Glycerol	Applichem
Hepes	Applichem
HybondTM XL membrane	Amersham
Hydrochloric acid (HCl)	Merck
Isopropanol	Merck
L-Glutamine	Invitrogen
Low Range PFG Marker	BioLabs
MEM Non-essential amino acids	Invitrogen
Mesa Green qPCR Mastermix	Eurogentec
MgCl2 25mM	Fermentas
Phenylmethanesulfonyl fluoride (PMSF)	Sigma
Pluronic acid F-127	Sigma
qPCR Mastermix Plus	Eurogentec
Random hexamer primer	GE-Healthcare
Retinoic acid (RA)	Sigma
RNA storage solution	Ambion
Sodium dodecyl sulfate (SDS)	Applichem
SephadexTm G-50	Amersham
Sodium Acetate (NaAc)	Ambion
Sodium hydroxide (NaOH)	Applichem
TRI Reagent	Sigma
Tris	Applichem
Trypsin 0,5% EDTA, white	Invitrogen
Water for embryo transfer	Sigma
Xylenol orange	Sigma

5.1.3 Enzymes and buffers

5x GoTaq flexi buffer	Fermentas
Restriction digest Buffer1	BioLabs
Buffer EcoRI	Fermentas
Restriction digest Buffer O	Fermentas
Restriction digest Buffer R	Fermentas
EcoRI	Fermentas
GoTaq Polymerase	Fermentas
Klenow Fragment	Fermentas
MluI	Fermentas
NotI	Fermentas
PacI	BioLabs
Proteinase K	Applichem
PstI	Fermentas

5.1.4 Kits

DNA-free™ Kit	Ambion
RevertAid™ First Strand cDNA Synthesis Kit	Fermentas

5.1.5 Solutions and Media

ES cell medium:

FBS	30 ml
Gentamycin	1 ml
L-glutamine	2 ml
MEM NEAA	2 ml
β-mercaptoethanol	400 µl
Sodium pyruvate	2 ml
DMEM w/o Hepes	up to 200 ml
LIF	variable amount

RA differentiation medium:

ES cell medium without LIF with 4 µl RA per 200 ml medium

EB differentiation medium:

ES cell medium without LIF

Freezing medium:

FBS	80%
DMSO	20%

DNA lysis solution:

1% SDS

0,5 mg/ml Proteinase K

1xTEN pH 9.0 (250mM Tris pH 9.0; 100mM EDTA pH 8.0; 200mM NaCl)

PFGE DNA lysis solution:

100mM EDTA

10mM Tris

20mM NaCl

0,1 mg/ml Proteinase K

1 % SDS

LS mix:

25 ml 1M Hepes pH 6.6

1 ml OL (50 units of hexamer primers dissolved in 1,6 ml TE pH 8.0)

25 ml TM (250mM Tris pH 8.0; 25mM $MgCl_2$; 50mM β-mercaptoethanol)

CTG mix:

100µM dCTP

100µM dGTP

100µM dTTP

2 mg/µl BSA

Denaturing solution:

0.5M NaOH

1.5M NaCl

Church Buffer:

250mM Na_2HPO_4

7% SDS

1mM EDTA

Church Wash:

1% SDS

20mM Na_2HPO_4

TE buffer:

10mM Tris HCl pH 8.0

1mM EDTA

TAE buffer:

40mM Tris base

20mM Acetic acid

1mM EDTA pH 8.0

TBE buffer:

90mM Tris base

90mM boric acid

2mM EDTA pH 8.0

10x loading buffer for agarose gel:

0.125g 0.5% Xylenol orange

7.5ml 30% Glycerol

17.5ml 1x TAE

5.1.6 Probes and primers

Southern blot hybridization probes:

AirT (chr17:12,746,083-12,746,525)

NEi (chr17:12,768,435-12,769,450)

OT2.4 (chr17:12,937,225-12,939,421)

Primers for non-quantitative PCR:

Ex12cDNA

Ex12cDNA-F: 5'-TTCACAGGTGAGGTGGACTG-3'

Ex12cDNA-R: 5'- CCGTGCAGTTCTCTCCTTCT-3'

Taqman qPCR assays:

Airn

Airn-TQF: 5'-GACCAGTTCCGCCCGTTT-3'

Airn-TQR: 5'-GCAAGACCACAAAATATTGAAAAGAC-3'

Airn-TQ probe: 5'-FAM-TACAAGTGATTATTAACTCCACGCCAGCCTCA-TAMRA-3'

CyclophilinA

CycloA-F: 5’-AGGGTTCCTCCTTTCACAGAATT-3’

CycloA-R: 5’-GTGCCATTATGGCGTGTAAAGTC-3’

CycloA probe: 5’-FAM-TCCAGGATTCATGTGCCAGGGTGG-TAMRA-3’

SybrGreen qPCR assays:

CKO recombination

CKO F^{Fl}: 5’-AGGGTTTGGCGCTATCCT-3’

CKO F^{Δ}: 5’-TTGAACACATGGGATGGAGT-3’

CKO R: 5’-CACCCTCAATTCCGATCAT-3’

Ex12qSNP

WtSeFCG: 5’-TGGCCTTGCCCTCCTGC-3’

MutSeFCG: 5’-CTGGCCTTGCCCTCCTGT-3’

GeSeR: 5’-GCTATGACCTGTCTGTGTTGGCT-3’

Ki-67

Ki-67 F: 5’-CAG-TAC-TCG-GAA-TGC-AGC-AA-3’

Ki-67 R: 5’-CAG-TCT-TCA-GGG-GCT-CTG-TC-3’

PCNA

PCNA F: 5’-AAT-GGG-GTG-AAG-TTT-TCT-GC-3’

PCNA R: 5’-CAG-TGG-AGT-GGC-TTT-TGT-GA-3’

5.2 Methods

5.2.1 ES cell culture

The undifferentiated ES cells were cultured with ES cell medium containing LIF in the incubator (37°C, 5% CO_2) on irradiated mouse embryonic fibroblast feeder cells. Once the ES cells reached a certain density, they were feeder depleted and transferred on gelatinized 15cm dishes to eliminate feeder cells. Feeder depleted ES cells were fed every day with fresh ES cell medium until confluent enough to set up a differentiation experiment. The ES cells were trypsinized and counted using the CASY® Cell Counter (Schärfe System GmbH). The required numbers of cells were transferred on gelatinized 10cm dishes for monolayer differentiation by retinoic acid (RA) addition or on AggreWell™ plates for embryoid bodies (EBs) differentiation (see below). The day the differentiation started was set as day 0 and the cells were harvested either on day 0, 5, 9, 13 or 17. Initial seeding densities were optimized to allow ~80% confluent cultures at the differentiation end point.

Gelatinizing dishes

10 or 15cm dishes were covered with 4ml or 10ml 0.5% gelatin in DPBS, respectively, and incubated for at least 10min in the incubator. Gelatin was removed before plating the cells.

Thawing cells

Frozen cells were stored in CryoTubes™vials (Nunc) in liquid nitrogen tanks. The tubes were transferred from the liquid nitrogen on to dry ice and thawed quickly by gently shaking the tubes in a 37°C waterbath. Once completely thawed, the cells were resuspended in 10ml of ES cell medium and spun at 1050rpm for 5min. The cell pellet was resuspended in ES cell medium and the cell suspension was plated on 10cm dishes.

Trypsinizing cells

To detach ES cells from the culture dish, the cells were first washed with DPBS. 1ml or 2ml trypsin were then added to 10 or 15cm dishes, respectively, and the dishes were incubated for 3min in the incubator. Single cells were examined under the microscope and resuspended in the appropriate amount of ES cell medium. The single cell suspension was then directly plated on a new dish or counted.

Freezing cells

Undifferentiated ES cells on a 10cm dish were trypsinized and resuspended in 9ml ES cell medium. The cell suspension was spun at 1050rpm for 5min and the pellet was resuspended in 1.5ml ES cell medium. 0.5ml of cells were transferred to each cryotube and 0.5ml freezing medium was added. Tubes were placed in a freezing container to guarantee slow freezing, and stored at -80°C for 24h. Afterwards the tubes were transferred to liquid nitrogen tanks.

Feeder depletion

Undifferentiated ES cells cultured on growth-inhibited feeder cells on 10cm dishes were trypsinized and resuspended in 9ml ES cell medium. The cell suspension was transferred to a non-gelatinized 10cm dish and incubated for 20min in the incubator. During this time, feeder cells start attaching to the surface of the dish whereas ES cells still float in the medium. The medium was then carefully removed and transferred to a Falcon tube. The ES cells were counted and approximately 2-3x10^6 cells were plated on gelatinized 15cm dishes.

ES cell differentiation in monolayer with RA

3.5x10^5, 4x10^5 or 5x10^5 feeder-depleted ES cells for d5, d9 or d13/17 samples, respectively, were plated on gelatinized 10cm dishes with 10ml RA differentiation medium. Two dishes were used for each time point of differentiation to harvest either DNA or RNA. The cells were fed every second day with RA differentiation medium. 5x10^6 cells were used for isolating DNA and RNA for d0 samples.

ES cell differentiation via embryoid body (EB) formation

EBs were generated using AggreWell™ plates (Stemcell Technologies). The 8 central wells of the plate contain approximately 1200 microwells each to generate EBs. The wells were first rinsed with DPBS and 1ml of 5% pluronic acid (dissolved in DPBS and autoclaved before usage) was added per well. The plate was spun at 2250x g for 3min and then incubated for 30min at room temperature. The pluronic acid was removed and the wells rinsed once with EB differentiation medium. 1ml EB differentiation medium was then added and the plate was spun again at 2250x g for 3min. To obtain EBs consisting of ~2000 cells each, a total of $2.4x10^6$ ES cells were seeded per well and the cell suspension was added dropwise into the wells. The final volume of medium in each well was 2ml. The plate was spun at 100x g for 3min and incubated for 8hrs in the incubator. The EBs were harvested by gently pipetting up and down with a 5ml pipette and the EB suspension passed through an inverted 40µl cell strainer to remove single cells. The wells were washed three times with 2ml of EB differentiation medium and cells poured again through the cell strainer to collect all EBs. Then the strainer was turned right-side up in a fresh 50ml Falcon tube and washed with 25ml EB differentiation medium to collect the EBs. The cell suspension was poured in a T75 ultra-low adherence flask and moved from side to side to evenly distribute the EBs. Cells were fed every second day with EB differentiation medium. Approximately half of the EBs in one flask were used for DNA and the other half for RNA isolation.

TAM treatment

Cre-dependent recombination of the two loxP sites was induced by adding 1µM TAM dissolved in 96% EtOH to the cell culture medium. To exclude a side effect of the ethanol on the cells, control samples were treated with vehicle only. Undifferentiated ES cells were treated for 48h with TAM to generate the control cell line. In differentiated cells the TAM treatment started either on d5, 9 or 13 and new TAM was added with fresh medium after 48hrs. After an additional 48hrs the cells were harvested.

5.2.2 Isolation of genomic DNA

Cell pellets (from d0 samples and EBs) were resuspended in 1ml of DNA lysis solution. For cell monolayers the medium was removed, 1ml of DNA lysis solution was added and the cells were scraped from the dishes. The cell lysate was incubated at 55°C overnight or for two nights in the case of EBs. 400µl saturated NaCl solution per 1ml of DNA lysis solution was added and mixed well by hand for 20 seconds. The tubes were then spun for 12min at 14000rpm at RT. The supernatant, approximately 700µl, was split into two new tubes containing 600µl cold isopropanol and shaken until a precipitate formed. Tubes were spun for 12min at 14000rpm at 4°C. The supernatant was discarded and the pellet was washed with 800µl 70% EtOH, incubated at RT for 15min and shaken by hand every 5min. Tubes were spun again for 10min at 14000rpm at 4°C. EtOH was carefully removed and the DNA pellet was dissolved in 30-100µl TE and incubated at 55°C overnight. The DNA concentration was determined on the next day using the NanoDrop ND-1000 Spectrophotometer (Peqlab).

5.2.3 Southern blotting

<u>DNA digest</u>

Approximately 20µg of isolated DNA were digested either in 35µl (single digest) or 50µl (double digest) volume with 2µl restriction enzyme and the corresponding buffer at 37°C overnight. 10x loading buffer was added to the digested DNA and the samples, together with a 1kb ladder, were loaded on a 0.8% agarose gel in 1xTBE. Electrophoresis was performed at 5V/cm for 3½h. Afterwards the gel was stained in ethidium bromide for 1h and with the UVsoloTS imaging system a photo was taken under UV light. To denature the DNA the gel was shaken two times in denaturing solution for 30min.

<u>Blotting</u>

A plastic tray was filled with the denaturing solution used for the gel and a glass plate was placed on it. 2 sheets of 3MM Whatmann paper were soaked in

denaturing solution and placed on the plate so that the ends dipped into the solution in the tray. At every step of assembling the blot a plastic pipette was used to roll out air bubbles. The gel was turned upside down and placed on the 3MM paper. The Hybond XL blotting membrane was placed in a clean tray, activating by soaking for 1min in MQ H_2O and then soaked for 5min in denaturing solution. The membrane was placed on top of the gel. To avoid buffer short circuits the area around the membrane was covered with plastic stripes. On top another 2 sheets 3MM Whatmann paper soaked in denaturing solution were placed. 10-15cm of green paper towels with a glass plate and a blotting weight of approximately 500g were added on top. After 1-2 days the blot was disassembled and shaking for 2min in Na_2HPO_4 neutralized the membrane.

Probe labeling and cleaning

20ng of probe were filled up with H_2O to a final volume of 14µl. The DNA was denatured for 5min at 100°C and placed on ice for 2-3min. 2-4µl α^{32}P-dATP were mixed with 20µl LS, 6µl CTG and 1µl Klenow fragment and added to the denatured probe. The labeling reaction was incubated for at least 6hrs up to 18hrs at RT. Afterwards the probe was diluted with 60µl TE and non-incorporated nucleotides were removed by centrifugation at 300rpm for 3min at RT on a sephadex column.

Hybridization of the blot

The membrane was pre-hybridized with Church buffer in a hybridization tube for at least 30min at 65°C in a hybridization oven, constantly rotating. The labeled probe was denatured for 5min at 100°C and placed on ice for 2-3min. The pre-hybridization buffer was discarded and 20ml Church buffer with the labeled probe were added to the membrane and hybridization carried on for at least 18hrs in the hybridization oven. Afterwards the membrane was washed twice for 30min with pre-warmed Church wash.

Exposing and scanning

After washing, the membrane was sealed in plastic foil and exposed to an imaging plate for 1 to 7 days. The plate was scanned with the Typhoon Trio Variable mode Imager (GE-Healthcare), intensity of the bands was detected and calculated with ImageQuant TL. Images were adjusted linear for brightness and contrast using Adobe Photoshop.

5.2.4 Pulsed field gel electrophoresis (PFGE)

For PFGE analysis, $1x10^6$ undifferentiated ES cells were seeded on 15cm dishes and differentiated with RA differentiation medium for 13 or 17 days.

Isolation of genomic DNA for PFGE

Harvested cells were counted with the CASY® Cell Counter, spun at 1050rpm for 5min and the cell pellet was resuspended in DPBS to obtain a concentration of $1x10^7$ cells/ml. The cell suspension was equilibrated to 42°C in a waterbath and an equal volume of 1% Biozym Gold Agarose in DPBS, also at 42°C, was added to it. Agarose blocks were made and once solidified they were dropped in Falcon tubes containing 50ml PFGE DNA lysis solution. The agarose blocks were incubated overnight at 55°C while gently shaking. The blocks were then washed five times with 50ml TE and twice with 50ml TE containing 1mM PMSF for 30min at 55°C while gently shaking. At the end, the blocks were stored in 50ml TE at 4°C.

DNA Digest for PFGE

The DNA was digested in the agarose blocks in a total volume of 250µl, using 2µl restriction enzyme and 25µl of the appropriate buffer, overnight at 37°C. The three digests were performed separately. The gel blocks were washed three times with 50ml TE for several hours at RT, while gently shaking, to remove residual buffer before setting up the next digest. The agarose blocks were loaded on a 1% agarose gel in 0.5x TBE together with the Low Range PFG Marker. The electrophoresis was performed with 6V/cm, with the switch time ramping from 1-12sec, at 14°C for 20h. Southern blotting was performed as described above.

5.2.5 RNA isolation

For cell monolayers the medium was removed, 1ml TriReagent was added and the cells were scraped from the dish. The cell pellets of d0 samples and EBs were resuspended in 1ml TriReagent. Cell suspensions were transferred to RNA tubes and incubated for 5min at RT. 100µl BCP were added to the tubes, mixed and incubated for 10min at RT. The cell lysate was centrifuged at 12000g for 15min at 4°C. The upper aqueous phase, containing the RNA, was transferred to new RNA tubes and 500µl isopropanol were added. The mixture was vortexed and incubated for 5min at RT. The tubes were again centrifuged at 12000g for 8min at RT. The supernatant was discarded and the pellet was washed twice with 75% EtOH, followed by centrifugation at 7500g for 5min. The remaining EtOH was removed, the pellet was air dried and resuspended in 20-100µl RNA storage solution. RNA concentration was determined using the NanoDrop ND-1000 Spectrophotometer (Peqlab).

5.2.6 cDNA preparation

6µg of isolated RNA was DNAseI treated with the DNAfree™ kit (Ambion) in a total volume of 33µl and cDNA was prepared with the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas) following the provided protocol. Two plus reverse transcriptase (+RT) and one minus reverse transcriptase (-RT) reactions were made. After reverse transcription the cDNA was diluted 1:6 with water for embryo transfer.

5.2.7 Non-quantitative PCR

PCR was performed in a total volume of 25µl containing 5µl GoTaq Flexi buffer (5x), 1.25µl forward and reverse primer (10µM), 0.5µM dNTPs (10mM), 2µl MgCl2 (25mM), 0.1µM GoTaq polymerase (5U/µl) and 6µl cDNA. The PCR was performed on both +RT and –RT samples.

The following PCR program was used: 3min at 95°C, 35 cycles of 45sec at 95°C, 45sec at 59°C and 45sec at 72°C, 5min at 72°C.

The PCR products were mixed with 10x loading buffer and loaded on a 2% agarose gel in 1x TAE. Electrophoresis was performed with 4V/cm for $1^{3}/_{4}$hrs and afterwards

the gel was stained in ethidium bromide for 1hr. Under UV light a photo of the gel was taken with the UVsoloTS imaging system and brightness and contrast were linear adjusted with Adobe Photoshop.

5.2.8 Digest of PCR product

Half of the +RT PCR product was digested in a total volume of 30µl containing 1µl restriction enzyme and the corresponding buffer over night at 37°C. After digestion, PCR products were run on an agarose gel as above.

5.2.9 Quantitative PCR (qPCR)

The reactions were performed in optical 96-well reaction plates in a total volume of 25µl per well. The 7900HT Fast Real Time PCR system (Applied Biosystems) machine and the Sequence detection system version 2.3 (Applied Biosystems) software were used.

TaqMan assays were performed with 12.5µl universal MasterMix buffer (2x), 2.25µl forward and reverse primer (10µM), 0.1µl probe (50µM) and 5µl cDNA with following conditions: 2min at 50°C, 10min at 95°C, 40 cycles of 15sec at 95°C and 1min at 60°C.

For SYBRgreen assays 12.5µl MESA Green buffer (2x), 0.25µl forward and reverse primer (10µM) and 5µl cDNA (RT-qPCR) or 5µl gDNA (10ng/µl, qPCR) were used. Conditions were as follows: 2min at 50°C, 10min at 95°C, 40 cycles of 15sec at 95°C and 1min at 60°C (*Ki-67* and *PCNA*) or 65°C (Ex12SNP and CKO recombination), and an additional dissociation step of 1min at 65°C.

To calculate the relative amount of cDNA or gDNA a standard curve was carried out for every plate. The standard curve derived from a serial dilution of wt embryo cDNA, plasmid DNA or wt gDNA. +RT reactions were performed in triplicates and as a control –RT in duplicates. Results were either normalized to a housekeeping gene or displayed relative to another qPCR result. The data were analyzed using MS Office Excel.

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Education

Since October 2006	University of Vienna Magister in Genetics - Microbiology Second stage of degree: since October 2008 Molecular Genetics and Pathology First stage of degree: completed in 2008 Biology
2001 -2006	High school for high-performance sport, St.Pölten Graduaded with high school diploma

Research experience

Diploma thesis November 2011 to December 2012	CeMM Research Center for Molecular Medicine of the Austrian Academy of Sciences, Vienna Austria Group Denise Barlow Project: Control of *Igf2r* imprinted silencing in embryonic stem cell differentiation

Publication

Santoro F, Mayer D, Klement RM, Warczok KE, Stukalov A, Barlow DP, Pauler FM (in press) Imprinted Igf2r silencing depends on continuous Airn lncRNA expression and is not restricted to a developmental window. *Development*

Work experience

2004 - 2011	Gymnastic Coach at the Union St.Pölten
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