Investigating the relationship between *Airn* RNA and *Tcp-1* and *Sod2* genes in order to identify the functional role of *Airn* in newborn mice.

1. Background

Imprinted genes are expressed depending on their parent-of-origin (Santoro et al., 2013). The imprint control element (ICE) exhibits parent-of-origin specific epigenetic modifications and regulates imprinted gene expression (Zwart et al., 2001). On chromosome 17 there is an ICE that contains the promoter of the Antisense *Igf2r* RNA (*Airn*) gene (Zwart et al., 2001). The maternal chromosome 17 ICE acquires a DNA methylation imprint during oogenesis, therefore *Airn* is only expressed from the paternal chromosome. Transcription of the *Airn* gene produces a long and unstable RNA (Santoro et al., 2013). There are four known imprinted genes on chromosome 17: *Airn*, *Igf2r*, *Slc22a2* and *Slc22a3* (Santoro et al., 2013). *Airn* acts in cis to silence *Igf2r*, *Slc22a2* and *Slc22a3* genes on the paternal chromosome (Nagano et al., 2008). *Slc22a2* and *Slc22a3* are only imprinted in extraembryonic tissues and their expression is regulated in a transcription-independent manner (Nagano et al., 2008). In contrast, *Igf2r* is imprinted is all embryonic and extraembryonic tissues that co-express *Airn* and is regulated in a transcription-dependent manner (Sleutels, Zwart & Barlow, 2002). DNA methylation of the *Igf2r* promoter in 8.5 days post coitum (dpc) embryos results in stable gene repression however *Airn* continues to be expressed (Santoro et al., 2013).

Airn is expressed throughout development and is present in many adult mouse tissues (Hu et al., 1999). This suggests that *Airn* RNA may have other regulatory roles in mice in addition to regulating *Igf2r* expression. *Airn* is an unstable lncRNA, and if it is regulating gene expression it is likely acting in cis. *Tcp-1* and *Sod2* are located 56 and 148 bp downstream of the *Airn* gene, respectively and are tightly linked to *Igf2r* (Barlow et al. 1991). Barlow *et al.* studied 14.5 dpc embryos and found that *Tcp-1* and *Sod2* were not imprinted (1991). Imprinted gene expression pattern is known to change over time and it is possible that *Airn* regulates *Tcp-1* and *Sod2* expression later in development (Hu et al., 1999). Previous studies have demonstrated that *Airn*, *Tcp-1* and *Sod2* are present in heart, brain and lung tissue in young mice (Hiroshi

et al. 1992; Jones et al., 1995; Hu et al., 1999). In this study, we will look at *Airn* localization patterns in heart, brain and lung tissue from newborn mice in order to determine whether *Airn* may be involved in *Tcp-1* and *Sod2* regulation.

2. Research Question and Potential Impact

We will attempt to answer the following research question during this study: does *Airn* RNA localize at the *Tcp-1* and *Sod2* loci on chromosome 17 in heart, brain and lung tissue from newborn mice? Our understanding of the functional role of *Airn* is limited to a small window during embryogenesis. This research is novel because as of yet, we do not understand why *Airn* continues to be expressed after *Igf2r* acquires a DNA methylation mark. The continued expression of *Airn* in the embryo and in adult mice suggests that its expression is important for other cellular processes not associated with embryogenesis. If this study is successful in showing that *Airn* is localized at *Tcp-1* and *Sod2* loci then this would change the current way we think about imprinting and would suggest that imprinting is not limited to embryogenesis.

LncRNAs are important for imprinting and X-chromosome inactivation and they have received a great deal of attention recently as they have been linked to numerous serious congenital disorders and cancers (Lee & Bartolomei, 2013). *Xist* and *Kcq1ot1* are lncRNAs that have been associated with several diseases and are expressed in the developing embryo and in adult mice (Lee & Bartolomei, 2013). Like *Airn*, additional targets for these lncRNAs in adult mice have not been identified. While there has been a push to better understand the roles of lncRNAs and their relevance to understanding and treating various diseases, much regarding their function is unknown (Lee & Bartolomei, 2013). Therefore, this research is important because it will shed light on a novel role of lncRNAs in adults and may help researchers study diseases caused by the deregulation of lncRNA expression and develop novel treatments.

3. Hypothesis and Predictions

We hypothesize that *Airn* is localized at the *Tcp-1* and *Sod2* loci in heart, brain and lung tissue. In order to study the relationship between *Airn* and *Tcp-1* and *Sod2* genes, an RNA/DNA FISH assay will be performed. Using an RNA/DNA FISH assay we will be able to study the localization pattern of *Airn* RNA in the cell to see if *Airn* RNA associates with *Tcp-1* and *Sod2* loci. Additionally, we will calculate the cover index which measures the extent of overlap between fluorescent probes. If *Airn* is localized at the *Tcp-1* and *Sod2* loci, then by performing an RNA/DNA FISH experiment we expect to see a high level of overlap between *Airn* and *Tcp-1* and *Airn* and *Sod2* probes in heart, brain and lung tissue types.

4. Experimental Design

The model system being studied in this experiment is newborn mice. We used C57BL/c mice because they are an inbred mouse stock that is commonly used in research (Belknap, Crabbe & Young, 1993). Murine fetuses were carried to term and 60 newborn mice were sacrificed for tissue harvesting. We followed the dissection protocol used by Hu *et al.* (1999). Heart, brain and lung tissue samples were collected, frozen and stored at -80 degrees Celsius (Nagano et al., 2008). We mounted 14 um thick frozen tissue sections onto slides for the RNA/DNA FISH experiment.

In this study, we want to determine where *Airn* RNA is localized in relation to the *Tcp-1* and *Sod2* loci. To study this, we performed an RNA/DNA FISH experiment. An RNA/DNA FISH experiment allows us to a visualize the location of *Airn* RNA and the *Tcp*-1 and *Sod2* loci using labelled probes. Long DNA probes were designed to detect the *Tcp-1* and *Sod2* loci and a short DNA probe was used to detect *Airn* RNA (Barakat & Gribnau, 2014). Long probes contain more labelled nucleotides therefore they

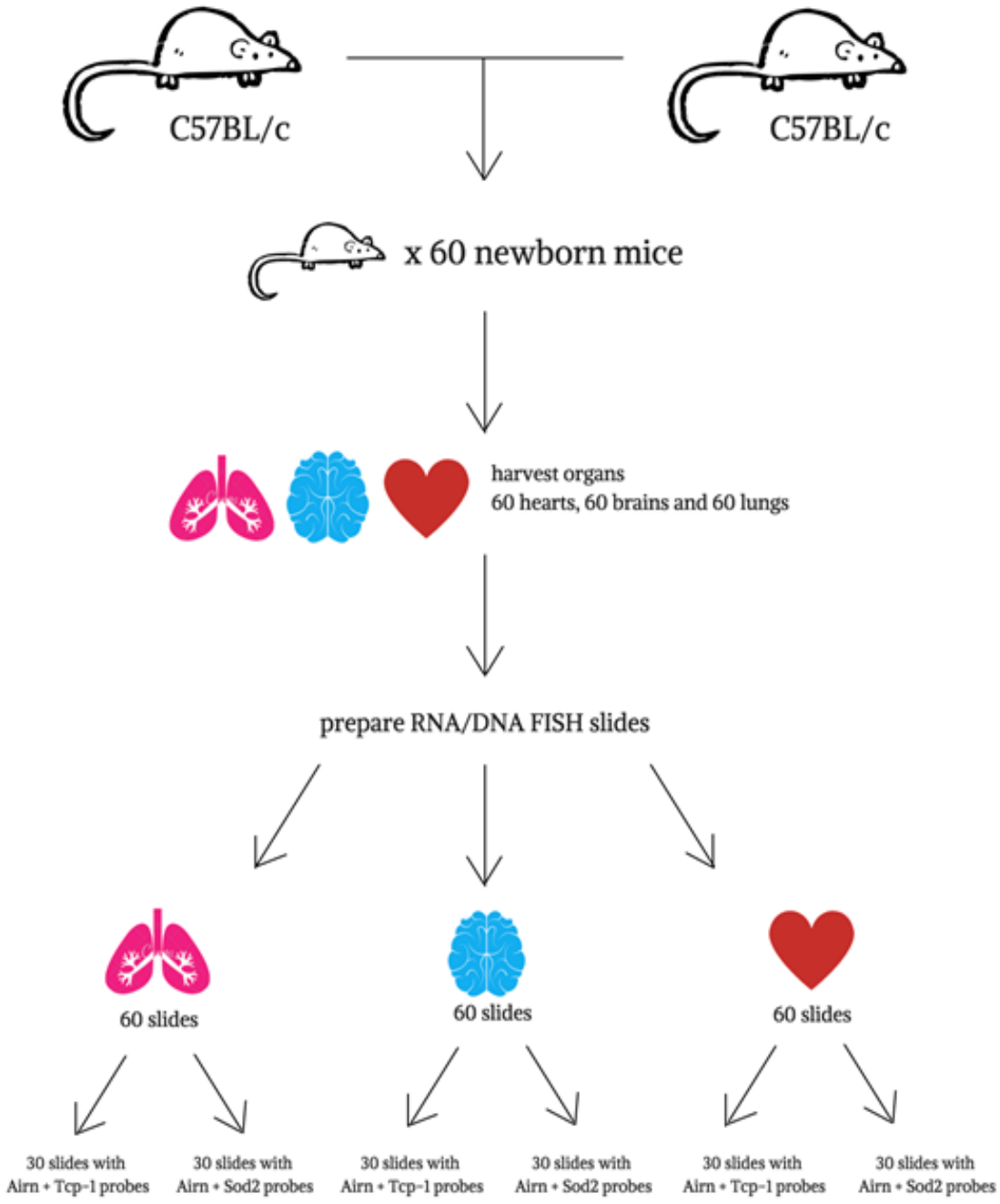

Fig 1. Illustration of the experimental design used in this study.

produce a stronger signal than short probes. Short probes are used to detect RNA because they produce weak signals and are advantageous because they minimize the detection of RNA probes binding DNA sequences. We prepared the RNA/DNA FISH slides for the hybridization step by following the protocol described by Barakat & Gribnau (2014). 30 slides of each tissue type were hybridized with *Airn* and *Tcp-1*

and *Airn* and *Sod2* probes. After the hybridization step, post hybridization washes were done to dissociate nonspecific interactions between the probes and off target nucleic acid sequences (Ransick, 2004). In order to visualize the probes, all the sections were incubated with a primary antibody and a secondary, conjugated antibody (Barakat & Gribnau, 2014). To detect the *Tcp-1* and *Sod2* probes, an additional incubation with a tertiary, conjugated antibody will be performed to amplify the fluorescent signal and make it easier to visualize the gene loci (Barakat & Gribnau, 2014). We added 4′,6’-diamidino-2-phenylindole (DAPI) fluorescent stain to the sections in order to visualize the nuclei of the cells (Barakat & Gribnau, 2014). We studied the nuclei of 150 cells for each tissue type. Section were imaged using a confocal microscope and the cover index for each of these nuclei was calculated using special software (Nagano et al. 2008).

5. Predicted Results and Conclusions

In this study, we collected data on the localization pattern of Airn RNA relative to the *Tcp-1* and *Sod2* loci as well as information on the tissue-specificity of Airn localization patterns. I will discuss these two results separately.

5.1 Cover Index Results

There are 3 possible cover index results (for the outcomes listed below, “gene” is used to refer to either the *Tcp-1* or *Sod2* gene depending on which RNA/DNA FISH slides are being studied):

1) RNA/DNA FISH results show that *Airn* RNA is localized at the gene locus and the average cover index is high

Based on these results, we can infer that *Airn* is associating with the gene locus in newborn mice. Moreover, because the cover index is high we can conclude that *Airn* is highly coating the gene (Nagano et al., 2008). This result demonstrates a potential regulatory role of *Airn* within the cell as a regulator of gene expression. While there is evidence that a high cover index is indicative of RNA-DNA interactions, a follow up RNA TRAP experiment will need to be performed to investigate the molecular relationship between

Airn and the gene locus. Results from an RNA TRAP experiment can determine whether *Airn* interacts with the gene sequence (Nagano et al., 2008). If the results from the RNA TRAP experiment show that *Airn* is interacting with the gene locus, this would be interesting because it suggests that the imprinted status of gene expression varies over time.

2) RNA/DNA FISH results show that *Airn* RNA forms a dispersed cloud near the gene locus and the average cover index in moderately high.

This result suggests that Airn RNA is moderately associated with the gene locus. Imprinted gene expression is known to change over time therefore a possible conclusion we can draw based on the localization pattern of *Airn* is that this result represents a time point just prior to or after *Airn* regulation of the gene (Hu et al., 1999). Nagano *et al.* found that in placental tissue from embryonic day 11.5 (E11.5), the cover index is higher than in placenta from E15.5 (2008). A follow up RNA/DNA FISH experiment should be performed in younger and older mice to study *Airn* localization to determine whether *Airn* RNA is associating with the gene locus.

3) RNA/DNA FISH results show that *Airn* RNA is not localized at the gene locus and the average cover index is low.

This result suggests that *Airn* is not associating with the gene locus. If the cover index is low then we can conclude that *Airn* is not coating the gene therefore it is unlikely that *Airn* is acting in cis to regulate gene expression at this time point. This result suggests that the gene is most likely not imprinted in newborn mice. While this result shows that Airn is not localized at the gene locus in newborn mice, a follow-up RNA/DNA FISH study should be performed in younger and older mice to see if *Airn* is localized at the gene locus at an earlier or later time point.

5.2 Tissue-Specific Results

It is possible that the *Airn* RNA localization pattern is similar in all the tissue types studied. This would indicate that the association between *Airn* and the *Tcp-1* and *Sod2* genes is conserved among the

tissues studied. Follow-up studies will have to be done to determine if *Airn* is interacting with and regulating *Tcp-1* and *Sod2* gene expression in order to conclude that *Airn* is involved in *Tcp-1* and *Sod2* regulation in heart, brain and lung tissue. It is possible that not all studied tissue types show the same *Airn* localization pattern. This kind of result suggests that tissue-specific factors influence whether *Airn* is associated with the *Tcp-1* and *Sod2* genes. Furthermore, a future study should be done to see if, in the tissue types where *Airn* does localize to the gene loci, Airn regulates gene expression. Hu *et al.* demonstrated that *Igf2r* is imprinted in all adult tissues that co-express *Airn* expect in the brain (1999). In the brain, *Igf2r* expression is biallelic even though *Airn* is expressed. Thus, a result where all the tissues show different localization patterns may indicate differences in gene regulation and imprinting status. *Sod2* encodes a superoxide dismutase enzyme that breaks downs reactive oxygen species and *Tcp-1* gene encodes a chaperone molecule that is involved in folding ~10% of cytosolic proteins (Zelko, Mariani & Folz, 2002; Posokhova et al., 2011). *Sod2* and *Tcp-1* are necessary for proper cellular functions and the deregulation of these genes has been associated with various diseases (Zelko, Mariani & Folz, 2002; Posokhova et al., 2011). In the tissue types where we see localization of *Airn* at the *Tcp-1* and *Sod2* loci, we can infer that regulation of these genes is very important in this tissue type and that expressing both alleles is not advantageous to the cell.

Probe Combination	Cover index		
	Heart Tissue	Brain Tissue	Lung Tissue
Airn + *Tcp-1* Probes	High	High	High
	Moderate	Moderate	Moderate
	Low	Low	Low
	High	Moderate	High
	High	Moderate	Moderate
	High	Low	Moderate
	High	Low	Low
	Moderate	High	High
	Moderate	High	Moderate
	Low	High	Moderate
	Low	High	Low
	Moderate	Moderate	High
	Low	Moderate	High
	Low	Low	High
Airn + *Sod2* Probes	High	High	High
	Moderate	Moderate	Moderate
	Low	Low	Low
	High	Moderate	High
	High	Moderate	Moderate
	High	Low	Moderate
	High	Low	Low
	Moderate	High	High
	Moderate	High	Moderate
	Low	High	Moderate
	Low	High	Low
	Moderate	Moderate	High
	Low	Moderate	High
	Low	Low	High

Table 1. A list of all the possible results from the RNA/DNA FISH experiment. RNA/DNA FISH slides were either hybridized with *Airn* and *Tcp-1* or *Airn* and *Sod2* probes. The cover index was calculated using a computer program and indicates the level of overlap between the two probes used.

6. Future Directions

Similar studies to the one we performed should be done to study the function of *Xist* and *Kcq1ot1* in newborn mice. In addition, if we are able to show that Airn associates with *Tcp-1* and *Sod2*, a next step we might consider taking is looking at younger and older mice to see when *Airn* begins and stops localizing at the *Tcp-1* and *Sod2* loci. Barlow *et al.* found that in 14.5 dpc embryos, *Airn* does not imprint *Tcp-1* or *Sod2*, therefore *Airn* may begin associating with *Tcp-1* and *Sod2* later in development.

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