CHARACTERIZATION OF GENES REQUIRED FOR PREIMPLANTATION EMBRYO DEVELOPMENT

Marc P Maserati, Jr, university of massachusetts, Amherst
CHARACTERIZATION OF GENES REQUIRED FOR PREIMPLANTATION EMBRYO DEVELOPMENT

A Thesis Presented

by

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Approved as to style and content by:

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Jesse Mager, Chair

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Joseph Jerry, Member

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Rafael Fis sore, Department Head,
Veterinary and Animal Science
DEDICATION

To my lovely wife and children; no matter the difficulty, there is always a way.
ACKNOWLEDGMENTS

I would like to thank Dr Jesse Mager for giving me an opportunity to join his research team studying preimplantation embryo development and hope we may find opportunities to collaborate on future projects. The lab comprises many outstanding individuals and I feel honored to have worked with them, most notably Mary Trask who has given advice and performed experiments towards the completion of this work, Melanie Walentuk who performed all of the immunofluorescence in the WDR74 paper as well as contributing her talents to the RNA processing and BCOR work. I would like to further acknowledge the efforts of Xaingpeng Dai who performed the initial pooled screen which resulted in the identification of 29 genes of interest for further evaluation. I would like to also thank Dr Hong Wei of Transova Genetics for supplying reagents and bovine embryos during our collaboration on the BCOR project.

Likewise I would like to thank Dr. Kimberly Tremblay and the members of the lab including Mara Isel Guerrero, Jesse Angelo and Si-Yeon Rhee for contributions both personal and professional toward the completion of this work.

It has been said that it takes a village to raise a child. The same is true for a graduate student within a university; the culmination of experiences shape students into professionals who, through work, will represent the university in all future positions. I am grateful for all the interactions I’ve had from faculty, staff and students alike.

This work was supported by a Massachusetts Life Sciences Grant and March of Dimes Research Grant to Jesse Mager.
ABSTRACT

CHARACTERIZATION OF GENE EXPRESSION REQUIRED FOR
PREIMPLANTATION EMBRYO DEVELOPMENT

SEPTEMBER 2013

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Directed by: Professor Jesse Mager

Preimplantation embryo development in the mouse is a time of rapid cellular
morphological and molecular changes leading to embryo implantation for the generation of
offspring. The Mager lab studies these events occurring between fertilization and implantation in
order to better understand the initial events which set the stage for all future aspects of
development. The result of this research impacts many scientific disciplines including *in-vitro*
based means of embryo culture, establishment of epigenetic marks, differentiation and cellular
reprogramming and can be used in translational research for the improvement of *in-vitro* culture
techniques and develop novel therapies such as cell replacement in the case of macular
degeneration (Bin, L., 2009).

Through the use of *in-vitro* embryo culture, RNA interference (RNAi) approaches and
daily observations, gene function required in preimplantation embryo development can be
determined. In the initial published body of work evaluating gene knockdown using our RNAi
approach (Maserati M 2011), WDR74 was characterized in preimplantation embryo
development. We now understand that WDR74 is implicated in RNA production and/or
stability as gene knockdown at the 1 cell stage significantly depletes mRNA within the embryo by the morula stage. Furthermore, double knockdown of Trp53 and Wdr74 results in a partial rescue of blastocyst formation suggesting p53 mediated apoptosis in the failure to make a blastocyst phenotype.

The initial characterization of 4 RNA processing genes (SF3b14, SF3b1/SAP155, Rpl7l1 and Rrp7a) required for blastocyst formation was later evaluated. The results of this work has been submitted for publication and will be published soon in the journal Zygote. SF3b14 and SF3b1, identified as being part of the splicesome complex, disproportionally contributes to gene transcription of those genes containing more than 1 exon verifying a role in RNA splicing. Rpl7l1, identified by GO terms as a possible ribosomal gene, was found to be present in the cytoplasm and, surprisingly, in the nucleus. It is surmised this gene influences polymerase 2 activity as Rpl7l1 gene knockdown embryos demonstrate reduced active polymerase 2 activity at the morula stage. Rrp7a was identified as being critical in blastocyst formation and is present in the cytoplasm while excluded from the nucleus. Based on location and GO terms, this suggests a role in translation. Taken together, these 4 genes act in 3 different ways impacting RNA production, splicing or translation promoting blastocyst formation in the mouse.

The final gene evaluated in this work was Bcl-6 corepressor (Bcor). As opposed to our previous work with RNA processing factors, this gene knockdown does not result in a failure to make a blastocyst. Bcor knockdown increases the rate of physiologically normal blastocysts in both murine and bovine models. Although further characterization must be done, temporary Bcor gene knockdown might be a useful improvement of in-vitro embryo culture systems including murine, bovine, equine and possibly even human.
This manuscript is divided into 4 chapters, the first of which is a review of preimplantation embryo development. This covers selected and relevant events between fertilization and just before implantation of the embryo into the uterus. I mainly focus on events after fertilization and the necessary changes required for zygotic genome transcription and lineage specification.

The second chapter characterizes WDR74, a gene we identified as critical in the formation of a blastocyst in a reverse genetic screen. As stated before, we assess WDR74 function with the developing embryo and conclude the protein plays a role in RNA production and/or stability of RNA transcripts. We also test to rescue blastocyst formation in WDR74 knockdown embryos in an attempt to further evaluate WDR74 function.

We continue the characterization of genes whose temporary reduction causes the failure of blastocyst formation in the third chapter. Here we report on four additional RNA processing genes in a body of work which has been published in the journal Zygote. Since these genes contained similar GO terms, we assumed they may all function in a similar way so they were assayed together as a group. As function of these genes were unknown, we determined protein localization within the cell, function in RNA splicing, alternative splicing and to determine if the failure to make a blastocyst is due to lineage specification.

In the final chapter, BCOR gene expression is characterized in preimplantation embryo development as in the former 2 chapters. However, the result of this gene knockdown does not lead to the failure to make a blastocyst, rather this improves the number of blastocysts formed during the correct physiological time; the same time that blastocysts form invivo. Undoubtedly, this could lead to possible commercial applications which are reviewed along with the
preliminary data we have been able to collect thus far. Specifically, the continuation of the BCOR gene knockdown research in preimplantation embryo development is pitched in the form of academic and international business collaboration with InvitroBrasil for the production of cloned bovine, equine and ICSI in equine.
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CHAPTER 1

INTRODUCTION

1.1 Preimplantation Embryo Development

Preimplantation embryo development is a carefully orchestrated series of events initiating with fertilization and concluding with formation of the blastocyst (Rossant, J 2009). Mammalian oocytes differ in terms of timing with the events of ovulation, oocyte nuclear and cytoplasmic maturation, fertilization and preimplantation embryo development; all more or less follow this ordered series of events with the exception of the dog (Reynaud, K 2006, Tadros, W 2009 and Rossant, J 2009). Therefore to understand mammalian embryo development in one species is to have a basic understanding of other mammalian species.

Fertilization is initiated with the sperm head fusing with the oocyte membrane and releasing a protein (PLCz) in to the oocyte cytoplasm which initiates a calcium release from the endoplasmic reticulum. This results in an oscillating calcium gradient which persists in a repeating degrading pattern until the first cell division (Malcuit, C 2006). The calcium signal initiates resumption of meiosis, formation and extrusion of the second polar body and cortical granule exocytosis to inhibit polyspermy. (Yoon, SY 2007; Ross, P 2009). The sperm haploid genome is released into the environment of the oocyte cytoplasm. The paternal nuclear material within the sperm is organized using protamines within the sperm to conserve space and ensure stability. Once in the oocyte, histones replace the protamine coincident but not because of the initiation of paternal genome demethylation at the one cell stage (Sassone-Corsi, P 2002) and (Santos, F 2002) and soon after forms the pronucleus.
Meanwhile in the maternal genome, upon resumption of meiosis the second polar body is released reducing the maternal genome to haploid. The 2 pronuclei join to form a new 2N genome called syngamy. The cell cycle then advances to the 2 cells stage where the embryo continues its rapid paternal genomic demethylation followed by a gradual maternal demethylation. Upon reaching the blastocyst stage, remethylation occurs in the ICM in a lineage specific manner (Santos, F 2002).

From the time of oogenesis until just after fertilization, protein production within the oocyte originates from the maternal RNA. The embryo genome is transcriptionally silent. Gradually, the embryo recruits and degrades maternal RNA transcripts in favor of transcripts of zygotic origin using the RISC complex to selectively degrade RNA transcripts of maternal origin. RNA transcription from the embryo, zygotic genome activation (ZGA), commences initially within the male pronucleus at the 1 cell stage with complete transcription by the 2 cell stage and throughout the genome as well (Schultz, RM., 2002). The cell cycle continues in development until the morula stage where lineage specification occurs.

1.1.1 Lineage specification

Post fertilization, the cell cycle resumes with even, reductive divisions until the blastocyst stage. As the embryo attains 16-32 cells, reorientation of blastomeres coincident with altered gene expression begins to distinguish 2 distinct cell types. The inner cells continue to express pluripotency genes with the addition of Nanog. The outer cells begin to express Tead4 and later, CDX2 which works to reduce Oct4 transcription. These differences become much more pronounced during the transitions between the morula and blastocyst stage as the hydrogen pumps activate transforming the compact ball of cells into a expanded structure with distinct
internal and external structures. This is accomplished by the development of tight gap junctions and ion channels which import sodium ions into and potassium out of the expanding blastocyst (Watson AJ., 2001). Thus, the initial cell differentiation event has taken place and is called lineage specification. Successful lineage specification results in a defined inner cell mass (ICM), trophectoderm (TE) and later the primitive endoderm (PE) (Cockburn, K 2010). These cells produce known mutually exclusive protein products and can be visually differentiated from each other using antibodies specific to these proteins via immunofluorescence. Cells of the ICM maintain expression of OCT4, SOX2 and NANOG expression while the outer TE cells produce TEAD4 and CDX2. The proteins produced by the ICM maintain pluripotency while TE expression of Cdx2 represses pluripotency genes leading to differentiation from the totipotent state. Mutually exclusive expression of OCT4 and CDX2 in the ICM and TE respectively signify completion of lineage specification (Cockburn, K 2010). While the ICM will develop into tissues used of embryo proper and specific extraembryonic membranes, the TE and PE will almost exclusively contribute to extra-embryonic tissues (Kwon, G 2008).

1.1.2 X-Chromosome inactivation

Somatic female cells contain 2 X-chromosomes, while males only contain 1. In order to compensate for the 2 fold increase in genes, female mammalian cells undergo a process called dosage compensation in which one X-Chromosome is rendered nearly transcriptionally inactive. The inactive X-chromosome was first observed in female feline motor neurons; described as a “Barr body” a dense heterochromatin mass closely associated with the nucleus (Barr, ML 1949). The Barr body was later to be recognized as the inactive X chromosome (review in Morey, C 2011). During preimplantation embryo development, female embryos at the 4 cell stage will begin to undergo paternal X-chromosome inactivation. By the blastocyst stage, the TE cells will
maintain the inactive paternal X– chromosome while the ICM will undergo paternal X-chromosome activation followed quickly by random X-chromosome inactivation (Kalantry, S., 2011). This is mediated by expression of 2 transcripts, \textit{Xist} and \textit{Tsix} which inactivate or activate transcription of the chromosome from which the RNA products originate (Ohhata, T., 2011). These transcripts are initiated by cis or trans activating factors such as in the case of RNF12 activation of \textit{Xist} transcription (Barakat, T 2011) which acts through degradation of the pluripotency factor Rex1 (Gontan, C 2012). \textit{Xist} functionally inactivates the chromosome from which it is expressed by physically coating the inactive X chromosome with the \textit{Xist} RNA transcript (Brown, CJ 1992)(reviewed in Kalantry, S 2011).

1.2 Reverse genetic screen

1.2.1 Introduction

This research endeavors to identify novel critical gene function during preimplantation embryo development by selectively reducing transcripts in using an RNAi approach and observing developmental phenotypes. Although there are several genome wide knockout programs currently ongoing (Guan, C 2010) (Austin, P 2004), this research differs from those attempts through the evaluation of gene function and are not limited to the determination of knockout lethal phenotypes. A reverse genetic screen was developed by Dr. Jesse Mager to study developmental and epigenetic events in preimplantation embryo development. The following sections describe the approach and implementation of the reverse genetic screen.

1.2.2 Candidate gene selection

The initial step in this screen was to identify genes of interest. As expression array data is accessible through on-line databases, a computer based screen was performed evaluating gene
expression over 3 different developmental stages using pooled expression array data. Pooling gene expression array data has been shown to increase reliability in expression array data (Mager, J 2006). The developmental stages evaluated in the expression arrays were arranged as follows; metaphase II until the 2 cell stage, 4 cell to the 16 cell stage, and early expanding blastocyst to late expanding blastocyst stages. Gene expression was then evaluated to identify those genes with a dynamic expression pattern as a temporal increase in expression may point to a critical function at this embryonic stage in development. Gene expression was determined absent or present (A or P) depending on the threshold of gene expression. As there are 3 different developmental stages defined, combinations of PPA, is indicative of high gene expression earlier on in development which tapers off at the blastocyst stage. This could indicate function in zygotic transcription initiation. APP indicates low gene expression in the initial stages of preimplantation embryo development while increasing at the 8 cell and blastocyst stages. This could indicate a function in blastocyst formation. Although there are 9 possible combinations, only 7 were used as AAA and PPP were omitted from consideration.

1.2.3 RNAi gene knockdown via microinjection

Knockdown of individual genes at the one cell stage was accomplished through the use of pulled glass pipets backloaded with dsRNA specific to the gene of interest. A Nikon Diaphot Mounted with eppendorf electrical micromanipulators held fine pulled pipets used for micromanipulation. dsRNA was injected into a secondary chamber formed by a larger than usual holding pipet which allows more volume to be introduced into the embryo as well as considerably reducing embryo lysis during injection (Figure 1). This secondary chamber is created through the deformation of the zona pellucida and oolemma as the holding pipet begins to aspirate the oocyte into the holder (Figure 1 C). The needle tip can then cut through the zona
pellucida and positioned within the inner chamber (Figure 1 C). A gentle piezo pulse will allow cutting of the oolemma at the tip and not along the sides of the needle minimizing the risk of lysis. dsRNA then displaced the cytoplasm in the chamber. The needle is quickly removed allowing the oolemma to reform intact (Figure 1 D). The reformation of the oolemma is helped by 2 factors based on the geometry of the secondary chamber. First, the elongation of the embryo allows the oolemma more surface area to interact, increasing the probability of reformation. Furthermore, due to the force of the dsRNA being introduced into the embryo and displacement of the cytoplasm, the outer edges of the oolemma are brought together increasing the probability of reformation. If done properly, lysis can be less than 1-2% of embryos injected which compares favorably with current techniques whereby 25% lysis is expected. (Nagy, A 2003).
1.2.4 Initial knockdown screen results

The initial screen identified 1200 genes which displayed a dynamic pattern of gene transcription. After eliminating genes which were already well characterized in preimplantation development or had obvious housekeeping functions, 400 were selected to be knocked down in 1 cell embryos in groups of 4-5 pooled dsRNAs. After injection, the embryos were evaluated daily for development up until the blastocyst stage to determine if the gene knockdown inhibited blastocyst formation or outgrowth. Those injected groups which resulted in a morphological change in development were chosen for further evaluation by evaluating lineage specification, zygotic genome activation and other gene specific assays to determine function. The initial
screen identified 29 genes which caused either a failure to make a blastocyst or if attained the blastocyst stage, failed to make an ES-like outgrowth *in-vitro* (Table 1).

**Table 1: Functional groups of genes identified in the initial reverse genetic screen**

<table>
<thead>
<tr>
<th>Results of the reverse genetic screen</th>
<th>Number of genes</th>
<th>Identified Genes</th>
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<td>Failure to make an outgrowth</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>Stem cell factors</td>
<td>2</td>
<td>Cxros1, Tbl1x1r1</td>
</tr>
<tr>
<td>Epigenetic factors</td>
<td>3</td>
<td>Suv39h1, Ube2a, Bcor</td>
</tr>
<tr>
<td>RNA factors</td>
<td>3</td>
<td>Puf60, Ptdc3, Asf1b</td>
</tr>
<tr>
<td>Protein factors</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Other factors</td>
<td>6</td>
<td>Tuba1a, Arg1</td>
</tr>
<tr>
<td>Unknown factors</td>
<td>4</td>
<td>1110003E01Rik, 17000250:D13Wsu50e, 1190002J23Rik, 1110054005Rik</td>
</tr>
<tr>
<td>Failure to make a blastocyst</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Stem cell factors</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Epigenetic factors</td>
<td>1</td>
<td>Nop2</td>
</tr>
<tr>
<td>RNA factors</td>
<td>7</td>
<td>Mxi1, Rpl7l1, Rrp7a, Sf3b1, Sf3b14, Wdr74</td>
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<tr>
<td>Protein factors</td>
<td>5</td>
<td>Uspl1, Itgae, P4Ha2/P4H1, Rtn4, Pbrm1</td>
</tr>
<tr>
<td>DNA factors</td>
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<td>Dck, Matr3</td>
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<td>Unknown factors</td>
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The distribution of outgrowth failure genes into groups based on function was somewhat evenly spread out between pluripotency, epigenetics and RNA production. As this group of genes are not required for making a blastocyst, their critical function could be implicated in differentiation, signal transduction, or cell migration as these functions are essential in the post implantation embryo. Interestingly, there were 6 factors which were completely unknown in function based on gene ontology (GO) terms and pubmed searches.
Among the genes characterized by failure to make a blastocyst, there was a bias towards RNA factors and protein modifying factors, basic functions required for cell division and development.

For my thesis research we characterize Wdr74 in preimplantation embryo development in chapter 2. Knockdown of this gene results in a failure to make a blastocyst and we evaluate how this failure occurs. This body or work resulted in a publication (Maserati, M 2011). In the third chapter, we investigate 4 RNA processing genes starting from the research platform used in the Wdr74 work to branch off into this further work. Finally in chapter 4 we investigate Bcor gene knockdown in preimplantation embryo development.
CHAPTER 2
WDR74 IS REQUIRED FOR BLASTOCYST FORMATION

2.1 Introduction

Preimplantation is a dynamic developmental period in which a combination of maternal and zygotic factors program the early embryo resulting in lineage specification and implantation. A reverse genetic RNAi screen in mouse embryos identified the WD Repeat Domain 74 gene (Wdr74) as being required for these critical first steps of mammalian development. Knockdown of Wdr74 results in embryos that develop normally until the morula stage but fail to form blastocysts or properly specify the inner cell mass and trophectoderm. In Wdr74-deficient embryos, we find activated Trp53-dependent apoptosis as well as a global reduction of RNA polymerase I, II and III transcripts. In Wdr74-deficient embryos blocking p53 rescues blastocyst formation and lineage differentiation. These results indicate that Wdr74 is required for RNA transcription, processing and/or stability during preimplantation development and is an essential gene in the mouse.

Preimplantation development in the mouse is a time of dynamic change in which the fertilized egg becomes a pluripotent embryo that subsequently develops into a blastocyst with two distinct cell lineages. This developmental period is characterized by three major transitions, each of which entails pronounced changes in the pattern of gene expression. The first transition is the maternal-to-zygotic transition (MZT) that serves three functions: (1) to destroy oocyte-specific transcripts, (2) to replace maternal transcripts that are common to the oocyte and early embryo with zygotic transcripts and (3) to facilitate the reprogramming of the early embryo by
generating novel transcripts that are not expressed in the oocyte (Latham, KE 1991). Zygotic gene activation initiates during the late 1-cell stage at some genes and throughout the genome by the 2-cell stage (Latham, KE. 1991), (Schultz,G 1993). Coincident with genome activation is the acquisition of a chromatin-based transcriptionally-repressive state (Nothias, JY 1995) (Schultz, RM 2002) and more efficient use of TATA-less promoters (Majumder S., 1994), which are likely to play a major role in establishing the appropriate patterns of gene expression required for proper development.

Up to the 8-cell stage, individual blastomeres are loosely associated. During compaction, adhesive interactions form between blastomeres to generate a tightly organized mass of cells (Fleming,TP 2001). Accompanying this morphological transition are pronounced biochemical changes through which blastomeres acquire characteristics resembling somatic cells, reflected in such features as ion transport, metabolism, cellular architecture, and gene expression patterns. The appearance of gap and tight junctions at the late 8-cell stage results in an epithelium that is essential for proper development (Fleming, TP 2001) (Kidder, GM 2001).

Following compaction, cleavage divisions allocate cells to the inside of the developing morula. These inner cells of the morula give rise to the inner cell mass (ICM) from which the embryo proper is derived. The outer cells differentiate exclusively into the trophectoderm (TE), which generates extraembryonic tissues. The TE is a fluid transporting epithelium that is responsible for forming the blastocoel cavity, which is essential for continued development and differentiation of the ICM (Biggers, DJ 1988) (Watson, AJ 1990). Overt cellular differentiation first occurs in the blastocyst and is characterized by differences in gene expression between the ICM and TE cells. For example, expression of Pou5f1 (Oct4) and Fgf4 (Nichols, J 1984) (Niswander, L 1992); Pesce, M 2001) become restricted to the ICM, while expression of Bex1,
the imprinted \textit{H19} gene and \textit{Cdx2}, an ortholog of the Drosophila homeotic Caudal (\textit{Cad}) gene, are restricted to the TE in the preimplantation embryo (Beck, F 1995) (Doherty, AS 2000) Williams, JW et al. 2002). Proper specification of these distinct lineages is required for blastocyst formation and facilitates hatching from the zona pellucida and implantation into the uterine epithelium reviewed in (Cockburn and Rossant 2010).

In order to expand our understanding of the genes required for these critical developmental events, we initiated an RNAi based screen during mouse preimplantation. Based on the simple assumption that a dynamic change in a given gene’s expression level may be indicative of temporal specific function, we analyzed published micro-array data sets (Hamatani, T 2004); (Zeng, F 2004) and (Zeng,F 2005) for genes with dynamic expression patterns during preimplantation stages. We first used an unbiased \textit{insilico} approach to merge published data sets in order to select genes based on preimplantation expression pattern alone, irrespective of associated gene ontology (Mager, J 2006). Genes with known roles during preimplantation and genes known to be required for cell viability were then removed as candidates. We next tested the preimplantation requirement of each of the remaining candidate genes by microinjection of gene-specific long double-stranded RNAs (dsRNAs) into fertilized 1-cell zygotes and subsequent culture \textit{in vitro} to the blastocyst stage, an approach which has been used successfully during preimplantation and does not elicit an interferon response or significant off-target effects (Stein, P 2005). We maximized our screening potential through injection of pools of up to 5 different dsRNAs in order to knockdown 5 separate genes simultaneously. Pooled dsRNAs that resulted in a phenotype were then injected singly to determine the gene responsible. Here we present data showing that the WD Repeat Domain 74 gene (\textit{Wdr74}) is required during preimplantation development.
*Wdr74* is characterized by six WD40 repeats, which are minimally conserved structural motifs of approximately 40 amino acids that often terminate in a tryptophan-aspartic acid (WD) dipeptide (Smith, TF 1999). To date, no studies have functionally characterized *Wdr74*. Analysis of the predicted Wdr74 protein using functional domain finding algorithms reveals no identifiable functional domains, besides the WD repeats, providing little insight. *Wdr74* is a single copy gene that is very well conserved across mammalian species with all vertebrates having one orthologous locus of at least 77% amino acid identity (Berglund, AC., 2008); (Penel, SA 2009). Interestingly, there are no homologous loci in mammals with significant amino acid similarities besides the WD repeat domains, indicating the unique nature of Wdr74. Although very little is known about *Wdr74*, the superfamily of WD40 repeat proteins have been implicated in a wide variety of cellular functions, such as cell division, cell fate determination, gene transcription, transmembrane signaling, mRNA modification, and vesicle fusion (Neer, EJ 1994). The function of WD-repeats has been shown to mediate protein-protein interactions during complex assembly (Lee, D 2001) possibly explaining the wide array of functions that are ascribed to genes containing them. Additionally, the importance of WD-repeat proteins is evident: the sequence has been conserved across all species in eukaryotes (reviewed in (Li,L 2001)), and WD repeat motifs have also been identified in prokaryotes as well (Janda, L., et al. 1996), (Hisbergues, M., et al. 2001). Here, we present data describing the developmental and molecular consequences of loss of *Wdr74* function.
2.2 Procedures for Wdr74

2.2.1 Embryo Production and collection

B6D2F1 females (Jackson Laboratories, Stock# 100006) received an intra-peritoneal injection of pregnant mare serum gonadotropin (PMSG; 5 IU/animal), followed by stimulation with human chorionic gonadotropin (hcG; 5 IU/animal) 48 hours later. Mice were mated with B6D2F1 males. Embryos (0.5dpc) were collected via flushing the infundibulum with M2 medium.

2.2.2 Microinjection

Injection and holding micropipettes were pulled from borosilicate capillary tubes with a Sutter p-87 glass puller (Sutter Instruments). The injection needle tip was cut to make a 1-2μM diameter opening and the needle was then bent to 15° using a microforge (DeFronbrun). Microinjection was performed using a piezo impact-drive injector (PrimeTech) mounted on a Diaphot inverted microscope (Nikon) with Hoffman Modulation Contrast objectives and condenser. Micromanipulation of embryos was performed using TransferMan NK 2 (Eppendorf,) in M2 medium (Chemicon) with 0.01% PVP (Sigma). Each embryo was injected with approximately 5pL dsRNA. After all embryos were injected, they were transferred into microdrops of M2 medium for 10 minutes at 37°C before deposition in KSOM medium for extended culture.
2.2.3 Embryo Culture

Embryos were cultured in 35mm petri dishes (Falcon) in 30-50µL drops of KSOM (Chemicon) under 3mL mineral oil (Fisher Scientific) in 5% CO₂, 5% O₂ and 90% N₂ at 37°C in a humidified incubator.

2.2.4 dsRNA in vitro transcription

For T7-RNA polymerase mediated double stranded RNA (dsRNA) production, Wdr74, Trp53 and Green Fluorescent Protein (GFP) specific PCR primers were designed using 40 base-pair oligos that contained the T7 binding sequences followed by gene specific sequences as follows: GfpF 5’- TAATACGACTCACTATAGGGCAATGAAGCAGCACGACTT -3’ and GfpR 5’-TAATACGACTCACTATAGGGTCAGGTTGGATATGTGCTG-3’, Wdr74F 5’ - TAATACGACTCACTATAGGGCAATGAAGCAGCACGACTT -3’ and Wdr74R 5’ - TAATACGACTCACTATAGGGCAATGAAGCAGCACGACTT -3’; Trp53F 5’ TTATACGACTCACTATAGGGCAATGAAGCAGCACGACTT -3’ and Trp53R 5’ - TTATACGACTCACTATAGGGCAATGAAGCAGCACGACTT -3’. All oligos were purchased from IDT. dsRNAs were created using the MEGAscript T7 in vitro transcription kit (Ambion) followed by DNase treatment (Roche) and purification using NucAway Spin Columns (Ambion) and Phenol/Chloroform extraction. dsRNAs were re-suspended in Nuclease-Free water (Integrated DNA Technologies) and diluted to 400ng/µL for microinjection. dsRNA was stored at -80°C until use.

2.2.5 Pyronin Y RNA staining

Embryos were fixed in 4%PFA for 10 minutes, rinsed twice in PBS with 0.3% PVP (PBS/PVP) and stained in PBS/PVP Hoechst (IDENT Hamilton Thorne) 40µg/mL for 10
minutes. Embryos were then directly transferred to PBS/PVP 5μM Pyronin Y (ACROS) for 10 minutes. The embryos were then rinsed three times in 400μL PBS plus 0.3% PVP before being mounted in VectaShield (Vector Labs) on glass slides and evaluated for epifluorescence with a TE2000S inverted microscope (Nikon). All photographs were taken using a Retiga EXi camera (Photometrics) using National Instruments Elements (National Instruments) image capture software under phase contrast and epifluorescence.

2.2.6 RNA Quantification

The Agilent RNA 6000 Pico kit was used according to manufacturer’s recommendations. RNA was extracted from embryos using the High Pure RNA Isolation Kit (Roche) and resuspended to 1.6 embryo equivalents per microliter (EE/μL). Samples were run in triplicate. RNA concentration was also assessed by NanoDrop spectrophotometer according to manufacturers’ instructions.

2.2.7 RNA isolation and cDNA synthesis

Total RNA was extracted from single and pooled embryos using the High Pure RNA Isolation Kit (Roche) according to manufacturer’s recommendations. First strand cDNA was prepared from total RNA using the qScript cDNA Synthesis Kit (Quanta) according to the manufacturers’ instructions.

2.2.8 Quantitative Real-Time PCR

Real-time RT-PCR was performed using 0.75 embryonic equivalents with Taqman probe based gene expression assays from Applied Biosystems as follows: 2X Quanta PerfeCTa Supermix Low ROX, 20X Vic-labeled ActB (#4352341E) or GapD (#4352339E), 20X Gene
Expression Assay (Bax Mm00432050_m1; miR-125 TM002198; miR-721- #TM001657; Oct4 Mm00658129_gH; Tead4 Mm01189836_m1; Trp53 Mm01731287; Wdr36 Mm00620161_m1; Wdr74 Mm00506573_m1; Rnu6 TM1973; Sno110 TM1230; Snord65 TM1228; Mvp Mm00453676_m1) and water to total 20µL. Reactions were run in triplicate on a Stratagene Mx3005p Real-Time PCR machine with a thermal profile of: 1 cycle of 50°C for 30 seconds, 1 cycle of 95°C for 2 minutes, then 40 cycles of 15 seconds at 95°C and 30 seconds at 60°C.

2.2.9 Reverse-Transcriptase PCR

PCR was performed using 0.75 embryonic equivalents, 2X RubyTaq mastermix, 10mM of both forward and reverse primers, and water to total 20µL. The Wdr74 forward primer used was 5’ CGGAATGATTGGCTTGATCT 3’ and the reverse primer was 5’ AGGGTACTTGGTTGGGCTCT 3’. PCR reactions were performed in a Biorad MyCycler machine for 35 cycles of 95°C for 30 seconds, 60°C for 30 seconds, 72°C for 30 seconds with a final extension of 2 minutes at 72°C.

2.2.10 Immunofluorescence

Embryos were fixed in 4% paraformaldehyde for 30 minutes at room temperature, washed three times in phosphate buffered saline (PBS) containing 0.1% Tween-20, and permeabilized with PBS containing 1% TritonX-100 for 30 minutes at room temperature. Embryos were then blocked for 1 hour at room temperature in 2% bovine serum albumin (BSA) in PBS. Primary antibodies were added in appropriate dilutions [1:200 rabbit anti WDR74 (Sigma SAB1102237); 1:50 rabbit anti-Trp53 polyclonal (Cell Signaling Technologies), 1:200]
rabbit anti-Ecadherin polyclonal (Abcam), 1:200 rabbit anti-Oct4 polyclonal (Abcam), 1:200 mouse anti-Cdx2 monoclonal (BioGenex), 1:200 rabbit anti-H3K4me3 polyclonal (Abcam), 1:200 Rabbit Anti-Fibrillarin (Abcam, ab5821), 1:100 Rabbit Anti-CTD PolII (Abcam, ab5095)] in PBS/BSA (2%) for 1 hour at room temperature. After the embryos were washed three times with PBS containing 0.1% Tween-20, secondary antibodies (Alexa Fluor 546 Anti Rabbit – Molecular Probes A-11035; Alexa Fluor 488 Anti Rabbit – Molecular Probes A-21206; Alexa Fluor 488 Anti Mouse – Molecular Probes A-11001) were added at a 1:200 dilution in PBS/BSA and incubated for 1 hour at room temperature. 4’,6-Diamidino-2-phenylindole (DAPI) was used as a marker to stain nuclear DNA at a concentration of 1:10,000 for 10 minutes, then the embryos were washed three times with PBS containing 0.1% Tween-20, and mounted in water under VWR micro cover glass. As negative controls, embryos were treated similarly, without primary antibodies. Embryos were imaged using a Retiga EXi camera (Photometrics) using National Instruments Elements (National Instruments) image capture software under phase contrast and epifluorescence.

2.2.11 Blastomere Counting

Double-blind cell counting was repeated by two individuals using DAPI-stained nuclei and the NIS Elements BR software.

2.3 Results

2.3.1 Wdr74 is required for blastocyst formation

In order to determine the temporal expression of Wdr74 during preimplantation development, we used both quantitative reverse-transcriptase PCR (qRT-PCR, Figure 1A) and intron spanning qualitative RT-PCR (Figure 2B) to visualize endogenous mRNA levels at
various preimplantation stages. As shown in Figure 2, Wdr74 is expressed at relatively low levels in MII oocytes and 1-cell embryos and increases through subsequent cleavage stage divisions. The peak of mRNA expression occurs at the morula stage, with a slight decrease in blastocyst embryos (Figure 2A), suggesting a key function during the morula to blastocyst transition. Additionally, immunofluorescence with an antibody directed against human WDR74 showed no specific signal until the 8 cell stage in wild type embryos (not shown), when weak nuclear localization is observed. In morula and blastocyst, Wdr74 is evident within nuclei of all cells (Figure 2K). This pattern of protein expression perfectly correlates with the endogenous RNA levels, and suggests no Wdr74 function until late 8cell/early morula stages.
Figure 2: Wdr74 is required for blastocyst formation

A. Quantitative RT-PCR analysis of endogenous Wdr74 mRNA during preimplantation development. B. RT-PCR with Wdr74 intron-spanning primers confirms relative abundance of transcripts observed by qRT-PCR. C-H. Microinjected and cultured embryos photographed at 36, 60, and 84 hours post fertilization. Control dsGFP-injected embryos show normal development and form blastocysts by 84 hpf (C-E). dsWdr74 injected embryos develop normally to the morula stage (F-G) but fail to make blastocysts (H). I. Quantification of percent 2 cell embryos that develop to the blastocyst stage by 84 hpf (# blastocysts/ # 2-cell x 100). J. qRT-PCR of Wdr74
transcripts indicates robust RNAi mediated knockdown due to microinjection of dsWdr74. K.

Immunofluorescence of Wdr74 in morula stage dsGFP embryos shows nuclear localization; which is drastically reduced in dsWdr74 embryos of the same stage (L). hpf, hours post fertilization. Results of student T-test shown, error bars represent standard deviation. All data shown normalized to embryo equivalents; MII, Metaphase II oocyte. Scale bar in F representative for C-H. K’ and L’ show DAPI signal (DNA) from the same embryos shown in K and L, respectively.

In order to remove Wdr74 activity, we injected dsWdr74 RNA into the cytoplasm of 1-cell embryos. In all experiments presented, control embryos were injected with dsGFP in order to similarly stimulate the RNAi machinery and show specific effects of loss of Wdr74. Wdr74 knockdown embryos (hereafter referred to as dsWdr74 embryos) were able to compact and develop normally to the morula stage with no obvious differences in morphology or rate of development compared to controls (compare Figure 2C and D with F and G). By 84 hours post fertilization in culture (hpf), 91% (89/98) of control embryos have initiated blastocoel formation (Figure 1E and I). However, only 4% (4/110) of the dsWdr74 embryos showed any evidence of a blastocoel cavity (Figure 1H,I). The majority of dsWdr74 embryos showed visible signs of degeneration by 84 hours post fertilization (necrotic and irregular cells, Figure 2H).

We confirmed robust knockdown of dsWdr74 by qRT-PCR with cDNA from pooled control and experimental embryos at the morula stage (Figure 2J). Furthermore, immunofluorescence showed drastic reduction in dsWdr74 morula stage embryos indicating functional knockdown of Wdr74 (compare Figure 2K and L), and consistent reduction of both mRNA and protein using this approach. As stated above, dsWdr74 embryos appear morphologically normal through the morula stage. However, immunofluorescence revealed that
the blastomeres of dsWdr74 morula have reduced E-Cadherin protein present at cell-cell contacts and appear somewhat disorganized relative to controls (compare Figure 3A-C and D-F). Nonetheless, the appropriate localization of E-cadherin suggests that knock-down morula blastomeres are contacting each other appropriately in preparation for compaction and cavitation. Consistent with a peak in Wdr74 activity during the morula to blastocyst transition (Figure 2), these results indicate that a molecular and cellular phenotype is present in dsWdr74 morula, 1 day before the embryos degenerate.

2.3.2 Lineage specification and apoptosis in dsWdr74 morula

In order to explore possible reasons for the blastocyst failure and embryo death we examined markers of lineage specification and apoptosis. As shown in Figure 2C, we find an overall decrease in both Oct4 and Tead4 transcripts, known markers of ICM and TE, respectively (results shown relative to embryo equivalence). Importantly, we also observe a reduction in the “housekeeping” transcripts, β-actin and Gapdh, within dsWdr74 embryos (Figure 3C). The reduced transcript levels could be the result of either reduced mRNA or reduced cell numbers per embryo. Counting of cells in dsGFP and dsWdr74 morula revealed no reduction in cell number in dsWdr74 morula (Figure 3D), suggesting either global reduction of transcription or a loss of mRNA due to defects in stability and/or processing of nascent transcripts.
A-B. E-cadherin (Cdh1) localization by immunofluorescence marks blastomere cell-cell adhesion as expected in dsGFP morula (A). E-Cadherin is appropriately localized but present at reduced in dsWdr74 morula (B).

C. qRT-PCR assays show reduced RNA polymerase II derived transcripts of Pouf51, Tead4, Actβ, GapdH, Bax, and Cdh1 but Trp53 shows an increase in transcripts in Wdr74-deficinet embryos.

D. The average number of cells in dsGFP
and dsWdr74 morula is not significantly different. E-F. Localization of Trp53 by immunofluorescence shows a marked increase of Trp53 protein in dsWdr74 embryos (compare F to E), consistent with the increase in Trp53 mRNA. Results of student T-test shown, error bars represent standard deviation. All data shown normalized to embryo equivalents. N.S., not significant. Scale bar in B and F representative for A-B and E-F, respectively.

Consistent with the blastomere cell death observed at 84 hours, we find an increase in transcripts of the apoptotic marker Trp53 (p53) in dsWdr74 morula (Figure 3C). However, we did not observe a similar increase in Bax levels, a gene thought to be downstream of Trp53 (Miyashita, S 1994). Instead, Bax transcripts show a similar decrease in abundance as Oct4, Tead4, Gapdh and β-actin. Supporting the qRT-PCR results, we observe increased Trp53 protein within the cells of dsWdr74 morula (compare Figure 3E and F).

2.3.3 Co-injection of dsWdr74 and dsTrp53 permits blastocyst formation

Because Wdr74-deficient embryos showed increased levels of p53, we attempted to block apoptosis by knocking-down p53 by co-injection of dsWdr74 and dsTrp53. Reduction of p53 in dsWdr74 embryos rescued blastocyst formation in 49% of embryos (Figure 4A-C). For this experiment we added an additional control of injecting dsTrp53 alone, which had no adverse consequences on development to the blastocyst stage (data not shown). All injection groups (dsGFP, dsWdr74, dsTrp53 and dsWdr74/dsTrp53) developed normally to the morula stage. qRT-PCR confirmed expected knockdown in each group (Figure 4D). As expected, dsWdr74 embryos failed to form blastocysts and remained at the morula stage with obvious morphological signs of dying cells. However, 49% (34/69) of the dsWdr74/dsTrp53 co-injected embryos were
able to successfully form blastocoel cavities with morphologically distinct ICM and TE cells during the culture period (asterisks, Figure 4B). This rescue of blastocyst formation (from 4% to 49%) suggests that dsWdr74 knockdown embryos are capable of differentiating ICM and TE cells, but fail to do so due to Trp53-induced apoptosis. However, immunofluorescent staining shows that the majority of TE cells in these “rescued” blastocysts express both Oct4 and Cdx2 (arrows, Figure 5) indicating that although morphological differentiation has occurred, molecular patterning has not been properly established. Furthermore, many cells within the ICM of dsWdr74/dsTrp53 blastocysts inappropriately express Cdx2. Consistent with reduced transcript levels in the absence of Wdr74, the levels of both Oct4 and Cdx2 protein were reduced in dsWdr74/dsTrp53 embryos. Many of these double knockdown “rescued” blastocysts contain morphologically abnormal and/or necrotic cells, indicating that while blastocyst formation does occur, viability was not rescued. It is also worth noting that Wdr74/Trp53-deficient blasts do not expand as wild type embryos do, and remain in “early” blastocysts stages, reminiscent of the Cdx2 null embryo phenotype, which form early blasts but fail to maintain epithelial integrity of the TE (Strumpf, D 2005).
Figure 4: Blocking *Trp53* permits blastocyst formation in *Wdr74*-deficient embryos

A-B. Morphological evaluation of dsWdr74-injected and dsWdr74 + dsTrp53 co-injected embryos at 84 hpf. dsWdr74 embryos do not develop past the morula stage (A). Reduction of *Trp53* permits differentiation of Wdr74-deficient blastocysts (B). C. Percent of 2-cell embryos reaching the blastocyst stage by 84hpf in dsGFP, dsWdr74 and dsWdr74 + dsTrp53 co-injected embryos. D. qRT-PCR confirms knockdown of *Wdr74* and *Trp53* as expected. Results of student T-test shown, error bars represent standard deviation. All data shown normalized to embryo equivalents.
Figure 5: Lineage specification in dsWdr74/dsTrp53 blastocysts

A-H. Immunofluorescence localizes Oct4 and Cdx2 to the inner cell mass and trophectoderm, respectively, in dsGFP embryos (A-E). In dsWdr74/dsTrp53 rescued blastocysts (2 shown, F-J and K-O), Cdx2 is expressed and Oct4 is reduced (but present) in some TE-like cells (arrows in F-O). Scale bar in K representative for all panels. DIC, differential interference contrast microscopy.

2.3.4 Global reduction of RNA in Wdr74 deficient embryos

Based on the observation of reduced transcripts of all genes examined (except Trp53) when Wdr74 is knocked-down (Figure 3), we assessed global RNA levels in morula stage embryos using Pyronin Y, a fluorescent molecule that binds RNA (Shen, H 2008). We find a drastic decrease in Pyronin Y fluorescence in morphologically normal dsWdr74 morula at 60hpf, indicating a significant reduction of RNA in Wdr74-deficient embryos (compare Figure 6A and B). It is worth noting that approximately 8 hours earlier (~52 hpf), no qualitative differences were detectable in Pyronin Y fluorescence, suggesting a rapid decrease in RNA at the time when dsWdr74 embryos begin to compact. To confirm and quantify these results, RNA was extracted.
from equal numbers of dsGFP and dsWdr74 embryos (50 each) and assessed using a Bioanalyzer. As shown in Figure 5C, 18S and 28S peaks are clearly observed in RNA extracted from dsGFP injected embryos at the morula stage. However, these peaks are entirely absent in RNA extracted from Wdr74-deficient morula (Figure 6D).

2.3.5 RNA quantification via Bioanalyser and Polymerase III transcription

As with the qPCR assays, we used equal numbers of embryo equivalents in order to compare dsGFP and dsWdr74 samples. For the Bioanalyzer results shown, precisely 1.7 embryo equivalents of total RNA was used for both dsGFP and dsWdr74, in triplicate (Figure 5C and D). Consistent with reduced but not absent gene specific transcripts (Figure 2C), many minor peaks are present in the electropherogram from the dsWdr74 extracted RNA, indicating that some RNA is present. Given these results, we also assessed RNA samples with a Nanodrop spectrophotometer, which indicated an extraction of ~5.8ng total RNA from each dsGFP morula, while only ~0.66ng was recovered from each dsWdr74 embryo. This reduction in total RNA is consistent with the qPCR results that show between 2 and 5-fold reduction of gene specific transcript
A-B. Hoechst 33322 (blue) and Pyronin Y (red) stains bind to DNA and RNA, respectively, in dsGFP (A) and dsWdr74 (B) embryos. Note the dramatic decrease in Pyronin Y fluorescence in the absence of Wdr74. C-D. Bioanalyzer electropherograms from total RNA extracted from dsGFP (C) and dsWdr74 embryos (D) show that 18s and 28s ribosomal RNAs are nearly absent in Wdr74-deficient embryos. E. Relative levels of microRNAs \textit{miR-125} and \textit{miR-721} and RNA polymerase III derived transcripts, \textit{Mvp}, \textit{Rnu6}, \textit{Snord110} and \textit{Snord65} in dsGFP and dsWdr74 morula. Results of student T-test shown, error bars represent standard deviation. All data shown normalized to embryo equivalents.
Since we observed reduction of transcripts generated by both RNA polymerase I (ribosomal RNAs) and RNA polymerase II (coding transcripts in Figure 2), we also assessed levels diverse types of RNA species. We examined two microRNAs (mir-125 and miR-721) expressed during preimplantation (Yang, Y 2008) and four RNA polymerase III dependent transcripts, major vault protein (Mvp), U6 small nuclear RNA (Rnu6), and two small nucleolar RNAs [Snord110 and Snord65 (Das,G 1988); Kickhoefer, VA 2003]. Consistent with a global RNA reduction, we observe significant decrease of all of these gene products in dsWdr74 embryos (Figure 5E).

2.4 Discussion of results and conclusions

Here we have shown the molecular and developmental consequences of loss of Wdr74 function in vivo. Our results indicate that Wdr74 is required during preimplantation development and suggest that this poorly studied protein plays an essential role in global RNA transcription, processing and/or stability across the genome. We observe a similar reduction of transcripts produced by all RNA polymerases (I, II and III) suggesting that Wdr74 may be a common protein component of all three polymerase complexes. Interestingly, a human WD containing protein, Wdr92, has been identified in as a common component of RNA polymerase complexes (reviewed in (Cloutier, P 2010). Furthermore, we observe reduction of both intron-containing and single-exon transcripts, indicating that the global reduction is not due to a failure of premRNA splicing which has also been shown required for blastocyst formation (Latham, KE 1991; Yonemasu,R 2005). Similarly, RNA polymerase I deficient embryos develop to morula that fail to form blastocysts (Chen, Li 2008), supporting the possibility that Wdr74 is an essential component of RNA polymerase complexes.
The increase in Trp53 mRNA and protein indicates that the basic cellular processes of transcription, mRNA processing and translation can (and does) occur in the absence of Wdr74. It does remain possible that Wdr74 is absolutely required for transcription or translation but that there is sufficient Wdr74 protein present at the time of mRNA knockdown to allow for transcription and/or translation of Trp53, a possibility consistent with the reduction, but not absence of Wdr74 protein in knockdown embryos. However, we do observe Cdx2 protein, albeit at reduced levels in dsWdr74/dsTrp53 embryos. Cdx2 protein is not consistently present at high levels in blastomeres until late morula/early blastocyst stage (Ralston, A 2008). This is precisely when we observe strongest reduction in Wdr74 transcripts in dsWdr74 embryos. Given that we do observe Cdx2 protein in Wdr74 deficient cells of “rescued” blastocysts (Figure 4), we conclude that Wdr74 function is not absolutely required for transcription or translation.

Similarly, we observe reduced but present E-cadherin protein, which is required during preimplantation for blastomere compaction and formation of functional TE (Riethmacher, D 1995). The reduction in E-cadherin may be one contributing factor of the failure to form blastocysts, as it is also required for a properly polarized TE (Kan, NG. 2007). However, because dsWdr74 blastomeres appropriately localize E-cadherin and knockdown embryos do compact normally, we believe that the reduction in E-cadherin is a consequence – not a cause of the phenotype in Wdr74-deficient embryos.

Another possible explanation for the reduction of RNA could be defects in nucleolus function/structure. We do not observe morphological defects in nucleolus formation or size based on visual morphology or Fibrillarin localization (not shown), nor do we observe overt cellular defects prior to day 3 in culture, the time at which dsWdr74 embryos have failed to form blastocysts. The fact that we observe a reduction in all transcripts and protein during a stage
when dsWdr74 embryos appear morphologically normal suggests that Wdr74 is essential during preimplantation for RNA transcription, processing or stability and that as proteins degrade below functional thresholds, Wdr74-deficient blastomeres undergo Trp53 dependent apoptosis. The rapid reduction of RNA coincident with Trp53 activation occurs at the precise developmental stage when we observe the highest Wdr74 transcript levels in wild-type embryos. As we do not observe appreciable levels of maternal transcripts or protein, our results indicate that Wdr74 is not required until the morula-to-blastocyst transition.

While the precise mechanism of Wdr74 remains to be determined, our results offer the following conclusions: 1) Wdr74 is expressed from the zygotic genome and is required for morula to blastocyst transition; 2) In the absence of Wdr74 function there is a global reduction of RNA transcripts from RNA polymerase I, II and III; 3) Loss of Wdr74 activates Trp53 dependent apoptosis; 4) When Trp53 function is blocked, dsWdr74 embryos are capable of morphological differentiation and blastocyst formation although molecular patterning of lineage specification remains defective.
CHAPTER 3

IDENTIFICATION OF 4 GENES REQUIRED FOR BLASTOCYST FORMATION

This work is published and can be found:


3.1 Introduction

Although genome wide transcriptome studies are readily available for many tissues and cell types, functional annotation of the entire mammalian genome remains a daunting challenge. Several consortium based genome wide knock out and mutagenesis projects are well underway, with the goal of generating mutant alleles for every gene in the mouse (Guan, C 2010), (Austin, CP 2004). While these resources are immensely valuable and continue to produce data through large-scale phenotype screening efforts, usually they will not functionally assess genes during development other than to document lethality when an essential gene is abrogated. We have undertaken an RNAi based screen during mouse preimplantation, aimed at identification of genes involved in epigenetic and developmental events during the first cell lineage decisions in vivo. During the initial phase of this screen we have identified many genes required for blastocyst formation. In an effort towards functional genome annotation, here we characterize four genes that are each essential during the morula to blastocyst transition in the mouse. This report documents the preimplantation requirement of Splicing Factor 3b, subunit 1 (Sf3b1 also called SAP155 and Sf3B155); 0610009D07Rik (hereafter called by the human orthologue gene name, Sf3b14, but also called HSPC175, Ht006, P14, SAP14, Sf3b14a)); Ribosomal RNA Processing 7 homolog A (S. cerevisiae, Rrp7a); and Ribosomal Protein L7-like 1 (Rpl7l1).
Sf3b1 has been shown *in vitro* to be an integral member of both the major and minor spliceosome and binds to both sides of the pre-mRNA branch site (Gonzani, O 1998), (Wang, C 1998) and (Das, BK 1999). Sf3b1 regulates ceramide Bcl-x alternative splicing through binding to ceramide-responsive RNA cis-element 1 in pre-mRNA, preventing splicing and resulting in an exon inclusion (Massiello, A 2006). Mice homozygous for a knock out allele of *Sap155* die at 16-32 cell stage, and interestingly, heterozygotes show homeotic transformations which are rescued in an MII null background (Isono, K 2005). U2snrp/Sf3b1 have been shown to interact with polycomb group genes ZFP144 and RNF2 (Isono, K 2005) possibly explaining the homeotic transformations due to shifts in hox gene expression boundaries. Although morula stage lethality was noted by Isono et. al, limited characterization of the preimplantation phenotype was performed (Isono, K 2005).

While genome wide expression analyses suggest that *Rpl7l1* transcripts are increased in the early gastrula organizer and during pancreas differentiation (Hoffman, BG 2008), (Tamplin, OJ 2008), there are currently no published reports specifically on *Rpl7l1* function. Similarly, the *Rrp7a* and *Sf3b14* genes have been annotated in cDNA sequencing projects (Okazaki, Y 2002), (Kawai, J 2001), but remain functionally undocumented.

Here, we report the preimplantation expression patterns of *Sf3b14*, *Sf3b1*, *Rpl7l1*, and *Rrp7a* and show that each is required for the morula to blastocyst transition. In the absence of each protein, cell fate decisions are initiated, suggesting cell lethal phenotypes rather than specific developmental requirements of these factors. We also report that zygotic transcription does initiate, but reduced mRNA and protein levels of several genes occurs in the absence of *Rpl7l1* and *Rrp7a* suggesting critical functions in mRNA translation and/or ribosome function during mammalian development. We also present data indicating an overall decrease in multi-
exon transcript levels in the absence of Sf3b14 and Sf3b1, confirming a role in RNA splicing for these genes in vivo.

3.2 Procedures

3.2.1 Embryo Production and collection

B6D2F1 females (Jackson Laboratories Stock 100006) received an intra-peritoneal injection of pregnant mare serum gonadotropin (PMSG; 5 IU/animal), followed by stimulation with human chorionic gonadotropin (hcG; 5IU/animal) 48 hours later. Mice were mated with B6D2F1 males. The following morning, females were removed from the stud cages and examined for the presence of a vaginal plug. If a plug was present, embryos (0.5dpc) were collected via dissection of the ampula in M2 medium followed by 5 minutes in 0.1% Hyaluronidase (Sigma) All animal studies were approved by the Animal Care and Use Committee, University of Massachusetts.

3.2.2 Microinjection and Embryo Culture

To create the microinjection needle (injector), borosilicate capillary tubes (Sutter Instruments) were pulled with a Sutter p-87 glass puller (Sutter Instruments) creating a fine tapered point. The needle tip was cut to make a 1-2μM diameter opening and the needle was then bent to 15° using a microforge (DeFronbrun). This needle was back filled with Mercury and rinsed with 10% PVP (Sigma) prior to use. The embryo holding glass needle (holder) was created using the same borosilicate capillary tubes and pulled as above, cut at 85μM, fire polished and bent to 15 degrees. Microinjection was performed using a piezo impact-drive injector (PrimeTech) mounted on a Diaphot inverted microscope (Nikon) with Hoffman Modulation Contrast objectives and condenser. Micromanipulation of embryos was performed.
using TransferMan NK 2 (Eppendorf,) in M2 medium (Chemicon) with 0.01% PVP (Sigma). Each embryo was injected with approximately 5pl dsRNA. Following microinjection, embryos were transferred into microdrops of M2 medium for 10 minutes at 37°C. Embryos were transferred to and cultured in 35mm petri dishes (Falcon) in 30-50μl drops of KSOM (Chemicon) under 3ml light mineral oil (Fisher Scientific) in 5% CO₂, 5%O₂ and 90% N₂ at 37°C in a humidified incubator.

3.2.3 dsRNA Production

For T7-RNA polymerase mediated double stranded RNA (dsRNA) production, Sf3b14, Sf3b1, Rpl7l1, Rrp7a and Green Fluorescent Protein (GFP) specific PCR primers were designed using 40 base-pair oligos that contained the T7 binding sequences followed by gene specific sequences as follows: GFP TAATACGACTCACTATAGGGCACATGAAGCAGCACGACTT and TAATACGACTCACTATAGGGTGCTCAGGTAGTGGTTGTCG;  Rpl7l1 TAATACGACTCACTATAGGGGCTTTGGAGGTGCCTGATAA and TAATACGACTCACTATAGGGGAGGAAAGGCACAGAAAT;  Rrp7a TAATACGACTCACTATAGGGCACCTTGTGAAGCGGAAT and TAATACGACTCACTATAGGGTCTGTGAAGGCCAAATCCTC; Sf3b1 TAATACGACTCACTATAGGGGTGTTGAGTTGGCAAACAAAG and TAATACGACTCACTATAGGGTCATTAGCGTTATGGGTGATGC. PCR products were amplified from genomic DNA and purified. dsRNAs were created using the MEGAscript T7 in vitro transcription kit (Ambion) followed by DNase treatment (Roche), purification with NucAway Spin Columns (Ambion) and then Phenol/Chloroform extracted. dsRNAs were re-
suspended in Nuclease-Free water (Integrated DNA Technologies) and diluted to 1µg/µL for microinjection. dsRNA was stored at -80°C until use.

3.2.4 RNA extraction and quantitative PCR

RNA extraction was performed with Roche High Pure RNA Isolation Kit (Roche). cDNA was synthesized with both random hexamers and oligo-dT primers as described previously [12]. Real-time RT-PCR was performed using 0.75 embryonic equivalents with Taqman probe based gene expression assays from Applied Biosystems as follows: 2X Quanta PerfeCTa Supermix Low ROX, 20X Vic-labeled ActB (#4352341E) or GapD (#4352339E), 20X Gene Expression Assays (Oct4 Mm00658129_gH; Cdx2 Mm01212280_m1; Nanog Mm01617761_g1; Sf3b1 Mm00473100_m1; Sf3b14 Mm01310843_m1; Rpl7l1 Mm00786031_s1; Rrp7a Mm00780551_s1; Peg3 Mm00493299_s1; Snrpn Mm02391920_g1) and water to total 20µL. Reactions were run in triplicate on a Stratagene Mx3005p Real-Time PCR machine with a thermal profile of: 1 cycle of 50°C for 30 seconds, 1 cycle of 95°C for 2 minutes, then 40 cycles of 15 seconds at 95°C and 30 seconds at 60°C.

3.2.5 Fixation, embedding, sectioning

Preimplantation embryos were embedded in a pellet of NIH 3T3 cells and prepared for histology by fixation in 4% paraformaldehyde (PFA) for 2 hours at room temp or overnight at 4C. Embryos were dehydrated through a series of ethanol washes; 15 minutes each in 25%, 50%, 75% ethanol diluted in phosphate buffered saline (PBS), followed by two 100% ethanol washes. Embryo/cell pellets were embedded and sectioned as previously described (Griffith, GJ 2011).
3.2.6 Immunohistochemistry

Sections were deparaffinized with three 5-minute xylene washes and rehydrated with three 5-minute washes in 100% ethanol, followed by successive 1-minute washes in 90%, 80%, 70% ethanol and then water. Antigen retrieval was performed by boiling for eleven minutes in 0.01M Tris Base pH 10.0 with 0.05% Tween20. After slides cooled to room temperature they were washed twice in PBT for 2 minutes and blocked with 0.5% milk in PBT for two hours at room temperature in a humidified chamber. Primary antibody was applied in 0.05% milk/PBT overnight at 4C in a humid chamber. Three 15-minute PBT washes preceded a 1-hour secondary treatment at 1:500 in 0.05% milk/PBT in a humid chamber at room temperature. Slides were washed in PBT for 15 minutes twice and then in PBS for fifteen minutes. Nuclei were stained with DAPI (Roche or Molecular Probes) in PBS (1:10,000) for 10 minutes and then rinsed with PBS. Slides were sealed and coverslipped with Prolong Gold (Invitrogen). Primary antibodies were used at the following concentrations: Phospho-p53 9284 Cell Signaling 1:200; Sf3b14 AP48076 AbGent 1:200; Sf3b1(SAP155) ab39578 abcam 1:200; Pol II phosphoS2 ab5095 abcam 1:200; Rpl7l1 16707-1-AP Proteintech Group 1:200; and Rrp7a sc-86109 Santa Cruz 1:200. AlexaFluor 488 Donkey anti-rabbit A21206 (molecular probes) was used as secondary antibody at 1:500.

3.2.7 Whole mount embryo immunofluorescence

Whole mount preimplantation embryo IF was performed as previously described12. Primary antibodies were used at the following dilutions: Oct4 sc-5279 Santa Cruz 1:200, Nanog RCAB0002P-F Cosmo Bio Co 1:200, Cdx2 AM392-5M BioGenex 1:1000. Secondary antibodies from Molecular Probes were used at 1:500: AlexaFluor 546 Donkey anti-goat

3.2.8 Imaging

Digital images of whole mount embryos and sectioned embryos were taken with a Nikon Eclipse TE2000-S inverted fluorescence microscope and QImaging Retiga Exi Fast 1394 camera with NIS-Elements BR Software.

3.3 Results and Discussion

3.3.1 $Sf3b14$, $Sf3b1$, $Rpl7l1$, and $Rrp7a$ required for blastocyst development

Single cell zygotes were microinjected with double-strand RNA (dsRNA) specifically designed in order to functionally knock-down (KD) each gene. While dsGFP injected control embryos develop normally to the blastocyst stage (Figure 7 A), injection of dsSf3b14 (B and K), dsSf3b1 (C and L), dsRpl7l1 (D and M) and dsRrp7a (E and N) resulted in nearly complete failure of blastocyst formation. Interestingly, embryos developed from 1 cell to morula without any observed morphological or developmental differences, including formation of compacted morula at 72 hours post fertilization (hpf). Despite the normal appearance at 72 hpf, KD of each of these four genes resulted in visibly degenerating embryos at 96hpf, when dsGFP control embryos had developed to the blastocyst stage (Figure 7 A-E).

Given the rapid and complete failure of blastocyst formation, we first asked if functional knock down of these critical factors results in increased apoptosis. Although Trp53 (p53) protein is present in blastomeres of the normal preimplantation embryo, it is present in its inactive form. IF detection with an antibody specific to active p53 (phosphorylated at serine 15) indicates
apoptosis has been initiated in many (but not all) blastomeres in dsSf3b14, dsSf3b1, dsRpl7l1 and dsRrp7a embryos (compare Figure 7F to 7G-J).

**Figure 7:** *Sf3b14, Sf3b1, Rpl7l1 and Rrp7a are required for blastocyst formation*

Evaluation of developmental progression at 24 (2-cell stage), 48 (8-cell stage), 72 (morula stage) and 96 (blastocyst stage) hours post fertilization, following microinjection of double stranded RNAs designed to target *Sf3b14* (A), *Sf3b1* (B), *Rpl7l1* (C) and *Rrp7a* (D). Note the morphologically normal progression until the compacting morula stage but failure to develop into blastocysts in each case. Consistent with apparent death of knock-down embryos at 96hpf, p53 is activated in many blastomeres indicating apoptosis (compare F with G-J). Quantification of blastocyst formation (K-N). Error bars represent SEM. Asterisks indicate T-test p values <0.01. Number of microinjected embryos are indicated for each group in charts K-N.
3.3.2 Gene knockdown confirmation and temporal gene expression

Since KD embryos failed to develop past the morula to blastocyst transition, we next sought to determine the normal expression pattern of each gene and validate gene specific KD. As shown in Figure 8A, Sf3b14 transcripts are barely detectable in ovulated oocytes (MII), 1-cell, 2-cell and 4-cell embryos, with a rapid rise in gene expression occurring between 8-cell, morula and blastocyst stages. Sf3b1 shows a similar expression profile, although appreciable maternal transcripts can be detected in the oocyte and zygote (Figure 8B). Rpl7l1 is not detected in MII or zygote stages, and steadily increases in expression levels from the 2-cell through blastocyst stage (Figure 8C). Rrp7a expression closely resembles that of Sf3b1, being present in the oocyte, reduced during cleavage stages, and then increasing dramatically during the morula and blastocyst stages. The dynamic expression profiles of identified genes was a criteria for candidate gene inclusion in our knock-down screen – the peak of expression in morula/blastocyst implies functional importance at that developmental window, and matches the observed blastocysts failure phenotypes observed.
Figure 8: Wild-type expression patterns and knockdown validation of *Sf3b14*, *Sfb31*, *Rpl7l1* and *Rrp7a*

RT-qPCR results show dynamic expression of each gene during preimplantation development (A-D). Quantitative assessment of knock-down efficiency resulting from gene specific dsRNA injection shows robust loss of mRNA for each targeted transcript (E-H). Detection of endogenous proteins by IF in control and KD embryos at the 8 cell stage show nuclear localization of Sf3b14 (I), Sf3b1 (K), nucleolar localization of Rpl7l1 (M) and cytoplasmic localization of Rrp7a (O). KD embryos show drastic reduction/absence of protein (J, L, N, and P). Error bars represent SEM. Asterisks indicate T-test p values <0.01.
Microinjection of dsSf3b14, dsSf3b1, dsRpl7l1 and dsRrp7a resulted in a drastic reduction of endogenous mRNA (figure 8E-H) and near complete loss of each protein at the 8-cell stage (figure 8I-P). We also confirmed mRNA knockdown at 72 and 96 hpf (data not shown) to ensure functional loss throughout the culture period, with similar results, indicating loss of gene function through 96 hpf. In addition to KD confirmation, IF on control embryos provided cellular localization information for each of these proteins, which has not been previously demonstrated in vivo. Sf3b14 and Sf3b1 are both uniformly localized to the nucleus in 8-cell embryos, with exclusion from the nucleolus (Figure 8I and K). Sf3b14 and Sf3b1 are thought to interact with each other in splicesome complexes (Schellenberg, MJ 2011) so the similar cellular localization supports these findings.

Rpl7l1 is also localized to blastomere nuclei, but appears in small foci concentrated specifically in or around the nucleolus – but not elsewhere within each nucleus (Figure 8M). Quite different from localization of the other three proteins, Rrp7a is found throughout the cytoplasm and is excluded from the nucleus of 8-cell blastomeres (Figure 8O). Gene ontology suggests that both Rpl7l1 and Rrp7A are functional components of ribosomes (Ashburner, M 2000). While the cytoplasmic localization of Rrp7a is consistent with this possibility, the nucleolar localization of Rpl7l1 suggests a non-ribosomal function of this protein. IF detection in knock-down embryos shows severely reduced/absent protein resulting from each dsRNA microinjection (Figure 8J, L, N and P), consistent with the reduction of each mRNA (Figure 8E-H). Given that we observe loss of protein at the 8-cell stage, it is interesting to point out that no developmental defects appear until the morula to blastocyst transition, 24-48 hours after we detect protein reduction. Together, these results suggest that the critical cellular functions of these gene products are not required until after morula compaction has occurred, or that there are
sufficient molecular redundancies of protein function (perhaps maternal components) that persist until the blastocyst stage.

3.3.3 Lineage specification in KD embryos

Blastomeres begin to molecularly differentiate at the 8-cell/morula stage into cells of the future inner cell mass (ICM) and trophectoderm (TE). Given the failure of KD embryos to form blastocysts, we assessed expression genes known to mark the future ICM and TE in order to ask if the absence of these four proteins disrupts cell fate decisions in the early embryo. All blastomeres initially express the Pou domain transcription factor, Pou5f1 (also known as Oct4), which becomes restricted to ICM cells coincident with expanded blastocyst formation (Nichols, J 1998). During early morula stages, the caudal-type homeodomain transcription factor, Cdx2, becomes variably expressed, with “Cdx2 low” cells marking the future ICM, and “Cdx2 high” cells marking the future TE (Strumpf, D 2005). Coincident with the variable expression of TE specific genes such as Cdx2 and Tead4 during the compacted morula stage, other ICM specific genes such as the homeodomain protein Nanog (which marks the future epiblast) are also variably expressed in individual blastomeres (Chambers, I 2003), (Dietrich, JE 2007) and (Mitsui, K 2003). Although normal blastomeres show uncoordinated and variable levels of these critical factors during the morula to blastocyst transition, mutually exclusive expression of Oct4/Nanog and Cdx2 is not observed until the late/expanding blastocyst stage (Strumpf, D 2005), (Dietrich, JE 2007) and (Plusa, B 2008). Due to the failure of blastocyst formation and obvious embryo death at 96hpf, we could not assess cell lineage at a stage when cells have mutually exclusive expression patterns. We did however assess the onset of lineage commitment by examining Oct4, Nanog, Cdx2 mRNA levels and protein localization in compacting morula in
order to determine if individual blastomeres show the expected variability in protein levels and if lineage specific transcription has been activated in KD embryos.

**Figure 9: Lineage specification in the absence of Sf3b14, Sfb31, Rpl7l1 and Rrp7a**

IF analysis of key lineage specific proteins Nanog, Cdx2 and Oct4 in KD embryos (A-J). RT-qPCR analysis of the same gene mRNAs in KD embryos (K). Error bars represent SEM. Asterisks indicate T-test p values <0.05.
As expected, at 72hpf we observe Oct4 and Nanog in all wild type blastomeres as well as a few cells with markedly higher Cdx2 levels in wild type morula (Figure 9A and F). Knockdown of Sf3b14 or Sf3b1 did not alter the protein localization of Oct4, Nanog or Cdx2, and we do observe variable levels of Nanog and Cdx2 in individual blastomeres (Figure 3B-C, G-H). However, the overall levels of both Nanog and Cdx2 are reduced relative to wild type controls, and many fewer “Cdx2 high” blastomeres are apparent in either dsSf3b14 or dsSf3b1 embryos. Knockdown of Rpl7l1 resulted in undetectable levels of Nanog and much reduced Cdx2 and Oct4 (Figure 9D and I). Similarly, knock down of Rrp7a resulted in very low levels of Nanog and Cdx2, although Cdx2 high/Oct4 low cells are obvious in dsRrp7a morula (Figure 9J). The presence of Cdx2 high/Oct4 low blastomeres suggests that dsSf3b1 and dsRrp7a embryos have functionally initiated TE lineage specification, while dsSf3b14 and dsRrp7a embryos have not (due to the absence of Cdx2 high/Oct4 low blastomeres).

Since each of these genes is implicated in cellular functions required for molecular gene expression (splicing or translation), we also assessed Oct4, Nanog, and Cdx2 mRNA levels in knockdown morula (Figure 9K). Consistent with only minor reduction in Oct4 protein observed by IF, Oct4 mRNA is modestly reduced in all KD morula. Also consistent with the reduction in Nanog and Cdx2 observed by IF in each case, both Nanog and Cdx2 are significantly reduced in all KD embryos.

The dramatic loss of transcripts suggests that the underlying defects resulting in blastocyst failure in KD embryos is likely due to defects in essential molecular biological processes, rather than a failure of specific developmental events. We therefore focused on the 8-cell stage to evaluate the cell biological consequences of loss of function of these genes, a
developmental time at which loss of protein has already occurred (Figure 8), but no obvious morphological phenotype is present.

3.3.4 RNA polymerase II activity and RNA abundance in KD embryos

In order to assess global transcriptional activity, we performed IF with an antibody that specifically detects RNA Polymerase II phosphorylated at serine 2 in the C-terminal domain, a post-translational modification that marks initiating polymerase (Komarnitsky, P 2000). As Sf3b14, Sf3b1, Rpl7l1 and Rrp7a proteins are implicated in post-transcriptional events (splicing or translation), we did not expect to observe differences in active Pol II levels or nuclear localization – which was the case for dsSf3b14 and dsRrp7a embryos (Figure 10 A,B and E). Surprisingly, we observed a reduction in active Pol11 in dsSf3b1 embryos (Figure 10C), and a near-complete absence of phospho-ser5 Pol II in dsRrp7l11 embryos (Figure 10D). Combined with the unexpected nuclear localization of Rpl7l1 (Figure 8M), our results implicate Rrpl7l1 either in phosphorylation of Pol II, polymerase complex formation or initiation of transcription. This observation also explains the reduction in all transcripts that we observe in dsRrp7l11 embryos; without active Pol II, a reduction in all Pol II dependent mRNAs would be expected.

We next asked if zygotic transcription was occurring in KD embryos by assessing mRNA levels of Peg3 and Snrpn, both maternally imprinted loci expressed exclusively from the paternal genome (Komarnitsky, P 2000), (Szabo, PE 1995) and (Kuroiwa, Y 1996) . All Peg3 and Snrpn transcripts after the 2-cell stage originate from the paternal allele and therefore serve as an indication of active transcription from the zygotic genome. Reduced but appreciable levels of both Peg3 and Snrpn transcripts are present in all KD embryos at the 8-cell stage. At the morula stage both Peg3 and Snrpn levels are further reduced in dsSf3b14, dsSf3b1, dsRpl7l1 and
dsRrp7a embryos. These results indicate that although transcription from the zygotic genome is occurring during cleavage stages, a steady decrease in zygotic transcript abundance occurs as development proceeds in the absence of these critical factors. The reduction of Peg3 and Snrpn is also consistent with the reduction of Nanog and Cdx2 mRNAs, further supporting the possibility that Sfb14, Sfb1, and Rrp7a are required post-transcription during nascent RNA processing. The loss of Peg3 and Snrpn transcripts in dsRpl7l1 is consistent with the lack of active Pol II observed in KD embryos.

**Figure 10: Transcription and splicing in the absence of Sfb14, Sfb1, Rpl7l1 and Rrp7a**

Assessment of active RNA polymerase II in wild-type and KD embryos at the morula stage (A-E). Quantitative assessment of Peg3 and Snrpn zygotic transcripts in 8-cell (F) and morula (G) KD embryos. Quantitative analysis
of multi-exon and single exon transcripts in KD embryos (H). Error bars represent SEM. Asterisks indicate T-test p values <0.01.

3.3.5 Pre-mRNA splicing in Sf3b14 and Sf3b1 KD embryos

Since Sf3b14 and Sf3b1 are implicated in the splicing of pre-mRNAs, we compared levels of single exon genes versus multi-exon genes in KD embryos. Previous studies have shown that defects in splicing machinery can result in specific reduction of multi-exon transcript abundance (Furumai, R 2010) while single-exon transcripts (that are not spliced) remain unchanged. To determine if Sf3b14 and Sf3b1 have a role in splicing during preimplantation embryo development, we assessed expression of additional multi-exon genes as well as several single-exon genes. Consistent with a role in production of properly spliced transcripts, each of the multi-exon genes that we examined (n=5) showed significant reduction in both dsSf3b14 and dsSf3b1 embryos (Oct4, Nanog, Cdx2 in Figure 9K, Peg3 and Snrpn in Figures 10F-G). However, we do not observe the same drastic reduction in single-exon transcripts. dsSf3b14 embryos show no significant reduction in any of the single-exon genes examined (n=3, Zsr1, H2be and Fut1, Figure 10I) and dsSf3b1 embryos have reduced H2be, but no reduction of Zsr1 and Fut1. The consistent reduction in multi-exon transcripts, but absence of reduction in single-exon transcripts supports a role for both Sf3b1 and Sf3b14 in pre-mRNA splicing during preimplantation development in vivo.

3.3.6 Alternative splicing of the Bcl-x gene

Alternative splicing is the generation of more than one RNA transcript from the same encoded DNA through the addition or deletion of introns or exons through the post transcriptional modification termed “splicing”. The advantage of alternative splicing is to create
more functional diversity of the genome’s 23,000 genes (Chen, L 2012). The prevalence of genes which undergo alternative splicing was initially estimated to be 35% of all genes (Mironov, A 1999) and more recently is up to 94% (Wang, ET 2008).

*Bcl*-x is expressed in 4 different transcripts alternatively spliced based on cell type and state of apoptosis and is detected in mouse and human preimplantation embryos. The *Bcl*-x (*L*) isoform promotes cell survival whereas expression of the *Bcl*-x(*s*) isoform promotes apoptosis (Perumalsamy, A 2009). Microinjection of the (*L*) isoform increases mouse embryo survival and prevents reactive oxygen species (ROS) build up in poor invitro culture conditions (Liu, X 2011). Knockdown of SF3b1 has been reported to increase the ratio of the apoptotic Bcl-X (s) Bcl-X (L) isoform over the pro-survival “L” isoform in mouse tissues. (Massiello, A 2006). SF3b1 has been shown to direct Bcl-X splicing through regulation of the 5’ splice site in the Bcl-x gene (Massiello, A 2006) by binding to the CRCE 1 DNA element and thereby promoting the expression of the Bcl-X (L) transcript. Here, we determined if SF3b14, Rpl7l1 and/or Rrp7a play a role in alternative splicing of this gene.

Gene knockdown was performed as in the previous experiments and in confirmation of the literature cited above, knockdown of SF3b1 does indeed result in the presence of Bcl-x(s) splice variant (Figure 11A). Interestingly, knockdown of Rpl7l1 and Rrp7a also produces Bcl-x(s) (Figure 11A). Even though SF3b1 and SF3b14 function together in the spliceosome for intron splicing, depletion of SF3b14 does not result in the production detectable Bcl-x (s) isoform. Although this finding is suggestive of these genes having a role in alternative splicing, apoptosis could be another reason for the presence of the Bcl-x(s) transcript (Perumalsamy, A 2009). To evaluate apoptosis in our knockdown embryos we assayed 8 cell embryos for p53 using qRT-PCR (data not shown) which results in no significant difference in the relative
abundance of p53 transcripts although analysis of p53 needs to be performed. Therefore, these results suggest SF3b1, Rpl7l1 and Rrp7a may play a role in Bcl-x alternative splicing.

**Figure 11: Bcl-x expression in RNA processing knockdown 8 cell embryos**

A) Bcl-x (L) and (s) isoform evaluation of 8 cell embryos knocked down with dsGFP or RNA processing genes.

### 3.4 Conclusions

Here we report loss of function preimplantation phenotypes when *Sf3b14, Sf3b1, Rpl7l1* and *Rrp7a* are functional abrogated. We report detailed embryonic expression patterns and show that each of these genes is required in the mammalian embryo for the morula to blastocyst transition. Towards functional annotation of these genes, we document for the first time 1) essential developmental requirement of *Sf3b14, Rpl7l1*, and *Rrp7a*; 2) *in vivo* nuclear localization Sf3b14, Sf3b1; nucleolar localization of Rpl7l1; and cytoplasmic localization of Rrp7a; 3) *in vivo* data supporting a role for Sf3b14 and Sf3b1 in splicing; 4) *in vivo* data implicating Rpl7l1 in initiation of Poll II and/or RNA transcription; and 5) activation of p53 dependent apoptosis in the absence of *Sf3b14, Sf3b1, Rpl7l1,* and *Rrp7a* gene function.
CHAPTER 4

BCOR RNAi INCREASES THE RATE OF PHYSIOLOGICALLY NORMAL BLASTOCYST FORMATION

4.1 Introduction

Preimplantation embryo development is a dynamic time of development and differentiation from one cell will arise more than 50 to form the inner cell mass, primitive endoderm and trophectoderm at the blastocyst stage. In an effort to discover novel genes required for this intricate process to occur, a reverse genetic screen was initiated to knockdown individual genes at the one cell stage and evaluate any resultant morphological, epigenetic or differentiation-based phenotypes.

This report documents the initial characterization of Bcor, Bcl6 corepressor, in preimplantation embryo development. Bcor knockdown enhances physiological normal embryo development in murine strains which are known for poor developmental potential. This improvement is also observed in embryos cultured in hyperoxia conditions. We further demonstrate that Bcor gene knockdown in bovine IVF embryos improves the prevalence of blastocysts on day 7 of in-vitro culture which is physiologically normal although no net increase in the overall number of blastocysts is observed. Taken together, Bcor gene knockdown in in-vitro conditions increases the rate of physiologically normal blastocysts.

Bcor was first identified as a corepressor of Bcl6. Bcl6 acts through interactions via its POZ domain which recruit SMRT and NCoR corepressors to bind the SIN3/HDAC complex in order to carry out gene repression/activation. Bcor acts by binding to the POZ domain of Bcl6 and by associating to class 1 and 2 HDACs repressing gene transcription in an epigenetic manner.
Huynh, K 2000). Bcor expression has been observed in the E7.5 ectoplacental cone and is then present throughout the embryo at E9.5. By E11.5, Bcor expression is found throughout developing organs of all three germ lineages (Wamstad, J 2007). Bcor gene functions in B-cell maturation by association with genes of proliferation and apoptosis regulation (Gearhart, MD 2006).

Human Bcor deletion, frameshift and nonsense mutations present as Oculofaciocardiodental disease or Lenz microphthalmia syndrome depending on the mutation. This X-linked affliction initiates during early embryo development affecting eye, face, heart and tooth development and is only observed in females due to presumed male homozygous lethality (Ng, D 2004, Horn, D 2005 and Cai, J 2010). Mouse Bcor gene knockdown during tooth development results in deformities similar to that documented in human mutation demonstrating a requirement for Bcor gene expression in the dental mesenchyme of E14.5 mouse embryos (Cai, J 2010). Functional analysis of human Bcor mutant mesenchymal stem cells in vitro demonstrate increased histone methylation of H3K4 and H3K36 along with increased expression of AP-2α, a target of Bcor gene repression, resulting in continuous tooth root growth (Fan, Z 2009). Bcor knockdown mouse ES cells have reduced efficiency for differentiation from pluripotent stem cells into mesoderm (Wamstad, JA 2008) which is suggestive of a role in the developmental abnormalities reported above.

We document that Bcor gene knockdown in preimplantation stage embryos enhances physiological timing of blastocyst formation in multiple mouse strains as well as in hyperoxia culture conditions. Blastocysts arising from both control and knockdown groups demonstrate inner cell mass and trophectoderm lineage specification and gene expression. Bcor knockdown can also increase the prevalence of physiologically normal blastocyst in bovine IVF embryos.
Taken together, these results suggest \textit{Bcor} gene knockdown increases physiologically normal blastocyst development.

4.2 Materials and Methods

4.2.1 Embryo Production and collection

B6D2F1 females (Jackson Laboratories Stock 100006) BALB females (Jackson Laboratories Stock 000651) DBA females (Jackson Laboratories Stock 000671) received an intra-peritoneal injection of pregnant mare serum gonadotropin (PMSG; 5 IU/animal), followed by stimulation with human chorionic gonadotropin (hcG; 5IU/animal) 48 hours later. Mice were mated with B6D2F1 males. The following morning, females were removed from the stud cages and examined for the presence of a vaginal plug. If a plug was present, embryos (0.5dpc) were collected via dissection of the infundibulum with M2 medium followed by 5 minutes in 0.1% Hyualronidase (Sigma) All animal studies were approved by the Animal Care and Use Committee, University of Massachusetts.

4.2.2 Microinjection

To create the microinjection needle (injector), borosilicate capillary tubes (Sutter Instruments) were pulled with a Sutter p-87 glass puller (Sutter Instruments) creating a fine tapered point. The needle tip was cut to make a 1-2μM diameter opening and the needle was then bent to 15° using a microforge (DeFronbrun). This needle was back filled with Mercury and rinsed with 10% PVP (Sigma) prior to use. The embryo holding glass needle (holder) was created using the same borosilicate capillary tubes and pulled as above, cut at 85μM, fire polished and bent to 15 degrees. Microinjection was performed using a piezo impact-drive injector (PrimeTech) mounted on a Diaphot inverted microscope (Nikon) with Hoffman
Modulation Contrast objectives and condenser. Micromanipulation of embryos was performed using TransferMan NK 2 (Eppendorf,) in M2 medium (Chemicon) with 0.01% PVP (Sigma). Each embryo was injected with about 5pL dsRNA. After all embryos were injected, they were transferred into microdrops of M2 medium for 10 minutes at 37°C before deposition in KSOM medium for extended culture.

4.2.3 Embryo Culture

M2 medium was used for microinjection and embryo extraction. Embryos were cultured in 35mm petri dishes (Falcon) in 30-50μl drops of KSOM (Chemicon) under 3ml light mineral oil (Fisher Scientific) in 5% CO₂, 5%O₂ and 90% N₂ at 37°C in a humidified incubator.

4.2.4 dsRNA Production

For T7-RNA polymerase mediated double stranded RNA (dsRNA) production, murine Bcor, bovine Bcor and Green Fluorescent Protein (GFP) specific PCR primers were designed using 40 base-pair oligos that contained the T7 binding sequences followed by gene specific sequences as follows: GFP TAATACGACTCACTATAGGGCACATGAAGCAGCAGCAGTTT
and TAATACGACTCACTATAGGGCTCTAGGTAGTGTTGTCG; murine Bcor
TAATACGACTCACTATAGGGGCTTGATCCACACCTG
and
TAATACGACTCACTATAGGGGCTGCTGGTGTTGTCG; bovine Bcor
TAATACGACTCACTATAGGGGTCGCTGGTGTTGTCG; dsRNAs were created using the MEGAscript T7 \textit{in vitro} transcription kit (Ambion) followed by DNase treatment (Roche) and purification using NucAway Spin Columns (Ambion) and Phenol/Chloroform
extraction. dsRNAs were re-suspended in Nuclease-Free water (Integrated DNA Technologies) and diluted to 1μg/μL for microinjection. The dsRNA was stored at -80°C until use.

4.2.5 RNA Quantification

The Agilent RNA 6000 Pico kit was used according to manufacturer’s recommendations. RNA was extracted from embryos using the High Pure RNA Isolation Kit (Roche) and resuspended to 1.6 embryo equivalents per microliter (EE/μL). RNA concentration was also assessed by NanoDrop spectrophotometer according to manufacturers instructions.

4.2.6 RNA extraction and RT-PCR

RNA extraction was performed with Roche High Pure RNA Isolation Kit (Roche). cDNA was synthesized with both random hexamers and oligo-dT primers as described previously1.

4.2.7 RT-PCR

RT-PCR was performed using 2 embryonic equilivants per Ruby Taq reactions. Primers used: B-actin forward AGCAAGCAGGAGTACGATGAGT, Reverse ATCCAACCGACTGCTGTCA; bovine Bcor forward AGAAGCCAAAGGCAACAGAA, bovine Bcor reverse GGATGGTTTCTCGCTGTGT; Reactions were placed in “My cycler” thermocyclers using the following thermal profile: 1 cycle of 95°C for 1 minutes, then 33 (for β-actin) 38 (for Bcor) cycles of 30 seconds at 95°C and 30 seconds at 60°C and 30 seconds at 72°C, and a final cycle of 72°C. PCR amplicons were electrophoresed through a 2% agarose gel at 80V for 45 minutes.
using a Thermo gel box. Bands were illuminated using a Syngene gel doc and band intensity was quantified with Gene Tools (Syngene software platform).

4.2.8 Quantitative PCR

Real-time RT-PCR was performed using 0.75 embryonic equivalents with Taqman probe based gene expression assays from Applied Biosystems as follows: 2X Quanta PerfeCTa Supermix Low ROX, 20X Vic-labeled ActB (#4352341E) or GapD (#4352339E), 20X Gene Expression Assays (Oct4 Mm00658129_gH; Tead4 Mm01189836_m1; Bcor Mm00551516_m1) and water to total 20μL. Reactions were run in triplicate on a Stratagene Mx3005p Real-Time PCR machine with a thermal profile of: 1 cycle of 50°C for 30 seconds, 1 cycle of 95°C for 2 minutes, then 40 cycles of 15 seconds at 95°C and 30 seconds at 60°C.

4.2.9 Whole mount embryo immunofluorescence

Performed as described (Griffith, GJ 2011). Primary antibodies were used at the following concentrations: Oct4 sc-5279 Santa Cruz 1:200, Cdx2 AM392-5M BioGenex 1:1000. Secondary antibodies from Molecular Probes were used at a 1:500 concentration: (AlexaFluor 488 Donkey anti-rabbit A21206, AlexaFluor 546 Donkey anti-mouse A10036

4.2.10 Imaging

Digital images of whole mount embryos and sectioned embryos were taken with a Nikon Eclipse TE2000-S inverted fluorescence microscope and QImaging Retiga Exi Fast 1394 camera fitted with a color-slider for use with brightfield images. All slides were imaged with NIS-Elements BR Software.
4.2.11 Bovine IVF

Oocytes, sperm and media sourced from TransOva Genetics (Long Prairie, MN) kindly provided in a joint collaboration, were shipped overnight in an incubator shipper at 38.5°C. After 18 hours post maturation (hpm), embryos were transferred into Fertilization medium along with sperm at a concentration of 1X10⁶ sperm/ml. for 18hrs. Embryos were gently stripped of the cumulus layer by aspiration and transferred into SOFaa for 1 hour before injection. Microinjection was performed using only embryos selected for having a second polar body. dsRNA was prepared at a concentration of 8ug/ul in nuclease free water prior to microinjection. Microinjection was performed in holding medium with injections lasting less than 1 hr for both groups. Embryos were transferred into SOFaa after 2X rinse in SOFaa and cultured in 5% O², 5% CO² and 90%N² until day 8. Media was prepared by TransOva Genetics.

4.3 Results and Discussion

4.3.1 Bcor gene knockdown improves the rate of physiologically normal blastocyst

Single cell B6D2F1 embryos were injected with dsRNA specific for dsGFP or dsBcor (from now on called dsGFP and dsBcor) followed up to day 4 of development (Figure 12 A-B). The initial RNAi screen identified Bcor as a failure to make a blastocyst yet upon further investigation, this proved not to be repeatable (data not shown). While performing the injection experiments testing the failure to make an outgrowth, we noticed an increase in the amount of dsBcor embryos making blastocysts 72 hrs post injection (Figure 12 G). To evaluate if this was a mouse background specific result, we injected dsGFP and dsBcor in the BALB and DBA mouse strains (Figure 12 C-D) and observed the same trend in the BALB background (Figure 12 H). Performing the same experiment using the DBA mouse strain yielded an increase in day
4 blasts which although is not physiologically normal in murine embryo culture, is a marked improvement in DBA blast development. To ensure gene knockdown was successful, we evaluated \textit{Bcor} gene expression using qRT-PCR (Figure 12 K) with 8 cell dsGFP and ds\textit{Bcor} embryos. Gene knockdown was consistently ~20% of dsGFP expression which demonstrating good gene knockdown. Temporal expression of \textit{Bcor} throughout preimplantation embryo development shows a dramatic increase at the 8 cell stage suggesting possible function at this step in preimplantation embryo development (Figure 12 J). We also note there is no morphological difference observed at this stage with reduced levels of \textit{Bcor}.
Figure 12: *Bcor* RNAi knockdown in murine embryo development

B6D2F1 (A-B), BALB (C-D) and DBA (E-F) dsGFP and dsBcor knockdown embryo development from 24 to 96 hrs post injection. The overall blastocyst rate of 2 cell embryos for B6D2F1, BALB and DBA (G, H and I respectively) demonstrate a significant difference in blastocysts being produced on day 3.5 which is physiologically normal in murine. Temporal *Bcor* gene expression shows increased abundance at the 8 cell stage (J). Representative gene knockdown evaluated at the 8 cell stage using qRT-PCR (K). Expression is normalized by GAPDH and to 1 embryo equivalence.
Although we are able to produce more physiologically normal blastocysts, we need to determine if these blastocysts are the same quality as in our control group. To assess the quality of dsBcor embryos, we decided to evaluate lineage specification, the first cellular differentiation event whereby an inner cell mass (ICM) and a trophectoderm layer (TE) are formed expressing mutually exclusive markers. Here, we compared OCT4 (ICM) and CDX2 (TE) immunofluorescence (Figure 13 A-F) with Oct4 (ICM) and TEAD4 (TE) qRT-PCR (Figure 13 G) for B6D2F1, BALB and DBA background. OCT4 and CDX2 demonstrate successful lineage specification based on mutually exclusive location of fluorescence. As observed in groups A-F, there are no instances of cells expressing both Oct4 and Cdx2 therefore lineage specification has occurred in every group. Based on qRT-PCR, we determine that although there are some groups with significant differences in gene expression, this observed difference could be due to a change in cell number in the ICM or TE. As the samples were normalized to 0.75 embryonic equivalence, a slight change in cell number could alter these results. Interestingly enough, the changes in gene expression seem to favor pluripotency though more experiments should be done to verify this claim.
**Figure 13: Lineage specification in dsGFP and dsBcor embryos**

B6D2F1, BALB and DBA embryo whole mount immunofluorescence (A-B, C-D and E-F respectively) of OCT4 in red as an ICM marker, CDX2 in green as a TE marker and DAPI nuclear stain. Based on mutually exclusive expression, lineage specification has occurred. qRT-PCR of Oct4 (ICM) and Tead4 (TE) show expression of both markers of lineage specification being produced (G).
4.3.3 Improved embryo development on day 7 of Bovine IVF

Having determined that physiologically normal embryo development in the mouse is improved with $B\text{cor}$ knockdown and the resulting blastocysts make ICM and TE with mutually exclusive markers, we were curious on the practical application of this finding. Since the embryos which were improved were cultured $\textit{invitro}$ we decided to evaluate bovine IVF as a commercial application of this approach as bovine embryo development is between 32 and 50%. We made bovine specific dsBcor, injected it into 1 cell bovine embryos and followed them through development. (Figure 14 C-C”). Development to blastocyst was compared to a control group which was not injected and to dsGFP injected embryos (Figure 4 A-A” and B-B” respectively). Knockdown of $B\text{cor}$ gene expression was confirmed by RT-PCR and normalized to bovine $\beta$-Actin (Figure 14 D). As was seen in the murine data, the presence of day 7 blastocysts was significantly improved in dsBcor embryos compared to dsGFP (Figure 14 E). Although there was no significant difference observed between control and dsBcor in terms of development overall, this could be due to the low number of embryos used in this comparison. Temporal $B\text{cor}$ gene expression between 2 cell and the blastocyst stage (Figure 14 D) display the same pattern as seen in the mouse (Figure 12 J) which may suggest a similar role in bovine as in mouse.
Control IVF embryos which were not injected were evaluated on day 4, 7 and 8 (A-A’’) resulting in the production of blastocysts by day 7. dsGFP and dsBcor injected bovine embryos were also evaluated on day 4, 7 and 8 (B-B’’ and C-C’’ respectively). Temporal expression of bovine Bcor in preimplantation development mirrors that observed in the murine (D). Overall blastocyst rate from all 3 groups result in no overall increase in the number of blastocysts but does result in an increase of physiologically normal blastocysts on day 7 (E). Bovine Bcor knockdown evaluated by RT-PCR and densitometer quantification (F).
4.4 Discussion of results and conclusions

Efforts to improve *in-vitro* embryo development outcomes have focused on selection of embryos for transfer based on timing (Sugimura, S 2012, Isom, SC 2012, Reijo Pera, RA 2011) additives in embryo culture medium (Nquyen, NT 2011, Lott, WM 2011, Maalouf, WE 2009) or novel culture conditions (Walter, EM 2004). Recently there have been reports of specific gene RNAi improving embryo culture blastocyst rates or physiological timing. There have also been studies to determine gene function using RNAi (Matoba, S 2011, Maserati, M 2011, Kawamura Y 2010).

Here we investigate a gene which when reduced in embryo development, improves the rate of blastocyst at the correct physiological time and stage; the same time as if it were cultured *in-vivo*. This observed improvement encompasses multiple mouse strains, oxygen concentration differences and species resulting in normal blastocysts which undergo lineage specification.

When evaluating outgrowths in the initial screen, we determined *Bcor* gene knockdown does continue at least up to 4 days past blastocyst (data not shown). Furthermore, since *Bcor* has been shown to interact with Polycomb group genes and alter histone methylation (Gearhart, MD 2006), the epigenetic impact of *Bcor* knockdown during this dynamic time in development must be investigated. To continue this work, one will need to produce ds*Bcor* embryos for transfer to generate live pups or calves as well as investigate the long term consequences, if any, of increased histone methylation on live births.

Based on this work we can speculate to some degree on what function Bcor performs in the developing embryo. In Wamstad, JA 2008, stem cells differentiated into a mesodermal lineage less efficiently in the absence of Bcor. Furthermore, Bcor is known to act as a repressor
of gene activity working in tandem with Bcl-6 during B-cell maturation. Therefore it is possible
Bcor functions as a repressor of gene function in preimplantation embryo development acting in
a way to ensure embryos which make blastocyst are well suited to continue in development
resulting in offspring having a higher chance of survival.

Depending on species, there are different approaches to guaranteeing survival of
offspring to the next generation. One approach used by insects produce vast quantities of young
without protection leaving only 1-2% of young to survive into adulthood. Mathematically, this
works as 1-2% of thousands still are hundreds which is enough to repopulate the niche they hold.
Mammals use another approach which puts more emphasis on the few live offspring which are
born, ensuring that a much higher percent live into adulthood and here is where I think Bcor fits
into the larger picture. Bcor gene repression helps to weed out weaker embryos ensuring those
which reach the blastocyst stage are the most robust of the group ensuring stronger and more
robust offspring.

Further evidence of this theory is increased housekeeping gene expression in Bcor
knockdown embryos as observed with B-actin and GAPDH. Increased gene expression can lead
to increased rate of cell cycle resulting in a higher cell number at the blastocyst stage. By
ensuring that only the best embryos survive, Bcor confers selective pressure on embryo
development, much like that observed in oogenesis the ovary. Since in-vitro techniques can
reduce this selection pressure but transferring only 1 excellent quality embryo per recipient, the
presence of Bcor in pre-implantation embryo development is not essential.

The improvement in embryo development observed in high oxygen cultures of Bcor
knockdown embryos could also work in hand to reduce the amount of reactive oxygen species
(ROS) damage. By reducing the rate of cell cycle in high oxygen cultures, the embryo reduces the amount of DNA exposed to ROS, reducing double strand breaks and the overall possibility of mutation. In this case, Bcor acts protectively to ensure offspring survival and reduce the rate of mutation. If these embryos were in-vivo and elevated amounts of oxygen can be used as an indication of stress, perhaps Bcor acts to slow or halt pregnancies when the mother is not in the best condition to be pregnant. In this case, Bcor would work to evaluate the mother’s condition as an indication of external environmental factors signaling the embryo to grow or to halt. Perhaps Bcor is involved with cases of spontaneous abortions.

Commercialization of Bcor can benefit many industries such as human and animal assisted reproductive services. A modest improvement of embryo development would be welcome in all areas of animal reproduction and financially lucrative.

In order to commercialize this technology, I proposed a 3 phase approach which would maintain gene knockdown as well as add temporal control. In the first phase, I would re-evaluate the results from the initial 400 genes and retest those genes which demonstrated no gene knockdown phenotype. All the individual genes would be knocked down individually in the B6D2F1 mouse strain and cultured in elevated oxygen as in these embryos it is easier to observe the embryo recovery and is most cost effective. Ones which demonstrate a significant increase in embryo development will be flagged for further evaluation with Bcor in phase 2.

Once these genes have been identified, we would like to test gene knockdown in different combinations and individually to determine if there is indeed an additive effect. The best combination of gene knockdown will then proceed to the next phase.
Phase 3 will require a biochemical partner, preferably a major pharmaceutical to identify combinations of small molecule inhibitors which can be added to the culture medium for improved development. These molecules will directly inhibit protein function for so long as the embryos are in the medium. Once the embryos are removed gene function can return.

I imaging the pharmaceutical will then proceed to use this proprietary mix of small molecule inhibitors to improve embryo culture medium used in human IVF clinics and in animal reproductive technologies. There is a high probability one or more large pharmaceuticals would finance the second and third phase for the exclusive rights to develop the technology for the clinic as this is a possible new direction for many pharmaceuticals in new drug development.

In order to begin this project, I propose the completion of the Bcor project in collaboration with InvitroBrasil in Mogi Mirim Brazil. Though this location lacks many of the essential tools required for manufacturing dsRNAs, the Mager lab contains all required tools and reagents. The major benefit of using Mogi Mirim is the volume of IVF embryos produced and the lower cost of embryo transfer and maintaining embryos. Furthermore, there is another application which would benefit from Bcor gene knockdown. Horse SCNT and ICSI are highly inefficient because culture conditions have not been optimized for this species. Using Bcor knockdown in addition to SCNT might increase the rate of physiological normal blastocysts in sub optimal culture media and allow more embryos to be transferred. Though different dsRNAs will have to be made, this is a good application for the temporary Bcor gene knockdown.
APPENDIX A

ADDITIONAL EXPERIMENTS AND FIGURES FOR CHAPTER 2
A.1. Testing Embryo Paraffin Embedding Protocol

Figure A 1: Sequential sections in embedded embryos in NIH3T3 cells. (Chapter 3)
A.2. Testing Embryo Paraffin Embedding Protocol Using OCT4 and CDX2 Antibodies

with OCT4 Knockdown Embryos

Figure A 2: Sequential sections of embedded Oct4 knockdown embryos in NIH3T3 cells.

(Chapter 3). Demonstrates observable gene knockdown in embryos.
A.3. Testing Pyronin Y Staining Protocol 1

RNA relative abundance via Pyronin Y (PY) / Hoechst (HXT) staining

**Experiment 3**: dsGFP and dsWdr74 8 cell embryos

Staining conditions depending on group:
- 10 minutes in 4% PFA
- 10 minutes in HXT
- 10 minutes in PY
- 5 Rinses in PBS-PVP
- Evaluation in drops under oil

![Image of stained embryos](image)

**Figure A 3**: Relative quantification of RNA in 8 cell embryos based on Pyronin Y staining.
RNA relative abundance via Pyronin Y (PY) / Hoechst (HXT) staining

**Experiment 4:** dsGFP and dsWdr74 morula stage embryos

Staining conditions:
→ 10 minutes in 4% PFA
→ 10 minutes in HXT
→ 10 minutes in PY
→ 3 X Rinses of ~300μl in PBS-PVP in depression glass
→ Evaluation in VectaShield at 40X

<table>
<thead>
<tr>
<th>dsGFP Morula</th>
<th>dsWdr74 Morula</th>
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<td>HXT 40X</td>
<td>HXT 40X</td>
</tr>
<tr>
<td>PY 40X</td>
<td>PY 40X</td>
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Figure A 4: Relative quantification of RNA in 8 cell control and Wdr74 gene knockdown embryos based on Pyronin Y staining demonstrating RNA reduction.
APPENDIX B

ADDITIONAL EXPERIMENTS AND FIGURES FOR CHAPTER 3
B.1. 5-MEC staining of 2 cell embryos injected with dsGFP or dsSF3b14

Figure B1: Embryos were stained with anti-5-Mec to determine methylation status as a previous experiment from Dr Xiangpeng Dai demonstrated a difference. As seen above, no difference was observed upon repeating the experiment.
B.2. Antibody Testing SF#B14 from ABGENT in Fixed/Sectioned NIH3T3 Cells

NIH3T3 cells embedded in paraffin
SF3b14 (AbGent) 1:200 overnight
Oct4 (Abcam) 1:200 overnight

Figure B 2: Initial test of AbGent SF3b14 antibody used in the publication. Using a known Oct4 antibody, we determined the SF3b14 antibody is very specific and is localized to the nucleus.
B.3. Additional Pictures of RPL7LI Knockdown Staining

Knockdown evaluation Rpl7l1
100X 8 cell embryos

dsGFP

dsRpl7l1

Figure B 3: Embedded and sectioned embryos were stained with anti Rpl7l1 (Chapter 3)
B.4. Additional Pictures of RRP7A Knockdown Staining

Figure B 4: Embedded and sectioned embryos were stained with anti Rrp7a (Chapter 3)
B.5. Additional Pictures of SF3B1 Knockdown Evaluation

Knockdown evaluation SF3b1
100X 8 cell embryos

dsGFP

dsSF3b1

Figure B 5: Embedded and sectioned embryos were stained with anti Sf3b1 (Chapter 3)

B 6: Embryo development in simultaneous RNA processing gene and p53 gene knockdown demonstrating partial blastocyst formation recovery.
B.7. Gene Knockdown Results of DGCR8 and YY1

09.09.11 injection dsGFP, dsDgcr8, dsYY1 and dsDgcr8/YY1 combo results:

<table>
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<th>Day 1</th>
<th>Day 2 (4)</th>
<th>Day 2 (8)</th>
<th>Day 3 (M)</th>
<th>Day 3 (BL)</th>
<th>Day 4 (M)</th>
<th>Day 4 (BL)</th>
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<th>% Blast D4</th>
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<td>15</td>
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<td>9</td>
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<td>14</td>
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Figure B 7: Gene knockdown of YY1, DGCR8 and a combination to determine if YY1 function in embryo development is regulated by microRNAs.
C.1. OXYGEN Knockdown of BCOR in Different Mouse Strains in 5% Oxygen

Figure C.1: Gene knockdown of different mouse strains at the once cell stage on development to the blastocyst stage. Those strains without error bars were tested one time. Though there are no error bars the visual increase in number of blastocysts suggest a trend similar to the statistically significant change observed in DBA and BALB mice.
C.2. Knockdown of BCOR in Different Mouse Strains in Elevated Oxygen

Figure C.2: Gene knockdown of different mouse strains at the once cell stage on development to the blastocyst stage. Those strains without error bars were tested one time.
C.3. Embryo Developmental Images of BCOR Knockdown in DBA Embryos

Figure C.3: DBA embryos development of bcor gene knockdown. 20X
C.4. Embryo Developmental Images of BCOR Knockdown in Balb Embryos

02.01.11 dsGFP vs dsBcor in “Poor Culture” BALB embryos

\[
\begin{align*}
\text{dsGFP} & \quad \text{dsBcor} \\
\text{Day 2 20X} & \\
\text{Day 3 20X} & \\
\text{Day 4 20X} & \\
\text{Day 5 20X} & \\
\%\text{Blast} & 2/7 \text{ or } 29\% \\
& 6/8 \text{ or } 75\%
\end{align*}
\]

Figure C.4: BALB embryos development of bcor gene knockdown. 20X
C.5. Endogenous Expression of Murine BCOR in Oocytes

Figure C.5: Expression of Bcor in B6D2F1 and DBA oocytes normalized to 1 embryonic equilivance. The results suggest there are
C.6. Embryo Development of Bovine IVF Embryos Injected with Bovine DSBCOR

Embryo Development
Fert 24hpm; Oocytes were incubated with sperm for 20hrs

<table>
<thead>
<tr>
<th>Fert date</th>
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<th>Groups</th>
<th>Injected</th>
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<th>Day 7 Morula</th>
<th>Day 7 Blast</th>
<th>Day 8 Morula</th>
<th>Day 8 Blast</th>
<th>Early pm Morula</th>
<th>Early pm Blast</th>
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<td>0</td>
<td>11</td>
<td>21%</td>
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dsGFP late day 8

ds4C03 late day 8

Figure C.6: The first set of experiments showing no developmental differences. IVF medium, supplies and protocols kindly donated by Hong Wei of Transova. Images are of day 8 embryos taken at 20X.
C.7. Gene Knockdown of Bovine BCOR Assessment at the 1 cell and 2 cell stage

Knockdown evaluation at 2 cell
4C03 expression normalized to B-Actin

Result: No gene knockdown detected

Figure C 7: In figure C5, there were no observed developmental differences. Here we show that although we injected dsBcor into these one cell oocytes, knockdown was not observed at the one cell or 2 cell stage. For the next experiments we injected at a concentration of 8ug/ul. Densiometer results normalized by bovine β-actin.
C.8. Gene Knockdown of Bovine BCOR Assessment at Blastocyst

Knockdown evaluation at late day 8 blastocyst
100usec exposure

![Image of 4C03 expression in day 8 blastocysts]

Result: No gene knockdown detected

Figure C.8: In figure C5, there were no observed developmental differences. Here we show that although we injected dsBcor into these one cell oocytes, knockdown was not observed at the blastocyst stage. For the next series of experiments we detected Bcor gene knockdown we injected dsBcor at a concentration of 8ug/ul. Densitometer results normalized to bovine β-actin.
C.9. Early BCOR Characterization Data: Endogenous

Preliminary Data:
qRT-PCR panel of Bcor \textit{in vivo} expression

Preliminary Data:
qRT-PCR panel of Bcor \textit{in vivo} expression

Figure C.9: Bcor expression between MII and Blastocyst stage embryos was evaluated via qRT-PCR relative to 0.75 Embryo Equivalence or GAPDH as observed below. Based on EE evaluation, Bcor expression peaks at the 2-cell and blastocyst stage.
C.10. Early BCOR Characterization Data: Knockdown Evaluation

Preliminary Data:
qRT-PCR validation of Bcor knockdown expression relative to GAPDH

- Knockdown is observed at the 8 cell stage
- Knockdown is still present after 4 days in outgrowth culture

Figure C.10: Gene knockdown observed at the 8-cell, blastocyst and day 4 of outgrowth.

Knockdown at the blastocyst stage looks significantly reduced and may be due to embryo to embryo variation than actual results.
C.11. Early BCOR Characterization Data: Development Data

Figure C.11: Temporary Knockdown of Bcor gene expression rescues embryo development in “poor embryo culture” experiments in DBA. More experiments later show significant rescue of blastocyst in BALB in later experiments.
C.12. Early BCOR Characterization Data: Lineage Specification

Figure C. 12: Lineage Day 5 B6D2F1 and BALB dsBcor and dsGFP embryos maintain lineage specification to ICM and TE.
Figure C.13: Day 5 DBA dsBcor and dsGFP embryos maintain lineage specification to ICM and TE.

Figure C.14: qRT-PCR of blastocysts selected from each group matched based on relative appearance. The letter A embryo from the dsGFP group looked similar to letter A from the dsBcor group ect. They were frozen individually and assessed for relative Bcor, Oct-4 (ICM) and Tead-4(TE) expression.
C.15. Early BCOR Characterization Data: Lineage Specification QRT PCR of BALB Embryos

Figure C.15: qRT-PCR of blastocysts selected from each group matched based on relative appearance. The letter A embryo from the dsGFP group looked similar to letter A from the dsBcor group ect. They were frozen individually and assessed for relative Bcor, Oct-4 (ICM) and Tead-4(TE) expression.
APPENDIX D

PROTOCOLS

D.1. Bacterial Transformation

Introduction:

Transformation is the introduction of a plasmid into bacteria. Here, we use chemically competent bacteria, which allow plasmids to enter the bacterium when heat shocked.

Chemicals/Equipment:

<table>
<thead>
<tr>
<th>Component</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>DH5α Competent bacteria</td>
<td>Made here (-80)</td>
</tr>
<tr>
<td>100mm LB-Agar plate</td>
<td>Made here (+4)</td>
</tr>
<tr>
<td>Autoclaved Glass beads</td>
<td>Made here</td>
</tr>
<tr>
<td>Water bath set to 42°C</td>
<td></td>
</tr>
<tr>
<td>SOC</td>
<td>Made here</td>
</tr>
<tr>
<td>Shaker incubator set at 37°C</td>
<td></td>
</tr>
<tr>
<td></td>
<td>and 250RPM</td>
</tr>
</tbody>
</table>

Procedure:

1. Thaw DH5α Competent cells on ice.

2. Add 1-2µl of appropriate plasmid and place back on ice for 15 minutes. The control cells receive NF-H₂O

3. Heat shock the cells by placing in the 42°C water bath for 30 seconds.

4. Put onto ice immediately.

5. Add 200µl SOC (if no SOC media, you can use LB broth containing no antibiotics) and place in the 37°C shaking incubator for one hour.

6. Make certain of the antibiotic resistance in your plasmid and use the appropriate plate. Plate out 20-50µl onto one LB using glass beads. Put glass beads onto a plate along with plasmid/competent cells and shake the plate back and forth to distribute evenly. Check your plasmid for the antibiotic needed, and make sure we have plates made. NOTE: If this is for a ligation reaction you can plate out all 200µl. Be sure to always include a “control” group (no DNA added).

7. Leave in 37°C oven overnight. Do not overgrow plates; this leads to satellite colony formation (which does not contain your plasmid).
8. Pick individual colonies with a pipette tip and put into tube of 3-5mL LB containing 100 μg/mL of antibiotic and shake at 37°C overnight. Again, do not grow for more than 18 hours otherwise you will have overgrown bacteria some of which will not contain your plasmid. Proceed to miniprep protocol or place in refrigerator.

D.2 Mini-Prep STET Protocol

STET is a quick protocol for isolation of plasmid DNA. Although the resultant DNA is not as clean as if isolated using the Qiagen Mini-prep kit, it is quite efficient and inexpensive. This protocol should be used in all plasmid identification experiments.

<table>
<thead>
<tr>
<th>Component</th>
<th>Company</th>
<th>Catalog #</th>
</tr>
</thead>
<tbody>
<tr>
<td>STET</td>
<td>Made here</td>
<td></td>
</tr>
<tr>
<td>NF-H2O</td>
<td>IDT</td>
<td></td>
</tr>
<tr>
<td>Lysosome (10mg/ml) in MilliQ H2O</td>
<td>Thermo Scientific</td>
<td>89833</td>
</tr>
<tr>
<td>RNase (1mg/ml) in MilliQ H2O</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.5ml Eppendorf tubes, Autoclaved</td>
<td>Axygen</td>
<td>MCT-150-C</td>
</tr>
<tr>
<td>PCR tubes (0.2ml capacity)</td>
<td>Axygen</td>
<td>PCR-0208-FCPC</td>
</tr>
<tr>
<td>Thermo-cycler or 37°C water bath</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100% Isopropanol (Kept at -20°C)</td>
<td>Fisher</td>
<td>BP2632-4</td>
</tr>
<tr>
<td>70% EtOH in MilliQ H2O</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1. Add ~1.4 ml liquid culture into a 1.5ml Eppendorf tube. Spin at 5000 RPM for 2 minutes. Aspirate the clarified LB medium and repeat if low yields are expected.

2. Make STET Master Mix; add 250ul to each pelleted sample and vortex to resuspend.

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount/Sample</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>STET Buffer</td>
<td>200μl</td>
<td>miniprep shelf</td>
</tr>
<tr>
<td>Lysosome (10mg/ml)</td>
<td>40μl</td>
<td>Green Rnase box, backup in bottom of F1</td>
</tr>
<tr>
<td>RNase A (1mg/ml)</td>
<td>10μl</td>
<td>Green Rnase box</td>
</tr>
</tbody>
</table>


4. Remove pellet with a toothpick and add 160μl of cold Iso-propanol; invert 3 times.

5. Incubate 10 min. at -20°C and then centrifuge 10 min at full speed.

6. Discard supernatant and wash with 300μl 70% EtOH and centrifuge for 5 minutes at 5000 RPM.
7. Dry pellet completely (~20 min) and resuspend in 30µl of NF- H₂O.

8. Nanodrop the re-suspended sample to get the DNA concentration and purity.

D.3 Restriction Digest Protocol

A restriction digest is used to cut specific sequences. Particular restriction enzymes recognize unique DNA sequences which aid in molecular cloning.

<table>
<thead>
<tr>
<th>Component</th>
<th>Company</th>
<th>Catalog #</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer 10X</td>
<td>NEB</td>
<td></td>
</tr>
<tr>
<td>Sterile H₂O</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BSA 10mg/ml (100X)</td>
<td>NEB</td>
<td>B9001S</td>
</tr>
<tr>
<td>1.5ml eppendorf tubes, Autoclaved</td>
<td>Axygen</td>
<td>MCT-150-C</td>
</tr>
<tr>
<td>PCR tubes (0.2ml capacity)</td>
<td>Axygen</td>
<td>PCR-0208-FCP-C</td>
</tr>
<tr>
<td>37°C water bath</td>
<td>Made here</td>
<td></td>
</tr>
<tr>
<td>1% Agarose Gel</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gel Electrophoresis chamber</td>
<td></td>
<td>In Mager lab</td>
</tr>
<tr>
<td>UV imaging</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Bluescript plasmid map.
Introductory notes:

1. Restriction enzymes are supplied in Units. 1U is defined as the amount needed to completely digest 1µg of DNA in one hour.

2. Always keep enzymes on ice. The activity of these expensive enzymes will diminish quickly if allowed to alternate between -20°C and Room Temperature.

3. All samples which use 2 enzymes must be run with the single cut as well for troubleshooting purposes. For example, if using HindIII and EcoRI on the same sample, cut once with HindIII, once with EcoRI, once with both and then uncut. All 4 will be run individually to ensure each enzyme cuts as expected. Always run uncut plasmid DNA as a control.

4. Restriction endonucleases (RE) have different levels of cutting activity depending on the 10X buffer used. For example, HindIII has 100% activity in buffer 2 and 50% in buffers 1 and 4. If HindIII is used in buffer 3, it will only display 10% activity. Choose your buffers carefully using the attached double digest chart.

D.4 Analytical Restriction Digest

a. Once the best buffers/enzyme match has been chosen, make a master mix. Each sample will be digested in 10 ul total.

<table>
<thead>
<tr>
<th>Per Sample</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>10X buffer</td>
<td>1.0 ul</td>
</tr>
<tr>
<td>10X BSA*</td>
<td>1.0 ul</td>
</tr>
<tr>
<td>enzyme^</td>
<td>0.5 ul</td>
</tr>
</tbody>
</table>

add the appropriate amount of water to 9.0 ul

* BSA is optional depending upon the enzyme used.

^ it may be more appropriate to add your enzyme after making a mastermix of the other listed components.

2. Carefully label eppendorf tubes with the sample number and enzyme to be used.

3. Add 9µl of the master mix to each tube.

4. Add 1µl DNA (at least 200ng/ul, if less than you need to use more DNA and adjust the mastermix).

5. Incubate the samples for 15 minutes-1hour at their optimal activity temperature. Usually this is 37°C but some enzymes work better at other temperatures (SmaI 25°C)
6. Run samples on a 1% Agarose gel (gel electrophoresis protocol) to observe plasmid fragment size.

D.5 Hemacytometer (Cell Counting)

Resuspended cells can be counted for exact seeding densities. Hemacytometers use a fine grid etched in the bottom of a glass/quartz chamber to count cells within 1cc.

Procedure:

1. Resuspend cells into a known volume
2. Place the quartz coverglass over the counting grid. Be certain to cover the two edges which seal the coverglass to the grid when the sample is loaded.
3. Add exactly 10ul or a well mixed sample to the notch of the hemacytometer.
4. Let the cells settle and count the center grid. **NOTE: Do not count cells over the left and upper most border.**
5. Multiply that number by $10^4$ to calculate the number of cells/ml.
6. Multiply by the known volume. This is the total amount of cells you have in suspension.

For example: If one counts 64 cells in the center grid, that equals $(64)(10^4)$ or 640,000 cells/ml. If you have resuspended your cells in 10ml, you have $(6.4X10^5)(10)$ or $6.4X10^6$ total cells.

D.6 Streaking Bacteria on Plates

Bacteria are streaked out on LB-agar plates to isolate single colonies.
Component | Company | Catalog #
--- | --- | ---
LB-Agar plates | Made here; stored at 4°C | 
p200 pipettor or bacterial inoculation loop | 
p200 tips | 
37°C dry incubator (on floor in rm 301) | 

Before you begin:

1. Always perform bacteria/plasmid based protocols in the designated areas.

2. Wear gloves and practice sterile technique.

Procedure:

1. Investigate the plasmid transformed into the bacteria stock you will be streaking out. Ensure the antibiotic resistance of the plasmid matches the antibiotic added to the LB-agar plate.

2. Label the LB-Agar plate with:
   a. Plasmid # (for example) \( \rightarrow \) #1
   b. Plasmid name FUG-Exm2
   c. Your initials MM
   d. The date 09.18.09

3. Find the frozen bacterial stock located in the -80°C freezer. Quickly scrape a visible quantity onto your p200 tip and gently scratch the top of the LB-Agar making as slight impression as possible. **Return stock to -80°C ASAP.** An inoculation loop/flame may be used as well flaming and cooling before extending a new streak.

4. Eject your tip, attach a fresh tip and proceed to streak once over the first area and continue the streak line to a sterile area of the plate.

5. Continue this cycle 3-4 times, streaking only once through the previously streaked area and continuing through to a sterile area.

6. Place the streaked LB-Agar plate inverted in the 37°C bacterial incubator overnight or for a minimum of 12 hours; until colonies appear.

7. Store the bacterial plate, inverted/wrapped in parafilm at 4°C for up to 1 month.

**D.7 Immunofluorescence of Pre-Implantation Stage Embryos**
Procedure:

1. **Fixation**: 4% PFA for 30 minutes at room temperature. Depression wells
2. **Wash embryos 3X in PBT.** 2-3 min/well Depression wells
3. **Permeabilization**: Place embryos into 0.5% Triton X-100 PBS 20-30 min Depression wells
4. **Wash embryos 3X in PBT.** 2-3 min/well Depression wells
5. **Block**: 2% BSA in PBS for 1 hr at RT Microdrop dish
6. **First Antibody**: 1 hr at RT or 4oC overnight. Antibody added to 2% Microdrop dish BSA in prescribed ratio. Embryos aspirated through 3 drops.
7. **Wash embryos 3X in PBT.** 2-3 min/well. Depression wells
8. **Second Antibody**: 1 hr at RT. Antibody and 1:100 DAPI added to 2% Microdrop dish BSA in prescribed ratio. Embryos aspirated through 3 drops.
9. **Wash embryos 3X in PBT.** 2-3 min/well. Depression wells
10. **Mount embryos** in 2.5uL water and cover with number 1 coverglass. Glass Slide

NOTE: Add sufficient Vaseline to prevent embryo flattening.
Mount embryos in VectaShield if imaging with the ApoTome.
Seal the edges of the slide with nail polish.
Store at 4°C, image ASAP!

**Media:**

**4% PFA:**

Add 4g PFA to 100ml PBS.
Heat to 56°C until in solution.
Aliquot 1ml/1.5ml eppendorf tube.
Store at -20°C until use.
**0.5% Triton X-100:**

Add 25uL Triton X 100 to 5 ml PBS

Gently rock for 20 minutes until in solution.

Make and use the same day; can be stored in the dark overnight.

**0.1% Tween-20 PBS (PBT):**

100ul Tween-20 into 100 ml PBS w/o Ca Mg

Mix well.

Store at RT

**2% BSA Block and antibody solution.**

Add 2g BSA over 100ml PBS.

Do not mix. Allow BSA to fall into PBS ~20min

Aliquot 1 ml into 1.5ml eppendorf tubes  Store at -20°C
D.8 Making Glass Injection Tools

These tools are used to inject dsRNA and other liquid based molecules into the cytoplasm of 1 cell embryos. These tools can also be adapted to be used for PN injection of DNA.

<table>
<thead>
<tr>
<th>Component</th>
<th>Company</th>
<th>Catalog #</th>
</tr>
</thead>
<tbody>
<tr>
<td>Borosilicate glass</td>
<td>Sutter</td>
<td>B100-75-100</td>
</tr>
<tr>
<td>Micropipet puller</td>
<td>Sutter</td>
<td>P-97</td>
</tr>
<tr>
<td>Microforge</td>
<td>Defronbrane</td>
<td></td>
</tr>
<tr>
<td>Mercury/elemental</td>
<td>EHS (recycled 3X distilled)</td>
<td></td>
</tr>
</tbody>
</table>

Before you begin:

1. Ask permission of the Fisore lab to use the
2. Wear nitrile gloves and handle mercury within the chemical safety hood.

Procedure:

1. Pull the pipets
   a. Insert a single pipet into the P-97 puller making sure both sides are equal in distance apart. There are black marks to align the needle.
   b. Load program 95 or 79 (same) and press the green button. The parameters for this code are “Heat 790, Pull 50, Vel 45, Time 210”
   c. The pipet is then removed and is ready for cutting.
2. Cut and bend the pipet
   a. The pulled glass pipet is loaded onto the metal spring loaded holder mounted on the right side of the light source.
   b. Using the size chart located on the front right of the DeFrombrune microforge machine, bring the needle into position using the adjustment knobs on the left and right of the light source.
   c. The heating element (platinum wire with a glass bead) should be located over the glass pipet which is coming in from the left through the oculars. In reality, the image through the oculars is inverted so with your eyes looking at the heating element directly, the needle should come from the right and be over the heating element.
   d. For injection needles the pulled pipet is cut at ~1-2 uM in diameter. This area though difficult to measure under 10X is easily seen as the first area behind the point that has a green sheen on the glass. Set the heat to ~7 and lower the glass to touch the pipet. Immediately turn off the heat and the needle will break in a perfect cross section.
e. Push the needle forward 1-1.5 cm using your finger and heat the element to bend the point up (observed thought the oculars). The needle is finished and is ready for Mercury and use.

f. Mercury is located in the chemical fume hood in the Fissore lab. Wearing Nitrile gloves (not latex) aspirate a small amount of mercury in the 1cc syringe with the spinal needle attached. Place no more than 1 cm mercury in the needle towards the large open end. You want the mercury to connect to the water or oil in the cell tram oil without air bubbles. Put the needle into a covered container for immediate use.

g. Discard in Mercury waste located in the Fissore lab fume hood.

h. For holding needles, measure for ~100uM and cut as above in step “d”. Fire polish tip to an opening of ~ 60uM. This is done by inverting the tip to be located directly over the heating element. Activate the heat and watch the tip of the holder begin to close. Using the measuring device in the oculars, stop the heat when the tip opening approaches 60uM. Return the needle to the horizontal position push the tip forward 1-1.5 cm. Bend the needle to 15%.

i. Discard holder in sharps container after use.

j. Store in a closed container at RT.

D.9 Embryo Injection of DSRNA

This protocol describes all events which need to take place for microinjection of dsRNA in 1 cell oocytes.

dsRNA

1. dsRNA is designed using the PRIMER 3 program using unique 21mer sequences to that gene. Searching the known genome of the species to be used should be done prior to making dsRNA.

2. Send sequences to IDT complete with the T7 primer attached.

3. Do a PCR using cDNA from the species of interest.

   a. PCR:

      i. Add 25 µl Ruby Taq, 1.25 µl of each primer (forward and reverse) as well as 5-10 µl cDNA. Fill to a total volume of 25 µl Cycle in MyCycler
Thermocycler to specifications based on the melting temp of the primers used.

As a default, I find 35 cycles at 57-60 degrees Centigrade over 35-45 cycles will produce enough T7DNA template for all injection experiments.

ii. Take 1μl of the unpurified product and run on a gel to verify expected amplicon size. 1% agar gels at 80V for 35-40 minutes will work well.

b. Purification:
   i. Purify the product using the PCR purification kit from Qiagen. After purification, adjust the concentration of the T7DNA to 40 ng/μl.

4. Making dsRNA (In-vitro Transcription)
   a. Using the MEGAscript Kit, add the following to a 0.2 μl PCR tube: 1 μl ATP solution, 1 μl CTP solution, 1 μl GTP solution, 1 μl UTP solution, 1 μl 10 X buffer, 4 μl T7DNA and 1 μl enzyme. Always run a T7GFP control.
   b. Wrap the tubes with parafilm and place them (designed T7DNA and T7GFP control) into an incubator set at 37 degrees C for 16-24 hours. The water bath is not as efficient and can introduce contamination. Do not place tubes on ice after in-vitro transcription!
   c. Run 0.5 μl of the product on a 2% gel to verify the dsRNA size.

5. DNAse treatment:
   a. add 9.5 μl in-vitro transcription product and 0.5 μl DNAse (from kit). Mix by flicking the tube and then place at 37 degrees centigrade for 30 minutes.

6. NucAway spin column purification:
   a. Add 650 μl NF water to each tube. Vortex for 30 seconds and tap the end of the tube on the table to free the air bubbles.
b. Remove the bottom stopped and centrifuge excess liquid into a 2 ml collection tube and spin at 750 g for 2 minutes. The tube is ready for use.

c. Place the column into a 1.5 ml collection tube. Add 20-100 μl sample to top of tube and spin for 750 g for 5 minutes. Your purified sample is located in the collection tube

7. Phenol/Cheroform extraction:
   a. Adjust the volume of the sample to 200 μl with NF-water.
   c. Centrifuge at maximum (~13,000 g) for 10 minutes.
   d. Transfer upper phase to new 1.5 ml eppendorf tube containing 200 μl chloroform. Vortex for 15 seconds.
   e. Centrifuge at maximum (~13,000 g) for 10 minutes.
   f. Transfer 200 μl upper phase to new 1.5 ml eppendorf tube containing 20 μl 3M sodium acetate. Vortex for 15 seconds.
   g. Add 660 μl ice cold 100% EtOH. Place tubes into -20 (4 hrs) or -80 (40 minutes) centigrade freezer.
   h. Centrifuge at maximum (~13,000 g) for 20 minutes.
   i. Rinse pellet with 70% EtOH. Spin for 5 minutes max speed.
   j. Remove supernatant and allow the tubes to dry ironed on a kimwipe for 30 minutes.
   k. Resuspend dsRNA with 10 μl NF-water
   l. Determine concentration of dsRNA using a nanodrop photospectrometer. Adjust the concentration to 1μg/μl. Aliquot 5 μl/tube. Store at -80 degrees centigrade.

8. Injection of 1 cell mouse embryos
a. B6D2F1 mice (B6 X DBA F1 8-12 weeks old) are superovulated with 10 IU PMSG followed by 10 IU hCG 48 hours later and mated with a stud male. Embryos are collected via discetion in M2 medium and transferred to 50 μl microdrops under oil prior to injection.

b. Prepare KSOM microdrops for embryo culture at least 3 hours before beginning the experiment.
   a. Add 7-8 ~25-30ul drops of KSOM into a 35mm petri dish overlayed with 3 ml mineral oil.
   b. Prepare 3 dishes per experiment to ensure enough for each group.

2. Set the needle for microinjection
   a. Once the injection needle has been back loaded with mercury, attach the needle to the piezo pipette holder ensure all air has been removed.
   b. Gently advance the mercury towards the tip while positioned over a 35mm dish containing a microdrop of 10% PVP overlaid with mineral oil.
   c. Under the 4X objective adjust the needle until you see it over the drop and move it into view. Use the PVP drop as a point of origin.
   d. Once the mercury is at the tip, place the needle in the PVP drop and aspirate to fill the tapered aspect of the needle. Leave it overnight.

Day of experiment:

1. Remove the embryos from the mice oviducts.
   a. Prepare the mice by cervical dislocation the plugged mice, open the mice ventrally, and cut out the oviducts.
   b. Remove the embryos by ripping the bulge in the oviducts containing the embryos.
   c. Collect all embryos via a P200 placing them in Hyaluranidase/M2 for 3 minutes at 37 degrees C.
   d. Rinse embryos through sequential M2 drops until there are no cumulus cells; about 5 (30ul) drops.
   e. Rinse embryos through one drop of KSOM and place into another KSOM drop until injection.

2. Arrangement of the plate:
   a. Petri dish: Invert the cover and use this to microinject upon. The reduced side height lends to better clearance of the injector and holder giving one more area for adjustment.
   b. M2/PVP- Add 400ul M2 and ~5uL 10%PVP into a 1.5ml eppendorf tube. Vortex briefly. Add 200ul of this mixture to the inverted top of a 100mm petri dish (Optilux is preferred but just any petri dish cover will due as long as it’s flat, clean and sterile.
c. dsRNA- Previously made and frozen at -80, thaw at RT, add 0.4ul of dsRNA to each group. Except in rare occasions, every experiment will include dsGFP as a control.
d. **10% PVP**- Add 15-25ul 10% PVP to the plate in order to better lubricate the injector needle.
e. Mineral Oil- Once all drops are placed using the suggested orientation on the next page, overlay the dish with Mineral oil to prevent evaporation.

NOTE: Overlay with mineral oil

3. Setting up the holder and initiation of microinjection.
   a. Place the microinjection plate on the Nikon Diaphot inverted microscope.
   b. Carefully add the mercury filled injector to the piezo ensuring no air bubbles are present between the mercury and the oil. Tighten until snug.
   c. Place the injector on the right micromanipulator arm. Do not adjust/tighten the clip as slight movement is required for the piezo to work properly.
   d. Viewing through the inverted microscope at 4X, move the 10% PVP drop into range. Adjust the focus until the edge of the drop is in crisp focus. This will be your point of reference setting up your injection tools.
   e. Now switch your gaze from the oculars to the micromanipulation arm holding the piezo and injection needle. Move the manipulation arm so as the tip is over the drop. Adjust the angle of the needle so as the tip end is parallel to the plate.
   f. Blow out all the air. Now move the needle into the drop while viewing it from the oculars on the microscope. You should see the shadow from the needle as it comes into view. Keep moving the needle down until it is clearly visible.
   g. Do the same for the holder
   h. Rinse needle in 10% PVP and keep in PVP until ready for microinjection.

4. Microinjection
   a. Aspirate dsGFP into the injector. Aspirate 2-3 complete turns over 2 minutes time to fill the injector.
   b. Reverse flow until the dsRNA is coming out very slowly. Evaluate this flow in the 10% PVP drop only.
c. Load a group of 10-50 1 cell embryos into the injection drop rinsing in M2 1X prior.

d. One by one, collect the embryo with the holder. Pull the embryo into the holder as seen in figure 1 in the text. Place the injector inside the holder and activate the piezo to lyse the oolemma and inject the dsRNA. Once the secondary chamber is filled, remove the injector and load another oocyte. Inject the entire group waiting 5 minutes to transfer into KSOM for culture.

e. Evaluate culture daily and record development.

5. Cryopreservation of embryos:
   a. Rinse embryo(s) in 0.3% PVP PBS 3X.
   b. Place in 1.5 ml eppendorf tube with minimal medium
   c. Place immediately into liquid nitrogen
   d. Store in -80 degree centigrade freezer until use.

6. Isolation of RNA from frozen of fresh samples
   a. Thaw embryo samples and add 200 μl PBS. Add 400 μl Lysis/Binding buffer (green cap)
   b. Prepare a High Pure Filter placing it into a collection reservoir.
   c. Add entire sample and centrifuge 15 sec at 8000 g
   d. Cut caps off filter unit and discard flow through liquid.
   e. Add 90 μl DNAse to sample and keep at RT for 15 minutes.
   f. Add 500 μl wash buffer I and centrifuge 15 seconds at 8000 g
   g. Remove flow though, add 500 μl wash buffer II and centrifuge again 15 seconds at 8000 g
   h. Remove flow through and add 200 μl wash buffer II. Centrifuge at maximum (13,000 g) for 5 minutes.
   i. Remove collection tube replacing it with a fresh 1.5 ml eppendorf tube.
   j. Add 30 μl NF-water, wait 2 min. and then centrifuge 1 minute at 13,000 g
   k. Store eluted RNA at -80 degrees centigrade until use.

7. Making cDNA of embryo samples:
   a. Thaw RNA samples (or use fresh from above protocol) adding 8 μl of qScript reaction mix and 2 μl of qScript RT enzyme.(qScript cDNA Synthesis kit)
   b. Mix by flicking the tube and place inside thermocycler using the following protocol to heat the tube and activate the RT to transcribe cDNA: 1 cycle at 22 degrees centigrade for 5 minutes; 1 cycle at 42 degrees centigrade for 30 minutes; 1 cycle at 85 degrees centigrade for 5 minutes and then off. Do not put on hold for 4 degrees centigrade as it can damage the thermocycler over prolonged periods of use.
   c. Store at -20 degrees centigrade until use.

8. qRT-PCR evaluation of samples
   a. Performed in triplicate of 20 μl volumes, use the serial pipettor to make the solution concentration the same in each tube.
   b. Calculate how many embryo equivalents (EE) you need to run per sample. For instance if the tube you just made cDNA originally held 40 embryos and your final volume of medium is 40, you already are at 1 embryo
equivalent per μl. If you had only 10 embryos then you will be at 4 μl per
embryo equivalent. (volume/number of embryos = EE). For convienence
sake, reactions are run at 1EE/ μl.

c. Calculate (1) 20 μl reaction by listing the following: 10 μl 2X master mix,
1 μl normalizing primer (GAPDH or β–actin) 1 μl gene expression
primer, 1 EE sample (1-8 μl) with the balance of NF-water.
d. Add 3X per tube and make up a master mix with all parts except DNA idf
you are testing different samples.
e. Carefully aliquot samples into 3X tubes.
f. Put into 3000mx cycler and input parameters based on primer melting
temps.
g. Run samples and analyze data
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